PhD thesis presented on the 31th of March 2017 at Hasselt University

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AGENTSCHAP
INNOVEREN &
ONDERNEMEN

Woord vooraf

Na een intensieve periode van vijf jaar die nodig was om deze thesis af te ronden kom ik toe aan het schrijven van dit dankwoord. Hierin wil ik stilstaan bij de mensen die mij in deze periode geholpen en gesteund hebben. Een thesis maak je nooit alleen, zonder jullie allen was het dan ook nooit gelukt! :-)

First and foremost, I would like to gratefully acknowledge the time spend for the critical evaluation of my PhD thesis by **all members of the jury**: Dr Hab. M. Wójcik, Prof. Dr N. Verbruggen, Dr T. Remans, Prof. Dr A. Cuypers and Prof. Dr F. Rineau. I would also like to thank the "Agentschap Innoveren & Ondernemen" (also formerly known as the "Agentschap voor Innovatie door Wetenschap en Technologie" (IWT)) for the financial support of my PhD project.

Ten tweede dank ik mijn **promotor** prof. dr. Jaco Vansgronsveld voor het vertrouwen dat u in mij had zodat ik deel mocht worden van uw onderzoeksgroep. U gaf me dan ook de mogelijkheid dit onderzoek uit te voeren in het Centrum Voor Milieukunde (Universiteit Hasselt). Ik wil u verder bedanken voor het brainstormen tijdens de overlegmomenten waarin ik vaak door uw kennis en wijze raad ondergedompeld werd. Ook dank ik u voor het nalezen van al mijn teksten.

Daarnaast wil ik ook graag mijn **co-promotor** Nele Weyens bedanken. Jij wist mij tijdens zowel mijn junior als seniorthesis te motiveren om een IWT-beurs aan te vragen. Verder wil ik je bedanken voor alle hulp en goede raad en het beantwoorden van mijn vele mailtjes. Ook na één van de vele tegenslagen in het labo wist je me terug richting te geven om weer voort te gaan. Natuurlijk ben ook jij bedankt voor het uren en uren nalezen en helpen sleutelen aan mijn thesis.

Ook mijn tweede **co-promotor** Wout Boerjan verdient een bedankje voor de samenwerking, het voorzien van de mutanten, de mogelijkheid om in zijn labo technieken aan te leren, het delen van zijn kennis omtrent de lignine mutanten en het nalezen van de thesis.

Graag zou ik tevens de **hele biologiegroep** bedanken. Jullie zijn met teveel om op te noemen maar allemaal bedankt om mij welkom te laten voelen, voor de leuke middagen en alle andere activiteiten.

Ik zou ook nog eens graag in de verf zetten dat ik met plezier deel was van de **groep "Bacteriën"**. Ik heb echt genoten van de leuke feestjes en teambuilding momenten. Dank je wel aan allen die zich ondertussen al doctor mogen noemen zoals onder

andere Joke, Sarah, Jolien, Bram, Sascha, Panos, Sofie en Nele E. Maar bedankt aan ook degenen die nog gaan volgen zoals onder andere: Inge, Wouter, Iva, Jordan, Valeria en Vincent. Jullie maakten het mogelijk al mijn plantenmateriaal van de zovele condities geoogst te krijgen waarvoor 1000 maal dank. Furthermore, I would like to thank all the foreign students who joined our lab for the nice times. Een speciale "dank u wel" gaat uit naar Bram voor het nalezen van mijn thesis in de laatste rechte lijn tot het versturen ervan naar de jury maar ook voor het beantwoorden van vele mails over de pyrosequencing data, diversiteitsindices, … (zelfs terwijl je even niet in dienst was). En Nele E. dank je wel voor de hulp met het verwerken van de pyrosequencing data met Mothur. Verder ook dank je wel Sascha voor de leuke momenten op het FEMS congres in Maastricht, de ervaring van het werken met *A. thaliana* die je met me gedeeld hebt en de hulp bij de inoculaties. Ik vond het zeer gezellig om samen met jou de bureauruimte te delen.

Ook een welgemeende dank je wel aan mijn beste **bureaugenootje Nelly**. Na twee jaar samen studeren in het middelbaar (Virga Jessecollege) deelden we drie bachelor en twee masterjaren aan Uhasselt en mochten we erna samen een bureau delen tijdens onze doctoraatsjaren. Dit leverde veel plezante momenten op. Ik wil je dan ook bedanken om vaak een luisterend oor te bieden, al denk ik dat wie ons kent natuurlijk weet dat jij eerder de prater bent en ik de luisteraar ;-). Ook nog eens bedankt voor de extra helpende handen tijdens de middagreceptie van mijn trouw.

Om verder te gaan: dank je wel Els, Heidi en Marijke J. om mij even deel te laten uitmaken van jullie **"oxidatieve stress" groep** en de fijne sfeer tijdens de POG congressen in Warschau en Verona. Els en Heidi, ook bedankt voor het antwoorden op de vele mails tijdens het voorbereiden voor de verdediging.

Ook wil ik enkele **collega's van VIB/Ugent** bedanken. Dank je wel Ruben Vanholme om je immense kennis te delen omtrent de (*ccr1*) mutanten. Daarnaast Rebecca Van Acker bedankt voor de hulp bij de thioacidolysis metingen. Verder wil ik Barbara De Meester bedanken voor de antwoorden op de vele mails over de *ccr1* mutanten. Barbara en Paula, eveneens dank je wel voor de leuke momenten op het lignine congres in Zweden.

Bovendien wil ik ook graag even de **praktische ondersteuning van Ann en Carine** (mama) in de bloemetjes zetten. Jullie boden jullie hulp aan bij de omvangrijke proeven door onder andere te helpen bij het zaaien, het onderhouden van de planten en het oogsten ervan. Ook alle bestellingen die jullie in goede banen leiden verdienen een dikke merci. Zonder jullie zou voor zoveel van ons een doctoraat maken

onmogelijk zijn! Daarnaast wil ik ook Natascha nog bedanken voor de gemaakte coupes.

Om stilaan af te sluiten zou ik ook graag **mama en papa** bedanken voor hun onvoorwaardelijke steun gedurende de afgelopen 5 jaar (maar natuurlijk ook de 24 jaar ervoor), de vele hartverwarmende telefoontjes als het schrijven even wat minder vlot verliep en vele opvangmomenten voor Tessa. Door ondertussen zelf een kindje te hebben begrijp ik eens te meer dat jullie echt veel voor mij gedaan hebben en nog steeds doen. Ook mijn **schoonfamilie** Anne, Rudy en omie zou ik willen bedanken voor de extra opvang voor Tessa als ik op zondagen weer moest verder schrijven aan mijn thesis. Ook de rest van de **familie** (bomma, bompa, oma, Jan, Katrien, …) bedankt voor het begrip dat ik wat minder tijd had in de weekenden om langs te komen en bedankt voor jullie interesse in mijn werk en steun.

Eveneens dank je wel **Thomas en Mimi** (en Frone). Om het met jullie eigen woorden te zeggen: je hebt vrienden IN het leven en je hebt vrienden VOOR het leven! ;-)

Als laatste maar zeker niet als minste wil ik **mijn man Tommy** bedanken om er ondertussen tien jaar, waarvan twee en half jaar getrouwd en iets meer dan één jaar met onze geweldige dochter Tessa, er voor mij te zijn. Zowel tijdens mijn studiejaren als doctoraatsjaren was jij mijn steun en toeverlaat. Dank je wel om mij te helpen relativeren indien er iets niet helemaal liep zoals ik wenste.

Kortom: lieve allemaal, heel erg bedankt!

Marijke Gielen – Maart 2017

Summary

Fossil energy has been exploited since the industrial revolution. However, because the stocks are finite and the energy demand is rising due to a growing world population, this would lead to energy insecurity and thus results in an increasing demand for alternative renewable power sources (*e.g.* biofuels: bioethanol, biodiesel and biogas). Second generation biofuels, produced from lignocellulosic biomass (poplar, switchgrass, *Miscanthus* sp. and others), are a good alternative to meet the demand for renewable energy sources. An additional advantage is that these crops can be grown on marginal and degraded lands (*e.g.* cadmium (Cd) contaminated soil). Using these types of lands for growing energy crops can reduce the conflict between the production of food and biofuels, which is a major concern for the first generation biofuels made from food crops. In these lignocellulosic energy crops, photosynthetically fixed carbon is accumulated in the cell walls of the plants. After harvesting these plants, the sugars from the cell walls can be extracted (saccharification) and fermented into bioethanol. However, the recalcitrance of lignin in the lignocellulosic biomass forms an obstacle by lowering the efficiency of the conversion process. Genetically modified (GM) energy crops with a lower lignin content and/or a different lignin composition can partially overcome this problem. However, modification of genes involved in the monolignol biosynthesis pathway can lead to accumulation of phenolic intermediates of this pathway and derivatives thereof (*e.g.* ferulic acid). These phenolic compounds are assumed to have negative effects on plant growth (*e.g.* for *CCR* down-regulated plants). Furthermore, these soluble phenolics can possess antimicrobial activity or may serve as new carbon sources for microbes living inside the plants. In this way, the plant microbiome, which is important for plant health and growth, may be affected in these lignin-reduced plants.

In our study we used *A. thaliana* as a model organism for woody plants and we selected 2 independent allelic T-DNA knockout mutants from 2 genes (*4-COUMARATE:COA LIGASE 1* (*4CL1*) and *CINNAMOYL COA REDUCTASE 1* (*CCR1*)) out of the 13 different genes which are known to be involved in the production of monolignols. Furthermore, we aimed to investigate if this lignin-reduced biomass can be grown on marginal, moderately metal-contaminated soils and therefore we selected Cd as contaminant for this study.

Summary

Our objective was to unravel the effects of **(A) the lignin-reduced genotype** and **(B) Cd exposure** on (i) the plant, including: plant growth **(subsection 3.2)**, elemental and Cd concentrations **(subsection 3.2)**, markers for stress responses associated with reactive oxygen species (ROS) (pigment concentrations, lipid peroxidation, enzyme capacities and gene expression levels; **subsection 3.3**) and lignin (concentration and composition; **subsection 3.4**) and (ii) the plant-associated microbiome, including: the culture-independent **(subsection 4.2)** and culture-dependent **(subsection 4.3)** bacterial endophytic communities and the phenotypic traits **(subsection 4.4)** of the cultivable communities. Furthermore, **(C)** we aimed to counteract the negative growth effects of both the genetic modification and Cd exposure **by inoculating promising endophytic strains (subsection 4.5).**

(A) Effect of the lignin-reduced genotype

In our study, we further supported the earlier observations that *ccr1* mutants display a retardation in development of the aerial parts **(subsection 3.2).** Concerning the effects of the genetic modification at the molecular level, no clear conclusion can be drawn with respect to the studied stress markers in *4cl1* and *ccr1* mutants since not all results pointed in the same direction **(subsection 3.3)**. In addition, we demonstrated that *4cl1* and *ccr1* mutants included lower lignin concentrations and compositional differences in their stems, as previously described in other studies **(subsection 3.4)**. Concerning the roots of lignin-reduced mutants, this is the first study. We found evidence that the lignin concentration in roots of *4cl1* and *ccr1* mutants was not affected though the lignin composition changed due to the genetic modification **(subsection 3.4).**

No prominent effects were observed on the endophytic communities of roots and leaves of the lignin-reduced mutants in comparison to wild type (WT) plants **(subsection 4.2 and 4.3)**. However, we found evidence that the stem endophytic communities of *ccr1* mutants were affected although this might be rather an effect of the developmental delay **(subsection 4.2)**. Next to the stem endophytic communities, also the seed communities of the *ccr1* mutants were changed (in comparison to WT plants) as a result of the genetic modification **(subsection 4.2 and 4.3)**. Furthermore, the cultivable communities of the lignin-reduced mutants indicated a different selection (in comparison to WT

plants) of several growth promotion traits and Cd tolerance in all investigated plant compartments (seeds, roots, leaves and stems) **(subsection 4.4)**.

(B) Effect of cadmium

A higher Cd concentration was observed in roots and leaves of lignin-reduced mutants in comparison to WT plants. Moreover, in roots the concentration of other elements (*e.g.* Ca, Zn) in WT plants seemed lower due to Cd exposure while no changes in elemental concentrations were found in roots of *ccr1* mutants exposed to Cd. In Cd-exposed plants, leaves of lignin-reduced mutants showed a higher elemental concentration in comparison to Cd-exposed WT plants **(subsection 3.2)**. The lignin concentration in stems of all studied genotypes, including WT, was not changed due to Cd exposure, which is important since no additional lignification is wanted for future applications. In roots, the lignin concentration increased in WT plants while rather a decrease after Cd exposure was observed in lignin-reduced mutants **(subsection 3.4)**. Moreover, the capacity of syringaldazine peroxidases (SPX) and the expression of phenylalanine ammonia-lyase 1 (*PAL1)*, corresponding to enzymes involved in lignification, supported this observation in roots **(subsection 3.3)**. The lignin composition in stems of WT plants exposed to Cd was changed; a limited decrease in %H was observed which remained unchanged after Cd exposure of lignin-reduced mutants. In roots of Cd-exposed plants the change in H units was different between WT plants (no change) and lignin-reduced mutants (increase) **(subsection 3.4)**. However, *ccr1* mutants appeared less sensitive to Cd exposure in comparison to WT plants as demonstrated by the growth **(subsection 3.2)** and several stress markers (*e.g.* increase in lipid peroxidation in WT plants though not in *ccr1* mutants, less inhibited pigment concentrations in *ccr1* leaves and oxidative stress marker gene 1 (*OX1*) expression levels increased in WT leaves though not in *ccr1* mutants, glutathione reductase 1 (*GR1*) expression levels increased in WT though not in lignin-reduced mutants) **(subsection 3.3).**

However, the Cd-induced effects that were observed at the plant level may not be related to differences in the bacterial endophytic communities since at that level only minor changes were observed which were only established for the *ccr1* mutants with a developmental retardation **(subsection 4.2, 4.3 and 4.4)**.

(C) Inoculation of growth promoting endophytic strains

In order to counteract the negative effects of both the genetic modification and Cd exposure on growth we selected promising endophytic strains based on (i) their *in vitro* growth promoting traits and Cd tolerance and (ii) their ability to use phenolic compounds (ferulic acid and *p*-coumaric acid), which are assumed to cause the growth retardation in *ccr1* mutants, as a sole C-source. In the non-Cd-exposed condition, we found 2 promising strains which may counteract the growth reduction due to the *CCR1* mutation **(subsection 4.5)**. Moreover, 14 strains promoted the growth of all plant genotypes through production of volatiles. In addition, we also found 4 endophytic strains which could counteract negative effects of Cd on root growth **(subsection 4.5)**. In future experiments, further optimisation of the inoculation strategy and exploitation of consortia should be considered. Furthermore, the observed growth promoting results should be validated using more realistic conditions of growing the plants (*e.g.* in sand in a greenhouse).

In conclusion, in **non-Cd-exposed plants** we demonstrated that genotypic modification lead to the most prominent effects on **the plant-associated bacterial communities**. We provided evidence that in non-exposed plants the endophytic bacterial communities of stems and seeds of the *ccr1* mutants were different from those of WT plants and that in stems this can be due to the developmental retardation of the mutants. Further, we demonstrated the presence of different relative abundances of plant growth promoting traits in lignin-reduced mutant communities in comparison to the communities associated with WT plants. In the **Cd-exposed plants**, the observed changes in endophytic communities could not be related to the most distinctive results noticed at the **level of the plant**. The most important differences were (i) the higher Cd concentration in roots and leaves of lignin-reduced plants while (ii) these plants appeared less sensitive to Cd exposure (in comparison to WT plants), in combination with (iii) an increased lignification of the roots of WT plants though not in lignin-reduced mutants.

We also demonstrated that **inoculation** of lignin-reduced mutants with beneficial bacteria can be a promising strategy to counteract the growth reduction caused by both the genetic modification and Cd exposure.

Samenvatting

Fossiele energiebronnen worden sinds de industriële revolutie op steeds grotere schaal aangewend. Omdat de voorraad van deze bronnen eindig is en de energievraag mede door de groeiende wereldbevolking blijft stijgen, zal dit leiden tot een onzekerheid in het energieaanbod. Dit zal leiden tot een alsmaar toenemende vraag naar alternatieve hernieuwbare energiebronnen (vb. biobrandstoffen: bioethanol, biodiesel en biogas). Tweede generatie biobrandstoffen worden geproduceerd uit lignocellulose biomassa (zoals onder andere populier en grassoorten (*Panicum virgatum*, *Miscanthus sp.* en andere)) en zijn een goed alternatief om mede de stijgende vraag naar hernieuwbare energie te beantwoorden. Een bijkomend voordeel is dat deze gewassen op marginale en gedegradeerde bodems (vb. cadmium (Cd) verontreinigd) geteeld kunnen worden. Het gebruik van dergelijk types land voor het groeien van energiegewassen kan het conflict tussen de productie van voedsel en biobrandstof verminderen, een conflict dat een groot probleem vormt voor de eerste generatie biobrandstoffen gemaakt uit voedsel. In deze lignocellulose energie gewassen worden grote hoeveelheden fotosynthetisch gefixeerde koolstof opgestapeld in de celwanden van de plant. Na het oogsten van deze planten kunnen de suikers uit celwanden geëxtraheerd worden (saccharificatie) en gefermenteerd naar bioethanol. Tijdens dit proces vormt de recalcitrantie van het lignine in de lignocellulose biomassa een obstakel door het verlagen van de efficiëntie van het omzettingsproces. Genetisch gemodificeerde (GM) energiegewassen met een lagere lignine-inhoud en/of een andere ligninesamenstelling kunnen dit probleem gedeeltelijk omzeilen. Modificatie van genen betrokken in de biosynthese van monolignolen kan echter leiden tot de accumulatie van fenolische intermediairen en afgeleiden daarvan (vb. ferulinezuur). Er wordt verondersteld dat deze ophoping van fenolische verbindingen de basis voor de negatieve impact op de plantengroei vormt (vb. in *CCR*-neergereguleerde planten). Daarenboven kunnen oplosbare fenolen antimicrobiële activiteit vertonen of kunnen ze als nieuwe koolstofbronnen aangewend worden door microben die leven in de plant. Op deze manier kan het microbioom, dat belangrijk is voor de gezondheid en groei van de plant, wijzigingen ondergaan in deze lignine-gereduceerde planten.

In onze studie werd *Arabidopsis thaliana* gebruikt als model organisme voor houtige planten en werden 2 onafhankelijke T-DNA knockout mutanten van 2 genen (*4-COUMARATE:COA LIGASE 1* (*4CL1*) en *CINNAMOYL COA REDUCTASE 1* (*CCR1*)) geselecteerd uit de 13 verschillende genen die betrokken zijn in de productie van monolignolen. Aangezien één van de doelstellingen van deze studie was om te onderzoeken of de lignine-gereduceerde planten gegroeid kunnen worden op marginale, matig metaal-verontreinigde bodems, selecteerden we Cd als contaminant voor onze studie.

Onze doelstelling was om de effecten te achterhalen van **(A) de lignine-gereduceerde genotypen** en **(B) Cd blootstelling** op **(i) de plant**, meer bepaald: groei van de plant **(subsectie 3.2)**, element en Cd concentraties **(subsectie 3.2)**, merkers voor stressresponsen geassocieerd met reactieve zuurstof species (ROS) (pigment concentratie, lipide peroxidatie, enzymcapaciteiten en genexpressie niveaus; **subsectie 3.3**) en lignine (concentratie en samenstelling; **subsectie 3.4**) en **(ii) het plantgeassocieerde microbioom**, meer bepaald: de cultuuronafhankelijke **(subsectie 4.2)** en cultuurafhankelijke **(subsectie 4.3)** bacteriële endofyten gemeenschappen en de fenotypische eigenschappen **(subsectie 4.4)** van de cultuurafhankelijke gemeenschappen. Verder was ons doel om **(C)** de negatieve effecten op de groei van zowel de genetische modificatie als de Cd blootstelling tegen te gaan door **veelbelovende endofytische bacteriestammen te inoculeren (subsectie 4.5)**.

(A) Effecten bij de lignine-reduceerde genotypen

In onze studie werd de eerdere waarneming betreffende de vertraagde ontwikkeling van de bovengrondse delen van de *ccr1* mutanten bevestigd **(subsectie 3.2)**. Aan de hand van de bestudeerde stressmerkers konden echter geen duidelijke conclusies getrokken worden over de effecten van de genetische modificatie op het moleculaire niveau. Dit omdat voor de *4cl1* en *ccr1* mutanten niet alle resultaten in dezelfde richting wezen **(subsectie 3.3)**. Verder toonden we aan, zoals reeds eerder beschreven in andere studies, dat *4cl1* en *ccr1* mutanten een lagere lignineconcentratie en gewijzigde ligninesamenstelling

bevatten in hun stengel **(subsectie 3.4)**. Dit is tevens de eerste studie naar de lignineconcentratie en -samenstelling in wortels van de lignine-gereduceerde mutanten. We vonden aanwijzingen dat de totale lignineconcentraties in wortels van *4cl1* en *ccr1* mutanten niet wijzigden maar de ligninesamenstelling wel veranderde ten gevolge van de genetische modificatie **(subsectie 3.4)**.

Op de samenstelling van de endofyten gemeenschappen van wortels en blaadjes van de lignine-gereduceerde mutanten werden geen significante effecten waargenomen in vergelijking met deze van wildtype (WT) planten **(subsectie 4.2 en 4.3)**. Daarentegen vonden we aanwijzingen dat de endofyten gemeenschappen van de stengels van *ccr1* mutanten beïnvloed werden, al leek dit eerder een effect van de vertraagde ontwikkeling **(subsectie 4.2)**. Naast verschillen in de stengel endofytische gemeenschappen, werden als gevolg van de genetische modificatie ook verschillen vastgesteld in de gemeenschappen van zaadendofyten van *ccr1* mutanten (ten opzichte van deze van WT planten) **(subsectie 4.2 en 4.3)**. Ook werd een verschillende selectie van verscheidene groeipromotie eigenschappen en Cd tolerantie in de cultiveerbare gemeenschappen van de lignine-gereduceerde mutanten (ten opzichte van deze van WT planten) in alle onderzochte planten compartimenten (zaden, wortels, blaadjes en stengels) gevonden **(subsectie 4.4)**.

(B) Effecten van cadmium

De Cd concentratie in wortels en blaadjes van lignine-gereduceerde mutanten verhoogde in vergelijking met WT planten. Verder verlaagden de concentraties van andere elementen (vb. Ca en Zn) in de wortels van WT planten na Cd blootstelling terwijl geen verandering in elementconcentratie werd waargenomen in de wortels van Cd-blootgestelde *ccr1* mutanten. Na Cd blootstelling vertoonden lignine-gereduceerde planten een hogere elementconcentratie in de blaadjes in vergelijking met Cd-blootgestelde WT planten **(subsectie 3.2)**. Bij geen van de bestudeerde genotypen (inclusief WT) werden de lignineconcentraties in de stengel beïnvloed door blootstelling aan Cd. Dit is belangrijk aangezien geen bijkomstige lignificatie gewenst is in toekomstige toepassingen. In wortels van Cd-blootgestelde WT planten verhoogde de lignineconcentratie tegenover eerder een daling in wortels van Cd-blootgestelde lignine-gereduceerde mutanten **(subsectie 3.4)**. Bovendien werd deze

waarneming in de wortels ondersteund door de capaciteit van syringaldazine peroxidasen (SPX) en de expressie van *PAL1,* gerelateerd aan enzymen betrokken in het lignificatie proces **(subsectie 3.3)**. De ligninesamenstelling in de stengel van WT planten werd beïnvloed door Cd blootstelling; een lichte verlaging van %H werd geobserveerd in Cd-blootgestelde WT planten terwijl deze parameter niet wijzigde in lignine-gereduceerde mutanten. In de wortels was de verandering van H eenheden ten gevolge van Cd blootstelling verschillend tussen WT (geen Cd-geïnduceerde verandering) en lignine-gereduceerde mutanten (Cd-geïnduceerde verhoging) **(subsectie 3.4)**.
Verder leken *ccr1* mutanten minder gevoelig voor Cd blootstelling in vergelijking met WT planten zoals blijkt uit hun groei **(subsectie 3.2)** en stressgerelateerde merkers (vb. verhoging van lipide peroxidatie in WT planten maar niet in *ccr1* mutanten, minder verlaging van de pigment concentratie in *ccr1* blaadjes en *OX1* expressie verhoogde in WT blaadjes maar niet in *ccr1* mutanten en glutathion reductase 1 (*GR1*) expressie verhoogde in WT maar niet in lignine-gereduceerde mutanten) **(subsectie 3.3)**.
De Cd-geïnduceerde effecten op het niveau van de planten werden echter niet gerelateerd aan verschillen in de bacteriële endofyten gemeenschap aangezien alleen maar matige veranderingen werden gevonden welke enkel bevestigd werden voor de *ccr1* mutanten met een ontwikkelingsachterstand **(subsectie 4.2, 4.3 en 4.4)**.

(C) Inoculatie en groeipromoverende endofyten stammen

Veelbelovende stammen van endofytische bacteriën werden geselecteerd met als doelstelling de negatieve effecten op de groei ten gevolge van zowel de genetische modificatie als de blootstelling aan Cd tegen te gaan. Deze selectie gebeurde op basis van (i) de *in vitro* groeibevorderende eigenschappen en Cd tolerantie en (ii) het vermogen om fenolische verbindingen (ferulinezuur en *p*-coumarinezuur) als enige C-bron te gebruiken. Fenolische verbindingen die accumuleren in de *ccr1* mutanten worden verondersteld mede verantwoordelijk te zijn voor de groeivertraging; bijgevolg zou een verlaging van de concentraties van deze stoffen de groei mogelijks kunnen verbeteren.
In de niet-Cd-blootgestelde conditie werden 2 veelbelovende stammen gevonden die de groeireductie door de *CCR1* mutatie kunnen tegengaan

(subsectie 4.5). Verder konden 14 stammen de groei van alle planten genotypen verbeteren door de productie van vluchtige stoffen. Daarnaast vonden we 4 endofytische stammen die de negatieve effecten van Cd op de wortelgroei verminderden **(subsectie 4.5)**.
In toekomstige experimenten dienen zowel een verdere optimalisatie van de inoculatiestrategie als het gebruik van consortia overwogen worden. Verder moeten de waargenomen groei promotie resultaten gevalideerd worden onder meer realistische groeicondities (vb. in zand in een serre).

Om te besluiten, bij de **niet-Cd-blootgestelde** planten leidt de genetische modificatie tot opvallende effecten in de plant-geassocieerde bacteriële gemeenschappen. We toonden aan dat in niet-Cd-blootgestelde planten de endofytische bacteriële gemeenschappen van de stengel en zaden van de *ccr1* mutanten verschillen van deze van de WT planten en dat in de stengel deze verschillen mogelijk door de ontwikkelingsachterstand van de mutanten verklaard kunnen worden. Verder demonstreerden we verschillen in relatieve abundantie van plantengroeibevorderende eigenschappen in lignine-gereduceerde mutanten gemeenschappen in vergelijking met de gemeenschappen geassocieerd met WT planten. Zo vonden we bijvoorbeeld een lagere relatieve abundantie van bacteriën die Cd tolerant, ACC deaminase producerend, siderofoor producerend, P oplossend of N fixerend zijn in de lignine-gereduceerde wortelgemeenschappen in vergelijking met de WT wortelgemeenschap. In **Cd-blootgestelde** planten konden de veranderingen in de endofyten gemeenschap niet gerelateerd worden aan de meest opvallende effecten op het **niveau van de plant**. De meest belangrijke verschillen waren (i) een hogere Cd concentratie in wortels en blaadjes van lignine-gereduceerde planten terwijl (ii) deze planten minder gevoelig leken voor Cd blootstelling (in vergelijking met WT planten), in combinatie met (iii) een verhoogde lignificatie in de wortels van WT planten maar niet in lignine-gereduceerde mutanten.
Inoculatie van lignine-gereduceerde mutanten met groeibevorderende bacteriën bleek een veelbelovende strategie om de groeireductie ten gevolge van zowel de genetische modificatie als Cd blootstelling tegen te gaan.

INDEX

SECTION 1
INTRODUCTION

1.1 Problem statement

Since the industrial revolution mankind has exploited fossil energy (*e.g.* coal, crude oil or natural gases). These fossil fuels were formed out of ancient photosynthetic products during geochemical processes and every year the solar energy photosynthetically captured during 550 years is used to supply the current global demand for fossil fuels (Ringsmuth *et al.* 2016). However, due to a **growing world population** and rapidly developing countries, the **energy demand is expected to raise** up to 50% by 2025. Since **fossil fuel** sources are **finite** (Ragauskas *et al.* 2006), this will eventually lead to **energy insecurity** (Littlewood *et al.* 2014, Shafiee and Topal 2009).

Next to the depletion of fossil fuels and growing world population, also **climate change** creates a need for new alternative and sustainable energy sources that reduce the ecological footprint *e.g.* CO_2 neutral biofuels (Littlewood *et al.* 2014, Meng and Ragauskas 2014, Ragauskas *et al.* 2006, Blanch 2012). In case fossil fuels are burnt, carbon that was fixed millions of years ago is released in the atmosphere in the form of CO_2. Although combustion of biofuels also results in the emission of CO_2, the emitted CO_2 was captured throughout the growth period of the photosynthetic organisms, hence the overall CO_2 emission balance can be neutral or even negative (Singh *et al.* 2011a, Naik *et al.* 2010, Karp and Richter 2011).

Alternative renewable power sources (*e.g.* wind, solar, hydro) are available, but options to transport the produced energy, which is possible for liquid fuels, are limited (Karp and Richter 2011). Renewable bioenergy can offer a solution for these issues. Bioenergy is the chemical energy stored in organic material that can be directly converted into useful energy sources. Bioethanol, biodiesel and biogas are the three main biofuel forms (Lopez-Bellido *et al.* 2014, Yuan *et al.* 2008). In European guidelines it is stated that by 2020, 10% of the transport fuels should originate from renewable sources (The Renewable Energy Directive (Directive 2009/28/EC)). Moreover, these biofuels can have additional advantages. For instance, bioethanol is a substitute for gasoline and has the

advantage that no adaptation of current engines is necessary in case it is blended with gasoline (FAO 2008, IEA 2011). Furthermore less CO, unburned hydrocarbons, carcinogens and sulphur oxide (causing acid rain) are produced during combustion (FAO 2008).

All these biofuels are produced from plants, hence are solar-powered by making use of photosynthesis (Ringsmuth *et al.* 2016). Biofuels production can be classified into 3 main classes: first, second and third generation biofuels, based on the biomass that is used (Singh *et al.* 2011a).

First generation (1G) biofuels are mainly made from food crops (*e.g.* corn, sugarcane and beets) that are grown on fertile land and require a lot of water, fertilisation and pesticides (Fiorese *et al.* 2013, Singh *et al.* 2011a). Consequently, **production of this biofuel competes with the food and feed production** and questions are raised concerning the overall sustainability since deforestation and subsequent effects on biodiversity are a potential result (Fiorese *et al.* 2013). Furthermore, food shortage and raising food prices can be a result and can have a huge impact in developing countries, where already more than 850 million people suffer from malnutrition and starvation (FAO 2007). Moreover, they are inefficient in producing energy and their capacity to reduce CO_2 emission is low. Therefore, the sustainability of first generation biofuels is an item for discussion (Lopez-Bellido *et al.* 2014). Finally, they are not cost-competitive with fossil fuels (Fiorese *et al.* 2013). Nevertheless, bioethanol from grain, sugarcane and corn is currently the most common biofuel (Chen and Fu 2016).

The **second generation (2G) biofuels**, made from lignocellulosic crops (*e.g.* fast growing trees in short rotation such as poplar or willow or grasses such as switchgrass, *Miscanthus* and giant reed) (Karp and Richter 2011) and organic rest streams, are more sustainable than 1G because of a better net greenhouse gas emission rate (FAO 2008, Morales *et al.* 2015, Gomez *et al.* 2008). Lignocellulose is the most abundant renewable source on earth in which fixed carbon is accumulated in the cell walls (Schroder *et al.* 2008, Karp and Richter 2011). Compared to the 1G crops, more biomass can be produced of 2G crops per hectare because the entire above-ground parts can be used (Lopez-Bellido *et al.* 2014). Moreover, handling costs can be reduced because these crops are

more robust to handle and species are selected for low external input such as low need for fertilisers (FAO 2008, Lopez-Bellido *et al.* 2014). On top of that, these crops might be grown on **marginal and degraded soils** where conventional agriculture for food crops is not possible, and can therefore **minimise the conflict with food production** (Murphy *et al.* 2011, Singh *et al.* 2011a, Milbrandt and Overend 2009, FAO 2008, Lopez-Bellido *et al.* 2014, Schroder *et al.* 2008). Even though these lands will be less productive, the additional environmental and social benefits (*e.g.* restoration of degraded land, carbon sequestration and job creation) make it a valuable strategy (Milbrandt and Overend 2009). The produced lignocellulosic biomass contains cellulose and hemicellulose which can be converted into sugars that can be fermented into bioethanol (Chen and Fu 2016). However, the **lignin component** in the lignocellulosic biomass is an **obstacle in the efficiency of producing bioethanol out of this biomass** (Van Acker *et al.* 2013).
The **third generation (3G)** biofuels is mainly based on microscopic organisms such as micro-algae (Singh *et al.* 2011b).

We need to move away from the first generation of biofuels and shift to the use of more advanced biofuels, such as second or third generation biofuels (Murphy *et al.* 2011). To make these crops economically valuable, the yield, resilience to biotic and abiotic challenges and biomass composition must be optimised (Ragauskas *et al.* 2006). For the optimisation, we will need to pay attention to the **cell walls, more specifically the lignin component** (Eudes *et al.* 2014).

1.2 The cell wall of lignocellulosic plants

Lignocellulosic plant cells are surrounded by two types of cell wall layers to maintain the rigidity and strength of the cell and to protect the cell against environmental stress. First, the **primary cell wall** is made during development. Subsequently, the **secondary cell wall** is deposited at the inside of the primary cell wall in fibres and tracheary elements to provide additional mechanical support and to secure the water-conducting capacity of the cell by providing imperviousness to the cell wall (Endler *et al.* 2011). These secondary cell walls are made from 3 main components: the polysaccharide polymers (i) **cellulose** and (ii) **hemicellulose**; and a matrix of other substances, mainly (iii) **lignin**

(Fig 1.1). Next to these 3 main components also other components such as pectin can be incorporated in the cell wall (Marriott *et al.* 2016). The cell wall composition can differ between species, tissues, cell types and even localisation around the cell (Zeng *et al.* 2014). For instance, for hardwood plants the composition is 40-55% (of the plants dry weight) cellulose, 20-35% hemicellulose and 18-25% lignin (Marriott *et al.* 2016). Lignin is covalently cross-linked to hemicellulose and the lignin-hemicellulose complex binds to cellulose by making hydrogen bonds (Park *et al.* 2014). These interactions make the cell wall very strong but also result into a **major problem** for the use of these lignocellulosic cell walls in bioethanol production. More specifically, **lignin impedes the enzymatic hydrolysis** of cellulose and hemicellulose into fermentable sugars (Van Acker *et al*. 2013).

1.2.1 Cellulose and hemicellulose

Cellulose is the most abundant polymer on earth and is the main component of the cell wall. It is a β-1,4 linked chain, without side-chains, of 7000-15000 **glucose monomers** (Meng and Ragauskas 2014, Gibson *et al.* 2012) (Fig 1.1). These chains are arranged in parallel, crystalline structures to form microfibrils by using inter- and intra-molecular hydrogen bonds and Van der Waals forces (Endler *et al.* 2011). Cellulose provides the skeleton of the cell wall (Delmer and Amor 1995). The cellulose fibres are oriented in different angles in each layer (Gibson *et al.* 2012) and a primary cell wall just contains 3-4 layers of these microfibrils while secondary cell walls can contain hundreds. Because cellulose can be enzymatically depolymerized into its glucose monomers, secondary cell walls are an **important component for energy production** (Marriott *et al.* 2016).

In contrast to the cellulose, the **hemicellulose** is a highly branched heteropolymer of 500-3000 pentose and hexose sugar monomers such as xylose, arabinose, mannose, glucose, galactose and sugar acids (Kricka *et al.* 2014) (Fig 1.1). The side-chain formation prevents the formation of a crystalline structure (Marriott *et al.* 2016). There exists a large variety of structural types, classified by the main type sugar in the polymer: xylans, xylogucans, mannans and mixed linkage β-glucans (Gibson *et al.* 2012, Endler *et al.* 2011, Hoch *et al.* 2007). Hemicellulose makes the matrix of the cell wall (Baucher *et al.* 2003) and

holds the microfibrils of the cellulose together at specific junctions by formation of hydrogen bonds (Marriott *et al.* 2016). Hemicelluloses can reduce accessibility of cellulose for cellulases, decreasing the saccharification efficiency (Marriott *et al.* 2016). To make the processing of lignocellulosic biomass into bioethanol more efficient, both cellulose and hemicellulose sugars may be targetted. However, hemicellulose is more difficult to use since it contains **different hexose and pentose sugar monomers** resulting in a different appropriate approach. In general, *Saccharomyces cerevisiae*, which are mostly used for the conversion of sugars into bioethanol, cannot metabolise the pentose sugar xylose, a major component of hemi-cellulose (Kricka *et al.* 2014). Genetically modified *Saccharomyces* that can ferment these pentose sugars may enhance bioethanol production (Wang *et al.* 2016).

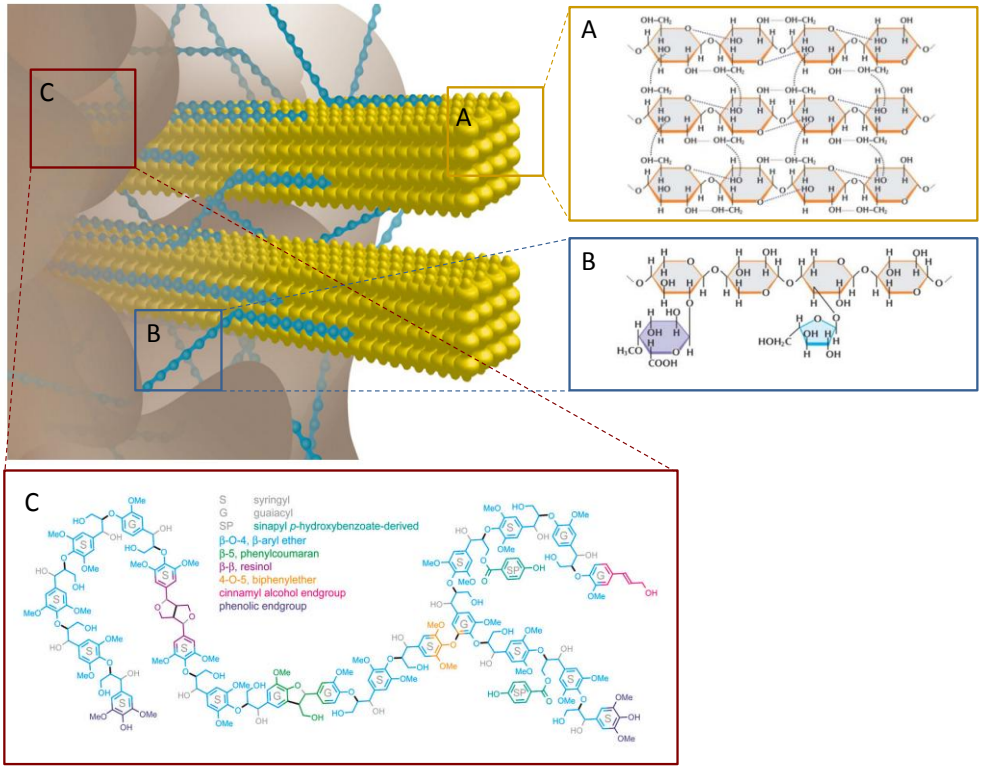


Figure 1.1: Structure of the cell wall (Marriott *et al.* 2016) including the chemical structure of (A) cellulose (Akhtar *et al.* 2016, (B) hemi-cellulose ((Akhtar *et al.* 2016) and (C) lignin (Vanholme *et al.* 2010).

1.2.2 Lignin

The word 'lignin' is derived from the Latin word "lignum" which means wood, although lignin is also found in non-woody plants (Lewis *et al.* 1990). It is the second most abundant biopolymer on earth next to cellulose (Zeng *et al.* 2014). Hence, it is a major carbon sink is vascular plants (Van de rest *et al*. 2006). The presence of lignin was a major step during the evolution of the higher plants since it has 3 major functions: (i) give the plant strength and rigidity to grow upward, (ii) development of a hydrophobic vascular system which enabled uptake and transport of water and nutrients and (iii) act as a defence mechanism against pathogens, insects and other herbivores by protecting the degradation of cell wall polysaccharides (Vanholme *et al.* 2008, Hatfield and Fukushima 2005, Brown *et al.* 2014). Furthermore, lignin is part of the Casparian strips forming a barrier for passive diffusion of ions across the roots apoplast (Piquemal *et al.* 1998).

1.2.2.1 Monolignol production

The composition of lignin molecules is highly variable between species, tissues, cell types and even within a cell wall (Vanholme *et al.* 2012b) and can be influenced by environmental conditions such as pathogen attack, photoperiod, temperature, hygrometry, wounding and abiotic stress but also climate and soil type (Ghosh *et al.* 2014, Baucher *et al.* 2003, Piquemal *et al.* 1998, Goujon *et al.* 2003). Lignin molecules are made by coupling *p*-hydroxycinnamyl alcohols (mainly *p*-coumaryl, coniferyl and sinapyl alcohol; the monolignols) into a biopolymer (Brown *et al.* 2014) (linkages displayed in Fig 1.3). From then the building blocks are called *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) (Ralph *et al.* 2004, Vanholme *et al.* 2010, Eudes *et al.* 2014). The monolignols differ in their number of methoxy substituents on the aromatic ring (Brown *et al.* 2014) (Fig 1.2). However, lignin has an enormous plasticity for its monomers (Barros *et al.* 2015). For instance, also other building blocks such as ferulic acid can be incorporated (Vanholme *et al.* 2012b).

Inside the cell, the different monolignols are made from phenylalanine in the phenylpropanoid pathway, which consists of a general phenylpropanoid and monolignol specific pathway (Fig 1.2).

The **general phenylpropanoid pathway** (Fig 1.2) delivers products to the monolignol specific pathway but also acts as input for other pathways such as flavonoid, benzenoid, coumarin, sinapate and ferulate ester production. It comprises a variety of reactions (Vanholme *et al.* 2012b). Phenylalanine ammonia-lyase (PAL) performs the first reaction in this pathway, the non-oxidative deamination of phenylalanine to cinnamate (Baucher *et al.* 2003). Subsequently, cinnamate-4-hydroxylase (C4H) hydroxylates the aromatic ring resulting in *p*-coumarate (Raes *et al.* 2003) and **4-coumarate:CoA ligase (4CL)** converts this substrate into a thioester yielding *p*-coumaroyl-CoA (Ehlting *et al.* 2001). Via hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase (HCT) the formation of quinic or shikimic acid ester derivatives is performed. Hydroxylation by *p*-coumarate 3-hydroxylase (C3H) and again transesterification by hydroxycinnamoyl transferase (HCT) to form caffeoyl-CoA are the subsequent steps (Vanholme *et al.* 2012b). An alternative route to form caffeoyl-CoA out of the quinic or shikimic acid esters makes use of caffeoyl shikimate esterase (CSE) and **4-coumarate:CoA ligase (4CL)** (Vanholme *et al.* 2013). The methylation of the 3-hydroxyl group of caffeoyl-CoA by caffeoyl CoA O-methyltransferase (CCoAOMT) produces feruloyl-CoA (Raes *et al.* 2003).

In the **monolignol-specific pathway** (Fig 1.2) feruloyl-CoA is converted into coniferyl and sinapyl alcohol and *p*-coumaroyl-CoA is converted into *p*-coumaryl alcohol. First, **cinnamoyl CoA reductase (CCR)** reduces the cinnamoyl esters *p*-coumaroyl-CoA and feruloyl-CoA to *p*-coumaraldehyde and coniferaldehyde, respectively (Lauvergeat *et al.* 2001). Two extra steps are necessary in the production route towards syringyl: (1) ferulate 5-hydroxylase (F5H) hydroxylating coniferaldehyde at the 5-position and (2) caffeic acid O-methyltransferase (COMT) to methylate this 5-hydroxyl group to provide sinapaldehyde (Raes *et al.* 2003). In the last step *p*-coumaraldehyde, coniferaldehyde and sinapaldehyde are further reduced to alcohols by cinnamyl alcohol dehydrogenase (CAD) (Baucher *et al.* 2003). The produced *p*-coumaryl, coniferyl and sinapyl alcohol can then be incorporated into the lignin polymer (Vanholme *et al.* 2012b).

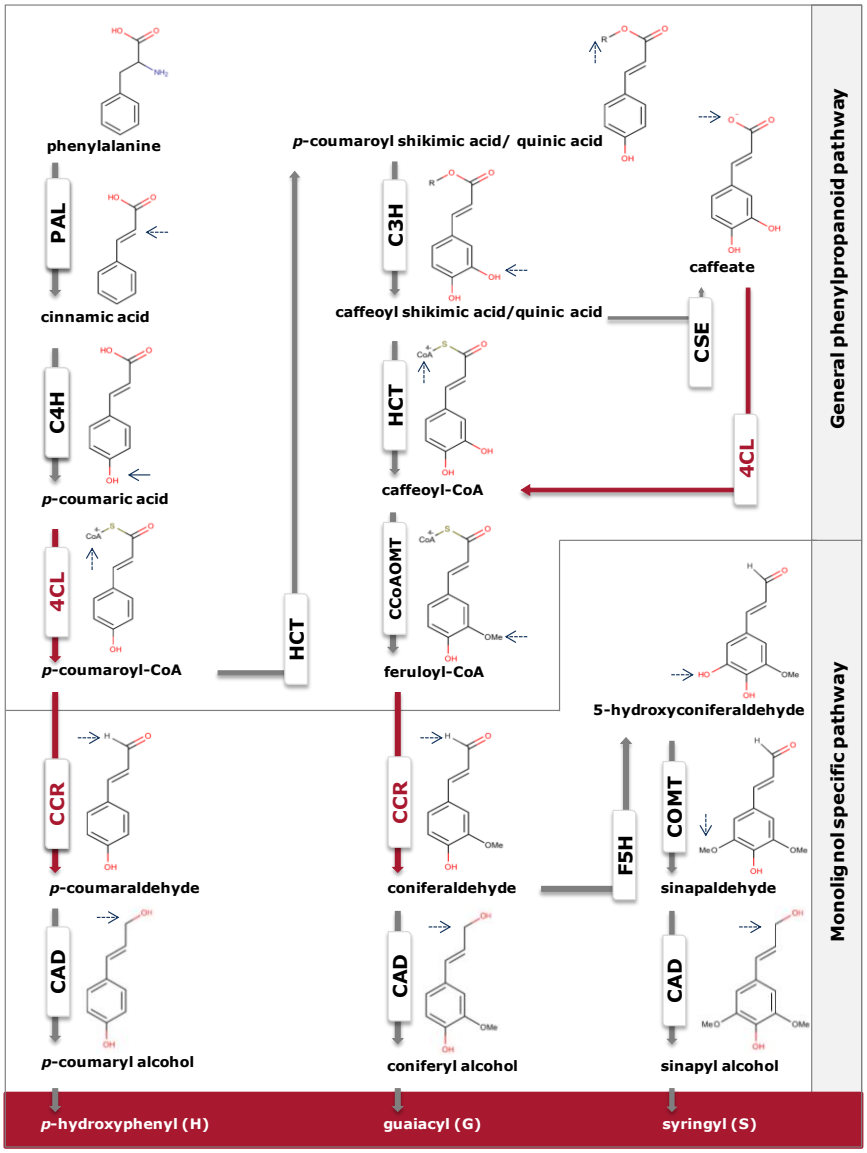


Figure 1.2: Predominant route of the monolignol biosynthetic pathway. Abbreviation of the different enzymes: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; HCT, *p*-hydroxycinnamoyl-CoA: quinate shikimate *p*-hydroxycinnamoyl transferase; C3H, *p*-coumarate 3-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyl transferase; CCR, cinnamoyl-CoA reductase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid/5-hydroxyconiferaldehyde O-methyl transferase; CAD, cinnamyl alcohol dehydrogenase; CSE, caffeoyl shikimate esterase. (Adapted from Vanholme *et al.* 2008, Vanholme *et al.* 2012b, Vanholme *et al.* 2013, Marriott *et al.* 2016)

The produced monolignols are not only used to produce lignin polymers but can also be incorporated into for instance lignans and neolignans (Lewis *et al.* 1990). Lignans are neolignans are derived from monolignol dimers and are thought to be involved in defence responses for their antioxidant properties (Wang *et al.* 2013, Dima *et al.* 2015).

1.2.2.2 Monolignol transport and coupling

The lignification process is not identical for every plant species (Vanholme *et al.* 2012b). Moreover, lignin deposition is needed in various **cell types** (*e.g.* roots endodermis displaying Casparian strips, stems tracheary elements and sclerenchym cells and seed coat cells) and is dependent on cell type and **developmental stage of the plant**. Moreover, several parameters (*e.g.* timing, amount, size of the polymer, monomeric composition, specific enzymes, subcellular location) influence the lignin polymer formation. Therefore, it is difficult to introduce one general mechanism for lignification and the process is still poorly understood (Barros *et al.* 2015).

The **monolignols are produced** in the protoplast, more specifically in the cytoplasm near the outer surface of the endoplasmatic reticulum (Wang et al 2013, Barros *et al.* 2015). However, also non-lignifying tissues can express genes involved in monolignol production. Hence it is suggested that cells can co-operate and monolignols are not necessarily produced in the cell where they will be deposited. An example is the extra lignification of tracheary elements after death of their cells (Barros *et al.* 2015).

After production, monolignols are **transported** to the cell wall where they can be deposited (Boerjan *et al.* 2003). The exact mechanism is still unclear though possible mechanisms include passive diffusion, vesicle associated exocytosis and active ATP-dependent transport (Barros *et al.* 2015). Mobilisation and storage of monolignols might be coupled with glycosylation/deglycosylation although it might also play no role in these processes (Boerjan *et al.* 2003, Vanholme *et al.* 2008).

Lignin **coupling** is orchestrated directly in the cell wall by oxidative polymerisation. **Laccases and peroxidases**, respectively using dioxygen (O_2)

or hydrogen peroxide (H_2O_2), activate the monolignols into **radicals** by dehydrogenation. These radicals are partly stabilised by resonance (Vanholme *et al.* 2012b, Boerjan *et al.* 2003). Several isoforms of laccases and peroxidases, more specifically class III type peroxidases, are available in the plant (Vanholme *et al.* 2012a, Weng and Chapple 2010). NADPH oxidases at the plasma membrane, also known as Respiratory Burst Oxidase Homologs (RBOH) in *A. thaliana*, form superoxide ($O_2^{\cdot-}$) that can be converted into H_2O_2 by superoxide dismutase (SOD). These H_2O_2 molecules are necessary for the peroxidases to produce monolignol radicals (Lee *et al.* 2013, Barros *et al.* 2015).

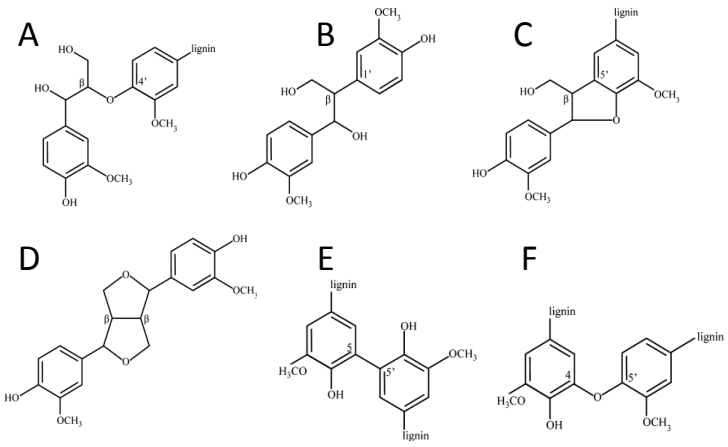


Figure 1.3: Major linkages that occur between monolignols within the lignin polymer: (A) β-O-4', (B) β-1', (C) β-5', (D) β-β , (E) 5-5', (F) 4-O-5 (Marriott *et al.* 2016).

The produced monolignol radicals, which can reallocate their radical charge (Vanholme *et al.* 2010), will be able to form **different linkages** including the condensed C-C linkages (*e.g.* 5-5', β-5', β-β and β-1') and non-condensed ether linkages (*e.g*. 4-O-5 and β-O-4') (Ralph *et al.* 2004) (Fig 1.3). The β-O-4' is the most common linkage (Wadenback *et al.* 2008) because monolignol radicals favour coupling at their β position (Vanholme *et al.* 2010). The various linkages are a result of differences in thermodynamic reactivity (Barros *et al.* 2015). Although the mechanism to control the polymerisation is poorly understood, the length of the polymer and proportion of linkages may be dependent on the availability and reactivity of the monolignols (Ralph *et al.* 2008). The end-wise addition of the monolignol radicals might occur independent of enzymes (Barros

et al. 2015). However, also proteins with a dirigent function may be involved (Vanholme *et al.* 2008).

1.2.2.3 Lignin degradation

There are three main reasons why **lignins** are extremely **difficult to degrade** enzymatically. (i) Firstly, although the monolignols are the main monomers, also other phenolic compounds can be incorporated into the polymer, a process which causes a lot of structural variation (Ralph *et al.* 2004). Hence, lignin is not one specific polymeric structure but a group of various structures with **different phenolic subunits and bindings**. (ii) Secondly, lignin is a **three-dimensional network** with chain branching and inter/intra chain coupling. This forms a limitation for the enzymes to enter the structure to degrade the different bindings. (iii) And thirdly, the aromatic lignin units will **react as non-phenolic** because of the occurrence of many ether linkages. Because of this non-phenolic property, it cannot be oxidised by low redox potential oxido-reductases (Ruiz-Dueñas and Martínez 2009).

1.2.2.4 Bioethanol production and lignin

To produce bioethanol out of biomass, several steps are necessary: (i) collection of the biomass, (ii), pre-treatment, (iii) enzymatic hydrolysis (iv) fermentation by making use of yeast and (v) distillation/rectification/dehydration (Meng and Ragauskas 2014). To reduce bioethanol production costs, pre-treatment, enzymatic hydrolysis and fermentation need to be further optimised.

Because of the highly crystalline cellulose structure which is protected by highly recalcitrant lignin, only 20% of the theoretical sugar yield is available for fermentation in untreated biomass (Singhvi *et al.* 2014). Several **pre-treatment** processes can be incorporated to make the sugars in the biomass more accessible including chemical (*e.g.* acids, alkali, salts and ionic liquids), physical (*e.g.* steam explosion, heat water and hydrothermal microwave) or biological approaches (Menon and Rao 2012, Wang *et al.* 2016). For the biological approaches, only a few microorganisms are known that can degrade lignin, though it is a rather time and energy consuming process. For instance, both White and Brown Rot fungi can decompose lignin via oxidative actions with

specialised metalloenzymes (Brown *et al.* 2014). Also some bacteria can break down the lignin polymers by using haem peroxidases but they have a lower oxidative power than the fungi (Brown *et al.* 2014). Most of these enzymes work extracellularly (Zeng *et al.* 2014) but the use of these enzymes is not commercially profitable because obtained enzyme levels are low and the enzymes are not active under extreme conditions (*e.g.* extreme pH and temperature) (Ruiz-Dueñas and Martínez 2009). However, all the proposed pre-treatments often produce polluting by-products, demand a lot of energy and time and/or make the processing of lignocellulosic biomass very expensive (Zeng *et al.* 2014, Singhvi *et al.* 2014, Park *et al.* 2012).

The **enzymatic hydrolysis** of the biomass into sugars can be optimised by selecting the most beneficial ratios of the enzymes (cellobiohydrolases-I, endo-1,4-β-glucanases, β-glucosidases and xylanases).

The **fermentation** is dependent on the enzymes of the used yeast, mostly *Saccharomyces cerevisiae*. Normally this species only uses hexoses, hence can only convert glucose, though transgenic strains can also use pentoses, whereby also hemicelluloses are available for conversion, making it more efficient in converting all available sugars. Another obstacle in the fermentation are the inhibitory products present due to the pre-treatment steps (Wang *et al.* 2016). Again, this points out the need to reduce the pre-treatments steps.

Because there exists no good alternative to sufficiently remove the lignin, **the use of modified plants with lower lignin or lignin that is easier degradable becomes an attractive solution** (Zeng *et al.* 2014, Blanch 2012, Vanholme *et al.* 2008).

1.3 Genetically modified plants for lignin content or composition

Both the **amount and composition of lignin** are identified as two influencing factors on the digestibility of lignin and consequently the sugar release upon enzymatic hydrolysis of the sugar polymers in biomass (Marriott *et al.* 2016).

A strong correlation between **lignin content** and saccharification efficiency was found using 20 *A. thaliana* mutants in 10 different genes of the monolignol biosynthesis pathway (Van Acker *et al.* 2013). A strong correlation between lignin content and saccharification efficiency was also demonstrated in alfalfa

down-regulated for 6 lignin biosynthetic genes (Chen and Dixon 2007). In contrast, the cellulose content seemed a less important factor determining saccharification efficieny based on research in *A. thaliana* and poplar plants (Van Acker *et al.* 2013, Van Acker *et al.* 2014). Hence, genetically modifying plants by down-regulating enzymes involved in the monolignol biosynthesis and consequently reducing the lignin content of cell walls is an appealing strategy to make sugars more accessible and pre-treatment steps less required.

Moreover, lignin **composition** and structure partily determine saccharification efficiency (Van Acker *et al.* 2013). An extra methoxy group means less reactive sites (Ziebell *et al.* 2010). The S units are the most methoxylated resulting in a blocked 5-position giving fewer 5-5 and β-5 linkages which are highly stable. Therefore, it is speculated that for instance in lignin molecules rich in S units more linear chains, showing less cross linkages, are formed (Studer 2011). Hence, increasing the **S/G ratio** can elevate the sugar that is released and the S/G ratio is an important parameter in the assessment of biomass (Ziebell *et al.* 2010, Li *et al.* 2003, Giordano *et al.* 2014). However, the S/G ratio is not always reported to have a big influence on saccharification (Van Acker *et al.* 2013, Davison *et al.* 2006).
Next to the S and G units, also **H units** can influence digestibility (Ziebell *et al.* 2010). Normally, H units are present at low levels although in for instance *A. thaliana cse* mutants the H units increased tremendously along with the cellulose to glucose conversion (Vanholme *et al.* 2013). H units may stop the polymerisation process resulting in shorter chains hence more easily extractable lignin. This is because the H radical is less stable during the polymerisation process because it has no methoxy groups (Sundin *et al.* 2014).
Additionally, more than 160 plant metabolites have been defined as possible **alternative lignin monomers** that can theoretically be incorporated into the lignin polymer. These non-conventional lignin monomers (*e.g.* ferulic acid) may influence the degradability of the lignin polymer (Vanholme *et al.* 2012b, Ralph *et al.* 2008).

The lignin content and S/G ratio might be regulated independently (Li *et al.* 2003). Although in most cases **both content and composition are altered**

upon genetic modification of genes in the monolignol pathway and effects cannot be separated (Marriot *et al.* 2016, Vanholme *et al.* 2008). All together, **these genetically engineered plants can have a great potential in reducing the costs of bioethanol production and can reduce pre-treatment steps** (O'Connel *et al.* 2012, Van Acker *et al.* 2014).

However, there are some issues inherent to the lignin modification. Plants have a great **ability to adapt to lower concentrations of lignin and a compositional shift of the different monolignols**, even without visible effects at plants level. Nevertheless, when lignin is reduced below a certain threshold, negative effects on plant growth and development can be observed. In order to survive, a minimum of lignin is needed (Zeng *et al.* 2014, Vanholme *et al.* 2008). Moreover, a problem with down-regulation of the monolignol biosynthesis genes is the simultaneous influence on other necessary **metabolites** (*e.g.* flavonoids, suberin, coumarin, lignan, tannins, hydroxycinnamates, anthocyanins, stilbene, *etc.*) which are also produced in side branches of the phenylpropanoid pathway (Eudes *et al.* 2014, Vanholme *et al.* 2008, van der Rest *et al.* 2006, Hu *et al.* 2011). The attenuation of lignin can also affect the **integrity of vessels** which can cause dysfunction of the vascular system and even collapse of the vessels (Eudes *et al.* 2014, Vanholme *et al.* 2008).

In our study we focus on *A. thaliana* in which at least 13 different genes are involved in the production of monolignols (Vanholme *et al.* 2012, Vanholme *et al.* 2013, Van Acker *et al.* 2013, Marriott *et al.* 2016) (Fig 1.2). A collection of 2 independent mutant alleles is available for 11 of these monolignol biosynthesis genes. Taking into account the practicality of the experiments, a selection of maximum 2 genes was possible hence we will also focus in the introduction on these 2 selected genes: ***4-COUMARATE:COA LIGASE 1* (*4CL1*) and *CINNAMOYL COA REDUCTASE 1* (*CCR1*)** (Fig 1.2 red boxes).

1.3.1 *4CL* down-regulation

The enzyme **4-COUMARATE:COA LIGASE** (4CL, EC 6.2.1.12) is involved in the general phenylpropanoid pathway (Xu *et al.* 2011) (Fig 1.2). It is the branch

point enzyme that channels the intermediates into specific monolignols and flavonoids (Gao *et al.* 2012). Recently, it has been reported that caffeoyl shikimate esterase (CSE) together with 4CL can bypass the second HCT reaction (Vanholme *et al.* 2013) (Fig 1.2). The 4CL enzyme uses 4-coumarate as main substrate, and forms 4-coumaroyl CoA thioester via a two-step ATP and Mg^{2+} dependent mechanism.

Several **isoforms** of 4CL have different substrate specificities and can also (next to 4-coumarate) use sinapinic acid, ferulic acid, 5-hydroxyferulate, caffeic acid or trans-cinnamic acid as a substrate (Raes *et al.* 2003, Yuan *et al.* 2014b, Li *et al.* 2015, Costa *et al.* 2005). These different 4CL isoforms are differentially transcribed in various organs and tissues and have a different time point of expression during development (Anterola and Lewis 2002, Hu *et al.* 1998). The *Arabidopsis thaliana* genome contains 14 *4CL* genes: 4 different isoforms of 4CL and 10 4CL like genes (Raes *et al.* 2003, Costa *et al.* 2005) which show different substrate specificities. Both At4CL1 and At4CL2 convert 4-coumarate while only At4CL1 is able to convert ferulate (Ehlting *et al.* 2001) and At4CL4 is the only 4CL enzyme that can activate sinapic acid (Li *et al.* 2015). At4CL3 is thought to be more associated with flavonoid biosynthesis (Ehlting et *al.* 2001, Li *et al.* 2015). Therefore, 4CL enzymes are classified into 2 groups: the class I enzymes involved in lignification and the class II enzymes involved in the **metabolism of other phenolic compounds** (Gui *et al.* 2011). The overall **expression** of *4CL* in *A. thaliana* seems highest in bolting stems, where large amounts of lignin accumulate (Lee *et al.* 1997). *4CL3* (class II) is expressed in various cell types while *4CL1* and *4CL2* (class I) are expressed in lignifying cells (Ehlting *et al.* 1999). 4CL1 is mostly involved in developmental lignification though 4CL1 or 4CL2 activity is necessary for normal plant growth (Li *et al.* 2015).

The down-regulation of *4CL* has been investigated using different plant species and techniques to down-regulate the activity of several 4CL isoforms. Suppression of 4CL in the **gymnosperm softwood tree** *Pinus radiata* shows a reduced lignin content though also displays a dwarf phenotype and collapsed water conducting elements (Wagner *et al*. 2009).

Angiosperm monocot species *Sorghum bicolor* (Saballos et al. 2012), switchgrass (*Panicum virgatum*) (Xu *et al.* 2011) and rice (*Oryza sativa*) (Gui *et*

al. 2011), down-regulated for *4CL*, show lower 4CL activity and reduced lignin contents. No growth effect was observed for Sorghum or Panicum though the *4CL* down-regulated rice showed a shorter plant height. No clear pattern in monolignol units was displayed since (i) in sorghum the subunits were not influenced, (ii) in switchgrass the G units decreased and (iii) in rice the G units increased. The **saccharification efficiency** of switchgrass was significantly enhanced due to the down-regulation in *4CL* (Xu *et al.* 2011).

Furthermore, **angiosperm dicot species** were studied. Holy basil (*Ocimum sanctum*) wherein a transient suppression was performed showed no effects due to the short down-regulated time (Ragstoni *et al.* 2011). Additionally, down-regulated *4CL* tobacco plants (*Nicotiana tabacum)* were obtained, resulting in plants with normal and brown xylem tissue (Kajita et al. 1996, Kajita *et al.* 1997a, Kajita *et al.* 1997b, Huan *et al.* 2012). The brownish tissues were reduced in lignin content and displayed lower S units. The brown colour was present due to linkage of *p*-coumaric, ferulic and sinapic acid to the cell wall with ester and ether linkages. Some lines, showed a dwarf phenotype.

Also ***Arabidopsis thaliana*** plants down-regulated for *4CL* were reported, mainly resulting in plants with a reduced lignin content (Lee *et al.* 1997, Van Acker *et al.* 2013, Vanholme *et al.* 2012a, Sanchez *et al.* 2006). Although no decrease in lignin content was present in *4cl2* mutants (T-DNA knockout mutants), decreases in lignin content were demonstrated in *4cl1* mutants (Van Acker *et al.* 2013, Vanholme *et al.* 2012a). Increases in S and decreases in G units, resulting in an increased S/G ratio, were reported for *4CL* down-regulated plants (Lee *et al.* 1997). Also a higher S and lower G units content (higher S/G ratio) was demonstrated for Arabidopsis *4cl1* (T-DNA knockout) mutants though again no effects were observed for *4cl2* mutants (Lee *et al.* 1997, Van Acker *et al.* 2013). No growth effect was observed due to the lower 4CL capacity (Lee *et al.* 1997, Van Acker *et al.* 2013, Vanholme *et al.* 2012a) though Sanchez *et al.* (2006) observed smaller stem growth and abnormal leaf shapes of *A. thaliana*. Ferulic acid and derivates accumulated in *4cl1* mutants and no real increase in cellulose content was observed in *4cl1* mutants of *A. thaliana* (Vanholme *et al.* 2012a, Van Acker *et al.* 2013). The *4cl1* mutants showed a **higher cellulose to glucose conversion** than wild type plants and these mutants were more

susceptible to acid pre-treatment with an effect of 36-76% more glucose yield than wild type (Van Acker *et al.* 2013).

Studies with **angiosperm hardwood trees** reduced for 4CL capacity in silver birch (*Betula pendula*) (Sutela *et al.* 2014), aspen *(Populus tremuloides)* (Li *et al.* 2003, Hu *et al.* 1999, Hancock *et al.* 2007, Roque-Rivera *et al.* 2011), Chinese white poplar *(Populus tomentosa*) (Tian *et al.* 2013, Jia *et al.* 2004), black cottonwood (*Populus trichocarpa*) (Min *et al.* 2012) and poplar *(Populus tremula x Populus alba)* (Voelker *et al.* 2010, Voelker *et al.* 2011, Kitin *et al.* 2010, Zhou *et al.* 2015) have also been published.
Reductions in lignin were observed for several of these *4CL* down-regulated trees (Li *et al.* 2003, Hu *et al.* 1999, Hancock *et al.* 2007, Roque-Rivera *et al.* 2011, Jia *et al.* 2004, Min *et al.* 2012, Zhou *et al.* 2015). Besides, field-grown poplars were reduced in lignin content in the study of Voelker *et al.* (2010) and for 5 of 14 tested field-grown lines of poplar in the study of Kitin *et al.* (2010). The *4CL* down-regulation resulted in an increase in cellulose content for several of these tree species (Li *et al.* 2003, Hu *et al.* 1999, Voelker *et al.* 2010) though a normal cellulose content was observed by Hancock *et al.* (2007) in aspen lines. No influence on S/G ratio was noted in some studies due to the *4CL* down-regulation (Hu *et al.* 1999, Hancock *et al.* 2007, Roque-Rivera 2011). In other studies, increases in S were reported in field-grown *4CL* down-regulated Chinese white poplar (Tian *et al.* 2013) and small increases in S/G ratio, though S units were highly variable, were observed in a field trial by Voelker *et al.* (2010). However, Zhou *et al.* (2015) reported a decrease of the S/G ratio.
No growth penalty was observed in many studies (Li *et al.* 2003, Hu *et al.* 1999, Jia *et al.* 2004) and in a field trial, using 1-year-old Chinese white poplar plants, stem diameter and plant height even increased for *4CL* down-regulated plants (Tian *et al.* 2013). On the contrary, in a field trial using *4CL* down-regulated poplar trees, 1 on 3 plants showed a reduced growth. Moreover, collapsed vessels could be observed and non-collapsed vessels were filled with phenolics (Kitin *et al.* 2010). Red-brownish coloured spots were observed in Chinese white poplar reduced for its 4CL activity (Jia *et al.* 2004) and patchy brown or reddish brown colour co-occurred with physiological abnormalities in *4CL* down-regulated poplars (Voelker *et al.* 2010). These brown spots showed a more

condensed lignin as a lower H+G+S content was released by thioacidolysis. Zhou *et al.* (2015) reported a transgenic line with uniformly brownish discolouration. These lines were uniformly down-regulated for the 4CL activity, which is mostly not the case in down-regulated trees. In the field trial of Voelker *et al.* (2010) no increase in ethanol yield of pre-treated wood was observed. However, total sugar yield was increased for *4CL* down-regulated black cottonwood (Min *et al.* 2012).

Anterola and Lewis (2002) plotted **different plant types** reduced in 4CL activity and concluded that the activity of 4CL needed to be reduced further than 60% to get a significant reduction in lignin content. This suggests that in wild type plants, more 4CL activity is provided than necessary.

1.3.2 *CCR* down-regulation

The first step of the monolignol specific pathway is executed by **CINNAMOYL COA REDUCTASE** (CCR, E.C.1.2.1.44) (Raes *et al.* 2003). It converts the cinnamoyl-CoA esters into cinnamaldehydes (Raes *et al.* 2003) in a NADPH-dependent conversion (Costa *et al*. 2003, Hu *et al.* 2011) (Fig 1.2).
CCR has an essential role in the monolignol biosynthesis in angiosperms (Wagner *et al.* 2013). *Arabidopsis thaliana* comprises 2 *CCR* genes and 9 *CCR*-like genes (Costa *et al.* 2003): AtCCR1 is involved in developmental lignification while AtCCR2 is involved in lignification in stress conditions especially pathogen responses (Lauvergeat *et al*. 2001). Also the maize genome contains 2 *CCR* genes though eucalyptus, poplar, tobacco, sugarcane and loblolly pine only display one single *CCR* gene (Boudet *et al.* 2000, Anterola and Lewis 2002, Shi *et al.* 2010). The expression of the different **isoforms** is dependent on developmental stage, organ and tissue (Costa *et al.* 2003).

Many species from several plant subdivisions are addressed in the research concerning the suppression of CCR capacity causing a **reduced lignin content and altered lignin composition**. The *CCR* down-regulated **gymnosperm softwood trees** Norway spruce (*Picea abies*)(Wadenback *et al.* 2008) and *Pinus radiata* (Wagner *et al.* 2013) showed reduced lignin content and less H and G

units. Also *CCR* down-regulated plants of the **angiosperm monocot** species maize (*Zea mays*) (Tamasloukht *et al.* 2011, Park *et al.* 2012) and dallisgrass (*Paspalum dilatatum*) (Giordano *et al.* 2014) were reported. The latter demonstrated a decrease in G units resulting in an increase in the S/G ratio and an enhanced digestibility. Furthermore, the saccharification efficiency of the *CCR* down-regulated line of the **angiosperm dicot** alfalfa (*Medicago sativa*) was significantly improved, again coinciding with an increased S/G ratio (Jackson *et al.* 2008). Further, tobacco (*Nicotiana tabacum*) had been frequently used in the study of *CCR* down-regulation (Piquemal 1998, Chabannes *et al.* 2001, Ralph *et al.* 1998, Dauwe *et al.* 2007, Ralph *et al.* 2008, Prashant *et al.* 2011, O'Connell *et al.*2002). Also in this plant species, an increased S/G ratio due to a decrease in G units was observed (Piquemal 1998, O'Connell *et al.*2002, Chabannes *et al.* 2001). Van der Rest *et al.* (2006) down-regulated *CCR* in tomato (*Solanum lycopersicum*). This species also exhibited a reduced lignin content, though this study was rather focussed on the secondary plant metabolites possessing antioxidant capacity.

The model plant ***Arabidopsis thaliana,*** down-regulated for *CCR,* was also used in many studies: the first *A. thaliana* lignin-reduced mutant for *CCR* was the irregular xylem 4 *irx4* mutant (Jones *et al.* 2001, Patten *et al.* 2005, Laskar *et al.* 2006). Further, also an antisense strategy (Goujon *et al.* 2003), T-DNA insertion mutants (Mir Derikvand *et al.* 2008, Ruel *et al.* 2009, Vanholme *et al.* 2012a, Van Acker *et al.* 2013, Gul and Whalen 2013) and mutants with a microRNA expressed against *CCR* (Smith *et al.* 2013) were used. Significant decreases in lignin content though identical S/G ratios compared to WT plants were observed in the mature stem stages indicating a delayed lignification pattern. This was accompanied with a later increase of phenolics in the *CCR* down-regulated plants (Jones *et al.* 2001, Laskar *et al.* 2006, Patten *et al.* 2005). On the contrary, in other studies decreases in S, hence decrease in S/G, and increases in H units were observed in *ccr1* mutants (Van Acker *et al.* 2013, Mir Derikvand *et al.* 2008, Smith *et al.* 2013). Moreover, increases in digestibility of lignin and saccharification with and without acid pre-treatment were reported (Goujon *et al.* 2003, Van Acker *et al.* 2013). Additionally, studies were performed using **angiosperm hardwood trees** birch (*Betula platyphylla* × *Betula pendula*) (Zhang *et al.* 2015), aspen (*Populus tremuloides*) (Leplé *et al.*

2007) and poplar (*Populus tremula x Populus alba)* (Van Acker *et al.* 2014). In the field-grown *CCR* down-regulated trees that displayed unequal levels of gene silencing in the study of Leplé *et al.* (2007), a decreased lignin content and a delay in the lignification pattern were reported. Though no significant influence on S/G was observed. Only for a specific line the S/G ratio was lower (Leplé *et al.* 2007). Van Acker *et al.* (2014) reported a field trial conducted in Belgium and France using a short rotation poplar culture. In the Belgian Field trial, ethanol production from the mutant biomass and WT biomass was equal. However, some problems such as unequal levels of gene silencing and growth reduction due to the *CCR* down-regulation are partly responsible for this observation. In the French field trial, one transgenic line delivered a 57% higher ethanol production compared to the WT. Especially in these hardwood trees "the unequal levels of silencing of the *CCR* gene" is still an obstacle in the production of *CCR* down-regulated plants (Leplé *et al.* 2007, Van Acker *et al.* 2014).
In addition, in many of these plant species down-regulated for *CCR*, a more condensed lignin was found as observed by decreased H+G+S levels derived in thioacidolysis assays (O'Connell *et al.*2002, Van Acker *et al.* 2013, Leplé *et al.* 2007).

Several generally observed properties of many above-mentioned *CCR* down-regulated plant species were shown. A generally observed consequence of down-regulation of *CCR* is an **impaired growth** (Ralph *et al.* 1998, Leplé *et al*. 2007, Patten *et al.* 2005, Van Acker *et al.* 2013, Van Acker *et al.* 2014, O'Connell *et al.*2002, Mir Derikvand *et al.* 2008, Dauwe *et al.* 2007, Jones *et al.* 2001, Goujon *et al.* 2003, Chabannes et al. 2001, Prashant et al. 2011, Ruel *et al.* 2009). Also the weight was negatively influenced (Van Acker *et al*. 2013, Van Acker *et al*. 2014). However, *CCR* down-regulated plants without a growth inhibition were also reported such as a maize mutant in the study of Tamasloukht *et al*. (2011) and one line of alfalfa by Jackson *et al.* (2008). Moreover, Smith *et al.* (2013) reported an *Arabidopsis* mutant silenced for monolignol production in the lignifying cells with a reduced lignin content although no growth inhibition and Zhang *et al.* (2015) even demonstrated that *CCR* down-regulated birch lines containing less lignin showed increased growth and fresh weight. Recently, a transgenic plant type of *A. thaliana*,

p3xSNBE:*CCR1* in *ccr1-6* was constructed, with a growth of the stem which is comparable to wild type plants while the lignin content is comparable with the *ccr1-6* mutant (personal communication with De Meester and co-workers). This was achieved by introducing a chimeric gene composed of an artificial vessel-specific promoter (McCarthy *et al.* 2011) fused to the *CCR1* coding sequence. In this way, the *CCR1* expression in the vessels is restored while the plant still lacks *CCR1* expression in other cell types. Additionally, these p3xSNBE:CCR1 in *ccr1-6* mutants show an increased saccharification efficiency in comparison to the wild type.

CCR down-regulated plants are known to be developmentally delayed (Patten *et al.* 2005, Gul and Whalen 2013) and show a **delayed senescence** (Mir Derikvand *et al.* 2008, Prashant et al. 2011). They also show a **reduced seed production** (van der Rest et al. 2006, Mir Derikvand et al. 2008, Jones *et al.* 2001, Gul and Whalen 2013) and need a longer period to produce viable seeds (Gul and Whalen 2013).
Furthermore, **collapsed xylem vessels** are a result of the *CCR* down-regulation in several species (Ralph *et al.* 1998, Patten *et al.* 2005, Van Acker *et al.* 2014, Mir Derikvand *et al.* 2008, Goujon *et al.* 2003, Prashant *et al.* 2011, Ruel *et al.* 2009). Water transportation induces compressive forces and by consequence the vessels of *CCR* down-regulated plants are unable to retain their shape (Piquemal 1998). This dysfunction in the xylem may be the cause of the growth retardation. However, the inhibited growth may rather be caused by secondary effects than effects of lignin down-regulation (Dauwe *et al.* 2007, Mir Derikvand *et al.* 2008, Piquemal 1998, Goujon *et al.* 2003).

As a consequence of the blockage of the CCR step, precursors in the monolignol pathway may accumulate (Leplé *et al.* 2007, Vanholme *et al*. 2012a) and the cinnamic acid:cinnamaldehyde ratios might increase (Leplé *et al.* 2007). Down-regulation of *CCR* results in an **accumulation of** for instance **ferulic acid** (Vanholme *et al.* 2012a, Mir Derikvand *et al.* 2008, Dauwe *et al*. 2007, Giordano *et al.* 2014) and growth problems of the *CCR* down-regulated plants may be due to the accumulation of these phenolic compounds (Goujon *et al.* 2003) as demonstrated for ferulic acid by Xue *et al.* (2015).

Next to the above-mentioned precursors, also different ferulate derivates *e.g.* ferulic acid hexoside, feruloyl malate and feruloyl glucose, vanillic acid glucoside and sinapic acid glucoside can accumulate (Dauwe *et al.* 2007; Leplé *et al.* 2007, Mir Derikvand *et al.* 2008, Wagner *et al.* 2013, Chabannes *et al.* 2001, Jones *et al.* 2001). These products are formed in a **detoxification** attempt. Glycosylation is a pathway to detoxify the accumulating acids such as vannilic acid, sinapic acid and caffeic acid (Leplé *et al.* 2007). The production of ferulic acid hexoside might be an attempt to detoxify the ferulic acid accumulation (Dauwe *et al.* 2007).
However, signalling pathways can also be involved in response to a defective cell wall as observed by PAL up-regulation (Leplé *et al.* 2007, Dauwe *et al.* 2007).

Another route to reduce levels of ferulic acid, or other compounds that accumulate due to an incomplete monolignol biosynthesis, can be their **deposition in the cell wall** (Van Acker *et al.* 2013, Mir derikvand *et al.* 2008, Piquemal 1998). A commonly observed phenomenon in various *CCR* down-regulated plant species is a **brownish colouration** of the xylem (Van Acker *et al.* 2014, Chabannes *et al.* 2001, Park *et al.* 2012, Piquemal 1998, O'Connel *et al* 2002). It is thought that the colour change is due to the oxidation of accumulated phenolic compounds or the incorporation of ferulic acid (FA) into the polymer (Van Acker *et al.* 2014, Park *et al.* 2012). In some non-uniformly down-regulated plant types, this discolouration is associated with a reduction in lignin content (Van Acker *et al.* 2014, Leplé *et al.* 2007). **Incorporation of ferulic acid** in the cell wall can give rise to new thioacidolysis products such as β–O– 4-FA-I, β–O–4-FA-II, and bis-β–O–4-FA units (Leplé *et al.* 2007, Van Acker et al. 2013, Ralph *et al.* 2008, Van Acker *et al.* 2014, Ruel *et al*. 2009) and these new structures can result in new branch points in the lignin polymer (Ralph *et al.* 2008).

Moreover, contradicting observations were reported concerning **cellulose**. Increases in cellulose due to *CCR* down-regulation were reported by Park *et al.* (2012), Prashant *et al.* (2011) and Leplé *et al.* (2007) and, in some studies, were thought to compensate for the reduced lignin content. On the other hand,

decreases in cellulose due to *CCR* downregulation were reported by Jones *et al.* (2001), Van Acker *et al.* (2013) and Vanholme *et al.* (2012a). Furthermore, no effects on cellulose were noticed in field-grown *CCR*-downregulated poplar by Van Acker *et al*. (2014). However, not only cellulose content but also crystallinity of cellulose can influence saccharification. In a study measuring cellulose and lignin levels in a set of 20 Arabidopsis mutants, cellulose content was not correlated with saccharification yield (Van Acker *et al.* 2013).

Moreover, various other plant properties are reported to be influenced by the *CCR* down-regulation. For instance, ascorbate metabolism, stress responses, central carbohydrate metabolism, respiration and photorespiration can be altered (Leplé *et al.* 2007, Dauwe *et al.* 2007). Also, remodelling of hemicelluloses (Leplé *et al.* 2007) and starch (Dauwe *et al.* 2007) were suggested. Furthermore, chlorophyll content can be increased (Dauwe *et al.* 2007).

1.4 *Arabidopsis thaliana* as a model organism for woody plants

Arabidopsis thaliana is known as the standard model plant in biology (Koornneef and Meinke 2010), and was chosen as model organism for woody plants in our investigation. It has a short generation time, small plant size hence limited requirements for growth and it can produce a large number of seeds by self-pollination. *A. thaliana* has a diploid genome containing 5 chromosomes which are fully sequenced (Koornneef and Meinke 2010, The Arabidopsis Genome Initiative 2000, Weigel and Mott 2009). An enormous amount of information (*e.g*. the identity of genes, proteins and metabolites) about this organism is available by many publications and is summarized in a database known as "The Arabidopsis Information Resource" (TAIR) (Koornneef and Meinke 2010).

In addition, *A. thaliana* is also accepted as a good model system to understand secondary xylem formation including lignin biosynthesis, deposition and function in woody plants (Nieminen *et al.* 2004, Goujon *et al.* 2003). Although it is a herbaceous plant with a ground rosette, it develops interfascicular fibres and vessels with secondarily thickened, hence lignified, cell walls rich in G and S units in the inflorescence stem (Ehlting *et al.* 2005, Goujon *et al.* 2003). Especially during more mature life stages, the secondary xylem of *A. thaliana*

closely resembles the anatomy of wood of angiosperm trees (Chaffey *et al.* 2002). Moreover, also the root tissue of *A. thaliana* is able to undergo secondary growth hence secondary xylem formation (Nieminen *et al.* 2004).

An additional advantage is the large collection of genetically modified plants, including lignin-reduced mutants, that are available (Sessions *et al.* 2001, Alonso *et al.* 2003, Tissier *et al.* 1999). These can be ordered via seed stock centres such as the European *Arabidopsis* Stock Centre (NASC) in Nottingham and the *Arabidopsis* Biological Resource Centre (ABRC) in Ohio (Koornneef and Meinke 2010). For instance, 2 independent mutant alleles for 11 different genes of the phenylpropanoid and monolignol biosynthetic pathway have been made homozygous for further study (Van Acker *et al.* 2013, Vanholme *et al.* 2012a, Vanholme *et al.* 2013): phenylalanine ammonia lyase (*PAL1, PAL2*), cinnamate 4-hydroxylase (*C4H),* 4-coumarate:CoA ligase (*4CL1*, *4CL2),* caffeoyl-CoA O-methyltransferase 1 (*CCOAOMT1*), cinnamoyl-CoA reductase 1 (*CCR1),* ferulate 5-hydroxylase 1 (*F5H1*), caffeic acid O-methyltransferase (*COMT)*, cinnamyl alcohol dehydrogenase (*CAD6*) and caffeoyl shikimate esterase (*CSE*). In our study, we selected two independent allelic mutants for 2 genes involved in the monolignol biosynthesis: ***4-COUMARATE:COA LIGASE* (*4CL1*) and *CINNAMOYL COA REDUCTASE* (*CCR1*)** (Fig 1.2 red boxes).

1.5 Marginal lands

The increased demand for biofuel production and the competition with food crops for good arable lands make the approach of using marginal soils to cultivate biofuel crops highly attractive (Schröder *et al.* 2008, Lopez-Bellido *et al.* 2014, Gelfand *et al.* 2013, Moura *et al.* 2010, Remans *et al.* 2012). Next to mechanical stresses (*e.g.* wind and gravity) plants growing on these marginal soils can deal with problems such as salinity, drought, extreme temperatures (cold/heat), high UV, low quality of soil (*e.g.* less nutrients), steep terrains and contamination (*e.g.* organics or **toxic metals**)(Milbrandt and Overend 2009, Ruttens *et al.* 2011, Osakabe *et al.* 2011). Because plants cannot escape these detrimental conditions, different defence mechanisms have evolved. However, in most cases this results in **oxidative stress responses**, accumulation of secondary metabolites (*e.g.* isoflavonoid and other phenolic phytoalexins,

flavonoid glycosides, **lignin** and related phenolic compounds) and consequently reduction in the yield of plants (Osakabe *et al.* 2011, Douglas *et al.* 1987).

However, more research concerning the stress responses of lignin-reduced plants can generate more insight in whether or not these plants are suitable to grow on marginal lands. In this PhD study, we selected cadmium (Cd) as an example for toxic metal contamination and *A. thaliana* was selected as a model organism for woody crops (Nieminen *et al.* 2004). We will investigate various responses of the lignin-reduced plants and their microbiome towards Cd toxicity.

1.5.1 Toxic metal contamination

Metal contamination is a global environmental issue and millions of hectares are affected (Wang *et al.* 2016). For instance, in the Northeast of Belgium and the South of the Netherlands, an area of about 700 km² is contaminated with toxic concentrations of metals (*e.g.* cadmium (Cd), lead (Pb), copper (Cu), zinc (Zn) and arsenic (As)) (Ruttens *et al.* 2011, Vangronsveld *et al.* 1995, Schreurs et al 2011). This contamination is a result of the historical activity of pyrometallurgical zinc smelters. Consequently, the agricultural lands and kitchen gardens cannot be used to cultivate food and feed crops (Ruttens *et al.* 2011). Growing lignocellulosic biomass such as short rotation coppice (SRC) or fast growing tree species (*e.g.* willow or poplar) for biofuel production on these contaminated soils, can be taken into consideration (Ruttens *et al.* 2011, Janssen *et al.* 2015). An extra advantage is the gradual removal of the toxic metals out of the soil. These plants, together with their microbiome, are able to accumulate considerable amounts of toxic metals in their above-ground (and thus harvestable) biomass (Ruttens *et al.* 2011, Kidd *et al.* 2009). Metals cannot be degraded though they can be removed in case the aerial parts are harvested (Lasat 2000), an application called phytoremediation, more specifically phytoextraction (Ruttens *et al.* 2011, Kidd *et al.* 2009). This phytoextraction process is an environmentally sustainable, solar-powered and cost-effective remediation technology to clean up soils (Ruttens *et al.* 2011, Lasat 2000). Moreover, it is almost impossible for such vast and diffusely contaminated areas to apply conventional technologies such as excavation. Hence phytoextraction can be a good alternative (Ruttens *et al.* 2011). Both soil and plant

characteristics are determinative in the success of the phytoextraction process. Soil characteristics such as pH or organic matter influence the trace element availability and mobility and plants characteristics such as tolerance, uptake rate at the roots, accumulation and translocation of metals to the above-ground parts are important factors (Thijs *et al.* 2016, Sessitsch *et al.* 2013, Remans *et al.* 2012, Gong *et al.* 2003). Moreover, long periods of time are required to clean soils by phytoremediation hence production of biofuel crops can be an additional economic value for the use of these lands during the phytoremediation process (Weyens *et al*. 2013, Schreurs *et al.* 2011).

We selected Cd as a model contaminant for our study since in Belgium, as already discussed above, a large part of the Northeast is contaminated with Cd due to the former Zn smelters (Hogervorst *et al.* 2007, Schreurs *et al.* 2011). Of all the trace elements in the contaminated area at the border of Belgium and The Netherlands (*e.g.* Cd, Zn, Pb, Cu and As), Cd is the only one with concentrations exceeding the threshold values for agricultural soils set by the Flemish legislation (Schreurs *et al.* 2011). In addition, Cd is toxic and harmful for human health even at low concentrations, making the remediation of Cd contaminated lands highly essential. Besides, a lot of information about Cd toxicity is available.

1.5.2 Cadmium

Cadmium (Cd) occurs naturally in the environment at relatively low concentrations. Due to weathering of sedimentary rocks, forest fires and eruption of volcanoes, Cd can spread in a natural way (WHO 2000, Dupae *et al.* 2014, Nagajyoti *et al*. 2010). However, anthropogenic activities (*e.g* traffic, waste incinerators, heating systems, cement factories and the use of phosphate fertilizers, pesticides and sewage sludge in agriculture) have led to dispersal of Cd into the environment in concentrations significantly exceeding the emission by natural sources (Gallego *et al.* 2012, Schellingen *et al.* 2014). Many of the activities resulting in Cd contamination were banned by changing to more environmentally friendly production processes. However, still a lot of historical pollution is persistently present (Schreurs *et al.* 2011). Moreover, still a lot of Cd is used especially for the production of nickel–cadmium batteries, and is

released into the environment (Gallego *et al.* 2012). However, in the European Union (EU) Battery Directive (2006/66/EC and 2013/56/EU) it is stated that the use of nickel-cadmium batteries is abandoned in the EU.

Cadmium is a **non-essential, non-redox-active element** (Kovacik *et al.* 2008b, Remans *et al.* 2012) and is one of the most hazardous inorganic contaminants of ecosystems (Dauthieu *et al.* 2009). Even in low concentrations it causes toxicity to human health, animals, plants and microorganisms (Suzuki *et al.* 2001, Dupae *et al.* 2014). Environmental Cd pollution is endangering public health since the element can be inhaled bound on contaminated dust (Hogervorst *et al.* 2007) and it can bio-accumulate into the food chain (Gallego *et al.* 2012). Cadmium is considered a class 1 human carcinogen by the International Agency for Research on Cancer (IARC, 1993) (Cailliatte *et al.* 2009) and it can have deleterious effects for human health, even at trace concentrations (Cuypers *et al.* 2010). Since it accumulates in the body, kidneys, bones, or lungs can be prone to Cd toxicity. Metals cannot be degraded and persist in the environment indefinitely (Rajkumar *et al.* 2009). Therefore, strategies for the clean-up of contaminated soils are necessary.

1.5.2.1 Uptake by plants

Uptake of Cd into the plant is performed at the roots where the negatively charged carboxyl groups of the root create cell wall exchange sites for cations (Dauthieu *et al*. 2009). Cd uptake is dependent on concentration in the soil and several soil characteristics such as pH, texture and organic matter determine the availability of the metal (Gallego *et al*. 2012). However, no specific **transporter** systems for Cd are known (Zhu *et al*. 2013, Dauthieu *et al*. 2009). Cd-ions can take advantage of cation transporters for essential elements with a broad substrate range (Herbette *et al.* 2006). For instance, ZIP (*e.g*. the Fe(II) transporter iron regulated transporter 1 (IRT1) in *A. thaliana*) and natural resistance-associated macrophage protein (Nramp) families of transporters can be used for the enterance of Cd into the root (Clemens and Ma 2016, Mendoza-Cózatl *et al.* 2011). However, most of the plant species retain the largest concentration at their roots although all species translocate some of the Cd-ions to the aerial parts via the xylem (Dauthieu *et al.* 2009).

The movement of Cd to the xylem can be prevented by transport of Cd into the root vacuole. In rice and *A. thaliana* plants, the heavy metal-transporting ATPase 3 (OsHMA3 and AtHMA3) can be involved in the movement of Cd into the vacuole (Clemens and Ma 2016, Mendoza- Cózatl *et al.* 2011). In addition, ABCC1 or 2 transporters in Arabidopsis can transport Cd-ions bound to phytochelatins and can be involved in vacuolar sequestration of Cd (Mendoza-Cózatl *et al.* 2011). Further, the root-to-shoot translocation of Cd in rice is dependent on the HMA2 homolog since these transporters can load the Cd-ions into the xylem. Moreover, also in *A. thaliana* HMA2 and/or HMA4 homologs are involved in xylem loading and may be required for Cd translocation (Clemens and Ma 2016).

1.5.2.2 Toxicity for plants

Several signs of **toxicity** might be visible after Cd exposure such as chlorosis (Van Belleghem *et al.* 2007) as a result of decreases in **chlorophyll content** (Suzuki *et al.* 2001, Cuypers *et al.* 2016), necrosis on the leaves and reduction of growth and biomass (Dupae *et al.* 2014). In addition, stomatal opening, transpiration, photosyntesis and water balance are altered after Cd exposure (Dauthieu *et al*. 2009, Yang *et al.* 2007).

At **cellular level** three main mechanisms of Cd-toxicity are proposed: (i) Cd can **bind to functional groups** since it has a high affinity towards sulphur or nitrogen within proteins of biomolecules and can subsequently inactivate or denaturate them (Villiers *et al.* 2011); (ii) Cd **can compete with other divalent elements** (*e.g.* Fe^{2+}, Mg^{2+}, Ca^{2+}) which act as co-factors in enzymes (and eventually substitute them) and by consequence obstruct their **functionality** (Cuypers *et al*. 2016) but this can also cause **deficiency** (*e.g.* Fe deficiency) due to competition for uptake at the roots since no transporter systems for Cd are known (Kupper *et al.* 2016, Zhu *et al.* 2013); (iii) it may cause imbalances in the production and quenching of **reactive oxygen species (ROS)** via indirect action. For instance, Cd can be detoxified by glutathione and phytochelatins, depleting the glutathione stock. It can stimulate ROS-producing enzymes such as NADPH oxidases (Cuypers *et al.* 2016). However, Cd is not redox reactive itself but can increase free Fe concentration by replacement in

various proteins. The increase in free redox-active metals can enhance ROS production via the Fenton reaction (Cuypers *et al.* 2010).

ROS is a collective name for several products originating from stepwise adding electrons to O_2. The O_2 molecule has two impaired electrons with the same spin quantum number (Demidchik 2015). By consequence, O_2 can accept electrons one at a time forming the superoxide radical ($O_2^{-}\cdot$) by accepting one electron and the peroxide ion (O_2^{2-}), which quickly forms hydrogen peroxide (H_2O_2), by subsequently accepting another electron. This H_2O_2 can be converted into hydroxyl radicals (·OH), which are highly reactive and toxic, via the Fenton reaction (Gill and Tuteja 2010). Next to these negative effects, ROS production can also possess a signalling function for instance in regulation of growth, development and defence pathways. However, in case the production of ROS is imbalanced, ROS and especially hydroxyl radicals can cause damage to proteins, lipids, carbohydrates and DNA (Mittler *et al.* 2004, Halliwell 2006). For instance, in the membrane, one hydroxyl radical can result in peroxidation of many polyunsaturated fatty acids because of a propagating chain reaction (Sharma *et al.* 2012). Peroxidation can lead to increasing membrane permeability due to chain breakage and is considered as the most damaging process to occur in living organisms (Gill and Tuteja 2010).

1.5.2.3 Defence mechanisms

In order to keep plants healthy, ROS and other oxidants need to be in balance with the **antioxidative defence enzymes and metabolites** (Smeets *et al*. 2008). The ROS-related gene network includes at least 152 genes in *A. thaliana* (Gadjev *et al.* 2006). Hence, many enzymes are operable (table 1.1). **Superoxide dismutases (SOD)** can **convert superoxide ($O_2^{-}\cdot$) to hydrogen peroxide (H_2O_2)**. Several classes of SOD are known using different metal cofactors in their active site: copper and zinc in CuZnSOD (plastidic or cytosolic, *CSD1, CSD2* and *CSD3* in *A. thaliana*), iron in FeSOD (plastidic, *FSD1*, *FSD2* and *FSD3* in *A. thaliana*), and manganese in MnSOD (mitochondrial, *MSD1* in *A. thaliana*) (Kliebenstein et al. 1998). The produced **H_2O_2 can be neutralized by catalases (CAT) or peroxidases (PX)** (Mittler *et al.* 2004). **Catalase** is a tetrameric iron porphyrin protein and the catalase gene family includes 3

members (*CAT1*, *CAT2* and *CAT3*) in the *Arabidopsis* genome. These isoforms are differently expressed throughout the lifespan of *A. thaliana*. For instance, *CAT2* expression decreased during senescence while *CAT3* is induced. Moreover, it is known that *CAT* expression can be enhanced by several environmental stresses (Du *et al.* 2008). Further, *Arabidopsis* encodes 73 peroxidases (Passardi et al. 2004) including for instance **guaiacol and syringaldazine peroxidases** (GPX and SPX), which respectively use a common artificial aromatic electron donor (guaiacol) (Vianello *et al.* 1997) and an analogue for lignin monomers (syringaldazine) (Pang *et al.* 1989).

Furthermore, the antioxidants ascorbic acid and glutathione are of great importance and their action is coupled via the ascorbate-glutathione cycle (Mittler *et al.* 2004). **Ascorbate peroxidases (APX)** convert H_2O_2 to H_2O by making use of ascorbate (AA) as reducing agent. Ascorbate can be recovered in a reaction coupled to the oxidation of glutathione (GSH). Levels of glutathione are restored by **glutathione reductase (GR)** (Mittler *et al.* 2004). However, in stress situations these enzymes and several other metabolites can be imbalanced leading to oxidative stress (Jozefczak *et al.* 2014).

Another defence mechanism to reduce metal toxicity is by chelating them in the cytosol (Clemens 2001, Hassinen *et al.* 2011) and transporting them into the vacuole (Anjum *et al.* 2015, Villiers *et al.* 2011). Compounds able to bind metals can be divided into 2 classes: thiol-based (*e.g.* metallothioneins, phyotochelatins) or non-thiol-based compounds (*e.g.* organic acids and amino acids) (Hassinen *et al.* 2011, Anjum *et al.* 2015). **Metallothioneins** are low weight proteins containing cysteine residues (Hassinen *et al.* 2011) and **phyotochelatins** are oligomers of glutathione (Anjum *et al.* 2015). Both classes of compounds can bind Cd since it has a high affinity toward S containing groups. These mechanisms are involved in metal homeostasis and tolerance to high concentrations of essential metals (Leszczyszyn *et al.* 2013). Moreover, their activity can have an impact on metal translocation to the aerial parts (Haydon and Corbett 2007). In addition, for instance organic acids such as phenolic compounds can chelate metals and scavenge ROS (Kovacik *et al.* 2008a, Pietta 2000, Pereira *et al.* 2009, Chaoui *et al.* 2005). These phenolic acids (*e.g.* sinapinic acid) can accumulate in Cd-exposed plants (Michalak *et al.* 2006, Sun *et al.* 2010).

To coordinate the plant defence, cells have evolved mechanisms to signal stress. These mechanisms operate in particular at their cell membranes since it is the first barrier (Chmielowska *et al.* 2014). For instance, **lipoxygenases (LOX)** can cause dioxygenation of polyunsaturated fatty acids generating hydroperoxy fatty acids. These products can activate downstream signalling via other molecules such as jasmonates or oxylipins (Smeets *et al.* 2008, Mochizuki *et al.* 2016). Another example are NADPH oxidases (*e.g.* **respiratory burst oxidase homologs (*RBOH*)** in *A. thaliana*). Here, $O_2^{-}\cdot$ is produced in the apoplast, which can convert spontaneously or by SOD activity to H_2O_2 (Liu and He 2016). H_2O_2 serves as signal molecule because of its relative stability (Mhamdi *et al.* 2010). *Arabidopsis* encodes 10 RBOH genes with different functions (Torres and Dangl 2005).

Table 1.1: Summary of the action of antioxidative enzymes and their corresponding genes included in our study design.

Annotation	Enzyme	Gene	Reaction
Superoxide dismutase	SOD	*FSD1* *CSD1* *CSD2*	$O_2^{-}\cdot + O_2^{-}\cdot + 2H^+ \rightarrow H_2O_2 + O_2$
Ascorbate peroxidase	APX	*APX1* *APX2*	$2Asc + 2H_2O_2 \rightarrow 2MDHA + 2H_2O$
Glutathione reductase	GR	*GR1* *GR2*	$GSSG + NAD(P)H \rightarrow 2GSH + NAD(P)^-$
Catalase	CAT	*CAT1* *CAT2* *CAT3*	$2H_2O_2 \rightarrow 2H_2O + O_2$
Guaiacol peroxidase	GPX	/	$Guaiacol_{(red)} + H_2O_2 \rightarrow guaiacol_{(ox)} + H_2O$
Syringaldazine peroxidase	SPX	/	$Syringaldazine_{(red)} + H_2O_2 \rightarrow Syringaldazine_{(ox)} + H_2O$

1.5.2.4 Cell wall response to Cd

Increased lignification due to biotic and abiotic stress factors is a well-known defence response (Moura *et al.* 2010, Osakabe *et al.* 2011, Yang *et al.* 2007). Moreover, lignification can influence the plant tolerance to Cd. For instance, overexpression of *CSE*, a gene involved in the monolignol pathway, made plants more tolerant to Cd (Gao *et al.* 2010). In line with this observation, many

Table 1.2: Studies concerning lignin visualisation and lignin content after Cd exposure. Abbreviations: h: hours, d: days, m: months.

Plant species	Cd salt	Concentration	Exposure duration	Response	Technique	Reference
			Roots			
Camellia sinensis	$Cd(NO_3)_2$	63 µM and 106 µM	45 d	↑	Spectrophotometrically	Zagoskina *et al.* 2007
Hordeum vulgare	$CdCl_2$	1 mM	72 h	↑	Microscopy	Valentovicova *et al.* 2009
Phragmites australis	$CdSO_4$	100 µM	21 d	↑	Microscopy	Ederli *et al.* 2004
Matricaria chamomilla	$CdCl_2$	3, 60 and 120 µM	7d	↑ from 60 µM	Thioglycolic acid reaction	Kovacik *et al.* 2008a and 2008b
Vicia sativa	$CdCl_2$	10, 25 and 50 µM	7 or 10 d	↑	Acetyl bromide and microscopy	Rui *et al.* 2016
Populus x canescens	$CdSO_4$	50 µM	24 d	↑	Acetyl bromide	Elobeid *et al.* 2012
Glycine max	$CdCl_2$	1 µM	48 h	↑	Thioglycolic acid reaction	Yang *et al.* 2007
Trifolium repens	$CdCl_2$	5 and 10µM	28 d	↑ from 10 µM	Method van soest *et al.*	Lambrechts *et al.* 2014
Pinus sylvestris	$CdSO_4$	5 and 50 µM	96 h	↑ from 50 µM	Thioglycolic acid method	Schutzendubbel *et al.* 2001
Zea mays	$Cd(NO_3)$	50 and 100 µmol	4d	↑/=	Microscopy	Lux *et al.* 2011
Matricaria chamomilla	$CdCl_2$	60 µM	7d	↑	Thioglycolic acid reaction	Kovacik *et al.* 2009 and 2011
Citrus paradisi	$CdCl_2$	5 and 10 µM	7 d	↑	Microscopy	Podazza *et al.* 2012
Oryza sativa	$CdCl_2$	150 µM	96 h	↑/=	Thioglycolic acid reaction	Chang *et al.* 2012
Glycine max	$CdCl_2$	5, 10, 15, 20 and 25 mg l^{-1}	48 h	↑	Thioglycolic acid reaction	Pawlak-Sprada *et al.* 2011
Glycine max	$CdCl_2$	25 to 100 mM	24h	↑	Thioglycolic acid reaction and Alkaline nitrobenzene oxidation	Finger-Teixeira *et al.* 2010
Zea mays	$CdCl_2$	100 mM	6 d	↑	Acidic hydrolysis	Schreiber *et al.* 1999
Triticum aestivum	$Cd(CH_3COO)_2$	1 mM	1d	↑	Microscopy	Bezrukova *et al.* 2011
Phragmites australis	$CdCl_2$	2 and 20 mg l^{-1}	2 m	=	Acetyl bromide	da Silva *et al.* 2014b
Juncus maritimus	$CdCl_2$	2 and 20 mg l^{-1}	2 m	=	Acetyl bromide	da Silva *et al.* 2014b
Lolium perenne	$CdCl_2$	25 and 50µM	28 d	=	Method van soest *et al.*	Lambrechts *et al*. 2014
Lupinus luteus	$CdCl_2$	5, 10, 15, 20 and 25 mg l^{-1}	48h	↓	Thioglycolic acid reaction	Pawlak-Sprada *et al.* 2011
Pseudotsuga menziesii	$CdCl_2$	15, 31 and 68 mg kg^{-1}	9 m	↓	Sequential extraction of cell wall constituents (acetic acid)	Astier *et al.* 2014

Plant species	Cd salt	Concentration	Exposure duration	Response	Technique	Reference
Leaves						
Camellia sinensis	$Cd(NO_3)_2$	63 µM and 106 µM	45 d	=	Spectrophotometrically	Zagoskina *et al.* 2007
Elodea nuttallii	$CdCl_2$	500 µg dm^{-3}	7 d	=	Microscopy	Larras *et al.* 2013
stems, bark and wood						
Camellia sinensis	$Cd(NO_3)_2$	63 µM and 106 µM	45 d	↑	Spectrophotometrically	Zagoskina *et al.* 2007
Populus x canescens	$CdSO_4$	50 µM	24 d	↑	Acetyl bromide	Elobeid *et al.* 2012
Phragmites australis	$CdCl_2$	2 and 20 mg l^{-1}	2 m	↑ from 20 mg l^{-1}	Acetyl bromide	da Silva *et al.* 2014b
Juncus maritimus	$CdCl_2$	2 and 20 mg l^{-1}	2 m	↑ from 20 mg l^{-1}	Acetyl bromide	da Silva *et al*. 2014b
Elodea nuttallii	$CdCl_2$	500 µg dm^{-3}	7 d	↑/=	Microscopy	Larras *et al.* 2013
Pseudotsuga menziesii	$CdCl_2$	15, 31 and 68 mg kg^{-1}	9 m	↓	Sequential extraction of cell wall constituents (acetic acid)	Astier *et al*. 2014

studies suggested that an increased lignification of the roots can be associated with a defence mechanism against metal stress (Cuypers *et al.* 2002, Chaoui *et al.* 2005, Yang *et al.* 2007, Kovácik *et al.* 2008b, Finger-Teixeira *et al.* 2010, Schützendübel *et al.* 2001 and many more). For instance, the capacity of enzymes involved in lignification, *e.g.* phenyl ammonia-lyase (PAL) (Pawlak-Sprada *et al.* 2011, Finger-Teixeira *et al.* 2010, Kovacik *et al.* 2008a, Kovacik *et al.* 2008b, Dai *et al.* 2006), many laccases (Yang *et al.* 2007) and cell wall bound peroxidases, can be significantly increased after Cd exposure (Moura *et al.* 2010, Finger-Teixeira *et al.* 2010, Yang *et al.* 2007). Moreover, the signalling of the lignin synthesis due to cell wall damage is ROS dependent (Sharma *et al.* 2012). PAL and phenolics may also have a part in detoxification of Cd-induced oxidative stress (Kovacik *et al.* 2008b). For instance, phenolic metabolites have hydroxyl groups with antioxidative properties (Kovacik *et al.* 2008a).

In table 1.2, the studies reporting lignin measurements after Cd exposure are summarised. Many species are included (although not our model organism *A. thaliana*). Roots are the first organs to encounter metal stress (Tamas *et al.* 2010, Zhu *et al.* 2013). A general tendency of increased root lignification is reported repeatedly (Table 1.2). It is suggested that the loss of antioxidant capacity due to Cd exposure can increase H_2O_2 levels that subsequently can act as signalling molecule to increase lignification (Chaoui *et al.* 2005). However, most of the time relatively high exposure, non-realistic concentrations and short exposure periods were studied and at lower concentration no effects were observed (Table 1.2). Furthermore, two studies reported decreases of lignification in the roots after Cd exposure (Pawlak-Sprada *et al.* 2011, Astier *et al.* 2014) (Table 1.2).

Lignification in the aerial parts after Cd exposure is less studied. Only 2 studies were found for leaves, though no extra lignification was observed in this compartment (Zagoskina *et al.* 2007, Larras *et al.* 2013) (Table 1.2). Further, in stems, increases in lignification were reported (Zagoskina et al. 2007, Elobeid et al. 2012, da Silva et al. 2014b, Larras et al. 2013). However, also for the stem, when lower concentrations were applied, no effects of Cd exposure were observed and even decreases were reported in *Pseudotsuga menziesii (*Astier et al. 2014) (Table 1.2).

The increased lignification after Cd exposure can be **beneficial** or **detrimental** for the plant. Increased lignification, especially in the roots, can be interpreted as a defence reaction to limit the entry of the toxic ions (Bielen *et al.* 2013). The roots apoplastic barriers (*e.g.* Casparian bands) restrict passive transport of ions across the root (Stoláriková-Vaculíková *et al.* 2015). Closer to the root apex, the development of cell walls with lignified secondary xylem elements has been observed after exposure to toxic metals (Stoláriková-Vaculíková *et al.* 2015).
Moreover, the cell wall plays an important role in the uptake of Cd-ions in the roots. The cell wall contains many negative carboxyl groups, which are good binding places for cations (Dauthieu *et al.* 2009, Yang *et al.* 2007, Gallego *et al.* 2012). Therefore, increased synthesis of lignin, hence more binding places for Cd-ions, can also be a manner to immobilise the toxic metal ions at the root level (Kovacik *et al.* 2008b).
On the other hand, in different studies correlations between growth reduction and increased lignificaton are observed since lignin can limit cell expansion and decrease the nutrient uptake (Bielen *et al.* 2013, Fan *et al.* 2006, Finger-Teixeira *et al.* 2010).

1.6 Endophytes

Microorganisms are omnipresent in the biosphere (Lemfack *et al.* 2014, Das *et al.* 2014). They have been present for over 3.8 billion years although it is estimated that still only 1% of all microbes is known (Das *et al.* 2014, Riesenfeld *et al.* 2004).
Eukaryotic organisms provide residence to microorganisms and are influenced by **complex interactions** with these **microbial communities** (Lareen *et al*. 2016). They are termed as the microbiome (van der Heijden and Hartmann 2016). Moreover, most of these microorganisms are **essential for the host organism** (Lemfack *et al.* 2014). For instance, in a human body more microbial cells than human cells are present and these can make up to 2 kg (Lemfack *et al*. 2014). The gut microbiota can affect health and nutritional state (Lareen *et al.* 2016) and these microbes can be influenced by the hosts diet (Lareen *et al.* 2016). The root system of plants, together with the xylem, is crucial for nutrient and water uptake. Similar to the human gut, these plant systems are colonized

by a complex microbial community (Lareen *et al.* 2016, Berg *et al.* 2014). The functions of the plant-associated microbiome are not just the sum of the individual components since it is a complex network in which strong interactions play (van der Heijden and Hartmann 2016).

Several plant niches can harbour plant-associated microorganisms including the exterior rhizosphere and phyllosphere as well as the interior endosphere (Weyens *et al.* 2009c). These microorganisms can colonize the plant through soil, wind, air or water (Berg *et al.* 2014) and can provide **beneficial characteristics** for the **plant health** (Rout *et al.* 2014).

The **rhizosphere** refers to the zone surrounding the roots, which is influenced by root exudates (Rout *et al.* 2014, Weyens *et al.* 2009c). The rhizosphere has a higher water holding capacity and increased nutrient availability compared to the bulk soil making it attractive for microorganisms (Rout *et al.* 2014). These rhizosphere microorganisms can colonise the root hairs to become endophytic (Hardoim *et al.* 2015).

The **phyllosphere** bacteria reside on all external surfaces of the aerial parts (Weyens *et al.* 2009c): on the leaves (phylloplane), on stems (caulosphere), on flowers (anthosphere) and on fruits (carposphere) (Compant *et al.* 2010). They must withstand rapid fluctuations in temperature, solar radiance and water availability (Hardoim *et al.* 2015, Weyens *et al.* 2009c). Phyllospheric bacteria can become endophytic via stomata or wounds (Hardoim *et al.* 2015).

The **endophytic** bacteria are defined to reside inside the host internal tissue in a commensal or beneficial way (Kaul *et al.* 2016, Taghavi *et al.* 2009), without negative influence for the host or causing visible signs of infection (Weyens *et al.* 2009c). These endophytes were considered contaminants for a long time (Berg *et al.* 2014). However they can **interact closely with the host plant** and display beneficial traits (Bulgarelli *et al.* 2013, Turner *et al.* 2013). In this interaction, the endophytes can make use of the photosynthetic products of the plants (Mendes *et al.* 2013) and the endophytes can enhance plant growth and development in several manners (Thijs *et al.* 2016).

Endophytes can colonize the plant via the rhizosphere or phyllosphere (Weyens *et al.* 2009c) or can be vertically transferred from the parent to the next generation through the seeds (Truyens *et al.* 2015). Endophytic bacteria can penetrate the endodermis and reach the xylem vessels, the main transport route to colonise the internal plant compartments (Compant *et al.* 2010). These bacteria can pass through the holes of the perforation plates between xylem elements. However, the transport through the vascular system is a slow process (Hardoim *et al.* 2015) and only few endophytes are able to pass several barriers to enter this route (Compant *et al.* 2010). Endophytes can be strictly bound to plants and reside inside the plant for the entire or a major part of their lifecycle inside the plant (obligate); though, others are mainly found outside the plant and can sporadically enter the plant (opportunistic). In between these two extreme situations also a group of "facultative" endophytes are classified (Hardoim *et al.* 2015). Endophytic bacteria can be **influenced by the environment** due to biotic and abiotic factors (Agler *et al.* 2016). For example, exposure to toxic concentrations of metals can influence the microbial endophytic communities in their composition and activity (Rajkumar *et al.* 2009). It is also known that endophytic communities can be influenced by genetic **variation of the host plants** (*e.g.* ecotypes, cultivars or genetically modified genotypes) (Bulgarelli *et al.* 2012, Beckers *et al.* 2016, da Silva *et al.* 2014a, Haney *et al.* 2015, Pieterse *et al.* 2016, Badri *et al.* 2013, Coleman-Derr *et al.* 2016). For instance, the available C-sources nourishing the endophytes and the cell wall composition can affect the community (Hardoim *et al.* 2008, Bulgarelli *et al.* 2012, Miedes *et al.* 2014). Therefore, a modification in the **lignin content and/or composition may also affect the endophytic communities**.

1.6.1 Phenolic compounds and endophytes

The available **carbon-sources** (C-sources) inside the host plant can give rise to community shifts based on the use of these nutrient sources (Hardoim *et al.* 2008). In lignin-reduced mutants, several C-sources, more specifically phenolic compounds (*e.g.* ferulic acid), are known to accumulate (Vanholme *et al.* 2012a). Moreover, the flavonoid levels can be altered due to a mutation of genes involved in the monolignol biosynthesis pathway since the monolignol and

flavonoid production pathways share the first steps (Ehlting *et al.* 1999). Furthermore, the endophytic communities of *CCR* down-regulated poplars, isolated via selective enrichment with ferulic acid, were demonstrated to more efficiently use ferulic acid as a C-source (Beckers *et al.* 2016).

Phenolic compounds have a common structure with an aromatic ring with one or more hydroxyl substituents. They can be divided into several classes such as phenolic acids, flavonoids, tannins, stilbenes, coumarins and lignans (Akyol *et al.* 2016). Some phenolic compounds can act as signalling molecules (Badri *et al.* 2013). Furthermore, many of the phenolic products of plants are **antimicrobial** agents (Turner *et al.* 2013, Rice-Evans *et al.* 1996, Akyol *et al.* 2016).

1.6.2 Techniques to identify endophytic communities

At present microorganisms can be detected via culture-dependent and independent techniques (Hardoim *et al.* 2008). For both approaches the identification of the community members can be performed based on the 16S rRNA gene which is highly conserved and contains hypervariable regions (Clarridge *et al.* 2004). Other genetic markers such as gyrA, gyrB, rpoA, rpoB, rpoC, rpoD, recA, recN, and ppk1 can also be used for identification (Das *et al.* 2014). Though the **16S rRNA gene** is the most suitable since (i) the gene has the same function in all microorganisms (ii) the sequence is conserved making use of universal primers possible but also contains 8 hyper-variable regions ranging in size from 50-100bp, (iii) the size of approximately 1500 bp is relatively easy to sequence and (iv) the gene contains enough information for identification (Clarridge *et al.* 2004, Rastogi and Sani 2011, Nikolaki and Tsiamis 2013).

Culture-based studies deliver bacterial collections that can be screened for phenotypic traits and can be exploited to improve plant growth by means of inoculation (Weyens *et al.* 2009c). However, it is estimated that only 1% of the total communities can be cultured in laboratory conditions (Riesenfeld *et al.* 2004, Gołębiewski *et al.* 2014) although they are viable in their natural environment (Rastogi and Sani 2011).

By consequence, culture-dependent techniques miss a great part of the endophytic diversity (Turner *et al.* 2013). Therefore, the **total communities**, directly sequenced from environmental samples without the need for cultivation,

can provide more information on the present communities. For this purpose, the next generation sequencing such as the 454 pyrosequencing technique, Illumina or ABI SOLiD can be applied (Das *et al.* 2014, Luo *et al.* 2012, Nikolaki and Tsiamis 2013). However, also the culture-independent techniques can be biased for instance by the DNA extraction, biases associated with the PCR such as the selected primer and they cannot differentiate alive from death cells (Turner *et al.* 2013, Lebeis *et al.* 2014, Rastogi and Sani 2011). Moreover, some researchers claim that the cultivable sub-community is the most active hence most important part of the total community (Gołębiewski *et al.* 2014). Therefore, the best way to approach bacterial communities is to apply both culture-dependent and independent assays (Lebeis *et al.* 2014, Rastogi and Sani 2011).

1.6.3 Plant growth promotion by endophytic bacteria

Endophytic bacteria use several direct and indirect mechanisms to enhance plant growth. These mechanisms can become of even more important in case the plants are under stress, like when they are grown on contaminated land (Kaul *et al.* 2016). Moreover, the beneficial endophytes can be exploited by means of inoculation to **improve yield of energy crops** on marginal lands (Schroder *et al.* 2008).

DIRECT - Biofertilisation

Nitrogen (N) is an essential element for plant development and is often a limiting factor for plant growth (Weyens *et al.* 2009c). Atmospheric nitrogen (N_2) cannot be directly used by plants (Santi *et al.* 2013). Therefore, ammonium or urea is included in most fertilisers (Noguero and Lacombe 2016). Endophytic diazotrophs displaying nitrogenase activity can fix N_2 into ammonium (NH_4^+) and nitrate (NO_3^-) and supply it to the plant (Hardoim *et al.* 2015, Ullah *et al.* 2015). Nitrogenase can be encoded by the Nif genes (Ryan et al. 2008, Laranjo *et al.* 2014) and the enzyme activity can be inhibited by O_2 (Goldberg *et al.* 1987). Diazotrophic bacteria can either form nodules on the root (close associations) or be loosely associated with the host plant (Weyens *et al.* 2009c). Furthermore, nitrate is a signalling molecule for example for lateral root growth, leaf development and flower induction (Noguero and Lacombe 2016). Endophytes

with nitrogenase capacity can decrease the need for chemical nitrogen fertilisers (Laranjo *et al.* 2014).

Iron (Fe) is important for plant growth since it is involved in many enzymatic reactions. However, in soils it often exists in insoluble ferric (Fe^{3+}) forms (*e.g*. as oxides, hydroxides, phosphates and carbonates) (Ma *et al.* 2016). Bacterial siderophores are low molecular weight chelating agents (200-2000 Da) (*e.g.* hydroxamates, catecholates and phenolates) with a high affinity towards Fe^{3+} (Zloch *et al.* 2016). Plants themselves also produce phytosiderophores, though the microbial siderophores have a higher affinity for iron. Moreover, plant roots can also take up the bacterial Fe-siderophore complex (i) via ligand exchange, directly form the Fe^{3+}-siderophore complex and/or (ii) by degradation of the organic chelate (Ma *et al.* 2016). Hence, endophytic bacteria can be used as a Fe fertilisation strategy.

Phosphorous (P) is essential for plant health. For instance, P is necessary for adenosine diphosphate and triphosphate (ATP) hence is involved in energy transfer processes during photosynthesis and P is part of DNA polymers. Soils have large reserves of P, though P is present under a form that is unavailable for plants (Rodríguez and Fraga 1999). P can be solubilised by endophytic bacteria via organic acids or extracellular phosphatases. These bacteria form the monobasic ($H_2PO_4^-$) or dibasic ($H_2PO_4^{2-}$) that can be absorbed by plants (Gopalakrishnan *et al.* 2015). Biofertilisation of P by endophytes is a possible strategy and can reduce the need for traditional phosphorous fertilisers (Weyens *et al.* 2009c).

Moreover, **organic acid** production by microorganisms is also a mechanism to mobilise other essential nutrients (Truyens *et al.* 2015, Weyens *et al*. 2013).

DIRECT - Phytohormone production

It is known that the typical plant hormones indole-3-acetic acid (IAA), cytokinins, gibberellins and volatile organic compounds can also be produced by endophytic bacteria (Hardoim *et al*. 2015). IAA is involved in many plant processes including cell division, root development, apical dominance, cell expansion and plant defence (Osakabe *et al*. 2011). Root elongation by endophytes due to **IAA** production has been proven by Patten and Glick (2002). However, high levels of IAA can cause developmental perturbations (Rout *et al.*

2014). **Also cytokinins** are involved in growth and development of the plant (*e.g.* cell division, root formation and vascular cambium activity) (Osakabe *et al*. 2011). Bacteria can also synthesize cytokinins and in this way increase levels of this plant hormone and consequently influence plant development (Arkhipova *et al*. 2005). **Gibberellins** have a role in the initiation of early flowering, improvement of crop yield and bigger fruit size and can also be produced by endophytes (Khan *et al.* 2014). Also bacterial **volatiles** can affect the hormones of the plant (Blom *et al.* 2011). For instance, organic volatiles can influences the plants signalling via cytokinins, ethylene, auxins, salicyclic acid, brassinosteroids, gibberellins, abscisic acid and jasmonic acid (Ryu *et al.* 2003, Ryu *et al.* 2004, Ryu *et al.* 2005, Cho *et al.* 2008, Bhattacharyya *et al.* 2015, Blom *et al.* 2011). Various volatile substances produced by bacteria such as 1-hexanol, indole, pentadecane and 2,3-butanediol are known (Blom *et al.* 2011, Ryu *et al.* 2003).

DIRECT - Counteracting ethylene production

Ethylene is considered a plant stress hormone. It modulates defence responses due to stress and can inhibit plant development and accelerate senescence (Schellingen *et al.* 2014). Moreover, it is involved in xylem formation (Glick *et al.* 2007). It is synthesised via the **precursor ACC**. Endophytes can produce ACC deaminases which lower the levels of the ACC precursor of ethylene (Belimov *et al.* 2009). ACC deaminase cleaves the ACC into α-ketobutyrate and ammonia (Belimov *et al.* 2005). The ammonia can again be used as a N source (Ma *et al.* 2016). Therefore, these endophytes might reduce the negative impacts of ethylene and by consequence enhance plant growth under stressful conditions (Ma *et al*. 2016).

INDIRECT

Various indirect plant growth promoting mechanisms are known. **Suppressing the phytopathogen activity** can result from a competition for space and nutrients with phytopathogens (Weyens *et al.* 2009c, Ma *et al.* 2016). Via production of siderophores, endophytes can also outcompete pathogens by reducing the available Fe for these organisms (Weyens *et al.* 2009c). Also via production of antibiotics or hydrolytic enzymes (chitinases, proteases and

glucanases), pathogens can be unable to grow (Ma *et al.* 2016). Moreover, induced systemic resistance (ISR) can be achieved leading to a higher tolerance of the plant towards pathogens (Hardoim *et al.* 2015). For instance, volatile bacterial compounds can evoke ISR (Ryu *et al.* 2004). ISR resembles the systemic acquired resistance (SAR), which results from pathogen infection. Both mechanisms are an induced resistance wherein the plant defence is preconditioned by the previous infection (Choudhury and Panda 2007).

1.6.4 Growth promotion after Cd exposure

Certain endophytes may **alleviate metal toxicity** or **alter metal uptake** by enhancing the metal availability.

Metal phytotoxicity can negatively influence plant health and growth. Therefore, the toxicity needs to be diminished to improve plant growth and make phytoremediation of contaminated soils more successful (Ma *et al.* 2016). Various endophytic mechanisms (*e.g.* efflux or intracellular sequestration such as via glutathione or metallothioneins), aimed to detoxify Cd for the bacteria itself, can reduce the metal phytotoxicity. However, the energy cost for such an efflux system is lower than complexation making the efflux system the main route of detoxification (Nies 1999). The nickel/cobalt/cadmium (ncc) resistance and cobalt/zinc/cadmium resistance (czc) system are well-known bacterial efflux pumps for Cd. The czc system is composed of several subunits and is driven by a proton motive force. CadA, a bacterial P-type ATPase efflux pump can detoxify Cd mainly in gram-positive bacteria (Nies 1999). After the efflux of the toxic Cd-ions, precipitation on the membrane takes place by binding to the hydroxyl, carbonyl, carboxyl, sulfhydryl, thioether, sulfonate, amine, amide, and phosphonate groups (Ma *et al.* 2016). Due to this, the toxic metals are less available for both the bacteria themselves, but also for their host plants. Moreover, bacteria are usually numerous, have a high surface to volume ratio and they display a net negative charge on the cell envelope making them capable to accumulate metal cations (Haferburg and Kothe 2007).

Endophytes may also influence the capacity of antioxidative enzymes (CAT, SOD, GPX, APX) of the plant inducing **defence mechanisms** (Ma *et al*. 2016).

Metals entering the endophytic cell can be sequestered and translocated into, for example, the vacuole. The use of plants to clean a soil from metal contamination (phytoremediation/phytoextraction) can be limited by the **availability of the metal** in the soil (Weyens *et al*. 2009b). The bioavailability is soil dependent (particle size, nutrients, pH, redox potential, organic matter content, *etc.*) but can be enhanced by endophytes. For instance, the production of siderophores or organic acids can make Cd more available for plants (Rajkumar *et al.* 2009). Organic acids can enhance the availability of Cd and nutrients by increasing the soluble forms (Weyens *et al.* 2013). Also biosurfactants (*e.g.* mycolic acid, glycolipids, lipopeptides, polysaccharide protein complexes, phospholipids, fatty acids) produced by endophytes can enhance metal bioavailability. These biosurfactants can desorb metals from the soil matrix making them more mobile. These endophytic strategies can **enhance Cd** uptake into the plant (Ma *et al.* 2016).

To remove the toxic metals out of the soil by phytoextraction, the metals need to be **translocated** from the root to the aerial parts. This process can also be enhanced by endophytes with a sequestration system (Weyens *et al.* 2009c).

In an ideal case, we can make use of **promising bacterial endophytes to inoculate the lignin-reduced mutants** in order to **improve plant growth** and **increase Cd uptake** while **Cd toxicity is counteracted**. In this way, biomass production on marginal lands could become more efficient and at the same time these contaminated soils could get remediated by phytoextraction.

SECTION 2
OBJECTIVES

Increasing energy demands, global warming and depletion of fossil fuels emphasize the necessity to move towards renewable energy sources (Van Acker *et al*. 2014, Ragauskas *et al*. 2006). However, **biofuel** (*e.g.* bioethanol) production, and especially biofuel from the first generation (*e.g.* corn, maize, sugarcane), is often competing with food production for available land, which is an unfavourable situation (Weyens *et al*. 2009c, Naik *et al*. 2010). Production of second generation biofuels derived from **lignocellulosic biomass** (*e.g.* poplar, switchgrass, *Miscanthus sp*., and others) is a valuable alternative to meet the increasing energy demands (Van Acker *et al*. 2014) without infringing on food production since it can be grown on marginal lands (*e.g.* land diffusely contaminated with **cadmium (Cd)** and other metals) (Singh *et al.* 2011a). Moreover, since plants sequester CO_2, the use of biofuels instead of fossil fuels might have a positive impact on CO_2 emission (Naik *et al*. 2010). However, lignocellulosic biomass contains relatively high fractions of **lignin** polymers, which are very recalcitrant and lower the enzymatic conversion efficiency of the biomass into fermentable sugars. Genetically modified crops with reduced lignin content or more easily degradable lignin molecules can be a partial solution to overcome this problem (Marriott *et al*. 2016).

In this PhD dissertation, we will work with three different aspects and the interaction between these factors: (A) *Arabidopsis thaliana* as model organism as well as lignin-reduced *A. thaliana* mutants, (B) cadmium as exposure and (C) bacterial endophytes.

(A) *Arabidopsis thaliana*: wild type and lignin-reduced mutants

A. thaliana is often used as a model organism for woody plants (Nieminen *et al*. 2004). Moreover, the information that is acquired based on research on *A. thaliana* can, to some extent, be extrapolated to more economically valuable species (*e.g.* poplar). Firstly, we selected two types of lignin-reduced mutants, which are inhibited in their monolignol production pathway and consequently contain less lignin or lignin molecules that are more easily cleavable (Van Acker

et al. 2013). The first type has a blockage (T-DNA knockout mutants) in the *4-COUMARATE:CoA LIGASE 1* gene (AT1G51680), encoding an enzyme of the general phenylpropanoid pathway. The second type is down-regulated (T-DNA knockout mutants) in the *CINNAMOYL-COA-REDUCTASE 1* (AT1G15950) gene, encoding the first enzyme of the monolignol specific branch of the monolignol pathway (Vanholme *et al.* 2012a). The selected *4cl1* mutants (*4cl1-1* (SALK_142526) (Alonso *et al.* 2003) and *4cl1-2* (SM_3_27345) (Tissier *et al.* 1999)) do not display a particular phenotype while the *ccr1* mutants (*ccr1-6* (GABI_622C01) (Sessions *et al.* 2002) and *ccr1-3* (SALK_123689) (Alonso *et al.* 2003)) are inhibited in their growth. Moreover, the lignin content in the stems of the *ccr1* mutants is more decreased than that of *4cl1* mutants (Vanholme *et al.* 2012a, Van Acker *et al*. 2013) and may in this way be of great importance. In some experimental set-ups, p3xSNBE:*CCR1* in *ccr1-6* is included which has a similarly decreased lignin content as *ccr1-6* mutants but without growth inhibition due to the fact that *CCR1* expression is restored in the vessels while the plant still lacks *CCR1* expression in other cell types (McCarthy *et al.* 2011, personal communication with De Meester and co-workers). We will address several **objectives at the level of the plant** as discussed below.

(B) Cadmium

Another essential choice for our study is the contaminant, for which the non-essential metal Cd is selected because in Belgium, more specifically the Northeastern part, a huge area is diffusely contaminated with Cd due to the historical activities of zinc smelters (Hogervorst *et al*. 2007).

(C) Bacterial endophytes

In this PhD dissertation, since bacterial endophytes are essential for the plants health (Berg *et al*. 2014, Hardoim *et al*. 2015) and can be applied to try to improve planth growth (Beckers *et al.*, *in preparation*), we address several **objectives at the level of the endophytic bacterial communities**.

Objective 1 and 2

Our **first objective** (subsection 3.2) is to characterise the **growth** of the selected lignin-reduced mutants with and without Cd exposure and the **second**

objective (subsection 3.2) is to verify if the **accumulation of Cd and other elements** is changed due to the mutation of the selected genes of the monolignol biosynthesis pathway.

It is known that the growth of some lignin-reduced mutants is inhibited (*e.g ccr1* mutants) while this is not the case for others (*e.g 4cl1* mutants) (Van Acker *et al*. 2013, Vanholme *et al*. 2012a). However, no information is available about their responses to toxic amounts of Cd. We hypothesize that these lignin-reduced mutants may react differently in their Cd accumulation and growth inhibition after Cd exposure since due to the changes in the composition of their cell walls also the potency to block Cd-ions from entering the plant cells can be affected. Moreover, since Cd can compete with essential elements for entering the cells, elemental accumulation can also be disturbed (Suzuki *et al.* 2001). Furthermore, because we will make use of a hydroponic cultivation system, it is essential to study the growth in non-exposed conditions as well.

Objective 3

The **third objective** (subsection 3.3) is to investigate several **stress responses** associated with reactive oxygen species (ROS) production which may operate in a different manner in the lignin-reduced mutants in the non-exposed condition. Furthermore, these same stress responses may be differently working under Cd exposure of the lignin-reduced mutants in comparison to wild type plants.

Cd exposure can strongly increase the cellular production of reactive oxygen species (ROS) in a non-redox reactive manner and can consequently "switch on" several defence mechanisms (Cuypers *et al.* 2011). Moreover, several links between ROS production and lignin can be made. The polymerisation of monolignols is a free-radical coupling process (Vanholme *et al.* 2008). Furthermore, in lignin-reduced mutants a variety of phenolic compounds are accumulating (Vanholme *et al.* 2012a) and these compounds can possess antioxidant capacity (Rice-Evans *et al.* 1996). We hypothesize that in both non-exposed and Cd-exposed situations, the cellular defence mechanisms in lignin-reduced mutants can be activated in another way.

Objective 4

The **fourth objective** (subsection 3.4) is to examine **lignification** in non-exposed and Cd-exposed conditions.

Lignin-reduced mutants are designed to have a lowered lignin content, which was already demonstrated for the *A. thaliana* stem compartment (Vanholme *et al.* 2012a). Furthermore, the lignin composition in lignin-reduced mutant *A. thaliana* stems is well studied (Van Acker *et al.* 2013). Nevertheless, it is essential to investigate these parameters again for the specific conditions of our hydroponics cultivation system with and without Cd-exposure. Moreover, no information is available yet on the lignification of roots, which are the first entry site for contaminants such as Cd-ions, of these lignin-reduced mutants. In addition, it has been demonstrated that for wild type plants root lignification can be triggered (Kovacik *et al.* 2008a) and lignin composition can also be altered under Cd-exposed situations (Finger-Teixeira 2010). However, since lignin-reduced mutants are inhibited in their ability to produce lignin, we hypothesize that lignin-reduced mutants will be able to a lesser extent (in comparison to wild type plants) to increase lignification after Cd exposure.

Objective 5

The **fifth objective** (subsection 4.2 and 4.3) is to characterise the **endophytic communities** in various plant parts (roots, leaves, stems and seeds) of the lignin-reduced mutants. Moreover, the effect of Cd exposure on these communities is studied. Both the culture-independent (total) and culture-dependent (cultivable) communities are addressed.

It is recognised that plants live in constant interaction with endophytic bacteria that are essential for the plants' health and growth (Berg *et al*. 2014, Hardoim *et al*. 2015) and genetic variation has been reported to impact the plant microbiome (Bulgarelli *et al*. 2012, Beckers *et al*. 2016b, da Silva *et al*. 2014a, Haney *et al*. 2015, Pieterse *et al*. 2016, Badri *et al*. 2013, Coleman-Derr *et al*. 2016). Also the plant physiology may be altered upon the presence of microorganisms (Agler *et al*. 2016). We hypothesize that the mutation of genes in the monolignol pathway can affect the endophytic communities of the lignin-reduced mutants (i) by accumulation of several phenolic compounds acting as a C-source for microorganisms (Beckers *et al*. 2016b) or exhibiting antimicrobial

action (Rice-Evans *et al.* 1996), (ii) by differences in cell wall composition inducing different colonisation processes (Bulgarelli *et al*. 2012) and (iii) by the vascular system which may collapse, especially in *ccr1* mutants, and form a barrier for further translocation of endophytes (Mir derikvand *et al*. 2008). Next to the plant genotype, also Cd exposure may affect the endophytic communities (Truyens *et al*. 2013, Truyens *et al*. 2016a, Croes *et al*. 2013). However, it is not known to which extent this will happen in lignin-reduced mutants.

Objective 6 and 7

The **sixth objective** (subsection 4.4) is to understand the selection of several growth promoting traits, Cd tolerance and use of phenolic compounds as a C-source by the isolated endophytes. The **seventh** and last **objective** (subsection 4.5) is to **counteract the negative effects on the growth** of both the *ccr1* mutation and Cd exposure by enriching promising endophytes selected based on their traits examined during the sixth objective.

Endophytic communities may include strains with promising characteristics such as plant growth promoting traits (*e.g.* production phytohormones, reduction of ethylene production or making more N, Fe and P available to the host plant) and Cd tolerance (Weyens *et al*. 2009c). Moreover, it can be hypothesized that bacterial strains possessing the ability to use ferulic acid as a C-source, may decrease the negative impact of ferulic acid on plant growth (Beckers *et al*. 2016b) since ferulic acid accumulation is assumed to be responsible for the growth inhibition in the *ccr1* mutants (Xue *et al*. 2015). Hence, it can be expected that promising endophytes may reduce the growth inhibition due to the mutation of genes of the monolignol synthesis pathway and/or Cd exposure.

Since endophytes evolve fast in their host plant, this can result in selection of various endophytes in the community (Hardoim *et al*. 2015). The selection of endophytes was demonstrated for several phenotypic traits under selective pressure of toxic amounts of metals (Truyens *et al*. 2013, Croes *et al*. 2013). Hence, we hypothesize that both the mutation of genes involved in the monolignol biosynthesis pathway, resulting in a lower lignin content, and Cd contamination can affect the presence of bacterial strains which display various plant growth promoting traits and Cd tolerance.

SECTION 3:
EFFECTS ON THE PLANT

The growing world population gives rise to an increasing demand for energy. Because the stocks of traditional fuels (*e.g.* oil, gas, coal) are limited (Ragauskas *et al.* 2006), **renewable biofuels** made from plant biomass (*e.g.* bioethanol) can contribute to a solution to this problem. However, also food supply is a growing problem and production of biofuels should not compete with food production. Therefore, lignocellulosic plants (*e.g.* poplar, switchgrass, *Miscanthus*, *etc.*) grown on marginal and contaminated soils (*e.g.* **cadmium (Cd)** contaminated soils), which cannot be used for food production, can minimise the competition (Singh *et al.* 2011). However, in the lignocellulosic biomass, lignin is an obstacle during the conversion of the biomass into biofuel. This problem can be partially overcome by making use of **lignin-reduced genetically modified plants** (Van Acker *et al.* 2014).
Within the perspective to cultivate commercially valuable lignocellulosic plants with reduced lignin content on Cd-contaminated lands, a lot of knowledge still needs to be gained about how these lignin-reduced plants will cope with Cd stress.

To study this, *Arabidopsis thaliana* is used as a model organism for woody plants (Nieminen *et al.* 2004) and knockout mutants for two different genes in the monolignol production pathway were selected. **The first selected gene, the *4-COUMARATE:CoA LIGASE 1* gene (AT1G51680),** is expressed during developmental lignin biosynthesis and the corresponding enzyme is involved in the formation of CoA esters of for instance *p*-coumaric acid (Vanholme *et al.* 2012a) and caffeate (Vanholme *et al.* 2013) in the general phenylpropanoid pathway. It is the branch point enzyme that channels the intermediates into specific lignin monolignols and flavonoids (Ehlting *et al.* 1999). *A. thaliana* possesses 4 isoforms of the 4-coumarate-CoA ligase (4CL1, 4CL2, 4CL3 and 4CL4), but 4CL1 is most involved during lignin biosynthesis (Li *et al.* 2015) and therefore selected in our study. The 2 available mutant alleles, *4cl1-1* (SALK_142526; Alonso *et al.* 2003) and *4cl1-2* (SM_3_27345; Tissier *et al.*

1999), were included because they show a 25% lower lignin content while they do not demonstrate a visible phenotype (no growth reduction) (Table 3.1).

Table 3.1: Selected *A. thaliana* mutants and their characteristics of growth (final height of the inflorescence stem), lignin content (measured by the acetyl bromide assay) and cellulose conversion in comparison to WT plants as (approximately) reported by Van Acker *et al.* (2013), Vanholme *et al.* (2012a) and personal communication with De Meester and co-workers).

Gene	AGI-code	Mutant allel	Growth (relative to WT)	Lignin (relative to WT)	Cellulose conversion (relative to WT)
4CL1	AT1G51680	*4cl1-1*	100%	75%	138%
		4cl1-2	100%	75%	132%
CCR1	AT1G15950	*ccr1-6*	65%	50%	349%
		ccr1-3	20%	50%	284%
		p3xSNBE:*CCR1* in *ccr1-6*	100%	55%	380%

Secondly, CINNAMOYL-COA-REDUCTASE 1 (AT1G15950) is the first enzyme of the monolignol specific branch of the pathway and is expressed during developmental lignin biosynthesis (Vanholme *et al.* 2012a, Mir Derikvand *et al.* 2008). The CCR enzyme catalyzes the conversion of cinnamoyl-CoA esters to their respective cinnamaldehydes. *A. thaliana* contains 2 *CCR* genes, *CCR1* and *CCR2*, and 5 *CCR*-like genes (Raes *et al.* 2003). While CCR1 is more involved in developmental lignin biosynthesis, CCR2 is related to stress-induced lignification (Lauvergeat *et al.* 2001). Two mutant alleles, *ccr1-6* (GABI_622C01; Sessions *et al.* 2002, Mir Derikvand *et al.* 2008) and *ccr1-3* (SALK_123689; Alonso *et al.* 2003, Mir Derikvand *et al.* 2008) are available. Although both mutants are known to have a significant developmental delay, they are interesting because their lignin content is strongly diminished (by 50%) in comparison to the wild type (Table 3.1). Hence *CINNAMOYL-COA-REDUCTASE 1* is the second selected gene for our study.

Moreover, **another transgenic plant type, p3xSNBE:*CCR1* in *ccr1-6*** *(*or abbreviated as *ccr1-6*/SNBE throughout our study*),* was constructed in the lab of Prof. Dr Wout Boerjan (VIB, Department of Plant Systems Biology) by Barbara De Meester with *ccr1-6* as origin. She introduced a chimeric gene composed of an artificial vessel-specific promoter (McCarthy *et al.* 2011) fused to the *CCR1* coding sequence to restore the *CCR1* expression in the vessels while the plant

still lacks *CCR1* expression in other cell types (personal communication with De Meester and co-workers). It has already been demonstrated that the growth of these plants is restored to the level of wild type plants while the lignin content is still as low as that of the non-complemented *ccr1-6* mutant (Table 3.1). Moreover, these plants show an increased saccharification efficiency in comparison to the wild type.

By making use of the selected *A. thaliana* mutants, our study highlights some first attempts to understand several effects, going from macroscopic (*e.g* plant growth) to microscopic (*e.g.* lignin coloration assays) and even molecular levels (*e.g.* gene expression levels), of the **genetic modification** of genes involved in the monolignol biosynthesis, resulting in a **reduced lignin content**, and **Cd exposure**.

First, the **growth** of the plant will be followed up during its complete life cycle. Also the fresh weight of the plants will be taken into account (Subsection 3.2). Furthermore, since Cd can influence the uptake and translocation of other essential micro- and macroelements (Suzuki *et al.* 2001) and no knowledge is available concerning element accumulation in the lignin mutants, also the **concentrations of Cd**, **micro- and macroelements** were investigated (Subsection 3.2).

Secondly, both the alteration of lignin content and Cd exposure can have an influence on **stress-related defence reactions associated with ROS production**. First of all, monolignol coupling is a free-radical coupling process (Vanholme *et al.* 2008) and interruption of the monolignol production can give rise to accumulation of phenolic compounds (Vanholme *et al.* 2012a), which can possess antioxidant capacity (Rice-Evans *et al.* 1996). Further, Cd administration is known to give rise to oxidative stress responses (involving reactive oxygen species (ROS)) in a non-redox reactive way (Cuypers *et al.* 2011). Moreover, toxic metals can be detoxified by different chelators (Clemens 2001). Good markers to investigate these stress responses are: pigment concentration, lipid peroxidation, capacity of enzymes with antioxidant functions and expression of genes involved in different ROS-related processes (hallmark

genes for oxidative stress, genes corresponding to anti- and pro- oxidative enzymes, genes corresponding to metal chelation and genes corresponding to enzymes involved in stress induced lignification) (Subsection 3.3).

Thirdly, stem and root lignification of the lignin mutants are investigated (Subsection 3.4). Although stem lignification of *A. thaliana* plants was already subject of several earlier studies (Vanholme *et al.* 2012a, Van Acker *et al.* 2013), the lignin concentration and composition still needs to be examined for our study design. Moreover, no reports are available on lignification in the roots in these lignin-reduced mutants. Furthermore, lignification can be induced by abiotic stresses including Cd exposure (Kovacik *et al.* 2008a). Withal, no extra stress induced lignin is desired since both **lignin content and composition** affect the saccharification and by consequence biomass processing efficiency (Van Acker *et al.* 2013). Therefore the knowledge about these parameters can provide initial but important indications concerning the potential of using Cd-contaminated lands for the cultivation of lignin-reduced crops.

Subsection 3.1
Materials and methods used in the plant section

3.1.1 A closer look into the plants

3.1.1.1 Meet the lignin-reduced mutants

Different *Agrobacterium* transferred DNA (T-DNA) insertion mutants of the *Arabidopsis thaliana* Col-0 ecotype with reduced lignin production, were selected from the collection from the European Arabidopsis Stock centre (NASC) and donated by the lab of Prof. Boerjan (VIB, Department of Plant Systems Biology). In our study, *4-COUMARATE:COA LIGASE 1* (*4CL1*) and *CINNAMOYL-COA REDUCTASE* 1 (*CCR1*)) for which two allelic mutants were available (*4cl1-1* and *4cl12*, *ccr1-6* and *ccr1-3*) were included. Also **p3xSNBE:*CCR1* in *ccr1-6*** *(*or abbreviated as *ccr1-6*/SNBE throughout our study*),* in which *CCR1* expression in the vessels is restored while the plant still lacks *CCR1* expression in other cell types by making use of an artificial vessel-specific promoter (McCarthy *et al.* 2011) fused to the *CCR1* coding sequence, was used in our study.

3.1.1.2 Genotyping of lignin-reduced mutants

To identify the homozygous mutant lines, the DNA was extracted from the wild type and transgenic plant leaves by using the Edwards extraction (Edwards *et al.* 1991). The first step was grinding the tissue in liquid nitrogen in a Retch Mixer Mill MM2000 (Retch, Haan, Germany) followed by adding 400 µl extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). After centrifugation (12 min, 16.110 g), the supernatant was precipitated using isopropanol, washed with 70% ethanol and air-dried. The dry DNA pellet was re-suspended in 50 µl nuclease-free water and used for the PCR reaction (Promega GoTaq™ Flexi DNA Polymerase kit, Madison, WI, USA). In a 25 µl reaction: 5 µl of green go taq flexi buffer, 1 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 µM of the appropriate forward and reverse primer (see Table 3.1.1), 1.25 U of the Go Taq polymerase and 1 µl of the DNA template were combined.

Table 3.1.1: Primers to prove the absence of the gene and presence of T-DNA in the transgenic lines as reported by Vanholme *et al.* (2012a) and personal communication with De Meester and co-workers.

Prove absence of	**Forward primer**	**Reverse Primer**	**Amplicon length in WT**
4CL1 in 4cl1-1	5′-CGCCACAAGAACAAGCAGTTT-3′	5′-TGAGTTTGGTGTTGGAGGCTT-3′	417 bp
4CL1 in 4cl1-2	5′-TGCCGAAGTTTGAGATCAATCTG-3′	5′-AATATGTCATCTGAGTAGCCAAA-3′	438 bp
CCR1 in ccr1-6	5′-TTGTTTTGATTGACAATTTGGA-3′	5′-GGGATTAGATAACGTCACGACA-3′	387 bp
CCR1 in ccr1-3	5′-CCGGTCTCAAGGTACTCGTC-3′	5′-GGATCATGGGACCAATTCAC-3′	395 bp
CCR1 in p3xSNBE:*CCR1* in *ccr1-6*	5′-TTGTTTTGATTGACAATTTGGA-3′	5′-GGGATTAGATAACGTCACGACA-3′	387 bp
Prove presence of	**Forward primer**	**Reverse Primer**	**Amplicon length in mutant**
T-DNA in *4cl1-1*	5′-CGCCACAAGAACAAGCAGTTT-3′	5′-GCCCTTTGACGTTGGAGTC-3′	340 bp
T-DNA in *4cl1-2*	5′-TTTCAGTAAGAGTGTGGGGTTTT-3′	5′-AATATGTCATCTGAGTAGCCAAA-3′	342 bp
T-DNA in *ccr1-6*	5′-TTGTTTTGATTGACAATTTGGA-3′	5′-ATATTGACCATCATACTCATTGC-3′	411 bp
T-DNA in *ccr1-3*	5′-CCGGTCTCAAGGTACTCGTC-3′	5′-CTGGCGTAATAGCGAAGAGG-3′	606 bp
T-DNA in p3xSNBE:*CCR1* in *ccr1-6*	5′-TTGTTTTGATTGACAATTTGGA-3′	5′-ATATTGACCATCATACTCATTGC-3′	411 bp
SNBE elements in p3xSNBE:*CCR1* in	5′-TTGCTTCAAAGCCAGCAAG-3′	5′-GTGACGCAGACGGTTTTTC-3′	265 bp

The PCR setup contained following cycling conditions: initial denaturation at 98°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C (for the *4cl1-2* and *ccr1-6* primer pairs) or 55°C (for the *4cl1-1* and *ccr1-3* primer pairs) for 30 s and extension at 72°C for 1 min. Afterwards a final extension step at 72°C for 1 min was performed.
The presence/absence of a DNA sequence of the desired gene or T-DNA insertion was explored by making use of gel electrophoresis (1.5% agarose gel, 1 kb plus ladder Invitrogen, 90 V for 2 h).

3.1.2 A closer look into the plant growth systems

Throughout this PhD project, 3 main plant cultivation systems were used: vertical agar plates, the Arasystem and the hydroponic cultivation system (Fig 3.1.1). Below the 3 systems are further discussed and an overview of all different experimental set-ups is provided in table 3.1.2.

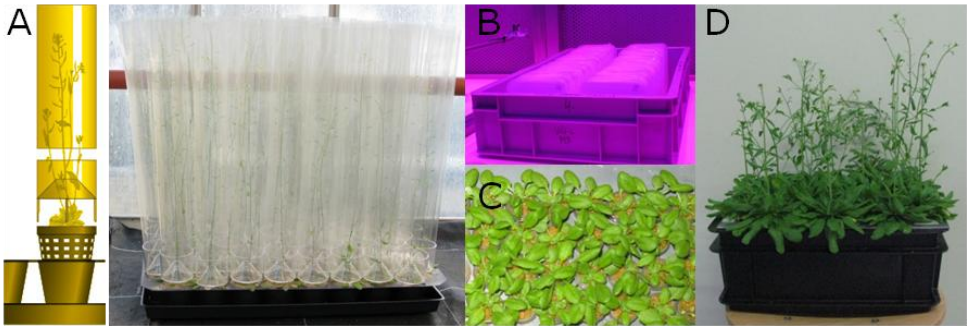


Figure 3.1.1: Representative pictures of the different growth systems (A) Arasystem for seed production, (B) vertical agar plates, (C) hydroponics system of *Arabidopsis thaliana* plants grown for 3 weeks, (D) hydroponics system for the cultivation of plants with an inflorescence stem.

3.1.2.1 Vertical agar plates

The first growth system is the vertical agar plate (VAPs) (Fig 3.1.1B) containing growth medium with agar which are used to monitor root growth (Table 3.1.2).
To set up VAPs, seeds were first surface sterilised with 0.1 NaOCl supplemented with 0.1% Tween 80 for 1 min. After thoroughly rinsing with distilled water, seeds were air-dried (Truyens *et al.* 2013). In order to obtain homogeneous germination, the dry seeds were incubated for 48 h at 4°C. By making use of a sterile toothpick, the seeds were transferred to 12 by 12 cm transparent dishes

(Remans *et al.* 2006) containing 40 ml 50-fold diluted Gamborg's B5-medium (Zhang and Forde 1998) with 1% agar. The medium composition was made up of macronutrients (0.5 mM KNO_3, 0.02 mM $MgSO_4 \cdot 7H_2O$, 0.02 mM $CaCl_2 \cdot 6H_2O$, 0.022 mM NaH_2PO_4, 0.94 µM $MnSO_4 \cdot H_2O$ and 0.02 mM $(NH_4)_2SO_4$); micronutrients (90 nm KI, 0.97 nm H_3BO_3, 0.14 nM $ZnSO_4 \cdot 7H_2O$, 2 nM $CuSO_4 \cdot 5H_2O$, 20.6 nM $Na_2SO_4 \cdot H_2O$ and 2.6 nM $CoSO_4 \cdot H_2O$); 3.6 µM $FeCl_3$; 2.56 nM 2-(N-Morpholino)ethanesulfonic acid hydrate (MES) and 15 mM sucrose. After performing a range finding experiment (paragraph 3.2.2.1) with concentrations of 3 and 5 µM $CdSO_4$, plates with and without 3 µM $CdSO_4$ were used. Plates were placed upright in a growth chamber (day/night temperatures of 22/18°C, 12 h photoperiod, 65% relative humidity and photosynthetic active radiation (PAR) of 170 µmol m^{-2} s^{-1}) equipped with blue, red and far-red Philips GreenPower LED modules to mimic the photosynthetic spectrum of sunlight. Optimas6 (Media Cybernetics, Rockville, MD, USA) was used to analyse primary root length (Truyens *et al.* 2013).

3.1.2.2 Arasystem

The Arasystem was used to produce seeds. Furthermore, lignification of roots and element concentration by the plants were studied using the Arasystem (Table 3.1.2).

Seeds of the selected genotypes were sown on Araflats of the Arasystem (Betatech, Gent, Belgium) containing quartz sand with a particle size of 0.4-0.8 mm. The Aratrays were filled and refilled with a modified Hoagland solution with macronutrients (505 µM KNO_3, 150 µM $Ca(NO_3)_2 \cdot 4H_2O$, 10 µM $NH_4H_2PO_4$ and 100 µM $MgSO_4 \cdot 7H_2O$); micronutrients (4.63 µM H_3BO_3, 0.91 $MnCl_2 \cdot 4H_2O$, 0.03 µM $CuSO_4 \cdot 5H_2O$, 0.06 µM $H_2MoO_4 \cdot H_2O$ and 0.08 µM $ZnSO_4 \cdot 7H_2O$) and 1.64 µM $FeSO_4 \cdot 7H_2O$ in 0.81 µM Na_2-EDTA. Plants were cultivated under greenhouse conditions (Fig 3.1.1A). At the moment of inflorescence formation, leaves were collected for genotyping and Arabases with Aratubes were installed to prevent cross-contamination and half of the trays were exposed to 3 µM $CdSO_4$, which was added to the nutrient solution. Once the plants were overblown, adding of nutrient solution and consequently also $CdSO_4$ exposure were stopped. The

plants containing the seeds were left to dry before collecting seeds. The total seed yield was weighed and the number of seeds per mg was determined.
In case the growth system was used to study lignification of roots and element determination, plants were grown for 1 week where after they were exposed via the roots to 3 µM $CdSO_4$ during 3 more weeks.

3.1.2.3 Hydroponics cultivation system

The third, and most used system throughout the study, is the hydroponics cultivation system. This cultivation system was used to follow up growth phases, lignification of the plant, lipid peroxidation, pigment concentration, capacities and gene expression of stress related enzymes (Table 3.1.2).

Seeds were surface-sterilised for 1 min with 0.1% NaOCl (supplemented with 0.1% Tween 80) and thoroughly rinsed with distilled water (Truyens *et al.* 2013). To ensure a homogenous germination, an incubation step of 48 h at 4°C was implemented. Seedlings were grown in a hydroponics system as optimised by Smeets *et al.* (2008), except that quartz sand with a particle size of 0.4-0.8 mm was used instead of Rockwool to support the seedlings (Fig 3.1.1C and D). The system contained a modified Hoagland solution with macronutrients (505 µM KNO_3, 150 µM $Ca(NO_3)_2 \cdot 4H_2O$, 100 µM $NH_4H_2PO_4$ and 100 µM $MgSO_4 \cdot 7H_2O$); micronutrients (4.63 µM H_3BO_3, 0.91 $MnCl_2 \cdot 4H_2O$, 0.03 µM $CuSO_4 \cdot 5H_2O$, 0.06 µM $H_2MoO_4 \cdot H_2O$ and 0.08 µM $ZnSO_4 \cdot 7H_2O$) and 1.64 µM $FeSO_4 \cdot 7H_2O$ in 0.81 µM Na_2-EDTA. A photoperiod of 12 h (otherwise deviations from the photoperiod are indicated) with 65% relative humidity was used and temperatures were 22°C during daytime and 18°C in the night. The spectrum of photosynthetic active radiation (PAR) of sunlight was mimicked by making use of the combination of blue, red and far-red led modules (Philips GreenPowed LED, 170 µmol m^{-2} s^{-1} at rosettes level). After 1 week of growth, seedlings were aerated at the level of the roots to avoid hypoxia.

The used Cd concentration was selected based on a range-finding experiment wherein plants were cultivated in hydroponics during 1 week in a non-exposed condition where after seedlings were exposed by adding 0, 3, 5, 7 or 10 µM $CdSO_4$ at their roots during 2 weeks (paragraph 3.2.2.1).

Table 3.1.2: Combination of growth systems, exposure times and genotypes.

Subsection	Experiment	[Cd] (µM $CdSO_4$)	Start exposure	Time of exposure	Extra information	WT	*4cl1-1*	*4cl1-2*	*ccr1-6*	*ccr1-3*	p3xSNBE:*CCR1* in *ccr1-6*
Vertical agar plates (VAPs)											
3.2	Range finding Cd concentration	0, 3 and 5	Day 7	2 weeks	/	x					
3.2	Growth phases	0 and 3	Day 0	2 weeks	same age	x	x		x	x	
Arasystem with sand											
3.2	Growth phases seed yield, Cd concentration	0 and 3	Stem formation	Until overblown	Same age	x	x	x	x	x	
3.2	Cd concentration, Element analysis	0 and 3	Day 7	3 weeks	Same age	x	x	x	x	x	x
3.4	Lignin staining roots sections (saffranine)	0 and 3	Day 7	3 weeks	Same age	x			x	x	

Subsection	Experiment	[Cd] (µM $CdSO_4$)	Start exposure	Time of exposure	Extra information	WT	*4cl1-1*	*4cl1-2*	*ccr1-6*	*ccr1-3*	p3xSNBE:*CCR1* in *ccr1-6*
Hydroponics											
3.2	Range finding Cd concentration	0, 3, 5, 7 and 10	Day 7	2 weeks	/	x					
3.2	Growth phases	0 and 3	Day 7	± 7 weeks	Same age	x	x		x	x	
3.3	Enzyme capacity, Gene expression (Real Time PCR), Pigment concentration	0 and 3	Day 7	2 weeks	Same age	x	x	x	x	x	
3.2	Cd concentration	0 and 3	7 days	2 weeks	Same rosette diameter	x	x	x	x	x	
3.4	Lignin concentration (AcBr), Lignin composition (Thioacidolysis), Lignin staining stem sections (Wiesner, Maule), Lignin staining of roots (phloroglucinol)	0 and 3	Stem formation	2 weeks	/	x					
3.2 3.3 3.4	Cd concentration, Lipid peroxidation (TBA), Lignin concentration (AcBr), Lignin composition (Thioacidolysis)	0 and 3	Stem formation	2 weeks	Same stem length	x	x	x	x	x	
3.2	Cd concentration	3	Day 7	2 weeks	Different light conditions (8 weeks 9 h and 2 weeks 16 h) Same age	x	x		x	x	
3.2	Cd concentration	3	Week 8/ stem formation	2 weeks	Different light conditions: (8 weeks 9 h and 2 weeks 16 h) Same age	x	x		x	x	

In case tissues of younger plant (roots and leaves) were needed, half of the plants of 1 week old (deviations from this growth system are indicated) were exposed to 3 µM Cd during 2 weeks where after they were harvested. In case plants with inflorescence were needed, plants were exposed to Cd from the start of stem formation onwards during 2 weeks where after harvest took place. Entire root systems and rosettes were sampled whereas from the inflorescence stem the bottom part starting from 1 cm up to 10 cm was harvested. For both growth systems, the modified Hoagland solution was refreshed twice a week to maintain nutrient availability and pH as constant as possible (Smeets *et al.* 2008). With every refreshment, the $CdSO_4$ concentration was again reset to 3 µM. Hence a rather long-term exposure of 2 weeks to Cd was used for both younger and older plants.

Because of a growth retardation of *ccr1* mutants (Vanholme *et al.* 2012a, Van Acker *et al.* 2013), a hydroponic system with similar developmental stages, wherein *ccr1* mutants are sown earlier (as discussed in paragraph 3.2.2.2C), was used to explore the cultivable and total endophytic communities (section 4), lignification of the plant and lipid peroxidation (as indicated in Table 3.1.2). Nevertheless, for the major part of the subsection on antioxidative defence, plants of the same age were used since age can have a significant effect on the antioxidative system (Moustaka *et al.* 2015). In this case, plants (leaves and roots) were cultivated for 3 weeks where after they were harvested.

3.1.3 Plant parameters under investigation

3.1.3.1 Growth phases

Vertical agar plates (see paragraph 3.1.2.1) were used to assess the early growth phases of the different genotypes with and without exposure to 3 µM $CdSO_4$ from the beginning of the experiment. In the plate-based phenotypic analysis 25 replicates per group were studied. The experimental design contained principal growth phase "zero" (seed germination) and "one" (leaf development), as described by Boyes *et al.* (2001), as well as stage R6 (root development). Hence, the study included following parameters: seed imbibition, radicle, hypocotyls and cotyledon emergence, the opening of the cotyledons, the emergence of rosette leaves (> 1 mm) and root length (Table 3.1.3).

Table 3.1.3: Growth stages included in our study as described by Boyes *et al.* (2001).

Growth stage	**Plate based assay**	**Hydroponics**
Principal growth stage 0	**Seed germination**	/
0.10	Seed imbibition	/
0.50	Radicle emergence	/
0.70	Hypocotyls and cotyledon emergence	/
R	**Root development**	/
R6	More than 50% of the seedlings have a primary root > 6 cm in length	/
Principal growth stage 1	**Leaf development**	**Leaf development**
1.00	Opening of the cotyledons	/
1.xx	Emergence of rosette leaves (> 1 mm) with xx the number of leaves from 02 until 26	
Principal growth stage 3	/	**Rosette growth**
3.20, 3.50, 3.70	/	Rosette growth of respectively 20, 50 and 70%
3.90	/	Rosette growth finished
Principal growth stage 5	/	**Inflorescence emergence**
5.10	/	Inflorescence emergence
Principal growth stage 6	/	**Flower production**
6.00	/	First flower open

In addition, plants were cultivated in a hydroponics system in order to explore differences in the subsequent growth phases of the lignin-reduced mutants in comparison to the wild type with and without Cd exposure. Seedlings were grown for 1 week (as described in paragraph 3.1.2.3) where after half of the plants were exposed to 3 µM $CdSO_4$ for the remaining time of the experiment. From that point the nutrient solution (without or with Cd) was renewed twice a week. In hydroponics, 28 replicates per group were studied. Different growth phases, as described in Boyes *et al.* (2001), were included in our study such as principal growth stages "one" (leaf development), "three" (rosette growth), "five" (inflorescence emergence) and part of "six" (flower production) (Table 3.1.3). To achieve this, leaf development and rosette growth were evaluated nearly daily from the start of the exposure until no additional growth was observed. Furthermore, emergence of the inflorescence, height of the primary inflorescence stem and total number of flowers and siliques were observed. To prevent contamination of the environment with the mutant seedstock, plants were harvested before rupture of the siliques.

Seeds were produced by using the Arasystem (as described in paragraph 3.1.2.2) to prevent contamination of the environment with the mutant seeds and to prevent cross-fertilization.

3.1.3.2 Element determination

During harvest, plant tissues were collected and rinsed in water after which samples were oven-dried at 60°C. Four digestion steps of 4 h at 120°C were applied: 3 with 65% HNO_3 and 1 with 37% HCl. The remainder was dissolved in 20% HCl and diluted to a concentration of 2% HCl. Cadmium concentrations, as well as macro- (Mg, P, Ca, S, K) and micronutrients (Zn, Cu, Mn, Na, Fe) were determined using the inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Agilent technologies 700 Series) (Truyens *et al.* 2014).

3.1.3.3 Enzyme capacities

Snap frozen leaves and roots of younger plants were homogenised by mortar and pestle in pre-cooled extraction buffer (0.1 M Tris-HCl, 1 mM EDTA, 1mM dithiotreitol, pH 7.8) with addition of a little sand and polyvinylpyrrolidon and filtered through a nylon mesh and centrifuged (10 min, 20,000 g, 4°C).

The capacities of catalase (CAT, EC1.11.1.6), glutathione reductase (GR, EC1.6.4.2), superoxide dismutase (SOD, EC1.15.1.1) and peroxidases (EC1.11.1.9) (ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and syringaldazine peroxidase (SPX)) were determined spectrophotometrically in the supernatant (25°C) as indicators for oxidative stress (Chaoui and El Ferjani 2005, Weyens *et al.* 2010).

CAT capacity was determined as the decrease of H_2O_2 at 240 nm. In case of GPX and SPX, capacities were determined by monitoring the formation of respectively oxidized guaiacol at 436 nm and oxidized syringaldazine at 436 nm. The GR capacity was determined by the reduction of GSSG in the presence of NADPH at 340 nm (Bergmeyer *et al.* 1974). By taking the ascorbate oxidation into account at 298 nm, APX capacity was monitored (Imberty *et al.* 1984) and the SOD capacity was determined at 550 nm as an inhibition of the reduction of cytochrome c (McCord and Fridovich 1969).

Afterwards enzyme capacities were calculated using the law of Lambert-beer:

$\Delta C/\Delta t = \Delta A/(\Delta t.\varepsilon.d)$

With ΔC difference in concentration, Δt difference in time, ΔA difference in absorption, ε (extinction coefficient) dependent on the enzyme and d (distance) equal to 1 cm.

3.1.3.4 Gene expression assay

Leaves and roots of younger plants were snap frozen in liquid nitrogen at harvest time. These tissues were grinded using 2 steel balls and the Retch Mixer Mill MM2000 (Retch, Haan, Germany). The total RNA was extracted using the RNaqueous total RNA isolation kit (Ambion, Belgium) and the sample purity and concentration was assessed spectrophotometrically on a Nanodrop ND-1000 (Thermoscientific, Wilmington, DE, USA). Genomic DNA contamination was removed by using the TURBO DNA-free kit (Ambion, Applied Biosystems, Foster City, CA, USA), which contains a DNase treatment. Subsequently single stranded cDNA was made from the treated RNA via the High-Capacity cDNA Reverse Transcription Kit (Ambion, Applied Biosystems, Foster City, CA, USA). In this step equal amounts of 1 µg RNA were added to get a similar concentration of cDNA in all samples. The obtained cDNA samples were $1/10^{th}$ diluted in TE buffer (1 mM Tris-HCl, 0.1 mM Na_2-EDTA, pH 8.0; Sigma Aldrich, Belgium) and stored at -20°C.

Gene-specific mRNA concentrations were determined using the 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Forward and reverse primers (300 nM) were designed and optimised via the Primer Express software (v2.0, Applied Biosystems, Foster City, CA, USA) (Table 3.1.4). Amplification was performed in optical 96 well plates containing a volume of 10 µl containing 2 µl of diluted cDNA sample, 5 µl of 2x Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.3 µl forward and 0.3 µl reverse primer and 2.4 µl RNase free H_2O. "No template controls" were added containing RNase free H_2O instead of cDNA samples. Following cycling conditions were applied: 20 s at 95°C, 40 cycles of 1 s at 95°C and 20 s at 60°C where after a dissociation curve was generated to examine the amplification specificity.

Table 3.1.4: List of genes and corresponding primers used in the real time PCR assay.

AGI	Annotation	Cell location	FW Primer sequences (5'-3') REV Primer sequences (5'-3')
Reference genes			
AT3G18780	*ACT2*	Cytoplasm / nucleus	CTTGCACCAAGCAGCATGAA CCGATCCAGACACTGTACTTCCTT
AT4G26410	/	Mitochondrion / nucleus	GAGCTGAAGTGGCTTCCATGAC GGTCCGACATACCCATGATCC
AT5G25760	*UBC21*	Peroxisome / nucleus	CTGCGACTCAGGGAATCTTCTAA TTGTGCCATTGAATTGAACCC
AT4G34270	TIP41-like protein	Cytoplasm / nucleus	GTGAAAACTGTTGGAGAGAAGCAA TCAACTGGATACCCTTTCGCA
AT5G55840	PPR superfamily protein	Mitochondrion	AAGACAGTGAAGGTGCAACCTTACT AGTTTTTGAGTTGTATTTGTCAGAGAAAG
AT2G28390	SAND family protein	Chloroplast / nucleus	AACTCTATGCAGCATTTGATCCACT TGATTGCATATCTTTATCGCCATC
AT5G08290	*YLS8*	Cytoplasm / nucleus	TTACTGTTTCGGTTGTTCTCCATTT CACTGAATCATGTTCGAAGCAAGT
AT5G15710	F-box protein	Nucleus	TTTCGGCTGAGAGGTTCGAGT GATTCCAAGACGTAAAGCAGATCAA
AT4G05320	*UBQ10*	Cytoplasm / nucleus	GGCCTTGTATAATCCCTGATGAATAAG AAAGAGATAACAGGAACGGAAACATAGT
AT1G18070	*EF-1a*	Cytoplasm / vacuole	TGAGCACGCTCTTCTTGCTTTCA GGTGGTGGCATCCATCTTGTTACA
Genes encoding oxidative stress hallmark proteins			
AT2G21640	*OX1* / unknown protein	Mitochondrion / nucleus	GACTTGTTTCAAAAACACCATGGAC CACTTCCTTAGCCTCAATTTGCTTC
AT2G43510	*OX2* / defensin like protein	Extracellular	ATGGCAAAGGCTATCGTTTCC CGTTACCTTGCGCTTCTATCTCC
AT1G19020	*OX3* / unknown protein	/	GAAAATGGGACAAGGGTTAGACAAA CCCAACGAAAACCAATAGCAGA
AT1G05340	*OX4* / unknown protein	Cytoplasm / nucleus	TCGGTAGCTCAGGGTAAAGTGG CCAGGGCACAACAGCAACA
AT1G57630	*OX5* / TIR family protein	Chloroplast	ACTCAAACAGGCGATCAAAGGA CACCAATTCGTCAAGACAACACC
Genes encoding ROS producing enzymes			
AT1G55020	*LOX1*	Plasma membrane	TTGGCTAAGGCTTTTGTCGG GTGGCAATCACAAACGGTTC
AT3G45140	*LOX2*	Chloroplast membrane	TTTGCTCGCCAGACACTTG GGGATCACCATAAACGGCC
AT5G51060	*RBOHC*	Plasma membrane	TCACCAGAGACTGGCACAATAAA GATGCTCGACCTGAATGCTC
AT5G47910	*RBOHD*	Plasma membrane	AACTCTCCGCTGATTCCAACG TGGTCAGCGAAGTCTTTAGATTCCT
AT1G19230	*RBOHE*	Plasma membrane	GTGATGCAAGATCAACCCTGA GCCTTGCAAAATGTGTTCTCA
Genes encoding antioxidative enzymes			
AT1G20630	*CAT1*	Peroxisome	AAGTGCTTCATCGGGAAGGA CTTCAACAAAACGCTTCACGA
AT4G35090	*CAT2*	Peroxisome	AACTCCTCCATGACCGTTGGA TCCGTTCCCTGTCGAAATTG
AT1G20620	*CAT3*	Peroxisome	TCTCCAACAACATCTCTTCCCTCA GTGAAATTAGCAACCTTCTCGATCA
AT1G07890	*APX1*	Cytoplasm	TGCCACAAGGATAGGTCTGG CCTTCCTTCTCTCCGCTCAA
AT3G09640	*APX2*	Cytoplasm	TTGCTGTTGAGATCACTGGAGGA TGAGGCAGACGACCTTCAGG
AT3G24170	*GR1*	Cytoplasm	CTCAAGTGTGGAGCAACCAAAG ATGCGTCTGGTCACACTGC
AT3G54660	*GR2*	Mitochondrion / Chloroplast	GCCCAGATGGATGGAACAGAT TAGGGTTGGAGAATGTTGGCG

AGI	Annotation	Cell location	FW Primer sequences (5'-3') REV Primer sequences (5'-3')
Genes encoding antioxidative enzymes			
AT4G25100	*FSD1*	Chloroplast	CTCCCAATGCTGTGAATCCC TGGTCTTCGGTTCTGGAAGTC
AT1G08830	*CSD1*	Cytoplasm	TCCATGCAGACCCTGATGAC CCTGGAGACCAATGATGCC
AT2G28190	*CSD2*	Chloroplast	GAGCCTTTGTGGTTCACGAG CACACCACATGCCAATCTCC
Genes encoding metal chelating enzymes			
AT1G07600	*MT1a*	/	AACTGTGGATGTGGCTCCTC CAGTTACAGTTTGACCCACAGC
AT1G07610	*MT1c*	/	GCATGGTCTCAAACCAAGGA TACGCAACACAATGCCAAGT
AT3G09390	*MT2a*	/	ACCCTGACTTGGGATTCTCC GCGTTGTTACTCTCCCCTGA
AT5G02380	*MT2b*	/	ACTCTTGTCCTCGGTGTTGC TTGCACTTGCAGTCAGATCC
AT3G15353	*MT3*	Extracellular	TCGACATCGTCGAGACTCAG CACTTGCAATTTGCGTTGTT
AT5G44070	*PCS1*	Chloroplast	TGGTGTTGAATGCTCTTTCTATCG GGTTCGCAGCAATCCAACAT
Genes encoding monolignol biosynthesis enzymes			
AT2G37040	*PAL1*	Cytoplasm	GCTTCATCCTTCTCGCTTCTG ACGGGTACGTTGCGCTACA
AT3G53260	*PAL2*	Cytoplasm	ACAGTTACGGAGTCACCACCG CCGGCGTTCAAAAATCTAATG
AT1G80820	*CCR2*	Cytoplasm	GAACCCAACTGATCCCAAGA ACAATGGCTTGAGTGTCACG

A manual cut-off of 0.2 Ct was used to extract Relative Real Time Data and relative gene expression data were generated by making use of the $2^{-\Delta Cq}$ method (Livak and Schmittgen 2001). The obtained data are presented with and without normalisation to get a sense of the grey zone in which the real condition will be located (Remans *et al.* 2014). The normalisation was performed organ specific by selecting at least 3 reference genes out of a set of 10 candidate genes (Remans *et al.* 2008) (Table 3.1.4) by making use of the GrayNorm algorithm (Remans *et al.* 2014) and samples were normalised according to the geometric average of the $2^{-\Delta Cq}$ values of these selected reference genes. To study the effect of the genotype without Cd exposure, the selection was performed within the root and leaf samples separately and selected genes are listed in table 3.1.5. To study the effect of Cd, within each plant compartment in combination with genotype the best combination of reference genes was selected and are also represented in table 3.1.5. Since in the study of the effects of Cd we were interested in whether or not there is an effect of Cd on the gene expression levels in the selected genotypes and no further comparisons between the different genotypes were aimed, we preferred to select the reference genes by

making use of the Graynorm algoritm in each plant genotype separately (*e.g.* WT versus Cd-exposed WT, *4cl1-1* versus Cd-exposed *4cl1-1*, etc.). In this way it was possible to minimise the effect of the normalisation and produce a higher experimental resolution, hence a lower level of uncertainty.

Table 3.1.5: Selected reference genes by the graynorm algoritm to study the effect of both genotype or Cd exposure.

Genotype effect roots		
Non-exposed plants		AT5G08290 (*YLS8*), AT4G05320 (*UBQ10*) and AT1G18070 (*EF-1a*)
Genotype effect leaves		
Non-exposed plants		AT3G18780 (*ACT2*), AT5G15710 (F-box protein) and AT1G18070 (*EF-1a*)
Cd effect roots		
Non- and Cd-exposed plants of:	WT	AT3G18780 (*ACT2*), AT5G15710 (F-box protein) and AT1G18070 (*EF-1a*)
	4cl1-1	AT5G55840 (PPR superfamily protein), AT5G08290 (*YLS8*) and AT4G05320 (*UBQ10*)
	ccr1-6	AT3G18780 (*ACT2*), AT5G55840 (PPR superfamily protein) and AT5G15710 (F-box protein)
	ccr1-3	AT3G18780 (*ACT2*), AT4G34270 (TIP41-like protein), AT2G28390 (SAND family protein), AT5G08290 (YLS8), AT5G15710 (F-box protein), AT4G05320 (*UBQ10*) and AT1G18070 (*EF-1a*)
Cd effect leaves		
Non- and Cd-exposed plants of:	WT	AT3G18780 (*ACT2*), AT4G34270 (TIP41-like protein), AT5G55840 (PPR superfamily protein) and AT5G15710 (F-box protein)
	4cl1-1	AT3G18780 (*ACT2*), AT5G25760 (*UBC21*), AT4G34270 (TIP41-like protein) and AT1G18070 (*EF-1a*)
	ccr1-6	AT3G18780 (*ACT2*), AT5G15710 (F-box protein) and AT1G18070 (*EF-1a*)
	ccr1-3	AT3G18780 (*ACT2*), AT2G28390 (SAND family protein), AT5G08290 (*YLS8*), AT5G15710 (F-box protein), AT4G05320 (*UBQ10*) and AT1G18070 (*EF-1a*)

3.1.3.5 Pigment concentrations

Snap frozen leaves of younger plants were grinded with mortar and pestle in the presence of $CaCO_3$, sand and acetone where after centrifugation (5 min, 20,000 g) was performed. $1/10^{th}$ diluted samples were measured spectrophotometrically at 663, 646 and 470 nm. The concentrations of chlorophyll *a*, *b* and total carotenes were calculated by using following formulas (Lichtenthaler and Wellburn 1981) and taking fresh weight and volume of supernatants into account:

Chlorophyll *a* (µg ml^{-1}): 12.21 A663 - 2.81 A646
Chlorophyll *b* (µg ml^{-1}): 20.31 A646 – 5.03 A663
Total carotenes (µg ml^{-1}): (1000 A470 – 3.27 chla – 104 chlb) /229

3.1.3.6 Lipid peroxidation analysis (TBA)

The concentration of thiobarbituric acid reactive metabolites (TBArm) in snap frozen roots, leaves and stems of older plants were determined spectrophotometrically as an estimation of lipid peroxidation. Snap frozen plant tissue was grinded in 0.1% trichloroacetic acid (TCA) by mortar and pestle and centrifuged (10 min, 20,000 g, 4°C). The samples were diluted in 0.5% TBA by adding 1 ml 0.5% TBA in 20% TCA to 400 µl supernatant and heated to 95°C for 30 min. After a cooling step of 5 min on ice, the samples were centrifuged again (10 min, 20,000 g, 4°C) and absorbance was measured at 532 nm. Absorbance was corrected for unspecific absorbance at 600 nm. Blanks with 0.1% TCA (without plant samples) were used as a reference (Dhinsda *et al.* 1981).

Concentrations were calculated by using the Lambert-Beer law:

$A = C \times \varepsilon \times d$

With A absorption, C concentration, ε (extinction coefficient) equal to 155 mM^{-1} cm^{-1} and d (distance)

3.1.3.7 Lignin staining of stem sections

Fresh stem samples were collected and the bottom 2 cm was placed in a 7% agarose solution. The agarose block was glued to a vibratome and sections of 100 µm were cut. Sections were placed onto a glass slide. During Wiesner staining (for cinnamaldehydes) the phloroglucinol solution (100 ml 1% phloroglucinol in 95% ethanol mixed with 16 ml 37% HCl) was dropped onto the stem section and sections were studied by microscopy after 2 min incubation at RT. Mäule staining (for S units) was performed by a sequence of treatments of the stem sections: 5 min with 1% $KMnO_4$, rinsing with water, 2 min with 37% HCl and neutralising with a drop of NH_4OH. Images of sections were taken immediately after Mäule staining (Sundin *et al.* 2014).

3.1.3.8 Lignin staining entire root (Wiesner)

Freshly harvested roots were placed into a dish with an excess of phloroglucinol solution (100 ml 1% phloroglucinol in 95% ethanol mixed with 16 ml 37% HCl), where after the result was photographed.

3.1.3.9 Microscopic sections and lignin staining of roots

Fresh roots were fixed for 4 to 24 h with a solution containing 5 ml formaldehyde, 5 ml concentrated acetic acid and 90 ml ethanol 70%. The samples were rinsed with 70 % ethanol and stored at 4°C. Samples were dehydrated at room temperature by the following steps: 2 h with solution A (45 ml ethanol (96%), 10 ml butanol, 45 ml water); 2 h with solution B (45 ml ethanol (96%), 20 ml butanol, 35 ml water); 2 h with solution C (50 ml ethanol (96%), 30 ml butanol, 20 ml water); 4 h with solution D (50 ml ethanol (96%), 40 ml butanol, 10 ml water); 12 h with solution E (50 ml ethanol (96%), 50 ml butanol) and 4 h with a 100% butanol with some eosine. Next, they were embedded in a solution of 50% paraffin and 50% butanol for 4 h (60°C) and three times 12 h in 100% paraffin.

Staining was performed with saffranin in combination with astra blue and was performed by incubating 5 min with 96% ethanol, 5 min with 85% ethanol, 15 min with saffranine (0.4% in 70% ethanol) followed by rinsing with 70% ethanol, 5 min with 85% ethanol, 20 min with astra blue (0.4% in 96% ethanol) followed by rinsing with 96% ethanol, 15 min with 100% ethanol and two steps of 15 min with xylene followed by enclosing in Depex mounting medium.

3.1.3.10 Preparation of cell wall residue

Harvested plant tissues (older plants) were stored at -80°C where after they were freeze-dried. Five replicates, containing 2 roots or stems (or leaves) each, were used per group. Whole root and leaf samples were crushed and respectively aliquots of 6 and 8 mg tissue were made. The freeze-dried bottom part of 10 cm (minus the lowest 1 cm) of the stem was cut in pieces of approximate 2-3 mm and aliquots of 5 mg were taken. A purified cell wall residual (CWR) was obtained by washing the samples with different reagents nearly at their boiling point for 30 min while shaking (850 rpm). The following sequence was used: water (98°C), ethanol (76°C), chloroform (59°C) and acetone (54°C) (Van Acker *et al.* 2013). For leaves, the ethanol step was executed several times until the green colour of samples disappeared. The washed samples were air-dried for at least 24 h.

3.1.3.11 Lignin concentration

Lignin concentration was quantified by the acetyl bromide (AcBr) assay optimised for small amounts of plant tissue (Van Acker *et al.* 2013). After dissolving the CWR (paragraph 3.1.3.10) in 100 µl freshly made 25% acetyl bromide in glacial acetic acid and adding 4 µl 60% perchloric acid, samples were incubated for 30 min at 70°C while shaking (850 rpm) and centrifuged (15 min, 16,110 g). Supernatant was separated from the pellet and the pellet was washed with 500 µl glacial acetic acid. The supernatant was mixed with 200 µl sodium hydroxide and 500 µl glacial acetic acid where after also the wash of the pellet was added. The total sample volume was brought to 2 ml with glacial acetic acid and an incubation step of 20 min at room temperature followed. Absorbance at 280 nm was explored using a Nanodrop ND-1000 (Thermoscientific, Wilmington, DE, USA) with at least 3 technical repeats per sample. The lignin concentration was calculated by making use of the Bouguer-Lambert-Beer law:

$A = C \times \varepsilon \times d$

With A absorption, C concentration, ε (extinction coefficient) equal to 23.55 l g^{-1} cm^{-1} and d (distance)

3.1.3.12 Lignin composition

Composition of lignin was determined by thioacidolysis (Van Acker *et al.* 2013) on the same CWR samples (paragraph 3.1.3.10) used for lignin concentration measurements. After adding a reaction mixture of 2.5% boron trifluoride etherate and 10% ethanethiol in dioxane (v/v) and removing air under liquid nitrogen vapure, samples were incubated for 4 h at 98°C. The reaction was stopped by incubating for 5 min at -20°C and an internal standard of 200 µl tetracosane in dichloromethane (5 mg ml^{-1}) was added. The pH was adjusted by adding 300 µl 0.4 M $NaHCO_3$. The organic phase was extracted after adding 1 ml dichloromethane and dried using a SpeedVac concentrator. Samples were kept at -20°C until further handling.

Samples were prepared for gas chromatography (GC) by adding 200 µl dichloromethane where after 20 µl of the dissolved samples were combined with 20 µl pyridine and 100 µl N,O-bis(trimethylsilyl)acetamide. An incubation step of

2 h at 25°C while shaking (750 rpm) was applied and samples were put into 100 µl GC vials. In the GC (Hewlett-Packard HP 6890 Series GC system (Agilent, Santa Clara, CA, USA) coupled with a HP-5973 mass-selective detector) samples were brought to 130°C for 3 min, encountered a temperature gradient (rise of 3°C per min till 250°C), were hold for 5 min at 250°C and cooled down with 50°C per min till 130°C (Van Acker *et al.* 2013). The lignin monomers (H, G and S as their trimethylsilyl (TMS) ether derivatives) involved in β–O–4-ether bonds, as well as ferulic acid (FA), estered ferulic acid and a CCR marker could be detected (Ralph *et al.* 2008) and calculated as µmol g^{-1} AcBr lignin.

3.1.3.13 Statistical analysis

Outliers in datasets were determined and excluded using the extreme studentised deviate method (Graphpad Software, Inc.) with a significance level of 0.05. Normality and homoscedasticity tests were implemented and transformations were performed if required in order to obtain normality. A log transformation was always applied for Real Time data. Data were tested either with one or two-way anova and the Tukey-Kramer post-hoc test using R (version 3.2.0, R development Core Team 2015) when data were normally distributed. In case no normality was obtained, a non-parametric Kruskal-Wallis test followed by the Wilcoxon rank sum test was used to explore the statistical significant differences between conditions.

Statistics on thioacidolysis data were performed by SAS Enterprise Guide 6.0. Outliers were removed based on box plots. An anova with mixed model analysis with post-hoc Dunnett T-test, using genotype as a fixed effect was applied. Corrections were made for any possible batch effect since samples were measured in 3 different batches.

Subsection 3.2
Growth characteristics of the lignin-reduced mutants with and without Cd exposure

Renewable biofuels (*e.g* bioethanol) are a possible solution to meet up with the high demand for energy and limited fossil fuels (Ragauskas *et al.* 2006). Moreover, it is profitable to use lignocellulosic biomass from lignin-reduced plants since they are more efficiently converted into fermentable sugars hence they yield more biofuel (Van Acker *et al.* 2014). An extra benefit can be obtained in case marginal soils (*e.g.* cadmium (Cd) contaminated soils) are used to produce the biomass because the competition with food production is lowered.

However, it is known that both the T-DNA knockout of genes involved in the monolignol biosynthesis (Van Acker *et al*. 2013, Vanholme *et al*. 2012a, Table 3.1 in the introduction of section 3) and Cd contamination (Keunen *et al.* 2011) can have negative influences on the growth of the plants. In addition, elemental accumulation can also be influenced upon Cd exposure since Cd can compete with nutrients for entering the plant (Suzuki *et al.* 2001). It can be hypothesized that the growth disturbance after Cd exposure can be different for these lignin-reduced mutants since they have another cell wall composition (Van de Mortel *et al.* 2008, Vanholme *et al*. 2012a). Hence the defence to stop Cd-ions can be lower for these lignin-reduced mutants resulting in a different mode of toxicity. Moreover, elemental accumulation may be altered in a similar manner.

In order to grow lignin down-regulated non-food-crops (*e.g.* poplar) on for instance cadmium (Cd) contaminated agricultural land, the knowledge about the possible effects on growth and nutrient/Cd accumulation is an advantage in developing applications. The model organism *Arabidopsis thaliana* is a good candidate to acquire this knowledge since it is a well-known model for woody plants (Nieminen *et al.* 2004).

For reasons of feasibility of the experiments in our research, we selected 4 Cd concentrations (3, 5, 7 and 10 µM $CdSO_4$) to perform a range finding experiment

with the aim of selecting 1 suitable concentration that will be used during the entire PhD dissertation. All selected Cd concentrations in the optimisation set-up are environmentally relevant as a range of 1 to 10 µM Cd was found in the pore water of Lommel-Maatheide, a Cd/Zn/Pb-contaminated site of a former Zn-smelter in the North of Limburg (Belgium) (Krznaric *et al.* 2009). Soils with pore water concentrations that are higher than 5 µM Cd are considered to be highly contaminated (Van Belleghem *et al.* 2007). Though, Keunen *et al.* (2011) already have proven the sublethal nature of all tested Cd concentrations.

We continued our research on *Arabidopsis thaliana* to unravel the effects of both the mutation of genes involved in the monolignol biosynthesis pathway (*4CL1* and *CCR1*) and the Cd exposure at the plant's growth stages covering the whole lifespan. Subsequently, the Cd and nutrient concentration in the different tissues of the lignin-reduced mutants with and without Cd exposure were explored.

3.2.1 Experimental design

In subsection 3.2, 3 different growth systems were applied: (i) a vertical agar plate assay to assess the early growth stages, (ii) plants grown in hydroponics to investigate growth characteristics of the subsequent of the growth stage, and (iii) the Arasystem to produce seeds.

First, a range finding experiment was performed to select 1 appropriate Cd concentration out of 4 selected Cd concentrations (3, 5, 7 and 10 µM $CdSO_4$). To investigate how plants cope with a rather long-term exposure to these Cd concentrations, seedlings were grown for 1 week in a hydroponics system (paragraph 3.1.2.3) without Cd exposure where after they were exposed for 2 weeks to Cd. Three weeks old plant organs were harvested and the fresh weight and Cd concentration of wild type plants (WT) after exposure to the 4 different Cd concentrations were compared with non-exposed control plants.
Subsequently, the range finding experiment to determine an adequate Cd concentration for the vertical agar plate assay was performed using concentrations of 0, 3 and 5 µM $CdSO_4$ with root length as a studied parameter.

Growth phases were assessed as described in paragraph 3.1.3.1. Growth of seedlings on vertical agar plates (VAPs) (paragraph 3.1.2.1) with and without 3 µM $CdSO_4$, as selected during the range finding experiment (paragraph 3.2.2.1), was observed to examine the early effects on growth: growth phase "zero" (seed germination) and "one" (leaf development), as described by Boyes *et al.* (2001), as well as stage R6 (root development) were assessed (paragraph 3.1.3.1). Subsequent growth phases were observed in hydroponics (paragraph 3.1.2.3). For this purpose, one-week-old hydroponically grown plants were or were not exposed to 3 µM $CdSO_4$, the concentration determined during the range finding experiment (paragraph 3.2.2.1). Twice a week the Cd concentration was reset to 3 µM. Using this experimental set-up, principal growth stages "one" (leaf development), "three" (rosette growth), "five" (inflorescence emergence) and part of "six" (flower production) as described by Boyes *et al.* (2001), were studied (paragraph 3.1.3.1). To produce seeds, plants were grown in arasystems (paragraph 3.1.2.2) and half of the trays were exposed to 3 µM $CdSO_4$ from the moment stem formation was initiated. The different systems wherein Cd concentration was investigated are further discussed in paragraph 3.1.2. Methods for determination of elemental and Cd concentrations are described in paragraph 3.1.3.2.

3.2.2 Results and discussion

3.2.2.1 Range finding of the cadmium concentration

For our study, we aimed to work with a concentration which results in significant Cd effects. Though we did not prefer a strong influence that can lead to many secondary effects derived from the growth inhibition itself.

Both roots and leaves of plants were significantly inhibited in their fresh weight for all tested Cd-concentrations. For our study, the inhibition of about 64% leaf growth that was obtained at 10 µM Cd was rather strong (Fig 3.2.1A) and root growth was inhibited for approximately 50% or more after exposure to 5 µM Cd or higher, which is a higher impact than aimed for our study (Fig 3.2.1B). Therefore, both the use of 5 and 7 µM Cd are excluded from our selection. Further, exposure to 3 µM Cd led to moderate yet significant effects on fresh weight of roots and leaves (Fig 3.2.1A and B).

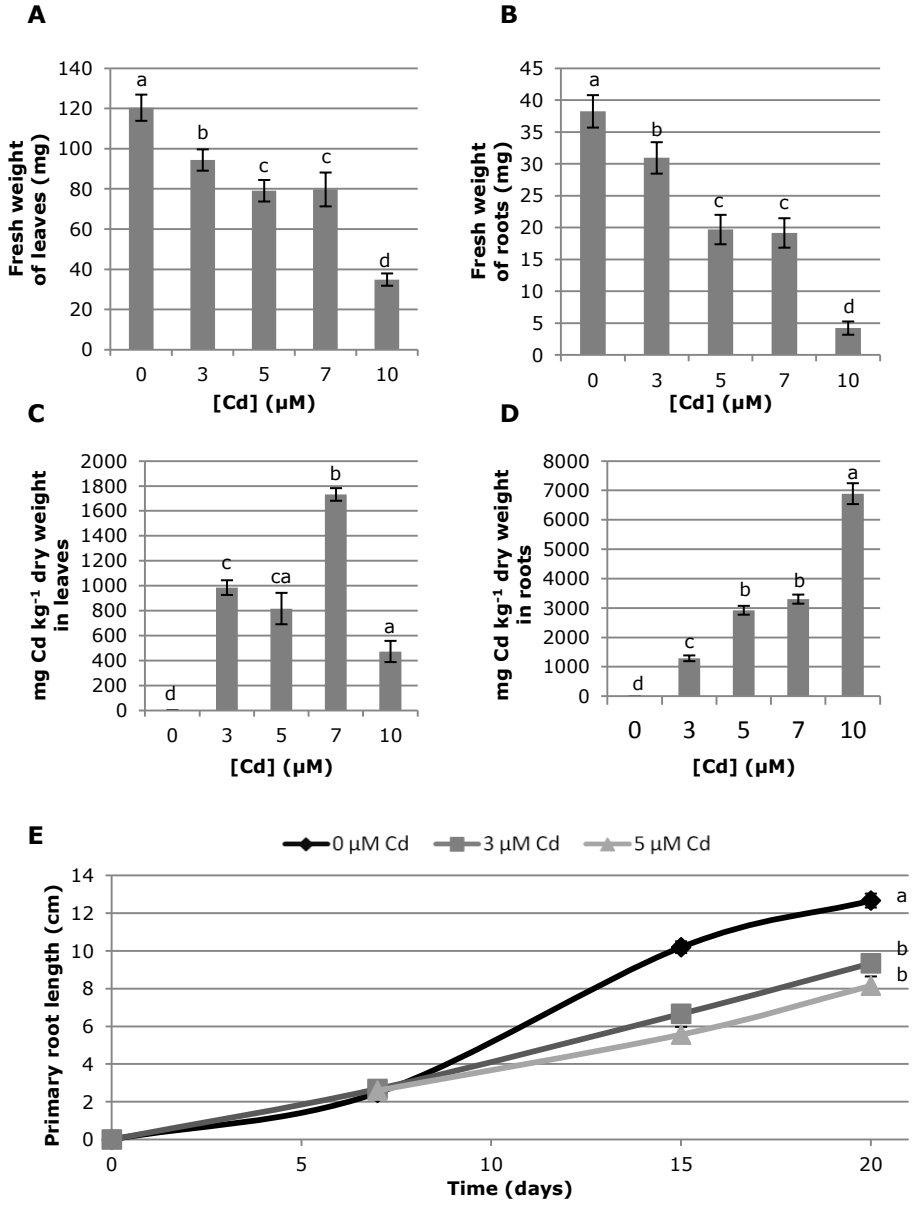


Figure 3.2.1: Range finding for the concentration of $CdSO_4$ with *Arabidopsis thaliana* WT plants grown for 1 week without and 2 weeks in the presence of 0, 3, 5, 7 and 10 µM Cd in hydroponics (n=3) and 0, 3 and 5 µM in vertical agar plates (VAP) (n=18). (A) Fresh weight of leaves (mg) in hydroponics system and (B) fresh weight of roots in hydroponics system. (C) Cd concentration (mg kg^{-1} dry weight) of leaves and (D) of roots in hydroponics system. (E) Primary root length (cm) on VAP. Standard errors are indicated in the error bars. Statistical significance (one way anova with subsequent Tukey-Kramer post-hoc test) indicated by letters: $p<0.05$.

As earlier observed in many studies where Cd was administered to plants (Lozano-Rodriguez *et al.* 1997, Herbette *et al.* 2006, Polec-Pawlak *et al.* 2005, Lux *et al.* 2011, and many others), Cd accumulated less in leaves in comparison to roots. The Cd concentration in roots changed in a dose-dependent way (Fig 3.2.1D) although no dose-dependent increase of Cd translocation to the leaves was observed (Fig 3.2.1C). The concentration of Cd in the leaves of the 10 µM-exposed plants was limited in comparison to the other used Cd exposures, an observation which most likely resulted from the very strong inhibition of root growth. Furthermore, Cd uptake is usually restricted to actively growing roots, which are probably less numerous in plants exposed to 10 µM Cd. However also defence mechanisms (*e.g.* lignification) can be induced in roots by Cd exposure which might reduce the translocation of Cd to the aerial plant parts (Lux *et al.* 2011). All these results obtained from the hydroponics experiment make exposure of 1-week-old *Arabidopsis* seedlings during 2 weeks to 3 µM Cd the best for our study design.

Likewise, the effect of Cd (3 and 5 µM $CdSO_4$) on the root growth of WT *Arabidopsis* plants was investigated using the vertical agar plate system. Both concentrations significantly inhibited root growth. As no significant differences were observed among the Cd concentrations, we decided to continue with 3 µM $CdSO_4$.

In conclusion, even though all tested concentrations were environmentally relevant, we chose to work with a sublethal concentration that had a significant impact on plant growth yet no excessive decrease in growth of both roots and above-ground plant parts. Hence, the concentration of 3 µM $CdSO_4$ was further used throughout our study.

3.2.2.2 Growth phases of the different genotypes

The growth of the *A. thaliana* lignin-reduced mutants has already been well described for plants grown on soil supplemented with 10% vermiculite (Vanholme *et al.* 2012a). In the current study, we investigated the growth of these mutants in a hydroponic growth system. Furthermore, no knowledge is available about the growth of these mutants under stress conditions.

Table 3.2.1: Growth phases of *Arabidopsis thaliana*, as described by Boyes *et al.* 2002, during the plate-based assay of the different lignin-reduced mutants and WT seedlings, with and without exposure to 3 µM $CdSO_4$. Data are calculated as the day a specific growth stage was reached and standard errors (n=25) are indicated (two way anova with subsequent Tukey-Kramer post-hoc test or, in case no normality was obtained, a non-parametric Kruskal-Wallis test followed by the Wilcoxon rank sum). Significant delays of the lignin-reduced mutants relative to WT plants are indicated in green ($p<0.05$, $p<0.1$). Significant earlier reaching the growth phase of the lignin-reduced mutants relative to WT plants are indicated in red ($p<0.05$). Significant effect of exposure to Cd, *i.e.* non-exposed genotype relative to the same genotype exposed to Cd are indicated in purple ($p<0.05$) for delays and orange ($p<0.05$) in case the stage was reached earlier.

Stage	**Description**	**The day a specific growth stage is reached**							
Principal growth phase 0	**Seed germination**	**Effect of genotype (values of plants in control conditions)**				**Effect of cadmium (values of plants exposed to 3µM $CdSO_4$)**			
		WT	***4cl1-1***	***ccr1-6***	***ccr1-3***	**WT**	***4cl1-1***	***ccr1-6***	***ccr1-3***
0.10	Seed imbibition	2.16 ± 0.07	2.28 ± 0.09	2.00 ± 0.00	2.00 ± 0.00	2.28 ± 0.12	2.64 ± 0.25	2.00 ± 0.00	2.08 ± 0.25
0.50	Radicle emergence	3.16 ± 0.07	3.28 ± 0.09	3.00 ± 0.00	3.00 ± 0.00	3.28 ± 0.12	3.68 ± 0.24	3.04 ± 0.04	3.08 ± 0.25
0.70	Hypocotyls and cotyledon emergence	4.08 ± 0.08	4.16 ± 0.11	3.64 ± 0.10	3.96 ± 0.04	4.08 ± 0.11	4.45 ± 0.24	3.84 ± 0.07	4.00 ± 0.20
Principal growth phase 1	**Leaf development**								
1.00	Cotyledons fully opened	4.40 ± 0.14	5.04 ± 0.15	4.00 ± 0.00	4.00 ± 0.00	4.52 ± 0.16	4.00 ± 0.25	4.00 ± 0.00	4.00 ± 0.29
1.02	2 rosette leaves > 1mm in length	9.04 ± 0.09	10.00 ± 0.17	8.36 ± 0.10	8.76 ± 0.17	10.44 ± 0.17	11.26 ± 0.13	10.08 ± 0.10	10.04 ± 0.13
1.04	4 rosette leaves > 1mm in length	13.56 ± 0.12	14.04 ± 0.21	12.72 ± 0.12	12.24 ± 0.09	15.48 ± 0.13	16.70 ± 0.19	15.00 ± 0.14	14.63 ± 0.10
Stage R	**Root development**								
R6	More than 50% of seedlings have a primary root of ≥ 6 cm in length	13.29 ± 0.21	14.25 ± 0.27	11.87 ± 0.17	11.90 ± 0.12	14.71 ± 0.24	15.40 ± 0.27	12.88 ± 0.22	12.00 ± 0.18

To this end, the results from the non-exposed plants are compared with data derived from Cd-exposed plants. In this way, both the effects of the genetic modification of genes involved in the monolignol biosynthesis, resulting in lower lignin contents, and Cd exposure were included in the growth phase experimental design.

(A) Effects of the lignin-reduced genotype – early growth phases

During the growth stage "zero" (seed germination) the *ccr1* mutants tended to be a bit earlier in their seed imbibition and emergence of radicle, hypocotyl and cotyledons (Table 3.2.1). Though, differences among genotypes were not significant.

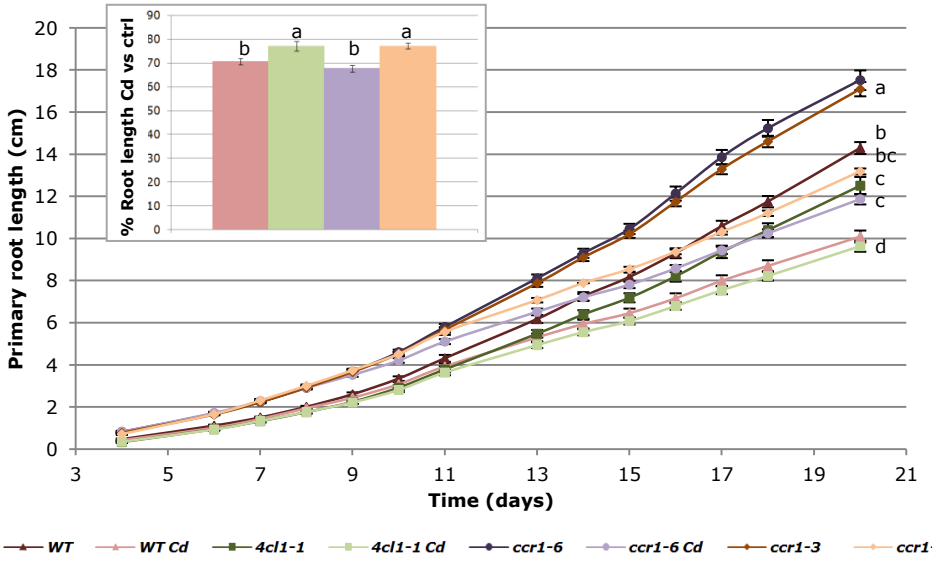


Figure 3.2.2: Root length (cm) on vertical agar plates per day and the percentage of growth of the roots of Cd-exposed seedlings in comparison to the non-exposed genotype at day 20. Standard errors (n=25) are indicated and statistical differences ($p<0.05$) (one or two way anova with subsequent Tukey-Kramer post-hoc test) of the endpoint of the measurement were indicated by letters.

Principal growth phase "one" (leaf development) was 1 day delayed for the *4cl1-1* mutants in comparison to the WT plants. The delay was significant for growth stage 1.02 (2 rosette leaves of more than 1 mm in length) and borderline significant ($p<0.1$) for 1.00 (cotyledons fully open). Besides, also root formation of the *4cl1-1*-mutant was delayed as the R6 stage (more than 50% of seedlings

have a primary root of ≥ 6 cm in length) was reached 1 day later in comparison to WT seedlings (Table 3.2.1). The primary root length of *4cl1-1* seedlings at the end of the experiment was significantly lower in comparison to those of WT plants (Fig 3.2.2). Overall, the data reveal that the *4cl1-1* mutant is delayed in its development during these early growth phases in the VAP-based assay. *4cl1-1* seeds were produced in the same tray at the same moment as WT seeds hence no effects of seed production can explain these differences. No reports of this early effect were found in literature. It is know that during development, 4CL expression is correlated with lignin deposition in cotyledons and roots, which occurs 2 to 3 days after germination (Lee *et al.* 1995). By consequence, it is more likely that the slight growth retardation is a secondary effect rather than an effect of the *4cl1* mutation by itself. Moreover, when both allelic lignin-reduced mutants for *4CL1* were studied, only for *4cl1-1* the delay was observed (data not shown) and the very early delay in root formation is outweighed during later growth stages. Further, no significant differences in fresh weight of roots of *4cl1-1* seedlings in comparison to WT seedlings were observed in later experiments (Fig 3.2.3E). Therefore, we may consider this temporal growth delay to be unimportant for the rest of our study.

In contrast, during these VAP-based assay, *ccr1* mutants were a little faster in their leaf and root development stages in comparison to WT seedlings. *ccr1-6* mutants were 1 day in advance ($p<0.05$) at the growth stages 1.02 (2 rosette leaves of more than 1 mm in length). Whereas *ccr1-6* and *ccr1-3* were 1 day in advance ($p<0.05$) at the growth stages 1.04 (4 rosette leaves of more than 1 mm in length). Root development was affected in the same manner: *ccr1-6* and *ccr1-3* seedlings reached stage R6 (more than 50% of seedlings have a primary root of ≥ 6 cm in length) almost 1 day earlier ($p<0.05$) in comparison to the WT seedlings (Table 3.2.1). Also at the end of the experiment the roots were significantly longer for both *ccr1* mutants in comparison to WT seedlings (Fig 3.2.2).

To summarise, the *ccr1 mutants* were more advanced in their development in the VAPs-based assay in comparison with WT seedlings. An observation which can be explained by multiple assumptions. (i) c*cr1* mutants may invest more

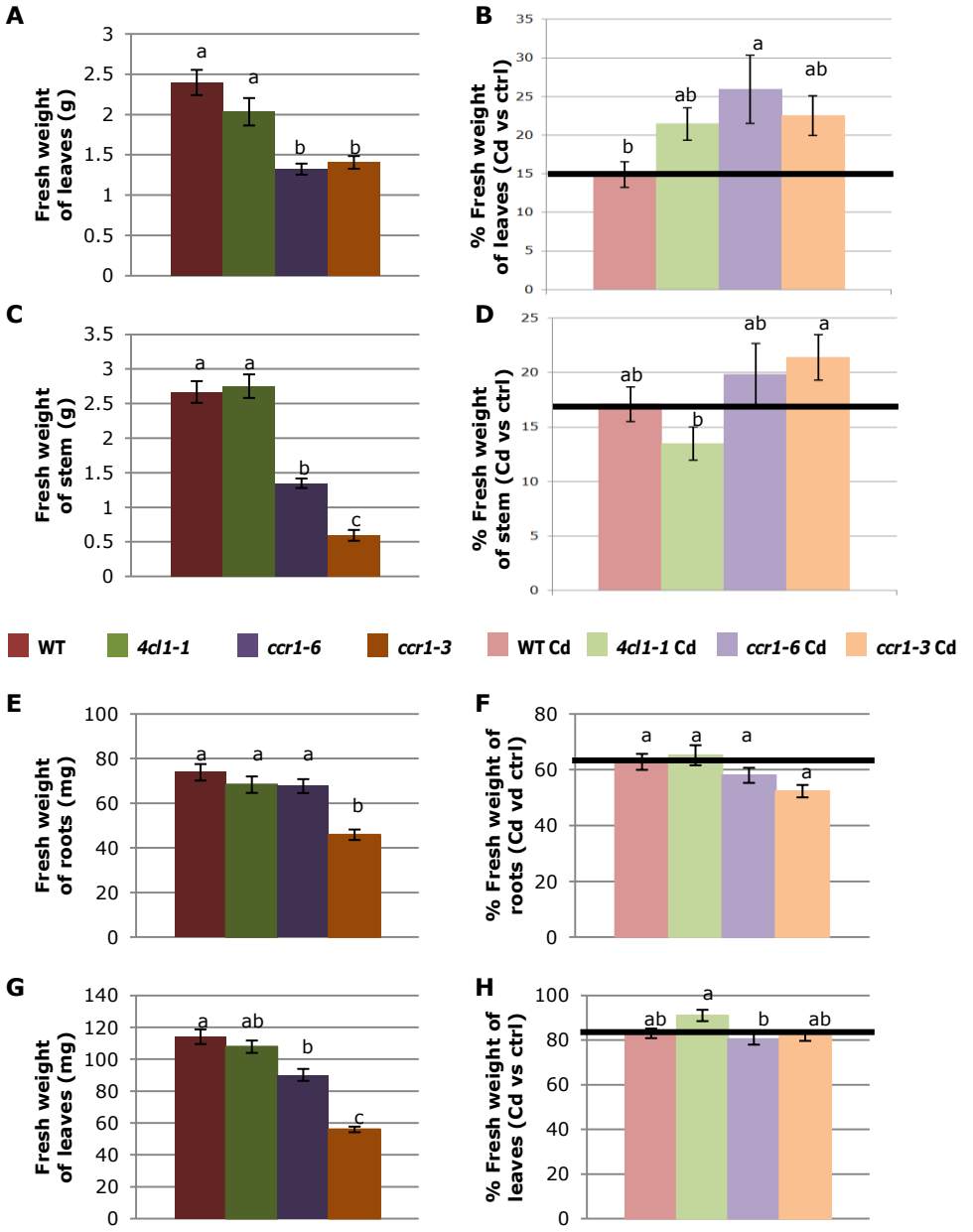


Figure 3.2.3: Measurements of fresh weight at day of harvest (A) of rosettes (day 59) (n=28), (B) percentage of the leaf fresh weight of Cd-exposed plants in comparison to the non-exposed genotype, (C) of stems (day 59) (n=28), (D) percentage of the stem fresh weight of Cd-exposed plants in comparison to the non-exposed genotype. (E) Fresh weight of roots at day 21 (n=30 or more), (F) percentage of the roots fresh weight of Cd-exposed plants in comparison to the non-exposed genotype, (G) fresh weight of rosette leaves at day 21, (F) percentage of the leaves fresh weight of Cd-exposed plants in comparison to the non-exposed genotype. Standard errors and statistical differences ($p<0.05$) (one way anova with subsequent Tukey-Kramer post-hoc test) by letters are indicated.

energy in one seed as these plants produce less seeds per plant (Fig 3.2.4A and E) and per silique (Gul and Whalen 2013). Moreover, one *ccr1* seed weighs more (less seeds could be counted in 1 mg seeds) in comparison to one WT seed (Fig 3.2.4C). Even if the higher weight per seed would be due to a higher water content within the seed, it would be advantageous during germination. However, the higher seed weight might also be due to the fact that the *ccr1 mutants* may store more reserves in their cotyledons and therefore develop more efficiently during the early growth phases. (ii) The bacterial community of these seeds can be influenced by the genotype or via vertical transmission. Some of these bacteria can possess indirect or direct growth promoting traits and therefore accelerate germination (see section 4) (Truyens *et al.* 2015). (iii) Maternal inheritance of some other internal features, as discussed in the review of Donohue *et al.* (2009), may have an influence on germination. For instance, inheritance of plastids, the maternal provision with nutrients, hormones, proteins transcripts or other compounds can have an influence on the germination of seeds. Or the maternally provided composition of seed coat which influences light permeability and therefore can cue earlier germination, can have an impact on seed germination. (iv) It is known that under stress conditions, cell wall structures are strengthened resulting in an increased rigidification which can reduce plants growth (Herbette *et al.* 2005, de Cássia Siqueira-Soares *et al.* 2013). Less lignification due to the mutation could cause a lower rigidification resulting in an enhanced growth. However, this hypothesis is contradicted by the slower growth in the first developmental stages of *4cl1-1* mutant in comparison to WT seedlings and may not explain the possible earlier germination of *ccr1* mutants.

(B) Effects of the lignin-reduced genotype – subsequent growth phases

In contrast to the early growth phases on the VAPs, in the hydroponics system (Fig 3.2.5) no differences between WT plants and *4cl1-1* mutants were detected in the subsequent growth phases. Our observation in *A. thaliana* supported the results of Van Acker *et al.* (2013) and Vanholme *et al.* (2012a) (Table 3.1 in the introduction of section 3). Moreover, Tian *et al.* (2013) noticed even an increased plant height of transgenic *4cl1* poplars.

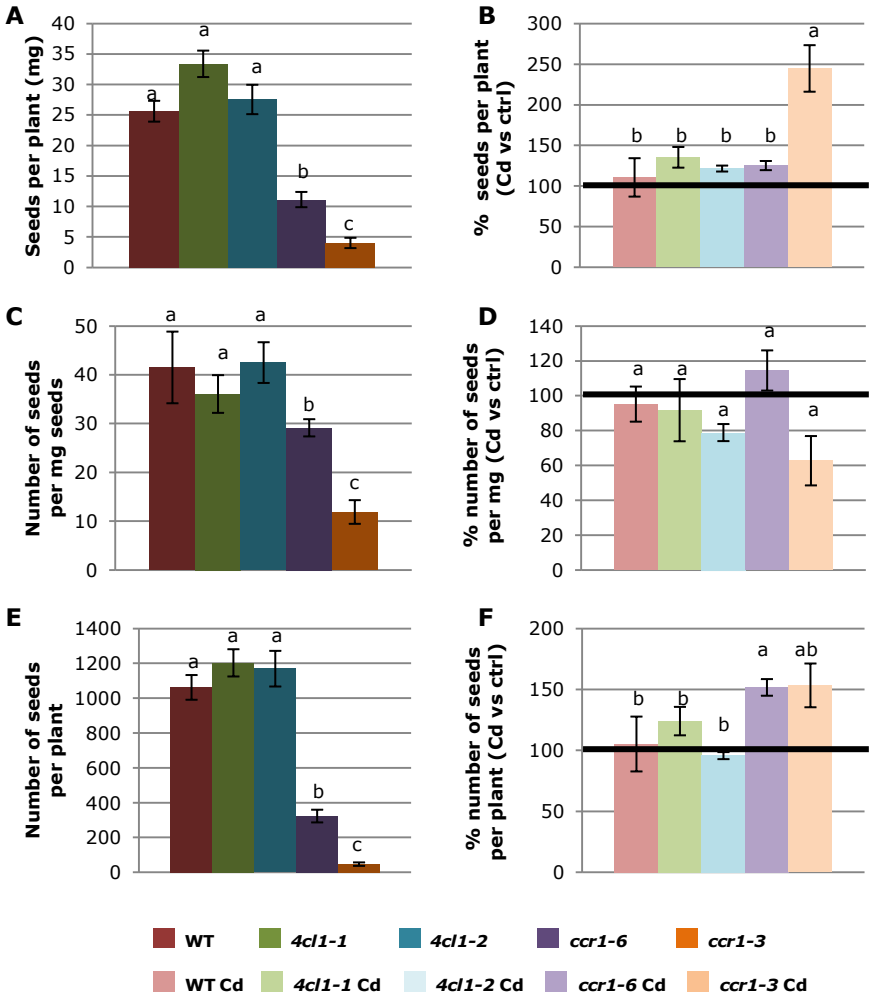


Figure 3.2.4: Seed yield from plants cultivated on sand. (A) seeds per plant (mg), (B) seeds per plant of Cd-exposed plants relative to the non-exposed genotype (%), (C) number of seeds in 1 mg, (D) number of seeds in 1 mg of Cd-exposed plants relative to the non-exposed genotype (%), (E) number of seeds per plant, (F) number of seeds per plant of Cd-exposed plants relative to the non-exposed genotype (%). Standard errors (n=3 or more) are indicated and statistical differences: $p<0.05$ (one way anova with subsequent Tukey-Kramer post-hoc test). Black line indicates the non-exposed situation.

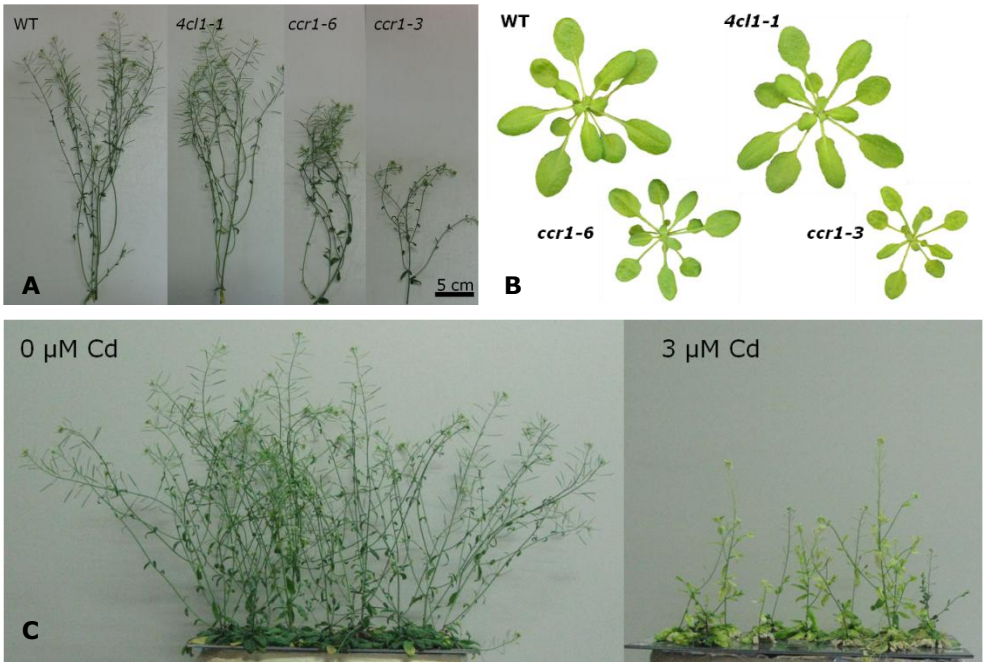


Figure 3.2.5: Representative pictures of *Arabidopsis thaliana* of (A) the inflorescence of the different genotypes, (B) leaves of the different genotypes at day 25, (C) the effect at day 58 on the inflorescence of exposure to 3 µM $CdSO_4$ (exposure from day 7 after germination).

On the other hand, *ccr1 mutants* were slower in leaf development in comparison to WT plants. The delay was significant once the plants attained growth phase 1.10 (10 rosette leaves more than 1 mm in length) and further on. Moreover, the *ccr1 mutants* did not reach all growth stages (*e.g* 1.26 (26 rosette leaves more than 1 mm in length)) (Table 3.2.2).

The total number of leaves that developed was significantly lower for *ccr1* mutants in comparison to WT plants (Fig 3.2.6A). During principal growth phase "three" (rosette growth) the retardation was not that pronounced and only *ccr1-3* mutants revealed a delay ($p<0.1$ for growth stage 3.20 and 3.50 and $p<0.05$ for growth stage 3.70) in comparison to WT plants (Table 3.2.2). Also the rosette diameter at the end of the experiment was less ($p<0.05$) for both *ccr1* mutants in comparison to WT plants (Fig 3.2.6B). The fresh weights of the *ccr1* mutants, measured at day 21 and at the end of the experiment, illustrated the same significant reduction in leaf development (Fig 3.2.3A and G).

Table 3.2.2: Growth phases of *Arabidopsis thaliana*, as described by Boyes *et al.* 2002, during the hydroponics-based assay of the different lignin-reduced mutants and WT plants, with and without exposure to 3 µM $CdSO_4$. Data are calculated as the day a specific growth stage was reached and standard errors (n=28) are indicated (two way anova with subsequent Tukey-Kramer post-hoc test or, in case no normality was obtained, a non-parametric Kruskal-Wallis test followed by the Wilcoxon rank sum). In case more than half of the replicates did not reach the stage, no value was calculated and ND (not determined) is indicated. Significant delays of the lignin-reduced mutants relative to WT plants are indicated in green ($p<0.05$, $p<0.1$). Significant earlier reaching the growth phase of the lignin-reduced mutants relative to WT plants are indicated in red ($p<0.05$). Significant effect of exposure to Cd, *i.e.* non-exposed genotype relative to the same genotype exposed to Cd are indicated in purple for delays ($p<0.05$).

Stage	**Description**	**The day a specific growth stage is reached**							
Principal growth phase 1	**Leaf development**	**Effect of genotype (values of plants in control conditions)**				**Effect of cadmium (values of plants exposed to 3µM $CdSO_4$)**			
		WT	***4cl1-1***	***ccr1-6***	***ccr1-3***	**WT**	***4cl1-1***	***ccr1-6***	***ccr1-3***
1.02	2 rosette leaves > 1mm in length	8.03 ±0.04	8.04 ±0.04	8.07 ±0.05	8.08 ±0.15	8.38 ±0.14	8.68 ±0.13	8.36 ±0.11	8.27 ±0.09
1.06	6 rosette leaves > 1mm in length	16.23 ±0.12	16.68 ±0.09	16.41 ±0.10	16.88 ±0.08	16.27 ±0.09	16.40 ±0.12	16.00 ±0.43	16.58 ±0.11
1.10	10 rosette leaves > 1mm in length	20.46 ±0.16	20.71 ±0.14	21.52 ±0.12	22.35 ±0.16	21.00 ±0.18	20.80 ±0.15	22.12 ±0.28	22.50 ±0.22
1.14	14 rosette leaves > 1mm in length	23.92 ±0.18	24.00 ±0.15	25.44 ±0.21	26.85 ±0.15	24.77 ±0.24	24.28 ±0.19	26.91 ±0.40	27.76 ±0.32
1.18	18 rosette leaves > 1mm in length	26.84 ±03.21	26.96 ±0.17	29.11 ±0.23	31.50 ±0.30	28.73 ±0.44	28.24 ±0.22	31.67 ±0.64	34.06 ±0.61
1.22	22 rosette leaves > 1mm in length	29.62 ±0.33	29.29 ±0.20	32.53 ±0.30	36.22 ±0.42	32.32 ±0.48	31.23 ±0.66	ND	ND
1.26	26 rosette leaves > 1mm in length	32.04 ±0.37	31.78 ±0.21	ND	ND	ND	ND	ND	ND
Principal growth phase 3	**Rosette growth**								
3.20	Rosette measures 20% of its final size	17.04 ±0.40	17.21 ±0.19	17.15 ±0.23	18.08 ±0.22	12.19 ±0.26	12.25 ±0.30	12.40 ±0.42	11.00 ±0.22
3.50	Rosette measures 50% of its final size	26.48 ±0.37	26.18 ±0.25	27.35 ±0.31	29.04 ±0.42	20.93 ±0.68	20.93 ±0.71	21.16 ±1.00	20.96 ±0.67
3.70	Rosette measures 70% of its final size	30.44 ±0.41	30.57 ±0.28	32.19 ±0.28	34.25 ±0.41	26.70 ±0.87	26.29 ±0.95	26.36 ±1.25	28.26 ±0.99
3.90	Rosette growth finished	40.60 ±0.64	41.36 ±0.49	42.08 ±0.52	42.96 ±0.51	39.70 ±0.91	38.61 ±1.12	38.48 ±1.83	41.35 ±0.93
Principal growth phase 5	**Emergence of inflorescence**								
5.10	Emergence of the inflorescence meristem	35.80 ±0.30	35.43 ±0.31	34.26 ±0.44	37.69 ±0.52	36.30 ±0.41	35.80 ±0.43	36.95 ±1.19	38.44 ±0.74
Principal growth phase 6	**Flower production**								
6.00	First flower open	43.54 ±0.37	43.39 ±0.46	42.56 ±0.57	47.19 ±1.13	49.44 ±1.19	49.04 ±1.38	47.55 ±1.50	47.80 ±1.39

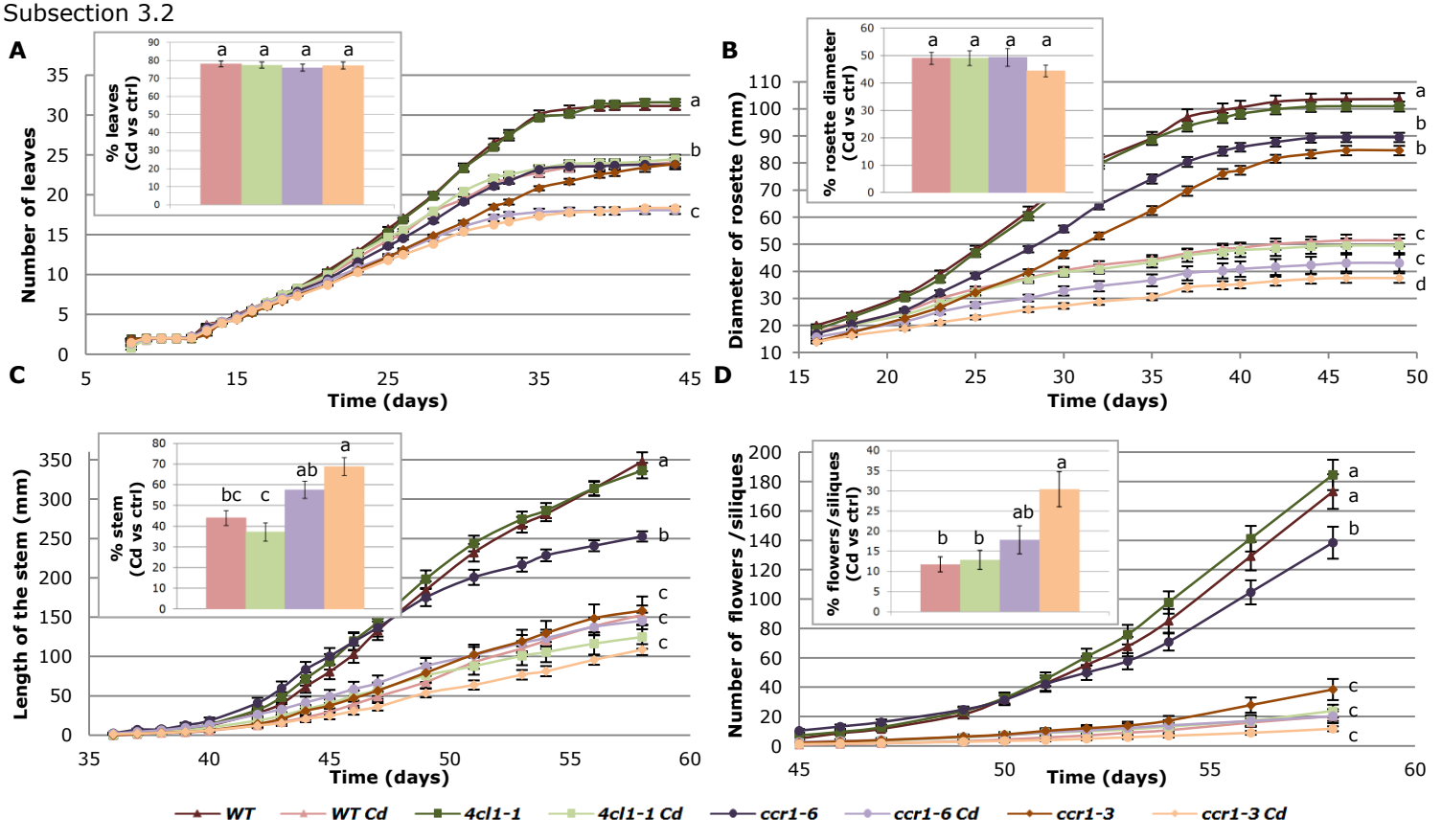


Figure 3.2.6: Both mutation of *4CL1* and *CCR1* and exposure to 3 µM $CdSO_4$ can have effects on (A) the number of leaves related to principal growth phase "one", (B) diameter of the rosette related to principal growth phase "three", (C) length of the stem related to principal growth stage "five" and (D) numbers of flowers and siliques related to principal growth phase "six". The above positioned boxes display the corresponding effect of Cd as a the percentage of the exposed plants in comparison to the non-exposed genotype. Standard errors (n=28) are indicated and statistical differences ($p<0.05$) (one or two way anova with subsequent Tukey-Kramer post-hoc test) of the endpoint of the measurement are indicated by letters.

Furthermore, root growth, assessed by the fresh weight at time point 21 days, was significantly lower for *ccr1-3* mutants though not for the other genotypes (Fig 3.2.3E).

No significant differences in emergence of the inflorescence (5.10) and opening of the first flower (6.00) were observed (Table 3.2.2). At the end of the experiment, both stem height and total number of flowers/siliques were significantly lower for *ccr1-6* and *ccr1-3* mutants in comparison to WT plants (Fig 3.2.6C and D). When taking the fresh weight of the stem into account, again *ccr1-6* and *ccr1-3* mutants were significantly decreased in growth (Fig 3.2.3C).

Moreover, also seed production from plants grown on sand was significantly negatively affected for both *ccr1* mutants as deduced from the average amount (mg) of seeds per plants and total count (number) of seeds per plant (Fig 3.2.4A and E). Interestingly, these mutants produced heavier seeds as less seeds were counted per mg seeds (Fig 3.2.4C).

Our data display a significant delay in the growth phases in hydroponics and a reduced reproductive capacity of the *ccr1* mutants in comparison with WT plants as already observed in several studies using plants with reduced CCR activity of a variety of plant species (Piquemal *et al.* 1998, Jones *et al.* 2001, Chabannes *et al.* 2001, Patten *et al.* 2005, van der Rest *et al.* 2006, Leplé *et al.* 2007, Mir Derikvand *et al.* 2008, Zhou *et al.* 2010, Vanholme *et al.* 2012a, Van Acker *et al.* 2013, Gul and Whalen 20130). The dwarfism was thought to be caused by collapsed xylem vessels and the perturbation in the flavonoid pool (Mir Derikvand *et al.* 2008). Though, it is known that ferulic acid, which is increased in *ccr1* mutants in comparison to WT plants, can decrease root and shoot growth (Devi and Prasad *et al.* 1996, Singh *et al.* 2014). Moreover, recently a dramatic increase in ferulic acid was proposed to be the cause of the dwarf phenotype (Xue *et al.* 2015).

(C) Consequence of the delayed development of ccr1 mutants for future experimental designs

Vanholme *et al.* (2012a) reported a correlation between the developmental stage with the lignin content of WT *A. thaliana* stems. Therefore, the delay in development in plants grown in hydroponics can have an effect on its

lignification and complicates the study of these *ccr1* mutants. Both, Vanholme *et al.* (2012a) and Van Acker *et al.* (2013), coped with the retardation of *ccr1* mutants by growing *ccr1-6* and *ccr1-3* mutants earlier but harvesting them at the same day. Hence, for our study, plants of a similar developmental stage were used to investigate lipid peroxidation (TBA), lignin concentration, lignin composition, Cd concentration and endophytic communities. To achieve this, the acquired knowledge from the growth phase experiment can be used. For experiments on younger plants (cultivation of roots and rosettes) a similar rosette diameter is desirable. Therefore, the *ccr1-6* mutants will be sown 2 days and *ccr1-3* mutants 4 days in advance (Fig 3.2.7A). For experiments on older plants (cultivation of roots, rosettes and stems) a similar stem length is desirable and *ccr1-6* and *ccr1-3* mutants will need to be sown respectively 1 and 10 days earlier (Fig 3.2.7B).

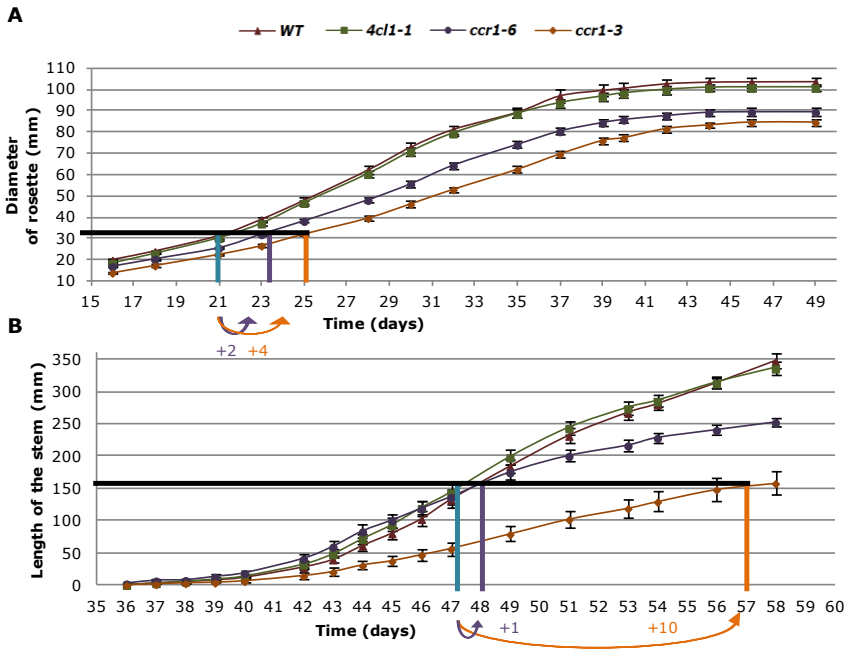


Figure 3.2.7: Optimisation of the growth system to obtain (A) rosettes of the same size (younger plants) and (B) comparable stem lengths (older plants). Black lines indicate the desirable growth at the day of harvest. Blue (WT plants, *4cl1-1* an *4cl1-2* mutants), purple (*ccr1-6* mutants) and orange (*ccr1-3* mutants) line indicates the age at the day of harvest to get to that particular growth stage. Purple and orange arrows indicate how many extra days (at least) consequently are necessary to achieve a comparable plant growth for *ccr1-6* and *ccr1-3* mutants.

(D) Effects of the Cd exposure

The effect of Cd on the growth stages of *A. thaliana* wild type plants was already described in the study of Keunen *et al.* (2011). However, the effects of Cd exposure on growth of lignin-reduced mutants was not yet considered in former studies.

As demonstrated in the study of Keunen *et al.* (2011), *A. thaliana* plants are negatively affected by Cd exposure in almost all investigated parameters related to vegetative growth and reproduction.

In our study, chlorosis, a frequently observed effect of Cd exposure (Van Belleghem *et al.* 2007, Sanità di Toppi and Gabbrielli 1999, Keunen *et al.* 2011), was observed in the VAP-based assay after approximately 11 days of exposure and no differences were found between plant genotypes (Fig 3.2.8). Though, in hydroponics the Cd-induced chlorosis was observed after 23.6 ± 0.3 days in WT plants and 23.9 ± 0.5 days in *4cl1-1* mutants (*i.e.* 17 days of exposure), in both *ccr1* mutants the colour change occurred significantly later at 25.6 ± 0.4 days in *ccr1-6* mutants and 25.9 ± 0.5 days in *ccr1-3* mutants: (*i.e.* after 19 days of exposure) (Fig 3.2.8), suggesting that the *ccr1 mutants* are less sensitive to Cd exposure. However, the starting point in chlorophyll concentration of the *ccr1* mutants may be different (Dauwe et al 2007) from WT plants which can influence this observation.

Because of the effects of the plant genotype on the studied growth phases, the best way to study the effects of Cd exposure on the growth phases is to compare for each genotype non-exposed and Cd-exposed plants.

In the VAP-based assay, growth stages 1.02 and 1.04 (2 and 4 rosette leaves more than 1 mm in length) were significantly delayed under Cd stress for all genotypes (Table 3.2.1). The relative growth of the roots of the exposed plants in comparison to the non-exposed plants was significantly higher for *4cl1-1* and *ccr1-3* mutants but no differences were observed for *ccr1-6* mutants in comparison to WT plants (Fig 3.2.2). Also the R6 stage (more than 50% of seedlings have a primary root of ≥ 6 cm in length) was delayed after Cd-exposure for WT, *4cl1-1* and *ccr1-6* plants though this was not the case for *ccr1-3* mutants (Table 3.2.1). Again, *ccr1-3* mutants seemed to be less sensitive to

Cd exposure. However, this was not the case for the other *ccr1* mutant (*ccr1-6)* for these parameters.

In hydroponics, no significant differences in Cd-induced root inhibition (% inhibition of fresh weight) were observed among the different genotypes (Fig 3.2.3F). Also no Cd-induced differences in reduction of the leaves fresh weight at day 21 (Fig 3.2.3H) and in decrease of the number of rosette leaves and diameter of the rosette at the end of the experiment were detected for the genotypes under investigation (Fig 3.2.6A and B). However, the leaf fresh weight at harvest time of *ccr1-6* mutants was significantly less ($p<0.01$) inhibited (Fig 3.2.3B). This is another indication that at least some of the lignin-reduced mutants may be more tolerant to Cd exposure.

Taking into account the reproductive systems, all studied genotypes could produce an inflorescence resulting in the formation of viable seeds. Therefore, we can conclude that the chosen concentration of 3 µM $CdSO_4$ was indeed sublethal as indicated in paragraph 3.2.2.1.

In the study of Keunen *et al.* (2011) it was demonstrated that Cd had a negative effect on the reproductive growth stages though in a different way than for the vegetative growth phases. Interestingly, reproduction of the *ccr1-3* mutants seemed to be less affected by Cd exposure since no significant delay in the opening of the first flower (growth phase 6.0) was observed while the delay was significant for all other studied genotypes (Table 3.2.2). Moreover, for the *ccr1-3* mutants the stem length inhibition was significantly lower from the decrease in WT plants and for *ccr1-6* mutants the same trend ($p=0.10$) was observed (Fig 3.2.6C). In the study of Keunen *et al.* (2011) a dose-independent decrease in flower/siliques production of 90% was reported after exposure to 5 and 10 µM $CdSO_4$. The strong decrease of approximately 90 % in WT plants was also observed in our study after exposure to 3 µM $CdSO_4$ (Fig 3.2.6D). The reproduction structures (flowers and siliques) of the *ccr1-3* mutants were less reduced (in comparison to WT plants) after Cd exposure (Fig 3.2.6D). Though, when taking the fresh weight of the stem into account, there were no significant differences in inhibition among genotypes (Fig 3.2.3D).

In plants grown in sand, the Cd-exposed *ccr1-3* mutants produced even significantly more seeds (weight) per plant in comparison to the non-exposed ones (Fig 3.2.4B) and this change was significantly different from the effect in WT plants. In addition, *ccr1-6* ($p<0.05$) produced more seeds (number) per plant (Fig 3.2.4F). We also investigated the seed's germination capacity, and this for both seeds produced in the presence of 3 µM Cd and seeds from non-exposed plants. However, no significant differences in germination were observed (data not shown).

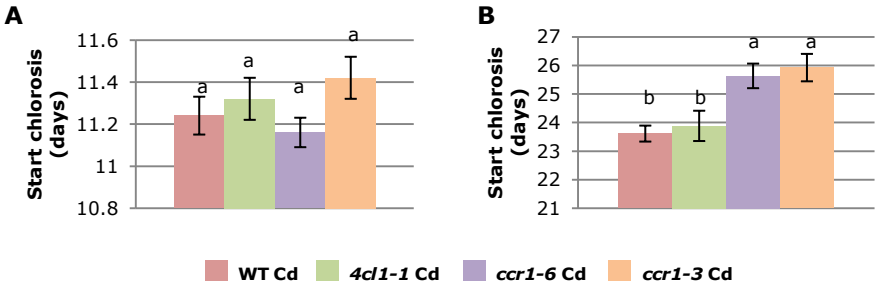


Figure 3.2.8: Start (days) of chlorosis after Cd exposure (A) in the VAP-based assay (n=25) and (B) in hydroponics (n=28) . Standard errors are indicated. Statistical differences ($p<0.05$) (one way anova with subsequent Tukey-Kramer post-hoc test) are indicated by letters.

All together, the *ccr1* mutants responded differently to Cd exposure in comparison to WT plants. In the root and leaf production, *ccr1* (or at least *ccr1-3*) mutants were less inhibited after Cd exposure.

Three hypotheses can be proposed to explain these findings:

(i) We can hypothesize that some phenolic products, that accumulate in the *ccr1* mutants, may possess ROS scavenging capacity. For example, in tomato plants with a reduced CCR activity, higher levels of soluble phenolics were found, which correlated with a higher antioxidative capacity (van der Rest *et al.* 2006).

(ii) Polyphenols are known to be metal chelators (Kovacik *et al.* 2008a, Tsao *et al.* 2010). By consequence, these organic acids may chelate Cd inside the plant and make it less toxic.

(iii) The initial growth conditions of *ccr1* mutants are not the same as for WT plants. Stress tolerance can depend on plant size, stomatal conductance and the interaction with the environment rather than the targeted metabolic process itself (Lawlor *et al.* 2013). In their review about drought stress resistance, Lawlor *et al.* (2013) stated that stress develops later in drought resistant GM

plants because of the distinct appearance of the plant (decreased total leaf area, decreased stomatal conductance, differences in photosynthetic capacity per leaf area unit, *etc.*) and therefore plants display a delayed stress onset. Indeed, the *ccr1* mutant has a lower evapotranspiration rate than WT plants under control conditions (Gul and Whalen 2013) and its delayed stress also may be explained by these effects on plant size. It this case the observed delay in stress will disappear when grown for longer periods.

Furthermore, also the reproduction of *ccr1 A. thaliana* plants seemed to be less affected, or even enhanced after Cd exposure. A similar stress-induced increase in seed production was observed in *apx2* mutants under heat stress (Suzuki *et al.* 2012). However, the underlying mechanism has still to be clarified.

3.2.2.3 Cd concentration

During our study, various growth systems were applied to produce seeds, younger (3 weeks old roots and leaves) and older plants (roots, rosettes and stems of plants which are already in the reproduction growth phases). Plants were exposed to 3 µM $CdSO_4$ for 2 or more weeks. In some cases, plants were sown at the same day and plants with the same age were produced, however in other cases an experimental set-up was adopted wherein same stem lengths at harvest were produced (see paragraph 3.2.2.2C for more information) (Vanholme *et al.* 2012a). Another growth system with different day lengths (light period of 9 h during eight weeks and 12 h during two weeks) was investigated though because of practical reasons the system was not further adopted in other experimental designs. In all these different growth systems, Cd concentrations were investigated.

The roots of both *ccr1* mutants contained significantly more Cd in comparison to these of WT plants and a similar trend was observed in both *4cl1* mutants (only significant for 4cl1-1 for one of the experiments) (Table 3.2.3). Also in leaves higher Cd concentrations were detected in the lignin-mutants; nevertheless the increases were not significant in each growth system (Table 3.2.3). Since the results appeared to be growth system dependent, no clear conclusions can be

Table 3.2.3: Cadmium concentrations (mg kg^{-1} dry weight) in roots, leaves, stems and seeds cultivated in various experimental designs with exposure to 3 µM $CdSO_4$. Standard errors are reported and significant differences (tested with one way anova with subsequent Tukey-Kramer post-hoc test) according to WT plants are indicated in red ($p<0.05$, $p<0.1$) for lower concentrations and green ($p<0.05$, $p<0.1$) for higher concentrations.

Experimental design and tissue	**WT**	***4cl1 -1***	***4cl1 -2***	***ccr1 -6***	***ccr1 -3***
Roots	**Cd concentration (mg kg^{-1} dry weight)**				
Growth system: hydroponics, same age (n=3) Exposure: from day 7 for 2 weeks to 3 µM $CdSO_4$	986 ±54	1349 ±35	1516 ±422	1479 ±134	1943 ±209
Growth system: hydroponics, same stem length (n=6) Exposure: from stem formation for 2 weeks to 3 µM $CdSO_4$	2305 ±70	2605 ±156	2896 ±480	2700 ±148	2682 ±55
Leaves	**Cd concentration (mg kg^{-1} dry weight)**				
Growth system: hydroponics, 8 weeks with 9 h daylight and 2 weeks with 16 h daylight, same age (n=3) Exposure: from day 7 for 8 weeks to 3 µM $CdSO_4$	1148 ±36	1115 ±71	ND	1442 ±104	1475 ±61
Growth system: hydroponics, 8 weeks with 9 h daylight and 2 weeks with 16weeks h daylight, same age (n=3) Exposure: from 8 weeks for 2 weeks to 3 µM $CdSO_4$	150 ±26	278 ±11	ND	358 ±46	400 ±10
Growth system: hydroponics, same age (n=3) Exposure: from day 7 for 2 weeks to 3 µM $CdSO_4$	734 ±196	991 ±202	1148 ±158	1191 ±196	1285 ±70
Growth system: hydroponics, same stem length (n=6) Exposure: from stem formation for 2 weeks to 3 µM $CdSO_4$	609 ±21	709 ±27	680 ±93	801 ±58	710 ±57
Stems	**Cd concentration (mg kg^{-1} dry weight)**				
Growth system: hydroponics, 8 weeks with 9 h daylight and 2 weeks with 16 h daylight, same age (n=3) Exposure: from day 7 for 8 weeks to 3 µM $CdSO_4$	810 ±22	930 ±11	ND	629 ±19	596 ±50
Growth system: hydroponics, 8 weeks with 9 h daylight and 2 weeks with 16weeks h daylight, same age (n=3) Exposure: from 8 weeks for 2 weeks to 3 µM $CdSO_4$	105 ±15	131 ±41	ND	67 ±4	152 ±4
Growth system: hydroponics, same stem length (n=6) Exposure: from stem formation for 2 weeks to 3 µM $CdSO_4$	1058 ±68	1128 ±78	986 ±109	1379 ±47	1169 ±170
Growth system: hydroponics, same stem length (n=5) Exposure: from stem formation for 2 weeks to 3 µM $CdSO_4$	417 ±24	415 ±31	440 ±23	437 ±40	665 ±51
Seeds	**Cd concentration (mg kg^{-1} dry weight or per 1 000 000 seeds)**				
Growth system: arasystem (sand), same age (n=3) Exposure: from stem formation until senescence to 3 µM $CdSO_4$	14.11 ±1.31	19.04 ±2.83	12.24 ±1.07	7.35 ±0.03	5.76 ±0.52
Per 1 000 000 seeds	0.36 ±0.03	0.58 ±0.09	0.37 ±0.03	0.21 ±0.001	0.77 ±0.07

drawn concerning translocation of Cd to the inflorescence and seeds of the lignin-mutants (Table 3.2.3).

Detoxification of Cd is very important for the plant to avoid free Cd-ions in the cells and consequently decrease Cd stress. The first site where Cd can be immobilized/accumulated, is in the root cell walls due to binding to carboxyl, phenolic, hydroxylic and carbonyl groups (Khan *et al.* 1984, Sanita di Toppi and Gabbrielli 1999, Van Belleghem *et al.* 2007, Babula *et al.* 2012, Lambrechts *et al.* 2013). In this regard, many studies demonstrated the use of lignin as a biosorbent for Cd and Cu in aquatic environments (Mohan *et al.* 2006). In roots, lignin can also directly bind Cd and in this way limit Cd uptake into the cell (Parrotta *et al.* 2015) which is rather a form of adsorption than absorption because the cell wall has a possible role to exclude Cd and therefore prevent toxicity (Larras *et al.* 2013, Parrotta *et al.* 2015). Because the used mutants in our study are inhibited in their lignin deposition, the cell walls will have another composition, hence another mode of adsorption might be active. Therefore, an increased deposition in the roots cell walls of the lignin-reduced mutants is not that likely since less lignin is available and the Cd-ions were also translocated to the leaves.

Different hypotheses can be made concerning the accumulation of Cd-ions and their subsequent translocation to the aerial parts in the lignin-reduced mutants.
(i) Only in root regions lacking Casparian bands (Babula *et al.* 2012), which are endodermal cells with a lot of lignin and suberin deposited in the radial and transverse cell walls, Cd can load into the xylem via apoplastic ways. However,most of the Cd must pass the root barrier symplastically. One of the mechanisms of the plant to prevent the uptake of Cd, is to strengthen the Casparian bands (Liska *et al.* 2016) and induce extra lignification (Schreiber *et al.* 1999, Schutzendubbel *et al.* 2001, Ederli *et al.* 2004, Herbette *et al.* 2006, Dauthieu *et al.* 2009) especially close to the root apex (Lux *et al.* 2011) to prevent apoplastic movement of Cd-ions towards the xylem. In a study of Akhter *et al.* (2014), barley contained a more effective barrier in its roots than lettuce and less translocation of Cd to the leaves was demonstrated. Considering the mutation in the monolignol biosynthesis pathway, one could hypothesize

that these mutants are not able to increase the lignification and therefore have less abilities to prevent incoming Cd-ions (see subsection 3.4). In some studies the abundance of lignin in the secondary cell wall is hypothesized to make plants more tolerant to Cd (Parrotta *et al.* 2015), which would imply that our lignin-reduced mutants would suffer more from Cd stress. As already discussed in paragraph 3.2.2.1 this was not the case.

(ii) Even though Cd can move as free ion, the accumulation can be influenced by different chelators originating from the plant itself (Sanita di Toppi and Gabbrielli 1999) *e.g.* metallothioneins (Lux *et al.* 2011), phytochelatins (Sanita di Toppi and Gabbrielli 1999) and organic acids (Babula *et al.* 2012, Van Belleghem *et al.* 2007). In order to detoxify, liganded Cd can also be deposited in the vacuole (Sanita di Toppi and Gabbrielli 1999, Ederli *et al.* 2004, Van Belleghem *et al.* 2007, Van De Mortel *et al.* 2008, Dauthieu *et al.* 2009, Lux *et al.* 2011).

The accumulation of different organic acids (cinnamic, *p*-coumaric, caffeic, ferulic, 5-hydroxyferulic, and sinapic acid) was found in the distinct *A. thaliana* lignin-reduced mutants (Vanholme *et al.* 2012a). Moreover, the cinnamic derivates, caffeic, ferulic and sinapic acid were found to be elevated after exposure to high Cd concentrations in *Matricaria chamomilla* roots (Kovacik *et al.* 2008a, Kovacik *et al.* 2009) and higher cinnamic acid concentrations were found in Cd tolerant plants when grown on contaminated soils in comparison with plants grown on non-contaminated soils (Marquez-Garcia *et al.* 2012). Additionally, these polyphenols are known to be metal chelators (Kovacik *et al.* 2008a, Tsao *et al.* 2010) and are even involved in root to shoot translocation in *Matricaria chamomilla* (Kovacik *et al.* 2009, Kovacik *et al.* 2011). The obtained knowledge indicates a possible role for phenolic intermediates of the monolignol biosynthesis pathway and derivatives thereof in the higher accumulation of Cd in the lignin-reduced mutants roots and leaves.

(iii) Furthermore, the endophytic or rhizospheric bacterial communities can influence Cd uptake by mobilising, extracting and translocating metals to the above-ground plant parts (Weyens *et al.* 2013, Babula *et al.* 2012, Truyens *et al.* 2013). These microbial endophytic communities change due to alterations in lignin, giving alterations of phenolic intermediates of the monolignol pathway and derivatives thereof which can be used by bacteria as carbon source (Beckers *et al.* 2016b). Consequently, these symbiotic bacteria can influence Cd uptake

and translocation. Possible effects on bacterial communities of the lignin-reduced mutants will be discussed in section 4.

(iv) NRAMP6 (natural resistance-associated macrophage protein) is a conserved bivalent metal transporter and this gene is located next to *CCR1* on chromosome 1 of the genome of *A. thaliana*. *NRAMP6* even possesses 474 bp that are antisense to the spliced gene *CCR1*. The mutant *nramp6-1* is more tolerant to Cd and over-expressors of NRAMP6 were hyper-sensitive to Cd (Cailiatte *et al.* 2009). Perhaps, the mutation of *CCR1* can have an influence on NRAMP6 activity and consequently the Cd toxicity or *vice versa*.

3.2.2.4 Element concentration

For normal growth and development, plants need both macronutrients (nitrogen (N), phosphorus (P), sulphur (S), potassium (K), calcium (Ca) and magnesium (Mg)) and micronutrients (copper (Cu), iron (Fe), manganese (Mn), zinc (Zn), chlorine (Cl), boron (B), nickel (Ni) and molybdenium (Mo)) (Nath and Tuteja 2016). Some of the above metals are essential for plant metabolism because of their function as cofactor in enzymes (*e.g.* copper (Cu), iron (Fe) and zinc (Zn), manganese (Mn)). However, plants need to balance these metals in a confined range because too low or high amounts can be detrimental for growth and development (Cuypers *et al.* 2016). Little is known about the incorporation of the essential macro- and micronutrients in lignin-reduced mutants with and without exposure to Cd. Moreover, under Cd stress, Cd-ions can compete with divalent metals (*e.g.* Zn^{2+}, Fe^{2+} and Ca^{2+}) resulting in inactivation of proteins in which the metals are essential to maintain their function (Suzuki *et al.* 2001) and consequently disturb cell functions. Therefore, in our study, we investigated the accumulation of a selection of these metals in the lignin-reduced mutants, either or not exposed to Cd.

In roots, no changes in elemental incorporation due to the mutation of *4CL1* or *CCR1* were observed in non-exposed plants.

When plants were exposed to 3 µM $CdSO_4$, WT plants revealed significant decreases in Ca, Zn and Cu (borderline significant; $p<0.1$) (Table 3.2.4). Cu deficiency in *A. thaliana* leaves, though not in roots, was also observed by Gielen *et al.* (2016) with exposure to 5µM Cd for 72h. Furthermore, Ca, Zn, Cu and Mn

were found to be decreased in maize roots after Cd exposure (Maksimovic *et al.* 2007). This can be explained by competition for the same transporters which can even result in nutrient deficiency. For instance, Ca competes with Cd at the Ca channels and Ca binding places at the plasma membrane (Nazar *et al.* 2012). Both the decrease in roots Zn and Cu concentration after Cd exposure of WT plants was not detected in all studied lignin-reduced mutants (Table 3.2.4). In plants, Zn and Cu are essential for the function of a large group of proteins including for example Cu-Zn superoxide dismutase. However, in too high concentrations, redox active metals such as Cu can also become toxic due to production of free radicals by means of the Fenton reaction (Hansch *et al.* 2009). Therefore, it is important to know if these ions are chelated (*e.g.* by metallothioneins or phytochelatins) or occur as free ions. As in WT plants, the Ca concentration in roots of the *ccr1-6*/SNBE mutants was significantly lowered after Cd exposure;. On the other hand, no differences were observed for both *ccr1* mutants (Table 3.2.4). Calcium plays a role in many processes. For instance, Ca can have an effect on rigification of cell walls since low Ca can make them more pliable and easily ruptured and also membrane structures are stabilised by binding Ca to phospoholipids (Hepler 2015). Possibly, c*cr1* mutants which already have less robust cell walls, could have more protection by these Ca ions under Cd stress in comparison to WT plants.

Moreover, Mn was lower after Cd exposure in both *4cl1* mutants (borderline significant, $p<0.1$) and the *ccr1-6*/SNBE mutants ($p<0.05$). Again, no Cd effect was detected in *ccr1* mutants (Table 3.2.4). Mn can have either a catalytic function in enzymes (*e.g*. Mn-superoxide dismutase) or can act as an activator of enzymes (*e.g.* for phenylalanine ammonia lyase) (Hansch *et al.* 2009). Furthermore, the K concentration of the *ccr1-6*/SNBE mutants decreased ($p<0.05$) after Cd exposure; but not for WT plants, *4cl1* and *ccr1* mutants (Table 3.2.4). K is not assimilated into organic matter and remains in the ionic form and is involved in many different metabolic processes (*e.g.* membrane transport and stress resistance) (Nath and Tuteja 2016). After Cd exposure, the *4cl1-1* roots showed also decreases in Mg (borderline significant, $p<0.1$) and Fe ($p<0.05$), though the decreases were not supported by the *4cl1-2* mutant and

Table 3.2.4: Elemental concentration (mg kg^{-1} dry weight) in roots and leaves of plants cultivated in sand exposed to 3 µM $CdSO_4$ for 3 weeks. Standard errors (n=4) are reported and significant differences (one way anova with subsequent Tukey-Kramer post hoc test) of the lignin-reduced mutants according to WT plants are indicated green (p<0.05, p<0.1) for higher concentrations. Significant differences after Cd exposure are indicated in orange for decreases (p<0.05, p<0.1).

ROOTS	*0 µM Cd*						*3 µM Cd*					
	WT	***4cl1-1***	***4cl1-2***	***ccr1-6***	***ccr1-3***	***ccr1-6 /SNBE***	**WT**	***4cl1-1***	***4cl1-2***	***ccr1-6***	***ccr1-3***	***ccr1-6 /SNBE***
Macronutrients (mg kg^{-1} dry weight)												
Mg	1802 ± 538	2226 ± 419	1908 ± 165	1421 ± 137	1329 ± 203	2295 ± 153	1337 ± 155	1369 ± 86	1647 ± 270	1495 ± 330	1350 ± 275	1898 ± 281
P	5965 ± 483	6541 ± 830	6454 ± 936	5261 ± 766	5594 ± 1105	6327 ± 512	4881 ± 730	5136 ± 386	5514 ± 986	5437 ± 579	5634 ± 1087	5574 ± 379
Ca	6556 ± 757	5955 ± 1044	6677 ± 1012	5551 ± 1277	5045 ± 1031	6692 ± 210	4179 ± 553	4685 ± 328	4684 ± 763	6393 ± 1634	7630 ± 2799	5112 ± 596
S	9628 ± 1140	12512 ± 1587	12221 ± 1980	8255 ± 779	9811 ± 1400	11634 ± 618	9043 ± 1275	10007 ± 811	10813 ± 2158	10587 ± 1074	11300 ± 2240	10105 ± 729
K	31669 ± 5017	40861 ± 5165	41409 ± 5981	29259 ± 2476	33494 ± 4256	41782 ± 1481	29670 ± 4017	34207 ± 2520	36017 ± 7044	36369 ± 3724	37622 ± 7603	34353 ± 3015
Micronutrients (mg kg^{-1} dry weight)												
Zn	162 ± 20	194 ± 75	137 ± 20	126 ± 41	108 ± 27	115 ± 7	79 ± 10	83 ± 14	94 ± 17	93 ± 12	132 ± 28	107 ± 19
Cu	30 ± 3	25 ± 5	25 ± 3	24 ± 4	24 ± 5	26 ± 3	19 ± 2	20 ± 1	22 ± 4	28 ± 3	30 ± 7	23 ± 3
Mn	953 ± 169	1131 ± 128	1293 ± 190	868 ± 73	1079 ± 193	1261 ± 63	654 ± 218	658 ± 157	700 ± 214	811 ± 140	882 ± 109	661 ± 59
Na	738 ± 86	603 ± 78	710 ± 94	674 ± 197	836 ± 157	786 ± 59	512 ± 90	647 ± 90	695 ± 117	751 ± 23	734 ± 171	559 ± 99
Fe	3753 ± 2497	6415 ± 995	5289 ± 1566	1985 ± 956	3865 ± 1102	7068 ± 1420	3228 ± 665	2194 ± 367	4571 ± 966	3301 ± 1721	2446 ±948	7031 ± 2402

LEAVES	0 µM Cd						3 µM Cd					
	WT	*4cl1-1*	*4cl1-2*	*ccr1-6*	*ccr1-3*	*ccr1-6 /SNBE*	WT	*4cl1-1*	*4cl1-2*	*ccr1-6*	*ccr1-3*	*ccr1-6 /SNBE*
Macronutrients (mg kg^{-1} dry weight)												
Mg	5093 ± 439	5089 ± 241	5120 ± 507	7236 ± 940	7110 ± 600	5421 ± 508	5025 ± 672	5120 ± 335	5713 ± 295	7073 ± 617	6991 ± 756	6207 ± 507
P	5782 ± 475	4978 ± 352	5556 ± 504	6900 ± 722	5737 ± 385	5288 ± 628	5797 ± 423	5875 ± 538	6509 ± 507	6301 ± 400	6767 ± 634	7101 ± 391
Ca	56092 ± 4430	53312 ± 4730	58785 ± 4479	75947 ± 6292	76054 ± 3934	55273 ± 4261	53995 ± 3452	57024 ± 2955	62367 ± 4160	78537 ± 4818	77944 ± 6691	58596 ± 3756
S	11244 ± 855	10612 ± 874	12158 ± 809	14129 ± 1060	14211 ± 1046	11269 ± 657	11951 ± 627	13753 ± 895	14620 ± 733	14622 ± 587	17314 ± 1332	14665 ± 911
K	23808 ± 1743	21272 ± 2092	24522 ± 1940	30499 ± 1890	30537 ± 1792	24762 ± 2172	24561 ± 1379	25363 ± 2220	27688 ± 3009	32632 ± 2292	34632 ± 3130	27967 ± 2259
Micronutrients (mg kg^{-1} dry weight)												
Zn	61 ± 10	66 ± 22	58 ± 13	77 ± 12	80 ± 13	48 ± 5	54 ± 4	59 ± 7	64 ± 7	84 ± 11	97 ± 6	65 ± 5
Cu	10 ± 1	10 ± 2	10 ± 1	15 ± 1	15 ± 2	10 ± 1	9 ± 0	10 ± 1	11 ± 1	15 ± 1	16 ±1	11 ± 1
Mn	280 ± 12	278 ± 13	300 ± 17	449 ± 45	419 ± 47	297 ± 17	216 ± 47	229 ± 41	249 ± 37	387 ± 57	388 ± 53	317 ± 42
Na	489 ± 62	499 ± 67	513 ± 34	552 ± 57	580 ± 21	487 ± 46	436 ± 38	502 ± 29	535 ± 28	644 ± 69	706 ± 90	520 ± 80
Fe	307 ± 102	420 ± 171	179 ± 19	375 ± 74	269 ± 31	230 ± 50	153 ± 17	239 ± 54	202 ± 44	314 ± 6	256 ±26	261 ± 106

by consequence it is not certain that the decrease is an effect of the mutation of the *4CL1* gene (Table 3.2.4).

In roots, no changes in elemental profile after Cd exposure were found for the *ccr1* mutants. This indicates that, though more Cd is taken up by roots (as discussed in paragraph 3.2.2.2), the Cd exposure has less effect on the accumulation of other elements in these *ccr1* mutants in comparison to WT.

In leaves, the opposite was observed. No significant changes in element profile were observed after Cd exposure. A small tendency to a higher S concentration was found for leaves of plants under Cd stress (Table 3.2.4), which is considered an important step in the defence to toxic concentrations of Cd (Nazar *et al.* 2012).

It is clear that the genotype was the main influencing factor for the elemental accumulation. However, Cd influenced the results in such a way that trends in elemental concentration which were observed in function of the genotype without Cd exposure became significant with Cd exposure. Similar to the concentration of Cd-ions (discussed in paragraph 3.2.2.2), the concentrations of Mg, Ca, S, K, Cu and Mn were higher in comparison with WT plants in leaves of both *ccr1* mutants in non-exposed plants. Though the increase in element concentrations was not observed for other lignin-reduced mutants.
The same genotype effects concerning the *ccr1* mutants were observed in Cd-exposed plants for Mg, Ca, S, K, Cu, and Mn. Moreover, also for Zn, Na and Fe higher concentrations in the Cd-exposed *ccr1* mutants in comparison to the Cd-exposed WT plants were observed (Table 3.2.4).
Also *4cl1-1*, *4cl1-2* and *ccr1-6*/SNBE mutants showed the same tendencies in Cd-exposed plants though only significant for the element S.

All these elements are required to maintain a normal plant metabolism (*e.g*. Cu-Zn superoxide dismutase, Mn-superoxide dismutase, metallothioneins, phytochelatins, catalase, peroxidase, *etc.*). However, some of these elements can also enhance free radical formation by the Fenton reaction and should

therefore be controlled by chelating mechanisms (*e.g.* organic acids, metallothioneins and/or phytochelatins). The increase is not surprising since we found an increased concentration of Cd in various experimental designs and plants can hardly differentiate in the uptake between metal ions. Three possible mechanisms may be responsible for the observation. (i) As discussed for Cd, one possible explanation can be the differential participation of the NRAMP transporters which are involved in divalent cation export (Nazar *et al.* 2012). However, also Na and K concentrations were increased, which indicates a more general system for accumulation of these elements. (ii) Another possibility can be the different permeability of the cell boundaries as a result of the lignin modification, making passage more easily. (iii) Lignin-reduced mutants were found to have increased concentrations of phenolic acids, which accumulate due to the blockage in the monolignol production (Vanholme *et al.* 2012a), which can chelate metals and thereby favour the entering of extra metals. (iv) Another explanation can be the
difference in development of these *ccr1* mutants (smaller rosette diameter (paragraph 3.2.2.2B)) which might influence the elemental accumulation.

However, hypotheses ii and iii are not supported by the *4cl1-1*, *4cl1-2* and *ccr1-6*/SNBE mutants in non-exposed conditions since in these mutants no differences in elemental concentrations (in comparison to WT plants) were observed while the lignin content is lower (only proven for stems of lignin-reduced plants (Van Acker *et al.* 2013, personal communication with De Meester and co-workers), Table 3.1 in the introduction of section 3). Moreover, in these mutants also phenolic compounds accumulate (only proven for leaves of *ccr1* mutants (Xue *et al.* 2015) and stems of lignin-reduced mutants (Vanholme *et al.* 2012a, personal communication with De Meester and co-workers)). Therefore, hypothesis iv (developmental effect: smaller rosettes) seems most likely.

Though in case plants were exposed to Cd the other lignin-reduced genotypes (*4cl1-1*, *4cl1-2* and *ccr1-6*/SNBE mutants) showed the same tendencies as *ccr1* mutants in comparison to WT plants and the above stated hypotheses (ii and iii) can be supported.

However, lignification of roots, the first entry site of these elements, should be examined in both non-exposed and Cd-exposed plants to understand the accumulation capacity towards these elements. Also information on the different

types of phenolic compounds and their concentrations inside the roots and leaves of lignin-reduced mutants in both non-exposed and Cd-exposed plants is needed to make further conclusions.

3.2.3 Conclusions

To investigate the effect of a genetic modification resulting in a reduced lignin content in combination with Cd exposure, different growth stages throughout the lifecycle of *A. thaliana* as well as Cd and nutrients concentrations were investigated. The most suitable Cd concentration for our study design was 3 µM $CdSO_4$.

On vertical agar plates, the *4cl1-1* mutant was slower in its root and leaf development in comparison to the WT. Though, during the later growth stages in hydroponics, no differences with WT plants were observed anymore. Therefore, the differences observed in the early growth stages seemed unimportant for our study. On the other hand, the development of *ccr1* mutants was altered in comparison to the WT *A. thaliana* plants. *ccr1* mutants took the lead on agar plates, revealing a faster development of both roots and leaves. But they lagged in development of rosettes and stems in hydroponics. Furthermore, they produced smaller rosettes (both in diameter and fresh weight) and stems (both in length and fresh weight) and fewer flowers, siliques and seeds. Also the root fresh weight of the *ccr1* mutants was lower. Therefore, to study the effects of both the genotype and Cd exposure on these plants, the differences in growth phase for *ccr1* mutants should be kept in mind or eliminated.

When plants were exposed to Cd, the lignin-reduced mutants (*ccr1* mutants and to a lesser extend *4cl1* mutants) took up more Cd in the roots and leaves in comparison to WT. Furthermore, not only Cd-ions were taken up by *ccr1* mutants in higher concentrations in comparison to WT plants but also the concentrations of several macro- and microelements were increased. This might be due to more permeable cell boundaries or the presence of metal chelating compounds (*e.g.* ferulic acid). Moreover, the lignin-mutants tended to cope better with the toxic Cd-ions since leaf chlorosis was delayed in hydroponics (for both *ccr1* mutants), inhibition of root growth was smaller on vertical agar plates

(certainly for *ccr1-3* mutants and also a trend for *4cl1-1* mutants), fresh weight of leaves was less inhibited after Cd (certainly for *ccr1-6* mutants and also a trend for *ccr1-3* and *4cl1-1* mutants*),* stem formation and flower production suffered less from Cd stress (certainly for *ccr1-3* mutants but also a trend in *ccr1-6* mutants) and seed production was even increased in both *ccr1* mutants after exposure to Cd. Moreover, in roots, Cd influenced the accumulation of other elements in WT plants, though no changes were observed for *ccr1* mutants. This may again be explained by a more permeable cell boundary of these mutants.
The different hypotheses to explain the observed effects of both the genetic modification, resulting in a reduced lignin content, and Cd exposure, *e.g.* the antioxidative defence systems, the presence of metal chelating compounds, and the influence on lignification should be further explored.

In the interest of growing lignin-reduced mutants on marginal soils to reduce the food-fuel competition, the *A. thaliana ccr1* mutants revealed a lot of promising traits such as lower sensitivity to Cd and higher concentration of Cd in the aerial parts. This can be an advantage in case *CCR1* down-regulated plants (*e.g.* poplar) will be used in phytoextraction applications while using the biomass to produce biofuel. However, *CCR1* down-regulated plants are impeded in their growth hence the increase in Cd concentration can have a low impact on the total metal amount removed. Therefore, first the negative influence of these *ccr1* mutants should be counteracted to make these plants economically attractive. Enhancing the growth of these *ccr1* mutants may be obtained by making use of endophytic bacteria with plant growth promoting capacity. Further investigation of the bacterial endophytic community is necessary to explore this option.

Subsection 3.3
The oxidative stress responses of lignin-reduced *Arabidopsis thaliana* with and without exposure to cadmium

In lignin-reduced mutants, different processes influencing the cellular redox state can be affected. Within the subset of investigated *Arabidopsis* mutants the monolignol production pathways are disturbed resulting in elevated levels of different upstream phenolic intermediates or derivatives thereof (*e.g. p*-coumaric acid in *4cl1* mutants, ferulic acid in *4cl1* and *ccr1* mutants and sinapinic acid in *ccr1* mutants) (Vanholme *et al.* 2012a). The accumulation of these phenolic compounds can result in the triggering of both anti- and pro-oxidant mechanisms (Sakihama *et al.* 2002). The different phenolic compounds can have radical scavenging properties and can therefore assist the cells defence mechanisms (Tsao *et al.* 2010, Marquez-Garcia *et al.* 2012, Rukkumani *et al.* 2014, Rui *et al.* 2016). Some phenolic compounds (*e.g. p*-coumaric acid and ferulic acid) are known to be allelopathic factors that can change membrane permeability, inhibit root growth and affect activities of stress-related enzymes (Li *et al.* 2010). Moreover, the phenolic compound ferulic acid has been proposed to be the main cause for growth retardation in *ccr1* mutants originating from its impact on ROS levels (Xue *et al.* 2015). Further, since monolignol and flavonoid production share the first steps of the pathway, disruption of the monolignol production can enhance flavonoid production by shuttling of intermediates in this direction (van der Rest *et al.* 2006, Fornalé *et al*. 2015). These flavonoids possess antioxidant capacity but can also exhibit signalling functions (Williams *et al.* 2004). Finally, lignin polymer production is a free-radical-coupling process wherein laccases and peroxidases take the leading role (Vanholme *et al.* 2008).

Besides lignin modification, also exposure to toxic concentrations of metals (*e.g.* Cd) can affect plant appearance and defence mechanisms. In the following, we will list the most relevant stress markers for our study:

(A) Pigment concentration

Chlorosis of leaves, resulting from a decrease in **chlorophyll concentration,** is a frequently observed symptom of toxic amounts of metals (including Cd) leading to an inhibition of the photosynthetic capacity (Van Belleghem *et al.* 2007, Cuypers *et al.* 2016).

(B) Lipid peroxidation

A second valuable marker to evaluate oxidative stress level is lipid peroxidation (Skórzynska-Polit *et al.* 2010, Cuypers *et al.* 2011). **Lipid peroxidation** is known to be a fast response to a broad spectrum of stimuli and is necessary for a rapid defence response without the need of large numbers of receptors (Spiteller 2003). Furthermore, toxic amounts of metals can alter membrane architecture (Chaoui *et al.* 1997, Spiteller 2003, Smeets *et al.* 2005) by changing the degree of lipid peroxidation (Jozefczak *et al.* 2014, Rui *et al.* 2016, Keunen *et al.* 2016a). Moreover, the radical-driven polymerisation of the monolignol building blocks takes place in the cell wall near the cell membrane (Vanholme *et al.* 2008) and non-enzymatically produced lipid peroxide radicals can attack phenolic compounds resulting in additional phenoxy radicals composing lignin like structures (Spiteller 2003).

(C) Hallmark genes for a general oxidative stress response

Also gene expression and their corresponding enzyme capacities can be altered due to toxic concentrations of metals. For instance, five **hallmark genes for a general oxidative stress response (*OX1*, *OX2*, *OX3*, *OX4* and *OX5*)** are up-regulated after exposure to various stresses such as ozone or treatment with ROS-generating agents (*e.g.* methyl viologen, 3-aminotriazole or a fungal toxin; Gadjev *et al.* 2006). Analysis of the expression levels of these stress markers can provide information about the strength of the general oxidative stress response due to the T-DNA knockout of *4CL1* or *CCR1* and Cd exposure.

(D) Activity of several antioxidative enzymes and the expression of the corresponding genes

During metal stress different reactive oxygen species (superoxide ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH\cdot$)) can be produced causing

damage to various cell components. After Cd exposure, this ROS production happens in a non-redox active manner (Sharma *et al.* 2012). When plants are exposed to Cd, the **activity of several antioxidative enzymes and the expression of the corresponding genes** can be altered in an attempt to restore the ROS equilibrium (Jozefczak *et al.* 2014). To gain insight into the role of oxidative stress in different lignin-reduced mutants, with and without Cd exposure, we analysed the capacity of various enzymes with antioxidant activity and measured the expression level of the corresponding genes. The superoxide ($O_2^{-}\cdot$), generated during signalling or under stress conditions, can be detoxified by **superoxide dismutases (SOD)** resulting in the formation of less toxic hydrogen peroxide (H_2O_2) (Kliebenstein *et al.* 1998). SOD gene expressions and capacities have been reported to increase under different metal stress conditions (*e.g.* Cd, Cu, Al, Mn, Fe and Zn) (Semane *et al.* 2007). In *A. thaliana*, different SODs are known such as the iron SOD (FSD1), copper-zinc SODs (CSD1 and CSD2*)* and manganese SOD *(*MSD1*)* (Mittler *et al.* 2004). The produced H_2O_2 can be further detoxified by catalases or peroxidases. H_2O_2 scavenging **catalases (CAT)** are mainly present in peroxisomes and take part in maintaining the cells redox balance (Mhamdi *et al.* 2010).

In addition, antioxidants such as ascorbic acid and glutathione are necessary during defence against metal stress. They are coupled by different enzymatic conversions in the ascorbate-glutathione cycle (Mittler *et al.* 2004). First, H_2O_2 is converted to H_2O by making use of ascorbate (AA) as reducing agent, a reaction performed by **ascorbate peroxidases (APX)**. The recovery of ascorbate is coupled with the oxidation of glutathione (GSH) (Cuypers *et al.* 2016). The cellular pool of glutathione is recovered in the reduced state by **glutathione reductases (GR)** (Mittler *et al.* 2004).

Also other peroxidases are important stress-induced players. For instance, **guaiacol peroxidases (GPX)**, which reduce H_2O_2, are present in the cell wall (Vianello *et al.* 1997) where they play a role in the cell wall lignification (Cuypers *et al.* 2002, Lee *et al.* 2007). Guaiacol is a common artificial aromatic electron donor and therefore peroxidases that oxidize guaiacol are referred to as guaiacol peroxidases (GPX) (Vianello *et al.* 1997)**.** Furthermore, syringaldazine is a monolingol analogue and *in vitro* all cell wall peroxidases can oxidise syringaldazine. Therefore, also **syringaldazine peroxidase (SPX)** capacity is

an interesting parameter with regard to oxidative stress and lignification (Pang *et al.* 1989).

(E) Expression of the genes corresponding to pro-oxidative enzymes

During metal stress, the production of reactive oxygen species (ROS) will not only indicate damage but will also play an important role in **signalling** with **pro-oxidative (ROS producing) enzymes** as major contributors (Cuypers *et al.* 2016). **Lipoxygenases (LOX)** cause lipid peroxidation but can also catalyse the dioxygenation of polyunsaturated fatty acids producing hydroperoxy fatty acids, which can activate downstream production of other signalling molecules (*e.g.* jasmonates and oxylipins) (Smeets *et al.* 2008, Mochizuki *et al.* 2016). Additionally, NADPH oxidases of *A. thaliana*, encoded by the **respiratory burst oxidase homologs (*RBOH*) genes,** can catalyse the production of $O_2^{-}\cdot$ and are located at the plasma membrane. The produced $O_2^{-}\cdot$ can be converted spontaneously or by SOD activity to H_2O_2 (Liu *et al.* 2016), which can function as signalling molecule to regulate hundreds of genes during various physiological and developmental states (Lee *et al.* 2013). *RBOHC, D* and *E* genes are known to be induced by a variety of stressors (Sagi and Fluhr 2006). Furthermore, a concurrent burst in the production of H_2O_2, most likely caused by the expression of *RHOH* genes and subsequent increased RBOH capacity (Barcelo 1998, Barcelo 2005), during lignification is proposed. For example, RBOHC was found to be involved in ROS production during cell wall modifications in root tips (Lee *et al.* 2013).

(F) Metal chelation

During metal stress, **chelation of metals** in the cytosol is a mechanism of detoxification and intracelllular binding of metals can influence plasma membrane passage (Clemens 2001, Hassinen *et al.* 2011). Both thiol-based (*e.g.* metallothioneins, phyotochelatins) and non-thiol-based (*e.g.* organic acids and amino acids) mechanisms are involved in the metal homeostasis and tolerance to high concentrations of metals (Hassinen *et al.* 2011, Anjum *et al.* 2015). The activity of metal sequestering pathways can have an influence on metal translocation to the upper parts of the plant (Haydon and Corbett 2007). The best-known metal chelating compounds are **metallothioneins (MT)**, which

are low molecular weight proteins containing cysteine residues. Under normal conditions MTs are involved in maintaining the homeostasis of essential metals (macro- and micronutrients) (Leszczyszyn *et al.* 2013). Furthermore, they can also be involved in ROS scavenging (Hassinen *et al.* 2011). Another group of well-described metal chelators are phytochelatins, which are produced by **phytochelatin synthase (PCS)**. Phytochelatins are oligomers of glutathione, which can be induced by Cd exposure. When bound to the metal, they can be transported into the vacuole for further detoxification (Anjum *et al.* 2015).

(G) Stress-related lignification

Finally, the expression of **genes corresponding to enzymes involved in stress-related lignification** may be affected by both the lignin modification (and the resulting metabolic and physiological changes) and exposure to Cd. During monolignol production, **phenylalanine ammonia lyase (PAL)** catalyses the first step of the general phenylpropanoid pathway. Increases in PAL activity have been reported under various stressors (Moura *et al.* 2010, Huang *et al.* 2010). Furthermore, PAL1 and PAL2 are involved in the production of flavonoids, substances with antioxidative capacities (Huang *et al.* 2010). Another gene involved in the monolignol pathway which can be induced during stress responses is **cinnamoyl CoA reductase 2 (*CCR2*)** (Lauvergeat *et al.* 2001). Furthermore, *CCR2* can also be used as an alternative route in *ccr1* mutants to bypass the lack of CCR1 capacity (Mir Derikvand *et al.* 2008).

Because metal concentrations are different in roots and leaves, it is likely that Cd can have a different influence on the redox balance in different tissues (Cuypers *et al.* 2011). Also Smeets *et al.* (2008) suggested that generation of superoxide due to Cd exposure is more prevalent in the roots while H_2O_2 can have a function in leaves. Therefore, these two plant organs will be discussed separately.

It can be hypothesized that both the genetic modification in the monolignol production, and by consequence lignin content, and exposure to toxic amounts of metals (*e.g.* Cd) can affect various oxidative stress responses. In this

subsection we aim to comprehend these responses by exploring the above described plant defence mechanisms.

3.3.1 Experimental design

Arabidopsis thaliana plants (WT, *4cl1-1*, *ccr1-6* and *ccr1-3*) were grown in a hydroponics system (see paragraph 3.1.2.3) for 1 week where after half of the plants were exposed to 3 µM $CdSO_4$ (see paragraph 3.2.2.1) during 2 weeks. At harvest, roots and rosettes were snap frozen in liquid nitrogen and stored at -70°C. Lipid peroxidation was measured in older plants with infloresence stems (see subsection 3.1.2.3). We evaluated (i) pigment concentration (see paragraph 3.1.3.5); (ii) lipid peroxidation (see paragraph 3.1.3.6); and (iii) differences in the capacities of stress-related enzymes (see paragraph 3.1.3.3) and their gene expression levels (see paragraph 3.1.3.4) were investigated. More details about the used assays are reported in subsection 3.1.

3.3.2 Results and discussion

3.3.2.1 Pigment concentration

Without Cd exposure, *ccr1* mutants tended to have a higher pigment concentration in comparison with the WT plants. The elevated pigment concentration was mainly due to higher concentrations of chlorophyll *a* (significant for *ccr1-6* mutants) (Fig 3.3.1A). Increases in chlorophyll *a* were previously observed in leaves of *CCR*-down-regulated tobacco plants (Dauwe *et al.* 2006) suggesting alterations at the level of photosystems and by consequence the photosynthetic capacity in *ccr1* mutants. No significant differences were observed for the *4cl1-1* mutants in comparison with the WT plants without Cd exposure.

After exposure to Cd, the pigment concentration in rosette leaves significantly decreased for all genotypes (Fig 3.3.1A). However, the decrease was significantly lower in the *ccr1-3* mutants in comparison to the decrease in pigment concentration due to Cd-exposure in WT leaves. Furthermore, the smaller inhibitory effect of Cd in *ccr1-6* ($p=0.38$) and *4cl1-1* ($p=0.20$) mutants in comparison to the inhibitory effect of Cd in WT plants was observed as a trend. The smaller inhibitory effect was mainly due to the significant difference

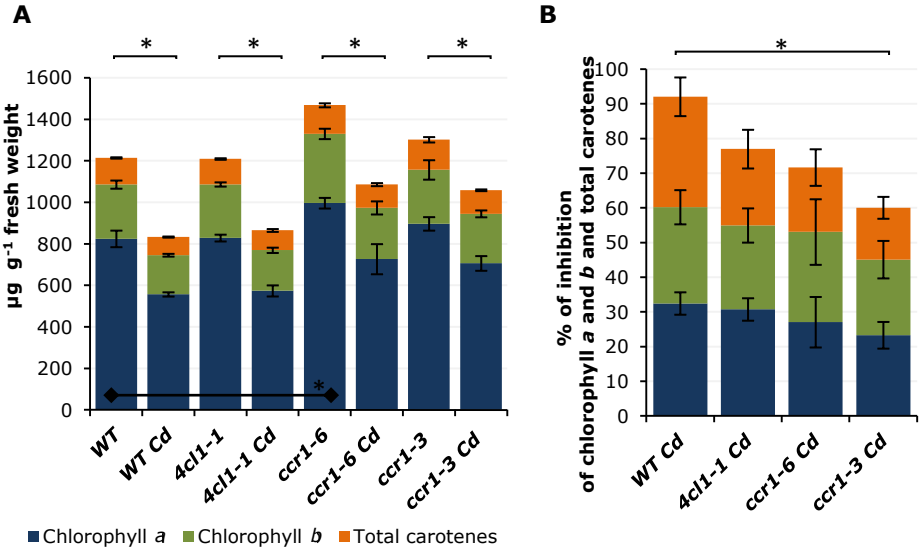


Figure 3.3.1: (A) concentration of chlorophyll *a*, chlorophyll *b* and total carotenes (in µg g^{-1} fresh weight); (B) percentage of inhibition of chlorophyll *a*, chlorophyll *b* and total carotenes due to Cd exposure (Cd-exposed in comparison to non-exposed plants). Data represent averages of five replicates with standard error. Statistical significance (one or two-way anova and the Tukey-Kramer post-hoc test): * $p<0.05$. Black bar indicated the significant difference in chlorophyll *a* between WT and *ccr1-6*.

in inhibition of the total level of lipid-soluble antioxidant carotenes between WT and *ccr1* mutants (*ccr1-6* ($p<0.05$) and *ccr1-3* ($p<0.05$))(Fig 3.3.1B). Carotenes play an important role in photo-protection of chlorophylls against photo-oxidative damage (Dai *et al.* 2006). Therefore, the lower inhibition of carotenes in *ccr1* mutants (as compared to WT plants) may potentially indicate that photosynthesis in these mutants will be affected less severely under Cd stress. Hence, we can tentatively speculate that lignin-reduced mutants are less affected by the toxic Cd-ions at the level of the leaves, where photosynthesis takes place. The lower stress level near the leaves was already evident from a delayed chlorosis during the growth phases assay (see paragraph 3.2.2.2D). However, in the review of Lawlor *et al.* (2013) a difference in photosynthetic capacity per leaf area unit was related to a delayed stress onset. Consequently, we must be cautious in interpreting the obtained results because the pigment concentrations of the *ccr1* mutants without Cd-exposure were already different indicating the possible existence of interaction effects between the lignin modification and Cd exposure.

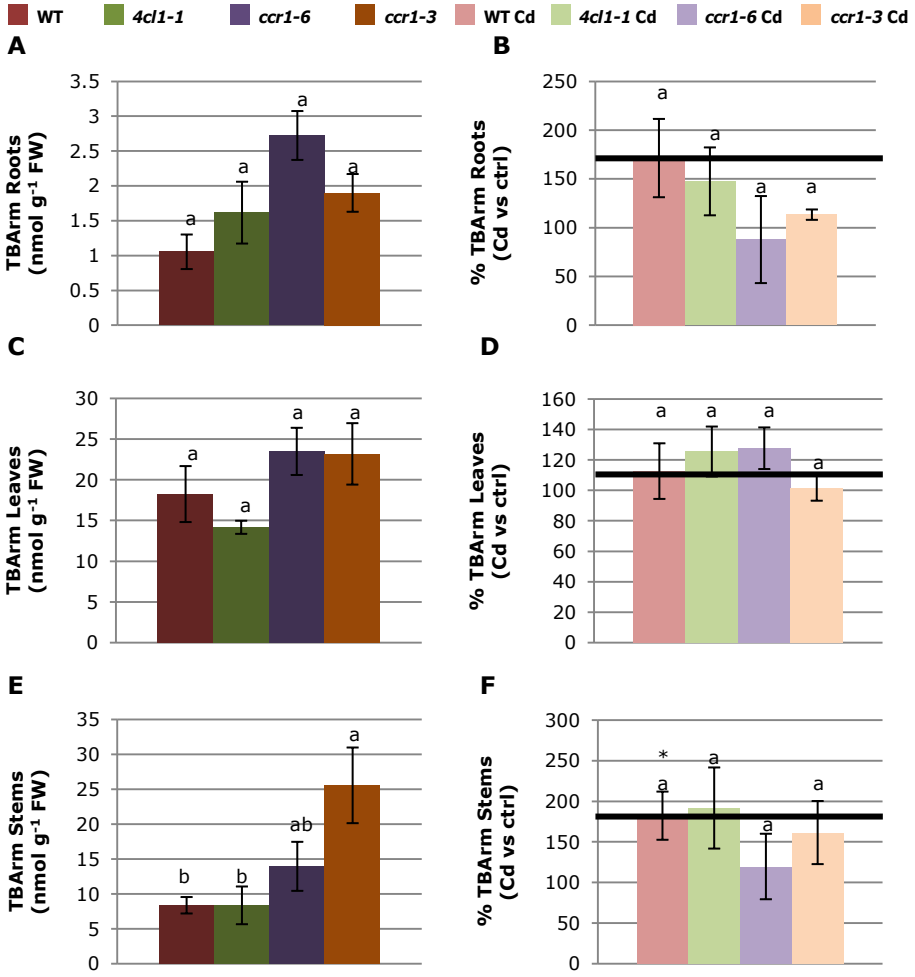


Figure 3.3.2: Lipid peroxidation (TBArm in nmol g^{-1} fresh weight (FW)) in (A) roots, (C) leaves and (E) stems with a similar development and the percentage of change due to exposure at the moment of stem formation for the remaining 2 weeks in (B) roots, (D) leaves and (F) stems. Data represent averages of four replicates with standard error. Statistical significance (one way anova and the Tukey-Kramer post-hoc test) between genotypes is indicated with letters: $p<0.05$, *: significant effect of Cd $p<0.05$.

3.3.2.2 Lipid peroxidation

Secondly, we evaluated the lipid peroxidation of WT plants and lignin-reduced mutants with and without Cd exposure. In the **non-exposed** plants, no significant differences were observed in the concentrations of TBA reactive molecules in the **roots and leaves** of lignin-reduced mutants in comparison to WT plants (Fig 3.3.2A and C). In the **stems**, *ccr1-3* mutants showed a significantly higher lipid peroxidation in comparison to WT stems (Fig 3.3.2E). Different explanations can be conceived for the increase in lipid peroxidation in *ccr1-3* mutants: (i) It can be an indication of difficulties during stem formation as *ccr1-3* mutants are known to produce a smaller stem and stem formation is slower in comparison to WT plants. Since the *ccr1-3* mutants display collapsed xylem vessels, and consequently may have less strength in their stems (Mir Derikvand *et al.* 2008), the plasma membrane structure can be altered and lipid peroxidation can be triggered (Spiteller 2003); (ii) Increases in lipid peroxidation products have already been associated with age and senescence (Spiteller 2003). Therefore, because our study design used plants with similar development, age may have influenced lipid peroxidation; (iii) Phenolic intermediates and derivatives thereof accumulating in lignin-reduced mutants can significantly enhance lipid peroxidation in a non-enzymatic way and are possibly the driving forces for these observations (Oracs *et al.* 2007, Singh *et al.* 2009, Li *et al.* 2010); (iv) Previously, increases in lipid peroxidation have been demonstrated to correlate with increased lignification (Lee *et al.* 2007, Rui *et al.* 2016). However, since the lignin content of the *ccr1-3* mutants is lower than in the WT, the increased lipid peroxidation cannot be linked to the lignin content in our study. Nevertheless, the increased lipid peroxidation can be an enzymatic signalling pathway to attempt to compensate for the reduced lignin deposition in *ccr1-3* mutants.

The increase in lipid peroxidation in different plant tissues due to Cd exposure is a well-known effect (Smeets *et al.* 2009). After **Cd exposure**, lipid peroxidation tended to increase in **roots** of WT plants and *4cl1* mutants. In *ccr1-6* and *ccr1-3* mutants, we did not observe any effect of the Cd-exposed plants compared to the non-exposed plants (Fig 3.3.2B) and *ccr1* roots tended to respond differently in comparison to WT plants considering lipid peroxidation after Cd exposure.

However, no strong conclusions can be drawn since lipid peroxidation can be an indication of oxidative damage or signalling and results were not statistically supported. These indications should be further investigated to reveal biological significance.

In the **leaves**, we observed highly comparable concentrations of TBA reactive molecules across all genotypes after Cd exposure (Fig 3.3.2D).

An increase in lipid peroxidation due to Cd exposure was observed in **stems** of WT plants though not in lignin-reduced mutants indicating that lignin-reduced mutants may be less sensitive to Cd-exposure. However, no notable differences in lipid peroxidation were detected due to the genetic modification to reduce the lignin content when the effects (%) of Cd were compared (Fig 3.3.2F).

On average, the results from the lipid peroxidation measurements indicate that the studied plant genotypes (WT plants, *4cl1* and *ccr1* mutants) did not experience strong Cd stress as only significant Cd-induced increases in lipid peroxidation were observed in stems of WT plants. The absence of significant increases or decreases in the lipid peroxidation may be related to the experimental exposure set-up: (i) The low significant results can be due to the moderate Cd concentration of 3 µM $CdSO_4$ used throughout the study. (ii) Due to the longer exposure time (2 weeks) to the moderate Cd concentration, plants could have adapted to a new physiological equilibrium and former damage could already have been repaired. However, reversely, these data may indicate that the *ccr1* mutant deals with the Cd stress in a different way than the WT. Possibly the distinct flavonoid pool with phenolic groups may quench some lipid radicals or chelate Cd ions (Skórzynska-Polit *et al.* 2010). In the future, investigating the abundances of possible Cd chelating phenolic compounds (*e.g.* ferulic acid, sinapinic acid, *etc.*) in these lignin-reduced mutants under Cd stress may help explain the differences in response to Cd in comparison to WT plants.

3.3.2.3 Capacities of stress related enzymes and their corresponding gene expression

(A) Effects of the lignin-reduced genotype in roots

The influence of the genetic modification of *4CL1* and *CCR1* in roots are summarised in figure 3.3.3. Detailed information about the numerical data and statistical significances can be found in supplemental table 3.3.1 for enzymatic capacities and supplemental table 3.3.3 for gene expression. Both not normalised and graynorm normalised data are analysed to estimate the "gray zone" of uncertainty due to both potential technical and biological variation (Remans *et al*. 2014).

***Hallmark oxidative stress marker genes* (*OX1*, *OX2*, *OX3*, *OX4* and *OX5*)**

Due to the *ccr1* mutation, *OX4* (cytoplasm) and *OX5* (plastid) mRNA levels were significantly lower (although not statistically significant for *OX5* in *ccr1-3* mutants with graynorm normalisation ($p=0.15$)) as compared to the levels of WT roots (Fig 3.3.3). Hence, we can hypothesize that, under normal conditions, *ccr1* mutants experience lower stress levels resulting in lower mRNA levels of *OX* genes at their roots in comparison to WT plants. *OX5*, belongs to a group of genes classified for their role in defence, was down-regulated in the stems of *4cl1* and *ccr1* mutants (Vanholme *et al.* 2012a). However, no differences in mRNA levels of the *OX* genes were observed in *4cl1-1* mutants. These differences in defence response between *4cl1-1* and *ccr1* mutants might be an additional explanation why some lignin-reduced mutants develop to a lesser extend in comparison to others. However, it is difficult to separate the cause from the effect since the delayed senescence can have an impact on the expression of these stress-related genes as reviewed by Lawlor *et al.* (2013). Hence, in *ccr1* mutants the delayed senescence may also influence the expression of genes.

ROS scavenging enzymes

A higher APX capacity in roots of *ccr1-3* mutants was observed (Fig 3.3.3). Therefore, it is possible that the ascorbate-glutathione cycle in the cytosol was more active in this type of mutant. A possible explanation for these observations

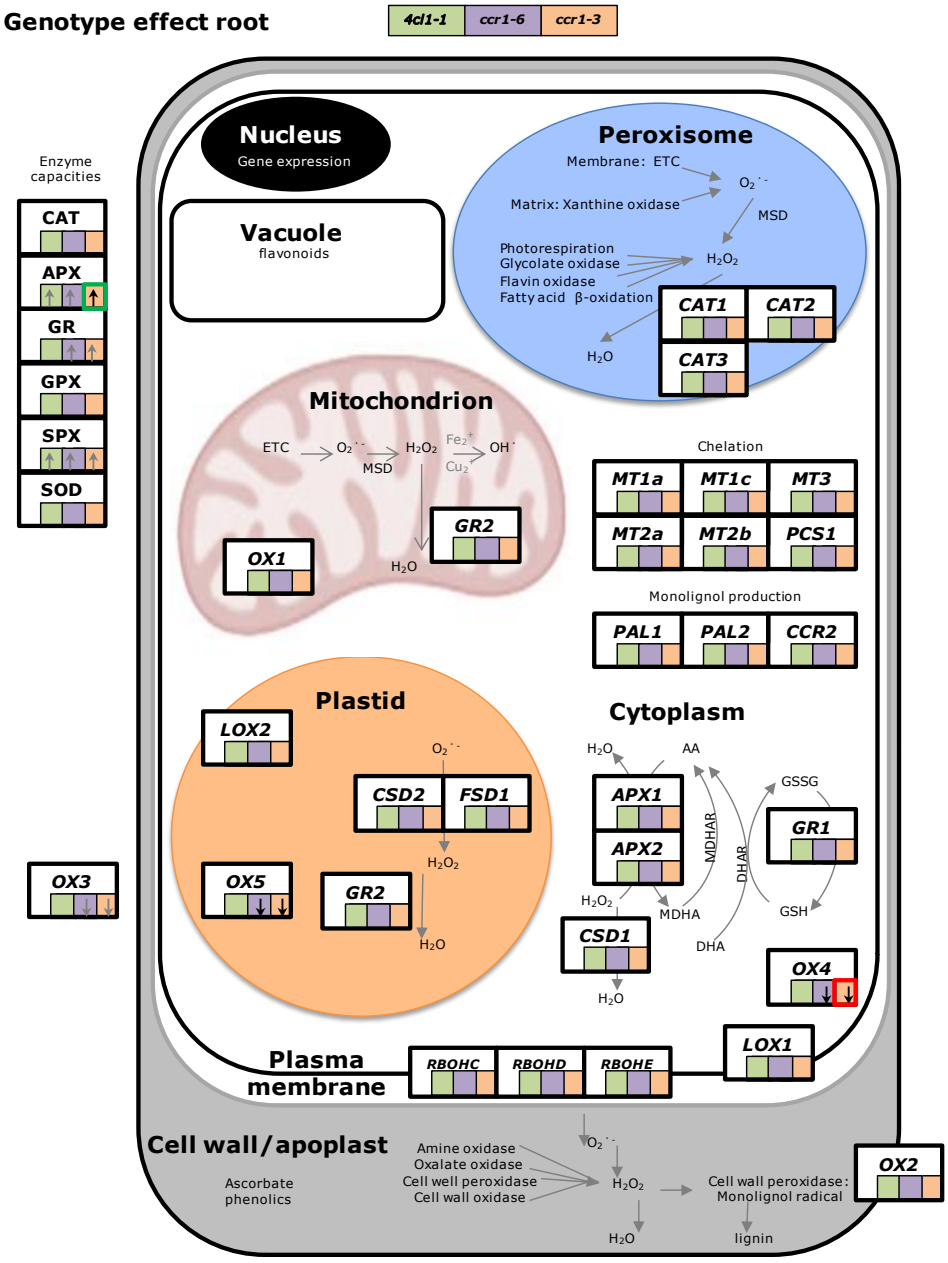


Figure 3.3.3: Overview of differences originating from the **T-DNA knockout of *4CL1* or *CCR1*** on enzyme capacities and differential expression levels of genes in the **root** under control conditions compared with WT plants. *OX3* is placed outside the cell because no specific localisation is known. Red or green framed arrows indicate significant differences in enzyme capacity or in case of gene expression significant differences for both normalisations, black arrows without a box indicate significant differences in gene expression for only one of both normalisations, grey arrows indicate other trends.

Abbreviations in fig 3.3.3: 4-coumarate:CoA ligase (4CL), ascorbic acid (AA), ascorbate peroxidase (APX), catalase (CAT), cinnamoyl-CoA reductase (CCR), Cu-Zn type superoxide dismutase (CSD), dehydroascorbate (DHA), dehydroascorbate reductase (DHAR), electron transport chain (ETC), Fe type superoxide dismutase (FSD), guaiacol peroxidase (GPX), glutathione reductase (GR), reduced glutathione (GSH), oxidized glutathione (GSSG), hydrogen peroxide (H_2O_2), lipoxygenases (LOX), monodehydroascorbate (MDHA), monodehydroascorbate reductase (MDHAR), manganese superoxide dismutase (MSD), metallothioneins (MT), superoxide radical ($O_2^{-}\cdot$), hydroxyl radical (OH·), oxidative stress marker gene (OX), phenylalanine ammonia-lyase (PAL), phytochelatin synthases (PCS), respiratory burst oxidase homologs gene (RBOH), syringaldazine peroxidase (SPX)

can be the induction of capacities of ascorbate peroxidases and other peroxidases by fenolic acids such as ferulic acid (Vianello *et al.* 1997, Devi and Prasad *et al.* 1996, Singh *et al.* 2014). Indeed, ferulic acid has been found to be increased in *4cl1* and *ccr1* stems (Vanholme *et al.* 2012a). However, Xue *et al.* 2015 did not demonstrate higher concentrations of ferulic acid in roots of *ccr1 A. thaliana* mutants in comparison to WT plants.

ROS producing enzymes, metal chelation and lignification

The perturbation in the lignin biosynthesis had little to no effect on ROS producing enzymes, metal chelation potential and lignification in the roots of the *4cl1-1*, *ccr1-6* and *ccr1-3* mutants (Fig 3.3.3).

(B) Effects of the lignin-reduced genotype in leaves

Detailed information about the influence of the *4CL1* and *CCR1* mutations are presented in supplemental table 3.3.1 for enzymatic capacities and supplemental table 3.3.3 for not normalised and graynorm normalised gene expression. All these data are summarised in figure 3.3.4.

Hallmark oxidative stress marker genes (OX1, OX2, OX3, OX4 and OX5)

In the leaves, in conjunction with the results from the roots, *ccr1* mutants displayed slightly lower expression values of some oxidative stress marker genes in comparison with WT plants (*OX2, OX3, OX5)* (Fig 3.3.4). As discussed in paragraph 3.3.2.3A, these lower levels of expression of oxidative stress marker genes can possibly be due to a delay in expression of stress genes influenced by the delay in senescence of *ccr1* mutants.

Subsection 3.3

ROS scavenging enzymes

The expression of *CAT2,* whereof the enzymatic isoform is most operative in the peroxisome, was significantly higher in *ccr1-3* mutants while the expression of *CAT3* tended to be lower (only significant with graynorm normalisation (not normalised: p=0.38)). *CAT2* capacity decreases during the lifecycle in *A. thaliana* leaves (Zimmerman *et al.* 2006). Considering the retardation in growth of the rosettes of the *ccr1-3* mutants, the elevated levels of *CAT2* mRNA might be due to the difference in growth stage. However, CAT capacity can also be induced by addition of ferulic acid as demonstrated in mung bean hypocotyls and *Zea Mays* roots and shoots (Devi and Prasad 1996, Singh *et al.* 2014). Consequently, the increased ferulic acid concentration (Xue *et al.* 2015) in the *ccr1-3* mutants might be responsible for the slight increase in the *CAT2* expression.

The GR capacity was slightly higher in the leaves of the *ccr1-6* mutants. Furthermore, we observed higher mRNA levels ($p<0.05$) of *GR1* (cytosolic) in *4cl1-1* mutants in comparison to WT plants and *GR2* expression was higher in leaves of *ccr1-3* mutants ($p<0.05$) (Fig 3.3.4). In the leaves, significantly higher levels in *APX1* mRNA were observed in all lignin-reduced mutants in comparison to the WT plants though *ccr1-6* mutants did only reach a significance level of $p<0.1$ and *APX2* expression was only higher ($p<0.05$) in *ccr1-3* mutants and not for other genotypes (Fig 3.3.4).

Most likely, the presence of ferulic acid, a compound which is more available in lignin-reduced mutants (proven in stems by Vanholme *et al.* (2012a) and leaves by Xue *et al.* (2015)), induces the oxidation of ascorbate and up-regulation of different ROS scavenging enzymes (Singh *et al.* 2009) and may indirectly cause the higher gene expression levels of ascorbate peroxidases.

To summarize, like in the roots, the ascorbate-glutathione cycle in the cytosol may be more active in lignin-reduced mutants comparison to WT plants. Though, no elevated stress levels could be observed in the expression of the *OX4* marker, indicating no extra cytosolic stress (Fig 3.3.4). An alteration in the ascorbate-glutathione cycle leading to photo-oxidative stress has already been suggested in *CCR* down-regulated tobacco plants (Dauwe *et al.* 2007).

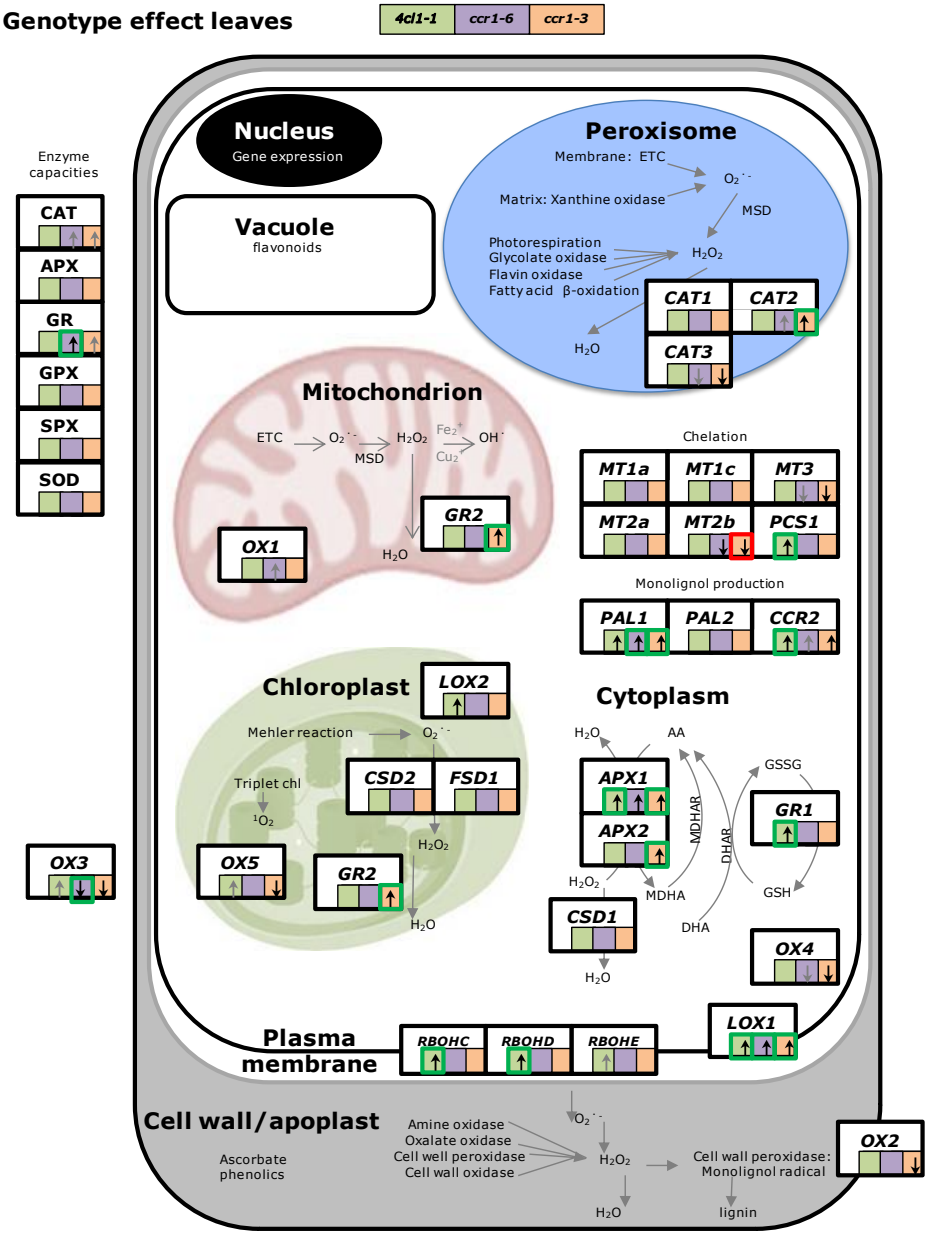


Figure 3.3.4: Overview of differences originating from the **T-DNA knockout of *4CL1* or *CCR1*** on enzyme capacities and differential expression levels of genes in the **leaves** under control conditions compared with WT plants. *OX3* is placed outside the cell because no specific localisation is known. Red or green framed arrows indicate significant differences in enzyme capacity or in case of gene expression significant differences for both normalisations, black arrows without a box indicate significant differences in gene expression for only one of both normalisations, grey arrows indicate other trends. Abbreviations: see figure 3.3.3.

ROS producing enzymes

LOX1 gene expression was significantly higher in *4cl1-1*, *ccr1-6* and *ccr1-3* leaves and *LOX2* also displayed higher expression values in *4cl1-1* leaves (borderline significant) in comparison to WT plants (Fig 3.3.4). Furthermore, significant higher levels in *RBOHC* and *D* mRNA were observed in the *4cl1-1* mutants (Fig 3.3.4). Since the lignin content is lower in *4cl1-1* mutants (although not proven for leaves), it can be assumed that the extra *RBOH* induction in comparison to the WT plants has a role in signalling to increase cellular defence functions rather than supporting the polymerisation of lignin by supplying the necessary superoxide ($O_2^{\cdot-}$) that can be converted into H_2O_2 which is needed for the peroxidases to produce monolignol radicals. Both *LOX* and *RBOH* gene expression patterns suggest that the lignin-reduced mutants experience some level of stress possibly related to the perturbation in the lignin biosynthesis, which requires the induction of stress-related signalling pathways.

Metal chelation

No differences in mRNA levels of *MT1a*, *MT1c* and *MT2a* were observed in leaves. However, both *ccr1* mutants showed lower gene expressions for *MT2b* and *MT3* (Fig 3.3.4). Several hypotheses could potentially explain these lower mRNA levels: (i) metallothioneins are essential for homeostasis of essential metals (*e.g.* Cu) (Leszczyszyn *et al.* 2013) and therefore a different translocation of essential metals to the leaves can be related to the change in *MT* expression in *ccr1* mutants; (ii) differences in organic acids (*e.g.* ferulic acid) in comparison to WT plants, which also scavenge metals and ROS, can perhaps alter the need for *MT* expression in metal scavenging processes; (iii) since *MT3* gene expression levels are particularly enhanced in senescence of leaves (Leszczyszyn *et al.* 2013), and *ccr1* mutants are delayed in their growth and senescence, observed differences could be due to a difference in growth stage.

Next to the effect on MTs in the *ccr1* mutants, a higher gene expression ($p<0.05$) of *PCS1* was observed in the *4cl1-1* mutant in comparison to the WT plants (Fig 3.3.4). In addition to metal chelation, the PCS enzymes also serve a role during detoxification of electrophilic compounds by glutathionylation through catabolising the glutathione conjugates to γ-Glu-Cys derivates (Blum *et al.* 2010). Perhaps the leaves of *4cl1-1* mutants are actively defending

themselves against accumulation of potentially harmful compounds from the interrupted monolignol pathway.

Lignification

PAL1 (significant for both *ccr1* mutants) and *CCR2* (significant for *4cl1-1* mutants) revealed higher mRNA levels in leaves of lignin-reduced mutants in comparison to WT plants (Fig 3.3.4). The up-regulation of *PAL1* and *CCR2* were already demonstrated in stems of *A. thaliana 4cl1* and *ccr1* mutants (Vanholme *et al.* 2012a). They postulated that the increase is a reaction to compensate for the lignin reduction. Although, phenolics (*e.g.* ferulic acid and *p*-coumaric acid) may also increase PAL activity (Li *et al.* 2010, Moura *et al.* 2010).

(C) Effects of cadmium in roots

The effects of Cd in roots of both WT plants and the lignin-reduced mutants (*4cl1-1, ccr1-3, ccr1-6)* are displayed in figure 3.3.5. Detailed information can be found in supplemental table 3.3.2 for enzymatic capacities and supplemental table 3.3.4 for not normalised and graynorm normalised gene expression.

Hallmark oxidative stress marker genes (OX1, OX2, OX3, OX4 and OX5)

After Cd exposure, the steady-state presence mRNA levels of the *OX2* gene (encoding an extracellular defence like protein) were elevated ($p<0.05$) in the roots of all plant genotypes (not significant for *4cl1-1* mutants) (Fig 3.3.5). In the roots of the *ccr1* mutant, the expression of *OX3* and *OX5* (plastid) were significantly ($p<0.05$) up-regulated after Cd exposure. Similar, elevated gene expressions of *OX5* were also observed in WT plants although only significant for graynorm normalised data (*OX5*: not normalised $p=0.18$) (Fig 3.3.5). These increases in *OX2*, *OX3* and *OX5* were already detected after short term exposure of wild type plants to 5 µM Cd (Jozefczak *et al.* 2015). Cd exposure can give rise to stress in the roots, which is the first site where Cd enters the plant. Higher stress levels observed in the *ccr1* mutants, as evaluated by the *OX2*, *OX3* and *OX5* gene expressions, may possibly be related to a higher concentration of toxic Cd ions in the roots of the *ccr1* mutants (paragraph 3.2.2.3).

Subsection 3.3

ROS scavenging enzymes

Cd-exposed WT plants and *4cl1-1* mutant roots showed significant increases in CAT capacity, a well-known Cd-effect in roots (Cuypers *et al.* 2011, Smeets *et al.* 2009), whereas no changes were observed in the roots of *ccr1* mutants. However, gene expression of *CAT1*, *CAT2* and *CAT3* did not support the raise in enzyme capacity for WT plants and *4cl1-1* mutants (Fig 3.3.5). Here, it appears that the main ROS defence systems in peroxisomes of the *ccr1* mutants are less needed, which could lead to a lower Cd-induced stress level in the peroxisomes. Peroxisomes are organelles involved in metabolic processes (*e.g.* fatty acid β-oxidation, photorespiration, and metabolism of reactive oxygen species (ROS) and reactive nitrogen species (Rodríguez-Serrano *et al.* 2009) but peroxisomes can also act as a sink for H_2O_2 produced outside the peroxisomes (Mhamdi *et al.* 2010). Consequently, the source of the perceived stress can also be located elsewhere in the cell.

Further, *FSD1* (plastid) expression was higher for all genotypes after Cd exposure although only significantly for both *ccr1* mutants and borderline significant for WT plants (Fig 3.3.5). These increases are in agreement with earlier studies where increases in *FSD1* expression were mainly found in roots of wild type plants (Smeets *et al.* 2009, Cuypers *et al.* 2011, Opdenakker *et al.* 2012, Keunen *et al.* 2013, Schellingen *et al.* 2015, Jozefczak *et al.* 2015, Smeets *et al.* 2013). It is likely that, in contrast to the peroxisomes, root plastids of *ccr1* mutants experience a higher stress level since both *FSD1* and *OX5* (genes of which the proteins are active in plastids) are significantly increased. The best-known effect of Cd on plastids is the change in lipid composition and degree of peroxidation in plastids (Filek *et al.* 2010). However, for *ccr1* mutants (in crude extracts) we did not observe a significant effect of Cd exposure on the lipid peroxidation.

Furthermore, the GR capacity was significantly higher in crude root extracts of Cd-exposed WT plants, *4cl1-1* and *ccr1-6* mutants in comparison to non-exposed conditions; this is a known Cd effect for WT plants (Smeets *et al.* 2009, Cuypers *et al.* 2011). However, in *ccr1-3* mutants no prominent GR capacity change after Cd exposure was observed (Fig 3.3.5). The enzyme capacity determinations were performed in crude root extracts and by

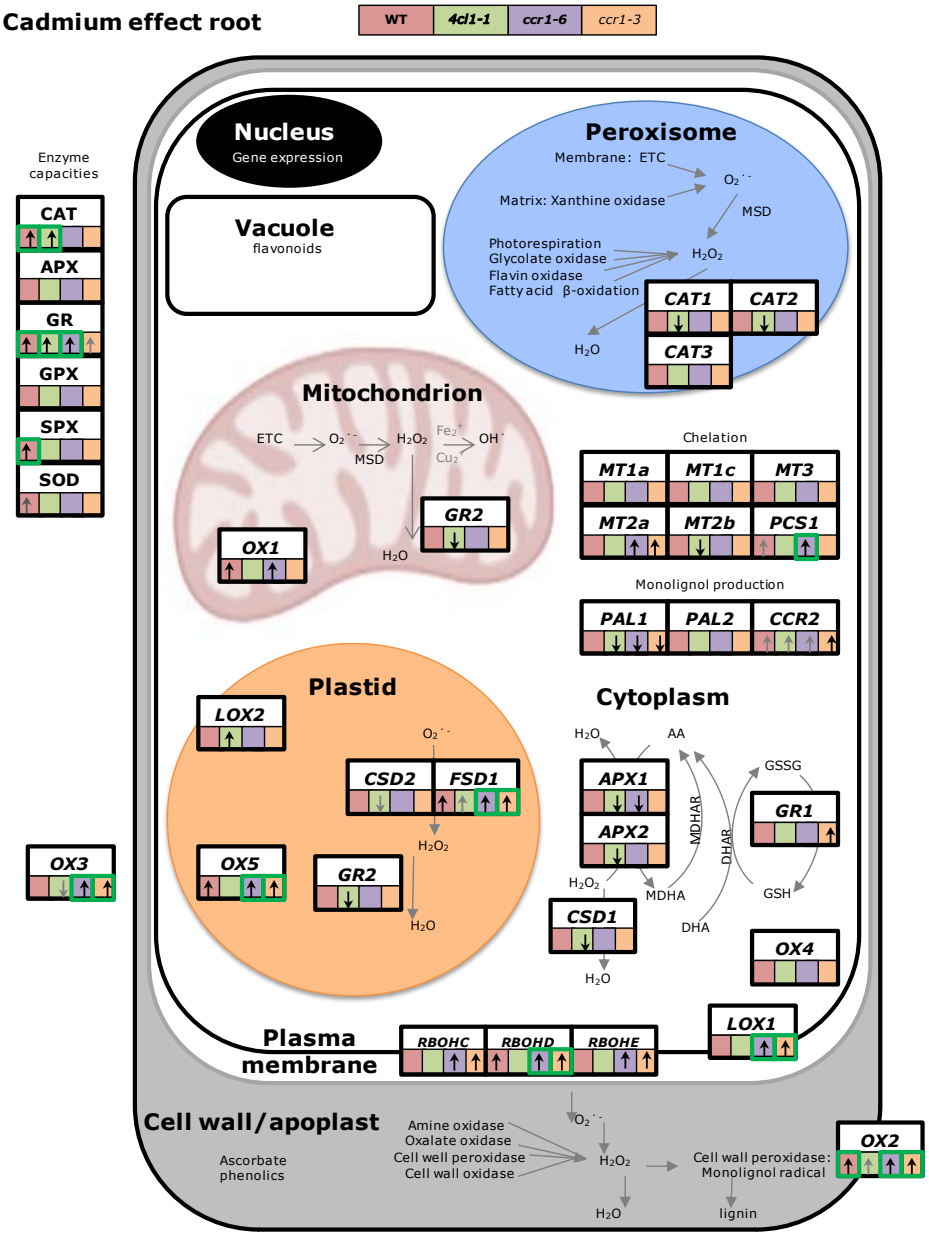


Figure 3.3.5: Overview of differences originating from **cadmium exposure** in enzyme capacity and differential expression levels of genes at the **roots**. *OX3* is placed out of the cell because no specific localisation is known. Red or green framed arrows indicate significant differences in enzyme capacity or in case of gene expression significant differences for both normalisations, black arrows without a box indicate significant differences in gene expression for only one of both normalisations, grey arrows indicate trends. Abbreviations: see figure 3.3.3.

consequence it is impossible to even hypothesize about the cellular compartment(s) where the GR capacity is increased. Moreover, the gene expression levels of *GR* did not support the observed raise in enzyme capacity.
However, it is difficult to estimate the stress levels based on these expression levels of *APX, GR* and *OX* genes in the cytoplasm and mitochondria because gene expression levels and enzyme capacities in these compartments do not display clear patterns.
For the SPX capacity a significant increase was detected in WT plants after Cd exposure which was less or even not existing in the lignin-reduced mutants (Fig 3.3.5). Syringaldazine is an analogue to lignin monomers, therefore the activity of this peroxidase is coupled to the lignification process. Here it can be presumed that in WT plants, lignification in Cd-exposed conditions could be altered whereas the lignification effect could be less in lignin-reduced mutants.

ROS producing enzymes

Cadmium exposure is known to increase *LOX1* expression in WT plants (Schellingen *et al.* 2015, Opdenacker *et al.* 2012, Keunen *et al.* 2013, Smeets *et al.* 2013). In our study, these increases in *LOX1* expression were only significant in *ccr1-6* and *ccr1-3* roots. *LOX2* expression was only slightly increased in *4cl1-1* mutants (not normalised $p<0.05$, graynorm $p<0.1$) (Fig 3.3.5). However, lipid peroxidation measurements indicated a more modest increase due to Cd in *ccr1* mutants in comparison to WT plants (no significance) (see paragraph 3.3.2.2). It might be the case that the lipid peroxidation in WT plants is due to damage and in *ccr1* mutants it is rather involved in signalling. Also, the expression of all analysed *RBOH* genes (*RBOH C, D* and *E*) was significantly up-regulated after Cd exposure in *ccr1* mutant roots (Fig 3.3.5) as observed for WT plants after Cd exposure by Smeets *et al.* (2009), Opdenacker *et al.* (2012), Cuypers *et al.* (2011), Schellingen *et al.* (2015a), Smeets *et al.* (2013), Keunen *et al.* (2013). In our study, *ccr1* roots may encounter another level of stress at their plasma membrane related to the alteration of the lignin biosynthesis, in comparison to WT. Perhaps the high Cd concentration (as discussed in paragraph 3.2.2.3) can be the cause for a higher stress response.

Metal chelation

No conclusions about the need for both *MT* and *PCS1* expression and by consequence possible changes in protein activity of the corresponding enzymes under Cd stress in lignin-reduced mutants can be drawn.

Lignification

In roots, *CCR2* mRNA levels tended to be higher (although only significant for non normalised data in *ccr1-3* mutants) after exposure to 3 µM $CdSO_4$ for all plant genotypes (Fig 3.3.5). The expression of *CCR2* has already been associated to biotic stress (Lauvergeat *et al.* 2001) and also under the abiotic stress of nitrogen depletion, increased expression of the *CCR2* gene was observed (Olson *et al.* 2007). However, no relation with metal toxicity could be found in literature. Next to *CCR2*, also *PAL1* can be involved in abiotic environmental stressors (Olsen *et al.* 2008). In soybean and *Matricaria chamomilla,* PAL activity can be induced in roots by Cd toxicity but no augmentation due to Cd was observed in Lupine (Pawlak-Sprada *et al.* 2011). However, no increases in the expression of *PAL1* and *PAL2* were shown in WT plants and even decreases in *PAL1* were observed in *ccr1* mutants (Fig 3.3.5). *PAL1* is involved in both monolignol and flavonoid production. Therefore, both production pathways can be influenced dissimilarly in lignin-reduced mutants in comparison to WT plants by Cd exposure.

(D) Effects of cadmium in leaves

Detailed information about the effect of Cd in leaves can be found in supplemental table 3.3.2 for enzymatic capacities and supplemental table 3.3.5 for not normalised and graynorm normalised gene expression. All effects are summarised in figure 3.3.6.

Hallmark oxidative stress marker genes (OX1, OX2, OX3, OX4 and OX5)

In contrast to the roots, the gene expression of *OX1* (corresponding enzyme activity located in the mitochondria) was significantly increased in WT plants and *4cl1-1* leaves after Cd exposure and no significant results were obtained for *ccr1* mutants (Fig 3.3.6). The effect of Cd on *OX1* in WT plants was already observed after exposure for 48h (Schelllingen *et al.* 2015, Jozefczak *et al.* 2015). The

4cl1-1 mutant had reduced ($p<0.05$) expression levels of *OX3* (cellular localisation unknown) and *OX5* (corresponding enzyme activity located in the chloroplast) as compared to the non-exposed *4cl1-1* mutants (Fig 3.3.6). These results suggest that lignin-reduced mutants (both *4cl1* and *ccr1* mutants) were less affected after exposure to Cd as compared to the WT plants. In paragraph 3.2.2.3 it was demonstrated that leaves of mutants contained higher levels of Cd. Therefore, it is very likely that Cd is present in a chelated form or located in the vacuole.

ROS scavenging enzymes

In leaves, only *4cl1-1* mutants displayed a higher CAT capacity (borderline significant) after Cd exposure (Fig 3.3.6). Increased CAT capacity after Cd exposure was already described in WT plants Semane *et al.* (2007). The higher CAT capacity suggests another level of H_2O_2 in the peroxisomes of these *4cl1-1* mutants due to Cd exposure. Furthermore, in the *4cl1-1* mutant a significant increase of *CAT2* expression in combination with a decrease in *CAT3* mRNA level was observed (Fig 3.3.6). In leaf cell peroxisomes, CAT2 is considered to act as the major H_2O_2 detoxifying enzyme (Kangasjarvi *et al.* 2012). It is likely that the increase in CAT capacity in *4cl1-1* mutants is due to the CAT2 isoform. For all tested lignin-reduced mutants the *CAT1* expression was significantly decreased (*ccr1-3* mutants only confirmed by graynorm normalisation) after Cd exposure though not in WT plants (Fig 3.3.6). This decrease in *CAT1* expression may indicate a lower need for defence against Cd toxicity and by consequence a lower stress level in the lignin-reduced mutants.

In our study, no significant differences in SOD capacity were observed in leaves after exposure to 3 µM Cd in all the genotypes. No significant results were obtained for *FSD1* expression and decreases in *CSD1* and *2* expression were detected in *ccr1-6* mutants and *ccr1-3* mutants (Fig 3.3.6). In WT plants, it has been suggested that oxidative stress in leaves is not caused by H_2O_2 after short term exposure to Cd (Smeets *et al.* 2008, Smeets *et al.* 2009, Cuypers *et al.* 2011, Schellingen *et al.* 2015). Our data supports this, using a longer exposure time of 2 weeks to Cd, since no differences in SOD capacity and no differences or lower levels of gene expression of corresponding genes were observed.

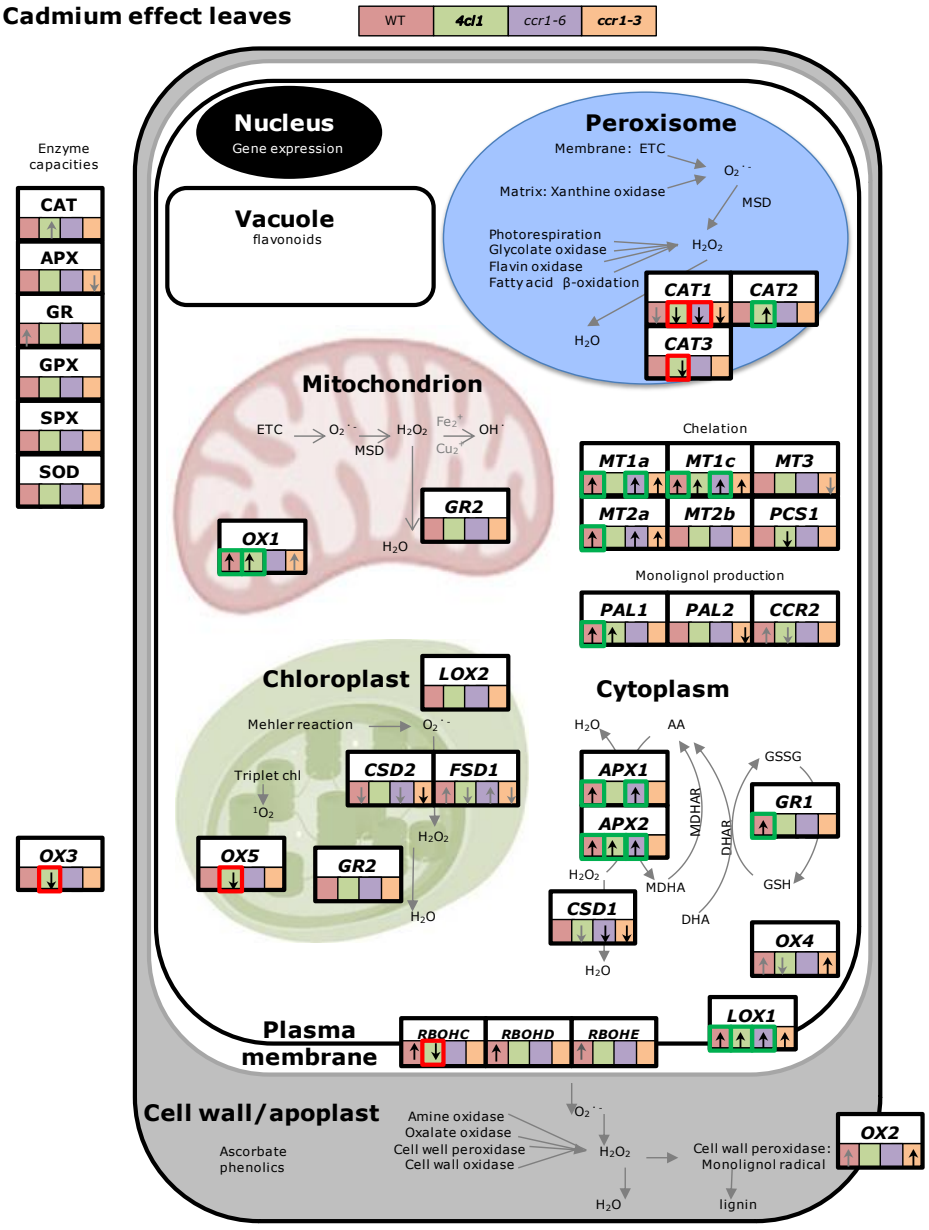


Figure 3.3.6: Overview of differences originating from **cadmium exposure** in enzyme capacity and differential expression levels of genes at the **leaves**. *OX3* is placed out of the cell because no specific localisation is known. Red or green framed arrows indicate significant differences in enzyme capacity or in case of gene expression significant differences for both normalisations, black arrows without a box indicate significant differences in gene expression for only one of both normalisations, grey arrows indicate trends. Abbreviations: see figure 3.3.3.

Subsection 3.3

A slight, though not significant (p=0.13) increase of GR capacity, a well-known effect of Cd (Chaoui *et al.* 1997, Semane *et al.* 2007), was observed in WT plants. The increase of GR capacity was absent in *ccr1* mutants (significant difference in comparison to WT plants) (supplemental table 3.3.2)). These observations were supported by *GR1* gene expression, which was significantly up-regulated in WT plants (as also demonstrated by Smeets *et al.* 2008, Smeets *et al.* 2009 and Schellingen *et al.* 2015) and no changes were detected in lignin-reduced mutants (Fig 3.3.6). No Cd-induced alterations were observed in *GR2* mRNA levels (corresponding enzyme activity located in the chloroplast/mitochondrion) (Fig 3.3.6), as already reported for WT plants by Schellingen *et al.* (2015a/b) after 72 h exposure to 5 and 10 µM Cd. These results indicate that the cytosolic *GR1* plays a major role during Cd responses of WT plants though not for lignin-reduced mutants. Again, a lower Cd stress in *ccr1* leaves can be suggested.

APX capacity was found to be increased after short-term exposure (24 h) (Cuypers 2011) and after a 1 week of exposure to 1 µM Cd though not for 10 µM Cd exposure (Semane 2007). In our study, no Cd-induced changes were observed on the APX capacity in leaves (Fig 3.3.6). These results suggest that, after a rather long-term exposure (2 weeks), the levels of APX are restored to the normal levels. Also significant increases in *APX2* were observed in leaves of WT plants (as reported by Cuypers *et al.* 2011, Keunen *et al.* 2013) and the *4cl1-1* and *ccr1-6* mutants while no changes were detected in the *ccr1-3* mutants. Furthermore, significant increases in *APX1* levels were observed in leaves of WT plants (as reported by Smeets *et al.* 2008, Smeets *et al.* 2009 and Cuypers *et al.* 2011) and *ccr1-6* mutants while no changes were found in leaves of the *4cl1-1* and *ccr1-3* mutants (Fig 3.3.6). Since both GR and APX (enzyme capacities and corresponding gene expression levels) did respond differently in the *ccr1-3* mutant (in comparison to WT), it can be that the ascorbate-glutathione cycle could be less important for the response of the *ccr1-3* mutant to Cd stress. However, this effect on the ascorbate-glutathione cycle was not observed for the *ccr1-6* mutant; therefore, no overall conclusion can be drawn (Fig 3.3.6).

ROS producing enzymes

In the leaves of all genotypes, *LOX1* gene expressions levels were significantly higher after Cd exposure (Fig 3.3.6) (as also reported by Smeets *et al.* 2008, Smeets *et al.* 2009, Keunen *et al.* 2013 and Schellingen *et al.* 2015 for leaves of WT plants). Also *RBOHC and D* genes tended (borderline significant) to be up-regulated in the leaves of WT plants after Cd exposure as reported for WT plants by Keunen *et al.* (2013). However, in the lignin-reduced mutants no increases were observed and even a significant decrease was found for mRNA levels of *RBOHC* in *4cl1-1* mutants (Fig 3.3.6). These results suggest that the used lignin-reduced mutants experience another stress level compared to WT plants at their cell boundaries, most likely related to the perturbation in the lignin biosynthesis.

Metal chelation

When plants were exposed to 3 µM Cd, various *MT* genes were up-regulated in the different plant genotypes. Up-regulation of *MT1a* expression was significant in WT plants and *ccr1-6* mutants, borderline significant in *ccr1-3* mutants, up-regulation of *MT1c* expression was significant in WT plants and *ccr1-6* mutants and borderline significant in *ccr1-3* and *4cl1-1* mutants; up-regulation of *MT2a* expression was significant in WT plants and borderline significant in *ccr1-6*. No changes were observed for *MT2b* and *MT3* expression levels. Also no Cd-induced changes were detected on the expression level of *PCS1* level in leaves of WT plants (as observed under Cd stress by Jozefczak *et al.* 2015). In *4cl1-1* mutants, *PCS1* was down-regulated (borderline significant for both normalisations) although the *4cl1-1* mutant showed very high expression without exposure which may be unnecessary under Cd exposure (Fig 3.3.6). To conclude, the *MT* and *PCS1* genes appear to participate in the same manner or even to a minor extend to implement Cd detoxification in lignin-reduced mutants compared to WT.

Lignification

After Cd exposure, the expression level of *PAL1* was significantly increased in leaves of WT plants and borderline significant for the *4cl1-1* mutant while no changes were observed for the *ccr1* mutants (Fig 3.3.6). Increased PAL activity due to Cd exposure was also observed in fonds of an aquatic fern (Dai *et al.*

2016). Again suggesting that the used lignin-reduced mutants experience less stress, the need for a Cd-induced increase in lignification is thus lower.

3.3.3 Conclusions

Plant stress responses consist of many players which can be switched on and off by means of damage and signalling in different plant organs and cellular compartments. Therefore, the puzzle to understand what happens during these stress responses is very complex. Here we tried to understand which defence responses are activated in mutants of the lignin biosynthesis pathway, without and with Cd exposure.

In **roots of non-exposed plants**, no effect on lipid peroxidation was observed (Fig 3.3.2). The activity of the ascorbate-glutathione cycle may be higher in roots of *ccr1-3* mutants (increases of APX capacity). Moreover, the expression of stress-related marker genes (*OX4* and *5*) was lower in roots of *ccr1* mutants in comparison to WT plants (Fig 3.3.3).

On the other hand, **non-exposed *ccr1* leaves** contained more pigments (especially chlorophyll *a* for *ccr1-6*) suggesting differences in photosynthetic capacity (Fig 3.3.1). This, together with the growth retardation makes it hard to study these mutants. As suggested by Lawlor *et al.* (2013) some stress responses can be partly explained by the difference in overall plant appearance.
We observed elevated levels of stress defence mechanisms related to the peroxisomes of *ccr1-3* leaves and the ascorbate-glutathione cycle in lignin mutant leaves. However, oxidative stress marker genes were slightly lower in the *ccr1* leaves in comparison to WT plants. Based on the effects of the genotypic modification on *MT2b* and *MT3* expression in leaves, metal chelation may be less required in *ccr1* mutants in comparison to WT plants indicating a possible existence of alternative routes to maintain homeostasis of essential elements. Moreover, all tested lignin mutant leaves showed a different need for stress signalling at their cell boundaries (expression levels of *LOX1* and *RBOH*). Also expression of *PAL1* and *CCR2* was higher in the leaves of the lignin-reduced mutants as a possible attempt to restore lignin content (Fig 3.3.3).

In **stems**, *ccr1-3* mutants showed a higher lipid peroxidation (Fig 3.3.2) which can be an indication of damage or it can have a signalling function.

It can be concluded for both roots and leaves that in non-exposed conditions lignin-reduced mutants and particularly *ccr1* mutants, differences in the capacities of the defence system against oxidative stress were revealed. However, it is difficult to conclude whether these lignin-reduced mutants are less or more stressed compared to WT.

Although differences in responses to **Cd exposure** were observed among genotypes **in roots** (Fig 3.3.5), it is too simplistic to claim that one genotype is less sensitive to Cd toxicity at their roots. In plastids of *ccr1* roots, indications for a higher stress levels were observed (*FSD1*, *OX5* expression). Furthermore, it may be that *ccr1* roots were experiencing the toxicity at their cell boundaries differently from WT plants (*LOX1*, *RBOHC*, *D* and *E*) though no Cd effect on lipid peroxidation (related to LOX) was detected. However, in roots of Cd-exposed WT plants lipid peroxidation rather increased (Fig 3.3.2). After Cd exposure, SPX capacity only increased in WT roots while *PAL1* expression remained unchanged in WT plants but rather decreased in *ccr1* mutants, suggesting a different response of lignification after Cd exposure. The higher capacities of CAT in roots after Cd exposure in WT plants and *4cl1-1* mutants, which was not observed for *ccr1* mutants, can indicate genotype differences in defence reactions near the peroxisome.

In contrast to what we observed in the roots, **in leaves** (Fig 3.3.6) all measured parameters point in the same direction and stress levels tended to be lower in *ccr1* mutants (and partly in *4cl1* mutants) due to **Cd exposure**. Pigment concentrations were less diminished by Cd toxicity in lignin mutant leaves (Fig 3.3.1). The *OX* (no reaction in *OX1* for *ccr1* mutants while it increased in WT plants and down-regulation of *OX3* and *5* for *4cl1-1* mutants), *CAT1* (down-regulated for lignin mutants though not for WT), *CSD1* (slightly down-regulated for lignin mutants though not for WT), *GR1* (up-regulated for WT plants but not for mutants) and *RBOH* (slightly up-regulated for WT plants but not for mutants)

gene expression levels in leaves suggest that, after Cd exposure, lignin-reduced mutants experienced less stress than the WT plants.

Also genes involved in lignification (*PAL1* and *CCR2*) were up-regulated in leaves of WT plants which was not the case for the lignin-reduced mutants. However, *MT* genes and *PCS* genes changed in the same or even a lesser extent in comparison to leaves of WT plants. Therefore, detoxification by these gene products may not explain the observed difference in response to Cd exposure. Moreover, in **stems** of Cd-exposed plants, lipid peroxidation was increased in WT plants though not in lignin-reduced mutants indicating the lignin-reduced mutants may be less sensitive to Cd exposure.

Consequently, it is more likely that other chelating molecules are present in these mutants to detoxify toxic Cd ions. Good candidates are the phenolic intermediates and derivatives thereof, which are found to be higher in the lignin-reduced mutants (Vanholme *et al.* 2012a, Xue *et al.* 2015). In future experiments the presence of different phenolic compounds with antioxidant and metal-chelating capacities and their abundances should be explored.

Supplementary information

Supplemental table 3.3.1: Enzyme capacities in **roots and leaves without Cd exposure** indicated in units gram^{-1} fresh weight (± standard error) (n=5). Significant (one way anova and the Tukey-Kramer post-hoc test) higher enzyme capacities due to the genotype are indicated in green ($p<0.05$) and lower enzyme capacities in red ($p<0.05$).

Effect of genotype	**ROOT**				**LEAVES**			
Enzyme	**WT**	***4cl1-1***	***ccr1-6***	***ccr1-3***	**WT**	***4cl1-1***	***ccr1-6***	***ccr1-3***
CAT	9.57 ± 0.84	8.00 ± 0.34	10.11 ± 0.24	10.23 ± 1.41	43.73 ± 5.84	46.66 ± 10.02	59.80 ± 5.33	69.29 ± 12.40
APX	0.13 ± 0.02	0.19 ± 0.02	0.18 ± 0.01	0.22 ± 0.02	0.26 ± 0.06	0.34 ± 0.09	0.19 ± 0.02	0.32 ± 0.08
GR	0.24 ± 0.02	0.19 ± 0.03	0.29 ± 0.02	0.28 ± 0.01	0.99 ± 0.14	1.17 ± 0.13	1.53 ± 0.10	1.37 ± 0.13
GPX	191 ± 33	166 ± 24	223 ± 29	200 ± 19	27.69 ± 3.26	37.76 ± 8.29	21.60 ± 1.56	22.50 ± 3.88
SPX	16.30 ± 1.90	22.64 ± 4.29	22.67 ± 3.59	25.12 ± 1.68	21.17 ± 4.85	25.09 ± 3.15	18.00 ± 1.08	20.15 ± 3.56
SOD	6.53 ± 0.78	7.09 ± 1.18	8.62 ± 0.96	8.47 ± 0.63	12.93 ± 1.12	13.67 ± 1.22	15.6 ± 0.98	13.22 ± 0.71

Supplemental table 3.3.2: Enzyme capacities in **roots and leaves with Cd exposure** relative to the non-exposed genotype (± standard error) (n=5). Significant differences (two way anova and the Tukey-Kramer post-hoc test) due to Cd are indicated in purple ($p<0.05$, $p<0.1$) for increases and orange ($p<0.05$) for decreases. Significant differences in Cd-effects of lignin-reduced mutants in comparison to WT plants are indicated with * ($p<0.05$).

Effect of Cd exposure	**ROOT**				**LEAVES**			
Enzyme	**WT**	***4cl1-1***	***ccr1-6***	***ccr1-3***	**WT**	***4cl1-1***	***ccr1-6***	***ccr1-3***
CAT	1.53 ± 0.13	1.47 ± 0.07	**1.14 * ± 0.04**	**1.09 * ± 0.03**	1.13 ± 0.14	1.57 ± 0.08	1.09 ± 0.10	1.04 ± 0.09
APX	1.57 ± 0.37	1.04 ± 0.22	1.27 ± 0.01	0.85 ± 0.13	0.80 ± 0.13	0.80 ± 0.26	1.07 ± 0.35	0.55 ± 0.08
GR	1.73 ± 0.10	1.95 ± 0.15	1.66 ± 0.15	1.26 ± 0.09	1.35 ± 0.09	1.15 ± 0.04	**0.93 * ± 0.07**	**0.98 * ± 0.06**
GPX	1.27 ± 0.18	1.42 ± 0.11	0.94 ± 0.07	0.97 ± 0.09	0.79 ± 0.06	0.67 ± 0.10	1.19 ± 0.19	1.05 ± 0.13
SPX	1.82 ± 0.22	1.27 ± 0.19	1.25 ± 0.09	**0.85 * ± 0.08**	0.79 ± 0.16	0.73 ± 0.13	1.28 ± 0.20	0.86 ± 0.11
SOD	1.56 ± 0.17	1.35 ± 0.28	1.29 ± 0.12	0.98 ± 0.15	0.98 ± 0.10	0.91 ± 0.11	0.77 ± 0.07	1.07 ± 0.05

Supplemental table 3.3.3: Gene expression in **roots and leaves** of the lignin-reduced mutants relative to the WT plants (±standard error) (n=5) for not normalised and graynorm (Gray) normalised measurements. Significant (one way anova and the Tukey-Kramer post-hoc test using log transformed data) lower mRNA levels in comparison to WT plants are indicated in red (p<0.05, p<0.1) and higher mRNA levels in green (p<0.05, p<0.1).

		ROOT				LEAF			
Gene	**Normalisation**	**WT**	***4cl1-***	***ccr1-6***	***ccr1-3***	**WT**	***4cl1-1***	***ccr1-6***	***ccr1-3***
Genes encoding oxidative stress hallmark proteins									
OX1	No	1.00 ± 0.11	1.21 ± 0.22	1.14 ± 0.29	1.08 ± 0.11	1.00 ± 0.12	1.25 ± 0.22	1.32 ± 0.13	1.34 ± 0.20
	Gray	1.00 ± 0.18	0.71 ± 0.13	0.95 ± 0.16	1.09 ± 0.26	1.00 ± 0.12	1.17 ± 0.09	1.40 ± 0.11	1.18 ± 0.08
OX2	No	1.00 ± 0.44	1.27 ± 0.43	0.73 ± 0.17	0.85 ± 0.42	1.00 ± 0.36	1.15 ± 0.24	0.49 ± 0.20	0.18 ± 0.06
	Gray	1.00 ± 0.61	0.82 ± 0.41	0.85 ± 0.33	0.83 ± 0.44	1.00 ± 0.30	1.33 ± 0.28	0.64 ± 0.25	0.18 ± 0.05
OX3	No	1.00 ± 0.34	2.77 ± 1.08	0.25 ± 0.06	0.37 ± 0.11	1.00 ± 0.25	3.07 ± 1.15	0.24 ± 0.13	0.30 ± 0.17
	Gray	1.00 ± 0.17	1.70 ± 0.69	0.36 ± 0.14	0.46 ± 0.16	1.00 ± 0.19	3.44 ± 1.37	0.29 ± 0.16	0.35 ± 0.11
OX4	No	1.00 ± 0.23	0.80 ± 0.11	0.42 ± 0.04	0.34 ± 0.05	1.00 ± 0.33	2.02 ± 0.82	0.31 ± 0.12	0.24 ± 0.05
	Gray	1.00 ± 0.21	0.61 ± 0.15	0.47 ± 0.11	0.37 ± 0.10	1.00 ± 0.27	2.43 ± 1.07	0.38 ± 0.16	0.24 ± 0.02
OX5	No	1.00 ± 0.39	1.87 ± 0.65	0.18 ± 0.04	0.25 ± 0.05	1.00 ± 0.29	2.96 ± 1.05	0.38 ± 0.26	0.21 ± 0.15
	Gray	1.00 ± 0.39	1.21 ± 0.42	0.26 ± 0.11	0.31 ± 0.08	1.00 ± 0.23	3.48 ± 1.33	0.47 ± 0.32	0.17 ± 0.11
Genes encoding ROS producing enzymes									
LOX1	No	1.00 ± 0.27	1.09 ± 0.31	0.77 ± 0.11	0.56 ± 0.07	1.00 ± 0.18	1.97 ± 0.33	2.02 ± 0.29	3.23 ± 0.45
	Gray	1.00 ± 0.42	0.83 ± 0.36	0.67 ± 0.16	0.51 ± 0.12	1.00 ± 0.13	2.05 ± 0.39	2.20 ± 0.20	3.02 ± 0.20
LOX2	No	1.00 ± 0.49	0.35 ± 0.20	0.98 ± 0.50	0.70 ± 0.25	1.00 ± 0.27	2.77 ± 0.82	0.93 ± 0.14	1.26 ± 0.09
	Gray	1.00 ± 0.62	0.31 ± 0.21	0.85 ± 0.42	0.72 ± 0.32	1.00 ± 0.23	3.05 ± 0.90	1.09 ± 0.17	1.45 ± 0.44
RBOHC	No	1.00 ± 0.22	1.01 ± 0.22	0.74 ± 0.18	0.67 ± 0.09	1.00 ± 0.26	10.85 ± 4.13	0.68 ± 0.27	0.84 ± 0.31
	Gray	1.00 ± 0.26	0.74 ± 0.27	0.74 ± 0.22	0.72 ± 0.17	1.00 ± 0.12	11.93 ± 4.42	1.48 ± 0.19	1.33 ± 0.18
RBOHD	No	1.00 ± 0.16	2.25 ± 0.73	0.64 ± 0.09	0.55 ± 0.05	1.00 ± 0.15	2.00 ± 0.27	0.76 ± 0.11	1.09 ± 0.22
	Gray	1.00 ± 0.13	1.36 ± 0.41	0.64 ± 0.13	0.59 ± 0.14	1.00 ± 0.11	2.09 ± 0.37	0.84 ± 0.12	0.96 ± 0.08
RBOHE	No	1.00 ± 0.21	1.02 ± 0.24	0.93 ± 0.22	0.76 ± 0.18	1.00 ± 0.23	1.50 ± 0.21	0.91 ± 0.21	1.58 ± 0.22
	Gray	1.00 ± 0.27	0.80 ± 0.30	1.00 ± 0.34	0.84 ± 0.24	1.00 ± 0.18	1.63 ± 0.33	1.00 ± 0.20	1.51 ± 0.09
Genes encoding antioxidative enzymes									
CAT1	No	1.00 ± 0.25	1.15 ± 0.21	0.91 ± 0.19	0.88 ± 0.14	1.00 ± 0.20	1.66 ± 0.39	0.87 ± 0.11	0.82 ± 0.13
	Gray	1.00 ± 0.09	0.80 ± 0.18	0.90 ± 0.18	1.01 ± 0.29	1.00 ± 0.13	1.77 ± 0.44	0.99 ± 0.12	0.75 ± 0.02
CAT2	No	1.00 ± 0.18	1.28 ± 0.24	1.26 ± 0.27	1.04 ± 0.11	1.00 ± 0.09	1.02 ± 0.13	1.24 ± 0.07	1.71 ± 0.24
	Gray	1.00 ± 0.23	0.96 ± 0.34	1.29 ± 0.38	1.08 ± 0.25	1.00 ± 0.10	1.00 ± 0.13	1.31 ± 0.03	1.54 ± 0.12
CAT3	No	1.00 ± 0.20	1.04 ± 0.22	0.94 ± 0.18	0.84 ± 0.09	1.00 ± 0.24	1.30 ± 0.09	0.57 ± 0.06	0.61 ± 0.14
	Gray	1.00 ± 0.13	0.78 ± 0.21	0.91 ± 0.21	0.89 ± 0.21	1.00 ± 0.15	1.42 ± 0.15	0.67 ± 0.08	0.56 ± 0.07

APX1	No	1.00 ± 0.18	1.33 ± 0.31	1.46 ± 0.43	1.13 ± 0.09	1.00 ± 0.17	2.05 ± 0.28	1.26 ± 0.06	1.62 ± 0.22
	Gray	1.00 ± 0.34	0.74 ± 0.18	1.10 ± 0.23	1.01 ± 0.22	1.00 ± 0.08	2.16 ± 0.33	1.42 ± 0.08	1.51 ± 0.05
APX2	No	1.00 ± 0.21	1.56 ± 0.25	0.76 ± 0.12	0.81 ± 0.12	1.00 ± 0.12	0.92 ± 0.11	1.06 ± 0.14	2.73 ± 0.46
	Gray	1.00 ± 0.21	1.01 ± 0.18	0.84 ± 0.19	0.89 ± 0.29	1.00 ± 0.21	0.85 ± 0.10	1.05 ± 0.13	2.29 ± 0.24
GR1	No	1.00 ± 0.20	1.00 ± 0.25	1.12 ± 0.23	0.95 ± 0.12	1.00 ± 0.20	1.71 ± 0.24	1.22 ± 0.13	1.57 ± 0.23
	Gray	1.00 ± 0.35	0.69 ± 0.27	0.99 ± 0.28	0.93 ± 0.23	1.00 ± 0.13	1.79 ± 0.30	1.36 ± 0.10	1.46 ± 0.05
GR2	No	1.00 ± 0.12	1.08 ± 0.19	1.11 ± 0.14	0.92 ± 0.14	1.00 ± 0.12	1.02 ± 0.11	1.25 ± 0.17	1.66 ± 0.20
	Gray	1.00 ± 0.23	0.76 ± 0.24	1.08 ± 0.23	0.99 ± 0.25	1.00 ± 0.08	1.03 ± 0.12	1.35 ± 0.12	1.54 ± 0.11
FSD1	No	1.00 ± 0.53	1.81 ± 0.43	0.90 ± 0.38	0.89 ± 0.46	1.00 ± 0.31	4.42 ± 1.89	0.23 ± 0.05	1.00 ± 0.74
	Gray	1.00 ± 0.65	1.47 ± 0.65	0.70 ± 0.32	0.51 ± 0.05	1.00 ± 0.40	4.55 ± 2.17	0.23 ± 0.06	0.76 ± 0.46
CSD1	No	1.00 ± 0.14	1.18 ± 0.22	1.19 ± 0.20	1.14 ± 0.09	1.00 ± 0.14	1.33 ± 0.36	1.05 ± 0.07	0.97 ± 0.10
	Gray	1.00 ± 0.18	0.73 ± 0.16	1.13 ± 0.23	1.20 ± 0.27	1.00 ± 0.07	1.32 ± 0.36	1.18 ± 0.10	0.93 ± 0.09
CSD2	No	1.00 ± 0.22	1.34 ± 0.37	1.27 ± 0.43	1.09 ± 0.10	1.00 ± 0.10	0.91 ± 0.23	1.41 ± 0.14	1.45 ± 0.17
	Gray	1.00 ± 0.10	0.76 ± 0.12	1.08 ± 0.13	0.99 ± 0.22	1.00 ± 0.12	0.82 ± 0.18	1.48 ± 0.19	1.33 ± 0.18
Genes encoding enzymes involved in chelation									
MT1a	No	1.00 ± 0.13	0.80 ± 0.18	0.92 ± 0.22	0.82 ± 0.08	1.00 ± 0.18	1.23 ± 0.14	0.91 ± 0.14	0.99 ± 0.15
	Gray	1.00 ± 0.29	0.53 ± 0.20	0.80 ± 0.25	0.79 ± 0.19	1.00 ± 0.17	1.18 ± 0.10	0.98 ± 0.17	0.89 ± 0.09
MT1c	No	1.00 ± 0.17	0.88 ± 0.21	0.87 ± 0.29	0.65 ± 0.07	1.00 ± 0.28	0.84 ± 0.18	0.81 ± 0.08	0.96 ± 0.15
	Gray	1.00 ± 0.20	0.61 ± 0.23	0.73 ± 0.22	0.64 ± 0.14	1.00 ± 0.26	0.79 ± 0.11	0.87 ± 0.11	0.88 ± 0.13
MT2a	No	1.00 ± 0.15	0.58 ± 0.13	0.55 ± 0.09	0.75 ± 0.21	1.00 ± 0.21	1.75 ± 0.43	0.85 ± 0.15	0.80 ± 0.12
	Gray	1.00 ± 0.20	0.40 ± 0.11	0.55 ± 0.15	0.58 ± 0.09	1.00 ± 0.13	1.94 ± 0.51	1.00 ± 0.18	0.79 ± 0.06
MT2b	No	1.00 ± 0.10	1.30 ± 0.29	1.19 ± 0.42	1.18 ± 0.10	1.00 ± 0.19	0.83 ± 0.17	0.56 ± 0.03	0.61 ± 0.14
	Gray	1.00 ± 0.21	0.75 ± 0.14	0.87 ± 0.11	1.10 ± 0.23	1.00 ± 0.16	0.80 ± 0.10	0.61 ± 0.04	0.55 ± 0.05
MT3	No	1.00 ± 0.12	1.54 ± 0.51	1.63 ± 0.81	0.89 ± 0.11	1.00 ± 0.16	0.69 ± 0.30	0.52 ± 0.14	0.47 ± 0.24
	Gray	1.00 ± 0.30	0.74 ± 0.13	0.92 ± 0.09	0.73 ± 0.12	1.00 ± 0.24	0.67 ± 0.05	0.58 ± 0.10	0.42 ± 0.03
PCS1	No	1.00 ± 0.18	1.68 ± 0.23	1.09 ± 0.20	0.91 ± 0.14	1.00 ± 0.16	2.69 ± 0.30	0.67 ± 0.14	0.73 ± 0.24
	Gray	1.00 ± 0.28	1.04 ± 0.21	1.05 ± 0.25	0.95 ± 0.24	1.00 ± 0.09	2.85 ± 0.41	0.77 ± 0.16	0.63 ± 0.14
Genes encoded monolignol biosynthesis enzymes									
PAL1	No	1.00 ± 0.19	1.35 ± 0.32	1.66 ± 0.48	1.54 ± 0.19	1.00 ± 0.11	1.65 ± 0.35	2.05 ± 0.12	3.46 ± 0.49
	Gray	1.00 ± 0.36	0.96 ± 0.38	1.58 ± 0.54	1.44 ± 0.35	1.00 ± 0.08	1.60 ± 0.31	2.21 ± 0.12	3.11 ± 0.19
PAL2	No	1.00 ± 0.16	0.92 ± 0.23	0.93 ± 0.24	1.31 ± 0.21	1.00 ± 0.16	0.92 ± 0.15	0.93 ± 0.12	1.31 ± 0.13
	Gray	1.00 ± 0.33	0.84 ± 0.29	1.13 ± 0.35	1.12 ± 0.31	1.00 ± 0.17	0.83 ± 0.12	0.91 ± 0.07	1.12 ± 0.10
CCR2	No	1.00 ± 0.22	1.09 ± 0.23	0.84 ± 0.18	0.69 ± 0.19	1.00 ± 0.44	3.73 ± 1.08	1.35 ± 0.30	2.35 ± 0.73
	Gray	1.00 ± 0.37	0.70 ± 0.20	0.77 ± 0.22	0.72 ± 0.22	1.00 ± 0.36	4.27 ± 1.19	1.60 ± 0.36	2.10 ± 0.51

Supplemental table 3.3.4: Gene expression in **roots with Cd exposure** relative to the non-exposed genotype (±standard error) (n=5) for not normalised and graynorm (Gray) normalised measurements. Significant (one way anova for the non-exposed versus the Cd-exposed genotype using log transformed data) decreases of the mRNA levels of the exposed in comparison to non-exposed genotype are indicated in red (p<0.05, p<0.1) and increases in green (p<0.05, p<0.1).

ROOTS		**0 µM Cd**				**3 µM Cd**			
Gene	**Normalisation**	**WT**	***4cl1-1***	***ccr1-6***	***ccr1-3***	**WT**	***4cl1-1***	***ccr1-6***	***ccr1-3***
Genes encoding oxidative stress hallmark proteins									
OX1	No	1.00 ± 0.11	1.00 ± 0.18	1.00 ± 0.25	1.00 ± 0.10	2.41 ± 0.74	1.32 ± 0.43	1.93 ± 0.41	1.54 ± 0.36
	Gray	1.00 ± 0.06	1.00 ± 0.18	1.00 ± 0.07	1.00 ± 0.14	2.06 ± 0.37	1.90 ± 0.70	1.99 ± 0.39	1.27 ± 0.09
OX2	No	1.00 ± 0.38	1.00 ± 0.34	1.00 ± 0.23	1.00 ± 0.50	24.36 ± 9.73	38.25 ± 25.15	39.46 ± 21.20	64.16 ± 17.08
	Gray	1.00 ± 0.39	1.00 ± 0.49	1.00 ± 0.32	1.00 ± 0.51	25.65 ± 8.39	50.30 ± 31.72	32.88 ± 16.99	53.53 ± 20.39
OX3	No	1.00 ± 0.34	1.00 ± 0.39	1.00 ± 0.23	1.00 ± 0.31	1.59 ± 0.41	0.36 ± 0.15	5.83 ± 1.86	3.29 ± 0.48
	Gray	1.00 ± 0.24	1.00 ± 0.41	1.00 ± 0.31	1.00 ± 0.30	1.46 ± 0.41	0.57 ± 0.25	5.08 ± 1.59	3.04 ± 0.61
OX4	No	1.00 ± 0.24	1.00 ± 0.14	1.00 ± 0.10	1.00 ± 0.14	0.92 ± 0.24	0.74 ± 0.32	1.12 ± 0.24	1.74 ± 0.33
	Gray	1.00 ± 0.16	1.00 ± 0.25	1.00 ± 0.14	1.00 ± 0.20	0.89 ± 0.22	0.87 ± 0.38	1.06 ± 0.23	1.51 ± 0.35
OX5	No	1.00 ± 0.39	1.00 ± 0.35	1.00 ± 0.22	1.00 ± 0.22	2.50 ± 0.59	1.06 ± 0.53	9.34 ± 3.56	7.04 ± 2.79
	Gray	1.00 ± 0.26	1.00 ± 0.36	1.00 ± 0.34	1.00 ± 0.22	2.51 ± 0.40	1.60 ± 0.81	8.21 ± 3.20	6.13 ± 2.69
Genes encoding ROS producing enzymes									
LOX1	No	1.00 ± 0.27	1.00 ± 0.29	1.00 ± 0.14	1.00 ± 0.13	2.34 ± 0.60	2.03 ± 1.09	2.97 ± 0.98	4.55 ± 0.81
	Gray	1.00 ± 0.33	1.00 ± 0.44	1.00 ± 0.14	1.00 ± 0.17	1.98 ± 0.48	1.90 ± 1.03	2.64 ± 0.76	4.02 ± 0.88
LOX2	No	1.00 ± 0.56	1.00 ± 0.63	1.00 ± 0.57	1.00 ± 0.47	1.24 ± 0.52	5.51 ± 2.01	1.76 ± 0.64	1.42 ± 0.31
	Gray	1.00 ± 0.66	1.00 ± 0.77	1.00 ± 0.53	1.00 ± 0.56	1.09 ± 0.50	4.48 ± 2.22	1.42 ± 0.43	1.14 ± 0.30
RBOHC	No	1.00 ± 0.22	1.00 ± 0.21	1.00 ± 0.24	1.00 ± 0.13	1.46 ± 0.38	0.89 ± 0.31	2.10 ± 0.49	2.36 ± 0.42
	Gray	1.00 ± 0.15	1.00 ± 0.35	1.00 ± 0.22	1.00 ± 0.17	1.36 ± 0.28	1.09 ± 0.45	1.96 ± 0.31	2.08 ± 0.36
RBOHD	No	1.00 ± 0.16	1.00 ± 0.32	1.00 ± 0.14	1.00 ± 0.09	1.80 ± 0.45	0.63 ± 0.23	2.93 ± 0.85	3.49 ± 0.83
	Gray	1.00 ± 0.04	1.00 ± 0.31	1.00 ± 0.10	1.00 ± 0.17	1.69 ± 0.26	0.90 ± 0.34	2.69 ± 0.58	2.93 ± 0.53
RBOHE	No	1.00 ± 0.21	1.00 ± 0.24	1.00 ± 0.23	1.00 ± 0.23	1.96 ± 0.53	1.71 ± 0.80	2.37 ± 0.64	2.65 ± 0.36
	Gray	1.00 ± 0.15	1.00 ± 0.37	1.00 ± 0.24	1.00 ± 0.29	1.69 ± 0.43	1.90 ± 0.91	2.15 ± 0.52	2.47 ± 0.48
Genes encoding antioxidative enzymes									
CAT1	No	1.00 ± 0.25	1.00 ± 0.19	1.00 ± 0.20	1.00 ± 0.16	1.04 ± 0.30	0.43 ± 0.17	0.99 ± 0.15	1.06 ± 0.16
	Gray	1.00 ± 0.08	1.00 ± 0.23	1.00 ± 0.11	1.00 ± 0.21	0.95 ± 0.28	0.58 ± 0.24	0.97 ± 0.10	0.94 ± 0.15
CAT2	No	1.00 ± 0.18	1.00 ± 0.18	1.00 ± 0.22	1.00 ± 0.11	0.96 ± 0.24	0.46 ± 0.19	0.73 ± 0.13	0.98 ± 0.19
	Gray	1.00 ± 0.10	1.00 ± 0.35	1.00 ± 0.20	1.00 ± 0.15	0.89 ± 0.15	0.50 ± 0.22	0.68 ± 0.08	0.86 ± 0.15
CAT3	No	1.00 ± 0.20	1.00 ± 0.21	1.00 ± 0.19	1.00 ± 0.11	0.96 ± 0.26	0.60 ± 0.24	0.78 ± 0.21	1.19 ± 0.22
	Gray	1.00 ± 0.05	1.00 ± 0.26	1.00 ± 0.17	1.00 ± 0.14	0.92 ± 0.17	0.70 ± 0.30	0.71 ± 0.11	1.06 ± 0.21

APX1	No	1.00 ± 0.18	1.00 ± 0.24	1.00 ± 0.29	1.00 ± 0.08	0.96 ± 0.28	0.48 ± 0.15	0.71 ± 0.11	1.09 ± 0.29
	Gray	1.00 ± 0.22	1.00 ± 0.25	1.00 ± 0.11	1.00 ± 0.12	0.80 ± 0.15	0.66 ± 0.25	0.73 ± 0.06	0.89 ± 0.05
APX2	No	1.00 ± 0.21	1.00 ± 0.16	1.00 ± 0.16	1.00 ± 0.15	0.75 ± 0.15	0.43 ± 0.07	1.05 ± 0.12	1.12 ± 0.15
	Gray	1.00 ± 0.13	1.00 ± 0.20	1.00 ± 0.16	1.00 ± 0.26	0.82 ± 0.12	0.55 ± 0.14	0.95 ± 0.09	0.98 ± 0.16
GR1	No	1.00 ± 0.20	1.00 ± 0.25	1.00 ± 0.21	1.00 ± 0.13	1.19 ± 0.34	0.87 ± 0.29	1.20 ± 0.19	1.44 ± 0.18
	Gray	1.00 ± 0.24	1.00 ± 0.38	1.00 ± 0.18	1.00 ± 0.19	0.99 ± 0.21	0.98 ± 0.38	1.13 ± 0.10	1.28 ± 0.21
GR2	No	1.00 ± 0.12	1.00 ± 0.18	1.00 ± 0.12	1.00 ± 0.15	0.95 ± 0.26	0.53 ± 0.18	0.85 ± 0.18	1.09 ± 0.18
	Gray	1.00 ± 0.10	1.00 ± 0.31	1.00 ± 0.09	1.00 ± 0.20	0.81 ± 0.19	0.63 ± 0.24	0.78 ± 0.10	0.99 ± 0.18
FSD1	No	1.00 ± 0.53	1.00 ± 0.24	1.00 ± 0.42	1.00 ± 0.51	4.91 ± 1.50	1.97 ± 0.63	4.90 ± 0.80	2.90 ± 0.56
	Gray	1.00 ± 0.57	1.00 ± 0.46	1.00 ± 0.38	1.00 ± 0.32	4.85 ± 0.41	1.96 ± 0.75	5.37 ± 0.74	3.13 ± 0.34
CSD1	No	1.00 ± 0.14	1.00 ± 0.18	1.00 ± 0.17	1.00 ± 0.08	0.85 ± 0.16	0.50 ± 0.09	0.89 ± 0.08	0.95 ± 0.21
	Gray	1.00 ± 0.10	1.00 ± 0.21	1.00 ± 0.07	1.00 ± 0.15	0.86 ± 0.04	0.67 ± 0.18	0.88 ± 0.08	0.77 ± 0.05
CSD2	No	1.00 ± 0.22	1.00 ± 0.27	1.00 ± 0.33	1.00 ± 0.09	1.00 ± 0.29	0.50 ± 0.06	0.82 ± 0.13	1.23 ± 0.45
	Gray	1.00 ± 0.07	1.00 ± 0.15	1.00 ± 0.09	1.00 ± 0.08	1.01 ± 0.08	0.74 ± 0.15	0.89 ± 0.11	0.92 ± 0.04
Genes encoding enzymes involved in chelation									
MT1a	No	1.00 ± 0.13	1.00 ± 0.22	1.00 ± 0.24	1.00 ± 0.10	0.99 ± 0.27	0.79 ± 0.28	1.12 ± 0.24	1.07 ± 0.28
	Gray	1.00 ± 0.18	1.00 ± 0.36	1.00 ± 0.19	1.00 ± 0.16	0.87 ± 0.24	1.03 ± 0.43	1.07 ± 0.17	0.88 ± 0.14
MT1c	No	1.00 ± 0.17	1.00 ± 0.23	1.00 ± 0.33	1.00 ± 0.11	0.82 ± 0.24	0.75 ± 0.32	1.20 ± 0.29	1.28 ± 0.47
	Gray	1.00 ± 0.09	1.00 ± 0.35	1.00 ± 0.19	1.00 ± 0.12	0.78 ± 0.21	1.00 ± 0.46	1.20 ± 0.22	0.95 ± 0.12
MT2a	No	1.00 ± 0.15	1.00 ± 0.22	1.00 ± 0.17	1.00 ± 0.29	1.29 ± 0.49	1.03 ± 0.34	1.69 ± 0.27	2.41 ± 1.07
	Gray	1.00 ± 0.26	1.00 ± 0.27	1.00 ± 0.21	1.00 ± 0.13	1.06 ± 0.43	1.19 ± 0.43	1.51 ± 0.17	1.91 ± 0.47
MT2b	No	1.00 ± 0.10	1.00 ± 0.22	1.00 ± 0.35	1.00 ± 0.09	0.81 ± 0.29	0.55 ± 0.14	1.04 ± 0.19	1.08 ± 0.34
	Gray	1.00 ± 0.08	1.00 ± 0.19	1.00 ± 0.12	1.00 ± 0.10	0.70 ± 0.19	0.75 ± 0.22	1.12 ± 0.13	0.86 ± 0.05
MT3	No	1.00 ± 0.12	1.00 ± 0.33	1.00 ± 0.50	1.00 ± 0.12	1.04 ± 0.30	0.44 ± 0.13	0.66 ± 0.21	1.14 ± 0.46
	Gray	1.00 ± 0.18	1.00 ± 0.17	1.00 ± 0.24	1.00 ± 0.04	0.90 ± 0.13	0.66 ± 0.22	0.75 ± 0.18	0.84 ± 0.06
PCS1	No	1.00 ± 0.18	1.00 ± 0.14	1.00 ± 0.19	1.00 ± 0.15	1.84 ± 0.52	0.64 ± 0.26	1.58 ± 0.19	1.06 ± 0.27
	Gray	1.00 ± 0.16	1.00 ± 0.20	1.00 ± 0.15	1.00 ± 0.19	1.55 ± 0.37	0.84 ± 0.37	1.52 ± 0.16	1.16 ± 0.41
Genes encoded monolignol biosynthesis enzymes									
PAL1	No	1.00 ± 0.19	1.00 ± 0.24	1.00 ± 0.29	1.00 ± 0.12	0.79 ± 0.18	0.49 ± 0.23	0.44 ± 0.10	0.64 ± 0.14
	Gray	1.00 ± 0.25	1.00 ± 0.39	1.00 ± 0.28	1.00 ± 0.18	0.72 ± 0.09	0.51 ± 0.24	0.40 ± 0.09	0.51 ± 0.06
PAL2	No	1.00 ± 0.16	1.00 ± 0.19	1.00 ± 0.21	1.00 ± 0.19	0.87 ± 0.23	0.69 ± 0.36	0.70 ± 0.21	0.77 ± 0.10
	Gray	1.00 ± 0.24	1.00 ± 0.35	1.00 ± 0.22	1.00 ± 0.23	0.70 ± 0.19	0.76 ± 0.40	0.64 ± 0.17	0.68 ± 0.12
CCR2	No	1.00 ± 0.22	1.00 ± 0.21	1.00 ± 0.22	1.00 ± 0.27	4.54 ± 2.00	2.27 ± 1.73	4.38 ± 2.08	4.14 ± 1.21
	Gray	1.00 ± 0.26	1.00 ± 0.28	1.00 ± 0.19	1.00 ± 0.26	3.96 ± 1.90	2.79 ± 2.09	3.87 ± 1.72	3.92 ± 1.37

Supplemental table 3.3.5: Gene expression in **leaves with Cd exposure** relative to the non-exposed genotype (±standard error) (n=5) for not normalised and graynorm (Gray) normalised measurements. Significant (one way anova for the non-exposed versus the Cd-exposed genotype using log transformed data) decreases of mRNA levels of the exposed in comparison to non-exposed genotype are indicated in red (p<0.05, p<0.1) and increases in green (p<0.05, p<0.1).

LEAVES		**0 µM Cd**				**3 µM Cd**			
Gene	**Normalisation**	**WT**	***4cl1-1***	***ccr1-6***	***ccr1-3***	**WT**	***4cl1-1***	***ccr1-6***	***ccr1-3***
Genes encoding oxidative stress hallmark proteins									
OX1	No	1.00 ± 0.12	1.00 ± 0.16	1.00 ± 0.10	1.00 ± 0.15	6.15 ± 1.41	2.86 ± 0.64	1.36 ± 0.18	1.54 ± 0.31
	Gray	1.00 ± 0.15	1.00 ± 0.11	1.00 ± 0.08	1.00 ± 0.08	5.70 ± 1.00	2.88 ± 0.55	1.41 ± 0.25	1.50 ± 0.24
OX2	No	1.00 ± 0.36	1.00 ± 0.19	1.00 ± 0.39	1.00 ± 0.31	2.57 ± 1.08	1.12 ± 0.24	1.05 ± 0.29	2.16 ± 0.48
	Gray	1.00 ± 0.34	1.00 ± 0.19	1.00 ± 0.40	1.00 ± 0.29	3.17 ± 1.44	1.11 ± 0.24	1.06 ± 0.34	2.27 ± 0.51
OX3	No	1.00 ± 0.25	1.00 ± 0.33	1.00 ± 0.54	1.00 ± 0.55	1.18 ± 0.40	0.25 ± 0.07	1.08 ± 0.14	0.76 ± 0.20
	Gray	1.00 ± 0.22	1.00 ± 0.33	1.00 ± 0.55	1.00 ± 0.51	1.14 ± 0.32	0.24 ± 0.07	1.06 ± 0.15	0.84 ± 0.21
OX4	No	1.00 ± 0.33	1.00 ± 0.36	1.00 ± 0.40	1.00 ± 0.20	1.95 ± 0.49	0.31 ± 0.08	1.40 ± 0.43	1.98 ± 0.44
	Gray	1.00 ± 0.31	1.00 ± 0.37	1.00 ± 0.41	1.00 ± 0.12	2.01 ± 0.42	0.31 ± 0.08	1.45 ± 0.50	1.88 ± 0.23
OX5	No	1.00 ± 0.29	1.00 ± 0.32	1.00 ± 0.69	1.00 ± 0.71	0.83 ± 0.15	0.17 ± 0.07	0.33 ± 0.07	0.48 ± 0.11
	Gray	1.00 ± 0.26	1.00 ± 0.32	1.00 ± 0.69	1.00 ± 0.69	0.87 ± 0.12	0.17 ± 0.08	0.33 ± 0.08	0.58 ± 0.19
Genes encoding ROS producing enzymes									
LOX1	No	1.00 ± 0.18	1.00 ± 0.17	1.00 ± 0.14	1.00 ± 0.14	3.49 ± 0.42	1.95 ± 0.31	1.84 ± 0.17	1.81 ± 0.36
	Gray	1.00 ± 0.17	1.00 ± 0.19	1.00 ± 0.09	1.00 ± 0.07	3.54 ± 0.53	1.90 ± 0.24	1.86 ± 0.14	1.65 ± 0.10
LOX2	No	1.00 ± 0.27	1.00 ± 0.30	1.00 ± 0.16	1.00 ± 0.07	1.25 ± 0.13	0.68 ± 0.16	1.26 ± 0.25	1.72 ± 0.34
	Gray	1.00 ± 0.24	1.00 ± 0.31	1.00 ± 0.16	1.00 ± 0.25	1.33 ± 0.20	0.65 ± 0.15	1.26 ± 0.26	1.42 ± 0.16
RBOHC	No	1.00 ± 0.26	1.00 ± 0.38	1.00 ± 0.39	1.00 ± 0.37	10.01 ± 6.47	0.09 ± 0.02	0.41 ± 0.13	1.74 ± 0.35
	Gray	1.00 ± 0.24	1.00 ± 0.37	1.00 ± 0.40	1.00 ± 0.34	9.03 ± 5.79	0.09 ± 0.02	0.41 ± 0.12	1.98 ± 0.57
RBOHD	No	1.00 ± 0.15	1.00 ± 0.14	1.00 ± 0.14	1.00 ± 0.20	1.41 ± 0.12	0.75 ± 0.12	1.24 ± 0.14	0.90 ± 0.16
	Gray	1.00 ± 0.15	1.00 ± 0.14	1.00 ± 0.14	1.00 ± 0.12	1.45 ± 0.17	0.74 ± 0.09	1.22 ± 0.10	0.89 ± 0.09
RBOHE	No	1.00 ± 0.23	1.00 ± 0.14	1.00 ± 0.23	1.00 ± 0.14	1.54 ± 0.20	1.13 ± 0.26	1.39 ± 0.27	1.33 ± 0.26
	Gray	1.00 ± 0.22	1.00 ± 0.16	1.00 ± 0.20	1.00 ± 0.06	1.65 ± 0.33	1.08 ± 0.22	1.40 ± 0.26	1.24 ± 0.10
Genes encoding antioxidative enzymes									
CAT1	No	1.00 ± 0.20	1.00 ± 0.24	1.00 ± 0.12	1.00 ± 0.16	0.73 ± 0.06	0.50 ± 0.06	0.65 ± 0.06	0.71 ± 0.14
	Gray	1.00 ± 0.16	1.00 ± 0.24	1.00 ± 0.13	1.00 ± 0.07	0.79 ± 0.11	0.50 ± 0.05	0.64 ± 0.04	0.69 ± 0.07
CAT2	No	1.00 ± 0.09	1.00 ± 0.13	1.00 ± 0.06	1.00 ± 0.14	1.18 ± 0.14	1.40 ± 0.12	1.19 ± 0.12	1.17 ± 0.23
	Gray	1.00 ± 0.07	1.00 ± 0.15	1.00 ± 0.03	1.00 ± 0.08	1.19 ± 0.12	1.38 ± 0.08	1.18 ± 0.10	1.07 ± 0.08
CAT3	No	1.00 ± 0.24	1.00 ± 0.07	1.00 ± 0.11	1.00 ± 0.23	0.68 ± 0.04	0.66 ± 0.11	0.88 ± 0.06	0.98 ± 0.21
	Gray	1.00 ± 0.20	1.00 ± 0.08	1.00 ± 0.11	1.00 ± 0.17	0.72 ± 0.10	0.66 ± 0.10	0.88 ± 0.07	0.92 ± 0.09

APX1	No	1.00 ± 0.17	1.00 ± 0.13	1.00 ± 0.05	1.00 ± 0.14	1.54 ± 0.16	0.87 ± 0.08	1.29 ± 0.07	1.06 ± 0.21
	Gray	1.00 ± 0.13	1.00 ± 0.16	1.00 ± 0.06	1.00 ± 0.05	1.59 ± 0.19	0.87 ± 0.09	1.28 ± 0.08	0.98 ± 0.05
APX2	No	1.00 ± 0.12	1.00 ± 0.12	1.00 ± 0.13	1.00 ± 0.17	2.04 ± 0.19	2.93 ± 0.99	2.65 ± 0.15	0.85 ± 0.12
	Gray	1.00 ± 0.19	1.00 ± 0.13	1.00 ± 0.12	1.00 ± 0.07	1.99 ± 0.29	2.83 ± 0.88	2.66 ± 0.14	0.85 ± 0.13
GR1	No	1.00 ± 0.20	1.00 ± 0.14	1.00 ± 0.10	1.00 ± 0.15	1.71 ± 0.11	1.04 ± 0.18	1.08 ± 0.13	1.12 ± 0.19
	Gray	1.00 ± 0.16	1.00 ± 0.15	1.00 ± 0.07	1.00 ± 0.04	1.81 ± 0.23	1.02 ± 0.15	1.07 ± 0.10	1.09 ± 0.11
GR2	No	1.00 ± 0.12	1.00 ± 0.10	1.00 ± 0.13	1.00 ± 0.12	1.17 ± 0.08	1.37 ± 0.20	1.14 ± 0.15	0.93 ± 0.13
	Gray	1.00 ± 0.10	1.00 ± 0.13	1.00 ± 0.09	1.00 ± 0.04	1.21 ± 0.13	1.34 ± 0.16	1.14 ± 0.13	0.90 ± 0.09
FSD1	No	1.00 ± 0.31	1.00 ± 0.43	1.00 ± 0.22	1.00 ± 0.45	1.59 ± 0.18	0.31 ± 0.09	1.72 ± 0.31	0.74 ± 0.09
	Gray	1.00 ± 0.37	1.00 ± 0.44	1.00 ± 0.27	1.00 ± 0.17	1.54 ± 0.18	0.30 ± 0.10	1.68 ± 0.33	0.44 ± 0.06
CSD1	No	1.00 ± 0.14	1.00 ± 0.27	1.00 ± 0.07	1.00 ± 0.11	0.86 ± 0.11	0.76 ± 0.05	0.73 ± 0.07	0.83 ± 0.13
	Gray	1.00 ± 0.10	1.00 ± 0.27	1.00 ± 0.09	1.00 ± 0.06	0.88 ± 0.11	0.77 ± 0.06	0.76 ± 0.10	0.78 ± 0.06
CSD2	No	1.00 ± 0.10	1.00 ± 0.25	1.00 ± 0.10	1.00 ± 0.12	0.73 ± 0.16	1.15 ± 0.19	0.85 ± 0.05	0.76 ± 0.15
	Gray	1.00 ± 0.09	1.00 ± 0.23	1.00 ± 0.13	1.00 ± 0.09	0.69 ± 0.11	1.20 ± 0.23	0.84 ± 0.07	0.68 ± 0.03
Genes encoding enzymes involved in chelation									
MT1a	No	1.00 ± 0.18	1.00 ± 0.12	1.00 ± 0.16	1.00 ± 0.15	2.33 ± 0.46	1.43 ± 0.27	1.90 ± 0.27	2.00 ± 0.42
	Gray	1.00 ± 0.15	1.00 ± 0.11	1.00 ± 0.18	1.00 ± 0.08	2.33 ± 0.41	1.44 ± 0.27	1.94 ± 0.38	1.82 ± 0.09
MT1c	No	1.00 ± 0.28	1.00 ± 0.21	1.00 ± 0.09	1.00 ± 0.16	2.98 ± 0.70	1.95 ± 0.48	2.04 ± 0.33	2.12 ± 0.60
	Gray	1.00 ± 0.23	1.00 ± 0.18	1.00 ± 0.13	1.00 ± 0.11	2.99 ± 0.54	2.01 ± 0.54	2.09 ± 0.45	1.80 ± 0.22
MT2a	No	1.00 ± 0.21	1.00 ± 0.25	1.00 ± 0.17	1.00 ± 0.15	1.79 ± 0.30	0.85 ± 0.09	1.44 ± 0.15	1.53 ± 0.35
	Gray	1.00 ± 0.17	1.00 ± 0.26	1.00 ± 0.18	1.00 ± 0.03	2.02 ± 0.47	0.83 ± 0.07	1.43 ± 0.17	1.35 ± 0.13
MT2b	No	1.00 ± 0.19	1.00 ± 0.21	1.00 ± 0.05	1.00 ± 0.17	0.75 ± 0.18	0.82 ± 0.12	1.00 ± 0.12	1.07 ± 0.22
	Gray	1.00 ± 0.14	1.00 ± 0.17	1.00 ± 0.07	1.00 ± 0.10	0.74 ± 0.11	0.85 ± 0.15	1.01 ± 0.15	0.98 ± 0.03
MT3	No	1.00 ± 0.27	1.00 ± 0.15	1.00 ± 0.13	1.00 ± 0.13	0.70 ± 0.19	0.87 ± 0.24	0.95 ± 0.19	0.85 ± 0.15
	Gray	1.00 ± 0.21	1.00 ± 0.12	1.00 ± 0.18	1.00 ± 0.07	0.69 ± 0.13	0.91 ± 0.28	0.96 ± 0.24	0.78 ± 0.07
PCS1	No	1.00 ± 0.16	1.00 ± 0.11	1.00 ± 0.20	1.00 ± 0.32	1.29 ± 0.13	0.60 ± 0.14	1.26 ± 0.14	0.93 ± 0.36
	Gray	1.00 ± 0.13	1.00 ± 0.13	1.00 ± 0.21	1.00 ± 0.26	1.31 ± 0.11	0.59 ± 0.14	1.24 ± 0.11	0.87 ± 0.25
Genes encoded monolignol biosynthesis enzymes									
PAL1	No	1.00 ± 0.11	1.00 ± 0.21	1.00 ± 0.06	1.00 ± 0.14	2.36 ± 0.25	1.86 ± 0.37	1.19 ± 0.19	0.96 ± 0.18
	Gray	1.00 ± 0.11	1.00 ± 0.22	1.00 ± 0.06	1.00 ± 0.08	2.34 ± 0.17	1.85 ± 0.33	1.15 ± 0.15	0.89 ± 0.06
PAL2	No	1.00 ± 0.15	1.00 ± 0.16	1.00 ± 0.13	1.00 ± 0.10	1.09 ± 0.11	1.29 ± 0.28	0.93 ± 0.16	0.72 ± 0.11
	Gray	1.00 ± 0.14	1.00 ± 0.14	1.00 ± 0.08	1.00 ± 0.05	1.07 ± 0.14	1.29 ± 0.24	0.92 ± 0.14	0.68 ± 0.06
CCR2	No	1.00 ± 0.44	1.00 ± 0.29	1.00 ± 0.22	1.00 ± 0.31	2.16 ± 0.65	0.44 ± 0.13	1.04 ± 0.36	1.37 ± 0.40
	Gray	1.00 ± 0.40	1.00 ± 0.28	1.00 ± 0.23	1.00 ± 0.27	2.39 ± 0.81	0.43 ± 0.13	1.06 ± 0.41	1.35 ± 0.26

Subsection 3.4
What happens to lignin in the lignin-reduced plants exposed to Cd?

Increased lignification has been associated with many different biotic (infections by fungi, pathogenic bacteria and viruses) and abiotic stressors (*e.g.* mineral deficiency, drought, ultraviolet-B radiation, *etc.*) (Bhuiyan *et al.* 2009, Moura *et al.* 2010). More specifically, after exposure to different metal stressors (excess of Zn, B, Cu, Al, Cd), the phenolic metabolism in *Matricaria chamomilla* roots seemed highly metal specific (Kovacik *et al.* 2008a). In roots, the effects of metal exposure on lignin amount and deposition have been extensively studied for various concentrations and exposure duration with various cadmium (Cd) salts. In general, based on literature, exposure to Cd salts seems to increase lignification in the root tissues (Table 1.2 in section1). In contrast, the responses of lignification to Cd exposure in aerial plant parts are less studied. The observed effects are more variable amongst plant species and study-design and range from increases in the lignin amount to no change and even decreases in the lignin amount (Table 1.2 in section1).

In our experiments, *Arabidopsis thaliana* is used as a model for woody plants (Nieminen et al 2004) to evaluate the effects of Cd on lignification in the roots and stems of WT and lignin-reduced mutants. Up till now, no studies reported on the effects of Cd exposure on lignification within different plant organs of *A. thaliana*. However, Herbette *et al.* (2006) and Van de Mortel *et al.* (2008) did suggest that lignification in roots and leaves should increase since they observed that genes of the lignin biosynthesis were up-regulated after Cd exposure. Furthermore, Keunen *et al.* (2016a) reported elevated levels of phenylalanine in roots and leaves of 3 weeks old *A. thaliana* plants after exposure to 5 and 10 µM $CdSO_4$ during 24 or 72 h. Phenylalanine is the sole precursor of the monolignol (and flavonoid) biosynthesis and thus elevated levels of phenylalanine represent a good indication of the link between metal exposure and increased lignification. Therefore, we hypothesized that Cd will significantly influence lignification in the roots, leaves and stems of WT *A. thaliana* plants. Furthermore, to gain insight into the stress-related responses of several lignin-reduced mutants (*4cl1-1, 4cl1-2, ccr1-6* and *ccr1-3*), we explored the effects of Cd exposure on the lignin concentration and composition in these mutants. Indeed, lignification in the

stems of these different lignin-reduced mutants has been extensively studied (Van Acker *et al.* 2013, Vanholme *et al.* 2012a) but little is known about stress-related responses of lignin concentration and composition of these mutants. Furthermore, no studies concerning roots, the main entry location for Cd-ions to the plant, of these lignin-reduced plants were found.

3.4.1 Experimental design

Plants were cultivated in a hydroponic system (paragraph 3.1.2.3) and were exposed to 3 µM $CdSO_4$ from the start of stem formation (47 days after sowing for WT, *4cl1-1* and *4cl1-2*; 50 days for *ccr1-6* and 57 days for *ccr1-3*) during 2 weeks. First, the acetyl bromide (AcBr) assay (paragraph 3.1.3.11) was performed on cell wall residues (CWR) (paragraph 3.1.3.10) for the 3 plant organs (roots, leaves and stems) from WT plants. Also stem sections of the bottom part were stained using Wiesner and Mäule and studied by microscopy (paragraph 3.1.3.7). Furthermore, fresh whole root tips were stained using a phloroglucinol solution (Wiesner) (paragraph 3.1.3.8). In a next step, also lignin-reduced mutants (*4cl1-1, 4cl1-2, ccr1-6* and *ccr1-3*) were examined by using the AcBr (paragraph 3.1.3.11) and thioacidolysis assays (paragraph 3.1.3.12) on CWR (paragraph 3.1.4.10). We opted to work with plants with the same stem height as discussed in paragraph 3.2.2.2C. Furthermore, to understand the effects on the root tip, sections were made from plants of the same age grown on sand for 1 week without Cd where after half of the plants were exposed during 3 weeks to 3 µM $CdSO_4$. These root tips were stained with saffranin and astra blue and studied by light microscopy (paragraph 3.1.3.9).

3.4.2 Results and discussion

3.4.2.1 Lignification after Cd exposure

It has become apparent that cell walls, and especially lignin molecules, have a diverse composition in different organs and tissues of plants (Parrotta *et al.* 2015). Therefore, we will discuss the above and belowground parts separately.

The aerial tissues (**leaves and stems**) of WT *A. thaliana* plants exposed to 3 µM Cd (from the start of stem formation for two weeks) showed no differences in

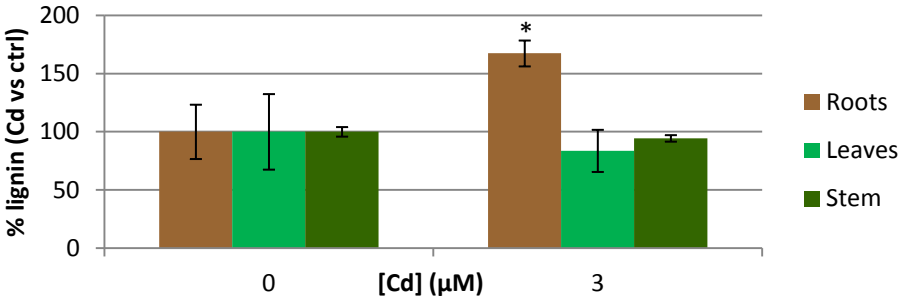


Figure 3.4.1: Lignin concentration determined by the acetyl bromide assay in WT roots, leaves and stems of *A. thaliana* plants exposed to 3 µM $CdSO_4$ for two weeks from stem formation onwards. Data represent 3 replicates with the standard error. Statistical significance (one way anova) *: p= 0.06.

lignin concentration in comparison with the respective tissues of control (non-Cd-exposed) plants (Fig 3.4.1). The absence of changes in the lignin concentration after Cd exposure was also observed by Larras *et al.* (2013) in the waterweed *Elodea nuttallii*. However, detailed microscopic analysis did reveal increases in lignification in some regions of the stems. Therefore, we studied lignification in the stems by staining of microscopic sections taken from the bottom part of the stem with both Wiesner (to stain cinnamaldehydes) and Mäule (to stain S units) staining. Based on these staining methods, we did not observe any increase in lignification in the stems after exposure to Cd (Fig 3.4.2). Since the ultimate objective of future studies is to cultivate genetically modified plants (*e.g.* poplar) with lower lignin content on contaminated soils, increases in lignin concentration after exposure to Cd are not desirable. Therefore, these preliminary results are very promising. In contrast, in other plant species such as *Phragmites australis, Juncus maritimus* stems (exposed for 2 months to 20 mg l^{-1} $CdCl_2$) (da Silva *et al.* 2014b) and callus cultures derived from stems of *Camellia sinensis L.* (exposure of 45 days to concentrations of 6.3 x 10^{-5} M or 10.6 x 10^{-5} M $Cd(NO_3)_2$) (Zagoskina *et al.* 2007) increases of lignification were observed after exposure to Cd. Moreover, for poplar (*Populus x canescens*) increased lignification was reported after 24 days exposure to a relatively high experimental concentration of 50 µM $CdSO_4$ (Elobeid *et al.* 2011). On the other hand, in the gymnosperm *Pseudotsuga menziesii* a decrease in lignin amount was reported after 9 months of exposure to 100 µM $CdCl_2$ (Astier *et al.* 2014). Because of these large differences in responses of lignification in

different study designs, plant species and Cd concentrations, more experiments should be performed with poplar, or other economically relevant plant species, exposed to environmentally relevant Cd concentrations in order to better understand the effects of Cd on lignification in field conditions.

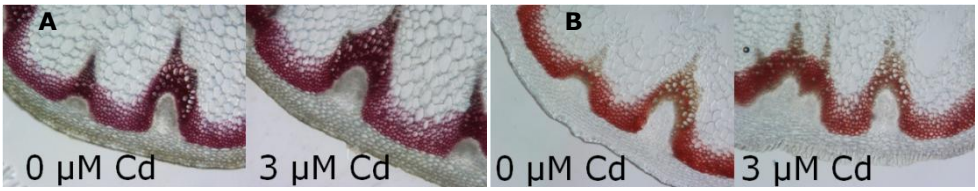


Figure 3.4.2: Representative pictures of lignin staining of cross sections of stems from WT plants exposed to 0 and 3 $CdSO_4$ for two weeks (A) Wiesner staining, (B) Mäule staining.

Considering that in leaves lignin is only found in the vascular tissues, reliable measurements of lignin concentration are difficult to generate on crude leaf samples. This became apparent by the large error bars for leaf samples (Fig 3.4.1). Therefore, only measurements in stems (from the above ground tissues) are interesting for the remainder of our study.

In contrast to the aerial parts, the lignin concentration in **roots** increased after exposure to 3 µM $CdSO_4$ (Fig 3.4.1). Spiteller *et al.* (2003) explained the stress-induced increase in the lignin concentration by the raise of peroxyl radicals, which can subduct a H-atom from phenolic groups of lignin precursors. In case two of these radicals meet, a dimer is formed which again can be attacked by a peroxyl radical producing lignin-like structures. Because the root tip is the most important location where Cd-ions can enter the plant, staining of the whole roots with phloroglucinol (to stain cinnamaldehydes) was performed (Fig 3.4.3). Using this staining method, the increase in lignification in the roots after exposure to $CdSO_4$ was clear as compared to the root tips of the WT plants (Fig 3.4.3). Similar results obtained with the acetyl bromide assay, which is the simplest and fastest method to evaluate lignin in herbaceous tissues (Moreira-Vilar *et al.* 2014), were already observed in many plant species such as *Vicia sativa* (Rui *et al.* 2016) and *Populus x canescens* (Elobeid *et al.* 2011). However, no differences in the lignification were reported in *Phragmites australis* and *Juncus maritimus* after exposure to 2 and 20 mg l^{-1} $CdCl_2$ (da Silva *et al.* 2014b). Again,

because of the plant species-related variance in the response to Cd exposure, it is difficult to extrapolate results concerning lignification and more experiments should be performed using environmentally relevant conditions and a commercially valuable species (*e.g.* poplar).

It has been suggested that the thickening of cell walls is a defence mechanism to reduce uptake of these unwanted and toxic metals in *A. thaliana* (Van de Mortel *et al.* 2008) and even in the more Cd resistant plants *Salix matsudana* (Yang *et al.* 2015). It can be explained by thickening of the Casparian strips and thereby forming a better barrier to block the apoplastic way of loading Cd to the xylem. Consequently, less toxic ions can enter the plant. Even though it is proposed that also suberin plays a significant role in Casparian strips, in *A. thaliana* the lignin barrier can be made without suberin (Naseer *et al.* 2012). Moreover, the extra lignin can bind Cd and thereby inhibit entry into the cell and even make plants more tolerant to the metal stress (Parrotta *et al.* 2015, Herbette *et al.* 2006). This extra tolerance to Cd by lignin deposition in cell walls of roots was also demonstrated in *Triticum aestivum L.* (Bezrukova *et al.* 2011).

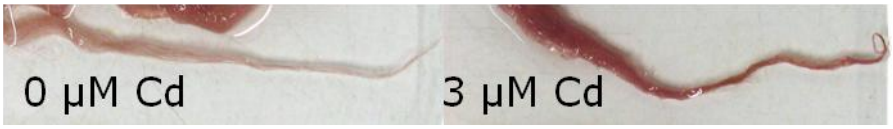


Figure 3.4.3: Representative pictures of staining with phloroglucinol of root tips from *A. thaliana* plants exposed to 0 and 3 $CdSO_4$ from stem formation for two weeks.

3.4.2.2 Lignin concentration in lignin-reduced mutants

Stems

In our study, using hydroponics and a similar development of the stem relative to WT plants (*ccr1-6* and *ccr1-3* were sown earlier) without Cd exposure, the stems of the *A. thaliana* mutants contained less lignin ($p<0.05$) though no difference between *4cl1* and *ccr1* mutants was observed (Fig 3.4.4C). In accordance with the results from the WT plants paragraph 3.4.2.1, the lignification in lignin-reduced mutant stems did not change when exposed to 3 µM Cd (Fig 3.4.4D). A result which is promising since no extra lignin is wanted in the conversion of biomass into biofuel. Of course, these results should be re-evaluated in commercially valorisable short rotation coppice plant.

Subsection 3.4

The lignin amount in the stems of the different selected *A. thaliana* mutants (*4cl1-1, 4cl1-2, ccr1-6* and *ccr1-3*) were already thoroughly investigated (Vanholme *et al.* 2012a). Vanholme *et al.* (2012a) demonstrated that both allelic variants of T-DNA mutants for *4CL1* and *CCR1* had significantly less lignin in their stems (when grown in soil with at first short day (9 h light) where after a transition to long-day conditions (16 h light) was applied). Van Acker *et al.* (2013) obtained similar results on stems of completely senescent plants.

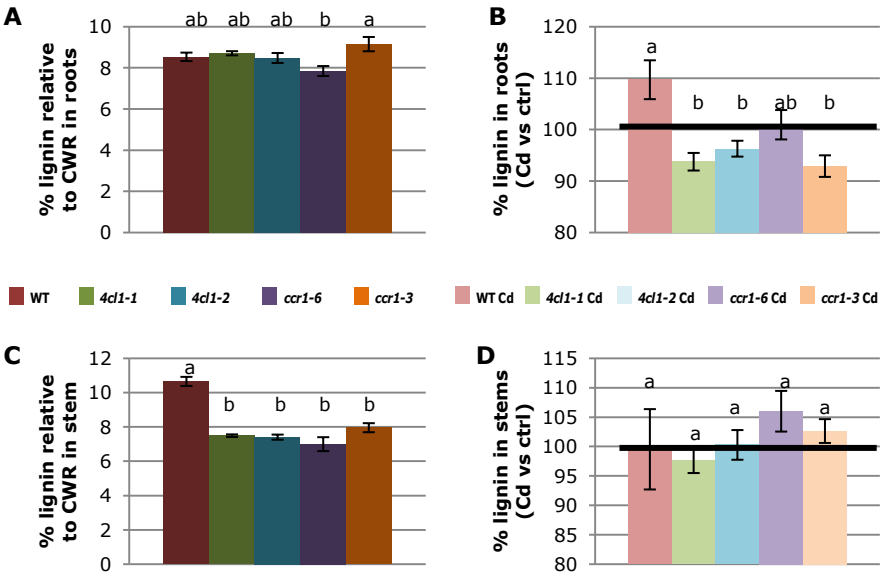


Figure 3.4.4: Lignin concentration (%) relative to the CWR determined by the acetyl bromide assay in (A) roots and (C) stems of non-exposed WT and mutant *A. thaliana* plants and the percentage of lignin concentration (determined by the acetyl bromide assay) of plants exposed to 3 µM $CdSO_4$ for 2 weeks from the start of stem formation in comparison to the corresponding non-exposed plant genotype in (B) roots and (D) stems. Data represent 5 replicates with the standard error. Statistical significances (one way anova and subsequent Tukey-Kramer post hoc test) indicated by letters $p<0.05$.

Roots

In contrast to the stems, no differences between the WT and lignin-reduced mutants roots were observed in the lignin concentration in absence of Cd (Fig 3.4.4). Since age can have an effect on the lignification of roots (Turner *et al.* 2005, Ranathunge *et al.* 2011), the differences in lignin concentration among *ccr1* mutants (Fig 3.4.4A) can possibly originate from the study design in which

ccr1-3 was sown ten days in advance to get a similar development of the stem as WT plants.

Due to the slight age effect, the best way to look into the Cd effect is to compare the percentages of changes of the non-exposed relative to the exposed for each genotype (Fig 3.4.4 B and D). As already mentioned in 3.4.2.1, after exposure to Cd, the lignin concentration in the roots of *A. thaliana* WT plants increased while the lignin concentration in the *A. thaliana* mutants (*4cl1-1, 4cl1-2* and *ccr1-3*) showed significant decreases in the lignin concentration when exposed to 3 µM Cd (Fig 3.4.4B). This decrease in lignin concentration could possibly be linked to interference of Cd-ions with enzymes of the alternative routes of lignification, which may be used in the *A. thaliana* mutants.

Lignin concentration in *ccr1-6* roots did not change after Cd exposure. Furthermore, differences in response were significant among *4cl1-1, 4cl1-2* and *ccr1-3* roots in comparison with WT plants (Fig 3.4.4B). Roots are the first location of entry of Cd and lignification is a 'first line' defence mechanism to stop Cd-ions from loading to the xylem (Tamas *et al.* 2010, Zhu *et al.* 2013). Due to the lack in increasing lignification in the roots of lignin mutants after Cd exposure, Cd-ions may enter the plant roots more easily compared to WT plants. This is important knowledge in case commercially valuable plants will be used on contaminated soils by making use of phytoremediation.

Subsequently, we studied (microscopically) the root tips of WT and *ccr1 A. thaliana* mutants with and without Cd exposure. In roots, Cd-ions can be transported radially from the rhizodermis through the cortex and endodermis up to the xylem via apo- (through the cell wall) or symplastic (through the cytoplasm) movement (Zanella *et al.* 2016). Plant roots can develop barriers to limit the apoplastic fluxes which can be enhanced after exposure to Cd. Young actively growing roots contain less lignin and are therefore expected to be more penetrable by Cd-ions (Lux *et al.* 2011).

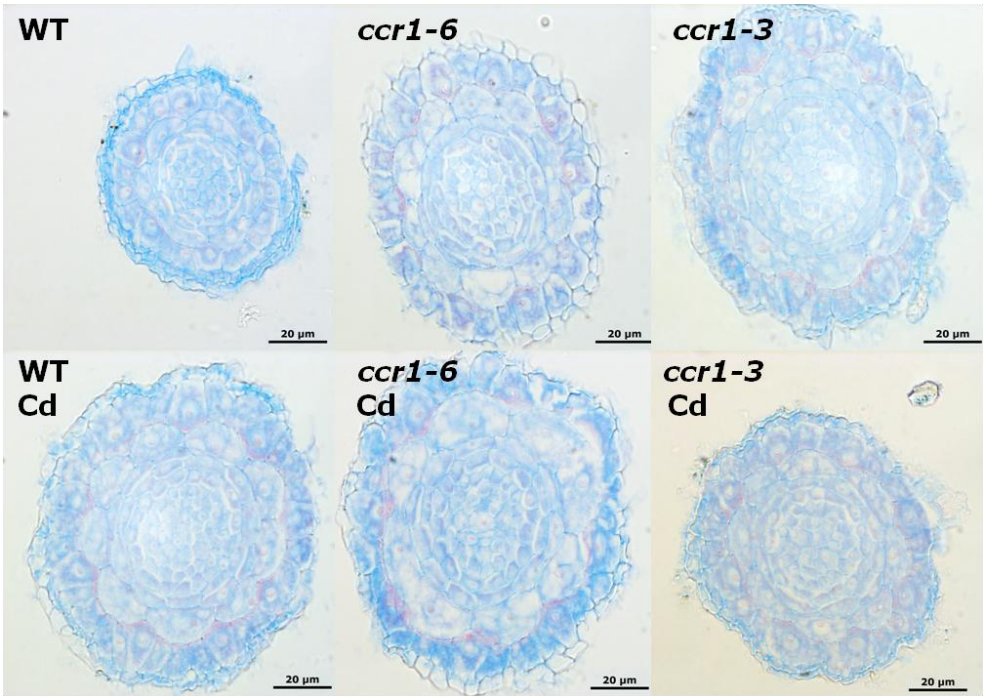


Figure 3.4.5: Representative picture of cross sections of root tips of WT, *ccr1-6* and *ccr1-3* roots from *A. thaliana* plants exposed to 0 and 3 µM $CdSO_4$ stained with saffranin (to colour lignin red) and astra blue (blue).

In the microscopic sections, both nuclei and lignin were stained by saffranin (red colour). The zones with the highest intensity of saffranin colour (and thus the highest lignin concentration) were found near the cell walls of outer cortex cells of Cd-exposed WT roots (Fig 3.4.5). Previously, a similar increase in lignin in the cortex was reported in wheat plants exposed to aluminium oxide nanoparticles (Yanik and Vardar 2015). Furthermore, increased lignin deposition due to Cd toxicity was found in the cortical cells of tobacco roots (Zanella *et al.* 2016). The cortical apoplast is considered as an early sequestration site for Cd in roots (Van Belleghem *et al.* 2007) and thickening of cell walls is an important strategy to limit access of toxic ions (Zanella *et al.* 2016). However, saffranin staining of cortex cell walls was also found in roots of non-exposed WT plants and lignin-reduced mutants (with and without Cd exposure). However, in the lignin-reduced mutants, the lignin staining looked more irregular after exposure to Cd and consequently defence against incoming Cd-ions appeared not as strong as compared to the Cd-exposed WT plants (Fig 3.4.5). These observations are

compatible with the data generated by the acetyl bromide measurement that was discussed above (Fig 3.4.4B).

3.4.2.3 Lignin composition in lignin mutants

Lignin content is the main influencing factor in the saccharification yield of *A. thaliana* plants without acid pre-treatment. However, the composition of the lignin molecules can also play a key role in determining saccharification yield (Van Acker *et al.* 2013). Lignin composition can also be affected by different biotic and abiotic stressors (*e.g.* toxic metals) although only a limited amount of studies take the proportions of the different monolignol building blocks into account (Moura *et al.* 2010, Boudet *et al.* 1998).

Effect of mutation of monolignol biosynthesis genes on stems lignin composition

Our study showed lower amounts of all main monolignols (H+G+S) in stems of lignin-reduced mutants (except *4cl1-1* mutants) in comparison to WT in similar conditions (Table 3.4.1). The lower monolignol amount in *A. thaliana* lignin mutants were already reported by Van Acker *et al.* (2013) and Mir Derikvand *et al.* (2008) for *ccr1* mutants. Vanholme *et al.* (2012a) demonstrated lower amounts of all oligolignols for *ccr1* and *4cl1* mutants. Also in *CCR* down-regulated poplar a lower H+G+S content was demonstrated (Leplé *et al.* 2007). In contrast, in a field study using transgenic *CCR* down-regulated poplars (Van Acker *et al.* 2014) such an effect was not observed. The H+G+S concentration expressed on a lignin amount basis is a good parameter to evaluate thioacidolysis yield, in which selectively the *β*-O-4 bonds are cleaved and the non-condensed fraction is released. The more condensed C-C bonds are not cleaved and do not give rise to detected monomers during the thioacidolysis assay. By consequence, the condensation degree of the lignin polymer can be evaluated by H+G+S concentrations (Leplé *et al.* 2007). It can be concluded that the condensation degree of all lignin-reduced *A. thaliana* mutants in our study rose due to the mutation, which can result in a lignin that is less easily cleavable (Nanayakkara *et al.* 2011) and can have a negative effect on saccharification. Nevertheless Van Acker *et al.* (2013) reported better saccharification rates for both *4cl1* and *ccr1* mutants of *A. thaliana*. This is

because next to lignin composition also lignin content is a major influencing factor and lignin content is reduced in all these mutants.

The H concentration in *4cl1* mutants was significantly higher while it was significantly lower in *ccr1* mutants in comparison to WT stems (Table 3.4.1). Albeit the percentage of H lignin is very low in stems in comparison to G and S units, the percentage of H units was significantly higher for all studied lignin mutants in comparison to WT stems. These findings are in agreement with the study of Mir Derikvand *et al.* (2008) and Van Acker *et al.* (2013). The increase in CCR2 capacity as an alternative route could be responsible for the higher H concentration in the *ccr1* mutants (Van Acker *et al.* 2013) though the role of CCR2 in the *ccr1* mutants still needs to be clarified.

The G monolignol concentration, as also observed by Van Acker *et al.* (2013), was reduced for all lignin-reduced genotypes. In the percentages, only in both *4cl1* mutants a significantly lower percentage G was observed in comparison to WT plants. Concerning the results on %G, in the *ccr1* mutants no definitive conclusions could be drawn since the allelic variants of *ccr1* showed different results (Table 3.4.1). Again these results are similar to those observed by Van Acker *et al.* (2013) albeit using a different cultivation system.

S units were significantly lower in the *ccr1* mutants in comparison to WT stems (Table 3.4.1). Lower S units can possibly be ascribed to a slow development of these mutants (Van Acker *et al.* 2013). For *ccr1* mutants the results in %S were unclear so no conclusions can be drawn (Table 3.4.1). No effect on S concentration was observed in the *4cl1* mutants. However, in *4cl1* mutants the S unit percentages were significantly higher (Table 3.4.1) in comparison to WT plants, as also previously reported by Van Acker *et al.* (2013).

A higher S/G ratio was present in the *4cl1* mutants in comparison to WT stems (Table 3.4.1), identical to Van Acker *et al.* (2013) in control conditions. In our study, no conclusions could be drawn about the S/G ratio in *ccr1* mutants.

The degree of methoxylation of the monolignol units is determinative for the depolymerisation of the lignin polymer. An additional methoxy group results in a diminished number of reactive sites and by consequence less possible combinations for polymerisation (Marriot *et al.* 2016). G units tend to make more C-C linkages whereas S units preferentially make *β*-O-4 linkages which

gives a rather non-condensed polymer. Therefore, the more S is present, the more easily the polymer can be degraded (Moura *et al.* 2010; Leplé *et al.* 2007). The higher S/G ratio as observed in *4cl1* can thus be an advantage during saccharification. In the paper of Van Acker *et al.* 2013, the cellulose conversion was reported to be increased for the *4cl1* mutants though here the lignin content itself seemed to play a larger role than the S/G ratio. Therefore, only considering lignin composition to draw conclusions about the possible influence on saccharification is too simplistic.

Effect of Cd exposure on lignin composition in stems

In general, Cd had a decreasing effect on total H+G+S concentration across all genotypes (Table 3.4.1). Significant differences in the total H+G+S concentration were observed for the *4cl1-1* and *ccr1-3* mutants.

However, when the change due to Cd exposure in lignin mutants was compared with the change in WT plants, significant lower percentages of H+G+S remained for the *4cl1-1* and both *ccr1* mutants after Cd exposure (Fig 3.4.7H). Therefore, it can be that due to Cd toxicity the lignin in the mutants becomes more condensed and thus less cleavable. A significant decrease in H units concentration was observed after Cd exposure in WT, *4cl1-2* , and *ccr1-6* plants, but not in *ccr1-3* mutants wherein no change was detected (Table 3.4.1 and Fig 3.4.7B).

Only in WT plants a significant decrease of %H was observed (Table 3.4.1). Even though these H units make up just a small part of the lignin polymer, differences in H units can have an effect on the degradability of the biopolymer. When exposed to Cd, levels of G units were further significantly reduced in lignin-reduced mutants (except for *4cl1-2* mutants) (Table 3.4.1 and Fig 3.4.7D). S unit levels of the plants decreased significantly in the *4cl1-1* mutant (Table 3.4.1) and *4cl1-1* and both *ccr1* mutants showed a higher decrease as calculated as a percentage of S units remaining after Cd exposure in comparison with changes due to Cd in WT stems (Fig 3.4.7F). Most plant genotypes did not show any effects in %G and %S under Cd stress. Although *4cl1-1* mutants were increased in %G and decreased in %S resulting in a decrease of the S/G ratio in comparison to the non-exposed plants, it was not significant for the other allelic form (*4cl1-2)* (Table 3.4.1 and Fig 3.4.7J).

Table 3.4.1: Composition of CWR from **stems**: : *p*-hydroxyphenyl (H), guaiacyl (G), syringyl (S), ferulic acid (FA) addition product, CCR marker, estered FA, and H+G+S are expressed in µmol g^{-1} AcBr lignin. %H, %G, and %S are molar percentages. Standard errors (n=5) are reported. Significant (anova with mixed model analysis with post-hoc Dunnett T-test, using genotype as a fixed effect) decreases ($p<0.05$) and increases ($p<0.05$) between the non-exposed lignin mutants according to WT. Effects of the Cd treatment of the Cd-exposed in comparison to the non-exposed same genotype are indicated in purple ($p<0.05$) for increases and orange ($p<0.05$) for decreases.

STEM	0 µM $CdSO_4$					3 µM $CdSO_4$				
	WT	***4cl1-1***	***4cl1-2***	***ccr1-6***	***ccr1-3***	**WT**	***4cl1-1***	***4cl1-2***	***ccr1-6***	***ccr1-3***
H+G+S	4691 ± 559	5278 ± 110	3474 ± 159	1350 ± 226	2251 ± 162	4231 ± 252	3390 ± 36	3462 ± 196	809 ± 38	1514 ± 149
H	26.7 ± 1.3	61.6 ± 1.7	53.3 ± 3.8	10.1 ± 0.8	18.9 ± 2.4	16.7 ± 1.9	38.9 ± 0.5	37.1 ± 2.0	5.1 ± 1.4	17.2 ± 1.6
G	3669 ± 390	2791 ± 160	2155 ± 144	1332 ± 228	1707 ± 115	2991 ± 324	2091 ± 43	2063 ± 27	676 ± 42	1177 ± 112
S	996 ± 169	2224 ± 138	1385 ± 85	203 ± 77	469 ± 68	948 ± 44	1214 ± 10	1460 ± 109	126 ± 10	321 ± 39
%H	0.54 ± 0.03	1.28 ± 0.06	1.28 ± 0.14	0.90 ± 0.15	0.96 ± 0.09	0.41 ± 0.02	1.15 ± 0.03	1.08 ± 0.05	0.74 ± 0.08	1.14 ± 0.05
%G	78.77 ± 1.37	54.95 ± 1.94	56.30 ± 2.68	85.49 ± 2.72	76.04 ± 0.84	76.59 ± 0.95	61.93 ± 1.23	56.84 ± 1.19	83.78 ± 1.25	77.80 ± 0.98
%S	20.68 ± 1.35	43.78 ± 1.99	42.31 ± 2.75	13.72 ± 2.81	23.00 ± 0.90	22.98 ± 0.94	36.92 ± 1.22	42.08 ± 1.22	15.49 ± 1.29	21.05 ± 0.95
S/G	0.264 ± 0.021	0.806 ± 0.066	0.764 ± 0.089	0.164 ± 0.040	0.303 ± 0.015	0.301 ± 0.016	0.599 ± 0.032	0.743 ± 0.037	0.186 ± 0.018	0.271 ± 0.015
CCR marker	1.42 ± 0.61	2.33 ± 0.72	1.95 ± 0.46	17.28 ± 7.27	17.71 ± 5.43	1.41 ± 0.34	1.52 ± 0.61	1.40 ± 0.37	7.07 ± 3.05	9.48 ± 1.84
Estered FA	0.51 ± 0.04	0.55 ± 0.07	0.45 ± 0.13	8.37 ± 0.91	6.21 ± 1.72	0.55 ± 0.12	0.31 ± 0.01	0.54 ± 0.19	3.88 ± 0.33	4.92 ± 0.76
FA addition product	0.25 ± 0.04	0.26 ± 0.01	0.21 ± 0.04	4.29 ± 0.55	2.96 ± 0.31	0.12 ± 0.04	0.17 ± 0.04	0.15 ± 0.02	1.08 ±0.12	2.19 ± 0.32

Table 3.4.2: Composition of CWR from **roots**: *p*-hydroxyphenyl (H), guaiacyl (G), syringyl (S), ferulic acid (FA) addition product, CCR marker, estered FA, and H+G+S are expressed in µmol g^{-1} AcBr lignin. %H, %G, and %S are molar percentages. Standard errors (n=5) are reported. Significant (anova with mixed model analysis with post-hoc Dunnett T-test, using genotype as a fixed effect) decreases ($p<0.05$) and increases ($p<0.05$) between the non-exposed lignin mutants according to WT. Effects of the Cd treatment of the Cd-exposed in comparison to the non-exposed same genotype are indicated in purple ($p<0.05$) for increases and orange ($p<0.05$) for decreases.

ROOT	0 µM $CdSO_4$					3 µM $CdSO_4$				
	WT	***4cl1-1***	***4cl1-2***	***ccr1-6***	***ccr1-3***	**WT**	***4cl1-1***	***4cl1-2***	***ccr1-6***	***ccr1-3***
H+G+S	1676 ± 205	1437 ± 187	898 ± 58	788 ± 15	719 ± 28	1158 ± 115	975 ± 128	1130 ± 125	923 ± 83	973 ± 90
H	72.8 ± 11.0	40.2 ± 6.4	26.8 ± 1.5	29.0 ± 2.2	30.0 ± 2.1	62.4 ± 3.6	62.3 ± 5.7	77.2 ± 11.8	71.1 ± 5.0	69.8 ± 8.4
G	1482 ± 178	1267 ± 168	803 ± 63	714 ± 16	653 ± 27	1123 ± 57	873 ± 118	1005 ± 111	748 ± 37	855 ± 79
S	101.4 ± 25.8	148.3 ± 10.4	68.2 ± 11.0	45.2 ± 2.5	40.3 ±3.9	55.7 ± 10.1	47.3 ± 3.2	53.1 ± 2.2	28.0 ± 4.8	43.3 ± 2.4
%H	3.65 ± 0.79	2.42 ± 0.25	3.01 ± 0.15	4.16 ± 0.30	4.59 ± 0.22	6.16 ± 0.52	6.53 ± 0.30	6.73 ± 0.39	8.72 ± 0.53	7.12 ± 0.41
%G	88.50 ± 0.23	88.17 ± 1.11	89.09 ± 1.79	89.61 ± 0.68	90.64 ± 0.15	89.95 ± 0.51	90.12 ± 0.48	88.89 ± 0.52	87.29 ± 1.13	87.73 ± 0.80
%S	7.17 ± 0.55	8.96 ± 0.78	7.90 ± 1.67	5.66 ± 0.26	4.86 ± 0.22	4.70 ± 0.53	4.17 ± 0.15	3.91 ± 0.25	3.33 ± 0.10	5.03 ± 0.46
S/G	0.081 ± 0.006	0.102 ± 0.010	0.090 ± 0.021	0.063 ± 0.003	0.054 ± 0.002	0.053 ± 0.006	0.047 ± 0.001	0.044 ± 0.003	0.038 ± 0.001	0.057 ± 0.005
CCR marker	0.77 ± 0.24	0.89 ± 0.06	0.42 ± 0.17	3.83 ± 1.64	1.30 ± 0.69	0.24 ± 0.03	0.31 ± 0.014	0.33 ± 0.10	1.88 ± 0.71	2.52 ± 0.25
Estered FA	1.02 ± 0.30	0.58 ± 0.24	0.18 ± 0.10	2.04 ± 0.46	1.54 ± 0.20	0.41 ± 0.12	0.21 ± 0.09	0.52 ± 0.10	1.40 ± 0.54	2.67 ± 0.29
FA addition product	0.59 ± 0.11	0.35 ± 0.08	0.14 ± 0.05	0.67 ± 0.14	0.71 ± 0.15	0.43 ± 0.16	0.12 ± 0.03	0.21 ± 0.06	0.83 ± 0.01	0.80 ± 0.08

In conclusion, Cd did not have clear influences on the monolignol units in stems though slight changes such as differences in %H were observed between WT (decrease) and lignin-reduced mutants (no significant change).

Effect of mutation of the monolignol biosynthesis genes on roots lignin composition

Since root biomass is not harvested, it is not useful for biomass production and processing. By consequence, little to no knowledge is available concerning the lignin composition in roots of lignin-reduced mutants for any plant species. However, roots are the first plant tissue to encounter soil nutrients and by extension the potential contamination present in the soil. Analysis of our results did show the difficulty to prove differences in the lignin composition among genotypes and treatments, probably because our samples existed of a mixture from low lignified tissue at the tip to more lignified closer to the hypocotyl.

As observed in stems, the main monolignols (H+G+S) were significantly lower in the roots of the lignin-reduced mutants in comparison to WT plants (Table 3.4.2). So, the lignin of roots of lignin mutants can be more condensed in comparison to WT plants.

In contrast to the stems, the H concentration in roots tends to be lower in all lignin-reduced mutants (although no significance was shown). Similar to stems, G units concentration in roots of the lignin-reduced genotypes (except for *4cl1-2* mutants) was significantly lower as compared to the WT plants. In roots, also S units were significantly lower in the *ccr1* mutants as compared to the WT plants (Table 3.4.2). Taken together, all the main monolignol units contributed to the lower H+G+S concentration.

Differences among genotypes in %H, %G and %S and S/G were not observed in roots (Table 3.4.2).

To summarise the effects of the genotype on lignin composition of the roots, lignin can be more condensed in the lignin mutants as H+G+S decreases; no significant additional conclusions can be drawn. Moreover, these findings in roots can also have implications on soil ecology because in commercial applications roots are not harvested, and more condensed lignin will be more difficult to degrade by fungi (Gul *et al.* 2014).

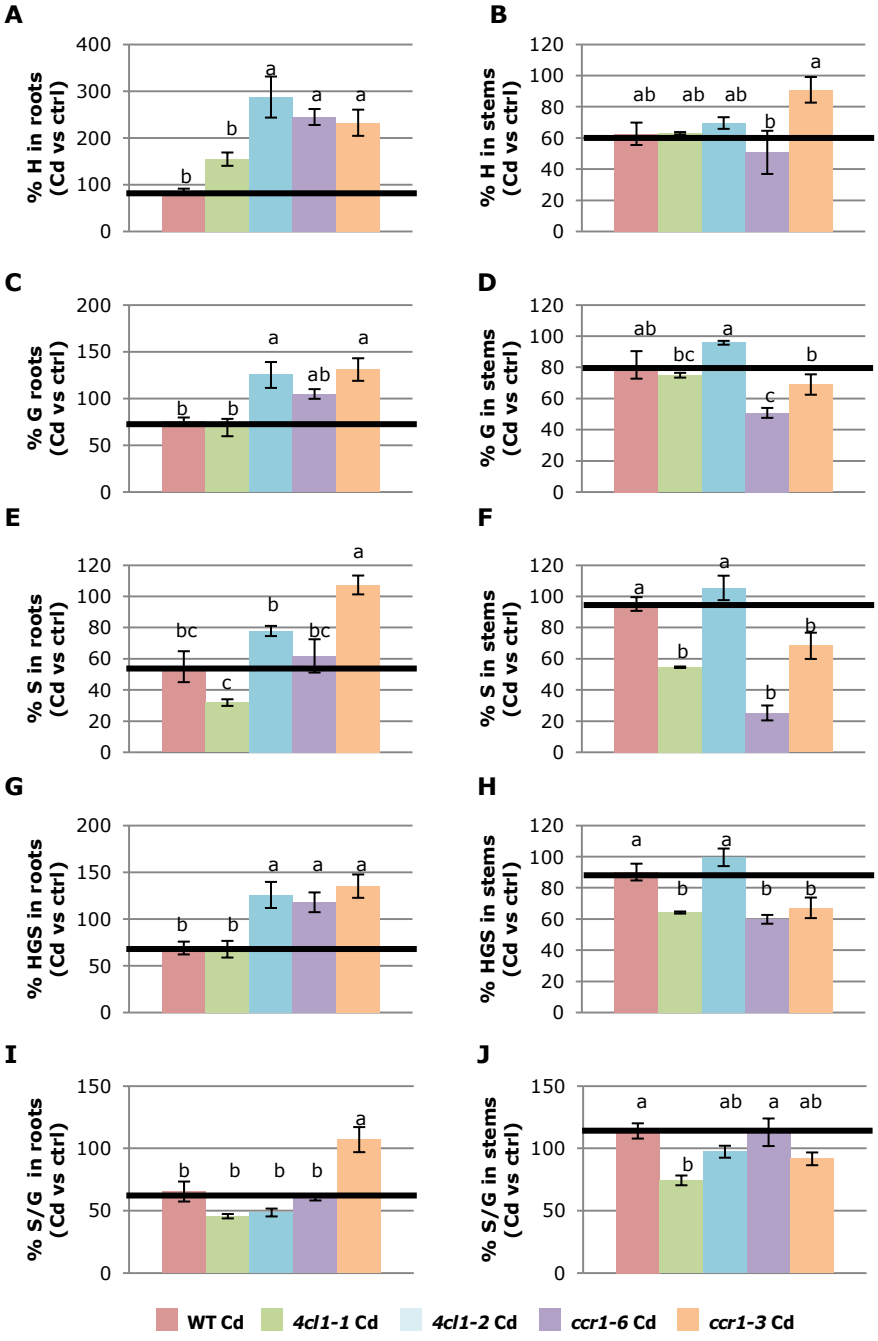


Figure 3.4.7: Differential changes upon exposure to 3 µM $CdSO_4$ in lignin composition in roots (A,C,E,G,I) and stems (B,D,F,H,J) with %H (A,B), %G (C,D), %S (E,F) and %H+G+S calculated using the lignin concentrations (µmol g-1 AcBr lignin) (reported in table 3.4.1 and 3.4.2) and %S/G (I,J) calculated by S/G values (reported in table 3.4.1 and 3.4.2). Data represent 5 replicates with the standard error. Significance (one way anova with subsequent Tukey-Kramer post hoc test) indicated by letters $p<0.05$.

Subsection 3.4

Effect of Cd exposure on lignin composition in roots

Monolignolic changes might occur when plant roots are exposed to Cd. An altered chemical composition of endodermal lignin after Cd exposure has been suggested by Lux *et al.* (2011) in *Zea mays* roots. However, only Finger-Taxeira (2010) reported on the effect of Cd exposure on monolignol composition. Finger-Taxeira (2010) used very young (3 days) soybean roots that were exposed for a period of 24 h to rather high experimental Cd concentrations (25, 50, 75 and 100 µM $CdCl_2$). In that study, H+G+S and S amount, determined by taking the CWR into account, only increased when the seedlings were exposed to the highest Cd concentration (Finger-Taxeira 2010). When their results were determined by taking the lignin content into account, they reported a decrease in total H+G+S amount, which indicates an increased condensation (Cabane *et al.* 2012). However, in our study no significant Cd effects on the total main monolignols (H+G+S) in roots were observed for WT plants (Table 3.4.2). Probably because our study-design used more mature plants with a rather long (2 weeks) and environmentally relevant exposure to 3 µM Cd. However, in the *4cl1-2* (not for *4cl1-1*) and both *ccr1* mutants, H+G+S were significantly higher after Cd exposure in comparison to changes in WT roots (Fig 3.4.7G). A trend for a decrease of S units (concentration and percentage) after Cd exposure was observed in all genotypes except *ccr1-3* mutants used in our study and this percentage of change due to Cd was significantly different for *ccr1-3* mutants (Fig 3.4.7E). However, because the allelic variants did not show the same tendency, no conclusion can be drawn about a distinct response of lignin mutants in comparison to WT plants (Table 3.4.2 and Fig 3.4.7E). No general effect of Cd was observed on G monolignol concentration and percentage of G (Table 3.4.2). This was in agreement with the results of Finger-Taxeira *et al.* (2010). Though, the increase in G units due to Cd was more pronounced in the *4cl1-2* (not for *4cl1-1* mutants) and *ccr1-3* mutants in comparison to the changes due to Cd in WT plants (Fig 3.4.7C). Unlike in stems of Cd-exposed plants, in the roots of all lignin mutants the H concentration was significantly increased (Table 3.4.2 and Fig 3.4.7A) and mean values in percentage of H were almost doubled ($p<0.05$) in all genotypes including WT plants upon Cd exposure (Table 3.4.2). As *CCR2* expression is increased as a response to various stressors (Boudet *et al.* 2000) and it is suggested that CCR2 may contribute to

H-lignin formation (Zhou *et al.* 2010) the increase in H may result from an increase of CCR2 capacity. Furthermore, an increase in H units in roots exposed to Cd was also reported when soybean roots were exposed to 75 and 100 µM Cd for 24 h (Finger-Taxeira 2010). In the study of Cheng *et al.* (2014), more %H was found in rather metal tolerant mangrove species in comparison with more sensitive pioneer species. Together with our results, the increase in H concentration after Cd exposure may play a role in the defence against metal contamination.

Moreover, reduced S/G ratios in roots were found when plants were exposed to Cd (for all genotypes except *ccr1-3*) (Fig 3.4.7I). Because the degree of methoxylation of the used monolignols is altered in favour of G, it might be possible that the produced lignin polymers will be more difficult to degrade. On the other hand, the H units may make the lignin polymers shorter since H units may stop the polymerisation and polymers become more easily extractable (Sundin *et al.* 2014). Although roots are not harvested for economic purposes, the changes in monomers can have effects on ecological soil processes, though this still needs to be further investigated.

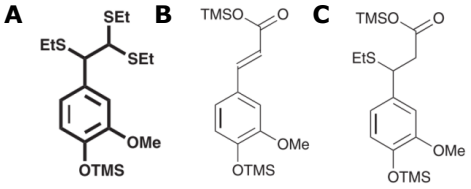


Figure 3.4.6: Non-conventional lignin units: (A) CCR marker (also known as bis-β–O–4-FA or G-CHR-CHR2 with R= EtS), (B) esterified ferulic acid (also known as β–O–4-FA-I or G-CH= CH-COOH) and (C) ferulic acid addition products (also known as β–O–4-FA-II or G-CHR-CH2-COOH with R= EtS) (Van Acker *et al.* 2013, Ralph *et al.* 2008)

CCR marker, FA addition products and FA estered

In addition to the traditional monolignols, also non-conventional lignin units such as the CCR marker, ferulic acid addition products and estered ferulic acid, which are specifically incorporated in cell walls of the *ccr1* mutant, were analysed (Van Acker *et al.* 2013) (Fig 3.4.6). The presence of the CCR marker (1,2,2-trithioethyl ethylguaiacol) gives evidence for ferulic acid incorporation in lignin (Ralph *et al.* 2008) and the incorporation of ferulic acid can result in more

branching points which can render a more readily cleavable lignin polymer (Ralph *et al.* 2008).

Elevated levels of CCR marker in combination with ferulic acid (FA) addition products and esterified ferulic acid were found in *ccr1-6* and *ccr1-3* stems and roots of plants exposed and not exposed to Cd in comparison to WT plants. The concentrations of these FA related products in roots was not that high as in stems (Table 3.4.1 and 3.4.2). Increases of CCR marker in combination with ferulic acid addition products and esterified ferulic acid have been reported in *CCR* deficient plants of *A. thaliana* (Van Acker *et al.* 2013, Ralph *et al.* 2008), poplar (Van Acker *et al.* 2014, Ralph *et al.* 2008, Leplé *et al.* 2007) and tobacco (Ralph *et al.* 2008) and as observed by Van Acker *et al.* (2013), our results showed that the CCR marker was the most abundant of all three measured ferulic acid derivatives (Table 3.4.1 and 3.4.2).
Nonetheless, they were found to a lesser extend in *ccr1* stems when plants were exposed to 3 µM $CdSO_4$ (Table 3.4.1 and 3.4.2). By consequence Cd might influence the degradability of these stems. In roots, Cd effects on the ferulic acid and CCR marker were inconclusive and only esterified ferulic acid was decreased due to Cd in *ccr1-6* (Table 3.4.2).

3.4.3 Conclusions

Roots of lignin-reduced mutants showed the same lignin concentration in comparison to WT plants (Fig 3.4.4A). However, no other studies were available to put the results in a larger context. Furthermore, in roots of WT plants the lignin concentration increased after Cd exposure (Fig 3.4.2 and Fig 3.4.4C). However, roots of the lignin mutants showed no change or even a lower lignification when plants were exposed to low Cd concentrations (Fig 3.4.4C). The lower lignin concentration will probably give rise to a higher permeability for Cd-ions in roots of lignin mutants in comparison to WT roots whereby more Cd-ions can be translocated into other plant tissues.
The total H+G+S concentration was increased in Cd-exposed *4c1-2, ccr1-6* and *ccr1-3* mutants, resulting in a less condensed lignin in comparison to Cd-exposed WT plants. Also S/G ratios were decreased under Cd stress (not the case for *ccr1-3*) (Fig 3.4.7I). These differences in lignin composition all suggest

that lignin might be more difficult to decompose in lignin mutant roots after Cd exposure. Moreover, the increase in %H may be a part of a defence mechanism against Cd stress in all investigated genotypes (Table 3.4.2). Although in case H concentration was studied, only increases were observed in lignin mutants (Table 3.4.2).

In **stems**, the formerly found lower lignin concentrations of the selected lignin-reduced mutants (Vanholme *et al.* 2012a, Van Acker *et al.* 2013) were also observed in our study-design. Our results also endorsed the observations of Vanholme *et al.* (2012a) and Van Acker *et al.* (2013) about monomer composition in stems. Furthermore, no increase in lignin was observed after Cd exposure of all genotypes including the WT (Fig 3.4.4D). The lack of increase in lignification when plants are grown in the presence of Cd is positive in the scope of our study, since more lignin in economically relevant plants (*e.g.* poplar) is not desirable with respect to the conversion of biomass into biofuel. Furthermore, the lignin composition was not strongly changed due to Cd exposure. The %H was significantly decreased for WT after Cd exposure but this was not the case for lignin mutants (Table 3.4.1). Even though %H is very low compared to %G and %S, it may have an influence on the degradability of lignin polymers.

In future experiments, these results should be validated in poplar plants in more genuine field conditions. Moreover, saccharification tests should be included to explore the effects on lignin degradability due to Cd exposure in both WT and plants with a reduced lignin content.

Subsection 3.5
EFFECTS ON THE PLANT: conclusions

In *Arabidopsis thaliana* plants, the **reduction of the lignin content via genetic modification** (*e.g.* down-regulation of 4 coumarate:CoA ligase (*4CL1)* or cinnamoyl Coa reductase (*CCR1)*) and **cadmium (Cd) exposure** can affect various plant processes going from macroscopic to cellular and molecular levels. We started with investigating the effects on the growth stages throughout the lifecycle of *A. thaliana*. Secondly, stress responses were studied since both genetic modification of the lignin biosynthesis genes and Cd exposure can lead to generation of reactive oxygen species (ROS). Thirdly, the selected lignin-reduced *A. thaliana* mutants are designed to contain less lignin, but since lignification was reported to be influenced by Cd exposure (Finger-Teixeira *et al.* 2010, Elobeid *et al.* 2012), lignin concentration and composition were monitored.

3.5.1 Effects of the lignin-reduced genotype

The lignin-reduced genotype had an effect on various parameters at the level of the plant (related to growth, stress responses and lignin) as summarised in figure 3.5.1. In the early growth stages on vertical agar plates *4cl1-1* roots and leaves developed slower (Fig 3.5.1). These observed differences in the *4cl1-1* mutant were not yet described in literature. In the later growth stages no differences were detected in hydroponics (Fig 3.5.1), which corresponds with the observations of Vanholme *et al.* (2012a) and Van Acker *et al.* (2013). Therefore, the growth retardation in the early growth phase of *4cl1-1* seedlings was no further complication during the rest of our study. In contrast, the early developmental stages on vertical agar plates of *ccr1* seedlings were accelerated in comparison to the *A. thaliana* wild type (WT) plants (Fig 3.5.1). However, both *ccr1* allelic types were delayed in rosette and stem development in hydroponic culture (Fig 3.5.1). Further, they produced smaller roots, rosettes and stems and less flowers, siliques and seeds. Although the seeds of *ccr1* mutants were heavier since less were present in 1 mg (Fig 3.5.1). The differences in seed characteristics might be the reason for the faster development of the *ccr1* seedlings during the early growth stages. Because of

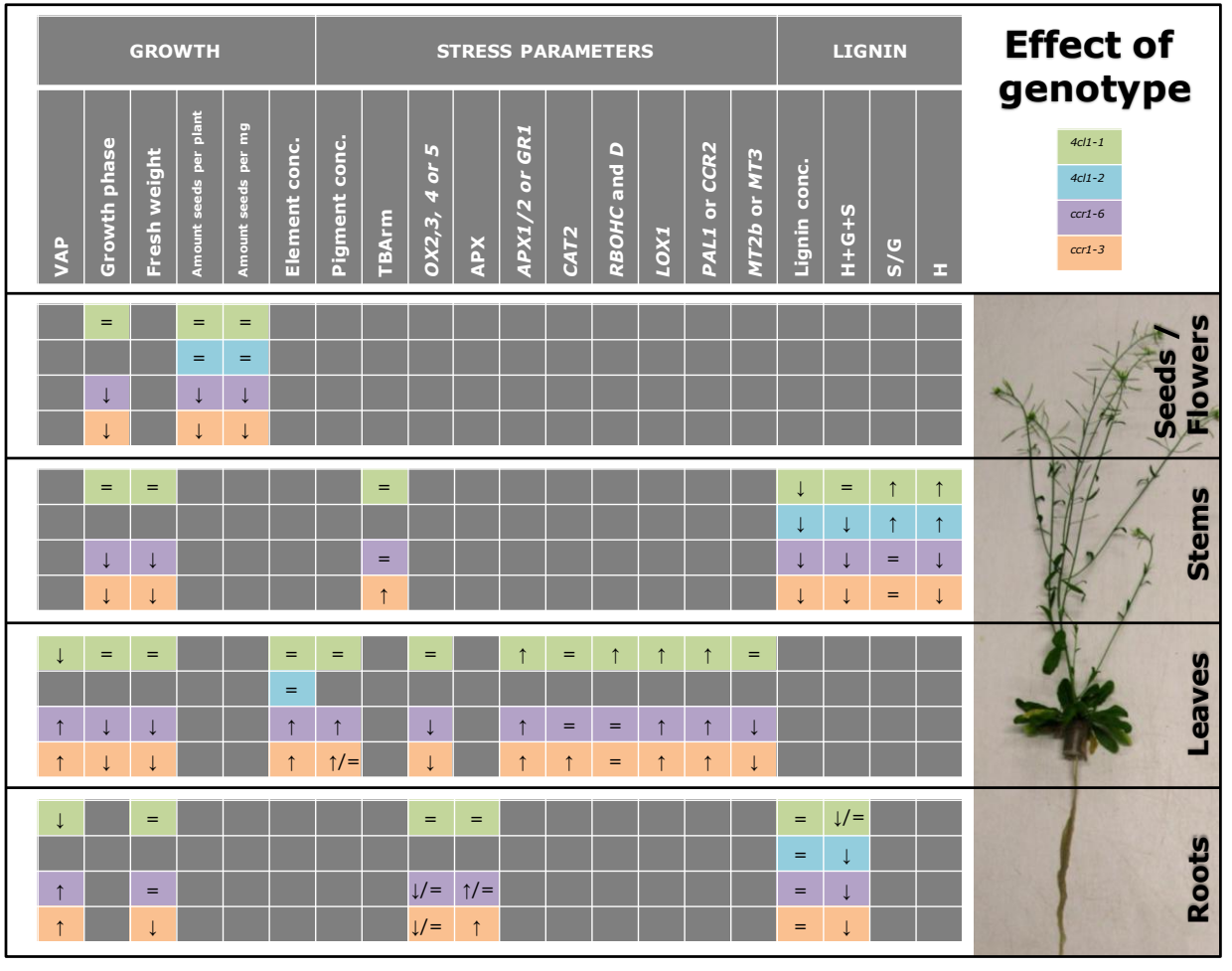


Figure 3.5.1: Summary of the outcome related to growth, stress responses and lignin in the lignin –reduced mutant roots, leaves, stems and seeds compared to WT plants due to the absence of the function of selected genes (*4CL1* and *CCR1*). Only measurements which are discussed in the text are presented. Conc. = concentration.

these significant differences in growth and development, the observed **differences in growth phases for *ccr1*** should be kept in mind or eliminated to investigate both the effects of the plant genotype and Cd exposure.

Below ground

In roots, no significant effects due to the plant genotype were observed in the lipid peroxidation analyses (TBArm) (Fig 3.5.1). In case lignin was quantified by the acetyl bromide assay, no significant differences in lignin concentration were present between the lignin-reduced mutants and WT roots (Fig 3.5.1). Therefore, the attempt to increase lignification despite the modification seems successful in the roots. However, the lignin was more condensed in lignin-reduced mutants in comparison to WT plants since a lower H+G+S concentration was measured during the thioacidolysis assay (Fig 3.5.1). The more condensed lignin, making the lignin polymers more difficult to degrade, may have an ecological impact and should therefore be further explored. Further, the capacity of APX, related to the ascorbate-glutathione cycle, was slightly higher in *ccr1-3* roots (Fig 3.5.1), suggesting a need for defence against ROS in the cytoplasm. However, oxidative stress-related marker genes (*OX 4* and *5*) were lower in *ccr1* mutants in comparison to WT plants (Fig 3.5.1) which could indicate that the defence systems are working properly and reducing the stress levels in a proper way.

Above ground

The pigment concentration of leaves of *ccr1* mutants tended to be elevated in comparison to the WT plants (significant ($p<0.05$) for chlorophyll *a* in *ccr1-6*) (Fig 3.5.1). Differences in photosynthetic capacity could be expected since leaves of *ccr1* mutants contain more pigments (especially chlorophyll *a*), which can influence other plant processes. The difference in pigment concentration along with the delay in development, makes that these mutants are difficult to study and we should keep in mind the differences in appearance of the plants, as suggested by Lawlor *et al*. (2013), in our study of stress responses of the *ccr1* mutants. Nonetheless, *CAT2* expression related to the peroxisomal defence mechanisms was to be higher in *ccr1-3* leaves (Fig 3.5.1). However, differences in *CAT* expression might be explained by the differences in the developmental

stage. Furthermore, the demand for the ascorbate-glutathione cycle was higher in leaves of *4cl1-1 (APX1* and *GR1* expression $p<0.05$) and both *ccr1* mutants (*ccr1-6*: *APX1* expression $p<0.1$ and *ccr1-3: APX1* and *APX2* expression $p<0.05$) (Fig 3.5.1). These observations may be explained by the oxidation of ascorbate by ferulic acid or other intermediates, and derivatives thereof, from the monolignol pathway, which accumulate in the lignin-reduced mutants. *OX* genes *3, 4* and *5* in leaves were differently activated in *ccr1* and *4cl1-1* mutants. In the *ccr1* mutant less mRNA from these marker genes was found in comparison to WT while in the *4cl1-1* mutant no significant effects were found (Fig 3.5.1). Further, *LOX1* (for all mutants) and *RBOHC* or *D* (for *4cl1-1* mutants) were slightly higher in the leaves of lignin mutants (Fig 3.5.1). This together with the higher mRNA levels of *PAL1* and/or *CCR2* in the lignin mutant leaves (Fig 3.5.1) could indicate attempts to enhance the perturbed lignification which was already suggested for these mutants by Vanholme *et al.* (2012a). They also found higher expressions of *PAL1* and *CCR2* in stems of lignin-reduced mutants in comparison with WT plants. However, no link to lignification can be made in our study since no measurements of the lignin concentration of the lignin-reduced mutants leaves were included.

In addition, the *ccr1* mutants took up more macro- and micro-nutrients in their leaves in comparison to WT plants (in both *ccr1-6* and *ccr1-3:* Mg, K, Cu ($p<0.1$) and Ca, Mn ($p<0.05$)) (Fig 3.5.1). Moreover, gene expression related to metal chelating proteins (*MT2b* and *MT3*) was lower in leaves of *ccr1* mutants in comparison to WT plants (Fig 3.5.1), suggesting that these lignin mutants have alternative routes to maintain homeostasis of essential elements (*e.g.* easier passable cell boundaries due to a higher permeability of the cell wall itself or differences in membrane transport). Another hypothesis is that the presence of organic acids (*e.g.* ferulic acid) can exert metal chelating capacity.

In stems, a higher lipid peroxidation level in *ccr1-3* was found in comparison to WT stems (Fig 3.5.1) which can be either by damage or can be part of a signalling function. Furthermore, a lower lignin concentration in stems of the lignin mutants and alterations in monolignol composition (as reported by Vanholme *et al*. 2012a, Van Acker *et al*. 2013) were also observed in our study (Fig 3.5.1).

3.5.2 Effects of exposure to cadmium

In figure 3.5.2, all the effects of Cd exposure of both WT and lignin-reduced plants related to growth, stress responses and lignin are summarized. When exposed to Cd, the *ccr1* mutant and to a lesser extend also the *4cl1* mutant took up more Cd-ions in their roots and translocated more Cd to the leaves (Fig 3.5.2). Since it was described that the abundance of lignin in the secondary cell wall can make plants more tolerant to Cd (Parrotta *et al*. 2015), it can be expected that lignin mutants with a lower lignin content could be more sensitive to Cd toxicity. However, rather the opposite was observed for the selected lignin-reduced mutants as discussed below.

Roots

In case of exposure to Cd on vertical agar plates, roots of *ccr1-3* and *4cl1-1* mutants were less inhibited ($p<0.05$) than the roots of WT plants (Fig 3.5.2). Furthermore, the presence of Cd-ions affected the elemental (micro- and macro-nutrients) concentrations of WT roots while no influences of Cd were observed on the elemental concentrations in roots of *ccr1* mutants (Fig 3.5.2). This support the above-mentioned hypothesis of the more penetrable cell boundary or presence of different metal chelators in the *ccr1* mutants.

Furthermore, we observed differences among the different plant genotypes in their capacities of stress-related enzymes and gene expression levels in roots in response to Cd exposure. However, in the roots no clear trend in response to Cd toxicity could be assigned. Higher stress-related gene expression at the cell boundaries (*OX2*) was measured for all plant genotypes including WT ($p<0.05$; though not significant for *4cl1-1*) and the *OX3* gene was only elevated after Cd exposure in *ccr1* mutant roots ($p<0.05$). However, GR capacity was elevated in roots of all genotypes ($p<0.05$) except the *ccr1-3* mutants (Fig 3.5.2). Also elevated gene expression levels (in comparison to changes in WT after Cd exposure) corresponding to enzymes which are active in plastids (*FSD1*, *OX5* expression $p<0.05$ in *ccr1-6* and *ccr1-3*) and cell boundaries (*LOX1* $p<0.05$, *RBOHC* $p<0.1$ and *D* $p<0.05$ in *ccr1-6* and *ccr1-3*) revealed that Cd toxicity was experienced differently by *ccr1* roots (Fig 3.5.2). However, levels of TBA reactive compounds did not increase in the *ccr1* mutants after Cd exposure (Fig 3.5.2).

Further, no Cd induced changes in CAT capacity were observed in roots of *ccr1* mutants while CAT capacity increased in roots of WT plants and *4cl1-1* mutants ($p<0.05$) (Fig 3.5.2.). The CAT capacity data suggests a lower need for defence against ROS near the peroxisomes of *ccr1* mutant roots.

The SPX capacity was increased in WT after Cd exposure ($p<0.05$) though not in lignin-reduced mutant roots (Fig 3.5.2). The higher syringaldazine peroxidase capacity can be an indication of peroxidase capacity needed for lignin biosynthesis. Furthermore, *PAL1* gene expression tended to decrease in roots of *ccr1* mutants but not in WT after Cd exposure (Fig 3.5.2.). These changes in SPX capacity and *PAL1* expression can be related to the observations concerning lignin concentration. In roots of WT plants lignification increased after Cd exposure while no changes or even decreases in lignin concentration were observed in the lignin mutants demonstrating differences in response to Cd of WT plants in comparison to lignin-reduced mutants ($p<0.05$ for *4cl1-1*, *4cl1-2* and *ccr1-3*) (Fig 3.5.2.). This supports the hypothesis that Cd concentrations are higher in roots and leaves of *A. thaliana* lignin-reduced mutants due to a more permeable passage.

Cadmium exposure also affected the monolignol composition. The S/G ratio decreased after Cd exposure for all genotypes except *ccr1-3* and increases in %H were observed in all genotypes including WT (Fig 3.5.2), suggesting that the alterations in lignin composition could be a general defence mechanism against Cd toxicity in roots to produce lignin molecules which are more difficult to decompose.

Leaves

A general pattern of lower stress levels was observed in leaves of *ccr1* mutants and partly also in the *4cl1-1* mutants as compared to the WT plants. Leaves of hydroponically grown *ccr1* mutants revealed a delay in chlorosis after Cd exposure in comparison to WT plants (Fig 3.5.2). An observation which was supported by the quantification of the pigment concentrations, which were less inhibited after Cd exposure in the lignin-reduced mutants in comparison to the WT plants (Fig 3.5.2.). Moreover, fresh weight of leaves of the lignin mutants was also less affected in response to Cd exposure (Fig 3.5.2). Other indications of a lower stress in lignin mutants leaves are the gene expressions of *OX* (*OX1*

not affected for *ccr1* mutants while increased ($p<0.05$) in WT plants and *OX3* and *5* down-regulated for *4cl1-1* mutants ($p<0.05$)), *CAT1* (down-regulated for lignin-reduced mutants and no effect for WT plants), *CSD1* (slightly down-regulated for *ccr1* mutants and no effect for WT plants), *GR1* (up-regulated for WT plants ($p<0.05$) but not for lignin-reduced mutants) and *RBOH* (up-regulated for WT plants ($p<0.1$ for isoforms C and D) but not for lignin-reduced mutants) (Fig 3.5.2). Furthermore, *APX1* and *2* gene expressions were less enhanced by Cd exposure in leaves of *ccr1-3* mutants. However, this observation was not supported by the results for *ccr1-6* mutants and can therefore not be considered as an effect of the down-regulation of the *CCR1* gene.
Genes involved in stress-related lignification (*PAL1*) were increased in leaves of WT plants after exposure to Cd, but not for the lignin-reduced mutants (Fig 3.5.2), which could suggest a lower increase in lignin due to Cd exposure in comparison to WT. Lignification measurements of leaves were not included in our study.

Expressions of genes corresponding to enzymes involved in metal chelation were investigated because of the higher concentration of Cd in the leaves in combination with a lower Cd toxicity. However, the results concerning the genes corresponding to enzymes involved in metal chelation (*MT* and *PCS* genes) could not help explain the observed differences among the lignin-reduced genotypes and WT plants in respect to Cd accumulation and detoxification since they were regulated in the same manner (Fig 3.5.2). Again, a possible explanation might be the existence of a dissimilarity in anti-oxidant or chelating, and thereby detoxifying, molecules among lignin-reduced mutants and WT plants. Various phenolic substances, which are found to be increased in lignin-reduced mutants, could be involved in the detoxification.

Stems

Also the effects of Cd exposure on the reproductive structures were investigated. Stem formation and flower production of *ccr1* mutants were less inhibited due to Cd exposure in comparison to the inhibition of WT plants (Fig 3.5.2). The production of seeds from *ccr1* mutants was even increased after Cd exposure (Fig 3.5.2).

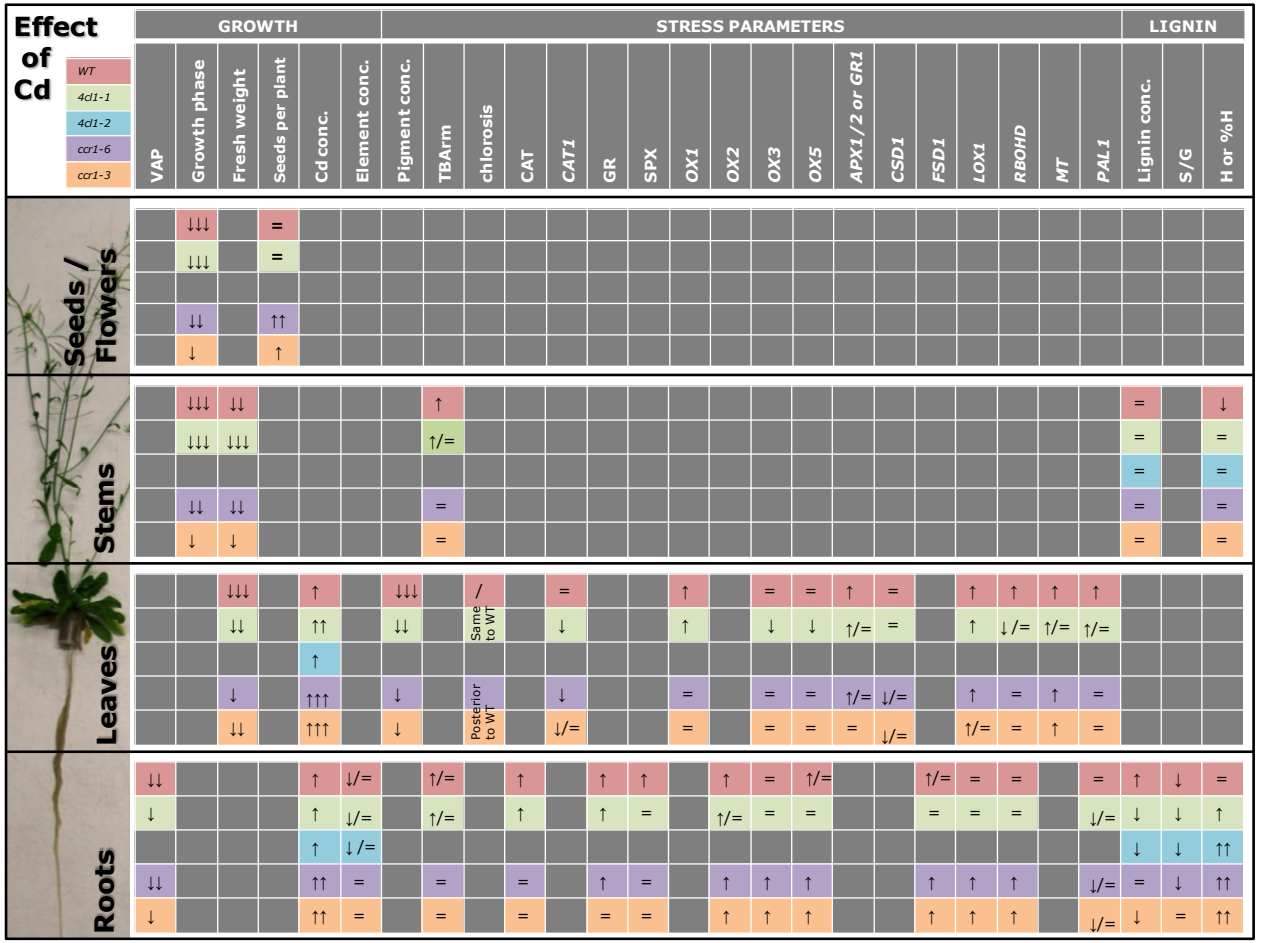


Figure 3.5.2: Summary of the outcome related to growth, stress responses and lignin in the WT plants and lignin-reduced mutant roots, leaves, stems and seeds due to exposure to 3µM $CdSO_4$. Only measurements which are discussed in the text are presented. Conc. = concentration.

In stems of all tested plant genotypes (including WT), no increases in lignin concentrations were observed (Fig 3.5.2). This is a promising result since no extra lignin is wanted in case of field applications. Furthermore, Cd did not have a big influence on the lignin composition in stems. The main effect was the significant decrease of %H in stems of WT plants but not for lignin-reduced mutants (Fig 3.5.2). The H unit is just a minor monomeric building block of lignin, although it may have an influence on the degradation of the formed lignin biopolymer. The degradation potential should be explored in future saccharification tests.
Due to Cd exposure of the plants, in stems of WT plants an increased lipid peroxidation level was observed while in lignin-reduced mutants no significant changes after Cd exposure were observed.

3.5.3 General

The *ccr1* down-regulated mutants are difficult to study since they show a retardation in development (Fig 3.5.1). However, lignin-reduced mutants and especially *ccr1 d*own-regulated mutants display different defence responses, in particular near cell boundaries of leaves, when compared to WT *A. thaliana* plants. Since these responses are just a small aspect within the complete physiological response of the plants, it is difficult to draw a comprehensive conclusion about whether WT or lignin-reduced mutants are more stressed.

In case the transgenic lignin-reduced plants were exposed to Cd, they could cope better with Cd toxicity while more Cd was translocated to roots and leaves (Fig 3.5.2). Not only the Cd concentration was increased but also concentrations of other elements were found to be higher in the *ccr1*-mutants (in non-Cd-exposed conditions) in comparison to WT plants (Fig 3.5.1). Furthermore, lignification did not increase in roots and stems after Cd exposure (Fig 3.5.2).

The most probable hypothesis is a more permeable cell wall barrier because of the lower lignin concentration, supplemented with the presence of chelating or anti-oxidant phenolic intermediates of the monolignol biosynthesis pathway (or derivatives thereof) which can lower Cd toxicity and facilitate Cd accumulation.

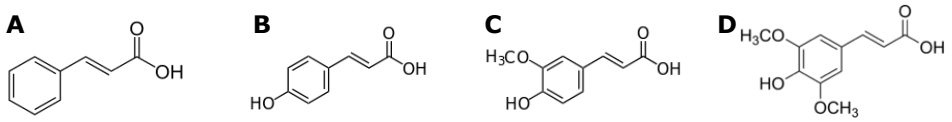


Figure 3.5.3: Phenolic compounds found to be increased in *A. thaliana* stems (Vanholme *et al.* 2012a) (A) cinnamic acid, (B) *p*-coumaric acid, (C) ferulic acid and (D) sinapinic acid.

Good candidates can be the phenolic acids with at least one carboxyl group (*e.g.* cinnamic acid*, p*-coumaric acid, ferulic acid and sinapinic acid (Fig 3.5.3)) which are good metal ligands. They can carry the metal through the xylem (translocation) or into the vacuole and play a role in detoxification (Anjum *et al*. 2015). Furthermore, different phenolic metabolites have anti-oxidative properties acting as reducing agent, hydrogen donor and singlet oxygen quencher (Rice-Evans *et al*. 1996, Marquez-Garcia *et al*. 2012) due to their hydroxyl groups (Kovacik *et al*. 2008a). The degree of methoxylation can help determine the antioxidative capacity of these compounds. Therefore, for example ferulic acid is a stronger antioxidant compared to *p*-coumaric acid (Fig 3.5.3) (Rice-Evans *et al.* 1996). Soluble phenolics can be increased in plants exposed to high concentrations of Cd (Kovacik *et al*. 2008b, Michalak *et al.* 2006, Sun *et al.* 2010). Moreover, these phenolic compounds (*e.g.* cinnamic acid, *p*-coumaric acid, ferulic acid and sinapinic acid) were found to be increased due to the interruption of the monolignol pathway in stems (Vanholme *et al*. 2012a) and leaves (Xue *et al*. 2015) of *A. thaliana* lignin-reduced mutants. Also in stems and leaves of *CCR* transformed tomato plants total phenolic compounds were enhanced because the substrate for chalcose synthase, coumaroyl-CoA esters, used in the first step in flavonoid synthesis, are accumulating (van der Rest *et al*. 2006). Moreover, in the study of lignin down-regulated tomato, the increased total level of phenolic compounds was correlated with a significant increase in total antioxidant capacity (van der Rest *et al*. 2006).

During abiotic and biotic stress responses also hydroxycinnamic acid amides (conjugated forms of cinnamic acid*, p*-coumaric acid, ferulic acid and sinapinic acid) play an important role. The 4Cl1 enzyme is essential for the synthesis of these compounds (Macoy *et al*. 2015). Therefore, it can be hypothesized that these phenolic compounds can be related to the differences in stress responses of both *4cl1* and *ccr1* mutants. However, in our study no measurements of the

phenolic compounds and their antioxidative capacity were included. These measurements (concerning the present phenolics and their abundances) can provide new information on how phenolics play a role in the defence of these lignin-reduced mutants and should be included in future studies to understand both the detoxification and accumulation of Cd-ions.

In future experiments, our promising results should be validated by making use of commercially valuable crops (*e.g.* poplar) in field conditions. Also the hypothesis of phenolic compounds should be further examined. Moreover, the mutation of the monolignol synthesis genes can have effects on the plant-associated bacterial communities (Beckers *et al*. 2016b), hence further information concerning these communities, with or without exposure to Cd, should be obtained.

SECTION 4
EFFECTS ON THE BACTERIAL COMMUNITY

All plants, including the lignin-reduced mutants, live in constant mutualistic interaction with microorganisms, collectively termed the plant microbiome (Berg *et al*. 2014). **Endophytic bacteria**, residing within the host plant without causing harm (Taghavi *et al*. 2009), represent an important part of the plant microbiome. In this interaction, plants nourish the endophytes with their photosynthetically fixed carbon sources and provide habitats for microbes (Thijs *et al*. 2016, Berg *et al*. 2014, Mendes *et al*. 2013). In return, bacteria can for example render protection against pathogens (induction of the plant immune system and/or production of hydrolytic enzymes, etc.), enhance plant nutrition (by fixation and mobilisation of unavailable nutrients such as nitrogen and phosphorus) and stimulate plant growth (through the production of phytohormones such as cytokinins), which are all beneficial for plant health and productivity (Thijs *et al*. 2016, Berg *et al*. 2014, Mendes *et al*. 2013). Moreover, the endophytic communities of plants are referred to as a second genome and an extension of the host phenotype; information about these communities can contribute to better understand plant growth and health (Berg *et al*. 2014, Hardoim *et al*. 2015).

It is known that the plant natural **genetic variation** (ecotypes, cultivars) or genetic modification can affect **the microbiome** (Bulgarelli *et al*. 2012, Beckers *et al*. 2016b, da Silva *et al*. 2014a, Haney *et al*. 2015, Pieterse *et al*. 2016, Badri *et al*. 2013, Coleman-Derr *et al*. 2016) though these microorganisms may also affect the physiology of their host (Agler *et al*. 2016). Furthermore, the photosynthetically produced **carbon sources** (C-sources) produced by plants are interesting nutrient sources for bacterial endophytes and may affect the community composition (Hardoim *et al*. 2008). In the lignin-reduced mutants, the mutation in genes of the monolignol production pathway results in the accumulation of many phenolic intermediates and derivatives thereof. For example, ferulic acid or *p*-coumaric acid were increased in the *A. thaliana* mutants and other plant species (Vanholme *et al*. 2012a, Leplé *et al*. 2007) and may serve as C-source for the associated microorganisms. Moreover, phenolic

compounds are assumed to be used either as a substrate or signalling molecule for microbes (Badri *et al*. 2013). Beckers *et al*. (2016b) demonstrated that endophytes of *CCR* down-regulated poplar isolated by selective enrichment with ferulic acid, were more able to efficiently use ferulic acid (enriched in *CCR* down-regulated poplar) as a C-source in comparison to the endophytes of WT plants. Furthermore, lignin and flavonoid production share the first steps in their pathway (Ehlting *et al.* 1999) and flavonoids (Weston and Mathesius 2013) and other phenolic compounds (Rice-Evans *et al*. 1996), can have **antimicrobial action**. In this way, also flavonoid production and consequently the bacterial community may be changed due to the mutation.
Also the **cell wall can affect microbial colonisation pathways** and is an important factor in determining which bacteria can enter the plant and become endophytic (Bulgarelli *et al*. 2012). Cell walls of lignin-reduced mutants may be more accessible to microbial degradation (Miedes *et al*. 2014). Moreover, strengthening of the cell wall by lignin is an important defence mechanism against pathogens (Compant *et al*. 2010, Bennett *et al*. 2015) and this defence capacity may be lower in lignin-reduced mutants. Lignin-reduced mutants may comprise different traits since the vascular system can collapse and in this way block the passage of microbes through the xylem (Mir derikvand *et al*. 2008).

Moreover, not only the host plant but also **environmental factors** may affect **the composition of the microbial communities** associated with the plant (Agler *et al*. 2016). Exposure to toxic concentrations of metals, such as cadmium (Cd), may affect these bacterial communities (Truyens *et al*. 2013, Truyens *et al*. 2016a, Croes *et al*. 2013). As our final aim is to grow lignocellulosic plants on marginal lands (*e.g*. Cd-contaminated land), plant-associated bacteria might play an important role in increasing the yield on these poor and hostile soils.

Bacterial endophytes can enhance plant growth either in a direct (*e.g.* enhancing available N, P and Fe, production of phytohormes, counteracting stress-induced ethylene) or an indirect way (*e.g.* making these plants less susceptible for phytopathogens) (Weyens *et al*. 2009c).

It is assumed that these microbes evolve fast in the host plant resulting in a selection of various endophytes with the most advantageous features to survive in the niche (Hardoim *et al*. 2015). Previously, examples of such a selection for beneficial phenotypic traits by plants under specific selective pressures have been demonstrated (Beckers *et al*. 2016b, Truyens *et al*. 2013, Croes *et al*. 2013). However, mechanisms affecting the selection of the microbial communities are still under strong debate (Agler *et al*. 2016).

In our study, *Arabidopsis thaliana* was selected as a model organism for woody plants (Nieminen *et al.* 2004) and two allelic T-DNA knockout mutants of two genes, ***4-COUMARATE:CoA LIGASE 1*** **(AT1G51680) and CINNAMOYL-COA-REDUCTASE 1 (AT1G15950)** of the monolignol production pathway were selected.

From all this information, we can hypothesize that both the genetic modification (*i.e.* plant genotype) as well as Cd exposure can affect the composition of the bacterial endophytic community. To investigate these effects, we designed experiments at three different levels:

(A) Firstly, we studied both the **culture-independent** and **culture-dependent bacterial communities** present in the roots, leaves, stems and seeds of the selected lignin-reduced mutants grown with and without Cd-exposure in subsection 4.2 and 4.3. The culture-independent bacterial communities will deliver the bigger picture to compare all communities and evaluate the effect of the plant genotype and Cd exposure. On the other hand, the culture-dependent bacterial communities can provide additional information concerning phenotypic traits of the endophytic communities (see level B) as well as provide promising bacterial strains for future inoculation applications.

(B) Secondly, we hypothesised that these lignin-reduced mutants with and without Cd exposure harbour different selective pressures toward endophytes and hence can select different bacterial phenotypes including **plant growth promoting traits, Cd tolerance and the use of phenolic compounds as a**

C-source. We evaluated, within the culture-dependent bacterial communities, the relative abundancy of several beneficial phenotypic traits in subsection 4.4.

(C) Thirdly, both the genetic modification and Cd exposure can negatively affect plant growth. To counteract the negative effects on the plant growth, we selected endophytes (from within the culture-dependent communities) with promising *in vitro* plant growth promoting traits to evaluate their ***in vivo*** **growth promoting capacity** with and without Cd exposure to counteract the negative plant growth effects (subsection 4.5).

Subsection 4.1
Materials and methods used in the bacterial section

4.1.1 Plant genotypes

To study the effects of the lignin modification on the culture-dependent and culture-independent bacterial communities, we selected 2 genes (*CINNAMOYL COA REDUCTASE 1* (*CCR1*) and *4-COUMARATE:COA LIGASE 1* (*4CL1*) of the monolignol production pathway of which 2 different T-DNA knockout mutants were available (*4cl1-1*, *4cl1-2*, *ccr1-6* and *ccr1-3*). These lignin mutants were selected from the collection of the European *Arabidopsis* Stock centre (NASC) and donated by the laboratory of Prof. Dr Boerjan (VIB, Department of Plant Systems Biology). Furthermore, we included 1 mutant wherein the monolignol expression was restored in the xylem vessels while the plant still lacks *CCR1* expression in all other cell types (p3xSNBE:*CCR1* in *ccr1-6* or abbreviated *ccr1-6/*SNBE). The genotyping of the selected mutants is presented in paragraph 3.1.1.2.

In table 4.1.1 the different plant growth systems are presented in combination with the chosen lignin-reduced genotypes used in section 4.

4.1.2 The culture-independent endophytic community

Plant material

Seeds of the selected *A. thaliana* genotypes (WT, *4cl1-1, 4cl1-2, ccr1-6* and *ccr1-3*) were produced in the Arasystem filled with sand as described in paragraph 3.1.2.2. From the start of stem formation, half of the trays were exposed to 3 µM $CdSO_4$. Seeds were collected at the end of the life cycle and allowed to maturate for at least 2 weeks at room temperature. Samples for culture-independent bacterial community determination were stored at -70°C until further processing.

Roots, leaves and stems of older *A. thaliana* plants (WT, *4cl1-1, 4cl1-2, ccr1-6*, *ccr1-3* and *ccr1-6*/SNBE) were produced in a hydroponics growth system as described in paragraph 3.1.2.3. Since no growth retardation was observed for *4cl1-1*, *4cl1-2* and SNBE/*ccr1-6* mutants, they were grown at the same moment as the WT plants. Because of the growth retardation, *ccr1-6* and *ccr1-3* mutants were grown respectively 3 and 10 days in advance to obtain equally developed

Table 4.1.1: Combination of growth systems, exposure times and genotypes used in section 4.

Subsection	Experiment	[Cd] (µM $CdSO_4$)	Start exposure	Exposure duration	Extra information	WT	*4cl1-1*	*4cl1-2*	*ccr1-6*	*ccr1-3*	p3xSNBE:*CCR1* in *ccr1-6*
Culture independent endophytic communities											
4.2	Seed community	0 and 3	Stem formation	Until overblown	Same age	x	x	x	x	x	
	Roots, leaves and stems (of older plants) communities	0 and 3	Stem formation	3 weeks	Same age and stem length	x	x	x	x	x	x
Culture dependent endophytic communities											
4.3 4.4	Seed community	0 and 3	Stem formation	Until overblown	Same age	x	x	x	x	x	
	Roots and leaves (of younger plants) communities	0 and 3	Day 7	2 weeks	Same rosette diameter	x	x	x	x	x	
	Roots, leaves and stems (of older plants) communities	0 and 3	Stem formation	2 weeks	Same stem length	x	x	x	x	x	
Inoculation experiments											
4.5	Inoculation	0 and 3	Day 7	2 weeks	VAP, same age	x			x	x	
	Co-cultivation	0	/	/	Separated Petri dishes with two different media, same age	x			x	x	

stems (see subsection 3.2.2.2C) (indicated with L throughout the discussion of the results). As an extra reference condition, in the experimental design *ccr1-6* and *ccr1-3* mutants were also grown simultaneously with WT plants to get equally old plants (indicated with O throughout the discussion of the results). From the start of stem formation, plants were exposed to 3 µM $CdSO_4$ during 3 weeks.

Sample preparation and DNA extraction

After at least 2 weeks maturation of the seeds at room temperature (20°C), aliquots of ±30 mg dry seeds from all conditions were stored at -70°C until further processing. Harvested root, leaf and stem (only the lowest 10 cm minus 1 cm) samples were pooled per 2 plants and stored in 10 mM $MgSO_4$ at 4°C. They were further processed within 36 h for roots, 60 h for leaves and 72 h for stems. Three biological replicates (each consisting of 2 plants) were included for all conditions. All plant organs (total number = 5 genotype x 2 treatments x 3 biological replicates for seeds; 8 genotype groups (6 of the same age and 2 of the same stem length) x 2 treatments x 3 biological replicates for the other 3 plant compartments = a total of 174 samples) were surface sterilised to remove epiphytic or other non-target DNA. This was done by several rinsing steps: sterile deionised water during 1 min, 70% (v/v) ethanol for 30 s, 1 min 1% NaOCl (supplemented with 0.1% Tween 80) and 70% (v/v) ethanol during 30 s and finally 5 times thoroughly rinsing in sterile deionised water. Samples were homogenized by mortar and pestle in sterile 10 mM $MgSO_4$. For each sample 3 aliquots were frozen at -80°C. After centrifugation (13,400 rpm; 30 min) to collect all cells in the pellet, total genomic DNA of the pellet was extracted using the Invisorb Spin Plant Mini Kit (Stratec, Biomedical AG, Birkenfeld, Germany) in 3 separate aliquots to minimise extraction bias (Op De Beeck *et al.* 2014). The kit was selected based on the optimisation from 3 kits to extract bacterial DNA from plant samples (Beckers *et al*. 2016a). After extraction, the 3 aliquots of the same sample were pooled to 1 sample.

Preparation of the amplicon libraries and 454 sequencing

Primer 799F (5'-AACMGGATTAGATACCCKG-'3) (Chelius and Triplet 2001) and 1193R (5'-ACGTCATCCCCACCTTCC-3') (Bodenhausen *et al*. 2013) were selected

to amplify the V5-V7 hypervariable region of the 16S rRNA gene. These primers were optimised to avoid amplification of *A. thaliana* chloroplast 16S rDNA because of a 2 bp mismatch (Truyens *et al*. 2016a) at the 3' end of the 799F primer.

The first PCR reaction was set–up in 25 µl using the FastStart High Fidelity PCR system (Roche, Applied Science, Mannheim, Germany). Per sample the PCR mastermix contained 2.75 µl 10x FastStart High Fidelity reaction buffer with 1.8 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µm forward primer, 0.4 µM reverse primer, 1.25U FastStart High Fidelity Taq DNA polymerase, 18.75 µl Rnase free water and 1 µl DNA template (±10 ng DNA). Following PCR conditions in the Techne TC-5000 thermocycler (Bibby, Scientific Limited, Staffordshire, UK) were applied: an initial denaturation step of 3 min at 94°C, 35 cycles of 1 min at 94°C, 60 s at 53°C and 60 s at 72°C followed by a final elongation step of 10 min at 72°C.

Mitochondrial DNA was removed from the PCR product by gel purification. PCR products were run on a 1.5% agarose gel (90 V, 2 u) and bacterial DNA (394 bp) was picked out of the gel. The mitochondrial DNA (± 800 bp) was discarded with the gel (Truyens *et al*. 2016a). PCR amplicons were extracted from the gel pieces by the QiaQuick gel extraction kit (Qiagen, Venlo, The Netherlands).

During the second PCR, primer 799F was fused to the Roche 454 pyrosequencing adaptor A (Roche, Applied Science, Mannheim, Germany) including a sample-specific 10 bp barcode (multiplex identifiers, MIDs) to make sure that sequences can be linked to the samples and hence can be identified after sequencing. The reverse primer (1193R) was fused to adaptor B (Roche Applied Science, Mannheim, Germany). The mastermix composition and cycling conditions were identical to the first PCR except the number of cycles was reduced to 25.

The QiaQuick PCR purification kit (Qiagen, Venlo, The Netherlands) was used to remove unbound nucleotides, primer dimers and short amplicons. After sample purification, DNA quantity was determined by the Quant-IT Picogreen dsDNA assay (Invitrogen, Carlsbad, CA, USA) using a Fluorostar Omega Plate reader (BMG Labtech, Orthenberg, Germany). Amplicon libraries (3 for root samples, 2 for leaf samples and 3 for seed combined with stem samples) were made with every sample in an equimolar concentration. Quality of the amplicon libraries

was tested by an Agilent 2100 Bioanalyser system (Agilent Technologies, Diegem, Belgium). Amplicon libraries were sent to Macrogen (Seoul, South-Korea) for sequencing on a Roche 454 Genome Sequencer FLX using Titanium technology (Roche, Applied Science, Mannheim, Germany). Each amplicon library was sequenced on 1/8th of a picotiter plate.

Data analysis

The raw pyrosequencing data were processed in Mothur (version 1.34.4) following the Standard operating procedure (SOP) available on http://www.mothur.org/wiki/454_SOP (Schloss *et al*. 2009, Schloss *et al*. 2011). Data were denoised and primer sequences, barcodes, sequences with homo-polymers longer than 8 bp and sequences shorter than 200 bp were removed. An alignment of the unique sequences with known sequences in the SILVA rRNA database was performed (Pruesse *et al*. 2007). The Uchime tool detected and removed the chimeric sequences (Edgar *et al*. 2011). Sequences identified as Archaea, mitochondria, chloroplast, unknown and singletons were removed from the dataset. Sequences were grouped into operational taxonomic units (OTUs) based on a 97% selection criterion and taxonomic groups (based on The Ribosomal Database Project (RDP)) were assigned with a 80% bootstrap cut-off value (Wang *et al*. 2007). All samples were subsampled to 990 sequences and OTU richness (S_{obs} function of "Mothur", number of observed OTUs), inverse Simpson diversity index (Simpson 1949, Hill 1973) and Shannon diversity (Hill 1973) based on 10,000 iterations were used to estimate community diversity. Furthermore, good coverage rates were calculated based on 10,000 iterations.

Diversity indices:

$$Inverse\ simpson\ Index: 1/\frac{\sum_{i=1}^{S_{obs}} n_i(n_i-1)}{N/N-1)}$$

$$Shannon\ diversity: -\sum_{i=1}^{Sobs} p_i\,(\ln p_i)$$

S_{obs} = number of different observed OTU in the dataset
n_i = number of sequences of the ith OTU in the dataset
N= total number of OTUs in the dataset
p_i= n_i/N

Statistics

Statistical significance in normally distributed (with or without transformation) datasets was tested with one or two way ANOVA and the Tukey-Kramer post-

hoc test in statistical program R (version 3.2.4, R development Core Team 2015).

Non-metric multi-dimensional scaling (nMDS) plots and cluster diagrams based on Bray-Curtis similarity were made on square root transformed data using Primer7 (version 7.0.5, Primer-E Ltd.). Also SIMPER dissimilarity based on Bray-Curtis similarity values were calculated in Primer7 (version 7.0.5, Primer-E Ltd.). ANalysis Of SIMilarity (ANOSIM) is a distribution-free method of multivariate data to analyse variation in species abundance and community composition and has some similarity to an ANOVA test (Clarke 1993). The ANOSIM analysis was performed using "R" (version 3.2.4) using 10,000 permutations.

4.1.3 The cultivable endophytes

4.1.3.1 Isolation procedure

Plant material

The Arasystem filled with sand was used to cultivate plants for **seed** (WT, *4cl1-1, 4cl1-2, ccr1-6* and *ccr1-3*) production (paragraph 3.1.2.2). Half of the trays were exposed to 3 µM $CdSO_4$ from the start of stem formation. Seeds were collected when mature.

Seeds from all genotypes (WT, *4cl1-1, 4cl1-2, ccr1-6*, *ccr1-3*), also used for determination of cultivable and total endophytic seed community, were germinated in a hydroponics growth system (see paragraph 3.1.2.3). Because of the retardation in development, *ccr1* mutants were grown earlier to allow the harvest of these plants on the same day. More information about the growth retardation can be found in subsection 3.2.2.2C. In order to produce **younger** plants (3 weeks old) with a similar rosette diameter, *ccr1-6* and *ccr1-3* mutants were sown respectively 2 and 4 days ahead of the other genotypes. Half of the plants were exposed to Cd after 1 week (for WT plants) for an exposure time of 2 weeks. **Roots and leaves** were harvested and the remainder of the non-exposed plants were further grown to obtain **older** plants (47 days old for WT plants). In addition, cultivation of *ccr1-6* and *ccr1-3* mutants was started respectively 3 and 10 days earlier in order to obtain similar stems. Half of the plants were exposed to 3 µM $CdSO_4$ from start of stem formation (as optimised for WT plants) during 2 weeks when **roots, leaves and stems** were harvested.

Sample preparation

Bacterial isolation was performed at 3 growth stages (1: seeds, 2: roots and leaves of younger plants, 3: roots, leaves and stems of older plants).

Dry seeds were stored for at least 2 weeks at room temperature (20°C) for further maturation before further processing. Samples of roots, leaves and stems were stored in 10 mM $MgSO_4$ at 4°C and further processed within 12h for roots and leaves and 36h for stems.

In the study design, 2 replications of approximately 30 mg seeds originating from at least 8 plants were included. For the younger tissues 2 replications of 5 whole roots of younger plants (± 250 mg) and rosettes of younger plants (± 500 mg) were analysed. Likewise, 2 replicates of 2 roots of older plants (±2 g), leaves of older plants (±1 g) and the bottom 10 cm (minus 1 cm) of the stem (±500 mg) were included. In case both replications were similar, 1 was further processed. In case some differences were observed, mainly in morphologically similar bacterial types which were found in low amounts, both repetitions were merged into 1 for further analysis.

All plant organs (total number = 5 genotype x 2 treatments x 2 biological replicates x 6 different plant tissues (seeds, roots of younger and older plants, leaves of younger and older plants and stems) = a total of 120 samples) were surface-sterilised using 0.1% NaOCl supplemented with 0.1% Tween 80. For seeds the sterilisation time was 1 min where after 4 rinsing steps with sterile deionised water and 4 incubation steps of 5 min in sterile deionised water were performed (Truyens *et al.* 2013). All other plant organs were sterilised during 3 min and 3 rinsing steps with sterile deionised water. Sterility was verified by plating the last rinsing water on 869 rich medium containing 10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 g glucose D+ and 0.345 g $CaCl_2 \cdot 2H_2O$ per litre (pH 7.0) supplemented with 1.5% agar (Mergeay *et al*. 1985).

Cultivable endophytes were isolated by adding an adequate amount of $MgSO_4$ to the sample in a mortar before crushing by pestle. A dilution series of 0 to 10^{-2} was plated on $1/10^{th}$ diluted 869 rich medium for seeds, roots and leaves of younger plants and leaves of older plants. For stems and roots of older plants the dilution series were adapted to respectively 10^{-1} and 10^{-3}. Plates were incubated during 1 week at 30°C where after colony forming units (cfu) per

morphologically different strain were counted and calculated per g fresh weight tissue. For each different type 5 to 10 technical repetitions were further purified and stored in a glycerol stock (15% (w:v) glycerol, 0.85% (w:v) NaCl) at -40°C.

4.1.3.2 Genotypic characterisation of the cultivable endophytes

Total genomic DNA was extracted from the purified morphologically different strains using the DNeasy blood and tissue kit (Qiagen, Venlo, The Netherlands). DNA concentration and purity were investigated using a Nanodrop ND-1000 spectrophotometer (Thermoscientific, Wilmington, DE, USA). Subsequent amplification of the 16S rRNA gene was performed by making use of the 26F bacterial specific primer (5'-AGAGTTTGATCCTGGCTCAG-3') and universal 1392R primer (5'-ACGGGCGGTGTGTRC-3') (Weyens *et al*. 2013). A mastermix containing 1.8 mM high fidelity PCR buffer, 1.8 mM MgCl2, 0.2 mM dNTPs, 0.4 µM 26F, 0.4 µM 1392R and 1.25U high fidelity Taq Polymerase (Invitrogen, Ghent, Belgium) was added to 1 µl of the total genomic bacterial DNA sample. Following PCR amplification settings were used: initial denaturation of 5 min at 95°C, 30 amplification cycles of 1 min at 94°C, 30 s at 52°C and 30 s at 72°C and a final elongation of 10 min at 72°C in an Eppendorf Mastercycler Gradient (Eppendorf, Rotselaar, Belgium). To identify identical genomic strains, amplified ribosomal DNA restriction analysis (ARDRA) was performed by adding a mastermix containing 2.9 µl NEB buffer 1, 0.3 µl restriction enzyme $HpyCH_4$ IV (New England biolabs, Ipswich, MA, USA), 1.1 µl Rnase and 4.3 µl Rnase free water to 20 µl PCR product. After an incubation step of 2h at 37°C, samples were separated by gel electrophoresis (1.5% agarose gel, 90V, 2h). Samples containing the same band pattern are of the same bacterial genus. Only 1 representative strain sample was selected for further sequencing. The selected PCR products were purified and bi-directionally sequenced by Macrogen Inc. (Amsterdam, The Netherlands). Derived sequences were blasted against the Ribosomal Database Project (RDP-II)(Cole *et al.* 2005).

Inverse Simpson diversity index (Simpson 1949, Hill 1973) and Shannon diversity (Hill 1973) were calculated per condition (see formulas paragraph 4.1.2). Both indices are nonparametric measures and imply no assumption about species abundance distribution (Kwak and Peterson 2007).

4.1.3.3 Phenotypic characterisation of the cultivable endophytes

All isolated pure bacterial cultures which were stored in the glycerol stock collection, were grown in liquid 869 rich medium (Mergeay *et al*. 1985). Subsequently, the bacteria were washed twice (3000 g, 20 min) in 10 mM $MgSO_4$ (Croes *et al*. 2013) and inoculated in the different media to examine them *in vitro* for different potential plant growth promotion traits. Also Cd resistance was evaluated.

Bacteria that did not re-grow in the regular 869 medium, were grown in liquid 869 medium supplemented with plant extract. In that way, plant molecules that may be necessary for the growth of symbiotic endophytes are present which can be beneficial for recultivating endophytes (Eevers *et al*. 2015). The plant extract was prepared by grinding 1 g of *A. thaliana* leaves in 25 ml sterile water with mortar and pestle where after it was filtered through a nylon mesh to remove larger pieces of tissue. The remaining fluid was added to 1 litre medium by filter sterilisation.

(A) Cadmium resistance

All bacteria were inoculated onto Petri dishes containing 284 minimal selective medium (Fig 4.1.2A) made up of macro elements (6.06 g Tris, 4.68 g NaCl, 1.49 g KCl, 1.07 g NH_4Cl, 0.43 g Na_2SO_4, 0.2 g $MgCl_2{\cdot}6H_2O$, 0.03 g $CaCl_2{\cdot}2H_2O$, 0.04 g $Na_2HPO_4{\cdot}2H_2O$); 4.8 mg Fe(III)NH_4citrate; 1 ml of SI7 trace elements (containing per litre: 1.3 ml HCl, 144 mg $ZnSO_4{\cdot}7H_2O$, 100 mg $MnCl_2{\cdot}4H_2O$, 62 mg H_3BO_3, 190 mg $CoCl_2{\cdot}6H_2O$, 17 mg $CuCl_2{\cdot}2H_2O$, 24 g $NiCl_2{\cdot}2H_2O$, 36 mg $NaMoO_4{\cdot}2H_2O$) and several carbon sources (0.7 ml lactate, 0.52 g glucose, 0.66 g gluconate, 0.54 g fructose and 0.81 g succinate) per litre (pH 7.0 with HCl and NaOH) supplemented with 2% agar (Schlegel *et al*. 1961). One control condition without Cd was used and 2 concentrations (0.4 mM and 0.8 mM) of $CdSO_4$ were added to the medium to test the ability of bacterial strains to grow in the presence of toxic amounts of Cd. The growth was evaluated after 7 days of incubation at 30°C. Difficultly growing bacteria were investigated with 284 minimal selective medium with 1 g per litre *A. thaliana* leaf extract.

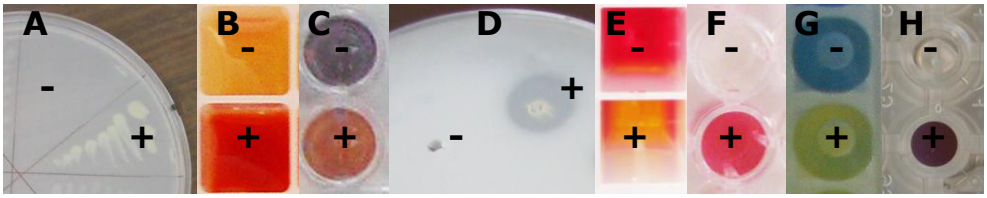


Figure 4.1.2: Representative pictures of *in vitro* phenotypic tests (negative:- or positive: +) to examine endophytic strains for (A) Cd resistance, (B) ACC deaminase production, (C) siderophore production, (D) phosphate solubilisation, (E) organic acids production, (F) IAA production, (G) nitrogen fixation and (H) their metabolic range.

(B) *In vitro* growth promotion tests

1-aminocylcopropane-1-carboxylate (ACC) deaminase activity

1-aminocyclopropane-1-carboxylase (ACC) is the precursor of the plant stress hormone ethylene, which inhibits plant growth. Endophytic bacteria may produce the enzyme ACC deaminase, which cleaves ACC and consequently can reduce ethylene production, which indirectly might promote plant growth under stressful conditions. The ability to produce ACC deaminase was investigated according to the slightly modified protocol of Belimov *et al*. (2009). In this test, the amount of α-ketobutyrate generated by hydrolysis of ACC is estimated. For this assay the exponentially growing bacteria are collected by centrifugation (3000 g, 15 min), washed twice in 0.1 M Tris-HCl buffer (pH 7.5) and resuspended in salt minimal medium containing 0.4 g KH_2PO_4 and 2 g K_2HPO_4 per litre (pH 7.4 with KOH) enriched by filter sterilisation to obtain the following amounts per litre: 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, 5 mg $FeSO_4 \cdot 7H_2O$, 2 mg H_3BO_3, 5 mg $ZnSO_4$, 1 mg Na_2MoO_4, 3 mg $MnSO_4$, 1 mg $CoSO_4$, 1 mg $CuSO_4$, 1 mg $NiSO_4$, 1 g glucose, 1 g sucrose, 1 g Na-acetate, 1 g Na-citrate , 1 g malic acid and 1 g mannitol (Belimov *et al*. 2005) with 5 mM ACC as the sole nitrogen source. After an incubation period of 4 days at 30°C, the bacteria were collected (3000 g, 15 min) and resuspended in 100 µl 1M Tris-HCl buffer (pH 8.5). Cells were ruptured by adding 3 µl toluene. Next, 100 µl 0.1 M Tris-HCl and 10 µl 0.5 M ACC were added and incubated for 30 min at 30°C, where after 400 µl 0.56 N HCl and 150 µl 0.2% 2,4 dinitrophenylhydrazine in 2 N HCl were added. After a last incubation period of 30 min at 30°C, 1 ml NaOH was added. Wells that coloured from yellow to brown are considered positive for ACC deaminase activity (Fig 4.1.2B). Because of interference of adding leaf extract in the testing

SMN medium, the difficultly growing bacteria were investigated by making them active in 869 with 1 g per litre plant extract but testing them without plant extract in the SMN medium.

Siderophore (SID) production

The active cultures were inoculated into 3 different 284 minimal media composed of macro elements (6.06 g Tris, 4.68 g NaCl, 1.49 g KCl, 1.07 g NH_4Cl, 0.43 g Na_2SO_4, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.03 g $CaCl_2 \cdot 2H_2O$, 0.04 g $Na_2HPO_4 \cdot 2H_2O$); 1 ml of SI7 trace elements (containing per litre: 1.3 ml HCl, 144 mg $ZnSO_4 \cdot 7H_2O$, 100 mg $MnCl_2 \cdot 4H_2O$, 62 mg H_3BO_3, 190 mg $CoCl_2 \cdot 6H_2O$, 17 mg $CuCl_2 \cdot 2H_2O$, 24 g $NiCl_2 \cdot 2H_2O$, 36 mg $NaMoO_4 \cdot 2H_2O$) and carbon sources (0.7 ml lactate, 0.52 g glucose, 0.66 g gluconate, 0.54 g fructose and 0.81 g succinate per litre) (pH 7.0 with HCl and NaOH) supplemented with 2% agar (Schlegel *et al*. 1961). The medium was supplemented with 0 µM (Fe limiting conditions, inducing siderophore production), 0.25 µM (mimics Fe conditions in the rhizosphere) or 3 µM Fe(III)NH_4citraat (sufficient Fe) (Croes *et al*. 2013). After 4 days of incubation (30°C) the production of bacterial siderophores was evaluated using the CAS reagent (Schwyn and Neilands 1987 adapted). 100 µl of CAS was added to 100 µl of bacterial supernatants. After 4h, positive, siderophore producing bacteria colour orange (Fig 4.1.2C). For bacteria that were not growing in the standard media, *A. thaliana* leaf extract was added to the 284 testing medium.

Phosphate (P) solubilisation

Phosphate solubilisation was tested in Petri dishes containing National Botanical Research Institute's phosphate growth medium (NBRIP) (per litre: 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl and 0.1 $(NH_4)_2SO_4$; 1.5% agar) (Nautiyal 1999). Actively growing bacteria were washed twice with 10 mM $MgSO_4$ where after aliquots of 50 µl were inoculated into holes of 2 mm diameter in the growth medium. For positive P solubilising bacteria, the presence of a clear hallow was observed after incubation for 1 week at 30°C (Fig 4.1.2D).

Subsection 4.1

Organic acid (OA) production

Bacteria were transferred to sucrose tryptone medium containing 20 g sucrose, 5 g tryptone and 10 ml SET trace elements (per litre: 20 mg $NaMoO_4.H_2O$, 200 mg H_3BO_4, 20 mg $CuSO_4.5H_2O$, 100 mg $FeCl_3$, 20 mg $MnCl_2.4H_2O$ and 280 mg $ZnCl_2$) per litre and incubated for 4 days at 30 °C. To evaluate the production of organic acids, 100 µl 0.1% v/v alizarine red S was added (Cunningham and Kuiack 1992), a pH indicator that will colour red when the medium is more basic and yellow if it is more acid so consequently positive bacteria for organic acid production are coloured yellow. Since plant extract interfered with the test, difficultly growing bacteria were made active in presence of 1 g per litre *A. thaliana* leave extract in 869 rich medium but no plant extract was added to the sucrose tryptone test medium (Fig 4.1.2E).

Indol-acetic acid (IAA) production

The active bacterial cells were added to $1/10^{th}$ diluted 869 rich medium (containing 10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 g glucose D+ and 0.345 g $CaCl_2 \cdot 2H_2O$ per litre (pH 7.0) for the undiluted medium) (Mergeay *et al*. 1985) supplemented with 0.5 g per litre L-tryptophane, the precursor of indole acetic acid (IAA) and incubated for 4 days at 30 °C. One ml Salkowski reagens (containing 50 ml 35% $HClO_4$ and 1 ml 0.5 $FeCl_3$) was added to 500 µl of supernatant of these bacteria (Gordon and Weber 1951). IAA producing bacteria coloured pink after 20 min of incubation at room temperature (Fig 4.1.2F). Difficultly growing bacteria were made active in 869 rich medium with 1 g per litre plant extract but no leaf extract was added during the assay to avoid interference with testing results.

Nitrogen fixation

Nitrogenase activity was tested in a semi-solid malate-sucrose medium (10 g sucrose, 5 g L-malic acid, 0.36 g $MgSO_4 \cdot H_2O$, 0.01 g $FeCl_3$, 0.1 g NaCl, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.1g K_2HPO_4, 0.4 g KH_2PO_4, 0.002 g $Na_2MoO_4 \cdot H_2O$ per litre; pH 7.2) modified from Döbereiner (1989) (Xie *et al*. 2006). In the medium 15 mg bromothymol blue per litre was added as a pH indicator (Nabti *et al.*, 2007). The pH indicator will allow the visualisation of the growth as the acidification by

respiration of the sugars, turning the growth medium from blue to yellow bacterial (Croes *et al.* 2013)(Fig 4.1.2G).

(C) Use of phenolic C-sources

Phenolic compounds, ferulic acid and *p*-coumaric acid, are known to be increased in the *ccr1* mutants or *4cl1* mutants of *A. thaliana* (Vanholme *et al*. 2012a) and ferulic acid is assumed to be involved in the growth reduction of these plants (Xue *et al*. 2015). Therefore, Biolog MT2 plates (Biolog Inc., Hayward, CA, USA) were utilised to estimate the use of these 2 phenolic compounds as a C-source. The assay was performed in a selected subgroup of bacterial strains. Endophytes testing positive for the trait may be involved in the internal breakdown in the plant of these substances in that way lowering the negative effects of for example accumulating ferulic acid. A general accepted carbon source mix (C-mix) was included as a control to explore the bacterial growth under the used growth condition.

The Biolog MT2 assay consists of a pre-coated 96 well plate, which contains tetrazolium violet redox dye. In a buffered nutrient medium, that was developed and optimised for a wide variety of bacteria, the dye reduces to the purple coloured formazan in the presence of a respiring bacterial strain (Garland *et al*. 1991). After adding the C-source and a bacterial suspension, the formation of purple formazan (Fig 4.1.2H), which is directly related to bacterial respiration (degradation of the C-source), is measured spectrophotometrically at 595 nm.

Bacterial preparation

Pure bacterial cultures were grown for at least 24 h in liquid 869 rich medium containing 10 g tryptone, 5 g yeast extract, 5 g NaCl 1 g, glucose D+ and 0.345 g $CaCl_2 \cdot 2H_2O$ per litre (pH 7.0) (Mergeay *et al*. 1985). To remove the C-source, the exponentially growing bacteria were washed twice (4000 rpm, 20 min) in PBS buffer containing 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4 \cdot 2H_2O$ and 0.24 g KH_2PO_4 per litre (pH 7.4).

All bacterial strains were diluted in PBS buffer to an optical density of 0.20 (at 660 nm). Before adding the bacteria to the MT2 plates, an incubation step of 18h at 30°C was implemented to completely deplete the cultures from the former C-source.

Subsection 4.1

Plate preparation

Each well of the MT2 plates was filled with 120 µl bacterial suspension and 30 µl of a 5 mM concentrated stock solution in 1% DMSO (pH 7) of the selected C-source (ferulic or *p*-coumaric acid) to obtain a concentration of 1 mM C-source in each well. Bacterial respirations of each C-source were tested in triplicate. Every plate also included negative controls containing only PBS or the selected C-source in combination with sterile PBS. For each strain a positive control was included. These were grown in presence of a general accepted C-source mix with a concentration of 0.5 mM of every C-source (lactate, glucose, gluconate, fructose and succinate in a 2.5 mM stocksolution with pH 7) in each well. Also 0.2% DMSO was present in each well in analogy to the selected phenolic acids.
Plates were incubated at 30°C in a sealed plastic bag containing a water-soaked paper towel to minimise evaporation from the wells. Absorbance (595 nm) was obtained after 0, 3, 6, 18, 24, 48, 72, 120, 144 and 288 h of incubation using a Fluorostar Omega Plate reader (BMG Labtech, Orthenberg, Germany).

Data processing

The raw absorbance data were standardised by individually subtracting the measured values at the time points of interest from the value before incubation (0 h). For every measuring point the net area under the absorbance versus time curve was evaluated by means of a trapezoidal approximation (Guckert *et al*. 1996). Afterwards the data were corrected for the Average Well Colour Density (AWCD) over the 96 well plate to eliminate the effect of the plate. The obtained value takes the lag phase, development of the purple colour as well as the maximum absorbance into account and represents the degradation capacity. Per bacteria/C-course combination the average of 3 repetitions was calculated.

$$\sum_{i}^{4}((v_i + v_{i-1})/2)*(t_i + t_{i-1}) = 50$$

4.1.4 Inoculation experiments

15 bacterial strains, isolated from different *A. thaliana* compartments (seeds, roots, leaves and stems) and genotypes (WT, *4cl1* or *ccr1* mutants) with or without Cd-exposure, were selected based on their performance in the above-

described *in vitro* tests (metabolic capacities, *in vitro* growth promoting capacities and Cd resistance). Selected bacterial strains were grown in 869 liquid rich medium (Mergeay *et al*. 1985) for at least 24 h while shaking at 30°C. The bacterial cells were harvested by centrifugation (3000 g, 20 min) and resuspended in 10 mM $MgSO_4$ to obtain a final OD (660 nm) of 1.

Seeds were surface-sterilised on a paper filter for 1 min in a 0,1% (w/v) NaOCl solution supplemented with 0.1% Tween 80, followed by 4 brief rinse cycles and 4 rinse cycles of 5 min each in sterile distilled water. Afterwards the seeds were dried in a laminar air flow (Truyens *et al*. 2013). The dry seeds were kept in darkness at 4°C for 48 h to obtain a homogenous germination.

In planta endophytic growth promoting performance was assessed by inoculation of plants grown on vertical agar plates (VAPs). The effect of bacterial volatiles on the plant growth was examined using the co-cultivation assay.

4.1.4.1 Vertical agar plates

The dry seeds were sown on 12 cm by 12 cm plates containing 40 ml 50-fold diluted Gamborg's B5-medium (Zhang and Forde 1998) with 1% agar. The medium composition consisted of macronutrients (0.5 mM KNO_3, 0.02 mM $MgSO_4 \cdot 7H_2O$, 0.02 mM $CaCl_2 \cdot 6H_2O$, 0.022 mM NaH_2PO_4, 0.94 µM $MnSO_4 \cdot H_2O$ and 0.02 mM $(NH_4)_2SO_4$); micronutrients (90 nm KI, 0.97 nm H_3BO_3, 0.14 nM $ZnSO_4 \cdot 7H_2O$, 2 nM $CuSO_4 \cdot 5H_2O$, 20.6 nM $Na_2SO_4 \cdot H_2O$ and 2.6 nM $CoSO_4 \cdot H_2O$); 3.6 µM $FeCl_3$ and 2.56 nM 2-(N-Morpholino)ethanesulfonic acid hydrate (MES). To ensure a good germination, the plates were supplied with 10 g sucrose per litre medium.

Subsequently the plates were placed at a vertical position for 7 days in growth chambers equipped with blue, red and far-red Philips GreenPower LED modules to mimic the photosynthetic spectrum of sunlight (day/night temperatures of 22/18°C, 12 h photoperiod, 65% relative humidity and photosynthetic active radiation (PAR) of 170 µmol m^{-2} s^{-1}).

Equally germinated seedlings were selected for transfer to vertical agar plates with the same B5/50 medium with or without 3 µM $CdSO_4$ (for optimisation see paragraph 3.2.2.1) and without sucrose addition. The upper 2 cm zone of these agar plates was cleared from the medium so that leaf growth could not be

troubled. All plates, except for controls without bacterial inoculation, were provided with 400 µl of a bacterial inoculum (final concentration of 10^4 cfu ml^{-1}). 30 seedlings (6 plates containing 5 plants) were used for the non-inoculated conditions and 15 seedlings (3 plates containing 5 plants) were inoculated for every selected strain. Root growth was monitored for 10 days after which the plates were scanned (Fig 4.1.3A). Only the endpoint measurement of the primary root was analysed using Rootnav (Pound *et al*. 2013). Results were reported relative to the non-inoculated group to eliminate the possible batch effects.

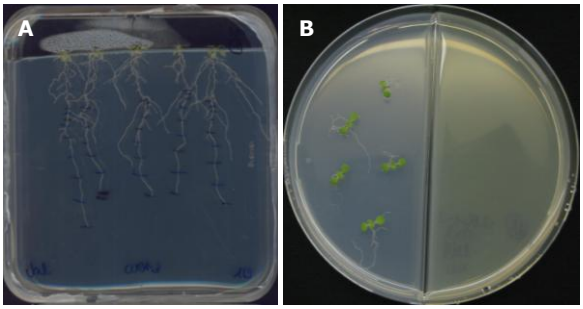


Figure 4.1.3: Representative picture for *in planta* growth promoting capacity tests. (A) vertical agar plates and (B) co-cultivation assay before inoculation.

4.1.4.2 Co-cultivation assay

The dried seeds were sown on 12 cm by 12 cm plates containing ¼ Murashige-Skoog (MS) Medium (Murashige and Skoog 1962) supplemented with 10 g per litre sucrose to ensure a good germination and placed horizontally in growth chambers (with blue, red and far-red Philips GreenPower LED modules to mimic the photosynthetic spectrum of sunlight and photosynthetic active radiation (PAR) of 170 µmol m^{-2} s^{-1}, day/night temperatures of 22/18°C, 12 h photoperiod, 65% relative humidity) for 3 days.

After germination, 5 seedlings per plate were transferred to the side of round Petri dishes (100x 15mm) containing MS medium without sucrose. The other side contained 869 rich medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 g glucose D+ and 0.345 g $CaCl_2 \cdot 2H_2O$ per litre (pH 7.0) (1.5% agar)) (Mergeay *et al*. 1985) for later inoculation. The plant side was separated from the bacterial side with a slide to assure that bacterial products could only be transferred through air (Fig 4.1.3B). The plates were photographed to demonstrate the

homogenous leaf surface at this time point. They were placed again horizontally into the growth chambers for 5 days to adapt to the medium before inoculation. For every bacterial strain, the rich medium of 3 plates was inoculated with 100 µl of the bacterial inoculum (final concentration of 10^8 cfu ml^{-1}) without making any direct contact with the plantlets. 6 non-inoculated control plates were included. Plates were sealed with Parafilm to avoid efflux of volatile substances. After 7 days of growth, fresh weight of the leaves was determined and leaf surface was estimated by means of a scan that is analysed using Adobe Photoshop (version 14.0).

4.1.4.3 Statistical analysis

Statistical analysis was performed in R 3.2.0 (The R Foundation for Statistical Computing Platform). Normal distribution of the data was tested by Shapiro Wilkes test. A parametrical ANOVA, followed by a Tukey-Kramer post-hoc test was used for normally distributed data. Other results were analysed by the Kruskal Wallis Rank Sum Test followed by the Pairwise Wilcoxon rank Sum Test.

Subsection 4.2
Composition of the culture-independent endophytic bacterial communities of lignin-reduced *A. thaliana* plants with and without Cd exposure

It is estimated that only up to 1% of the endophytic community is likely to be cultivable in laboratory settings (Torsvik and Øvreås 2002, Alain and Querellou 2009, Eevers *et al*. 2015). In this way, culture-dependent bacterial isolation techniques are biased in identifying all community members and consequently underestimate community diversity. A culture-independent approach is considered to be more sensitive to detect shifts in community composition (Lebeis 2014).

Hence, we evaluated the differences in culture-independent (total) endophytic communities of seeds, roots, leaves and stems of *Arabidopsis thaliana* lignin-reduced genotypes (T-DNA knockout mutants for *4-COUMARTE:COA LIGASE 1* (*4CL1*), the *4cl1-1* and *4cl1-2* mutants, or *CINNAMOYL-COA REDUCTASE 1* (*CCR1*), *ccr1-6* and *ccr1-3* mutants) in comparison with wild type (WT) plants. Another *ccr1-6* lignin mutant in which lignin is restored in the vessels though not in other cell types was used (p3xSNBE:*CCR1* in *ccr1-6* indicated with *ccr1-6*/SNBE). Furthermore, the effects of cadmium (Cd) exposure on the total endophytic bacterial communities of all investigated *A. thaliana* genotypes were examined.

4.2.1 Experimental design

The total endophytic communities of roots, leaves and stems (3 biological repetitions: pooled samples of 2 roots, leaves or stems) were investigated in WT, *4cl1-1, 4cl1-2, ccr1-6, ccr1-3* and *ccr1-6*/SNBE plants grown in a hydroponics system as described in paragraph 4.1.2. As explained in subsection 3.2.2.2C, *ccr1-6* and *ccr1-3* mutants are delayed in their development. Because the developmental stage of the host plant can influence endophytic bacterial communities (Yuan *et al*. 2015), both plants of the same age and the same stem length (older plants) were used in the study design. Also the moment of sampling (Agler *et al*. 2016) can influence the endophytic community. Therefore, some seeds of *ccr1-6* and *ccr1-3* mutants were sown earlier to harvest roots,

leaves and stems for the selected conditions at the same moment. Half of the seedlings were exposed to 3 µM $CdSO_4$ from the start of stem formation (as observed for WT plants during optimisation) during 3 weeks.

Seed endophytes (3 biological repetitions: pooled samples of ±30 mg seeds) were investigated from WT, *4cl1-1, 4cl1-2, ccr1-6* and *ccr1-3* plants which were grown simultaneously in Arasystems filled with sand (paragraph 4.1.2). Half of the seedlings were exposed to 3 µM $CdSO_4$ from the start of stem formation until overblown.

The total endophytic communities were investigated by making use of 454 pyrosequencing of a PCR derived amplicon library, with primer 799F and 1193R (Chelius and Triplet 2001, Bodenhausen *et al.* 2013) of the V5-V7 hypervariable region of the 16S rRNA gene, with optimised approach to reduce co-amplification of chloroplast and mitochondria (Beckers *et al*. 2016a). More details are presented in paragraph 4.1.2.

4.2.2 Results and discussion

4.2.2.1 The different plant compartments

In our study, 8 amplicon libraries were sequenced, each on $1/8^{th}$ of a picotiter plate. Three amplicon libraries (each amplicon library containing 16 samples resulting in 48 root samples) were composed of root samples and generated a total of 421,681 reads with an average length of 382 bp. Leaf samples were divided in 2 amplicon libraries (each amplicon library containing 24 samples resulting in 48 leaf samples) with a total of 288,139 reads with an average length of 376 bp. The seed and stem samples were combined in 3 amplicon libraries (each amplicon library containing 26 samples resulting in 48 stem samples and 30 seed samples) and generated a total of 451,978 reads with an average length of 391 bp. Sequences were processed using Mothur (version 1.34.4). The Standard Operating Protocol was followed as described in http://www.mothur.org/wiki/454_SOP (Schloss *et al*. 2009, Schloss *et al*. 2011). After assigning the reads to the samples, quality trimming (removal of the primer and barcode sequence) of the sequences, denoising and removal of chimeras, for roots, leaves, stems and seeds respectively 302,298; 196,085; 203,580 and 144,765 high quality sequences were obtained for further analysis.

Without singletons, which were found 0.72% or lower in the dataset (Table 4.2.1), along all libraries a total of 348 different OTUs were found.

(A) Non-template DNA

Sequencing of archaeal DNA amplicons is not aimed in our study design. Furthermore, assuming that the actual sequencing depth in 454 pyrosequencing is reduced in case non-target sequences such as chloroplast or mitochondrial 16S rRNA are co-amplified, to study the plant microbiome it is required to select primer pairs which have a low affinity to these non-target sequences. This was achieved by selecting primers containing mismatches with the chloroplast 16S rRNA gene. Mitochondrial amplicons were eliminated by post-PCR gel purification. Also the coverage rate and obtained phylum spectrum resulting from primer selection are important elements in metabarcoding studies. The primer selection was already performed for the analyses of the rhizosphere, and the root, leaf and stem endophytic community of poplar (Beckers *et al*. 2016a) and for seed endophytes of *A. thaliana* (Truyens *et al*. 2016a and b). Based on their findings and optimisation on test samples (data not shown), primers 799F (Chelius and Triplet 2001) and 1193R (Bodenhausen *et al*. 2013) were selected in our study.

Table 4.2.1: Co-amplification of non-target 16S rRNA, unclassified bacterial reads and singletons within each investigated plant compartment presented as average percentage with standard error.

	Seed	Root	Leaf	Stem
Archaea	0.00% ± 0.00%	0.00% ± 0.00%	0.00% ± 0.00%	0.00% ± 0.00%
Chloroplast	0.47% ± 0.42%	0.12% ± 0.06%	0.06% ± 0.01%	0.15% ± 0.02%
Mitochondria	0.05% ± 0.02%	0.00% ± 0.01%	0.01% ± 0.01%	0.07% ± 0.03%
Unknown	1.39% ± 0.59%	0.85% ± 0.61%	0.28% ± 0.06%	1.22% ± 0.53%
Singletons	0.72% ± 0.13%	0.32% ± 0.03%	0.44% ± 0.04%	0.38% ± 0.03%

After the 454 pyrosequencing and processing of samples making use of "Mothur", all non-target rRNA reads from Archaea, chloroplast, mitochondria, unknown bacterial sequences and singletons were removed. These removed sequences are presented in table 4.2.1 as the average relative percentages in all investigated plant compartments. No archaeal and only a small fractions of both chloroplast (below 0.50%) and mitochondrial (below 0.07%) rRNA sequences were found in seeds, roots, leaves and stems. The number of unknown bacterial sequences only represented a minor part (less than 1.39%) in all the plant

compartments. The least OTUs assigned to unknown bacterial sequences were found in leaves followed by roots, stems and seeds. Therefore, we can conclude that the used primers worked adequately in eliminating non-target sequences in our samples.

(B) Overall data description, richness and diversity

Rarefaction curves were assembled based on the number of observed OTUs (Fig 4.2.1). Furthermore, in "Mothur" Good's coverage rates were calculated making use of 10,000 iterations and are represented per plant compartment in Table 4.2.2. The sequencing depth was adequate for all investigated plant compartments since the average coverage rates were 97% or higher within every plant compartment so the endophytic microbiome could be reliably described.

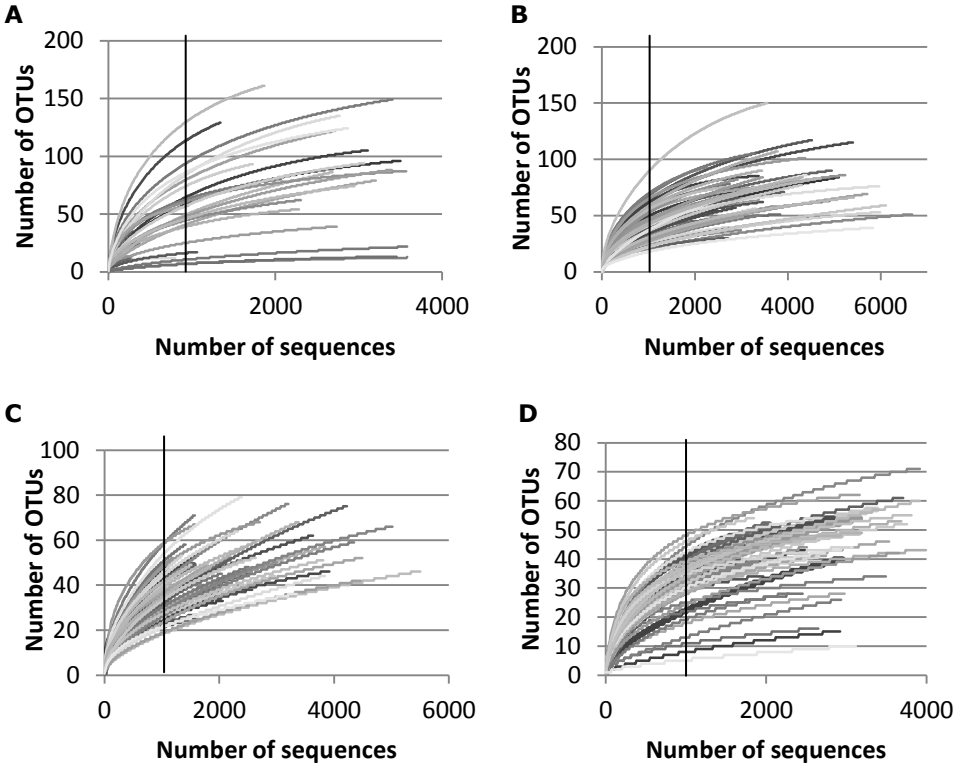


Figure 4.2.1: Rarefaction curves at the level of individual samples of (A) seeds, (B) roots, (C) leaves and (D) stems. The vertical line represents the subsampled number of sequences per sample (990).

Significant differences in observed OTU richness, Shannon and inverse Simpson diversity were observed in function of the plant compartment. The OTU richness was highest in seeds followed by roots and leaves and was lowest in stems. The Shannon and inverse Simpson diversity displayed a similar pattern (Table 4.2.2).

Table 4.2.2: Average Good's coverage estimates (%), Shannon and inverse Simpson diversity and OTU richness calculated in Mothur based on 10,000 iterations for a subsampling of 990 sequences. Standard errors are indicated. Letters: significant (one way anova with subsequent Tukey-Kramer post hoc test) differences $p<0.01$.

	Seed	**Root**	**Leaf**	**Stem**
Good's coverage	97.0% ± 0.65	98.0% ± 0.10	98.3% ± 0.07	98.7% ± 0.05
Shannon	1.86 ± 0.18 a	1.63 ± 0.08 ab	1.40 ± 0.08 bc	1.18 ± 0.45 c
Inverse Simpson	5.11 ± 0.80 a	3.47 ± 0.28 ab	2.87 ± 0.21 bc	2.81 ± 0.70 c
OTU richness	50.10 ± 6.08 a	41.46 ± 2.22 ab	34.57 ± 1.43 bc	30.68 ± 1.32 c

A higher diversity in seeds is not a generally supported observation in literature. However, in our study design, roots, leaves and stems were grown in a hydroponics system placed in plant growth chambers and roots were surrounded by 1/10 Hoagland nutrient solution (no real rhizosphere) whereas seeds were collected from plants cultivated in trays filled with sand in the greenhouse, which is a less controlled environment. Differences in environmental conditions can have significant effects on bacterial communities (Bulgarelli *et al*. 2012, Lundberg *et al*. 2012, Agler *et al*. 2016) and may explain the higher bacterial diversity and richness in the seeds.

Further, in the hydroponically grown plants, diversity and richness significantly decreased ($p<0.01$) from roots to stems (OTU richness, Shannon and inverse Simpson diversity indices). No significant differences in richness and diversity were observed between root and leaf tissues (Table 4.2.2) as reported by Bodenhausen *et al*. (2013). The root to shoot decrease in diversity is a generally accepted observation (Truyens *et al*. 2013, Croes *et al*. 2015, Beckers *et al*. 2016b) and can be associated with the translocation of bacterial endophytes that originate from the rhizosphere from roots to leaves and the need to cross several barriers during the translocation (Compant *et al*. 2010).

(C) Phylum level

Earlier studies investigated the microbiome of *A. thaliana* at phylum level in roots (Bressan *et al*. 2009, Lundberg *et al*. 2012, Bulgarelli *et al*. 2012, Bodenhausen *et al*. 2013, Schlaeppi 2014, Bai et al 2015) and leaves (Bodenhausen *et al*. 2013). Proteobacteria, Bacteroidetes and Actinobacteria (Lundberg *et al*. 2012, Bulgarelli *et al*. 2012, Bodenhausen *et al*. 2013, Schlaeppi 2014) were observed in high abundances suggesting similar host needs for *A. thaliana* plants (Lundberg *et al*. 2012). Moreover, it is demonstrated that Proteobacteria can colonise *A. thaliana* roots independently from the developmental stage (Yuan *et al*. 2015). However, we did not find information concerning the phylum composition of stems of *A. thaliana*.

Table 4.2.3: Three most abundant phyla per plant compartment and their average relative abundance.

Phylum	average abundance (%)	Phylum	average abundance (%)
Seed		**Root**	
Proteobacteria	67.89	Proteobacteria	92.09
Actinobacteria	13.51	Bacteriodetes	4.01
Bacteriodetes	7.88	Deinococcus-thermus	2.25
Total	89.28	Total	98.35
Leaf		**Stems**	
Proteobacteria	97.08	Proteobacteria	94.26
Actinobacteria	1.62	Bacteriodetes	3.30
Bacteriodetes	1.13	Firmicutes	1.42
Total	99.83	Total	98.98

In our study, the **root** endophytic community was dominated by members of Proteobacteria (92.09%), followed by Bacteriodetes (4.01%) and Deinococcus-thermus (2.25%) (Table 4.2.3). In the studies of Schlaeppi *et al*. (2014) and Bulgarelli *et al*. (2012), the majority of OTUs detected in the roots of *A. thaliana* belonged to Proteobacteria, Bacteroidetes and Actinobacteria. However, in our study, no OTUs of the phylum Actinobacteria were found at a relative abundance higher than 1% (Fig 4.2.4). The high dominance of Proteobacteria and low relative abundance of Actinobacteria and Bacteriodetes in roots of *A. thaliana* in its bolting stage was also reported by Yuan *et al*. (2015).

In the endophytic community of the **stems**, OTUs assigned to the phyla Proteobacteria (94.26%), Bacteriodetes (3.30%) and Firmicutes (1.42%) were most abundant. As observed in roots, no members of Actinobacteria were found in stems in a relative abundance higher than 1% (Fig 4.2.8). The high relative

abundance of Proteobacteria and Fimicutes was also observed in *Anthurium andraeanum* stems (Sarria-Guzmán *et al*. 2016)

In general, the total leaf and seed endophytic communities consisted of Proteobacteria, Actinobacteria and Bacteriodetes (Table 4.2.3). The presence of these 3 phyla in *A. thaliana* **leaves** sampled at various locations was already reported by Bodenhausen *et al*. (2013). However, both Actinobacteria (1.62%) and Bacteriodetes (1.13%) were less represented in leaves of our study and leaf communities were dominated by Proteobacteria (97.08%). The relative abundance of members of the phylum Firmicutes and Deinococcus-thermus, as observed by Bodenhausen *et al*. (2013), were very low and did not pass a relative abundance of 1% in any investigated condition (Fig 4.2.6).

Though the 3 most abundant phyla in **seeds** were the same as in leaves (Proteobacteria (67.89%), Actinobacteria (13.51%) and Bacteriodetes (7.88%)), the total seed endophytic community was less dominated by Proteobacteria (Table 4.2.3). In seeds, various other phyla were found in high relative abundances such as Deinococcus-thermus, TM7 and Firmicutes (Fig 4.2.10).

(D) Genus level

Differences in the endophytic community composition in function of the plant compartment, due to specific micro-environments (with for instance differences in available nutrients) were already previously reported (Beckers *et al*. 2016b, Bodenhausen *et al*. 2013, Zarraonaindia *et al*. 2015). Here we discuss the identified endophytic community compositions at the level of the genera among the studied plant compartments.

Samples were normalised to 990 reads and square root transformed where after the Bray Curtis similarity was calculated and overall similarities were presented in a non-metric multidimensional scaling plot (nMDS). In the nMDS plot, the different plant compartments visually group together with some overlap (Fig 4.2.2). The grouping was further supported by ANOSIM analysis (an analogue to the univariate ANOVA with R value <0.1: "similar", 0.1-0.25: "similar with some differences", 0.25-0.5: "different with some overlap", 0.5-0.75: "different", and 0.75-1 "highly different") (Clarke and Warwick 2001) and SIMPER dissimilarity.

Table 4.2.4: ANOSIM calculated with the Spearman Rank correlation and SIMPER dissimilarity scores calculated based on Bray Curtis Similarity of the comparisons of all investigated plant compartments. Significance: *: p-value 0.0089, **: p-value <0.00001.

	Comparison					
	Seed to Root	**Seed to Leaf**	**Seed to Stem**	**Root to Leaf**	**Root to Stem**	**Leaf to Stem**
ANOSIM (R-value)	0.552 **	0.540 **	0.574 **	0.093 *	0.208 **	0.230 **
SIMPER dissimilarity (%)	75.67	73.90	75.96	58.67	68.54	64.59

The seed endophytic community was significantly segregated from the communities of **all other plant compartments** with approximately a SIMPER dissimilarity of 75% and could be classified as "different" with an R value of approximately 0.55 ($p<0.00001$) (Table 4.2.4). The difference can be explained by the high relative abundance of *Rhizobium* (20.85%) and a member of the unclassified *Rhizobiales* (7.35%) in the seed community (Table 4.2.5). The presence of *Rhizobium* in several generations of seeds was already observed by Truyens *et al*. (2016a and 2016b). High relative abundance of Rhizobiaceae members was also observed in roots of bolting *A. thaliana* plants by Yuan *et al*. (2015). They hypothesized that plants select N fixing microbes in the later stages of development. Furthermore, members of the genus *Rhizobium* are known for their possible plant growth promoting traits (Truyens *et al*. 2016a and 2016b, Biswas *et al*. 2000, Chi *et al*. 2005, Gopalakrishnan *et al*. 2015).

Table 4.2.5: Three most abundant genera per plant compartment and their average relative abundance.

Genus	**average abundance (%)**	**Genus**	**average abundance (%)**
Seed		**Root**	
Rhizobium	20.85	*Pseudomonas*	31.85
Pseudomonas	18.30	*Acidovorax*	19.93
Uc Rhizobiales	7.35	Uc Oxalobacteraceae	16.42
Total	46.50	Total	68.20
Leaves		**Stems**	
Pseudomonas	59.56	*Alcaligenes*	44.45
Acidovorax	16.07	*Pseudomonas*	35.85
Stenotrophomonas	4.06	Uc Enterobacteriaceae	1.58
Total	78.00	Total	81.88

In all plant compartments, including seeds, more than 18% of the reads were assigned to *Pseudomonas* OTUs*,* although differences in relative abundance (seeds: 18.30%, roots: 31.85%, leaves; 59.56%, stems: 35.85%) were detected and could explain differences among plant compartments (Table 4.2.5). *Pseudomonas* members can display beneficial plant microbe interactions (*e.g.*

production of siderophores, phytohormones and antibacterial compounds) (Loper *et al*. 2012, Berg *et al*. 2014, Lebeis 2014, Truyens *et al*. 2016a). However, the possible over- or underestimation of these genera should be kept in mind since some bacterial strains just have one copy of the 16S rRNA gene while for example *Pseudomonas* strains enclose 5 copies (Bodenhausen *et al*. 2013, Berg *et al*. 2014). Moreover, biases towards less common genera could be forthcoming from the high dominance of *Pseudomonas* (Amend *et al*. 2010). The presence of *Pseudomonas* as *A. thaliana* seed endophyte was already mentioned by Truyens *et al*. (2016a and 2016b). Further, the genera *Acidovorax* and *Alcaligenes* were not part of the 3 most abundant genera in seeds as observed in other plant compartments (Table 4.2.5).

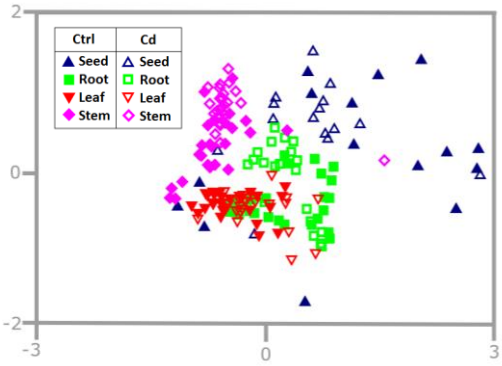


Figure 4.2.2: Similarity between total endophytic communities of selected plant compartments presented in a non-metric MDS plot based on Bray-Curtis similarity on square root transformed data. Kruskall stress formula 1, minimum stress 0.1, stress value 0.19.

In case the **stem** community was **compared to communities of root and leaf**, SIMPER dissimilarities of approximately 65% were found and could be classified as "similar with some differences" with the R value of approximately 0.2 ($p<0.00001$)(Table 4.2.4). Here the principal difference was the high occurrence of *Alcaligenes* in stems (44.45%) and *Acidovorax* in roots (19.93%) and leaves (16.07%) communities (Table 4.2.5). Members of the genus *Alcaligenes* were already identified as endophytes in for example perennial grasses (Leo *et al*. 2016), *Brassica napus* (Montalban *et al*. 2016) and *A. thaliana* (Bhattacharyya *et al*. 2015). A selection for N fixing microbes was observed by Yuan *et al*. (2015) in *A. thaliana* roots at later stages in the life cycle (bolting stages). Some members of the genus *Alcaligenes* are capable of N

fixation (Malik *et al*. 1981, You *et al*. 1993). However, further investigation needs to be performed to explain the selection of *Alcaligenes* as dominant stem endophyte.

In the comparison of the **root and leaf** bacterial communities, we observed a SIMPER dissimilarity of 58% and p-value (ANOSIM-result) smaller than 0.05 suggesting a significant difference in both communities. However, the R score (from the ANOSIM analysis) of 0.09 indicated (Clarke and Warwick 2001) highly similar communities, which could indicate the relation between the R score and the p-value is not always stable.
However, some differences were observed in average relative abundances of the endophytic communities from roots and leaves, which were dominated by *Pseudomonas* (roots: 31.85% versus leaves: 59.56%), *Acidovorax* (roots: 19.93% versus leaves: 16.07%) and OTUs from unclassified Oxalobacteraceae in roots (16.42%) or *Stenotrophomonas* in leaves (4.06%) (Table 4.2.5). Migration of endophytes from the roots to the leaves might be the cause of the similarity between the communities (Chi *et al*. 2005, Bodenhausen *et al*. 2013).
To further explain differences among genera, all genera which were found with a relative abundance of more than 1% are listed in supplemental table 4.2.1. Some genera were found in all plant compartments (*e.g. Alcaligenes*, *Acidovorax* or *Pseudomonas)*. Furthermore, some genera were more related to one plant compartment but were also observed in all other plant compartments to a lesser extend. For example *Bacillus*, *Sphingomonas* and *Nevskia* were more abundant in seeds; *Curvibacter*, *Pelomonas*, and *Acinetobacter* were mainly observed in roots; *Arthrobacter*, *Novosphingobium* and *Delftia* were more encountered in leaves and *Exiguobacterium, Staphylococcus* and *Halomonas* were more abundant in stems. Just some genera were exclusively found in 1 plant compartment (*e.g. Micromonospora* in seeds).

4.2.2.2 Effects due to the lignin-reduced genotype and Cd-exposure

In subsequent paragraphs, the differences due to the genotype with and without Cd exposure within each plant compartment were further analysed. First, we made a visual presentation of the differences among conditions by making use of a non-metric multidimensional scaling plot (nMDS) and hierarchical cluster

using Bray Curtis similarity based on OTUs (defined at a 97% similarity cut-off in Mothur in datasets which were normalised to 990 reads and square root transformed). The visual representation was further supported by making use of an ANOSIM analysis (calculated in "R") in combination with SIMPER dissimilarity (calculated in "Primer7"). Due to a low number of possible permutations in the dataset, p-values could only be derived in steps of 0.1 during the ANOSIM analysis. However, clear trends could be observed with a p-value of 0.1 (borderline significant) which may become significant in case more biological repetitions are used. Furthermore, the total community composition of each condition was visualised by making use of a bar plot. The colours of the bars (as used in the figures; a detailed legend is available in supplemental figure 4.2.1) of the discussed genera are given in parentheses throughout the next discussion.

Table 4.2.6: Diversity indices of total bacterial communities from *A. thaliana* WT plants and lignin-reduced mutant seeds, roots, leaves and stems. Averages and standard errors are indicated. Significance (two way anova with subsequent Tukey-Kramer test) $p<0.01$.

	Inverse Simpson		**Shannon**	
	0 µM Cd	**3 µM Cd**	**0 µM Cd**	**3 µM Cd**
		SEED		
WT	2.91 ± 1.54	9.75 ± 4.93	1.06 ± 0.80	2.59 ± 0.61
4cl1-1	2.71 ± 0.79	10.80 ± 2.64	1.53 ± 0.51	3.09 ± 0.34
4cl1-2	1.31 ± 0.32	5.84 ± 3.30	1.31 ± 0.15	1.82 ± 0.89
ccr1-6	3.31 ± 1.51	4.75 ± 0.86	1.69 ± 0.48	2.15 ± 0.23
ccr1-3	1.71 ± 0.59	4.66 ± 0.44	0.69 ± 0.41	2.25 ± 0.22
All genotypes	3.06 ± 0.55	7.16 ± 1.31	1.34 ± 0.21	2.38 ±0.23
		ROOT		
WT	2.80 ± 0.64	5.63 ± 1.90	1.39 ± 0.13	2.18 ± 0.28
4cl1-1 O	4.04 ± 2.04	3.51 ± 0.64	1.70 ± 0.63	1.83 ± 0.16
4cl1-2 O	4.18 ± 1.77	3.02 ± 1.65	1.73 ± 0.60	1.28 ± 0.55
ccr1-6 O	2.82 ± 0.65	4.83 ± 0.72	1.51 ± 0.22	2.06 ± 0.15
ccr1-3 O	5.01 ± 1.68	3.28 ± 0.19	1.93 ± 0.53	1.71 ± 0.06
ccr1-6*/SNBE *O	2.59 ± 0.60	2.68 ± 1.07	1.34 ± 0.11	1.33 ± 0.47
Ccr1-6 L	2.74 ± 0.31	3.68 ± 0.93	1.69 ± 0.17	1.80 ± 0.29
Ccr1-6 L	2.58 ± 0.84	2.06 ± 0.33	1.31 ± 0.34	1.19 ± 0.16
		LEAF		
WT	4.58 ± 1.29	2.84 ± 0.82	1.94 ± 0.28	1.52 ± 0.42
4cl1-1 O	2.26 ± 0.65	2.13 ± 0.52	1.26 ± 0.32	1.07 ± 0.27
4cl1-2 O	2.69 ± 0.60	3.02 ± 0.29	1.40 ± 0.31	1.46 ± 0.11
ccr1-6 O	4.08 ± 0.11	2.04 ± 0.16	1.70 ± 0.24	1.12 ± 0.17
ccr1-3 O	1.44 ± 0.11	1.99 ± 0.34	0.84 ± 0.13	1.06 ± 0.29
ccr1-6*/SNBE *O	1.46 ± 0.09	3.77 ± 1.31	0.86 ± 0.08	1.46 ± 0.48
Ccr1-6 L	3.33 ± 1.64	4.02 ± 0.29	1.45 ±0.47	2.05 ± 0.13
Ccr1-6 L	2.18 ± 0.35	4.15 ± 0.43	1.40 ± 0.25	1.86 ± 0.20
		STEM		
WT	1.22 ± 0.10	2.40 ± 0.23	0.51 ± 0.19	1.45 ± 0.03
4cl1-1 O	2.28 ± 0.49	2.74 ± 0.46	1.26 ± 0.32	1.45 ± 0.11
4cl1-2 O	2.49 ± 0.76	2.20 ± 0.20	1.07 ± 0.51	1.34 ± 0.12
ccr1-6 O	1.61 ± 0.05	2.00 ± 0.37	0.98 ± 0.05	1.17 ± 0.22
ccr1-3 O	2.43 ± 0.27	2.50 ± 0.43	1.28 ± 0.08	1.40 ± 0.24
ccr1-6*/SNBE *O	1.62 ± 0.37	2.95 ± 0.43	0.72 ± 0.29	1.59 ± 0.25
Ccr1-6 L	2.24 ± 0.28	2.05 ± 0.17	1.12 ± 0.16	1.20 ± 0.14
Ccr1-6 L	1.51 ± 0.30	2.66 ± 0.32	0.73 ± 0.27	1.59 ± 0.13

(A) Diversity

The diversity within each community was evaluated by making use of the inverse Simpson and Shannon diversities. No significant differences in the diversity of the total endophytic community were observed due to the lignin-reduced *A. thaliana* **genotype** in seeds, roots, leaves and stems. Also no significant effects due to **Cd exposure** of the plants were found in the separate genotypes (Table 4.2.6).

However, an overall significant increase ($p<0.01$) in inverse Simpson and Shannon diversities was observed (Table 4.2.6) in seeds after exposure of the parental plants to Cd which was also reported by Truyens *et al*. (2016a), though no further enhancing effects of Cd on endophytic diversity were found in literature. In soil and rhizosphere, no effects on total bacterial diversity were found after long term exposure to toxic concentrations of metals (Dell'Amico *et al*. 2005, Ellis *et al*. 2003). In all other plant compartments (roots, leaves and stems) no general effects of Cd exposure of the plants on diversity were observed (data not shown).

Table 4.2.7: ANOSIM calculated with the Spearman Rank correlation and SIMPER dissimilarity scores calculated based on Bray Curtis similarity at the bacterial genus level of all comparisons related to the developmental effect, genotype effect, cadmium effect or combined in the **root** compartment (n=3). Abbreviations O: *ccr1* mutants with a similar age in comparison to WT plants, L: *ccr1* mutants with similar stem length in comparison to WT plants, *ccr1-6*/SNBE: complemented *ccr1-6* mutant with restored lignin levels in the vessels. Significance: p-value = 0.1.

Genotype effect	**Comparison between non-exposed WT and lignin mutant**						
	4cl1-1	***4cl1-2***	***ccr1-6* O**	***ccr1-3* O**	***ccr1-6* /SNBE**	***ccr1-6* L**	***ccr1-3* L**
ANOSIM (R value)	0.037	-0.148	-0.296	0.148	-0.037	0.111	-0.370
SIMPER dissimilarity (%)	46.57	48.54	42.62	49.60	47.04	45.63	48.12
Genotype effect under Cd stress	**Comparison between Cd-exposed WT and lignin mutant:**						
	4cl1-1	***4cl1-2***	***ccr1-6* O**	***ccr1-3* O**	***ccr1-6* /SNBE**	***ccr1-6* L**	***ccr1-3* L**
ANOSIM (R value)	0.037	-0.074	0.185	0.407	-0.296	0.185	0.296
SIMPER dissimilarity (%)	42.45	47.33	45.49	48.46	50.03	47.11	52.00
Cd effect	**Comparison between non-exposed and Cd-exposed genotype:**							
	WT	***4cl1-1***	***4cl1-2***	***ccr1-6* O**	***ccr1-3* O**	***ccr1-6* /SNBE**	***ccr1-6* L**	***ccr1-3* L**
ANOSIM (R value)	0.296	0.630	0.037	-0.185	0.333	-0.370	-0.074	-0.333
SIMPER dissimilarity (%)	50.40	58.11	53.27	48.53	44.46	53.59	42.18	46.97

Developmental effect	**Comparison between:**			
	***ccr1-6* O and L**	***ccr1-3* O and L**	**Cd-exposed *ccr1-6* O and L**	**Cd-exposed *ccr1-3* O and L**
ANOSIM (R value)	0.407	0.074	-0.259	0.778
SIMPER dissimilarity (%)	47.60	53.80	43.09	60.40

(B) Culture-independent ROOT endophytes

Roots are the primary sites where soil and rhizosphere derived endophytes gain entry into the plant and are the primary organs to interact with the environment (Hardoim *et al*. 2015). After colonising the root, endophytes can inhabit the root or colonise the other plant compartments through the vascular system (Weyens *et al*. 2009b). Therefore, root endophytic communities will be further explored.

Phylum level

No differences were observed among all investigated groups at the phylum level. Meaning in roots all plant genotypes with or without Cd exposure selected for endophytic communities that are similar at phylum level.

ccr1 mutants - developmental effect?

In the study of Lundberg *et al*. (2012), samples of different developmental stages clustered together and no developmental effects could be observed. In contrast, Yuan *et al*. (2015) demonstrated that root communities changed during development of plants. Therefore, we should first examine the eventual effect of the difference in age among *ccr1* mutants.

Developmental effects (roots of plants with the same age in comparison to WT plants (O) relative to roots of plants with similar stem length in comparison to WT plants (L)) in the non-exposed *ccr1-6* mutants (R-value of 0.4, "different with some overlap") and Cd-exposed *ccr1-3* mutants (R-value 0.8 "highly different") communities were observed (Table 4.2.7). Hence, the difference in development affected the culture-independent endophytic communities of some conditions though it not influenced both allelic variants in the same treatment group of roots of *ccr1* mutants in the same way.

The effect of the lignin-reduced genotype

In roots, total endophytic communities did not reveal a specific clustering pattern (Fig 4.2.3) which was supported by ANOSIM R-values which suggested the communities to be "similar" or "similar with some differences" (Table 4.2.7). Most observed OTUs were assigned to *Pseudomonas* (bright green) as reported by Yuan *et al*. (2015) in roots of bolting *A. thaliana* plants and Lundberg *et al*. (2012). Also *Acidovorax* (light purple; Fig 4.2.4), unclassified Oxalobacteraceae

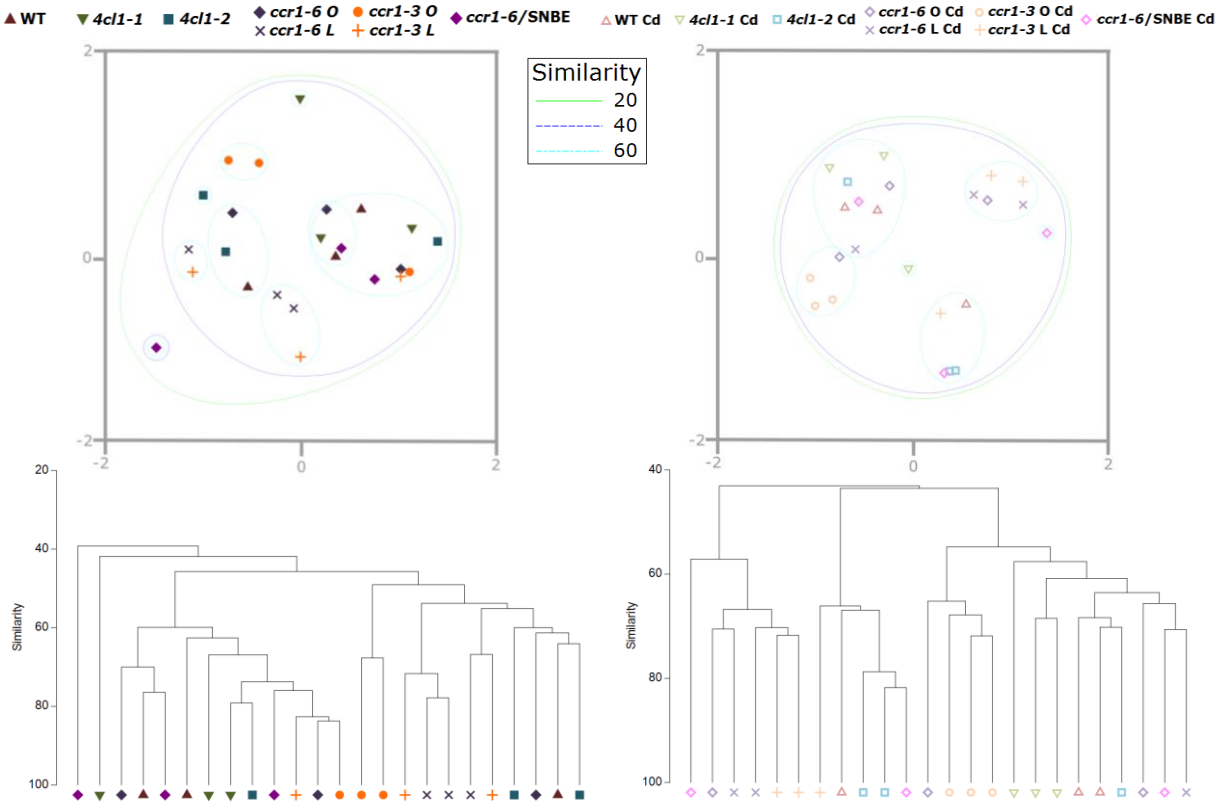


Figure 4.2.3: Similarity between total endophytic communities of **roots** of older plants presented in a non-metric MDS plot (Kruskall stress formula 1, minimum stress 0.1, stress value 0.14 for non-treated and 0.10 for roots of Cd-exposed plants) and hierarchical clustering of the samples based on Bray-Curtis similarity on square root transformed data. Abbreviations: O: *ccr1* mutants with a similar age in comparison to WT plants, L: *ccr1* mutants with similar stem length in comparison to WT plants, *ccr1-6*/SNBE: complemented *ccr1-6* mutant with restored lignin levels in the vessels.

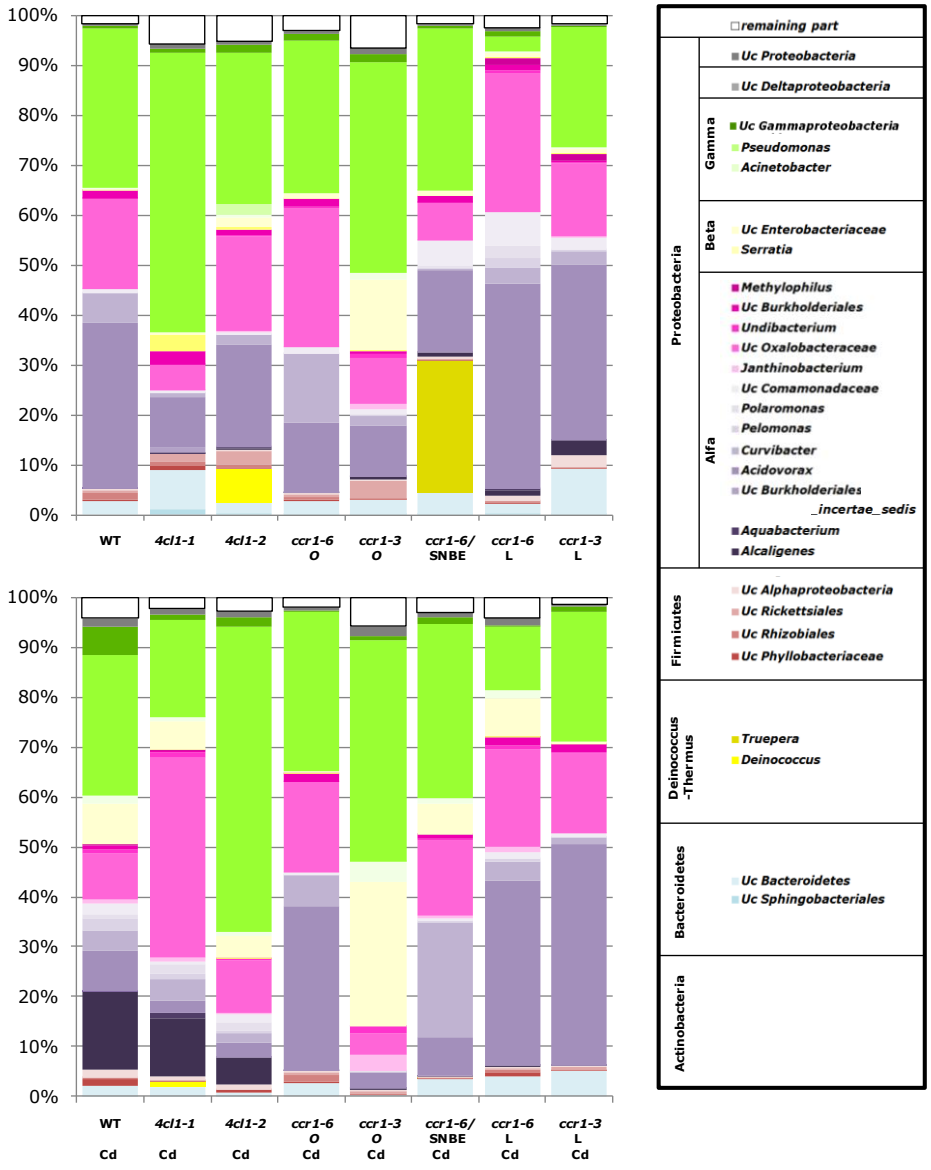


Figure 4.2.4: Total bacterial community composition presented as relative abundances of **roots** of older plants (n=3) from WT, *4cl1-1, 4cl1-2, ccr1-6, ccr1-3* and *ccr1-6*/SNBE plants with and without exposure to Cd. Abbreviations O: *ccr1* mutants with same age in comparison to WT plants and L: *ccr1* mutants with same stem length in comparison to WT plants. Uc: Unclassified. A detailed legend is provided in supplemental fig 4.2.1.

(pink; Fig 4.2.4), unclassified Bacteroidetes (light blue; Fig 4.2.4) and *Curvibacter* (light purple; Fig 4.2.4) were occurring in all *A. thaliana* genotypes root communities. Some genera were most abundant in 1 specific plant type such as *Deinococcus* (yellow; Fig 4.2.4) in the *4cl1-2* bacterial community, *Truepera* (dark yellow; Fig 4.2.4) in the *ccr1-6*/SNBE community, *Serratia* (ligh yellow; Fig 4.2.4) in the *ccr1-3* O community and *Alcaligenes* (dark purple; Fig 4.2.4) in the *ccr1-6* L and *ccr1-3* L community though these slight differences may not influence the ANOSIM R-value.

The effect of the lignin-reduced genotype under Cd exposure

In case Cd-exposed lignin-reduced mutants were compared to Cd-exposed WT plants, the root community of *ccr1-3* O mutants visually tended to show some differences as presented in the NMDS analysis (Fig 4.2.3). *ccr1-3* mutants (O and L) ANOSIM R-values were respectively approximately 0.4 and 0.3 classifying them as "different with some overlap". However, these differences only manifested themselves as trends and the SIMPER dissimilarity was only 45-52% (Table 4.2.7).

The most obvious differences explaining the observation, is the presence of the genus *Alcaligenes* (dark purple; Fig 4.2.4) in the Cd-exposed WT plants but minimal relative abundance in the root community of the Cd-exposed *ccr1* mutants. Also differences in relative abundance of *Pseudomonas* (bright green; Fig 4.2.4), *Acidovorax* (light purple; Fig 4.2.4)*, Curvibacter* and *Pelomonas* (both in light purple shades; Fig 4.2.4) can explain the observed trends in ANOSIM values. Under Cd stress, no other differences among the bacterial communities could be observed between WT plants and other lignin-reduced mutants.

The effects of cadmium

The Cd effect within each genotype only revealed a distinction between *4cl1-1* communities of non-exposed and Cd-exposed plants with an ANOSIM R-value of 0.6 ("different") and a 58% SIMPER dissimilarity. However, trends in Cd effects within communities of WT plants and *ccr1-3* O mutants were observed which also showed R-values of approximately 0.3 ("different with some overlap") but SIMPER dissimilarity was respectively only 50% and 44% (Table 4.2.7).

The Cd-effect in the *4cl1-1* root community can be explained by the higher relative abundance of the genera *Alcaligenes* (dark purple; Fig 4.2.4) and unclassified Oxalobacteraceae (pink; Fig 4.2.4) and the lower relative abundance of *Pseudomonas* (bright green; Fig 4.2.4), *Serratia* (light yellow; Fig 4.2.4) and *Acidovorax* (light purple; Fig 4.2.4). However, in WT plants and both *4cl1* mutants more *Alcaligenes* OTUs were found in the root communities in case plants were exposed to Cd. Because effects on the endophytic root communities were not observed in both allelic variants, no conclusions can be drawn concerning the Cd effect within these lignin-reduced mutants.

Table 4.2.8: ANOSIM calculated with the Spearman Rank correlation and SIMPER dissimilarity scores calculated based on Bray Curtis similarity at the bacterial genus level of all comparisons related to the developmental effect, genotype effect, cadmium effect or combined in the **leaf** compartment (n=3). Abbreviations O: *ccr1* mutants with a similar age in comparison to WT plants, L: *ccr1* mutants with similar stem length in comparison to WT plants, *ccr1-6*/SNBE: complemented *ccr1-6* mutant with restored lignin levels in the vessels. Significance: p-value = 0.1.

Genotype effect	**Comparison between non-exposed WT and lignin mutant**						
	4cl1-1	***4cl1-2***	***ccr1-6* O**	***ccr1-3* O**	***ccr1-6* /SNBE**	***ccr1-6* L**	***ccr1-3* L**
ANOSIM (R value)	0.111	0.111	-0.148	0.370	0.444	0.148	0.185
SIMPER dissimilarity (%)	45.19	47.04	39.63	47.62	45.92	47.68	48.22
Genotype effect under Cd stress	**Comparison between Cd-exposed WT and lignin mutant:**						
	4cl1-1	***4cl1-2***	***ccr1-6* O**	***ccr1-3* O**	***ccr1-6* /SNBE**	***ccr1-6* L**	***ccr1-3* L**
ANOSIM (R value)	0.185	0.148	0.259	0.185	0.148	0.519	0.185
SIMPER dissimilarity (%)	39.06	32.97	35.64	38.77	37.82	48.00	41.46
Cd effect	**Comparison between non-exposed and Cd-exposed genotype:**							
	WT	***4cl1-1***	***4cl1-2***	***ccr1-6* O**	***ccr1-3* O**	***ccr1-6* /SNBE**	***ccr1-6* L**	***ccr1-3* L**
ANOSIM (R value)	0.074	0.148	0.037	0.482	0.296	0.0741	0.779	0.148
SIMPER dissimilarity (%)	44.46	47.74	39.94	43.35	44.44	37.28	61.87	46.43
Developmental effect	**Comparison between:**							
	non-exposed *ccr1-6* O and L		**non-exposed *ccr1-3* O and L**		**Cd-exposed *ccr1-6* O and L**		**Cd-exposed *ccr1-3* O and L**	
ANOSIM (R value)	0.148		0.148		1.000		-0.074	
SIMPER dissimilarity (%)	51.03		45.54		54.68		42.75	

(C) Culture-independent LEAF endophytes

Leaf endophytic communities play a crucial role in plant health and development and are mainly derived from the environment (Bodenhausen *et al*. 2013, Truyens *et al*. 2016b). Hence, also information about the endophytic leaf communities is required.

Phylum level

At the phylum level, the *4cl1-1* (ANOSIM R-value 1 ("highly different"), supplemental table 4.2.2) and *ccr1-6* O (ANOSIM R-value 0.48 ("different with some overlap"), supplemental table 4.2.2) mutants did have distinct total endophytic leaf communities in comparison to WT plants because of the high relative abundances of members of respectively *Microbacterium* and *Arthrobacter* of the phylum Actinobacteria (Fig 4.2.6). Moreover, these same conditions revealed a Cd effect at the phylum level (*4cl1-1:* ANOSIM R-value 1 and *ccr1-6* O ANOSIM R-value 0.48 (supplemental table 4.2.2)).

ccr1 mutants - developmental effect?

No developmental effect was observed in the non-exposed *ccr1* leaf communities. After exposure to Cd, an effect of the plant development on the *ccr1-6* leaf community of Cd-exposed plants was observed (ANOSIM R score 1, "highly different") (Table 4.2.8). Because results were inconclusive between both allelic mutants, no further conclusions can be drawn.

The effect of the lignin-reduced genotype

The *ccr1-3* O and *ccr1-6*/SNBE but also *4cl1-2* mutants visually clustered apart from the leaf community of WT plants (Fig 4.2.5). Differences as explained by the ANOSIM R-value (±0.4, "different with some overlap") were observed in *ccr1-3* O and *ccr1-6*/SNBE mutants in comparison to the leaf community of WT plants. However, the related SIMPER dissimilarity of these comparisons to the community of WT plants were respectively 48% and 46% which indicates that more than half of the community should be similar (Table 4.2.8). Moreover, an effect of the *CCR1* down-regulation on leaves bacterial community was observed in *ccr1-3* O and *ccr1-6*/SNBE mutants but was not found in *ccr1-6* O leaves. Also no effect was observed for *ccr1-6* L and *ccr1-3* L mutant communities. By consequence no further conclusions could be drawn.

In the ANOSIM analysis the *4cl1* total leaf endophytic communities appeared comparable to WT plants with an R-score of 0.1 (Table 4.2.8). Hence, the endophytic community structure of the *4cl1* mutants leaves did not change.

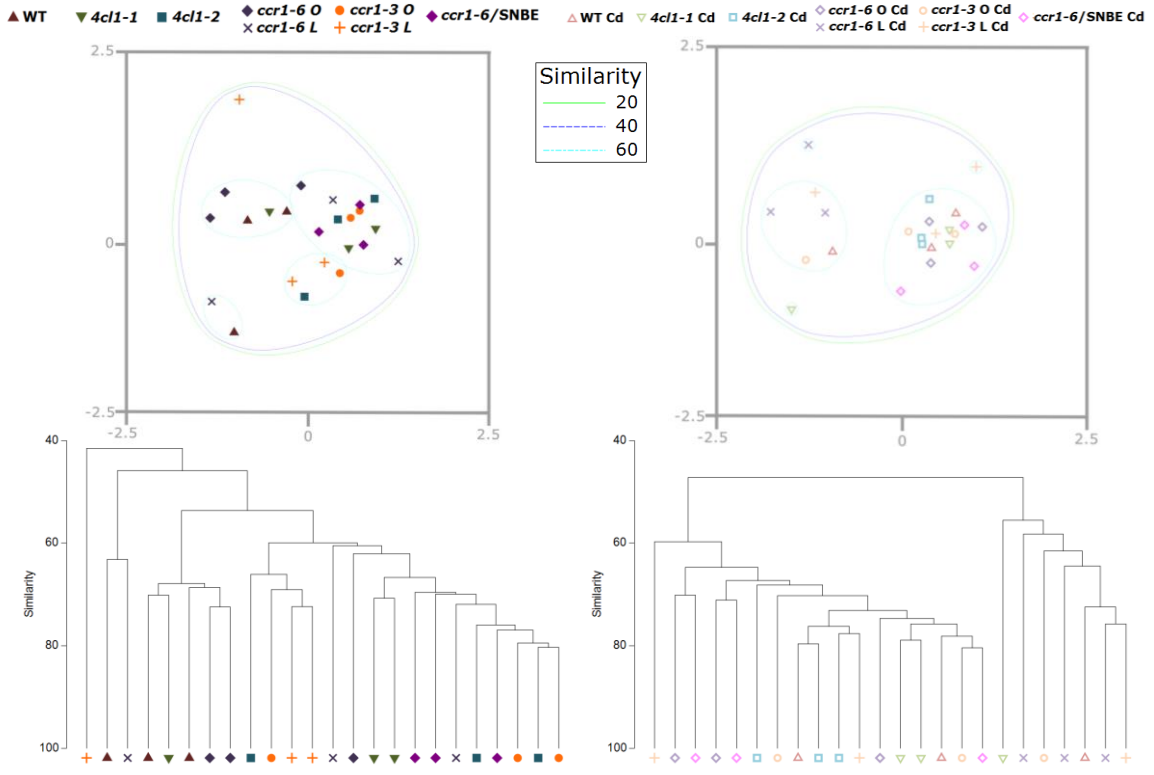


Figure 4.2.5: Similarity between total endophytic communities of **leaves** of older plants presented in a non-metric MDS plot (Kruskall stress formula 1, minimum stress 0.1, stress value 0.13 for non-treated and 0.10 for leaves of Cd-exposed plants) and hierarchical clustering of the samples based on Bray-Curtis similarity on square root transformed data. Abbreviations: O: *ccr1* mutants with a similar age in comparison to WT plants, L: *ccr1* mutants with similar stem length in comparison to WT plants, *ccr1-6*/SNBE: complemented *ccr1-6* mutant with restored lignin levels in the vessels.

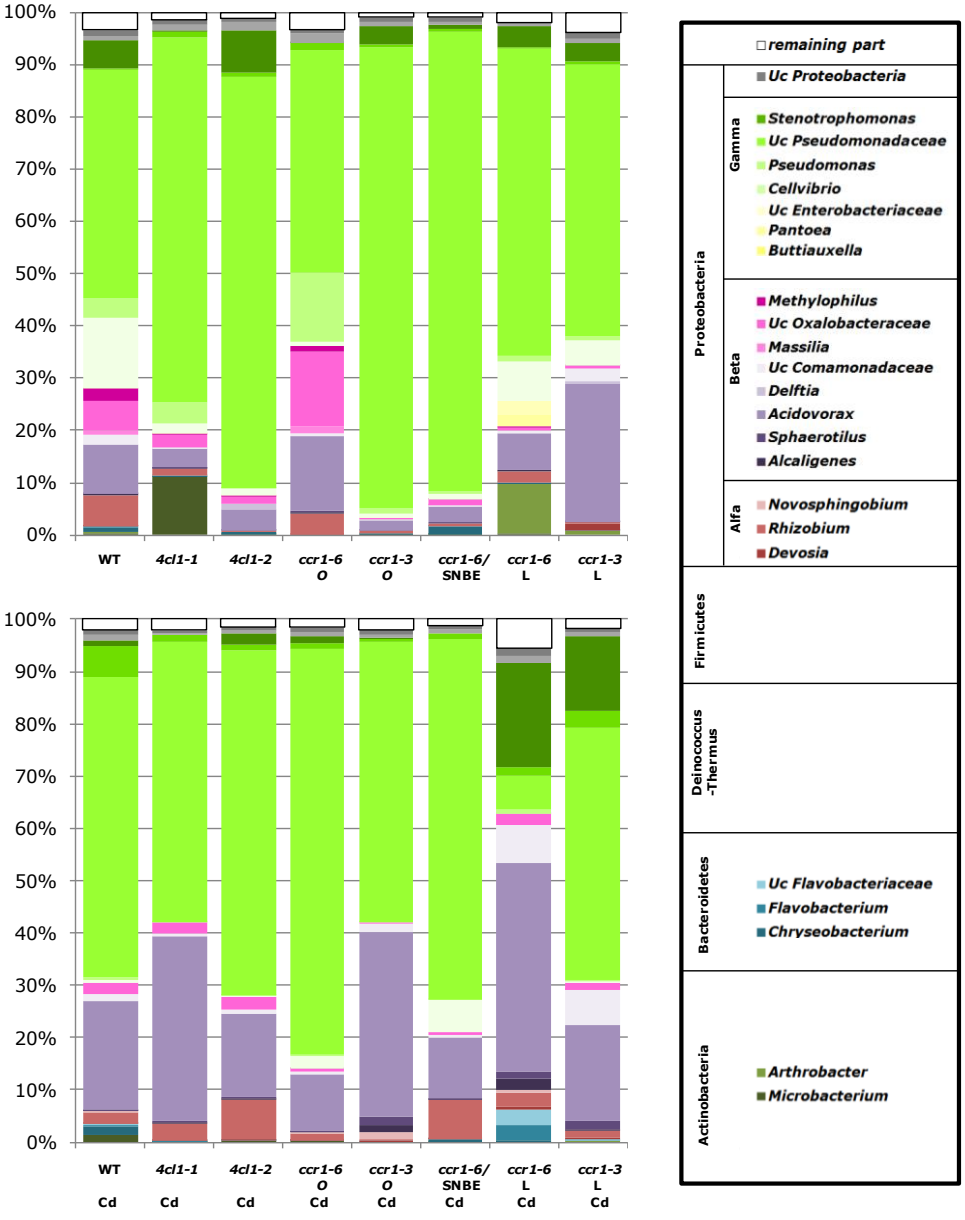


Figure 4.2.6: Total bacterial community composition (presented as relative abundances) of **leaves** of older plants (n=3) from WT, *4cl1-1, 4cl1-2, ccr1-6, ccr1-3* and *ccr1-6*/SNBE plants with and without exposure to Cd. Abbreviations O: *ccr1* mutants with same age in comparison to WT plants and L: *ccr1* mutants with same stem length in comparison to WT plants. Uc: Unclassified. A detailed legend is provided in supplemental fig 4.2.1.

Pseudomonas (bright green; Fig 4.2.6) dominated all total endophytic leaf communities in *A. thaliana* which was also observed by Agler *et al.* (2016) and Bodenhausen *et al*. (2013). The high relative abundance of *Pseudomonas* in aerial plant compartments was also reported in many other species such as poplar (Beckers *et al*. 2016b) and grapevine (Zarraonaindia *et al*. 2016). Furthermore, *Stenotrophomonas* (green; Fig 4.2.6) was also observed in the leaf endophytic communities of *A. thaliana* leaves by Agler *et al*. (2016). However, in the 3 genotypes which tended to cluster apart from WT plants (*4cl1-2, ccr1-6*/SNBE and *ccr1-3* O mutants) the dominance of *Pseudomonas* (bright green; Fig 4.2.6) in the community was even higher and could explain the separation from WT plants community during the clustering analysis. However, also other genera influenced the dissimilarity of these 3 groups in comparison to the WT endophytic community. For example in WT plants a higher occurrence of *Rhizobium* (light red; Fig 4.2.6; as observed in WT plants by Bodenhausen *et al*. 2013), *Cellvibrio* (light green; Fig 4.2.6), *Acidovorax* (light purple; Fig 4.2.6) and unclassified Oxalobacteraceae (pink; Fig 4.2.6; an observation which is in agreement with the findings of Bodenhausen *et al*. 2013) was found in the leaf community.

The effect of the lignin-reduced genotype under Cd exposure

The leaf endophytic communities of *A. thaliana ccr1-6* L mutants exposed to Cd visually separated from the total leaf endophytic communities of Cd-exposed WT plants (Fig 4.2.5) which was supported by the ANOSIM R-value of 0.5 ("different") with a SIMPER dissimilarity of 48% (Table 4.2.8). However, these results were not observed in the *ccr1-3* L mutant group.

The differences between the communities of the *ccr1-6* L mutant and WT plants exposed to Cd can be explained by high occurrences of *Stenotrophomonas* (green; Fig 4.2.6)*, Acidovorax,* unclassified Comamonadaceae (both in light shades of purple; Fig 4.2.6) and *Pseudomonas* (bright green; Fig 4.2.6) in the community of *ccr1-6* L mutants exposed to Cd.

The effects of cadmium

Cadmium influenced the total communities of the *ccr1-6* and *ccr1-3* leaves with a similar age in comparison to WT plants (O) with R-values of respectively 0.48 and 0.30 ("different with some overlap") and SIMPER dissimilarities of respectively 43% and 44%. Also the total leaf endophytic community of *ccr1-6* with a similar stem length in comparison with WT plants (L) was influenced by Cd with an ANOSIM R-value of 0.78 ("different") and a SIMPER dissimilarity of 62% (Table 4.2.8). However, the *ccr1-3* L mutant revealed no differences in community structure due to Cd-exposure of the plant.

Therefore, we can conclude that only the culture-independent community of the *ccr1* mutants with a similar age in comparison to WT plants (O) was influenced by Cd. In case plants with a similar stem length in comparison to WT plants (L) were used no Cd-induced differences were observed in the endophytic communities of the *ccr1-3* mutant while differences were found for the *ccr1-6* mutant.

In case *ccr1* seedlings were exposed to Cd, *Cellvibrio* (light green; Fig 4.2.6) almost disappeared from the endophytic community. Differences among the non-exposed *ccr1-6* O and Cd-exposed *ccr1-6* O condition can be further explained by an increase of *Pseudomonas* (bright green; Fig 4.2.6) and almost disappearance of unclassified Oxalobacteraceae (pink; Fig 4.2.6). However, the opposite was observed for *ccr1-6* condition with a similar stem length in comparison to WT plants (L). Here *Pseudomonas* (bright green; Fig 4.2.6) decreased and unclassified Oxalobacteraceae (pink; Fig 4.2.6) showed a limited increase in the leaf community. Other observed differences in the *ccr1-6* L mutant community after Cd exposure of the plant were the decrease of *Arthrobacter* (green; Fig 4.2.6), unclassified *Enterobacteriaceae, Buttiauxella* and *Pantoea* (all 3 in shades of yellow; Fig 4.2.6) while OTUs of *Acidovorax,* unclassified *Comamonadaceae* (both in light shades of purple; Fig 4.2.6), *Alcaligenes* (dark purple; Fig 4.2.6), *Stenotrophomonas* (green; Fig 4.2.6), *Flavobacterium* and unclassified *Flavobacteriaceae* (both in blue; Fig 4.2.6) increased. Also in the *ccr1-3* O mutant the relative abundance of *Pseudomonas* (bright green; Fig 4.2.6) was lower after Cd exposure of the plant. Furthermore, *Stenotrophomonas* (green; Fig 4.2.6) decreased in relative abundance while *Acidovorax (*light shade of purple; Fig 4.2.6), *Sphaerotilus* (both in shades of

purple; Fig 4.2.6), *Alcaligenes* (dark purple; Fig 4.2.6) and *Novosphingobium* (light red; Fig 4.2.6) increased.

(D) Culture-independent STEM endophytes

Stems of *A. thaliana* show secondary thickening and are therefore an excellent model to study lignification (Nieminen *et al*. 2004). Since lignification is highest in the xylem of stems (Boerjan *et al*. 2003) also the most prominent differences can be expected in stems. Moreover, in the study of Beckers *et al.* 2016b most differences were observed in the endophytic communities of stems of *CCR* down-regulated poplars.

Phylum level

In stems, no effects at the level of the phylum were observed due to genotype, Cd exposure or delayed development. Hence all *A. thaliana* plants across all conditions selected for the same endophytic communities at phylum level.

Table 4.2.9: ANOSIM calculated with the Spearman Rank correlation and SIMPER dissimilarity scores calculated based on Bray Curtis similarity at the bacterial genus level of all comparisons related to the developmental effect, genotype effect, cadmium effect or combined in the **stem** compartment (n=3). Abbreviations O: *ccr1* mutants with a similar age in comparison to WT plants, L: *ccr1* mutants with similar stem length in comparison to WT plants, *ccr1-6*/SNBE: complemented *ccr1-6* mutant with restored lignin levels in the vessels. Significance: p-value = 0.1.

Genotype effect	**Comparison between non-exposed WT and lignin mutant:**						
	4cl1-1	***4cl1-2***	***ccr1-6* O**	***ccr1-3* O**	***ccr1-6* /SNBE**	***ccr1-6* L**	***ccr1-3* L**
ANOSIM (R value)	0.037	-0.000	1.000	1.000	-0.074	0.482	-0.185
SIMPER dissimilarity (%)	41.89	46.23	61.11	56.28	35.96	46.36	32.86
Genotype effect under Cd stress	**Comparison between Cd-exposed WT and lignin mutant:**						
	4cl1-1	***4cl1-2***	***ccr1-6* O**	***ccr1-3* O**	***ccr1-6* /SNBE**	***ccr1-6* L**	***ccr1-3* L**
ANOSIM (R value)	0.037	-0.037	0.074	0.185	0.111	0.185	0.148
SIMPER dissimilarity (%)	51.85	49.83	49.12	54.00	49.09	48.48	47.34
Cd effect	**Comparison between non-exposed and Cd-exposed genotype:**							
	WT	***4cl1-1***	***4cl1-2***	***ccr1-6* O**	***ccr1-3* O**	***ccr1-6* /SNBE**	***ccr1-6* L**	***ccr1-3* L**
ANOSIM (R value)	0.519	0.185	0.444	-0.037	-0.074	0.556	0.444	1.000
SIMPER dissimilarity (%)	67.11	44.63	60.05	30.32	33.64	53.38	44.06	58.47
Developmental effect	**Comparison between:**							
	non-exposed *ccr1-6* O and L		**non-exposed *ccr1-3* O and L**		**Cd-exposed *ccr1-6* O and L**		**Cd-exposed *ccr1-3* O and L**	
ANOSIM (R value)	0.482		1.000		-0.074		0.370	
SIMPER dissimilarity (%)	45.68		54.24		29.03		40.36	

ccr1 mutants - developmental effect?

Age/development had a strong effect on the stem endophytic communities of non-exposed *ccr1-3* mutants (ANOSIM R-value 1, "highly different", SIMPER dissimilarity 55%). Also the bacterial communities of the non-exposed *ccr1-6* mutants and *ccr1-3* mutants exposed to Cd were influenced by the developmental stage (ANOSIM R-values of respectively 0.48 and 0.37, "similar with some differences", SIMPER dissimilarities of respectively 45% and 40%) (Table 4.2.9). In former studies, no developmental effects on communities of the stem compartment of *A. thaliana* were reported. However, in roots of *A. thaliana* (Lundberg *et al*. 2012, Yuan *et al*. 2015), stems of potato plants (van Overbeek and van Elsas 2008) and leaves of perennial wild mustard (Wagner *et al*. 2016) developmental effects were observed. For instance, Lundberg *et al.* (2012) showed a quantitative variation in the enrichment of specific bacteria in function of developmental stage and Yuan *et al.* (2015) demonstrated selection of specific micoorganisms depending on plant development in roots by supplying soil microbial slurry to *Arabidopsis* plants grown under specific sterile conditions.
In stems of non-exposed *ccr1-6* and *ccr1-3* mutants, more *Pseudomonas* (bright green; Fig 4.2.8) OTUs were found in the communities of plants with a similar stem length in comparison to WT plants (L) relative to plants which had the same age in comparison to WT plants (O). While *Alcaligenes* (dark purple; Fig 4.2.8) were more abundant in the stems communities of plants which did have the same age in comparison to WT plants (O) relative to plants with a similar stem length in comparison to WT plants (L). In *ccr1-6* mutants *Acidovorax* (light purple; Fig 4.2.8) was found in a high relative abundance in the community of stems with a similar length in comparison to WT plants (L) while in *ccr1-3* mutants the genus *Acidovorax* was more abundant in the plants which did have the same age in comparison to WT plants (O). In *ccr1-3* mutants exposed to Cd, differences in the total endophytic community were rather explained by a lower relative abundances of *Pseudomonas* (bright green; Fig 4.2.8), the presence of *Dysgonomonas* (blue; Fig 4.2.8) and *Staphylococcus* (orange; Fig 4.2.8) and higher relative abundances in *Aquabacterium* and *Alcaligenes* (both in shades of dark purple; Fig 4.2.8) in the stem community of plants with a similar length in comparison to WT plants (L).

The effect of the lignin-reduced genotype

The most distinct genotype effects were observed in the communities of stems of *ccr1* mutants with a similar age in comparison to WT plants (O). Both *ccr1-6 O* and *ccr1-3* O mutants communities did cluster apart from WT plants (Fig 4.2.7) which was supported by ANOSIM R-values of 1 "highly different" and SIMPER scores of respectively 61% and 56% for *ccr1-6* O and *ccr1-3* O mutants (Table 4.2.9). However, also the *ccr1-6* mutant with a similar stem length in comparison to WT plants (L) had an R-score of 0.48 "different with some overlap" in comparison to the WT plants stem community.

A higher relative abundance of *Acidovorax* (light purple; Fig 4.2.8) was only observed in the community of stems of *ccr1-3* O and *ccr1-6* L mutants, in comparison to stems of WT plants. The most obvious differences were the lower relative abundance of *Pseudomonas* (bright green; Fig 4.2.8) and higher relative abundances of OTUs of *Alcaligenes* (dark purple; Fig 4.2.8) in both *ccr1* mutants with a similar age relative to WT plants (O) and *ccr1-6* with a similar stem length relative to WT plants (L) in comparison to the WT endophytic community. Also in the bacterial communities of other lignin-reduced mutant stems, members of *Alcaligenes* (dark purple; Fig 4.2.8) appeared to be more numerous in comparison with the communities of WT plants. *Alcaligenes* species may use a range of organic and phenolic compounds including organic acids as their sole energy and growth sources (Clermont *et al*. 2001, Chaudhry and Chapalamadugu 1991, Thomas *et al*. 2002, Gallego *et al*. 2003) and may therefore be selected in stems of lignin-reduced mutants.

Several possible hypotheses could be developed to explain the observed community differences.

(i) Many other examples of plant genotype-induced effects on bacterial communities have been described (van Overbeek and van Elsas 2008, Germida and Siciliano 2001, Adams and Kloepper 2002, Lundberg *et al*. 2012, Bulgarelli *et al*. 2012, Weinert *et al*. 2011, Hur *et al*. 2011, Beckers *et al*. 2016b, Wagner *et al*. 2016). In our plant genotypes, changes in the monolignol production pathway due to the mutation in *CCR1*, lead to the accumulation of different phenolic compounds (*e.g.* ferulic acid) in the xylem (Vanholme *et al*. 2012a) and a changed composition of carbon sources is available for the endophytic

community. Beckers *et al*. (2016b) demonstrated that bacterial endophytes of *CCR* down-regulated poplar roots, leaves and stems showed a higher capacity to degrade ferulic acid. It is likely that in *A. thaliana* stems, the *ccr1* mutation influences the total endophytic community in a similar way. However, since the study design (using 454 pyrosequencing) only identified up to genus level, no further conclusions can be drawn about the species distribution and possible metabolic traits of these community members.

(ii) Some phenolic compounds will not only influence the available pool of C-sources inside the xylem but can also have antimicrobial action (Miedes *et al*. 2014, Weston and Mathesius 2013, Rout *et al*. 2014, Rice-Evans *et al*. 1996, Tilston *et al*. 2014). Hence, it is conceivable that some endophytes cannot be selected out of the environment since they are inhibited by the antimicrobial action of certain phenolic compounds.

(iii) The lignin reduction does not only interfere with the phenolic compounds inside the xylem but also affects the structural properties of the plants vasculature (Miedes *et al*. 2014). *ccr1* mutant *A. thaliana* plants show a collapsed xylem phenotype (mir Derikvand *et al*. 2008) which can affect the colonization throughout the plant (Miedes *et al*. 2014).

(iv) Cell walls of roots and stems show differences in lignin composition in comparison to WT plants (see subsection 3.4). Consequently, a different mode of selection of environmental bacteria to become endophytes can be a result of the cell wall which can be passed more easily. However, as discussed above, no big differences were observed in the culture-independent microbiome of roots and leaves.

(v) The development of plants can affect the microbial plant-associated communities (Lundberg *et al.* 2012, Yuan *et al.* 2015). Therefore, it is also likely that the delayed development of *ccr1* mutants influenced the microbiome of the stems since most differences were observed in the plants with a delayed development (same age in comparison to WT, indicated with O) and not in plants with the same stem length in comparison to WT plants (indicated with L).

The effect of the lignin-reduced genotype under Cd exposure

Under influence of Cd, the effect of the lignin reduction on the total stem endophytic community disappeared and no lignin-reduced mutant bacterial communities clustered apart (Fig 4.2.7 and table 4.2.9).

The effects of cadmium

The bacterial microbiome of almost all investigated genotypes with a similar development (WT, *4cl1-2*, *ccr1-6*/SNBE, *ccr1-6* L and *ccr1-3* L) were influenced (ANOSIM R-score 0.44-0.55, "different with some overlap") by Cd. However, no significant difference was observed in *4cl1-1* mutants (ANOSIM R-score 0.19, "similar with some differences") and the endophytic communities of *ccr1-6* and *ccr1-3* mutants with a delayed development (O) (ANOSIM R-score <0.1, "similar") did not change (Table 4.2.9).

In general, the observed differences after Cd exposure of the plants could be attributed to the lower relative abundances of *Pseudomonas* (bright green; Fig 4.2.8) and higher relative abundances of *Alcaligenes* (dark purple; Fig 4.2.8) in the stem communities after Cd exposure of the plants. Moreover, the lack of changes in the total endophytic communities of *ccr1-6* O and *ccr1-3* O mutants due to Cd exposure of the plants might be explained by the fact that *Alcaligenes* species already dominated the community in the non-exposed plants (Fig 4.2.8). It has been reported that members of the genus *Alcaligenes* can produce volatiles which can protect *A. thaliana* plants from salt stress (Bhattacharyya *et al*. 2015). Furthermore, IAA producing *Alcaligenes* strains, *Alcaligenes* strains with high radical scavenging activity (Chaudhuri *et al*. 2015) and strains of *Alcaligenes* resistant to cobalt, zinc and cadmium were described (Mergeay *et al*. 1985, Collard *et al*. 1994). Therefore, possibly some members of *Alcaligenes* can have comparable or other beneficial traits that can protect the plant in case of Cd exposure. In WT plants also increases in unclassified *Phyllobacteriaceae* and *Rhizobium* (both in shades of red; Fig 4.2.8) were observed after Cd exposure of the plants.

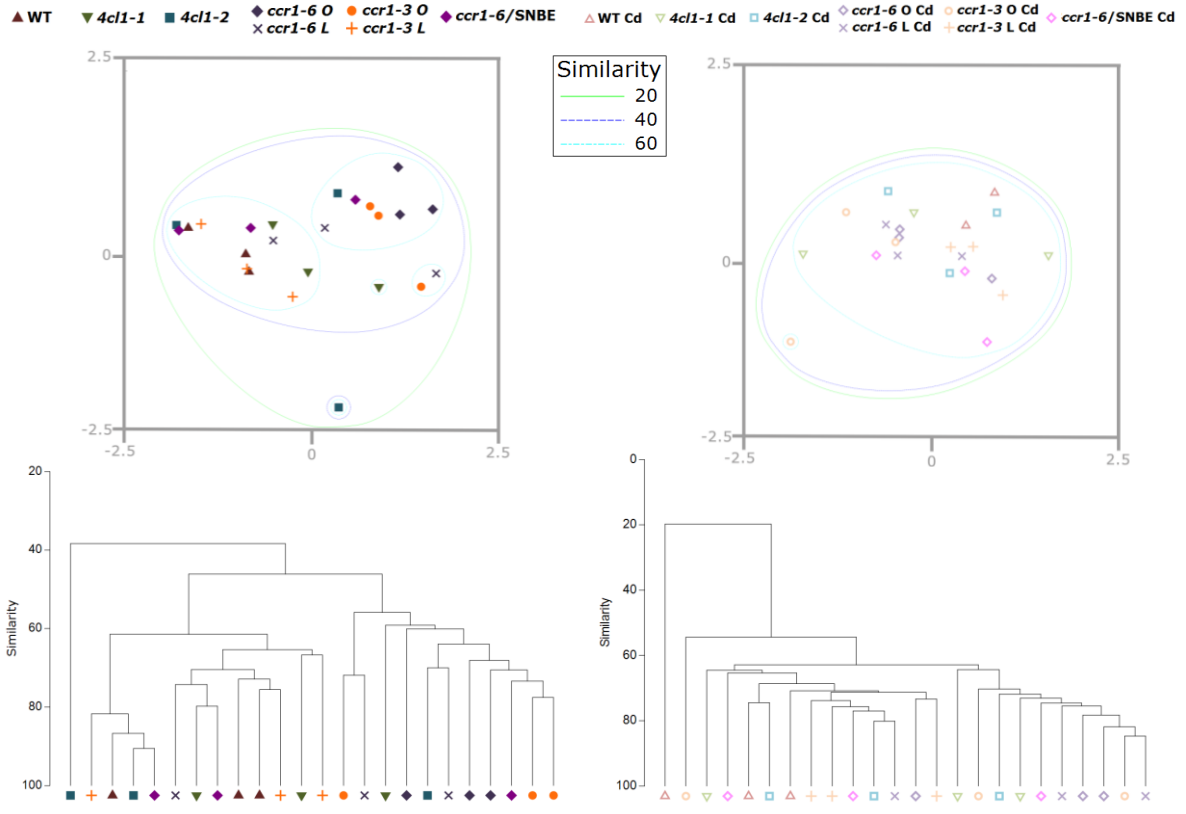


Figure 4.2.7: Similarity between total endophytic communities of **stems** presented in a non-metric MDS plot (Kruskall stress formula 1, minimum stress 0.1, stress value 0.10 for non-treated and 0.13 for stems of Cd-exposed plants) and hierarchical clustering of the samples based on Bray-Curtis similarity on square root transformed data. Abbreviations: O: *ccr1* mutants with a similar age in comparison to WT plants, L: *ccr1* mutants with similar stem length in comparison to WT plants, *ccr1*-6/SNBE: complemented *ccr1-6* mutant with restored lignin levels in the vessels.

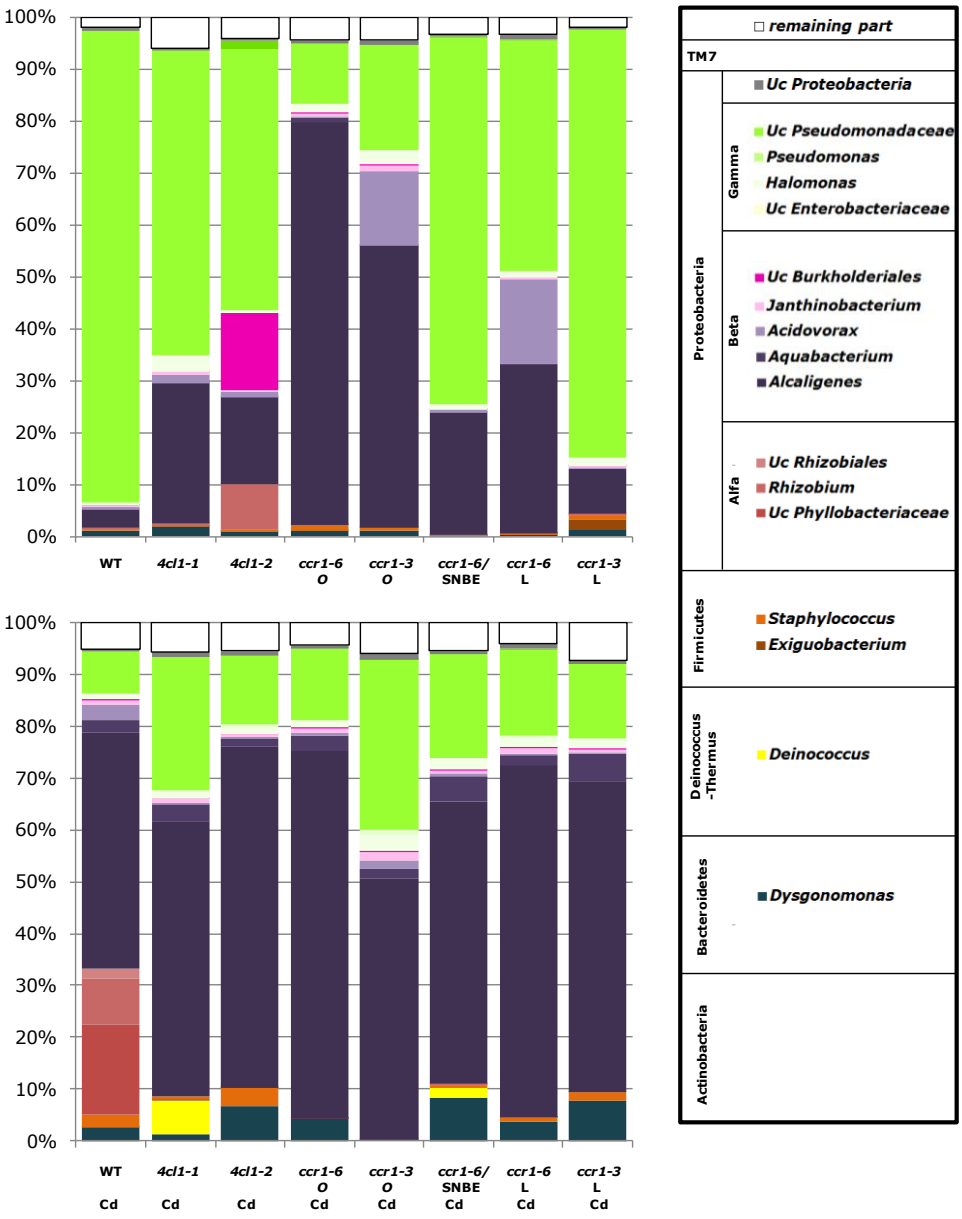


Figure 4.2.8: Total **stem** bacterial community composition (presented as relative abundances) (n=3) from WT, *4cl1-1, 4cl1-2, ccr1-6, ccr1-3* and *ccr1-6*/SNBE plants with and without exposure to Cd. Abbreviations O: *ccr1* mutants with same age in comparison to WT plants and L: *ccr1* mutants with same stem length in comparison to WT plants. Uc: Unclassified. A detailed legend is provided in supplemental fig 4.2.1.

(E) Culture-independent SEED endophytes

Seed endophytes can colonise the embryo and endosperm inside the seed coat and show features such as withstanding high osmotic pressure and possessing motility (Truyens *et al*. 2015, Pitzschke 2016). Seed-borne bacteria may influence seed germination and dormancy and are in this way important factors for seed quality (Williams *et al*. 2006, Johnstonz-Monje and Raizada 2011, Truyens *et al*. 2015, Glick 2014). Genotype effects on the seed cultivable endophytic communities were reported in cotton grown in growth chambers and field conditions by Adams and Kloepper (2002). Moreover, seed endophytes can have beneficial effects in case plants are exposed to Cd (Mastretta *et al*. 2009). However, seed endophytic communities are still largely unexplored (Truyens *et al*. 2015, Pitzschke 2016). For this reasons, also seed endophytic communities were addressed.

Table 4.2.10: ANOSIM calculated with the Spearman Rank correlation and SIMPER dissimilarity scores calculated based on Bray Curtis similarity at the bacterial genus level of all comparisons related to genotype effect, cadmium effect or combined in the **seed** communities (n=3). Significance: p-value = 0.1.

Genotypic effect	**Comparison between WT and:**			
	4cl1-1	***4cl1-2***	***ccr1-6***	***ccr1-3***
ANOSIM (R value)	0.037	0.222	0.704	1.000
SIMPER dissimilarity (%)	62.64	60.29	85.03	86.79
Genotypic effect under Cd stress	**comparison between WT Cd and:**			
	***4cl1-1* Cd**	***4cl1-2* Cd**	***ccr1-6* Cd**	***ccr1-3* Cd**
ANOSIM (R value)	0.148	0.296	0.074	0.037
SIMPER dissimilarity (%)	57.91	72.01	63.23	61.24

Cd effect	**Comparison between:**				
	WT and WT Cd	***4cl1-1* and *4cl1-1* Cd**	***4cl1-2* and *4cl1-2* Cd**	***ccr1-6* and *ccr1-6* Cd**	***ccr1-3* and *ccr1-3* Cd**
ANOSIM (R value)	1.000	1.000	0.370	-0.148	0.000
SIMPER dissimilarity (%)	85.91	68.59	69.76	63.71	70.28

Phylum level

At the phylum level, *ccr1-6* seed communities showed some differences (ANOSIM R-value 0.44 "different with some overlap", supplemental table 4.2.2) with WT plants due to the high relative abundance of Actinobacteria.

The effect of the lignin-reduced genotype

In the visual representation, both allelic forms of the communities of non-exposed *4cl1* mutants tended to group together with WT plants while the communities of non-exposed *ccr1* mutants grouped in a separate cluster (Fig 4.2.9). Only one sample of *4cl1-1* deviated from the pattern. The separation of both *ccr1* seed endophyte communities was supported by the ANOSIM R-values of 0.7 ("different") for *ccr1-6* mutants and 1 ("highly different") for *ccr1-3* mutants in comparison to WT seed communities. Furthermore, SIMPER dissimilarity scores of more than 85% were obtained for the endophyte communities of both *ccr1* mutants (Table 4.2.10). Both *4cl1* mutant seed communities classified as "similar" or "similar with some differences" in comparison to WT plants and SIMPER dissimilarity was in the order of 60% (Table 4.2.10).

These differences can be further explained by the composition of the communities, as presented in Fig 4.2.10. WT, *4cl1-1* and *4cl1-2* seed communities were dominated by the genus *Rhizobium,* OTUs classified as unclassified *Rhizobiales* and unclassified Alphaproteobacteria (red bars; Fig 4.2.10) while seeds of both *ccr1* mutants contained many OTUs assigned to *Pseudomonas* (bright green bar; Fig 4.2.10). As observed in seeds of *ccr1* mutants, *Pseudomonas* representatives were also observed in minor relative abundances in *4cl1* mutants. In seeds of WT plants the phylum Deinococcus-thermus (*Deinococcus* and *Meiothermus* (yellow bars; Fig 4.2.10)) was highly represented. Seeds of both *ccr1* mutants contained genera of the phylum Actinobacteria (dark green bars; Fig 4.2.10) (*Leifsonia*, *Microbacterium* and *Pseudoclavibacter* in the *ccr1-6* mutant community and *Microbacterium* in the *ccr1-3* mutant community). In the *ccr1-3* mutant seeds a large average relative abundance of *Chryseobacterium* (blue bar; Fig 4.2.10) was observed in the community, which was not present in all other *A. thaliana* genotypes.

In former studies concerning endophytes of WT *A. thaliana* seeds, *Rhizobium* and *Pseudomonas* were reported to be transferred from one generation through to the next (Truyens *et al*. 2016a). However, Truyens et al. (2016a) stated that it appears that vertical transmission of seed endophytes is not based on the actual genera but on their phenotypic traits. In future experiments, a more in

depth function-based metagenomics could reveal these differences in characteristics of the microbiome.

Several hypotheses can be made to explain the differences in seed endophytic community:

(i) Seed endophytes can be vertically transmitted from one generation to the next (Truyens *et al*. 2015, Berg *et al*. 2014). Since the origins of the different lignin-reduced *A. thaliana* seed stocks are different (Sessions *et al*. 2002, Alonso *et al*. 2003, Tissier *et al*. 1999), vertical transmission may influence the endophytic community of the investigated genotypes.

Seeds are particularly produced near the maternal surrounding plant organs and the seed coat is only maternally derived (Khan *et al*. 2014). In this way, it can be considered whether the vertical transmission is in particular maternally determined. However, endophytes can also be acquired by male gametes. For example, *Enterobacter chloacae* was found to be endophytic in mature pollen of Mediterranean pine (Madmony *et al*. 2005). However, in our study, the same *A. thaliana* plants fulfilled both the maternal and paternal role as our plants were self-pollinated (Abbott and Gomes 1989).

The vertical transmission, whether or not determined by the maternal plant, should be further explored in future experiments by making crosses between WT plants and plants with a reduced lignin content and subsequently studying the resulting seed endophytic communities.

(ii) Another route to gain endophytes can be the colonisation of the plant by bacteria via all kinds of plant surfaces (Truyens *et al*. 2015, Berg *et al*. 2014). Plants can select the seed endophytes which will be transferred to the next generation out of all endophytes recruited from the environment (Truyens *et al*. 2015). In our study, plants were grown in the same environment and were even placed in the same nutrient solution. Consequently, differences in endophytic community composition cannot be explained in this way. However, cell walls are an important aspect during colonisation since strengthening of the cell wall can be part of a plant-endophyte reaction (Compant *et al*. 2010). Moreover, cell wall features play a role in the selection of colonising endophytes (Bulgarelli *et al*. 2012). Cell wall characteristics of the lignin-reduced mutant *A. thaliana* plants are different from WT plants (Van Acker *et al*. 2013) and may affect the

selection of which environmental bacteria can become endophytic. However, only limited differences were observed in roots.

(iii) Furthermore, xylem vessels can be used by endophytes to migrate inside the plant (Truyens *et al*. 2015, Johnston-Monje and Raizada *et al*. 2011, Compant *et al*. 2008, Weyens *et al*. 2009b). Xylem vessels of plants with a reduced lignin content do not display the same features (*e.g.* collapse of the xylem vessels in *ccr1* mutants) (Mir Derikvand *et al*. 2008) as compared to WT plants. The blockage of vessels may also affect the translocation of endophytes to other plant parts through the plants xylem up to the seeds.

(iv) The monolignol production pathway is disturbed in *ccr1* mutants, leading to accumulation of phenolic intermediates of the monolignol biosynthesis pathway and derivatives thereof inside the xylem (Vanholme *et al*. 2012a) leading to changes in C-sources that can be used by endophytes (Beckers *et al*. 2016b). On the other hand, phenolics may influence the microbiome because of their potential antimicrobial action (Rice-Evans *et al*. 1996, Tilston *et al*. 2014). Furthermore, monolignols and flavonoids share the upper part of their production pathway altering the flavonoid pool in these *ccr1* mutants (van der Rest *et al.* 2006). Flavonoids may play a role in the plant's interaction with symbiotic bacteria like for example *Rhizobium* (Weston and Mathesius *et al*. 2012), an endophyte which is observed in high relative abundances in the non-Cd-exposed seeds of WT plants but not in *ccr1* mutants (Fig 4.2.10).

(iv) Less seeds are produced per *ccr1* mutant and silique (Mir Derikvand *et al*. 2008, Gul and Whalen *et al*. 2013) and seeds have a different appearance since seeds are heavier (see subsection 3.2). The difference in appearance could be due to a thicker seed coat, more energy storage in the endosperm and/or a higher water content. Although the characteristics responsible for these differences are not yet explored, these seed traits can possibly influence the endophytic community.

(v) Moreover, *ccr1* mutants develop slower (Vanholme *et al*. 2012a, Van Acker *et al*. 2013, Gul and Whalen *et al*. 2013, Mir Derikvand *et al*. 2008). A longer time period to form *ccr1* seeds is required (Mir Derikvand *et al*. 2008) which may also influence the colonisation of *ccr1* seeds. Development of roots can affect the endophytic community (Lundberg *et al.* 2012, Yuan *et al.* 2015).

Therefore differences in development may also be an influencing factor in seeds though the mechanism is unknown.

The effect of the lignin-reduced genotype under Cd exposure

The clustering due to the genotype disappeared after exposure to Cd in the plants (Fig 4.2.9) and ANOSIM values suggested that these communities were "similar" for *ccr1* seed endophytic communities and a trend to "similar with some differences" was observed for seeds of both *4cl1* mutants.

The effects of cadmium

The seed endophytic communities of WT plants of *A. thaliana* and the perennial grass *Agrostis capillaris* are known to be influenced by growing the plants on Cd-contaminated substrates (Truyens *et al*. 2014, Truyens *et al*. 2016a). However, the effect of Cd still needs to be elucidated for the plants with a reduced lignin content.

Only the seed communities of WT plants and *4cl1-1* mutants tended to be influenced by Cd-exposure of the parental plant with an ANOSIM R-value of 1 which means they are "highly different" to the communities of the non-exposed same genotype. The *4cl1-2* seed community tended to be "different with some overlap" (R-value 0.37). The SIMPER dissimilarity of WT plants due to Cd was the highest with 85% followed by the *4cl1-1, 4cl1-2* and *ccr1-3* mutants with approximately 70% and the *ccr1-6* mutant bacterial community was least influenced with a dissimilarity score of 63% (Table 4.2.10).

Total community compositions of seeds produced by Cd-exposed parental plants are presented in fig 4.2.10. The effects of Cd on both WT seeds and *4cl1-1* seeds (and *4cl1-2* seeds to a lesser extend) can be explained by the increase in *Pseudomonas* (bright green bars; Fig 4.2.10) and *Microbacterium* (dark green bar; Fig 4.2.10) and reduction in the relative abundance of *Rhizobium* (red bar; Fig 4.2.10) OTUs due to Cd exposure of the parental plants. Some *Rhizobium* species can increase metal resistance (Gopalakrishnan *et al*. 2015) although these species were out-competed by other genera with possibly more specialised features. For example, some species of the genus *Pseudomonas* are known to enhance biomass production of tobacco under Cd exposure (Mastretta *et al*. 2009); some members of the genus *Microbacterium* are known to express the

nickel-cobalt-cadmium or cobalt-zinc-cadmium resistance genes (Fierros-Romero *et al*. 2016) and others are even capable of enhancing plant biomass under non-exposed and metal-exposed conditions (Pereira *et al*. 2015).
In seeds of WT plants also the increase of *Chryseobacterium* (blue bar; Fig 4.2.10) and decrease of *Deinococcus* (yellow bar; Fig 4.2.10) may be influencing factors. Previously, a growth promoting *Chryseobacterium*, isolated from a metal-contaminated site, affected metal uptake and demonstrated plant growth promoting traits (Moreira *et al*. 2014).
Also the presence of other genera such as *Rothia*, *Nocardioides* (both in light green shades; Fig 4.2.10), *Granulicatella*, *Streptococcus* (both in light orange shades; Fig 4.2.10) and *Neisseria* (purper; Fig 4.2.10), which were only found in seeds of WT plants exposed to Cd, contributed to the dissimilarity between the seed endophytic communities of non-exposed and Cd-exposed plants. *Nevskia* (green; Fig 4.2.10) was only found in seeds of both Cd-exposed *4cl1* mutants and may help explain the R-value in the comparison between the endophytic communities of seeds of non-exposed and Cd-exposed *4cl1-1* mutants.
Another worth mentioning observation was the presence of *Alcaligenes* (dark purple bar; Fig 4.2.10) in all investigated Cd-exposed genotypes, as already observed in the stems of Cd-exposed plants (Fig 4.2.8).

The total endophytic seed communities of non-exposed *ccr1-6* and *ccr1-3* mutants already contained several OTUs of *Pseudomonas* (bright green; Fig 4.2.10), *Microbacterium* (dark green bar; Fig 4.2.10) (especially in *ccr1-6* mutants) and *Chryseobacterium* (blue bar; Fig 4.2.10) (especially in *ccr1-3* mutants), and were therefore less affected by Cd exposure of the plants. Although, in contrast to the non-exposed plants, the genus *Deinococcus* (yellow bar; Fig 4.2.10) was only represented in the Cd-exposed *ccr1* mutants. As observed in the seed endophyte communities of non-exposed *A. thaliana*, large relative abundances of *Rhizobium* and unclassified *Rhizobiales* (both in shades of red; Fig 4.2.10) were observed. Though in Cd-exposed condition they were most observed in the *4cl1-1*, *4cl1-2* and *ccr1-3* mutants.

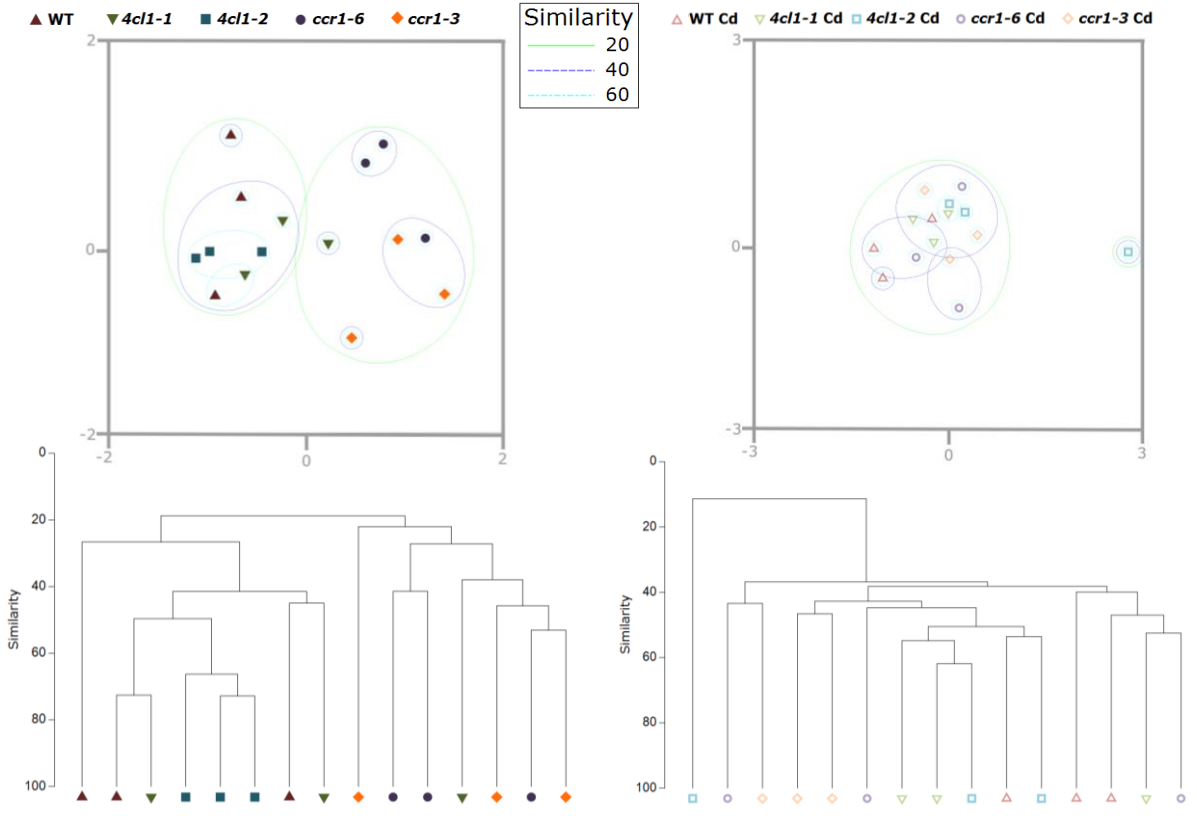


Figure 4.2.9: Similarity between total endophytic communities of **seeds** presented in a non-metric MDS plot (Kruskall stress formula 1, minimum stress 0.1, stress value 0.11 for non-treated and 0.13 for seeds of Cd-exposed plants) and hierarchical clustering of the samples based on Bray-Curtis similarity on square root transformed data.

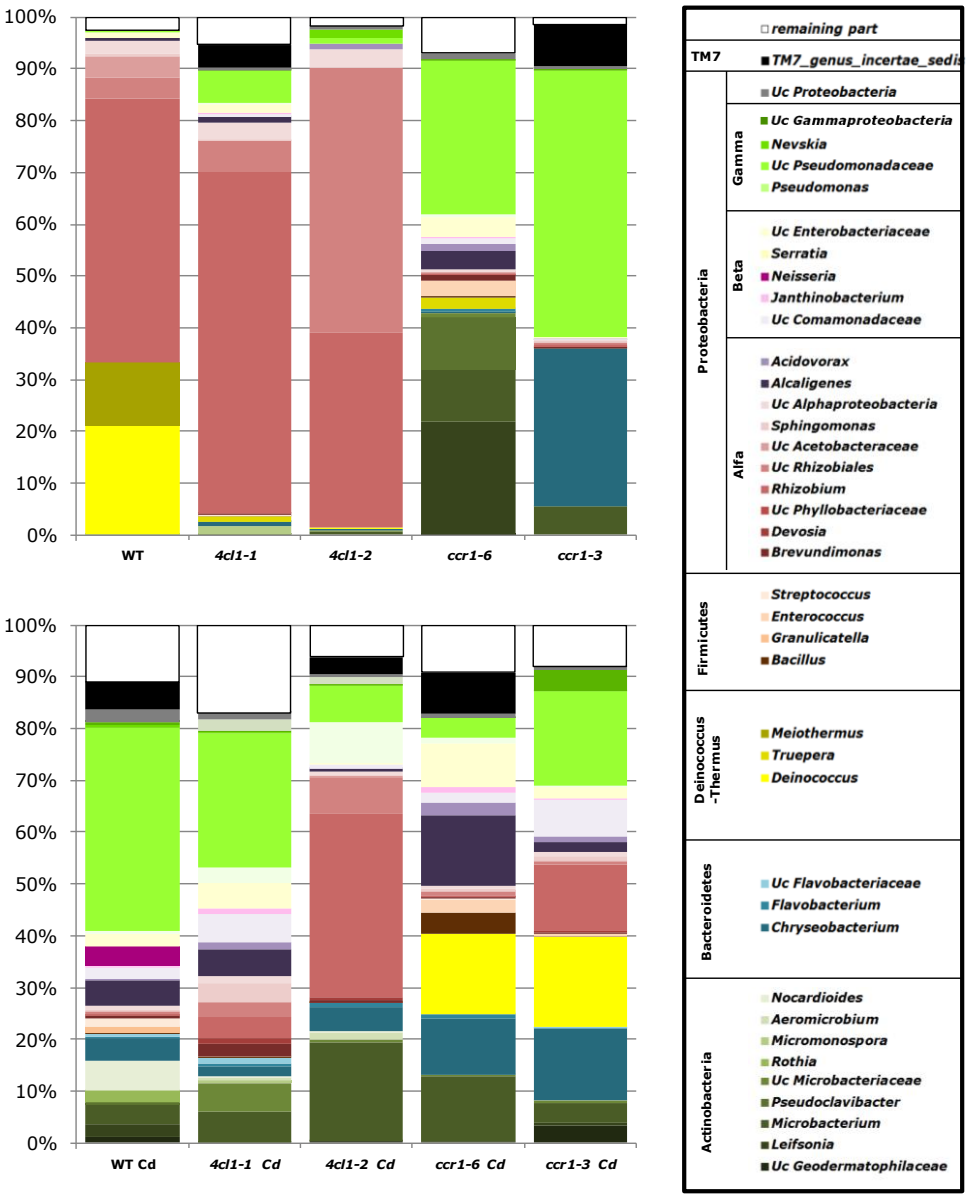


Figure 4.2.10: Total **seed** bacterial community composition (presented as relative abundances) from WT, *4cl1-1, 4cl1-2, ccr1-6*, *ccr1-3* plants with and without exposure to Cd. Abbreviation: Uc: Unclassified. A detailed legend is provided in supplemental fig 4.2.1.

We can conclude that the total seed endophytic community of WT plants was the most influenced by Cd exposure of the parental plants, followed by the *4cl1* mutants. In contrast, *ccr1* mutants did not experience an effect of Cd on the composition of the total seed endophytic community.

Several of the hypotheses that we mentioned above to explain the differences among the seed endophytic communities of WT plants and *ccr1* mutants may also explain the lower impact of Cd on the endophytic communities of *ccr1* seeds. For example, the differences in seed production (heavier seeds, longer time to produce seeds) or differences in colonisation route due to differences in cell wall composition and xylem characteristics of the *ccr1* mutan*t* in comparison to WT plants may explain these results. However, differences in the overall sensitivity of the plants to toxic amounts of Cd (as discussed in section 3) may also explain the differences in response of the endophytic community of WT seeds in comparison to the communities in *ccr1* seeds after Cd exposure of the parental plants.

4.2.3 Conclusions

Although most of the total endophytic communities were dominated by Proteobacteria (especially *Pseudomonas* in root, leaf, stem and *Rhizobium* in seeds), they differed between the different **plant compartments**. Furthermore, also **diversity and OTU richness** were different in function of the **plant compartment**. However, the diversity of the total endophytic communities was not influenced by the lignin-reduced **genotype** or **Cd-exposure.**

Effect of the lignin-reduced genotype

No plant genotype effects were observed on the total endophytic community of the roots of *A. thaliana*. Also no clear effect of the delayed development of *ccr1* mutants on the endophytic community was observed for both **roots and leaves**. Only limited differences in endophytic communities due to the genetic modification were observed in the leaves of *ccr1-3* O and *ccr1-6*/SNBE mutants in comparison to WT. However no clear conclusions can be drawn since these differences were not observed in the other allelic mutant community.

The total endophytic communities of the **stems** of *4cl1-1*, *4cl1-2*, *ccr1-6*/SNBE and *ccr1-3* L mutants did not differ from these of WT plants. On the other hand, the endophytic communities of stems of *ccr1-6* O and *ccr1-3* O mutants, which were of a similar age in comparison to those of WT plants, clustered apart from the WT community. Several differences due to the **genotype** (*e.g.* differences in cell wall composition, xylem vasculature or phenolic C-sources) may be the reason for the observations.
However, the total endophytic communities of the stems of *ccr1-6* and *ccr1-3* mutants differed between plants sown at different moments. Therefore, it is likely that the **delayed development** of *ccr1* mutants also had a significant influence on the total stem endophytic communities. The high relative abundance of *Alcaligenes* species in the stems of *ccr1* mutants with a similar age in comparison to the WT plants (O) is the most prominent difference. Moreover, only smaller differences in endophytic community in comparison with WT plants were observed for *ccr1-6* L stems and no effect was observed in *ccr1-3* L mutants, in case plants with a similar stem length in comparison to WT (L) were studied. In future experiments, a WT condition that is sown at a later moment, hence delayed in its development, can be added to further unravel the development related effect.

In comparison to WT plants, the endophytic communities of **seeds** of *4cl1* mutants were hardly affected by the genetic modification. These communities showed a high relative abundance of *Rhizobium,* which was not the case in the seed endophytic communities of *ccr1* mutants. As observed in stems of plants with a similar age in comparison to WT plants, in both *ccr1* allelic variants, the genetic modification had an impact on the total endophytic community of seeds. However, also vertical transmission of seed endophytes may influence the communities. We assume the vertical transmission to be of a minor importance since *4cl1* mutant seeds did not show differences in their total endophytic community in comparison with WT seeds. Moreover, it is difficult to conclude whether these effects are an actual effect of the genetic modification leading to the lignin reduction and accumulation of phenolic substances and flavonoids or whether it is due to the differences in development of the *ccr1* stems which may

lead to differences in colonisation of the seeds and differences in appearance of these seeds (see paragraph 3.2.2.2).

Effect of Cd

4cl1-1 **root** communities changed due to exposure of the plants to Cd. However, this was not the case for *4cl1-2* mutants. Hence no conclusions can be drawn concerning the effect of Cd on the root endophytic communities of *4cl1* mutants. In **leaves**, no effects of Cd exposure of the plant on the total bacterial endophytic community of *4cl1* mutants were observed.

The total **root** endophytic communities of Cd-exposed *ccr1* mutants (both for conditions with the same age and development) showed limited differences in comparison to the communities of Cd-exposed WT plants. However, the above is an effect of the genotype in combination with Cd and no noteworthy effect was observed for these *ccr1* mutants in case Cd-exposed plants were compared to non-exposed plants. However, in **leaves** the communities of *ccr1* mutants with a delayed development (O) and the *ccr1-6* L condition were affected by Cd exposure of the plants. A difference in Cd concentration of the leaves of *ccr1* mutants in comparison to WT leaves (subsection 3.2) may be the cause of these differences. However, no changes were observed in the *ccr1-3* L mutant leaves of Cd-exposed plants and no clear conclusion can be drawn.

The culture-independent communities of **stems** of plants of all genotypes (WT, *4cl1-2*, *ccr1-6*/SNBE, *ccr1-6*, *ccr1-3* and a tendency in *4cl1-1* mutants) in a similar development stage were affected by Cd. An increase in members of the genus *Alcaligenes* was the main change in community composition after Cd exposure of the plants. However, the communities of *ccr1-6* O and *ccr1-3* O mutants with a delayed development (same age in comparison to WT plants) were not changed after Cd exposure of the plants.

A higher dominance of *Alcaligenes* was observed both due to the *ccr1* genotype and Cd-exposure. In this way, it can be hypothesized that the effects of both factors - genotype and exposure to Cd - on the development of the stem may be the reason for the high relative abundance of *Alcaligenes*.

The seed endophytic communities of WT and *4cl1* mutants were affected by Cd-exposure of the parental plants whereas no effects were observed for seeds of *ccr1* mutants. One hypothesis to explain the observation can be that *ccr1* mutants appeared to be less sensitive to Cd-exposure (see section 3) which may also influence the seed endophytic community. It is hard to identify the factors that really influence the endophytic seed community because the differences in development of the stem during seed production cannot be eliminated. However, several hypotheses for the differences in colonisation of these seeds by endophytes should be further explored including the characteristics of the seed coat (*e.g.* thickness) and endosperm (*e.g.* presence of phenolic compounds).

In conclusion, many differences due to the genotype and/or Cd-exposure were observed. In future experiments, more information should be uncovered by expanding the sequencing depth to the species level or making use of a function-based metagenomic analysis. Moreover, the cultivable fraction of the endophytic communities must be evaluated and several phenotypic traits of the community members can be explored (*e.g.* growth promotion capacity, Cd tolerance and use of phenolic acids as a sole C-source).

Subsection 4.2

Supplementary information

Supplemental Table 4.2.1: Total endophytes of *A. thaliana* WT and lignin mutants in seeds, roots, leaves and stems with a relative abundance more than 1% indicated with x, in case they were found less abundant than 1% they are indicated with (x). Genera which were not found above the 1% cut-off in one of the plant compartments are not shown.

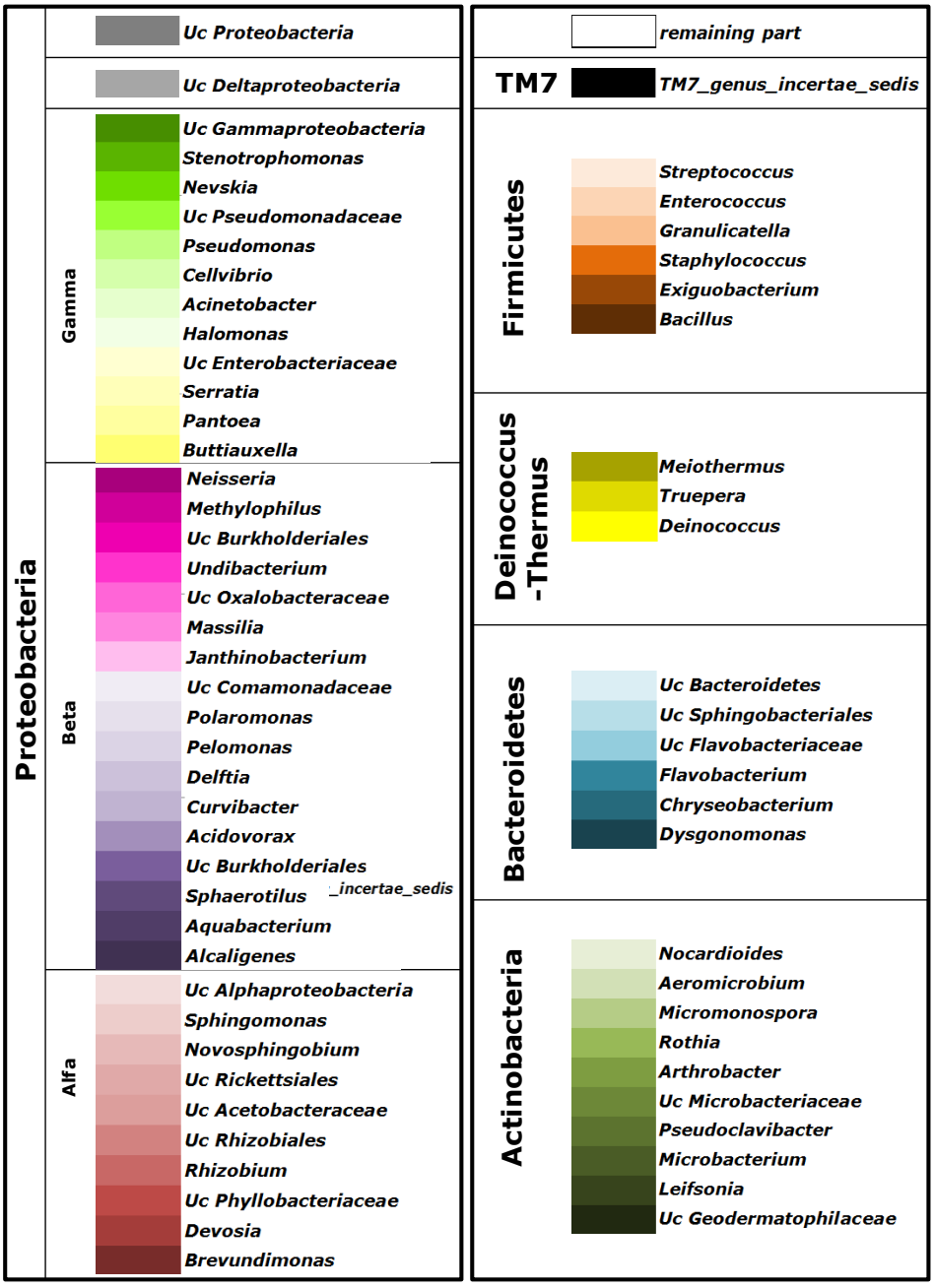
Phylum	Class	Order	Family	Genus	seed	root	leaf	stem
Acidobacteria	Actinobacteria	Actinomycetales	Geodermatophilaceae	*Uc Geodermatophilaceae*	x	(x)		(x)
			Microbacteriaceae	*Leifsonia*	x	(x)	(x)	(x)
				Microbacterium	x	(x)	x	(x)
				Pseudoclavibacter	x	(x)		
				Uc Microbacteriaceae	x	(x)	(x)	(x)
			Micrococcaceae	*Arthrobacter*	(x)	(x)	x	(x)
				Rothia	x	(x)		(x)
			Micromonosporaceae	*Micromonospora*	x			
			Nocardioidaceae	*Aeromicrobium*	x	(x)	(x)	(x)
				Nocardioides	x	(x)	(x)	(x)
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Dysgonomonas*	(x)			x
	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Chryseobacterium*	x	(x)	x	(x)
				Flavobacterium	x	(x)	x	(x)
				Uc Flavobacteriaceae	x	(x)	x	(x)
	Sphingobacteria	Sphingobacteriales	Uc Sphingobacteriales	*Uc Sphingobacteriales*	(x)	x	(x)	(x)
	Uc Bacteroidetes	Uc Bacteroidetes	Uc Bacteroidetes	*Uc Bacteroidetes*	(x)	x	(x)	(x)
Deinococcus-Thermus	Deinococci	Deinococcales	Deinococcaceae	*Deinococcus*	x	x	(x)	x
			Trueperaceae	*Truepera*	x	x	(x)	(x)
		Thermales	Thermaceae	*Meiothermus*	x	(x)		
Firmicutes	Bacilli	Bacillales	Bacillaceae 1	*Bacillus*	x	(x)	(x)	(x)
			Bacillales Incertae Sedis XII	*Exiguobacterium*	(x)	(x)	(x)	x
			Staphylococcaceae	*Staphylococcus*	(x)	(x)	(x)	x
		Lactobacillales	Carnobacteriaceae	*Granulicatella*	x	(x)		(x)
			Enterococcaceae	*Enterococcus*	x	(x)		(x)
			Streptococcaceae	*Streptococcus*	x		(x)	
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Brevundimonas*	x	(x)	(x)	(x)
		Rhizobiales	Hyphomicrobiaceae	*Devosia*	x	(x)	x	(x)
			Phyllobacteriaceae	*Uc Phyllobacteriaceae*	(x)	x	(x)	x
			Rhizobiaceae	*Rhizobium*	x	(x)	x	x
			Uc Rhizobiales	*Uc Rhizobiales*	x	x	(x)	x
			Acetobacteraceae	*Uc Acetobacteraceae*	x			

		Rickettsiales	Uc Rickettsiales	*Uc Rickettsiales*		x		
		Sphingomonadales	Sphingomonadaceae	*Novosphingobium*	(x)	(x)	x	(x)
				Sphingomonas	x	(x)	(x)	(x)
		Uc Alphaproteobacteria	Uc Alphaproteobacteria	*Uc Alphaproteobacteria*	x	x	(x)	(x)
	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Alcaligenes*	x	x	x	x
			Burkholderiales incertae sedis	*Aquabacterium*	(x)	x	(x)	x
				Sphaerotilus	(x)	(x)	x	(x)
				Uc Burkholderiales incertae sedis	(x)	x	(x)	(x)
			Comamonadaceae	*Acidovorax*	x	x	x	x
				Curvibacter	(x)	x	(x)	(x)
				Delftia	(x)	(x)	x	(x)
				Pelomonas	(x)	x	(x)	(x)
				Polaromonas	(x)	x	(x)	(x)
				Uc Comamonadaceae	x	x	x	(x)
			Oxalobacteraceae	*Janthinobacterium*	x	x	(x)	x
				Massilia	(x)	(x)	x	(x)
				Uc Oxalobacteraceae	(x)	x	(x)	(x)
				Undibacterium	(x)	x	(x)	(x)
			Uc Burkholderiales	*Uc Burkholderiales*	(x)	x	(x)	x
		Methylophilales	Methylophilaceae	*Methylophilus*	(x)	x	x	(x)
		Neisseriales	Neisseriaceae	*Neisseria*	x	(x)		
	Deltaproteobacteria	Uc Deltaproteobacteria	Uc Deltaproteobacteria	*Uc Deltaproteobacteria*	(x)	x	(x)	(x)
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Buttiauxella*		(x)	x	(x)
				Pantoea		(x)	x	(x)
				Serratia	x	x	(x)	(x)
				Uc Enterobacteriaceae	x	x	x	x
		Oceanospirillales	Halomonadaceae	*Halomonas*	(x)	(x)	(x)	x
		Pseudomonadales	Moraxellaceae	*Acinetobacter*	(x)	x	(x)	(x)
			Pseudomonadaceae	*Cellvibrio*	(x)	(x)	x	(x)
				Pseudomonas	x	x	x	x
				Uc Pseudomonadaceae	x	(x)	(x)	x
		Xanthomonadales	Sinobacteraceae	*Nevskia*	x	(x)	(x)	
			Xanthomonadaceae	*Stenotrophomonas*	(x)	(x)	x	(x)
		Uc Gammaproteobacteria	Uc Gammaproteobacteria	*Uc Gammaproteobacteria*	x	x	x	(x)
	Uc Proteobacteria	Uc Proteobacteria	Uc Proteobacteria	*Uc Proteobacteria*	x	x	x	x
TM7	TM7 class incertae sedis	TM7 order incertae sedis	TM7 family incertae sedis	*TM7 genus incertae sedis*	x	(x)		(x)

Subsection 4.2

Supplemental table 4.2.2: ANOSIM calculated with the Spearman Rank correlation calculated based on Bray Curtis similarity of all comparisons at the phylum level related to genotype effect, cadmium effect or combined in the root, leaf, stem and seed communities (n=3). Significance: p-value = 0.1.

Genotype effect	**Comparison between non-exposed WT and lignin mutant**						
	4cl1-1	***4cl1-2***	***ccr1-6* O**	***ccr1-3* O**	***ccr1-6* /SNBE**	***ccr1-6* L**	***ccr1-3* L**
Root	-0.037	-0.222	-0.370	-0.0.37	-0.185	-0.259	-0.037
Leaf	1.000	-0.074	0.481	0.074	0.074	-0.111	-0.037
Stem	-0.182	-0.259	-0.370	-0.111	-0.037	-0.111	-0.185
Seed	0.037	0.000	0.444	0.074	/	/	/
Genotype effect under Cd stress	**Comparison between Cd-exposed WT and lignin mutant:**						
	4cl1-1	***4cl1-2***	***ccr1-6* O**	***ccr1-3* O**	***ccr1-6* /SNBE**	***ccr1-6* L**	***ccr1-3* L**
Root	0.000	-0.074	-0.222	0.148	-0.111	-0.333	0.000
Leaf	0.185	0.222	0.148	0.037	0.222	-0.074	-0.111
Stem	-0.111	-0.148	-0.259	0.148	-0.037	-0.148	0.185
Seed	-0.037	-0.370	-0.148	0.185	/	/	/
Cd effect	**Comparison between non-exposed and Cd-exposed genotype:**							
	WT	***4cl1-1***	***4cl1-2***	***ccr1-6* O**	***ccr1-3* O**	***ccr1-6* /SNBE**	***ccr1-6* L**	***ccr1-3* L**
Root	-0.019	0.000	-0.037	0.037	0.074	-0.296	-0.296	0.037
Leaf	0.148	1.000	-0.074	0.556	-0.222	0.111	0.185	-0.037
Stem	0.074	-0.111	0.185	-0.074	-0.259	0.185	-0.148	0.407
Seed	0.407	0.222	0.000	-0.074	0.111	/	/	/



Supplemental figure 4.2.1: Colour scheme used to describe the total community composition in the bar plots presented in figure 4.2.4, 4.2.6, 4.2.8 and 4.2.10.

Subsection 4.3
Composition of the cultivable bacterial endophytic communities of lignin-reduced *A. thaliana* plants with and without Cd exposure

Beneficial bacterial endophytes can promote plant growth either in a direct (*e.g.* fixation and mobilization of unavailable nutrients) or indirect (*e.g.* modulation of the plant immune system) way. Furthermore, endophytic bacteria can even lower metal (*e.g.* cadmium (Cd)) phytotoxicity (*e.g.* by efflux and subsequently precipitation on the bacterial membrane (Nies 1999)) (Weyens *et al*. 2009b). Moreover, some bacterial endophytes may contain pathways to degrade several lignin-derived phenolic compounds such as ferulic acid (Beckers *et al*. 2016b), the phenolic compound that is thought to be the reason of the delayed development of *ccr1* mutants (Xue *et al*. 2015). In this way, enrichment of the innate endophytic community with promising bacteria, selected for specific beneficial traits, has the potential to overcome both the negative influence of Cd exposure (Weyens *et al*. 2009b) and the biomass impairment associated with the lignin-reduced genotypes (Beckers *et al*. *in preparation*). However, because of the need for testing these beneficial plant-growth promoting and phenolic degradation traits and further the bacterial inoculum preparation, good candidates for inoculation can only be selected from the cultivable fraction of the endophytic communities.

Considering these constraints, within subsection 4.3, the cultivable bacterial endophytic communities of seeds, roots, leaves and stems of *Arabidopsis thaliana* lignin-reduced genotypes (T-DNA knockout mutants for *4-COUMARATE:COA LIGASE 1* (*4CL1*) or CINNAMOYL-COA REDUCTASE 1 (*CCR1*)), with or without exposure to Cd, were compared to the bacterial communities of wild type (WT) plants. However, in these comparisons we should keep in mind the bias imposed by the cultivability of these endophytes (Ellis et al 2003).

4.3.1 Experimental design

Endophytes were isolated from seeds (±30 mg originating from at least 8 plants per sample) harvested from plants grown in an Arasystem filled with sand (see paragraph 4.1.3.1). From the start of stem formation, half of the trays were exposed to 3 µM $CdSO_4$ until overblown and seeds were collected.

Subsection 4.3

The seed stocks (WT, *4cl1-1, 4cl1-2, ccr1-6, ccr1-3*) (more information about the lignin-reduced mutants can be found in paragraph 3.1.1.1), from which cultivable endophytes were isolated, were used to grow the plants from which all other plant organs (roots of younger (5 plants per sample) and older (2 plants per sample) plants; leaves of younger (5 plants per sample) and older (2 plants per sample) plants; and stems (2 plants per sample)) were investigated.

Plants were grown hydroponically as described in paragraph 4.1.3.1.

In a first approach, plants with similar rosette size (younger plants in order to harvest roots and rosettes) were grown. Due to the delay in development, which may have effects on the bacterial endophytic communities (Lundberg *et al*. 2012), *ccr1-6* and *ccr1-3* mutants (as discussed in subsection 3.2) were sown respectively 2 and 4 days earlier in order to obtain rosettes with a similar diameter (see paragraph 3.2.2.2C). These plants were grown for 1 week (plus 2 and 4 days extra as indicated for *ccr1* mutants) after which part of the plants were exposed to 3 µM $CdSO_4$ during 2 more weeks. Plants were harvested after 3 weeks.

In a second approach, plants with a similar stem length (older plants in order to harvest roots, rosettes and stems) were cultivated. Again, the difference in development of *ccr1-6* and *ccr1-3* was circumvented by sowing them respectively 3 and 10 days earlier (see paragraph 3.2.2.2C) to obtain uniformly developed plants at the day of harvest. A similar stem length was the selection criterion for these bolting plants. Half of the plants were exposed to 3 µM $CdSO_4$ from the start of stem formation during 2 weeks after which plant organs were harvested.

The isolation procedure and genotypic determination of the obtained bacterial strains are explained in paragraph 4.1.3.1 and 4.1.3.2.

4.3.2 Results and discussion

4.3.2.1 Number of isolated endophytes

In general, the highest numbers of endophytes were isolated from the **seeds** as compared to the other plant compartments (roots, leaves and stems)(Table 4.3.1). This number is in accordance with the study of Truyens *et al*. (2013) and Truyens *et al*. (2016a). The WT plants of Truyens *et al*. (2013) contained 4.3 x 10^7 cfu g^{-1} FW and Truyens *et al*. (2016a) reported that this number of cfu

decreased with increasing the number of generation grown on sand to 5.7 x 10^6 cfu g^{-1} FW and 5.6 x 10^5 cfu g^{-1} FW for WT *A. thaliana* plants. Seed-borne endophytes can originate from (i) vertically transmission from the parent plants and (ii) colonization from the environment, including rhizosphere (root) and phyllosphere (*e.g.* phylloplane (leaf), caulosphere (stems), anthosphere (flower) and carposphere (fruit)), by crossing several barriers and translocation through the xylem up to the seeds (Berg *et al*. 2014, Truyens *et al*. 2015). The latter depends on the bacterial communities present in the environment at the start of the experiment. Therefore a possible explanation for the relatively high number of isolated seed endophytic bacteria (in comparison to the bacterial cell count in the other plant compartments) might be the greenhouse, in which the seeds were produced. The greenhouse environment may possibly also contain higher numbers of bacteria in comparison to the controlled cultivation room, where the plants were cultivated in hydroponics and sampled to represent the root, stem and leaf compartments.

The number of cultivable endophytes tended to be lower in the seeds of the lignin-reduced genotypes as compared to the WT plants (Table 4.3.1). In case bolting plants were exposed to 3 µM Cd, all selected *A. thaliana* genotypes tended to produce seeds with lower numbers of cultivable endophytes in comparison to non-exposed plants (Table 4.3.1). The lower numbers of seed-borne bacteria in all *A. thaliana* genotypes after Cd exposure might be explained by Cd toxicity. However, in previous studies, Cd exposure did not significantly affect the bacterial cell count under metal exposure in *A. thaliana* and *Agrostis capillaris* seeds (Truyens *et al*. 2013, Truyens *et al*. 2014). However, under Cd exposure of the parental plants, the lignin-reduced mutant seeds tended to contain higher numbers of cultivable endophytes in comparison to wild type seeds (Table 4.3.1).

In **roots and leaves** of younger and older non-exposed plants, the cultivable bacterial endophytes across all the genotypes ranged between 4.56 x 10^3 and 1.59 x 10^6 cfu g^{-1} FW.

In the **stems**, we routinely found the lowest number of endophytes across all the genotypes as compared to the roots and leaves (Table 4.3.1). The lower number of bacteria present in stems of plant species in comparison to roots is

generally accepted in literature (Weyens *et al*. 2014, Eevers *et al*. 2016 and Beckers *et al*. 2016b) suggesting that only a limited number of endophytic bacterial strains can be translocated up to the stem compartment by crossing several physiological barriers such as the endodermis and pericycle (Compant *et al*. 2010).

No genotypic effect on the numbers of isolated endophytes was observed in **roots, leaves and stems** (Table 4.3.1). Beckers et al. (2016b) reported qualitatively similar results for lignin-reduced poplars whereby no significant host genotype effects were observed in the bacterial cell counts in the rhizosphere soil, root, stems and leaves of *CCR* down-regulated poplars as compared to the WT poplar trees. In case Cd was added to the Hoagland solution, on average all plant genotypes (including WT) roots of younger plants tended to contain more bacterial endophytes as compared to the non-exposed plants (Table 4.3.1) though in roots of older plants, leaves of younger and older plants and stems no higher number of bacterial endophytes was observed (Table 4.3.1).

Table 4.3.1: Number of isolated cultivable bacterial endophytes from *A. thaliana* WT and lignin mutants in non-exposed situation and in combination with Cd-exposure.

		cfu g^{-1} fresh weight				
Plant compartment	**Cd (µM)**	**WT**	***4cl1-1***	***4cl1-2***	***ccr1-6***	***ccr1-3***
Roots (Younger plants)	**0**	1.39×10^4	1.42×10^4	6.93×10^4	3.61×10^4	4.56×10^3
	3	1.02×10^5	9.46×10^4	9.43×10^4	8.78×10^4	1.69×10^5
Roots (Older plants)	**0**	8.31×10^5	1.59×10^6	1.35×10^4	7.98×10^5	2.83×10^4
	3	9.01×10^5	5.03×10^5	5.50×10^5	2.32×10^5	1.14×10^5
Leaves (Younger plants)	**0**	1.87×10^5	7.91×10^4	4.93×10^5	1.64×10^5	4.43×10^5
	3	1.51×10^5	2.59×10^5	2.06×10^5	2.19×10^5	8.69×10^4
Leaves (Older plants)	**0**	7.05×10^4	4.18×10^4	5.13×10^5	1.12×10^5	2.67×10^5
	3	8.21×10^4	8.05×10^4	8.53×10^4	1.79×10^5	4.79×10^4
Stems	**0**	6.47×10^4	1.90×10^3	6.62×10^2	2.13×10^3	1.26×10^4
	3	5.99×10^2	2.95×10^3	8.10×10^2	1.27×10^3	4.55×10^2
Seeds	**0**	2.74×10^7	1.59×10^7	3.40×10^3	7.49×10^6	2.34×10^6
	3	2.34×10^3	2.18×10^6	3.68×10^4	3.91×10^4	6.73×10^4

4.3.2.2 Diversity

Shannon-Wiener and inverse Simpson diversity are commonly used indices to evaluate diversity in bacterial communities (Hill *et al.* 2003). The Shannon-Wiener diversity is sensitive to infrequent species while the inverse Simpson diversity is more influenced by more abundant species (Kwak and Peterson 2007). Therefore, to exclude bias associated with the selected community diversity estimators, both diversity indices were included in our study (Table 4.3.2).

When only **non-exposed WT plants** were considered, the highest diversity was observed in roots followed by leaves, stems and seeds (Table 4.3.2). A higher below ground diversity in comparison to the aerial compartments was also observed in other plant species (Zarraonaindia *et al*. 2015, Croes *et al*. 2015). The difference in diversity as a function of the plant compartment may go together with the stepwise endophytic colonisation from the rhizosphere in which first roots are colonised and some bacterial endophytes can be transferred to the upper parts through the xylem vessels. During the transfer, several barriers need to be crossed which indirectly select for the required physiological key traits (Compant *et al*. 2010).

Considering the effect of the plant genotype (lignin-reduced mutants), in **roots of younger plants**, the endophytic diversity tended to be lower due to the **T-DNA knockout** of *4CL1* and *CCR1* as compared to the roots of younger WT plants (Table 4.3.2). The mutation-related lower diversity was already observed in roots of *CCR* down-regulated poplars (Beckers *et al*.2016b). Cell walls represent a key aspect in the colonisation process (Bulgarelli *et al*. 2012). In this way, modifications in cell wall features may affect the endophytic community. Moreover, cell walls act as a passive barrier (Compant *et al*. 2010) making lignin-reduced mutant roots more susceptible for entering of bacteria (Miedes *et al*. 2014). However, also other characteristics of these mutants (*e.g.* different concentrations of phenolic compounds inside the xylem in comparison to WT plants which can serve as a C-source or can act in an antimicrobial way) may affect bacterial diversity (Beckers *et al*. 2016b). Although we observed a lower endophytic diversity observed in the roots of the younger lignin-reduced plants

as compared to the WT plants, the same observation was not apparent in the **roots of the older lignin-reduced plants.** Moreover, the decreasing tendency in diversity due to the genotype was not found in Cd-exposed conditions (Table 4.3.2).

Table 4.3.2: Diversity indices of isolated cultivable bacterial endophytes from *A. thaliana* WT and lignin-reduced mutant in combination with Cd-exposure.

Genotype	Inverse Simpson		Shannon-Wiener		Inverse Simpson		Shannon-Wiener	
	0 µM		0 µM	3 µM	0 µM	3 µM	0 µM	3 µM
	Roots of younger plants				Roots of older plants			
WT	4.07	1.73	1.73	0.75	2.06	2.42	0.78	1.13
4cl1-1	1.74	1.19	0.88	0.43	1.80	1.64	0.80	0.82
4cl1-2	3.02	1.10	1.29	0.25	2.91	1.64	1.26	0.77
ccr1-6	1.46	1.43	0.68	0.64	1.84	1.11	0.89	0.24
ccr1-3	1.46	1.79	0.88	0.97	2.69	2.53	1.23	1.23
	Leaves of younger plants				Leaves of older plants			
WT	1.83	3.64	0.71	1.49	1.57	6.59	0.88	1.98
4cl1-1	4.25	3.14	1.67	1.38	11.4	2.58	2.69	1.13
4cl1-2	3.87	2.40	1.44	1.31	5.37	2.88	1.98	1.35
ccr1-6	2.37	2.49	0.97	1.10	5.98	5.68	2.23	1.88
ccr1-3	1.96	4.86	0.93	1.73	1.79	6.03	1.02	1.92
	Stems				Seeds			
WT	1.02	5.59	0.06	1.83	1.00	1.82	0.00	0.80
4cl1-1	6.04	1.86	2.03	1.06	1.00	1.04	0.00	0.10
4cl1-2	4.15	8.82	1.68	1.28	3.27	1.23	1.38	0.46
ccr1-6	8.47	2.01	2.30	0.69	1.01	1.07	0.03	0.17
ccr1-3	3.85	1.00	1.67	0.07	1.25	3.24	0.52	1.27

In the aerial parts (**leaves of younger and older plants and stems)**, the diversity of cultivable endophytic bacteria tended to be higher in the lignin-reduced *4cl1* **mutants** (most distinct in the *4cl1-1* mutant) and *ccr1* **mutants** in comparison with WT plants (Table 4.3.2). No literature could be found for this observation; the underlying mechanisms are unknown. Moreover, Beckers *et al.* (2016b) rather observed a lower diversity in *CCR* down-regulated poplar endophytic communities. However, in that study endophytes were isolated by selective enrichment with ferulic acid and by consequence the differences in study design can explain these dissimilarities.

The cultivable endophytic communities of **seeds** not exposed to Cd showed low diversities. The highest diversity was observed in the *4cl1-2* **mutant** seeds (Table 4.3.2), from which only a low number of bacteria could be isolated (Table 4.3.1), but no general plant genotypic effect was found (Table 4.3.2).

Furthermore, in **roots, stems and seeds** of lignin-reduced mutants, no unambiguous effect of Cd was observed (Table 4.3.2). Only the cultivable bacterial leaf communities (both of younger and older plants) derived from **Cd-exposed** *4cl1* mutants tended to possess a lower diversity in comparison to the non-exposed ones (Table 4.3.2). Suggesting that Cd toxicity may act in a different manner to the endophytic community due to the *4cl1* mutation. In both leaves of younger and older plants of WT and *ccr1-3* the diversities increased after Cd exposure and in *ccr1-6* no notable Cd-effect was observed (Table 4.3.2). Consequently, the observed increases after Cd exposure of the plants cannot be considered as originating from the *CCR1* down-regulated variant.

To confirm the observed trends, all observed differences connected with the lignin genotype (lower diversity in roots and higher diversity in leaves and stems in comparison to WT plants) and with Cd-exposure (*e.g.* differences in *4cl1* leaves) should be further investigated using more replicates.

4.3.2.3 Community composition

The composition of different cultivable endophytic communities of roots of younger (Fig 4.3.1) and older (Fig 4.3.2) plants, leaves of younger (Fig 4.3.3) and older (Fig 4.3.4) plants, stems (Fig 4.3.5) and seeds (Fig 4.3.6) are discussed below. In case isolated genera are addressed, the corresponding colour (supplemental figure 4.3.1) of the figures is given in parentheses. More detailed information about phylum, class, order and family of the observed genera is presented in supplemental table 4.3.1.

(A) Cultivable ROOT endophytes

The most commonly used entry sites by bacteria to become endophytic are the roots from where they can spread into all other plant compartments via the xylem (Weyens *et al*. 2009b). Therefore, we started by exploring the cultivable endophytic community of roots. Cultivable endophytic communities of roots of younger and older plants revealed many correspondences and will be discussed together.

Subsection 4.3

The effect of the lignin-reduced genotype

In both roots of younger and older plants, ***Pelomonas*** (light purple; Fig 4.3.1 and Fig 4.3.2) dominated in all plant genotypes giving the cultivable endophytic root communities a high degree of similarity.

The genus *Pelomonas* was formerly known as a part of the genus *Pseudomonas*. Xie and Yokota (2005) reclassified them to a new genus with nitrogen fixing capacity. The genus *Pelomonas* is able to use complex carbohydrates as a carbon source (Doudoroff *et al*. 1956) but also contaminants, aromatic compounds (Gomila *et al*. 2007) and hemicellulose (Leung *et al*. 2016). All our derived 16S rDNA sequences mostly resembled the strains *Pelomonas puraquae* (AB698673, Tani *et al.* 2012) or *Pelomonas saccharophila* (AB495144, Tani *et al.* 2011). Endophytic *Pelomonas* strains were formerly isolated from sweet potato (Terakado-tonooka *et al*. 2008), narrowleaf cattail roots (Li *et al*. 2011) and tatsoi leaves (Koo *et al*. 2016). Furthermore, the motility due to a single polar flagellum provides them a suitable trait to become endophytic (Gomila *et al*. 2007). These endophytes may provide benefits towards their host plants by their ability of nitrogen fixation (Xie *et al*. 2005).

Of some other genera the presences differed in the studied plant genotypes. For example, in the communities of roots of younger plants, the genus ***Bacillus*** (brown; Fig 4.3.1) was observed in all plant genotypes but lowest relative abundances were observed in both *ccr1* mutants. *Bacillus* (brown; Fig 4.3.2) was still observed in the communities of roots of all older lignin-reduced mutants but not in WT plants which somehow differentiates the cultivable communities of lignin-reduced mutants from those of WT plants. *Bacillus* strains were isolated from several plant species including *A. thaliana* (Truyens *et al*. 2013, Truyens *et al*. 2016a) and the plant growth promoting capacity of a selected strain was demonstrated (Truyens *et al*. 2013). Other examples of differentiation between WT plants and the lignin-reduced mutants are ***Microbacterium*** (dark green; Fig 4.3.1) and ***Pseudomonas*** (light yellow; Fig 4.3.2) which were highly represented in the communities of respectively roots of younger and older WT plants though lower or not in lignin-reduced mutants. From *Pseudomonas* both pathogenic strains and strains possessing beneficial traits in their interaction with plants (Loper *et al*. 2012, Berg *et al*. 2014, Lebeis 2014, Hase *et al*. 2003,

Rocha *et al*. 2016) are known. Beckers *et al*. (*in preparation*) demonstrated promotion of *A. thaliana* root growth after inoculation of a *Microbacterium* strain that was isolated from poplar. Further, ***Acidovorax*** (purple; Fig 4.3.1) (a previously isolated *A. thaliana* endophytic genus (Truyens *et al*. 2013, Truyens *et al*. 2016 a and b)) was found in the bacterial community of roots of both younger *4cl1* mutant conditions. *Acidovorax* was not observed in non-exposed roots of younger plants of any of the other plant genotypes.

To summarise the genotypic effect in roots, a high relative abundance of *Pelomonas* dominated the cultivable root communities in the WT plants and the lignin-reduced mutants. However, interesting differences between WT and lignin-reduced genotypes were observed in the representation of isolates of other genera.

The effect of cadmium

In case plants were exposed to Cd, the cultivable communities in the roots of younger and older plants of *A. thaliana* mutants were even more similar because the genus ***Pelomonas*** (purple; Fig 4.3.1 and Fig 4.3.2) completely dominated all cultivable communities.

We also observed some noteworthy differences after exposure of the plants to Cd. For instance, ***Pseudacidovorax*** strains (light purple; Fig 4.3.1) were only found in the communities of young roots of *ccr1* mutants after exposure to Cd. The genus *Pseudacidovorax* was reported as an endophyte of potato roots and contains NifH genes (nitrogenase) (Pageni *et al*. 2014). However, no relation to metal stress was found in literature. Furthermore, ***Blastomonas*** (pink; Fig 4.3.1) showed the highest presence in the root community of younger Cd-exposed WT plants. In the community of roots of older Cd-exposed WT plants, ***Elizabethkingia*** (blue; Fig 4.3.2) and ***Acidovorax*** (purple; Fig 4.3.2) were found in a relatively high relative abundance. One endophytic strain of *Elizabethkingia* was reported in *Zea mays* (Kämpfer *et al*. 2015) though most references were found concerning pathogenic strains of strains of the genus *Elizabethkingia* for humans (Jean *et al*. 2014, Breurec *et al*. 2016, Lau *et al*. 2016). *Acidovorax* was already observed in seeds produced by Cd-exposed

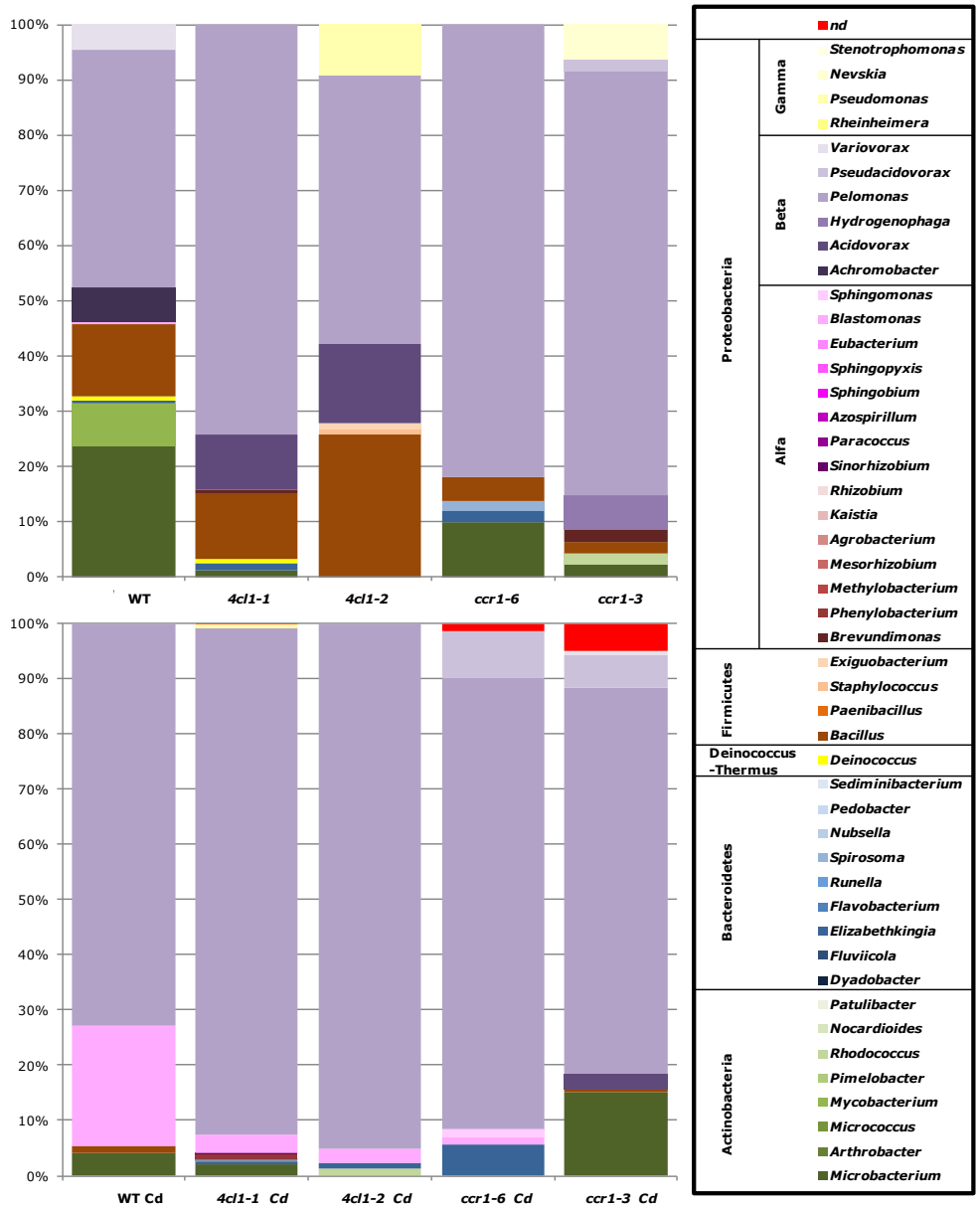


Figure 4.3.1: Cultivable bacteria (presented as relative abundances) isolated from **roots of younger** WT, *4cl1-1*, *4cl1-2*, *ccr1-6* and *ccr1-3* plants with and without exposure to Cd. nd: not determined. A detailed legend is provided in supplemental fig 4.3.1.

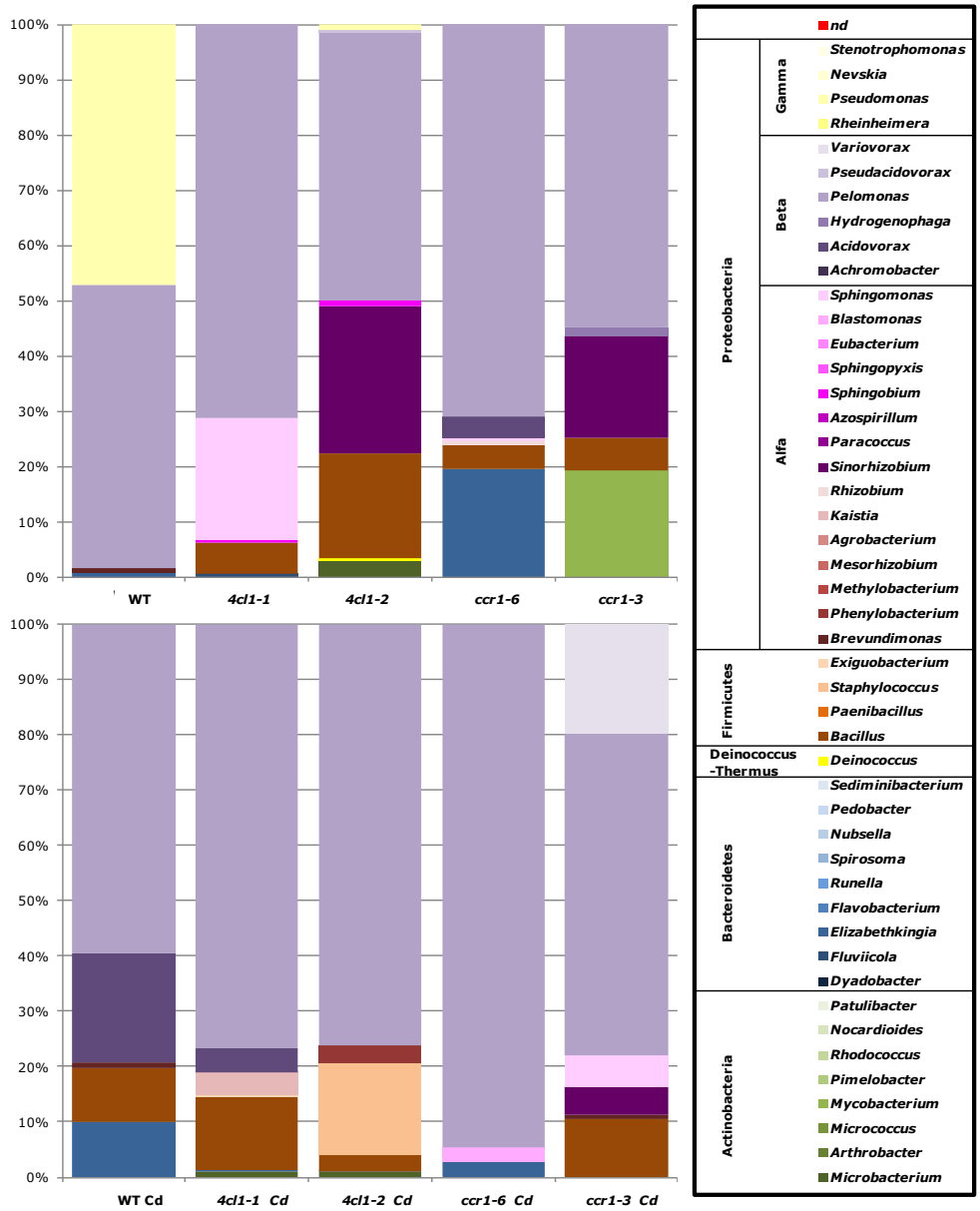


Figure 4.3.2: Cultivable bacteria (presented as relative abundances) isolated from **roots of older** WT, *4cl1-1*, *4cl1-2*, *ccr1-6* and *ccr1-3* plants with and without exposure to Cd. nd: not determined. A detailed legend is provided in supplemental fig 4.3.1.

parental plants and Cd resistance of isolated strains was demonstrated by Truyens *et al*. (2013).

In conclusion, under Cd exposure of the plants *Pelomonas* dominated the cultivable endophytic communities of roots of younger and older plants even more than under non-exposed conditions and made the cultivable endophytic communities of all studied genotypes highly similar.

(B) Cultivable LEAF endophytes

Bacterial communities in leaves are mainly formed through bacterial colonisation from the environment (*e.g.* rhizosphere or phylosphere) (Bodenhausen *et al.* 2013). Leaf endophytic communities are important for plant growth and health (Bodenhausen *et al*. 2013, Truyens *et al*. 2016b). In contrast to the roots, more differences as a function of the age of plants (leaves of younger versus older plants) were observed and are discussed below.

The effect of the lignin-reduced genotype

Like in the roots of younger and older plants, ***Pelomonas*** (light purple; Fig 4.3.3) strains were isolated from **leaves of younger plants** of all plant genotypes. The highest relative abundance was found in the WT endophytic community. Moreover, ***Pseudomonas*** (light yellow; Fig 4.3.3) was observed in leaves of younger plants of all genotypes and it even dominated the cultivable community from leaves younger *ccr1-3* mutants.

In **leaves of younger plants**, some genotype dependent tendencies were observed. For example, the genus ***Bacillus*** (brown; Fig 4.3.3) (as found in *A. thaliana* leaves by Kniskern *et al*. 2007) was only isolated from leaves of younger plants of all lignin-reduced mutants (both *4cl1* and both *ccr1*), suggesting a possible preference of *Bacillus* strains towards lignin-reduced mutants (which was also observed in roots of older plants). Beckers *et al*. (*in preparation*) demonstrated the ability of a *Bacillus* endophytic strain isolated from poplar to promote root growth of *A. thaliana ccr1-6* mutants after inoculation in a vertical agar plate assay. Furthermore, ***Brevundimonas*** strains (dark red; Fig 4.3.3) (as isolated from *A. thaliana* seeds by Truyens *et al*. 2016a and b) were observed in the highest relative abundances in the communities of

leaves of both younger *4cl1* mutants and were not detected in *ccr1* mutants. *Brevundimonas* strains (dark red; Fig 4.3.4) were also found in the communities of leaves of older *4cl1-1*, *4cl1-2* and *ccr1-6* mutants.

In **leaves of older plants** no similarity due to the dominance of *Pelomonas* (purple; Fig 4.3.4) was found anymore and clear cultivable community differences were observed as a function of the plant genotype. A dominance of *Pelomonas* (purple; Fig 4.3.4) was only observed in the community of the *ccr1-3* mutant (although *Pelomonas* was also isolated from all other lignin-reduced mutants). We selected plants with a similar stem length though not with a similar rosette growth. Hence, the high dominance in *ccr1-3* might also be due to differences in development of the rosette.
The genera ***Pseudomonas*** (light yellow; Fig 4.3.4) and ***Microbacterium*** (green; Fig 4.3.4) were more abundant in the leaves community of older WT plants. On the other hand, ***Variovorax*** (light purple; Fig 4.3.4), ***Deinococcus*** (bright yellow; Fig 4.3.4) and ***Pedobacter*** (light blue; Fig 4.3.4) were solely isolated from the leaves of older lignin-reduced mutants but not from WT plants. Endophytic *Deinococcus* members were also found in roots of rice (Sun *et al*. 2008, Raweekul *et al*. 2016), poplar stem (Beckers *et al*. 2016b) and *Deinococcus* members were found in phyllospheric communities of various tree species and *A. thaliana* leaves, which probably can be connected with their high capacity to withstand radiation including UV (Redford *et al*. 2010, Agler *et al*. 2016). Furthermore, **leaves** of older ***4cl1-1*** mutants showed a relatively high diversity (as discussed in paragraph 4.3.2.2) of endophytic bacteria, which were also isolated from several other lignin-reduced mutant communities but not from WT plants (*e.g. Sinorhizobium* (purple; Fig 4.3.4), *Rhizobium* (light red; Fig 4.3.4), *Exiguobacterium* (light orange; Fig 4.3.4), *Staphylococcus* (orange; Fig 4.3.4) and *Micrococcus* (green; Fig 4.3.4)).

To conclude, the cultivable endophytic communities of leaves of younger lignin-reduced mutants revealed some limited differences in comparison to those of WT plants, but at the same time also revealed some resemblances due to presence of *Pelomonas* and *Pseudomonas*. In leaves of older plants, the high dominance of *Microbacterium* in WT plants, *Pelomonas* in *ccr1-3* and high diversity in the

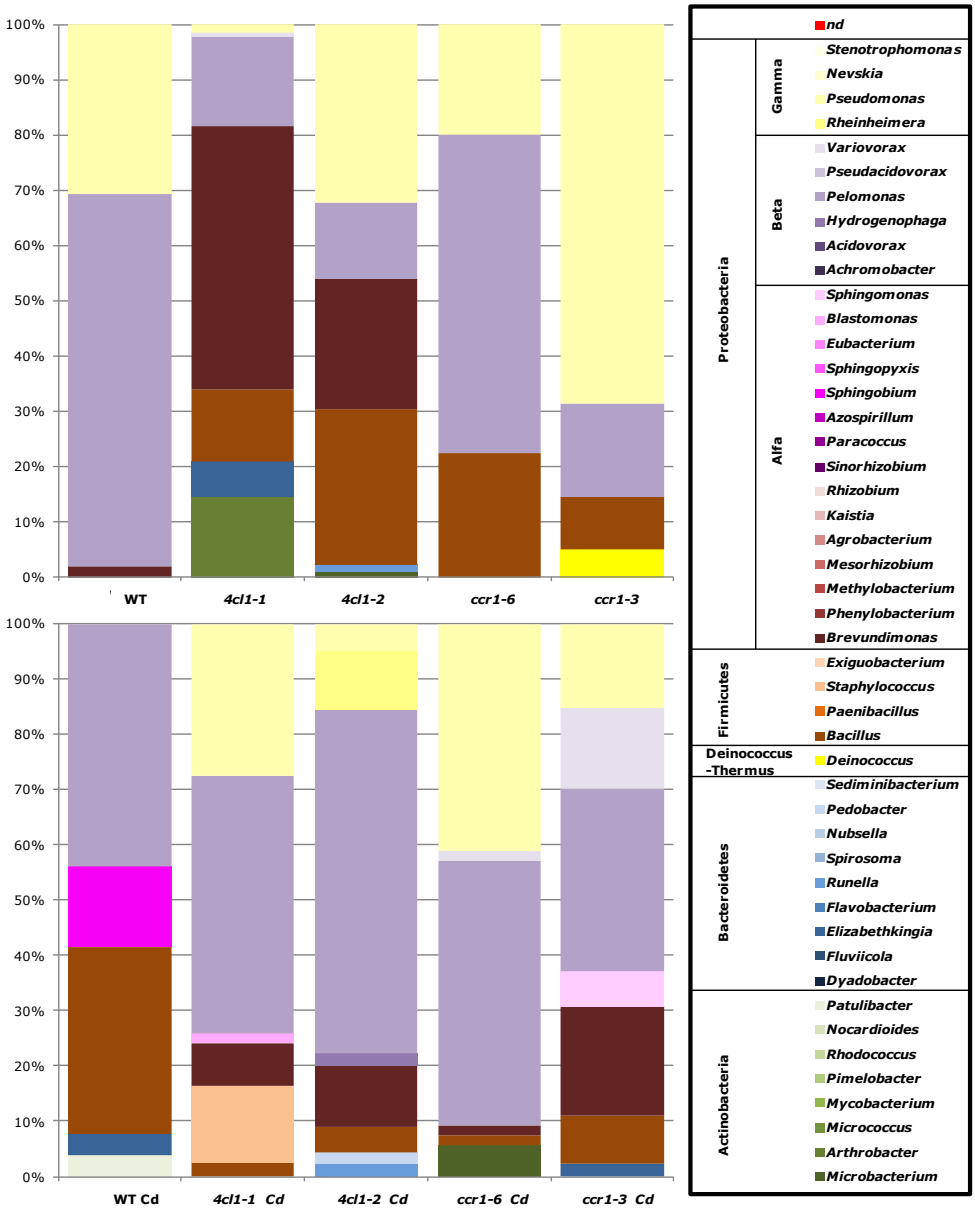


Figure 4.3.3: Cultivable bacteria (presented as relative abundances) isolated from **leaves of younger** WT, *4cl1-1*, *4cl1-2*, *ccr1-6* and *ccr1-3* plants with and without exposure to Cd. nd: not determined. A detailed legend is provided in supplemental fig 4.3.1.

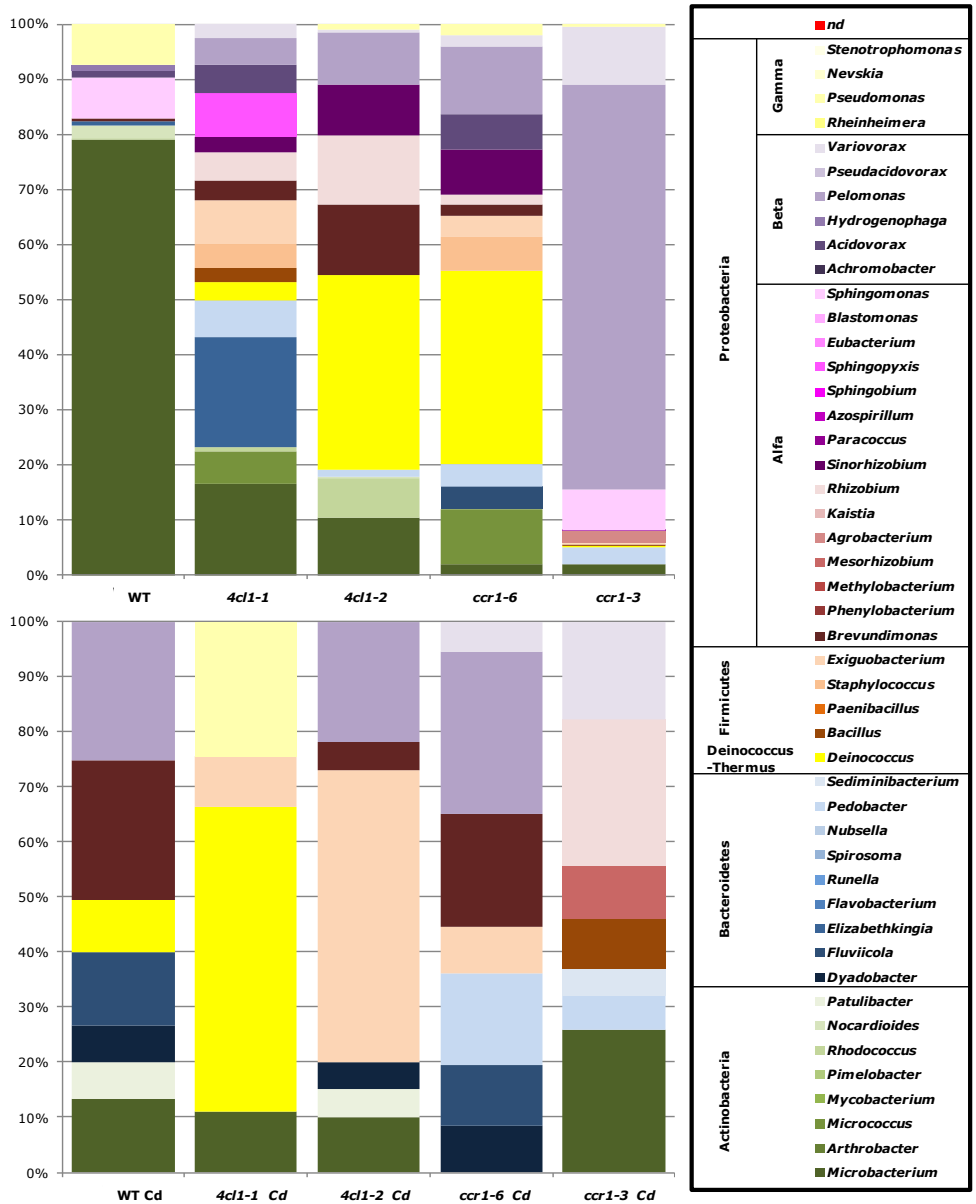


Figure 4.3.4: Cultivable bacteria (presented as relative abundances) isolated from **leaves of older** WT, *4cl1-1*, *4cl1-2*, *ccr1-6* and *ccr1-3* plants with and without exposure to Cd. nd: not determined. A detailed legend is provided in supplemental fig 4.3.1.

other lignin-reduced mutants differentiated the cultivable endophytic communities of older lignin-reduced mutants from those of leaves of WT plants.

The effect of cadmium

After exposure of the plants to Cd, some changes were observed in the composition of the cultivable endophytic communities of leaves of lignin-reduced mutants. Like in roots, the relative abundance of ***Pelomonas*** (purple; Fig 4.3.3) increased in the leaves communities of younger lignin-reduced mutants (except *ccr1-6*) while it decreased in WT plants after Cd exposure.

Pseudomonas (light yellow; Fig 4.3.3) disappeared (in comparison to the non-exposed plants) from the endophytic community of leaves of younger Cd-exposed WT plants but was still present in leaves of the **lignin-reduced mutants**. Furthermore, ***Variovorax*** (light purple; Fig 4.3.3) colonized leaves of younger Cd-exposed *ccr1* mutants (both *ccr1-6* and *ccr1-3*) and strains of the genus ***Brevundimonas*** (dark red; Fig 4.3.3) were found in the communities of all leaves of younger Cd-exposed lignin-reduced mutants. However, the latter genus was not detected in the similar leaf community of Cd-exposed WT plants (Fig 4.3.3). These three genera (*Pseudomonas, Brevundimonas* and *Variovorax)* were previously also isolated from seeds of non- and Cd-exposed *A. thaliana* plants (Truyens *et al*. 2016a and b). Another example of genera isolated only from one specific plant type after Cd exposure of the plants is ***Pedobacter*** (light blue; Fig 4.3.4) in leaves of older plants. In the cultivable communities of leaves of older Cd-exposed plants of both *ccr1* mutants the genus *Pedobacter* was observed while it was not detected (in comparison to the non-exposed plants) in the cultivable communities of *4cl1* mutants after Cd exposure. *Pedobacter* strains containing resistance genes towards metal toxicity were described by Lee *et al*. (2016). Under Cd-exposure of the plants, the above-mentioned genera might be more important in leaves of lignin-reduced mutants than in WT plants.

Microbacterium strains (green; Fig 4.3.4) were highly present in the leaves of non-exposed older WT plants. However, after Cd exposure of the plants their presence decreased in WT though for lignin-reduced mutants no general response to Cd was observed. Moreover, some genera were more present in the

leaves community of Cd-exposed **WT** plants compared to the communities of leaves of Cd-exposed lignin-reduced mutants. For instance ***Sphingobium*** strains (bright pink; Fig 4.3.3) were observed in leaves of younger Cd-exposed WT plants but not in lignin-reduced mutants. Strains of *Sphingobium* with genes encoding for metal resistance proteins were reported (Niharika *et al*. 2013). ***Bacillus*** (brown; Fig 4.3.3) was observed in high relative abundances in the community of leaves of younger Cd-exposed WT plants while it was not isolated from the leaves of non-exposed WT plants. However, in lignin-reduced mutant genotypes the genus *Bacillus* was observed in minor relative abundances in comparison to the non-exposed leaves of younger plants (Fig 4.3.3). Again highlighting some differences between the endophytic communities of WT plants and lignin mutants.

In conclusion, the high relative abundance of *Pelomonas* in the cultivable endophytic communities of the leaves of younger Cd-exposed plants resembled the cultivable endophytic communities present in the WT plants. However, the occurrence of the genera *Pseudomonas* and *Brevundimonas* in lignin-reduced mutants and *Sphingobium* in WT plants differentiated the WT and lignin-reduced plants. However, although an effect of Cd (*e.g.* decrease of *Microbacterium* in WT leaves) was observed for all the cultivable endophytic communities of the leaves of older plants of all studied genotypes, it is difficult to draw a general conclusion.

(C) Cultivable STEM endophytes

The lignin reduction might be an important driving factor for the composition of bacterial endophytic communities. Since lignification is highest in the xylem of stems (Boerjan *et al*. 2003), most differences can be expected in the communities of stems.

Effect of the lignin-reduced genotype

Pseudomonas (light yellow; Fig 4.3.5) strongly dominated (99.22%) the cultivable WT stem community. The genus *Pseudomonas* contains strains from pathogenic to plant growth promoting (Lebeis 2014). *Pseudomonas* strains were also isolated from stems of *4cl1-1* (29.23%), *4cl1-2* (49.33%) and the *ccr1-6*

mutants (5.88%), though in lower relative abundance; in stems of the *ccr1-3* mutant the genus *Pseudomonas* was not detected (Fig 4.3.5). In addition to *Pseudomonas*, the cultivable endophytic community of stems of WT plants consisted of 6 low abundant (lower than 0.4% of the community) genera (*Pedobacter, Sediminibacterium, Staphylococcus, Rhizobium, Azospirilium, Variovorax*) (Fig 4.3.5). Due to the high dominance of *Pseudomonas*, the cultivable stem endophytic community of WT plants looked highly different from those of all lignin-reduced mutants.

Examples of genera isolated from the stems of lignin-reduced mutants are ***Pedobacter*** (light blue; Fig 4.3.5) which was found in all lignin-reduced mutant stem communities and may be associated to the lower levels of lignin in the stems and ***Brevundimonas*** (dark red; Fig 4.3.5) which was only isolated from *4cl1* mutant stems and may be associated to the T-DNA knockout *A. thaliana* mutants of *4CL1*.
Furthermore, the high relative abundance of ***Sphingomonas*** (pink; Fig 4.3.5) in stems of the *ccr1-3* mutant should be mentioned, although it cannot be explained by the *ccr1* mutation itself since *ccr1-6* does not support the observation. Endophytes of the genus *Sphingomonas* were formerly observed in *A. thaliana* (Truyens *et al*. 2013, Truyens *et al*. 2016a, Kniskern *et al*. 2014, Agler *et al*. 2016) and field-grown *CCR* down-regulated poplar (Beckers *et al*. 2016b).

In conclusion, both the interference at the *4CL1* gene (the cultivable endophytic communities of these plants revealed some resemblances and some differences to those of stems of WT plants), and the *CCR1* gene (of which the cultivable stem endophytic communities clearly differentiated from those of WT stems), influenced the cultivable stem communities of the selected *A. thaliana* lignin-reduced genotypes in comparison to WT plants.

Effect of cadmium

The cultivable communities of stems of *ccr1-6* and *ccr1-3* mutants were very low in diversity in case the plants were exposed to Cd. Only 2 colony forming units (1 ***Mesorhizobium*** and 1 ***Kaistia*** strain (both in shades of red; Fig 4.3.5))

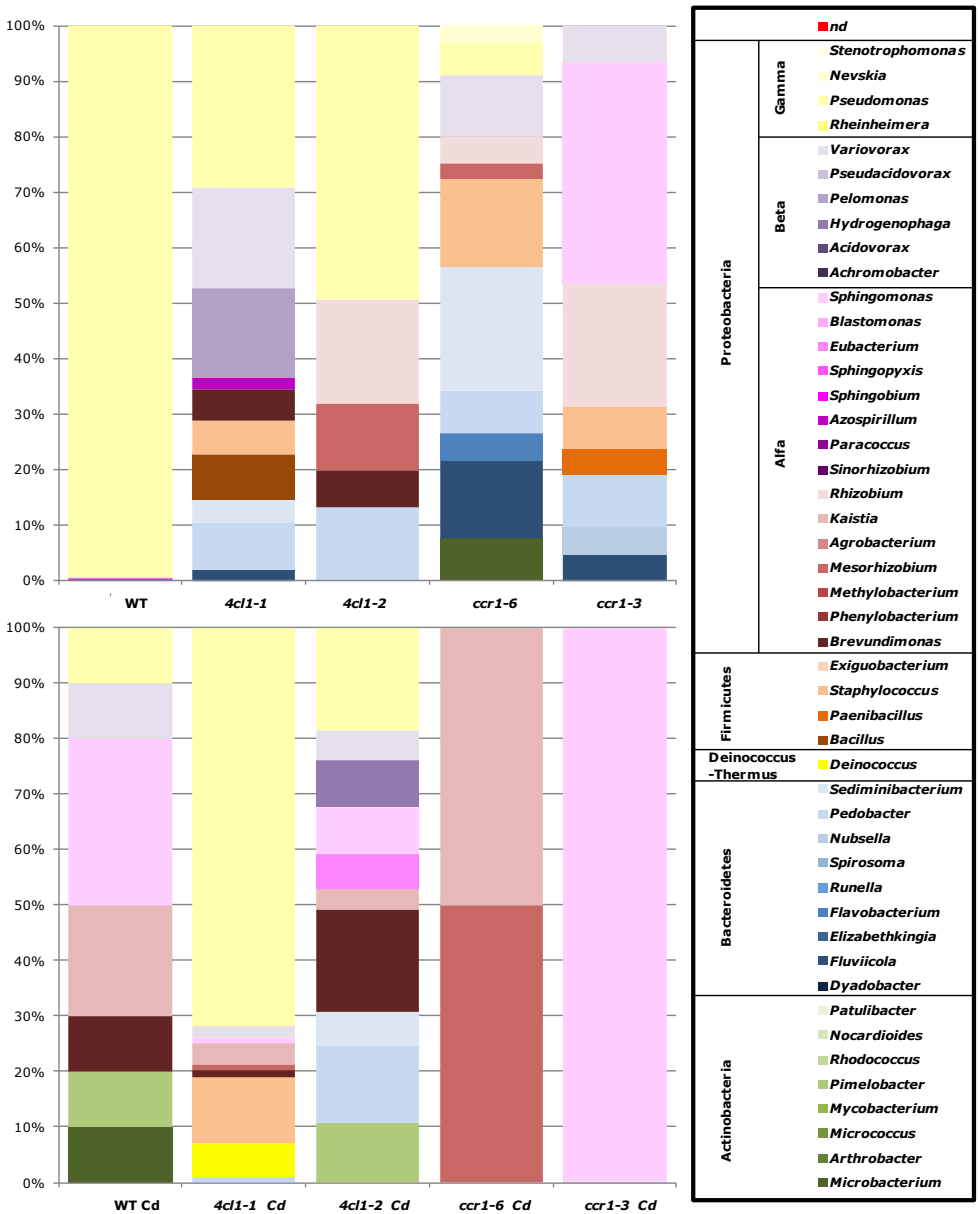


Figure 4.3.5: Cultivable bacteria (presented as relative abundances) isolated from **stems** of WT, *4cl1-1*, *4cl1-2*, *ccr1-6* and *ccr1-3* with and without exposure to Cd. nd: not determined. A detailed legend is provided in supplemental fig 4.3.1.

were isolated from the stems of Cd-exposed *ccr1-6* mutants. Metal-resistant strains of the genus *Mesorhizobium* are known and, *Mesorhizobium* species include nitrogenase genes in their genome, like the *Rhizobium* genus from which they were reassigned (Laranjo *et al*. 2014). *Kaistia* strains were previously isolated from root nodules of bur clover (Arone *et al*. 2014).

On the other hand, the endophytic community of stems of *ccr1-3* mutants exposed to Cd was dominated (99.92%) by ***Sphingomonas*** (pink; Fig 4.3.5). Interestingly, certain *Sphingomonas* were reported to enhance Cd uptake concurring with up-regulation of glutathione synthase (Pan *et al*. 2016). Cd resistance and plant growth promoting traits (*e.g.* N fixation) in *Sphingomonas* strains were demonstrated by Truyens *et al*. (2013). In our study, the genus *Sphingomonas* was also isolated in a high relative abundance from stems of non-exposed *ccr1-3* mutants, which may suggest that they could have an important role in the *ccr1-3* stems (Fig 4.3.5).

Despite the fact that both allelic forms of the *ccr1* mutants did not display the same genera in their cultivable community composition and the number of bacteria in stems of Cd-exposed *ccr1-6* mutants was very low, the cultivable endophytic communities of both *ccr1* mutants were rather different from the WT community under influence of Cd. However, *Sphingomonas* (pink) and *Kaistia* (red) were also isolated from the stems of WT plants and both *4cl1* mutants.

Microbacterium (green; Fig 4.3.5) was only isolated from stems of WT plants exposed to Cd. While ***Pseudomonas*** (light yellow; Fig 4.3.5), ***Variovorax*** (light purple; Fig 4.3.5) and ***Brevundimonas*** (dark red; Fig 4.3.5) were isolated from stems of WT plants and *4cl1* mutants exposed to Cd but were not detected in *ccr1* stems.

Altogether, after exposure to Cd of the plants, the cultivable endophytic stem communities of the lignin-reduced mutants tended to show differences in comparison to WT plants. The differences were the most prominent in the *ccr1* stem communities of Cd-exposed plants.

(D) Cultivable SEED endophytes

Bacterial seed endophytes can play an important role during germination and are in this way essential for plant health (Truyens *et al*. 2013).

Effect of the lignin-reduced genotype

Rhizobium (light red; Fig 4.3.6) was highly dominant in seeds communities of WT and *4cl1-1* but was also found in lower fractions in the communities of *4cl1-2* and *ccr1-3* mutants. This is in accordance with the high relative abundances of *Rhizobium sp.* that were found in several generations of *A. thaliana* WT seeds (Truyens *et al*. 2013, Truyens *et al*. 2016a and b). *Rhizobia* are well studied for their possible plant growth promoting traits (Biswas *et al*. 2000, Chi *et al*. 2005, Gopalakrishnan *et al*. 2015). In seeds of both *ccr1* mutants the genus ***Microbacterium*** (green; Fig 4.3.6) dominated the cultivable endophytic communities and might be essential for the *ccr1 A. thaliana* mutants. Both cultivable *4cl1* seed communities contained the genus ***Staphylococcus*** (orange; Fig 4.3.6) that was not isolated from other non-exposed seed. *Staphylococcus* strains were previously observed as seed endophytes in *A. thaliana* (Truyens *et al*. 2013, Truyens *et al*. 2016 a and b). ***Paenibacillus*** (orange; Fig 4.3.6) dominated the cultivable community of *4cl1-2* seeds but was also observed in a minor relative abundance in *ccr1-3* seeds. All above-mentioned genera may contain beneficial traits, which are selected in the different mutant plant genotypes.

We can conclude that the cultivable endophytic community of seeds of the *4cl1-1* mutant was similar to that of WT seeds while the community of the *4cl1-2* mutant revealed some differences in comparison to the WT community. The cultivable bacterial communities isolated from seeds of both *ccr1* mutants tended to be quite different from the WT seed endophytes.

Effect of cadmium

Microbacterium (green; Fig 4.3.6) strains were not observed in the communities of seeds produced by WT and *4cl1* mutants that were not exposed

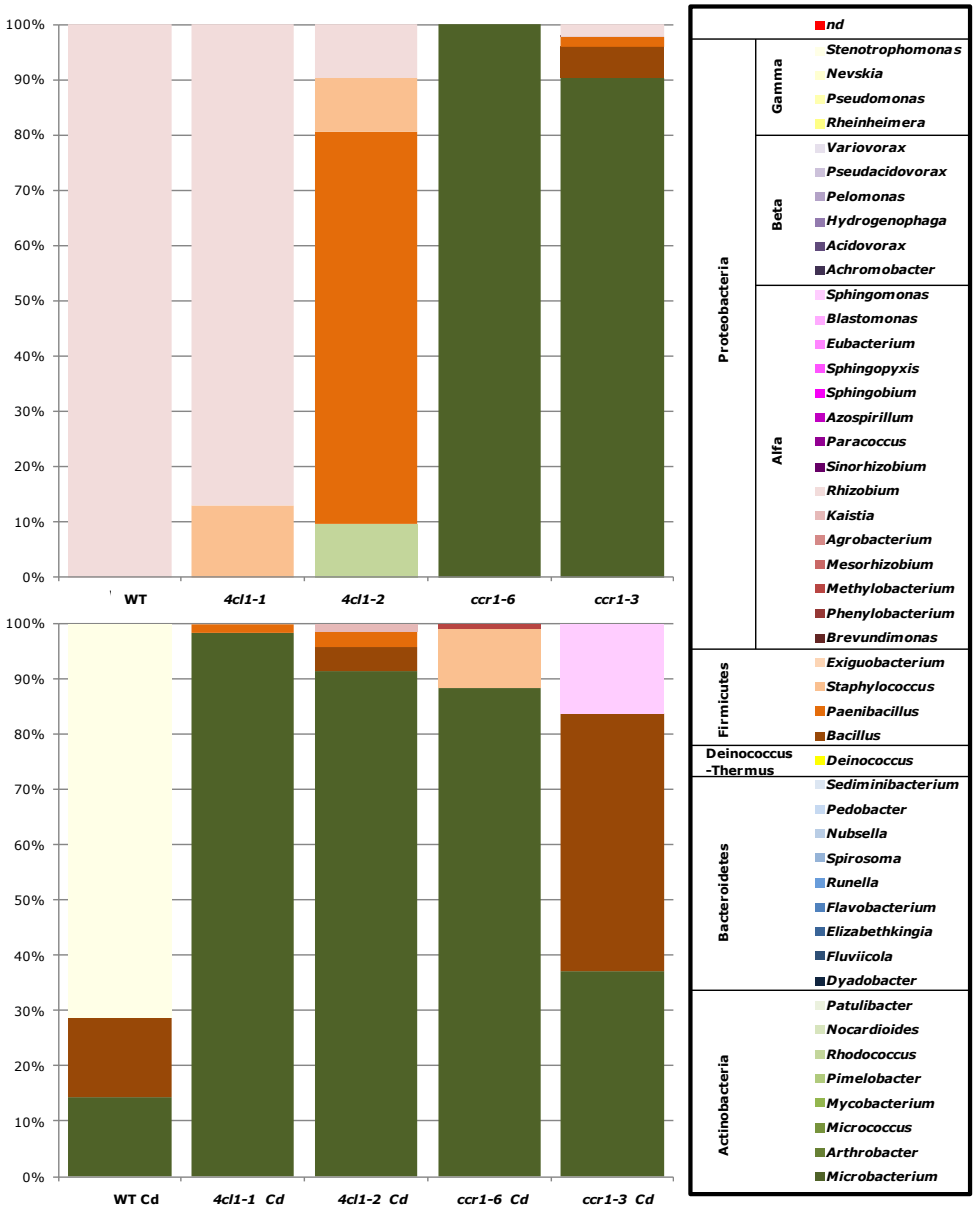


Figure 4.3.6: Cultivable bacteria (presented as relative abundances) isolated from **seeds** of WT, *4cl1-1*, *4cl1-2*, *ccr1-6* and *ccr1-3* with and without exposure to Cd. nd: not determined. A detailed legend is provided in supplemental fig 4.3.1.

to Cd. After Cd exposure of the parental plants, they appeared in low relative abundances in seeds of WT plants and even dominated the cultivable communities of seeds of both *4cl1* mutants. Also in *ccr1* seeds, *Microbacterium* (green; Fig 4.3.6) was still highly represented in the cultivable endophytic communities of seeds of Cd-exposed plants though it decreased in relative abundance in comparison to seeds of non-exposed plants. ***Bacillus*** (brown; Fig 4.3.6) was observed in the communities of seeds of several *A. thaliana* genotypes after Cd exposure of the parental plants and thus is not associated with a specific plant genotype. Though *Bacillus* and *Microbacterium* could not be linked to a specific plant genotype in seeds of Cd-exposed plants, both genera may contain promising traits that may assist plants to cope with Cd exposure. For example, several strains of the genus *Bacillus* with metal resistance genes have been described (Fierros-Romero *et al*. 2016). Moreover, endophytic *Bacillus* strains resistant to Cd were reported by Truyens *et al*. (2013). Furthermore, several metal resistant *Microbacterium* strains are known (Fierros-Romero *et al*. 2016, Sheng *et al*. 2008). ***Stenotrophomonas*** (light yellow; Fig 4.3.6) was only observed in seeds community of Cd-exposed WT plants (71.43%). Strains of *Stenotrophomonas* were also observed in low fractions in seeds of Cd-exposed *A. thaliana* by Truyens *et al*. (2013) and in roots of *CCR* down-regulated poplars by Beckers *et al*. (2016b). Metal-tolerant strains with plant growth promoting properties of *Stenothrophomonas* were also reported (Pages *et al*. 2008, Kartik *et al*. 2016). Remarkable is the relative high relative abundance of ***Sphingomonas*** (pink; Fig 4.3.6) in the seed community of Cd-exposed *ccr1-3* mutants. The genus *Sphingomonas* was also the dominant genus in stem communities (Fig 4.3.5) of non- and Cd-exposed *ccr1-3* mutants.

To conclude, under influence of Cd, the cultivable endophytic communities of all lignin-reduced mutants revealed a shift towards *Microbacterium* which tended to distinguish them from the cultivable seed community of WT plants that was more dominated by *Stenotrophomonas*.

4.3.2 Conclusions

Differences in amounts and diversity of cultivable bacteria were observed with respect to the different plant compartments. Furthermore, the lignin-reduced genotypes tended to decrease the diversity of the cultivable endophytic community in roots and to increase the community diversity in leaves and stems. No big effects of Cd exposure on diversity were observed. However, to make statistically supported claims the study design should be expanded by more repetitions.

Very **similar** community compositions were found for **roots of all younger and older plants** of all plant genotypes with and without Cd exposure: in each case, the genus *Pelomonas* was highly dominant. Nevertheless, some differences connected with the lignin-reduced genotype were observed at the level of less frequent genera in roots of younger (*e.g.* higher relative abundance *Microbacterium* in WT roots and *Acidovorax* exclusively in *4cl1* roots) and older plants (*e.g.* high relative abundances of *Pseudomonas* in WT roots and *Bacillus* exclusively in lignin-reduced mutant roots). In case plants were exposed to Cd, endophytic communities of roots of younger and older plants were even more similar because of an even higher dominance of *Pelomonas*.

Likewise, a high dominance of *Pelomonas* was observed in **leaves of younger plants** even though in this case also *Pseudomonas* was highly represented in all genotypes. Again, some differences in cultivable bacterial communities of the lignin-reduced mutants were observed (*e.g. Bacillus* in lignin-reduced mutants and high relative abundances of *Brevundimonas* in *4cl1* mutants). After Cd exposure of the plants, *Pelomonas* tended to increase in relative abundance in leaves of lignin-reduced mutants, which was not observed for WT plants. The cultivable communities of leaves of younger Cd-exposed lignin-reduced mutants tended to distinguish themselves from those of WT plants by higher relative abundances of *Pseudomonas* and *Brevundimonas* though a lower relative abundance of *Bacillus* was found in lignin-reduced mutants in comparison with WT. *Sphingobium* was only isolated from leaves of younger WT plant exposed to Cd.

Cultivable communities of **leaves of older plants** appeared rather **differently** affected by the lignin modification, resulting in a high dominance of *Microbacterium* in WT leaves and *Pelomonas* in leaves of *ccr1-3* mutants while the communities of leaves of older plants of other plant genotypes were more diverse in their inhabiting bacterial genera. The high dominance of *Microbacterium* diminished when WT plants were exposed to Cd. However, because of the occurrence of many different genera in several genotypes, general hypothesis about the effects of Cd exposure of the plants cannot be drawn for leaves of older plants.

Also the cultivable bacterial communities isolated from **stems** appeared to be **affected** by the down-regulation of genes encoding enzymes involved in the monoligninol biosynthesis pathway in *A. thaliana*. The cultivable communities in both the *4cl1* and *ccr1* tended to differ from the WT community which was completely dominated by *Pseudomonas*. Furthermore, *Brevundimonas* was exclusively found in *4cl1* while *Pedobacter* was only observed in stems of all lignin-reduced mutants. Under influence of Cd exposure of the plants, *4cl1* stem communities showed some differences in comparison to WT stems though the most distinct communities were observed in the *ccr1* mutants (*e.g.* dominance of *Sphingomonas* in *ccr1-3*).

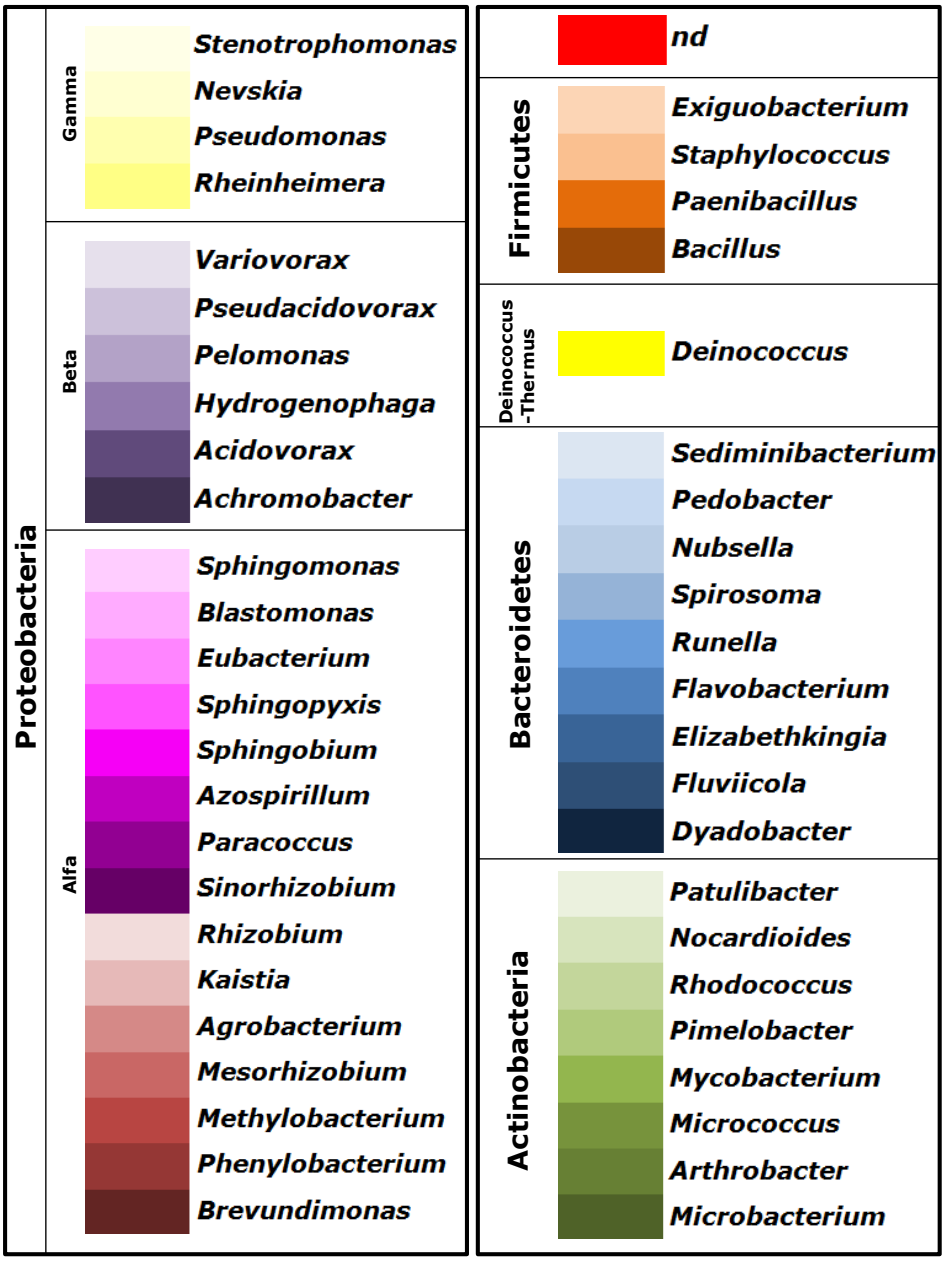
Cultivable endophytic communities isolated from **seeds** of the *A. thaliana* lignin-reduced genotypes under investigation tended to be **different**. WT and *4cl1-1* seed communities were dominated by *Rhizobium*, the *4cl1-2* communities by *Paenibacillus* and both *ccr1* communities by *Microbacterium,* again making the *ccr1* communities more different from the WT plants. Cd exposure of the plants made them select for *Microbacterium* in seeds of lignin-reduced mutants (supplemented by *Bacillus* and *Sphingomonas* in *ccr1-3*) while in Cd-exposed WT plants *Stenotrophomonas* was most abundant. In addition, the presence of *Sphingomonas* strains in seeds (and other plant parts) of the *ccr1-3* mutant underlines a specific selection by the *ccr1-3* mutant.

All observed differences in the cultivable endophytic community related to the genetic modification may result from various traits of the lignin-reduced mutants

(already discussed in subsection 4.2). For example, cell wall characteristics differ in lignin-reduced mutants resulting in different colonisation signals at their roots (Bulgarelli *et al*. 2012) and xylem vessels can be collapsed in *ccr1* mutants (Mir Derikvand *et al*. 2008) obstructing translocation of bacterial cells to other plant compartments. Furthermore, differences in development of *ccr1* mutants (mir Derikvand *et al*. 2001, Vanholme *et al*. 2012a), which cannot be completely eliminated, especially for seed production, may influence the endophytic bacterial communities of all plant compartments. Also accumulation of phenolic intermediates of the monolignol biosynthesis pathway and derivatives thereof that may serve as a C-source (Vanholme *et al*. 2012a, Beckers *et al*. 2016b) or antimicrobial agents (Rice-Evans *et al*. 1996) can affect the cultivable bacterial communities. Moreover, the seeds of lignin-reduced mutants have a different origin resulting in possible differences of seed endophytes that are vertically transmitted.

These endophytic bacterial communities, differing as a function of plant part, lignin-reduced genotype or Cd exposure, may contain diverse plant growth promoting properties, which will be further explored.

Supplementary information



Supplemental figure 4.3.1: Colour scheme used to describe the cultivable community composition in the bar plots presented in figure 4.3.1, 4.3.2, 4.3.3, 4.3.4, 4.3.5 and 4.3.6. nd= not determined.

Subsection 4.3

Supplemental Table 4.3.1: Cultivable endophytes of *A. thaliana* WT and lignin mutants in seeds, roots (of younger (Y) and older (O) plants), leaves (of younger (Y) and older (O) plants) and stems.

Phylum	Class	Order	Family	Genus	seed	root (Y)	root (O)	Leaf (Y)	Leaf (O)	Stem
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	*Microbacterium*	x	x	x	x	x	x
			Micrococcaceae	*Arthrobacter*				x		
				Micrococcus					x	
			Mycobacteriaceae	*Mycobacterium*		x	x			
			Nocardiaceae	*Pimelobacter*						x
				Rhodococcus	x	x			x	
			Norcadioidaceae	*Nocardioides*					x	
			Propionibacteriaceae	*Propionibacterium*						x
			Tsukamurellaceae	*Tsukamurella*	x					
		Rubrobacterales	Patulibacteraceae	*Patulibacter*				x	x	
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Dyadobacter*					x	
	Flavobacteria	Flavobacteriales	Cryomorphaceae	*Fluviicola*		x	x		x	x
			Flavobacteriaceae	*Elizabethkingia*		x	x	x	x	
				Flavobacterium						x
	Sphingobacteria	Sphingobacteriales	Sphingobacteriaceae	*Nubsella*						x
				Pedobacter				x	x	x
			Chitinophagaceae	*Sediminibacterium*					x	x
	Bacteroidetes bacterium	Bacteroidetes bacterium	Bacteroidetes bacterium	*Bacteroidetes bacterium*		x				
Deinococcus-Thermus	Deinococci	Deinococcales	Deinococcaceae	*Deinococcus*		x	x	x	x	x
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	x	x	x	x	x	x
				Paenibacillus	x					x
			Staphylococcaceae	*Staphylococcus*	x	x	x	x	x	x
			Bacillales Family XII. Incertae Sedis	*Exiguobacterium*		x	x		x	
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Brevundimonas*	x	x	x	x	x	x
			Caulobacteraceae	*Phenylobacterium*		x				

		Rhizobiales	phyllobacteriacea	*Mesorhizobium*					x	x
			Rhizobiaceae	*Agrobacterium*					x	
			Rhizobiaceae	*Kaistia*	x					
			Rhizobiaceae	*Rhizobiaceae bacterium*		x				
			Rhizobiaceae	*Rhizobium*	x		x		x	x
			Rhizobiaceae	*Sinorhizobium*			x		x	
		Rhodobacteriales	Rhodobacteraceae	*Paracoccus*					x	
		Rhodospirillales	Rhodospirillaceae	*Azospirillum*						x
			Rhodospirillaceae	*Kaistia*						x
		Sphingomonadales	Sphingomonadaceae	*Blastomonas*		x	x	x		
				Eubacterium						x
				Sphingobium			x	x		
				Sphingopyxis					x	
				Sphingomonas	x	x	x	x	x	x
		Caulobacterales	Caulobacteraceae	*Phenylobacterium conjunctum*			x			
	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Achromobacter*		x				
			Comamonadaceae	*Acidovorax*		x	x	x		
				Hydrogenophaga				x		
				Pelomonas		x	x	x	x	x
				Pseudacidovorax		x		x		
				Variovorax		x	x	x	x	x
		Rhodocyclales	Rhodocyclaceae	*Shinella*			x			
	Gammaproteobacteria	Chromatiales	Chromatiaceae	*Rheinheimera*				x		
		Pseudomonadales	Pseudomonadaceae	*Pseudomonas*		x	x	x	x	x
		Xanthomonadales	Sinobacteraceae	*Hydrocarboniphaga*		x	x		x	x
			Xanthomonadaceae	*Stenotrophomonas*	x					
			Sinobacteraceae	*Nevskia*						x

Subsection 4.4
Phenotypic characterisation of the cultivable bacterial endophytic communities of lignin-reduced *A. thaliana* plants with and without Cd exposure

As described in previous chapters, both (i) the genetic modification of genes involved in the lignin biosynthesis as well as (ii) exposure to cadmium (Cd) has the potential to negatively affect plant health, growth and development. In order to counteract or even eliminate the growth inhibition associated with lignin modification and/or Cd exposure, we can consider making use of **plant growth promoting endophytic bacteria** (Weyens *et al*. 2009c).

General plant growth promotion mechanisms of endophytic bacteria included: (i) increasing the plant nutrient uptake (organic acid (OA) and siderophore (SID) production, phosphate (P) solubilisation and nitrogen (N) fixation); (ii) production of regulators of plants growth such as auxins (indole-3-acetic acid (IAA) production) and (iii) suppression of the production of the stress hormone ethylene (by exerting 1-aminocylcopropane-1-carboxylate (ACC) deaminase capacity) (Weyens *et al*. 2009c). More specifically, in order to limit the growth inhibiting effect of the lignin modification (*i.e. ccr1* mutation), several bacterial endophytes may be used which "consume" **phenolic acids** (*e.g.* ferulic acid and *p*-coumaric acid) that are accumulated in the lignin-reduced mutants as carbon and/or energy source (Beckers *et al. in preparation*). Furthermore, the plant growth inhibition experienced after exposure to Cd (or other toxic metals) may be counteracted by inoculation of plants with bacterial endophytes with other beneficial traits such as bacteria equipped with a metal sequestration system that can lower the **Cd toxicity** towards the plant (Mastretta *et al*. 2009, Weyens *et al*. 2009c).

It is well established that specific endophytes (with specific phenotypic traits) can be selected in a host plant type whether or not under influence of contamination (Ma *et al.* 2016). Since endophytic communities may evolve relatively fast, bacteria equipped with the most beneficial traits for survival in a specific niche can also be quickly selected (Hardoim *et al.* 2015). Previously, the selection of phenotypic bacterial traits under influence of *CCR* down-regulation in poplar trees (Beckers *et al.* 2016b) and Cd exposure in *A.thaliana* (Truyens *et al.*

2013) and *Brassica napus* (Croes *et al*. 2013) have been described. Therefore, we can hypothesize that the most promising bacterial endophytes for our study will be encountered in Cd-exposed lignin-reduced mutants.

In our research, earlier isolated endophytes (from roots, leaves, stems and seeds) of WT and lignin-reduced (T-DNA knockout mutants for *4-COUMARATE:COA LIGASE 1* (*4CL1*) or *CINNAMOYL-COA-REDUCTASE 1* (*CCR1*)) *Arabidopsis thaliana* plants are examined for their *in vitro* plant growth promoting traits, Cd tolerance and use of phenolic acids as sole carbon-source (C-source). Furthermore, the relative abundance of different phenotypic traits in the endophytic community is examined for all conditions (different lignin-reduced mutants with and without Cd exposure). Based on the obtained information, promising bacterial strains are identified and selected for inoculation experiments (subsection 4.5).

4.4.1 Experimental design

Endophytes were isolated from seeds, roots (of younger (3 weeks old) and older (bolting plants of 47 days for WT plants) plants), leaves (of younger and older plants) and stems of WT plants and the selected lignin-reduced mutants (*4cl1-1, 4cl1-2, ccr1-6, ccr1-3*) grown with or without exposure to 3 µM $CdSO_4$. More information about the isolation procedure can be found in paragraph 4.1.2. These bacterial endophytes were *in vitro* examined for their Cd tolerance, growth promoting traits (1-aminocyclopropane-1-carboxylate (ACC) deaminase capacity, indole-acetic acid (IAA) production, siderophore (SID) production, phosphate (P) solubilisation, nitrogen (N) fixation capacity and organic acid (OA) production) and capacity to utilise ferulic acid and *p*-coumaric acid as sole C-source. These assays are described more in detail in paragraph 4.1.2.3.

4.4.2 Results and discussion

4.4.2.1 Cadmium tolerance

Many bacteria are tolerant to relatively high concentrations of toxic metals. These metal-tolerant bacteria may play a role in the mobilisation or immobilisation of these metals such as Cd (Belimov *et al*. 2005) and can reduce Cd phytotoxicity by use of sequestration systems and can even enhance translocation to the aerial parts. Both these traits can be advantageous in case

plants are used for phytoremediation purposes (Weyens *et al*. 2009b). In the following, we will discuss the Cd tolerance of the isolated bacterial endophytes in the roots (of younger and older plants) (Fig 4.4.1A and B), leaves (of younger and older plants) (Fig 4.4.1C and D), stems (Fig 4.4.1E) and seeds (Fig 4.4.1F) of WT plants and the selected lignin-reduced mutants (*4cl1-1, 4cl1-2, ccr1-6, ccr1-3*).

A subset of the endophytic strains (predominantly in the roots of the younger and older plants and leaves in the younger plants in all genotypes) was difficult to re-grow, especially on the 284 selective medium with added C-sources with and without Cd-exposure. Even on the same medium supplemented with plant extracts, many strains could not grow. Therefore, in the communities of **roots of younger and older plants** (Fig 4.4.1A and B) and **leaves of younger plants** (Fig 4.4.1C), a high relative abundance of the bacterial community remained in the status of "not determined" for the Cd tolerance trait (grey bars). Due to these cultivation limitations, it was difficult to draw definitive conclusions concerning the relative abundance of the microbial community that was Cd tolerant. The cultivation limitation was mainly due to a high relative abundance of *Pelomonas* species of which species with limited re-cultivability such as *Pelomonas aquatica* are known (Gomila *et al.* 2007). Seven incomplete (whole Genome Shotgun derived) genomes of *Pelomonas* species are available in the NCBI database of which 5 were isolated from *A. thaliana* roots (Bai *et al.* 2015). Genes encoding cobalt-zinc-cadmium (czc) resistance proteins were found in 2 strains (strains Root 1237 and Root 1444, Bai *et al.* 2015) and heavy metal transporters were reported in all 5 strains (strains Root 1237, Root 1444, Root 662, Root 1217 and Root 405, Bai *et al.* 2015). Although we were not able to test Cd tolerance for all our isolated *Pelomonas* strains, we can hypothesize that also some of them may contain resistance proteins since most of them were isolated from Cd-exposed plants. Consequently, some parts of the grey bars (Fig 4.4.1A, B and C), representing the non-determined strains, may possibly be tolerant to Cd and no clear conclusion is possible.
Also, but to a lesser extent than in roots and leaves of younger plants, some strains isolated from **leaves of older plants** and **stems** could not be re-grown on the 284 medium (Fig 4.4.1D and E). All bacterial strains isolated from **seeds** could grow on the control medium without Cd exposure (Fig 4.4.1F).

The effect of the lignin-reduced genotype

In order to investigate the effect of the plant genotype on the relative abundance of Cd tolerant strains, microbial communities of the different lignin-reduced genotypes were compared to the communities from WT plants in a non-exposed situation.

In **roots and leaves of younger plants** no clear plant genotype effects on the relative abundances of Cd tolerant strains in the communities were observed (Fig 4.4.1 A and C). Due to a large undetermined fraction in **roots of older plants** no conclusions can be drawn though the relative abundance of Cd tolerant endophytes appeared lower in the lignin-reduced mutants (Fig 4.4.1 B). In **stems and seeds** (Fig 4.4.1 E and F) from all selected lignin-reduced mutants, lower relative abundances of Cd tolerant bacteria were found in the communities in comparison to WT plants. In contrast, the relative abundances of Cd tolerant strains were higher in the **communities of leaves of older lignin-reduced mutant** in comparison to the WT community (Fig 4.4.1 D). Only for *ccr1-3* this higher relative abundance was not observed though a higher relative abundance of undetermined strains was found for this condition (Fig 4.4.1 D).

The effect of cadmium

In **roots of younger WT** plants exposed to Cd, a relative **increase** in the relative abundance of Cd tolerant strains was observed as compared to the non-exposed WT plants, although a high relative abundance of non-determined strains was present (Fig 4.4.1A). Increase of metal tolerance after metal exposure is a well-known phenomenon for soil bacteria (Diaz-Ravina and Baath 1996). Three mechanisms were proposed by Diaz-Ravina and Baath (1996) for the selection: (i) sensitive species cannot survive the toxic metal concentration, (ii) competitive abilities of the surviving bacteria will render a selection of tolerant species and (iii) adaptation of developing bacteria due to physiological and/or genetic changes. Moreover, increases of Cd tolerance in bacterial communities after Cd exposure of their host plants were also observed in *A. thaliana* seeds (Truyens *et al*. 2013, 2016a) and in *Brassica napus* plants grown on a contaminated field site (Croes *et al*. 2013).

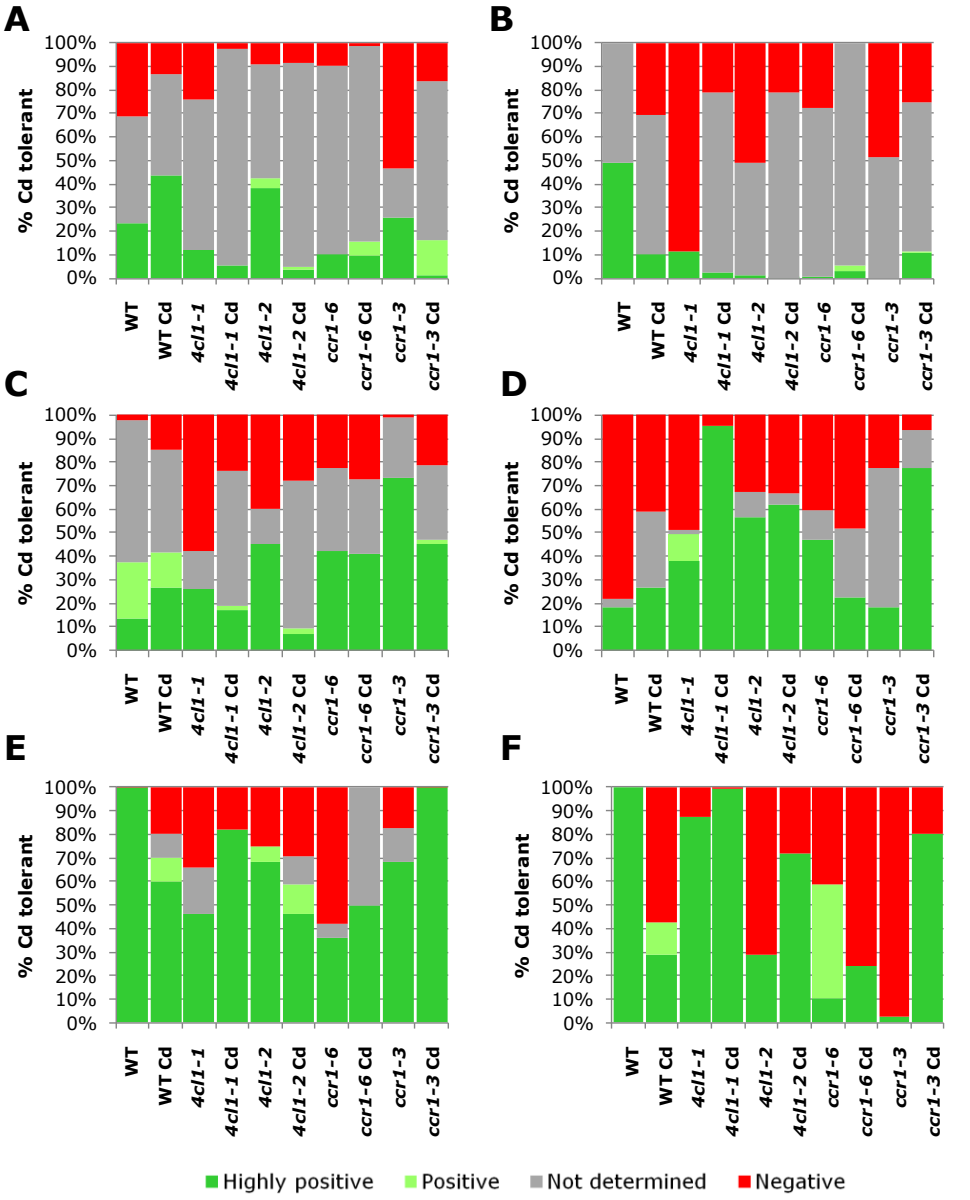


Figure 4.4.1: Relative abundances expressed in percentages of the community with **tolerance to 0.4 mM Cd** isolated out of WT, *4cl1-1, 4cl1-2, ccr1-6* and *ccr1-3* plants with and without exposure to 3µM $CdSO_4$ of (A) roots of younger plants, (B) roots of older plants, (C) leaves of younger plants, (D) leaves of older plants, (E) stems and (F) seeds.

In **stems and seeds** the relative abundance of Cd tolerant bacteria decreased in the **WT** plants community after Cd exposure as compared to the non-exposed WT plants (Fig 4.4.1E and F). These results are in accordance with the observations made in *Agrostis capillaris* seeds grown on Cd/Ni-contaminated lands though not the same genera were selected (Truyens *et al*. 2014). However, in the study of Truyens *et al*. (2016a) concerning *A. thaliana* seeds, the relative abundance of Cd tolerant bacteria was higher in seeds of Cd-exposed plants and became greater within subsequent generations grown on Cd-containing sand. Therefore, it can be hypothesized that more generations in presence of the selective pressure of toxic amounts of Cd may be necessary to enhance the selection for Cd tolerant strains in these communities.

In contrast to WT plants, **stem and seed** communities of **lignin-reduced mutants** rather increased in Cd tolerant strains after Cd exposure of the pants though this was not supported by all lignin-reduced genotypes hence **no definitive conclusion** can be drawn (Fig 4.4.1E and F). Further, the **roots of younger** lignin-reduced mutants did not show the same tendency in Cd-exposed conditions comparison to roots of younger WT plants for the presence of the Cd tolerance trait (Fig 4.4.1A). Moreover, for both **roots of younger and older plants** the high relative abundance of non-determined strains creates uncertainty about differences in response after Cd exposure between WT and lignin-reduced mutant communities concerning the presence of the Cd tolerance trait and **no further hypotheses** can be built (Fig 4.4.1B). Furthermore, **no definitive conclusions** could be made for the endophytic communities in **leaves of younger and older plants** since no clear effect of Cd exposure of the plants was observed on the relative abundance of Cd tolerant bacteria in the **WT** community and for **lignin-reduced mutants** communities**,** results were not similar for both allelic forms of *4cl1* and *ccr1* mutant leaves (Fig 4.4.1C and D).

4.4.2.2 1-aminocyclopropane-1-carboxylate deaminase (ACCd) activity

Ethylene is involved in plant stress responses (*e.g.* Cd toxicity) by for example inhibiting growth, and accelerating senescence. During developmental processes, ethylene is also involved in xylem formation (Glick *et al*. 2007). A possible strategy to promote growth of plants by bacterial endophytes is to

lower the production of stress ethylene by decreasing the levels of its immediate precursor ACC (Glick *et al*. 2007, Belimov *et al*. 2009). A decrease of ACC can be realized by ACC deaminase (ACCd) enzymes present in plant-associated microorganisms, which cleave ACC into ammonia and *α*-ketobutyrate (Sheng *et al*. 2008, Belimov *et al*. 2005). Moreover, the presence of higher levels of ACC resulting in enhanced ethylene biosynthesis can be correlated to a higher lignin content (Ievinsch *et al*. 1991). Therefore elevated levels of bacterial endophytes with ACCd capacity may indirectly affect lignification.

The effect of the lignin-reduced genotype

In comparison to the WT, the relative abundance of ACCd producing endophytes tended to be lower in the communities of both **roots of younger and older lignin-reduced mutants**, with exception for the *4cl1-2* mutant (Fig 4.4.2A and B). Also in **stems**, lower relative abundances of ACCd producing endophytes were observed for lignin-reduced mutants in comparison to WT plants (Fig 4.4.2E).

In both **leaves of younger and older plants,** higher relative abundances of ACCd producing bacteria were detected in the communities of lignin-reduced mutants, with exception for the *ccr1-3* community of leaves of older plants. However, the latter community contained the highest percentage of non-determined strains, which complicates the comparison (Fig 4.4.2C and D). Also in **seed** endophytic communities, higher relative abundances of ACCd producing strains were observed in the lignin-reduced genotypes though again a conclusion for *ccr1-3* is difficult due to the high relative abundance of undetermined strains (73.5%) for the ACCd trait (Fig 4.4.2F).

All plant tissues have the capacity to make ethylene though in most cases in very low amounts. The production of ethylene production can increase during developmental events (*e.g.* leaf and flower senescence and fruit ripening) and stress responses (Argueso *et al*. 2007, Rudus *et al*. 2013). Moreover, differences in expression of genes involved in ethylene production are tissue specific (Rudus *et al*. 2013). A continuous complex interaction exists between plants and microorganisms. Bacteria may affect the ethylene responses by lowering the perceived stress (Glick *et al*. 2007). Because of the interaction between bacteria

and plants, various causes and effects are hard to disentangle. Limited information is available about the ethylene household, and thus the role of ethylene in lignin-reduced mutants. Therefore, it is even harder to understand differences in selection for ACCd traits for the endophytic communities in the various mutant tissues. In literature, a link between high ethylene levels concurrent with ectopic deposition of lignin is described (Moura *et al*. 2010, Zhong *et al*. 2002) indicating that lignin deposition can be ethylene responsive. Moreover, some ethylene response elements are strongly regulated genes during secondary growth (Dharmawardhana *et al*. 2010). Though these effects are not studied for the *4cl1* or *ccr1* mutants, leaves of lignin-reduced mutants of *Brachypodium distachyon* with lower PHENYLALANINE AMMONIA LYASE activity showed up-regulated ethylene biosynthesis and signalling (Cass *et al*. 2015). Therefore, we can be hypothesize that in the leaves of *4cl1* and *ccr1 A. thaliana* mutants, the ethylene concentrations also changed. All together, **no definitive hypothesis** can be formulated based on the available information to explain the differences in responses in ACCd traits of the communities of roots and stems versus leaves and seeds in *A. thaliana* lignin-reduced mutants.

The effect of cadmium

Increases in ethylene synthesis under environmentally realistic Cd concentrations was reported by Schellingen *et al*. (2014). Furthermore, Keunen *et al*. (2016b) reviewed different aspects of ethylene synthesis and signalling under metal stress. These Cd-induced increases in ethylene levels, which negatively affect plant growth, can be (at least partly) diminished by the presence of bacteria containing ACCd enzymes (Glick *et al*. 2007).

In **roots of younger plants** and **leaves of younger and older plants,** no clear tendencies concerning ACCd were observed after Cd exposure of the plants on the endophytic communities of both WT and lignin-reduced mutants (Fig 4.4.2A,C and D).

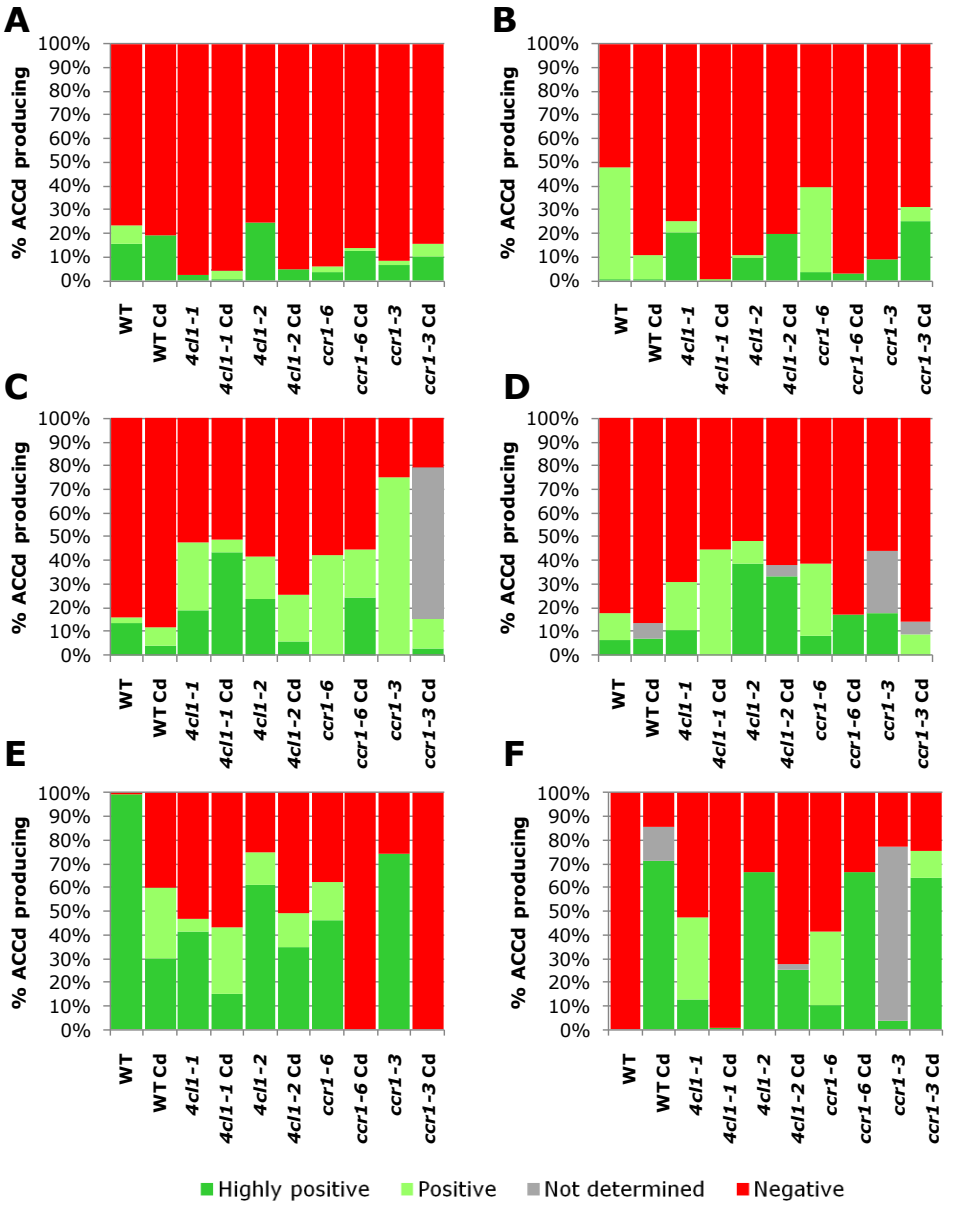


Figure 4.4.2: Relative abundances expressed in percentages of the community with **ACC deaminase capacity** isolated out of WT, *4cl1-1, 4cl1-2, ccr1-6* and *ccr1-3* plants with and without exposure to 3µM $CdSO_4$ of (A) roots of younger plants, (B) roots of older plants, (C) leaves of younger plants, (D) leaves of older plants, (E) stems and (F) seeds.

Proportionally less ACCd bacteria were found in **roots of older plants** of the Cd-exposed plants in comparison to non-exposed **WT** plants and no conclusion for **lignin-reduced mutants** can be drawn. (Fig 4.4.2B). In **stems**, almost **all** examined plant **genotypes,** including WT, displayed lower relative abundances of ACCd producing endophytes in their communities after Cd exposure (Fig 4.4.2E). In ***ccr1* mutant stem** communities, **the ACCd trait** was even **not detected** after Cd-exposure (Fig 4.4.2E).
The obtained result for older roots and stems was unexpected since stressful plant growth conditions are thought to increase ethylene levels resulting in higher relative abundances of ACCd producing bacteria as reported by Timmusk *et al*. (2011) for rhizosphere soil bacteria. Such an increase of ACCd producing strains due to exposure to toxic metal concentrations was also observed in bulk soil and rhizosphere soil on a metal contaminated site (Croes *et al*. 2013). However, endophytes are different from rhizosphere bacteria and ongoing physiological plant processes and plant niches are not comparable.

The **seed** endophytic communities of **WT plants** and ***ccr1* mutants** relatively increased in ACCd producing bacteria as compared to the non-exposed plants although the big "undetermined" fraction for the ACCd trait in the non-exposed *ccr1-3* mutants, make results for *ccr1-3* mutants inconclusive (Fig 4.4.2F). A strong increase in the relative abundance of endophytes with ACCd capacity in a WT *A. thaliana* seeds community was already reported for *A. thaliana* plants grown for several generations on Cd contaminated sand (Truyens *et al*. 2013 and 2016a). However, both allelic ***4cl1* mutants communities** of seeds revealed decreases in relative abundance of bacteria with ACCd capacity as compared to the non-exposed plants (Fig 4.4.2F). This probably could be attributed to a different selective Cd pressure present shaping these *4cl1* seed communities.

4.4.2.3 Indole-acetic acid (IAA) production

In addition to plants themselves, also bacterial endophytes can be able to synthesize the phytohormone IAA and may interfere with many plant processes (*e.g.* cell enlargement and division, tissue differentiation, and responses to light and gravity) (Spaepen *et al*. 2007, Gagne-Bourgue *et al*. 2012). Moreover,

increased root formation due to plant-associated bacteria with IAA biosynthetic capacity was demonstrated by Spaepen *et al*. (2007).

The effect of the lignin-reduced genotype

No general conclusions can be made for **roots of younger and older plants and leaves of older plants** since the allelic variants show differences in their relative abundance of IAA producing endophytes in the communities (Fig 4.4.3A, B and D).

The IAA producing capacity tended to be lower in the communities of *ccr1* mutant **leaves of younger plants** (Fig 4.4.3C). In plants, IAA is abundant in leaves of younger plants (Ongaro and Leyser 2008) and the regulation of leaf development is IAA dependant (Qin *et al*. 2005). However, the developmental rate of the *ccr1* mutants is not identical to WT plants (Van Acker *et al*. 2013), which might introduce a bias in the obtained results.

Also in **stem** development, IAA is a determining factor. For instance, branching of stems is regulated by auxins (Yruela 2015). Almost no IAA producing bacteria were present in **WT** stems (0.3%) and a higher relative abundance of the communities of **lignin-reduced mutants** contained the IAA production trait (Fig 4.4.3E). Interestingly, transcripts of auxin/indole-3-acetic acid and auxin response factor genes are less abundant in *4cl1* and *ccr1* stems (Vanholme *et al*. 2012a) indicating that the genetic modification of the *4CL1* and *CCR1* genes can affect the response to IAA. However, more information is necessary to understand the mechanism of the subsequent plant-bacterial interaction concerning IAA.

In contrast to the stems, all WT **seed** isolates (100%) could produce IAA *in vitro* (Fig 4.4.3F). The presence of IAA producing strains in the seed may enhance root growth after germination (Truyens *et al*. 2014). However, in *4cl1* mutant communities the relative abundance of the IAA production trait was lower (Fig 4.4.3F); and in *ccr1* mutant communities it was even lower (Fig 4.4.3F). Hence, our selected lignin-reduced mutants may not benefit as much as WT plants from these seed-borne endophytic traits.

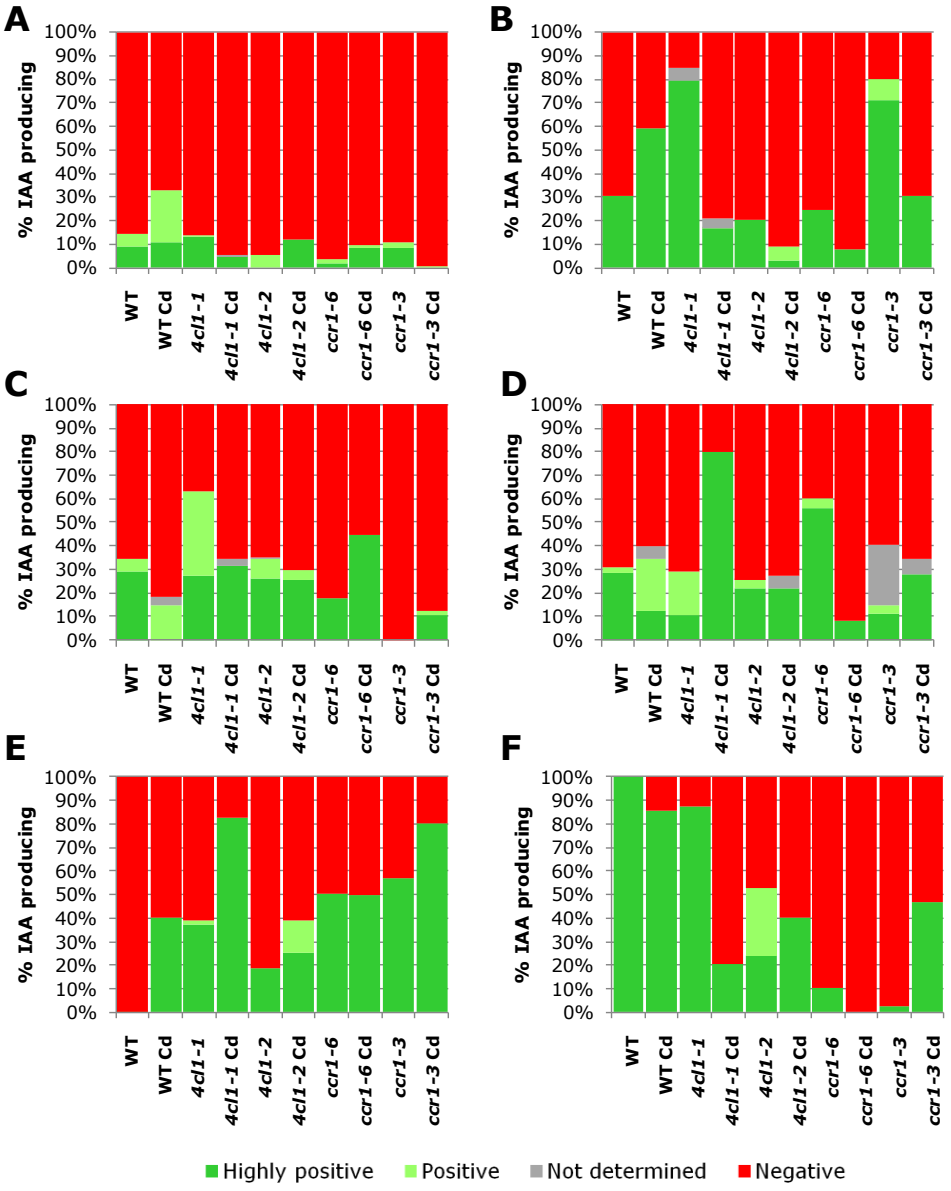


Figure 4.4.3: Relative abundances expressed in percentages of the community with **IAA producing capacity** isolated out of WT, *4cl1-1, 4cl1-2, ccr1-6* and *ccr1-3* plants with and without exposure to 3µM $CdSO_4$ of (A) roots of younger plants, (B) roots of older plants, (C) leaves of younger plants, (D) leaves of older plants, (E) stems and (F) seeds.

The effect of cadmium

Increases of IAA production in bacteria can be dependent on environmental cues (Spaepen *et al*. 2007) and increased root growth can be an advantage in the uptake of metals (*e.g.* Cd) which is an advantage in the clean-up of lands by making use of phytoremediation (Sessitsch *et al*. 2013).

No unequivocal patterns were observed for **roots of younger plants and leaves of older plants** (Fig 4.4.3A and D) while the relative abundance of endophytes capable of IAA production tended to be higher in the **WT roots of older plants** community as compared to the non-exposed WT plants and in the **lignin-reduced mutants** the relative abundance was lower after Cd exposure (Fig 4.4.3B). The relative abundances of endophytes with IAA producing capacity also tended to be higher after Cd exposure in **WT** and all **lignin-reduced mutant stem** communities (not true for *ccr1-6*) (Fig 4.4.3E). A high IAA producing relative abundance of endophytes was also detected in the communities of stems of yellow lupine grown on metal-contaminated soil (Weyens *et al*. 2013). This might suggest that containing a high relative abundance of IAA producing bacteria in the stem community may be beneficial in metal-exposed conditions.

The **Seed** endophyte communities contained lower relative abundances of IAA producing strains after Cd exposure of the plants (though not for *ccr1-3* mutants) (Fig 4.4.3F). Since IAA production was less detected in seeds of Cd-exposed plants, as also observed by Truyens *et al*. (2013) for WT seed communities, it may be expected that these traits are not important for seeds which develop in the presence of Cd.

4.4.2.4 Siderophore (SID) production

Endophytic bacteria producing SIDs may play a role in plant growth especially on contaminated land. These SIDs are of great importance in mobilising ferric iron (Fe^{3+}) for plants that encounter both Fe-deficiency and/or metal contamination (Zloch *et al*. 2016). Furthermore, they can chelate other metals (*e.g.* Cd), facilitating the uptake of such metals by plants (Dimpka *et al*. 2009) and in this way improving phytoextraction processes (Zloch *et al*. 2016).

The effect of the lignin-reduced genotype

No clear unambiguous differences in relative abundance of SID producing strains were observed between communities of **roots of younger and older WT plants and leaves of older WT plants** and **lignin-reduced mutants** (Fig 4.4.4A, B and D).

Relatively low numbers of SID producing endophytes were detected in the communities of **leaves of younger WT plants**. Higher relative abundances of SID producing bacteria, in comparison to WT communities, were noticed in all leaves of younger **lignin-reduced mutants** (Fig 4.4.4C).

WT stems displayed a community in which 99.5% was capable of producing SIDs *in vitro* (Fig 4.4.4E). The relative abundance of SID producing bacteria tended to be lower in **lignin-reduced mutant** communities of **stems** in comparison to WT plants (Fig 4.4.4E). In *Arabidopsis* stems it was demonstrated by Zhao *et al*. (2016) that the colonisation of for instance a *Burkholderia phytofirmans* strain from the rhizosphere to the root and shoot facilitated Fe uptake and translocation into the inflorescence stem. Suggesting a possible role of SID producing stem endophytic bacteria in the translocation of Fe into the stem. It can be hypothesized that the lignin-reduced mutants contain other translocation mechanisms for metals (*e.g.* chelating structures such as organic acids) whereby SID producing bacteria may not gain a similar competitive advantage in comparison to the endophytes in WT stem communities.

Also **roots of older** lignin-reduced mutants included a lower relative abundance of SID producing bacteria in comparison to WT plants (Fig 4.4.4B).

In **seeds**, the main iron storage takes place in the vacuole (Nouet *et al*. 2011, Kim *et al.* 2006) and this Fe storage is important for the germination of seedlings (Mary *et al*. 2015). As observed in stems, the **WT plants** community was composed of 100% bacteria capable of SID production (Fig 4.4.4E), suggesting an important role in helping seedlings after germination with their Fe balance. Seeds of **lignin-reduced mutants** (with exception of *4cl1-1*) displayed lower relative abundances of SID producing bacteria (Fig 4.4.4E) suggesting that they selected differently for the SID trait in their communities.

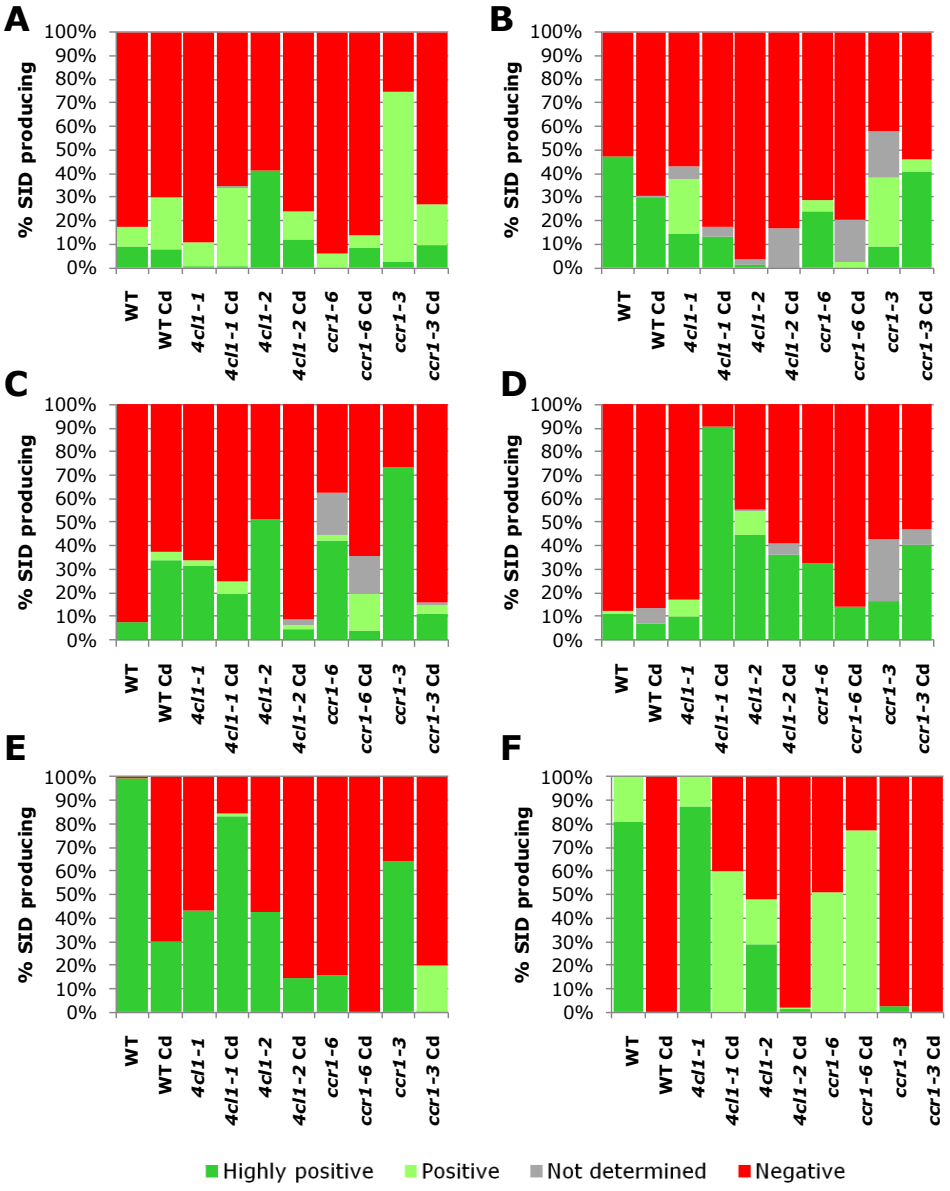


Figure 4.4.4: Relative abundances expressed in percentages of the community with **SID producing capacity** isolated out of WT, *4cl1-1, 4cl1-2, ccr1-6* and *ccr1-3* plants with and without exposure to 3µM $CdSO_4$ of (A) roots of younger plants, (B) roots of older plants, (C) leaves of younger plants, (D) leaves of older plants, (E) stems and (F) seeds.

The effect of cadmium

SID production can be stimulated in the presence of toxic amounts of metals (*e.g.* Cd) (Van der Lelie *et al*. 2000). Such a Cd-induced increase in SID producing bacteria was observed in communities of **roots and leaves of younger WT plants** (Fig 4.4.4A and C). However, the effect of Cd on the SID producing proportion in **roots of younger lignin-reduced mutants** was inconclusive since the endophytic communities of both allelic variants responded differently to Cd exposure of the plants (Fig 4.4.4A). In contrast to WT plants, SID producing relative abundances decreased in communities of **leaves of younger lignin-reduced mutants** after Cd exposure of the plants (Fig 4.4.4C).

Communities of **roots of older plants** were affected by Cd in a decreasing way concerning the SID trait in WT plants (Fig 4.4.4B). Also relatively less endophytes with SID production capacity were observed after Cd exposure of plants in the communities of **seeds and stems** of **WT plants** (Fig 4.4.4E and F). A decrease in SID producing bacteria in seeds of Cd-exposed plants was also observed by Truyens *et al*. (2013). For roots and leaves of older plants, stems and seeds no clear pattern was present and **no conclusions** can be drawn for the **lignin-reduced mutants**.

All together, it seems that, at least in our study, the SID production trait is of **limited importance**.

4.4.2.5 Phosphate (P) solubilisation

Phosphorous (P) is an important growth-limiting nutrient which is present in high amounts in soils though in insoluble non-bioavailable forms (Ullah *et al*. 2015). Unavailability of P is recognized to be a major growth-limiting factor for plants growing on many agricultural lands (Oteino *et al*. 2015). Microorganisms capable of converting the insoluble phosphates into accessible forms for plants can enhance plant growth (Oteino *et al.* 2015, Yazdani *et al*. 2009). The isolated bacterial strains were screened in the NBRIP broth assay. However, the used technique can only identify the most efficient phosphate solubilisers and other isolates may nevertheless be able to solubilise P to a lesser extent (Nautiyal *et al*. 1999). Therefore, we will only briefly discuss the results concerning P solubilisation.

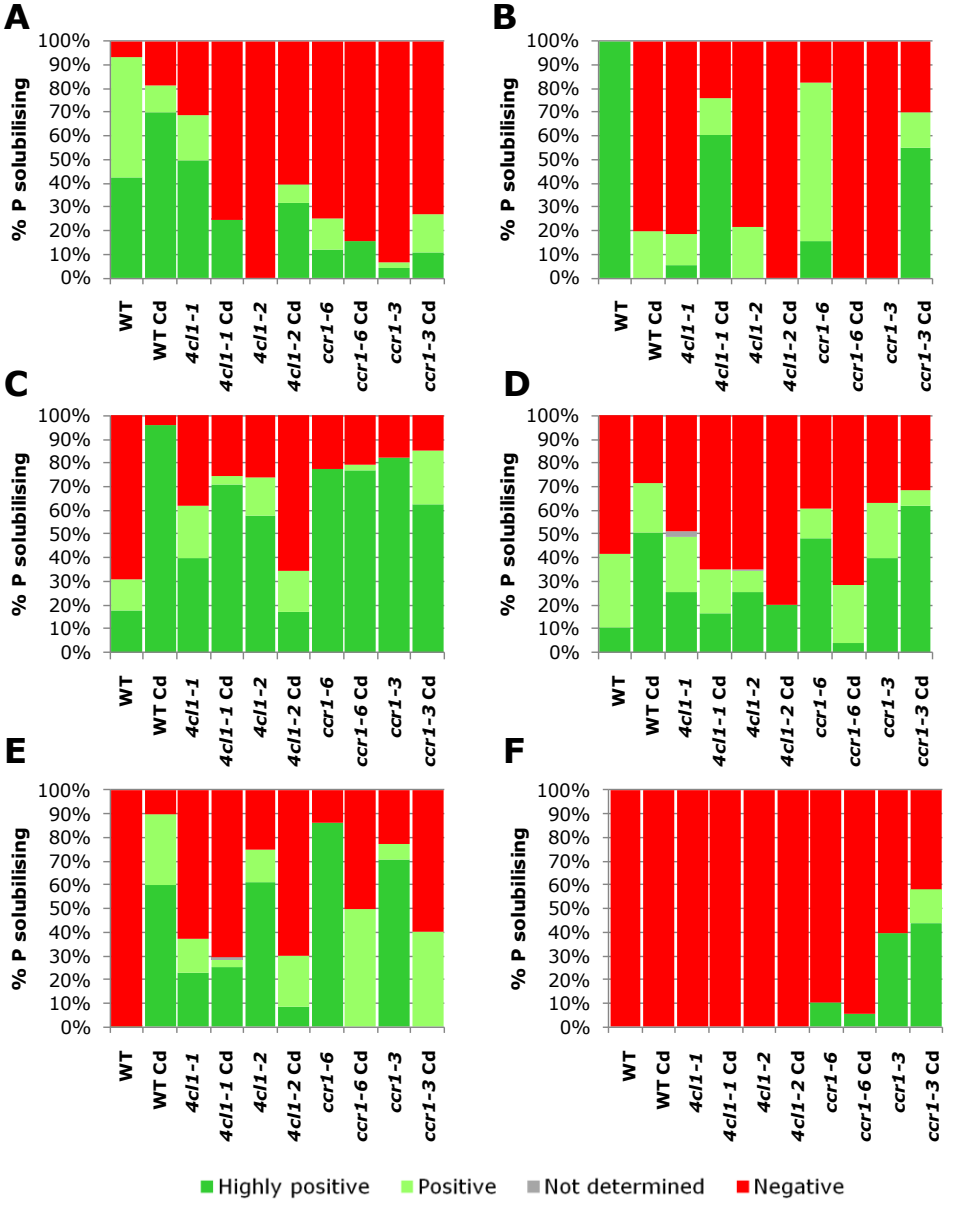


Figure 4.4.5: Relative abundances expressed in percentages of the community with **P solubilisation capacity** isolated out of WT, *4cl1-1, 4cl1-2, ccr1-6* and *ccr1-3* plants with and without exposure to 3µM $CdSO_4$ of (A) roots of younger plants, (B) roots of older plants, (C) leaves of younger plants, (D) leaves of older plants, (E) stems and (F) seeds.

The effect of the lignin-reduced genotype

Many reports are available about soil, rhizosphere or root endophytes with phosphate solubilisation capacity (Jilani *et al*. 2007, Yazdani *et al*. 2009, Oteino *et al*. 2013, Gull *et al*. 2004, Son *et al*. 2004). In the below-ground tissues (**roots of younger and older plants**), relative abundances of endophytes with P solubilisation capacity were lower in all selected **lignin-reduced mutants** when compared to WT plants (Fig 4.4.5A and B).

Endophytes with P solubilisation capacity can also be present in the leaf and stem compartment (Oteino *et al*. 2015, Ma *et al*. 2015). The P solubilisation capacity showed also some changes (higher relative abundances) in the **leaf, stem and seed** communities of lignin-reduced mutants (Fig 4.4.5C, D, E and F). Though their mechanism of action in these tissues is unknown.

The effect of cadmium

Many studies report the plant growth promoting capacity that soil and rhizosphere bacteria and root endophytes have through their P solubilising capacity in metal-contaminated soils (Yuan *et al*. 2014a, Babu *et al*. 2013, Jiang *et al*. 2008, Zhang *et al*. 2011, He *et al*. 2013, Mehta *et al*. 2014, Ullah *et al*. 2015, Gupta *et al*. 2002).

In **below-ground tissues (roots of younger and older plants)** the P solubilisation capacity decreased in **WT plants** when plants were exposed to Cd (Fig 4.4.5A and B). In the study of Croes *et al*. (2013), no effects on the root community of *Brassica napus* grown in metal-contaminated lands were observed. However, no conclusions can be drawn about the **lignin-reduced mutant** root communities since the allelic mutants for the same gene did not reveal the same Cd effects for the P solubilisation trait (Fig 4.4.5A and B).

4.4.2.6 Nitrogen (N) fixation capacity

Nitrogen is essential in plant development and a limiting factor in plant growth. Plants cannot use the atmospheric N_2 and can only take up N via their roots out of the soil in the form of ammonium or nitrates (Santi *et al*. 2013). Diazotrophic bacteria are able to fix the atmospheric nitrogen by nitrogenase activity, making

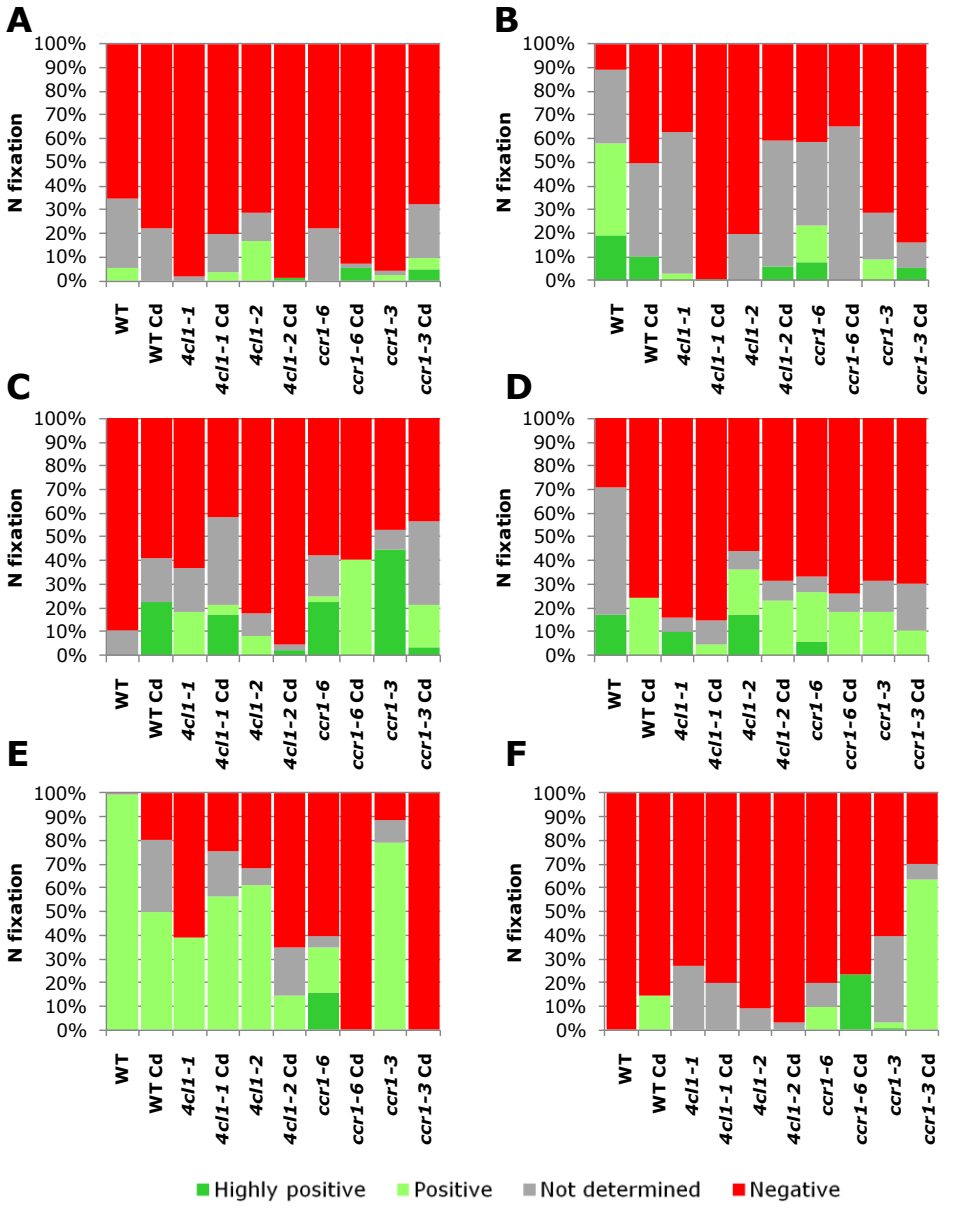


Figure 4.4.6: Relative abundances expressed in percentages of the community with **N fixation capacity** isolated out of WT, *4cl1-1, 4cl1-2, ccr1-6* and *ccr1-3* plants with and without exposure to 3µM $CdSO_4$ of (A) roots of younger plants, (B) roots of older plants, (C) leaves of younger plants, (D) leaves of older plants, (E) stems and (F) seeds.

it bioavailable for the plant, even in metal-contaminated conditions (Ullah *et al*. 2015).

The effect of the lignin-reduced genotype

In our study, low relative abundances of clearly positive N fixers (darker green in Fig 4.4.6) across all conditions were observed. Moreover, the relative abundance of endophytes with N fixation capacity was unaffected due to the lignin-reduced genotype in **roots of younger plants, leaves of older plants** and **seed** communities (Fig 4.4.6A,D and F). While lower relative abundances of endophytes with N fixation capacity were observed for all examined lignin-reduced mutant communities of **roots of older plants** and **stems** in comparison to the WT communities (Fig 4.4.6B and E).

The *in vitro* N fixing capacity was relatively higher in all lignin-reduced mutant **leaves of younger plants** communities in comparison to the WT community, where no *in vitro* N fixing bacteria were observed (Fig 4.4.6C). It is established that leaf endophytic bacteria with N fixing capacity are able to enhance plant growth and can promote seed production in *Jatropha curcas L.* (Madhaiyan *et al*. 2015). Hence the high relative abundance N fixating bacteria may be an advantage for the lignin-reduced mutants.

The effect of cadmium

No clear Cd effect was noticed in **roots and leaves of younger and older plants, stems and seeds** (Fig 4.4.7A,B, C,D, E and F). However, some bacteria were not capable to grow in the selected media and could not be characterised. This may impair the observed tendencies.

4.4.2.7 Organic acid (OA) production

Organic acid production and subsequent mobilisation of nutrients is a direct plant growth promotion mechanism (Truyens *et al*. 2015). Moreover, OAs can enhance metal (bio)availability and uptake, especially near the roots (Weyens *et al*. 2013) which might be favourable during phytoremediation (Ullah *et al*. 2015).

The effect of the lignin-reduced genotype

In general, the relative abundance of **roots, leaves and stem** endophytes with OA producing capacity was low (Fig 4.4.7A, B, C, D and E). No general effect related to the **lignin-reduced genotype** was detected in roots of younger and older lignin-reduced mutants in comparison to WT plants (Fig 4.4.7A, B).

Seed communities tended to have higher relative abundances of endophytes with OA producing capacity due to the **lignin-reduced genotype** (Fig 4.4.7F). The seed endophytes can migrate from the seed to the rhizoplane and the root where they may perform their growth promoting action (Puente *et al*. 2009). Hence, the higher relative abundance of OA producing endophytes in seeds may be an advantage after germination.

The effect of cadmium

Effects of Cd were rather limited and results for the allelic variants of the selected mutants were inconclusive in **root communities of younger and older plants** (Fig 4.4.7A and B). Hence no definitive conclusions can be drawn.

For **seeds** no *in vitro* OA producing bacteria were isolated for WT non-exposed plants communities and only low relative abundances (14.3%) for Cd-exposed ones (Fig 4.4.7F). These results are in agreement with the results of Truyens *et al*. (2013). Cd did have a different effect on ***ccr1*** **mutant's** endophytic communities in comparison to the ones from **seeds and stems** of WT plants or *4cl1* mutants. In *ccr1* the relative abundances for the OA production capacity tended to decrease in stems and seeds communities while no effects were observed in stems and slight increases of the relative abundances of endohytes with the OA production trait were observed in seeds for the WT plants and *4cl1* mutants (Fig 4.4.7E and F). Indicating that Cd exposure does not play a major role in the plants selection of the OA production in the endophytic community of *ccr1* seeds of plants grown in presence of toxic Cd concentrations.

Altogether, since no major increases in the relative abundance of OA producing bacteria after Cd exposure of WT and lignin-reduced plants were observed, we can speculate that the OA production trait is less important in all tested endophytic communities when plants are exposed to Cd.

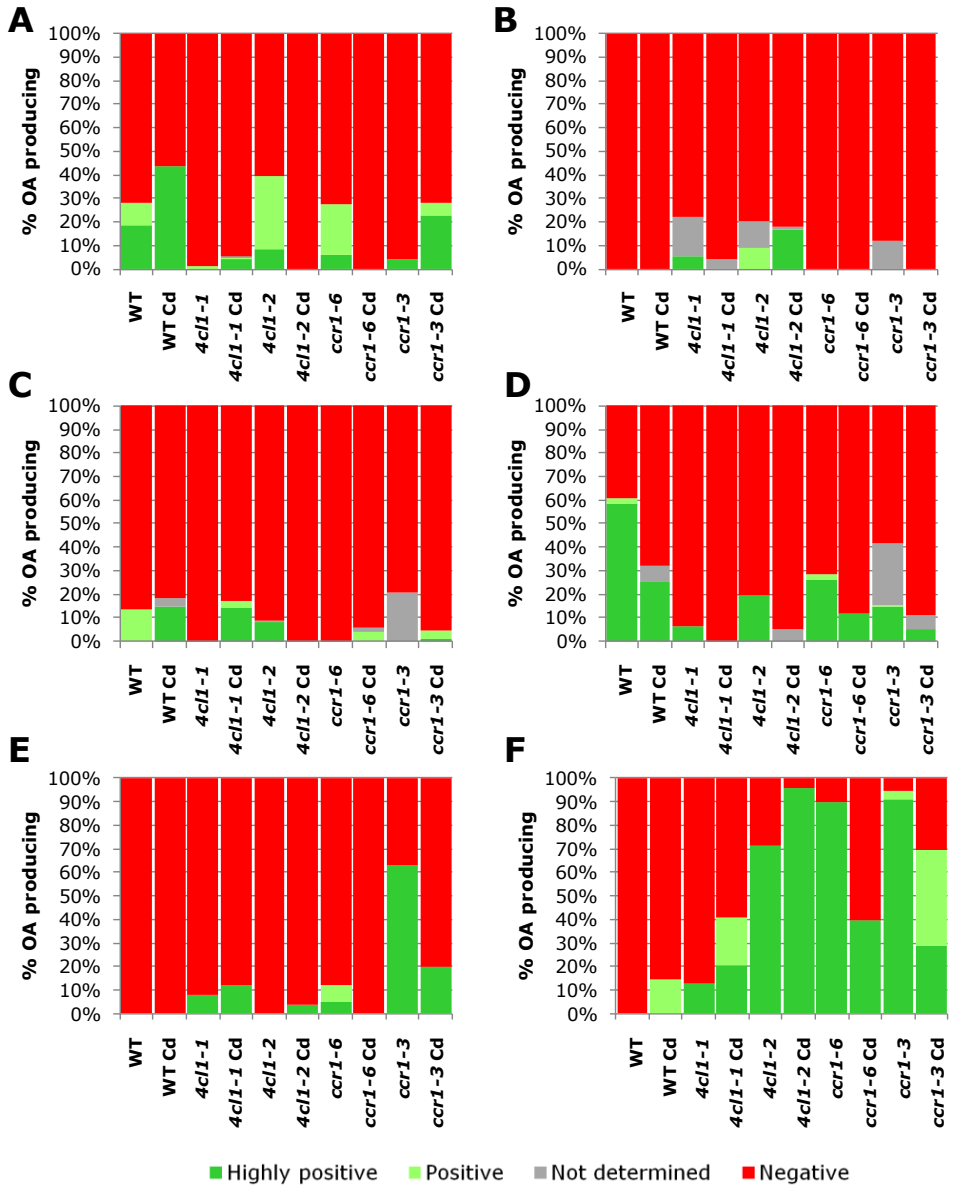


Figure 4.4.7: Relative abundances expressed in percentages of the community with **OA producing capacity** isolated out of WT, *4cl1-1, 4cl1-2, ccr1-6* and *ccr1-3* plants with and without exposure to 3µM $CdSO_4$ of (A) roots of younger plants, (B) roots of older plants, (C) leaves of younger plants, (D) leaves of older plants, (E) stems and (F) seeds.

4.4.2.8 Use of ferulic and *p*-coumaric acid as C-source

The mutations in *4CL1* and *CCR1* result in the accumulation of some phenolic intermediates of the monolignol production pathway and derivatives thereof in the xylem (*e.g.* ferulic acid or *p*-coumaric acid in *A. thaliana* mutants) (Vanholme *et al*. 2012a). Xue *et al*. (2015) indicated that the decreased growth of *ccr1 A. thaliana* mutant leaves is not caused by the lower lignin content but is a result of the high levels of ferulic acid. Furthermore, Beckers *et al*. (2016b) demonstrated a higher ferulic acid degradation capacity in bacterial endophytes isolated from *CCR* down-regulated poplar as compared to WT poplar trees. Suggesting that the different available phenolic C-sources in these *CCR* mutants affected the endophytic communities for this phenotypic trait. Furthermore, in case seedlings of *A. thaliana* are exposed to toxic amounts of Cd, phenolic acids concentrations (*e.g.* sinapinic acid) can also increase (Sun *et al*. 2010).
Additional degradation of ferulic acid by endophytic bacteria may be an advantage in lowering the stress that this molecule is causing in the *ccr1* mutants. In this regard, we selected the most promising endophytic bacteria based on all other *in vitro* growth promoting capacities and analysed their capacity for using ferulic acid and/or *p*-coumaric acid as sole C-source. **Because based on the presence of isolated genera the communities of seeds and stems were most affected, these communities were fully screened for the capacity to use these phenolic C-sources.**

The effect of the lignin-reduced genotype

Relatively low relative abundances of bacteria that tested clearly positive (dark green bars in Fig 4.4.8) for the use of ferulic or *p*-coumaric acid were observed in the **seed** endophytic communities. Most phenolic acid using bacteria were even observed in WT seeds. However, since no data are available concerning the presence of these substances in seeds, no clear conclusion can be drawn.

In **stems**, relatively more bacteria were able to use ferulic or *p*-coumaric acid as C-source in comparison to the endophytic communities of seeds. Though, most phenolic acid using bacteria were detected in the WT community and only low relative abundances were found in lignin-reduced mutants. However, the large relative abundance of bacteria identified as "not determined" during the Biolog test make it impossible to draw strong conclusions.

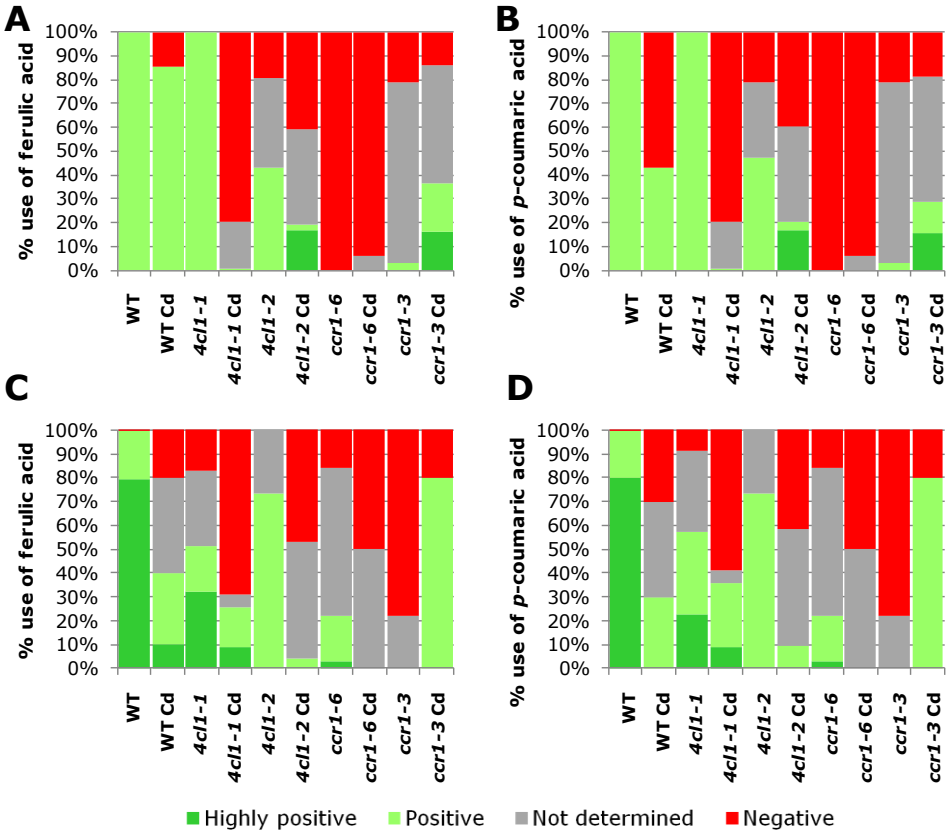


Figure 4.4.8: Relative abundances expressed in percentages of the community with **capacity to use ferulic acid** (A,C) or ***p*-coumaric acid** (B,D) **as sole C-source** isolated out of WT, *4cl1-1, 4cl1-2, ccr1-6* and *ccr1-3* plants with and without exposure to 3µM $CdSO_4$ of (A,B) seeds and (C,D) stems.

The effect of cadmium

The relative abundance of endophytes able to use ferulic or *p*-coumaric acid as a sole C-source tended to decrease in both **seeds** and **stems** in case plants were exposed to Cd with exception of *ccr1-3* for both seeds and stems where it increased (Fig 4.4.8). However, many bacteria were not determined during the Biolog test and conclusions are difficult to make based in these results.

The observations in seeds and stems of both non-exposed and Cd-exposed WT plants in comparison to lignin-reduced mutants were unexpected. We expected the bacterial endophytic communities of the lignin-reduced mutants to be more efficient in using the selected phenolic compounds, because ferulic acid and *p*-

coumaric acid accumulate in the xylem of these plant stems (Vanholme *et al*. 2012a) and communities may encounter a selective pressure (Beckers *et al*. 2016b). However, in our study of seed and stem endophytic communities, many bacteria were unable to grow in the assay as observed on the generally accepted C-sources (C-mix) which makes it difficult to interpret these results. Hence no general conclusion can be drawn.

4.4.3 Conclusions

We investigated the effects of both the genetic modification of the lignin biosynthesis as well as Cd exposure on *in vitro* growth promoting characteristics, Cd tolerance and the use of phenolic acids as a sole C source of the cultivable endophytic bacteria.

Most of the data suggest that the **endophytic communities of the lignin-reduced mutants select differently** for the studied plant growth promoting traits. In **non-exposed plants** the selection was the clearest and all lignin-reduced mutants selected equally for many of the investigated traits in all examined plant compartments. For instance, lower relative abundances of endophytes with Cd tolerance, ACC deaminase, SID production, P solubilisation and N fixation capacity were found in the endophytic communities of **roots** of younger or older lignin-reduced mutants. And in the **aerial parts** some plant growth promoting traits were found in higher relative abundances in the communities of the lignin-reduced mutants (*e.g.* endophytes possessing Cd tolerance in leaves of older plants, SID production and N fixation in leaves of younger plants, ACC deaminase production in leaves of younger plants and seeds, IAA production in stems and OA production in seeds) while some traits were lower in relative abundance in the communities of the lignin-reduced mutants (*e.g.* endophytes possessing Cd tolerance and SID production in stems and seeds, ACC deaminase and N fixation in stems and IAA production in seeds). However the selection of endophytes in these mutants were also affected by plant compartment and developmental stage revealing again the complexity of these plant-endophyte interactions.

Though, **after Cd-exposure** also examples of selection in the same manner as by the lignin-reduced mutants were observed. For instance, decreases in relative

abundances of SID and IAA producing bacteria in respectively leaves of younger and roots of older lignin-reduced mutants were observed while increases were found in WT plants. Lower relative abundances of OA producing endophytes in the *ccr1* communities of seeds after Cd exposure were observed while in WT plants and *4cl1* mutants displayed increases in the relative abundances of OA producing endophytes after Cd exposure. Furthermore increases in the relative abundance of IAA producing bacteria in *ccr1* mutants younger leaves communities were observed while decreases were obtained in other plant genotypes. However, results for most of the *in vitro* tested phenotypic traits were inconclusive and by consequence **changes in phenotypic traits resulting from Cd exposure seemed of minor importance in our study**.

To fully understand all ongoing processes in the plants selection of the most appropriate endophytic community on the right location, these interactions should be studied in more detail for both WT and lignin-reduced mutants. This may be examined in the total community wherein the cultivability of the endophytes does not produce a bias. However, out of these isolated and characterised strains the most promising bacterial strains can be selected, which is the main goal of our study, to perform an *in vivo* growth promotion assay to try to restore the growth which is negatively inhibited by both the genetic dification (resulting in a lignin reduction) and Cd-exposure.

Subsection 4.5
Counteracting negative growth effects due to both lignin reduction and cadmium exposure using plant growth promoting endophytes

The growth of *CCR1* down-regulated plants is decreased (Dauwe *et al*. 2007, Van Acker *et al*. 2013, Van Acker *et al*. 2014, Mir Derikvand *et al*. 2008, Leplé *et al*. 2007) and it is presumed that this is due to accumulating ferulic acid levels inside these lignin-reduced plants (Xue *et al*. 2015). Here, we will use *A. thaliana* as a model organism for woody plants (Nieminen *et al*. 2004) and aim to limit the effect of the genetic modification and **optimise the growth** of these lignin-reduced plants.

Microorganisms living in close contact with plants are of great importance for the plants health (Turner *et al.* 2013, Bulgarelli *et al.* 2013). A large range of direct and indirect plant growth promoting mechanisms of endophytes are well established (see section 1). Several of these direct mechanisms are related to (i) fixation and mobilisation of unavailable nutrients (organic acid (OA) production, siderophore (SID) production, phosphate (P) solubilisation and nitrogen (N) fixation), (ii) the production of plant hormones (indole-3-acetic acid (IAA) production) and (iii) the suppression of the stress hormone ethylene (1-aminocylcopropane-1-carboxylate (ACC) deaminase capacity). Moreover, also bacterial **volatiles** may play a major role in these plant-bacterial interactions (Blom *et al*. 2011). In subsection 4.4, we examined a large range of endophytic strains, isolated from various plant compartments of *A. thaliana* (WT and lignin-reduced mutants), on their capacity to produce several of the afore mentioned compounds.

We hypothesize that inoculation of the *ccr1* mutants with promising bacterial strains might improve, at least partly, restore the growth-impairment associated with down-regulation of *CCR*. It is known that in lignin-reduced mutants the accumulation of high levels of phenolic acids (*e.g.* ferulic acid) (Vanholme *et al.* 2012a) delay the growth of the plants as demonstrated for *A. thaliana* leaves by Xue *et al*. (2015). Therefore, also the use of phenolic compounds (ferulic acid and *p*-coumaric acid) as a sole C-source by the isolated strains was examined

(subsection 4.4). It can be hypothesized that enrichment of the community with the phenolic acid using endophytes may reduce levels of the phenolic compounds resulting in an improved plant growth (Beckers *et al*. 2016b).

Bacterial endophytes may also serve an important role in mobilising Cd-ions, hence increasing Cd uptake, and lowering the toxicity of Cd in Cd-exposed plants (Li *et al*. 2012). In this way, we also hypothesize that enrichment of the plants endophytic community with promising strains, displaying Cd tolerance (subsection 4.4), may lower Cd toxicity for the plant and by consequence increase plant growth in Cd-exposed conditions.

The used *in vitro* plant growth promoting assays (subsection 4.4) may give a good indication concerning the plant growth promotion potential of the selected bacterial strains. However, these bacterial assays may also fall short in predicting *in planta* growth promoting effects (Truyens *et al*. 2013, Weyens *et al*. 2013). Therefore, *in vitro* bacterial assays should be complemented with *in planta* examination. In subsection 4.5 we explore the capacity of several bacterial strains (selected based on their *in vitro* growth promoting capacities and metabolic traits to degrade phenolic acids) to improve growth of *A. thaliana* WT plants and *ccr1* mutants. This was either examined by a vertical agar plate system, to investigate the direct interaction of the microorganisms with the plant (Remans *et al.* 2012), and a co-cultivation assay (Ryu *et al.* 2003) to investigate the growth promotion induced by volatile organic signal molecules. The final aim is that after inoculation, both the negative effects of the lignin-reduced genotype as well as those induced by exposure to Cd will be, at least partly, alleviated.

4.5.1 Experimental design

Endophytic strains that were previously isolated from roots of younger and older plants, leaves of younger and older plants, stems and seeds of *A. thaliana* WT plants, *4cl1-1*, *4cl1-2*, *ccr1-6* and *ccr1-3* mutants, with or without Cd exposure were utilised for the inoculation experiments (see subsection 4.3). The isolated endophytic strains were examined *in vitro* for several plant growth promotion traits and Cd tolerance (subsection 4.4). From stems and seeds, all endophytes

were also examined for their ability to grow on ferulic or *p*-coumaric acid as a sole C-source. For the roots and leaves of younger and older plants, first a pre-selection was implemented based on their ability to grow in presence of Cd and *in vitro* plant growth promoting capacities. Only the most promising bacterial strains were included in the Biolog assay to evaluate the growth in presence of a sole phenolic acid (ferulic or *p*-coumaric acid) (see subsection 4.4). Out of the *in vitro* characterised strain collection, **15 strains were selected** for *in planta* growth promotion assays.

To evaluate the bacterial capacity to enhance root growth, 7 days old *A. thaliana* seedlings (equal germination) grown on **vertically positioned agar plates (VAPs)** (germinated on 1/50 Gamborg's B5-medium, 1% agar, 10 g L^{-1} sucrose) were transferred to VAPs (containing 1/50 Gamborg's B5-medium, 1% agar) with or without exposure to 3 µM $CdSO_4$. Plants were inoculated (400 µl, 10^4 cfu ml^{-1}) with one of the 15 selected bacterial strains. **Primary root growth** was analysed 10 days after inoculation (6 plates containing 5 plants for non-inoculated, 3 plates containing 5 plants for inoculated) (see paragraph 4.1.4.1).

Furthermore, the capacity of these endophytes to enhance plant growth by the production of bacterial volatiles was evaluated using a horizontally placed **co-cultivation assay**. For this purpose, 3 days old *A. thaliana* seedlings with equal leaf growth (germinated ¼ MS medium, 1.5% agar, 10g L^{-1} sucrose) were placed on one side (containing ¼ MS medium, 1.5% agar) of a divided Petri dish (Fig 4.5.3) and allowed to continue their growth for 5 days. Subsequently the other half of the Petri dish (containing 869 rich medium, 1.5% agar) was inoculated (100 µl, 10^8 cfu ml^{-1}) with one of the 15 selected strains. Both sides of the Petri dish were completely separated by a slide so that only volatile bacterial products could be transferred to the plants. Seven days after inoculation, **plant weight and leaf surface area** were evaluated (6 plates containing 5 plants for non-inoculated, 3 plates containing 5 plants for inoculated) (see paragraph 4.1.4.2).

Subsection 4.5

Table 4.5.1: Selected endophytic strains based on their *in vitro* plant growth promoting capacity, Cd tolerance and ferulic and *p*-coumaric acid degradation capacity. Cd: tolerance to 0.4 mM Cd is indicated with + and tolerance to 0.8 and 0.4 mM $CdSO_4$ with ++; ACCd: capacity to produce ACC deaminases *in vitro*; P: capacity to solubilise phosphate *in vitro*; IAA: capacity to produce IAA *in vitro*; OA: capacity to produce organic acids *in vitro*, SID; capacity to produce siderophores *in vitro* and N: capacity to fixate atmospheric N_2 *in vitro*. ND: Not determined. The Biolog response for ferulic acid, *p*-coumaric acid and C-mix was calculated by evaluating the net area under the absorbance versus time curve (average of 3 technical replicates) (Guckert *et al*. 1996).

Label	Origin		Genus	Cd	ACCd	P	IAA	OA	SID	N	Ferulic acid	*p*-coumaric acid	C-mix
B1	WT	root (older)	*Pseudomonas sp.*	++	+	+	-	-	+	+	64.83	29.71	86.25
B2	WT	root (older)	*Pseudomonas sp.*	++	+	+	-	-	+	-	62.68	30.45	85.38
B3	*ccr1-3*	leaf (older)	*Pseudomonas sp.*	++	+	+	-	-	+	+	36.38	29.24	59.06
B4	*4cl1-2*	stem	*Pseudomonas sp.*	++	+	+	-	-	+	-	71.80	44.56	84.18
B5	WT	stem	*Pseudomonas sp.*	++	+	-	-	-	+	+	254.40	254.44	340.62
B6	*ccr1-3* Cd	seed	*Sphingomonas sp.*	+	+	+	+	+	-	+	270.00	366.51	418.67
B7	*ccr1-3* Cd	seed	*Brevundimonas sp.*	-	+	+	+	-	+	-	43.07	53.59	96.66
B8	*ccr1-3*	leaf (older)	*Agrobacterium sp.*	++	+	+	+	+	+	+	11.53	23.26	144.64
B9	*4cl1-1* Cd	stem	*Deinococcus sp.*	++	-	+	+	-	+	+	15.19	7.52	96.09
B10	*4cl1-2*	seed	*Microbacterium sp.*	+	-	-	+	+	-	-	427.01	442.94	460.82
B11	*ccr1-3* Cd	seed	*Sphingomonas sp.*	+	+	+	+	+	-	+	37.20	22.47	26.51
B12	*4cl1-1*	seed	*Rhizobium sp.*	++	+	-	+	-	+	-	25.47	20.77	39.64
B13	*ccr1-3* Cd	root (older)	*Sphingomonas sp.*	+	-	+	-	-	+	+	27.49	29.34	100.76
B14	*ccr1-6*	stem	*Mesorhizobium sp.*	-	+	-	-	-	-	+	287.08	310.29	308.46
B15	WT Cd	stem	*Microbacterium sp.*	+	-	-	-	-	-	ND	287.75	103.12	215.18

4.5.2 Results and discussion

4.5.2.1 Selected strains

For our study, 15 strains were selected, out of the *in vitro* characterised strain collection, for *in planta* growth promotion tests. We aimed to select the most promising bacteria isolated from a wide variety of plant compartments (roots, leaves, stems and seeds), plant genotypes (WT, *4cl1-1*, *4cl1-2*, *ccr1-6* and *ccr1-3*) and Cd treatment (exposed to 0 µM or 3 µM $CdSO_4$). One selection criterion for suitable bacterial strains was a high capacity to use ferulic acid and *p*-coumaric acid as a sole C-source. Therefore, also 2 strains (B14 and B15) with a low level of *in vitro* plant growth capacity, though high capacity to use ferulic acid and *p*-coumaric acid as a sole C-source, were selected. Another criterion was containing several *in vitro* plant growth promotion (organic acid (OA) production, siderophore (SID) production, phosphate (P) solubilisation, nitrogen (N) fixation, indole-3-acetic acid (IAA) production and exerting 1-aminocylcopropane-1-carboxylate (ACC) deaminase capacity) capacity or Cd tolerance, resulting in a selection with almost all except 2 (B7 and B14) selected strains which were Cd-tolerant (Table 4.5.1).

4.5.2.2 Root growth enhancement

The effect of inoculation of the selected endophytes on growth and Cd toxicity was evaluated using a standardized test system based on root growth of *A. thaliana* in vertically positioned agar plates (Remans *et al.* 2012, Abbamondi *et al*. 2016).

(A) Effect of inoculation on root growth in non-exposed plants

In case plants were not exposed to Cd, all the selected *Pseudomonas* strains (B1-B5) significantly ($p<0.05$) **inhibited the growth** of the primary root in WT, *ccr1-6* and *ccr1-3* plants (Table 4.5.2). Some selected bacterial strains **only affected 1 or 2 of the genotypes**. For example, the selected *Rhizobium* (B12) and *Mesorhizobium* (B14) strains **inhibited** ($p<0.05$) the growth of the WT plants though the inhibition was not observed for the *ccr1* mutants and even an increasing tendency occurred for *ccr1-3* for strain B12 and both *ccr1* mutants for strain B14 (Table 4.5.2).

Subsection 4.5

The negative or non-existing impact of inoculation of strains that revealed promising *in vitro* growth promotion traits in situations without stress is a frequently observed event (Abbamondi *et al*. 2016, Remans *et al*. 2012, Beckers *et al*. *in preparation*, Haney *et al*. 2015, Hardoim *et al*. 2015). *In vitro* assays are an indication of the capacities of bacterial strains in ideal circumstances, wherein all necessary compounds are available in sufficient amounts. However, it is not obvious whether the endophytic strains also express these traits *in planta*. It is further known that, under specific conditions, bacteria with plant growth promotion capacities can become deleterious for their host plant (Hardoim *et al*. 2015). Furthermore, a compatible host plant genotype is required to enhance growth by bacterial strains (Haney *et al*. 2015). Still a lot of research is needed to understand under which conditions endophytes affect plant growth in a harmful, beneficial or neutral mode (Hardoim *et al*. 2015).

Table 4.5.2: Average growth (%) of the primary root relative to the non-inoculated similar genotype in **non-Cd-exposed plants** (±SE) (n=30 for non-inoculated and n=15 for inoculated). Significant (one way anova between non-inoculated and inoculated same genotype) decreases are in indicated in red (p<0.05) and increases in green (p<0.1).

		Average growth of the primary root relative to the non-inoculated similar genotype (%)								
		WT			***ccr1-6***			***ccr1-3***		
Non-inoculated		**100.00**	**±**	**4.05**	**100.00**	**±**	**3.38**	**100.00**	**±**	**3.13**
B1	*Pseudomonas sp.*	77.26	±	3.29	49.23	±	3.08	54.03	±	2.03
B2	*Pseudomonas sp.*	74.08	±	2.25	51.15	±	2.40	61.18	±	4.53
B3	*Pseudomonas sp.*	58.71	±	2.65	54.81	±	3.25	59.59	±	4.68
B4	*Pseudomonas sp.*	68.89	±	5.02	45.83	±	1.98	44.18	±	1.78
B5	*Pseudomonas sp.*	67.98	±	4.55	45.45	±	2.38	46.40	±	1.57
B6	*Sphingomonas sp.*	69.88	±	5.70	98.54	±	3.68	88.13	±	5.38
B7	*Brevundimonas sp.*	80.75	±	5.05	99.70	±	6.15	68.52	±	4.29
B8	*Agrobacterium sp.*	76.16	±	5.24	88.67	±	8.24	114.08	±	6.14
B9	*Deinococcus sp.*	61.78	±	3.40	100.39	±	6.24	74.64	±	4.79
B10	*Microbacterium sp.*	68.58	±	4.60	67.74	±	3.41	104.96	±	3.57
B11	*Sphingomonas sp.*	101.87	±	8.09	101.24	±	4.38	95.56	±	5.43
B12	*Rhizobium sp.*	86.40	±	8.52	99.05	±	5.52	106.87	±	5.27
B13	*Sphingomonas sp.*	74.64	±	3.92	75.18	±	3.97	110.63	±	2.68
B14	*Mesorhizobium sp.*	73.93	±	4.13	107.01	±	5.27	107.54	±	4.91
B15	*Microbacterium sp.*	124.04	±	8.57	115.00	±	4.04	111.75	±	5.10

For two of the selected strains, B8 (*Agrobacterium* sp.) and B15 (*Microbacterium sp*.), a borderline significant (p<0.1) **increase in the root growth** was observed for respectively *ccr1-3* (14.08% increase) and *ccr1-6* mutants (15.00% increase) (Table 4.5.2). For strain B8 the increase was only present for *ccr1-3* roots (Table 4.5.2). *In vitro*, bacterial strain B8 contained all investigated plant growth promoting traits (Table 4.5.1). Strains of *Agrobacterium* were

reported to induce growth of plants either in a direct way due to their own capacities (*e.g.* N fixation) or indirectly by stimulation of the growth of other beneficial bacteria in the community (Chihaoui *et al*. 2015). We only observed cultivable *Agrobacterium* strains in leaves of older *ccr1-3* mutants *(*subsection 4.3). This together with the observation of solely induced root growth on vertical agar plates after inoculation of *ccr1-3* seedlings may suggest a specific host-specific interaction between endophytic strain B8 and *ccr1-3* mutants. Similar effects of endophytic strains affecting only one *ccr1* mutant but not the other allelic variant nor WT plants were also observed by Beckers *et al*. (*in preparation*).

For strain B15, which increased the root growth of *ccr1-6* mutants ($p<0.1$), an increasing tendency was also detected in *ccr1-3* mutants (11,15% ± 5.10% longer primary root in comparison to non-inoculated plants, $p=0.12$) (Table 4.5.2). Out of the 15 selected bacteria, strain B15 was retained as the most promising endophytic strain for inoculation experiments in non-Cd-exposed conditions. Strain B15 belongs to the genus *Microbacterium* of which other plant growth promoting strains were described previously (Lin *et al.* 2011, Madhaiyan *et al.* 2010, Sheng *et al.* 2008). Moreover, it was repeatedly reported as an endophyte of *A. thaliana* (Agler *et al.* 2016, Truyens *et al.* 2016a and b). Beckers *et al*. (*in preparation*) demonstrated an improved root growth on vertical agar plates after inoculation of *A. thaliana* seedlings with a *Microbacterium* strain isolated from field-grown poplar trees (*Populus tremula* x *P. alba*). Strain B15 was selected for its high capacity to use phenolic substrates as a sole C-source and tested negative for almost all *in vitro* growth promoting traits included in our study (Table 4.5.1). The degradation capacity of persistent compounds may play a role in this observation though the precise mechanism of action is not clear. Here, insight in the possible degradation of the phenolic acids might contribute to the understanding of the mechanism.

Even though we only found limited growth promotion by the endophytes selected based on *in vitro* growth promoting capacity assays, it is possible that these strains render different growth promoting effects with a different concentration of the inoculum (cfu ml^{-1}), inoculation starting point and duration

(Truyens *et al*. 2013). Therefore, in future experiments, parameters determining the success of the inoculation should be unravelled.

Table 4.5.3: Average growth (%) of the primary root **with exposure to 3 µM $CdSO_4$** relative to the Cd-exposed non-inoculated similar genotype (±SE) (n=30 for non-inoculated and n=15 for inoculated). Significant (one way anova between non-inoculated and inoculated same Cd-exposed genotype) decreases are in indicated in red (p<0.05, p<0.1) and increases in green (p<0.05, p<0.1).

		Average growth of the primary root with exposure to Cd relative to the Cd-exposed non-inoculated similar genotype (%)								
		WT			***ccr1-6***			***ccr1-3***		
	Non-inoculated	**100.00**	**±**	**2.56**	**100.00**	**±**	**2.14**	**100.00**	**±**	**2.41**
B1	*Pseudomonas sp.*	92.42	±	5.32	96.53	±	4.19	85.00	±	6.29
B2	*Pseudomonas sp.*	90.32	±	6.46	89.39	±	4.63	101.58	±	6.49
B3	*Pseudomonas sp.*	95.80	±	4.75	96.59	±	4.64	86.88	±	4.57
B4	*Pseudomonas sp.*	104.60	±	5.54	100.39	±	7.23	82.64	±	3.85
B5	*Pseudomonas sp.*	104.21	±	5.96	94.08	±	6.54	91.14	±	4.98
B6	*Sphingomonas sp.*	99.80	±	4.40	108.41	±	3.59	95.70	±	4.55
B7	*Brevundimonas sp.*	101.16	±	4.37	105.20	±	6.28	89.27	±	3.27
B8	*Agrobacterium sp.*	100.06	±	4.15	109.41	±	7.26	91.23	±	5.61
B9	*Deinococcus sp.*	94.66	±	5.22	94.83	±	5.74	92.46	±	4.63
B10	*Microbacterium sp.*	106.55	±	6.44	93.94	±	5.26	77.93	±	7.02
B11	*Sphingomonas sp.*	105.45	±	8.55	126.09	±	9.07	85.45	±	5.41
B12	*Rhizobium sp.*	89.11	±	3.78	93.38	±	7.93	109.46	±	3.91
B13	*Sphingomonas sp.*	100.74	±	8.19	107.15	±	6.15	105.54	±	6.15
B14	*Mesorhizobium sp.*	93.73	±	3.51	105.84	±	7.09	98.53	±	4.94
B15	*Microbacterium sp.*	92.32	±	5.00	96.37	±	5.66	96.87	±	5.00

(B) Effect of inoculation on root growth of Cd-exposed plants

In case of Cd exposure, inoculation of the strains B6 (*Sphingomonas sp.*), B7 (*Brevundimonas sp.*), B8 (*Agrobacterium sp.*), B9 (*Deinococcus sp.*), B13 (*Sphingomonas sp.*), B14 (*Mesorhizobium sp.*) and B15 (*Microbacterium sp.*) **did not affect** primary root growth in comparison to the non-inoculated Cd-exposed plant genotypes (Table 4.5.3). The strains B1, B3, B4 and B5 (*Pseudomonas sp*.) and B10 (*Microbacterium sp.)* significantly **inhibited** primary root growth for the *ccr1-3* mutant in the Cd-exposed condition (Table 4.5.3). B2 (*Pseudomonas sp*.) decreased the primary root growth in the *ccr1-6* mutant (Table 4.5.3). In conditions of Cd-exposure, Truyens *et al*. (2013) also reported the possibility of negative effects (or no effects at all) on *A. thaliana* root growth on VAPs after inoculation of endophytes with promising *in vitro* results. Reasons are similar as discussed in the non-exposed situation: *in vitro* assays are performed in ideal laboratory circumstances and the traits may not

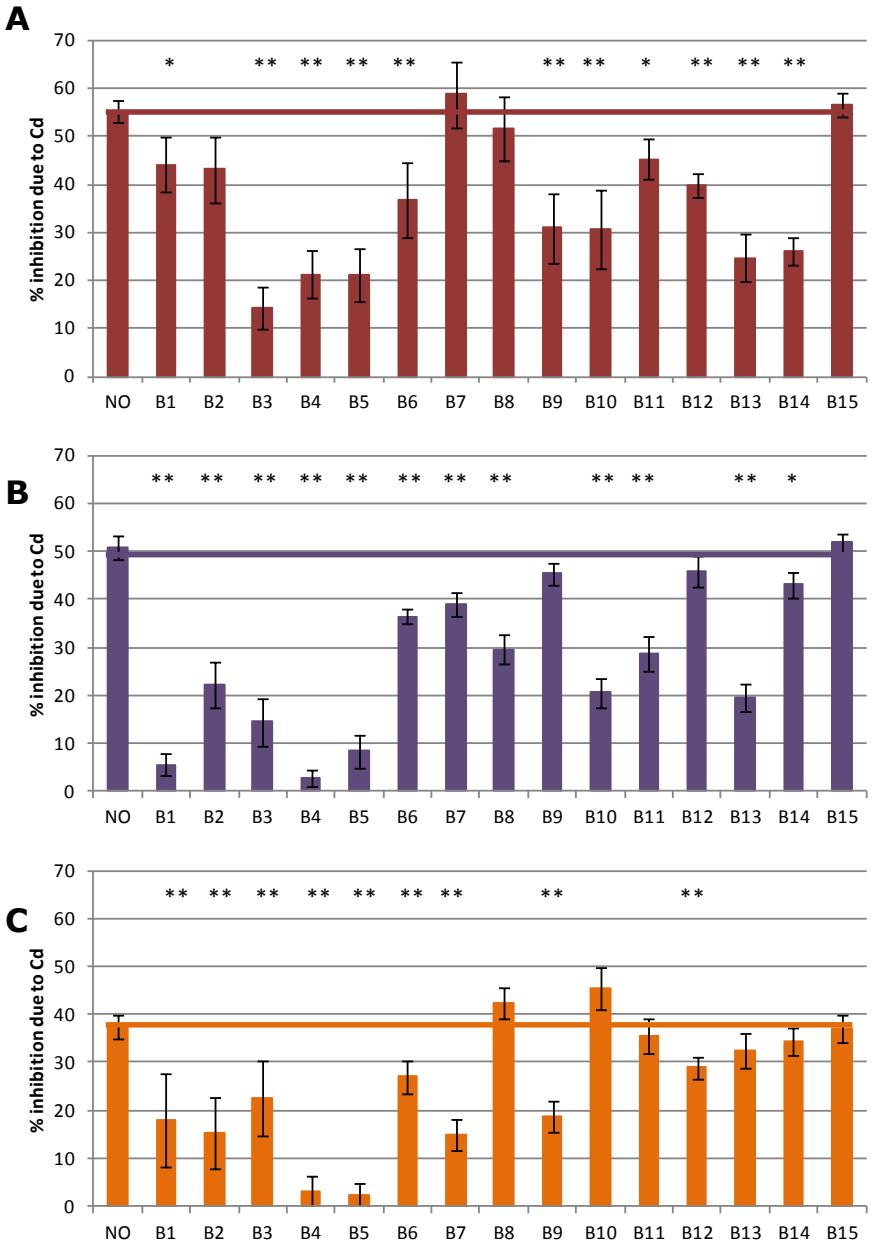


Figure 4.5.1: average relative inhibition (%) due to Cd exposure (±SE) (n=30 for non-inoculated and n=15 for inoculated) on primary roots grown on vertical agar plates (as calculated by the Cd-exposed situation in comparison to the non-exposed same inoculation condition) of (A) WT, (B) *ccr1-6* and (C) *ccr1-3* plants. Significance differences (one way anova between non-inoculated and inoculated same genotype) in relative inhibition of inoculated plants (B1-B15) in comparison to the the non-inoculated condition: $p<0.1$: *, $p<0.05$:**. Red, purple and orange line indicate the results of non-inoculated plants.

be expressed *in planta,* some bacteria may be deleterious under specific conditions (Hardoim *et al*. 2015) and host compatibility must be considered (Haney *et al*. 2015).

On the other hand, *Sphingomonas* strain **B11** significantly **increased** primary root growth of the ***ccr1-6*** mutant. However, this strain (B11) significantly decreased the growth of primary roots of *ccr1-3* in Cd-exposed conditions (Table 4.5.3). This is an unexpected result since strain B11 was isolated from seeds of Cd-exposed *ccr1-3* mutants so one would expect host compatibility (Table 4.5.1). Furthermore, endophytic strain **B12 enhanced** the primary root length in ***ccr1-3*** although the increase was not observed in WT or *ccr1-6* Cd-exposed roots (Table 4.5.3). Differences in effects of inoculated strains on growth of different *ccr1* mutants of *A. thaliana* were also observed by Beckers *et al*. (*in preparation*) in non-Cd-exposed conditions.

The **Cd-induced root growth inhibition** was significantly lower after inoculation with bacterial strains B1, B3, B4 and B5 (*Pseudomonas sp*. strains) for **all selected plant genotypes**. However, inoculation of these *Pseudomonas* strains resulted in a decreased root growth in the non-exposed condition (Table 4.5.2). Also the endophytic strain B6 (*Sphingomonas sp.*) showed the same significant decrease of inhibition after Cd exposure in all examined plant genotypes (Fig 4.5.1). For **B6** no significant difference with the non-exposed condition was observed hence the effect of B6 looks promising concerning the lowering of Cd-toxicity.

B8 (*Agrobacterium sp.*) was only able to reduce the Cd-induced growth inhibition on VAPs for the ***ccr1-6* mutant** primary roots ($p<0.05$) (Fig 4.5.1). Such an effect was not observed for *ccr1-3* (Fig 4.5.1) because strain B8 enhanced the growth under non-Cd-exposed conditions (Table 4.5.2). Furthermore, inoculation of endophytic strains B10 (*Microbacterium sp.*) ($p<0.05$), B11 (*Sphingomonas sp.*) ($p<0.05$ for *ccr1-6* and $p<0.1$ for WT), B13 (*Sphingomonas sp.*) ($p<0.05$) and B14 (*Mesorhizobium sp.*) ($p<0.05$ for WT and $p<0.1$ for *ccr1-6*) lowered the Cd toxicity in **WT plants and *ccr1-6* mutants** as observed by lower inhibition of the primary root growth (Fig 4.5.1). However, strains B10, B13 (and B14 for

WT) decreased the primary root growth in WT and *ccr1-6* non-exposed conditions and not in *ccr1-3* which can explain the difference with *ccr1-3* (Table 4.5.2). Indicating that we need to be careful in making conclusions about possible growth promotion of strains B10, B13 and B14. Strain **B11** did not affect growth of WT plants and *ccr1-6* mutants in the non-Cd-exposed situation (Table 4.5.2) and a lower Cd-induced growth inhibition, which confirms its promising effect concerning the lowering of Cd-toxicity.

A lower Cd-induced inhibition of primary root growth was observed after inoculation with B9 (*Deinococcus sp.*) and B12 (*Rhizobium sp.*) for **WT plants and *ccr1-3*** mutants ($p<0.05$) though not for *ccr1-6* mutants (Fig 4.5.1). A negative effect of the bacterial inoculation in the non-Cd-exposed situation, as observed by root growth inhibition, for B9 (WT plants and *ccr1-3* mutants) and B12 (only for WT plants) may have influenced the observation (Table 4.5.2). The effect of **B12 in *ccr1-3*** looks **promising** concerning lowering Cd-toxicity.

B2 (*Pseudomonas sp.*) and B7 (*Brevundimonas sp.*) lowered the inhibiting effect of Cd exposure on primary root growth in **both *ccr1* mutants** ($p<0.05$) (Fig 4.5.1). Though, the effect of B2 (for both *ccr1* mutants) and B7 (for *ccr1-3)* seems to be not advantageous since the root growth was inhibited under non-Cd-exposed conditions and may bias the observation (Table 4.5.2). However, inoculation with **B7 on *ccr1-6*** mutants might be **advantageous** in case of Cd exposure.

The most promising bacteria, improving root growth after Cd exposure, were B6 (*Sphingomonas sp.,* all genotypes), B7 (*Brevundimonas sp., ccr1-6*), B11 (*Sphingomonas sp.,* WT plants and *ccr1-6* mutants) and B12 (*Rhizobium sp., ccr1-3*). All these strains were isolated from **seeds of *A. thaliana*** indicating the significance of seed-borne endophytic bacteria as reviewed by Truyens *et al*. (2015). Furthermore, they all contained **ACC deaminase capacity** demonstrating the importance of ACC deaminase capacity in Cd-exposed conditions as observed by Truyens *et al*. (2016a).
Both promising *Sphingomonas sp.* strains (B6 and B11) were isolated from seeds of Cd-exposed *ccr1-3* mutants. *Sphingomonas* strains were previously

reported as *A. thaliana* endophytes (Truyens *et al*. 2013, Truyens *et al.* 2016a, Kniskern *et al.* 2014, Agler *et al.* 2016) containing Cd tolerance and plant growth promoting traits (*e.g.* N fixation) (Truyens *et al*. 2013, Truyens *et al.* 2016a). These strains (B6 and B11) displayed a similar profile of *in vitro* growth promoting traits (Table 4.5.1). Both bacterial strains contained the capacity to produce ACC deaminase, IAA and OA, P solubilisation and N fixation but tested negative for SID production. However, B6 seemed promising in all plant genotypes while the effect of B11 was unfavourable when inoculated in *ccr1-3*.

Also *Brevundimonas* strain B7 was isolated from seeds of Cd-exposed *ccr1-3* plants and showed *in vitro* capacity to produce ACC deaminase, IAA and SID and P solubilisation potential. *Brevundimonas* strains were formerly also reported as seed endophytes of *A. thaliana* (Truyens *et al.* 2016a and b) both in seeds of non-exposed and Cd-exposed plants. Furthermore, Singh *et al*. (2016) found *Brevundimonas* strains with plant growth promotion traits enhancing plant growth of rice grown in the presence of arsenic contamination. However, our endophytic strain B7 did not show *in vitro* tolerance to 4 mM $CdSO_4$. In the VAP assay a lower Cd concentration of 3µM was applied to which strain B7 may be resistant. Nonetheless, bacterial strain B7 did not show the same beneficial effect in *ccr1-3* inoculated roots exposed to Cd. Complex plant-bacterial interactions are functioning both in non-inoculated and inoculated conditions and root growth of *ccr1-3* was enhanced in the first moments after germination (see subsection 3.2). The difference in root growth may influence the plant-endophyte interactions. A different inoculation time or concentration might render different results (Truyens *et al*. 2013).

On the other hand, *Rhizobium strain* B12 appeared only advantageous after inoculation of Cd-exposed *ccr1-3* roots. *Rhizobium* strains were previously isolated as members of seed endophytic communities of *A. thaliana* (Truyens *et al.* 2013, Truyens *et al.* 2016a) and are well studied for their possible plant growth promotion (Biswas *et al.* 2000, Chi *et al.* 2005, Gopalakrishnan *et al.* 2015). Our B12 strain produced ACC deaminase, IAA and SID *in vitro* and was tolerant to 8 mM $CdSO_4$ which might explain the growth promoting effect.

4.5.2.3 Co-cultivation assay

The effect of bacterial volatiles on leaf growth was determined using divided Petri dishes wherein seedlings and bacterial strains were co-cultivated without being in direct contact with each other. In this assay, only gaseous compounds could be exchanged from the bacterial colonies to the plants.

Table 4.5.4: Average leaf area (cm^2) (±SE) (n=30 for non-inoculated and n=15 for inoculated) in the co-cultivation assay of WT, *ccr1-6* and *ccr1-3* plants. Significance (one way anova to compare non-inoculated and inoculated same genotype) increase $p<0.05$ of the inoculated plants in comparison to the non-inoculated. Significant (one way anova with subsequent Tukey-Kramer post hoc test) decrease of non-inoculated *ccr1* mutants relative to non-inoculated WT plants: $p<0.05$:*

		Leaf area (cm^2)								
		WT			***ccr1-6***			***ccr1-3***		
	Non-inoculated	**0.39**	**±**	**0.03**	**0.30**	**±**	**0.02 ***	**0.29**	**±**	**0.02 ***
B1	*Pseudomonas sp.*	1.61	±	0.08	1.21	±	0.17	1.21	±	0.13
B2	*Pseudomonas sp.*	1.90	±	0.22	1.86	±	0.13	1.59	±	0.22
B3	*Pseudomonas sp.*	1.24	±	0.22	1.46	±	0.16	1.34	±	0.18
B4	*Pseudomonas sp.*	1.81	±	0.17	1.40	±	0.15	1.60	±	0.17
B5	*Pseudomonas sp.*	1.76	±	0.22	1.60	±	0.21	1.76	±	0.18
B6	*Sphingomonas sp.*	1.26	±	0.09	1.31	±	0.13	1.55	±	0.13
B7	*Brevundimonas sp.*	1.92	±	0.12	1.96	±	0.18	2.06	±	0.13
B8	*Agrobacterium sp.*	2.08	±	0.12	1.43	±	0.21	1.69	±	0.14
B9	*Deinococcus sp.*	2.20	±	0.16	1.99	±	0.15	2.29	±	0.10
B10	*Microbacterium sp.*	0.77	±	0.07	0.37	±	0.05	0.35	±	0.03
B11	*Sphingomonas sp.*	1.10	±	0.13	0.86	±	0.11	1.20	±	0.13
B12	*Rhizobium sp.*	1.72	±	0.14	1.71	±	0.07	1.78	±	0.13
B13	*Sphingomonas sp.*	1.82	±	0.10	1.87	±	0.18	2.20	±	0.14
B14	*Mesorhizobium sp.*	1.59	±	0.14	1.45	±	0.11	1.55	±	0.18
B15	*Microbacterium sp.*	1.48	±	0.10	1.45	±	0.09	1.44	±	0.10

Almost all strains, except strain B10 (*Microbacterium sp.*), significantly ($p<0.05$) enhanced leaf growth irrespective of the plant genotype (Fig 4.5.3). For example, up to a 15-fold of the fresh weight (Fig 4.5.2) and a 7-fold the leaf area was observed (Table 4.5.4) in comparison to the non-inoculated *ccr1-6* mutants after inoculation of strain B2 in the bacterial compartment of the Petri dish.

These results demonstrate the possibility of volatiles to increase leaf fresh weight and area. Complex processes resulting in plant growth promotion by bacterial strains producing volatiles and affecting plant hormone signalling (*e.g.* cytokinin, ethylene, auxin, salicyclic acid, brassinosteroids, gibberellins, abscisic acid and jasmonic acid) were repeatedly reported in *A. thaliana* (Ryu *et al*. 2003, Ryu *et al*. 2004, Ryu *et al*. 2005, Cho *et al*. 2008, Bhattacharyya *et al*. 2015) and other plant species (Blom *et al*. 2011). Moreover, volatile bacterial compounds can evoke induced systemic resistance in plants (Ryu *et al*. 2004). Recently, it has been demonstrated that volatiles, produced by bacterial strains,

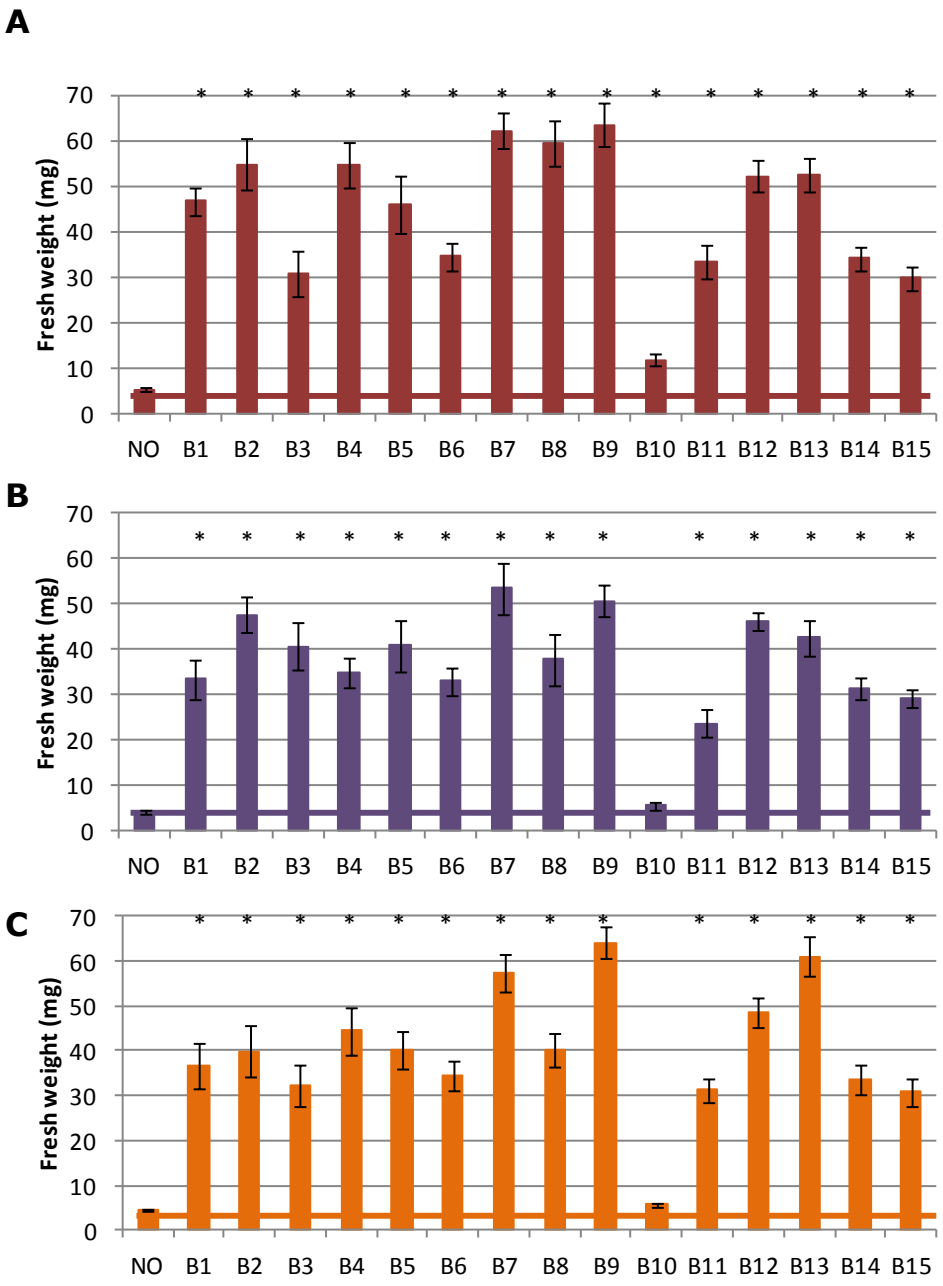


Figure 4.5.2: Average fresh weight of leaves (mg) (±SE) (n=30 for non-inoculated and n=15 for inoculated) in the co-cultivation assay of (A) WT, (B) *ccr1-6* and (C) *ccr1-3* plants. Significance (one way anova between non-inoculated and inoculated same genotype) displayed for the results of the plates inoculated with bacteria (B1-B15) in the comparison to the non-inoculated control group (NO): $p<0.05$:*. Red, purple and orange line indicate the results of non-inoculated plants.

can have promising effects on the soil bacterial diversity, its metabolic diversity and community function (Bhattacharyya and Lee 2016) indicating again the importance of the bacterial communities co-existing with plants and the complexity of plant-bacterial interactions. Bacterial plant growth promotion by volatile substances can be originating from various substances such as 1-hexanol, indole, pentadecane and 2,3-butanediol (Blom *et al*. 2011, Ryu *et al*. 2003). Recently, microbial volatiles were also brought together in a database (Lemfack *et al*. 2014).

However, to put these results into perspective, also CO_2 is produced by bacteria and may evoke plant growth responses by increasing chlorophyll content and photosynthetic efficiency, especially in closed systems such as in sealed Petri dish assays (Kai and Piechulla 2009), although CO_2 can only explain increases up to 25% (Blom *et al*. 2011). In a study of Lee *et al*. (2012), comparable levels of CO_2 were produced by non growth and growth promoting strains and the growth promoting strains were able to enhance growth in presence of 0.1 M $Ba(OH)_2$ which captures CO_2. Hence, we might conclude that elevated CO_2 levels most likely not explain the high increases in the leaf surface area we observed in our study and other volatile compounds must be responsible for the increases in leaf fresh weight and area.

However, in a more natural situation, which is not a closed system, volatile compound accumulation and consequently growth promotion is estimated to be lower (Blom *et al.* 2011). Nevertheless, great potential for bacteria producing volatiles in open-field conditions is suggested by Chung *et al*. (2016).

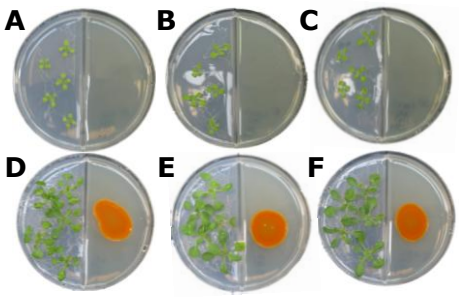


Figure 4.5.3: Representative pictures of the co-cultivation assay of the non-inoculated (A) WT, (B) *ccr1-6* and (C) *ccr1-3* and plates inoculated with strain B2 for (D) WT, (E) *ccr1-6* and (F) *ccr1-3*.

4.5.3 Conclusions

In our study, several strains enhanced the growth of primary roots after they were inoculated with direct contact with the roots. This was observed both in **non-Cd-exposed** (*Agrobacterium* sp. B8 in *ccr1-3* and *Microbacterium sp*. B15 in *ccr1-6* and trend in *ccr1-3*) and **Cd-exposed** (*Sphingomonas sp.* B6 in all genotypes, *Brevundimonas sp.* B7 in *ccr1-6*, *Sphingomonas sp.* B11 in WT and *ccr1-6* and *Rhizobium sp.* B12 in *ccr1-3*) conditions. Moreover, almost all endophytic strains significantly **enhanced leaf growth by producing volatiles**. However, the huge differences observed in our closed testing system are expected to be attenuated under real life conditions.

In our work, the first indication was delivered that these growth promoting effects can partly counteract both the negative effects of the mutation of genes involved in the lignin biosynthesis pathway and/or Cd exposure. However, in future experiments the potential of inoculation for these applications should be further explored. For example, consortia can be examined since different strains may reinforce the growth promoting capacity of each other (Thijs *et al*. 2016). Moreover, the vertical agar plates are a relatively sterile and well-controlled environment in which almost no competition with other bacterial strains is occurring which is influencing the composition of the community (Hardoim *et al*. 2015). Therefore, inoculations should be performed in more realistic conditions (*e.g.* in a greenhouse with sand as a substrate) (Truyens *et al*. 2013). Also other parameters such as dry weight, metal uptake, gene expression levels and enzyme activities of the plant and re-isolation (to demonstrate the endophytic nature of growth promotion of these strains) should be included in the experimental design. Furthermore, steps towards the use of inoculation of economically more valuable plant species (*e.g.* poplar) in field conditions should be taken.

Subsection 4.6

EFFECTS ON THE BACTERIAL COMMUNITY: conclusions

Both the genotype of the host plant and the environment can influence the bacterial endophytic communities (Bulgarelli *et al*. 2012, Agler *et al*. 2016). In section 4 we hypothesized that both (i) the T-DNA knockout mutation of *4-COUMARATE:COA LIGASE* (*4CL1)* or *CINNAMOYL COA REDUCTASE* (*CCR1),* encoding enzymes in the monolignol production hence resulting in plants with a reduced lignin content, and (ii) exposure to cadmium (Cd) can affect the endophytic communities.

We used *A. thaliana* as a model organism for woody plants (Nieminen *et al*. 2014) to investigate first the effects of both parameters (plant genotype and Cd) on the culture-independent (total) endophytic community. Secondly, the cultivable community was explored since the isolated endophytic strains may contain beneficial traits, which can be exploited to reduce the biomass-impairment associated with lignin modification and Cd exposure, and ultimately improve plant growth. Thirdly, we examined the isolated strains for *in vitro* plant growth promoting potential, their Cd tolerance and their capacity to use phenolic acids as sole C-source. Lastly, a subset of promising endophytic strains were examined for their *in vivo* plant growth promotion either (i) by investigating the effects of inoculation on root growth on vertical agar plates and (ii) leaf fresh weight and area in a co-cultivation assay in Petri dishes in which only the volatiles that are produced by the examined strains can reach the seedlings.

Since the *ccr1* mutants are retarded in their development (Van Acker *et al*. 2013), the developmental effects were circumvented by growing these plants earlier in order to obtain plants with a similar rosette diameter for younger plants or stem length for older plants. To investigate the culture-independent communities also *ccr1* mutants of the same age as WT plants were used. Furthermore, an additional mutant, **p3xSNBE:*CCR1* in *ccr1-6*** (or abbreviated as *ccr1-6/*SNBE) was included, in which *CCR1* expression is restored in the vessels while the plant still lacks *CCR1* expression in other cell types.

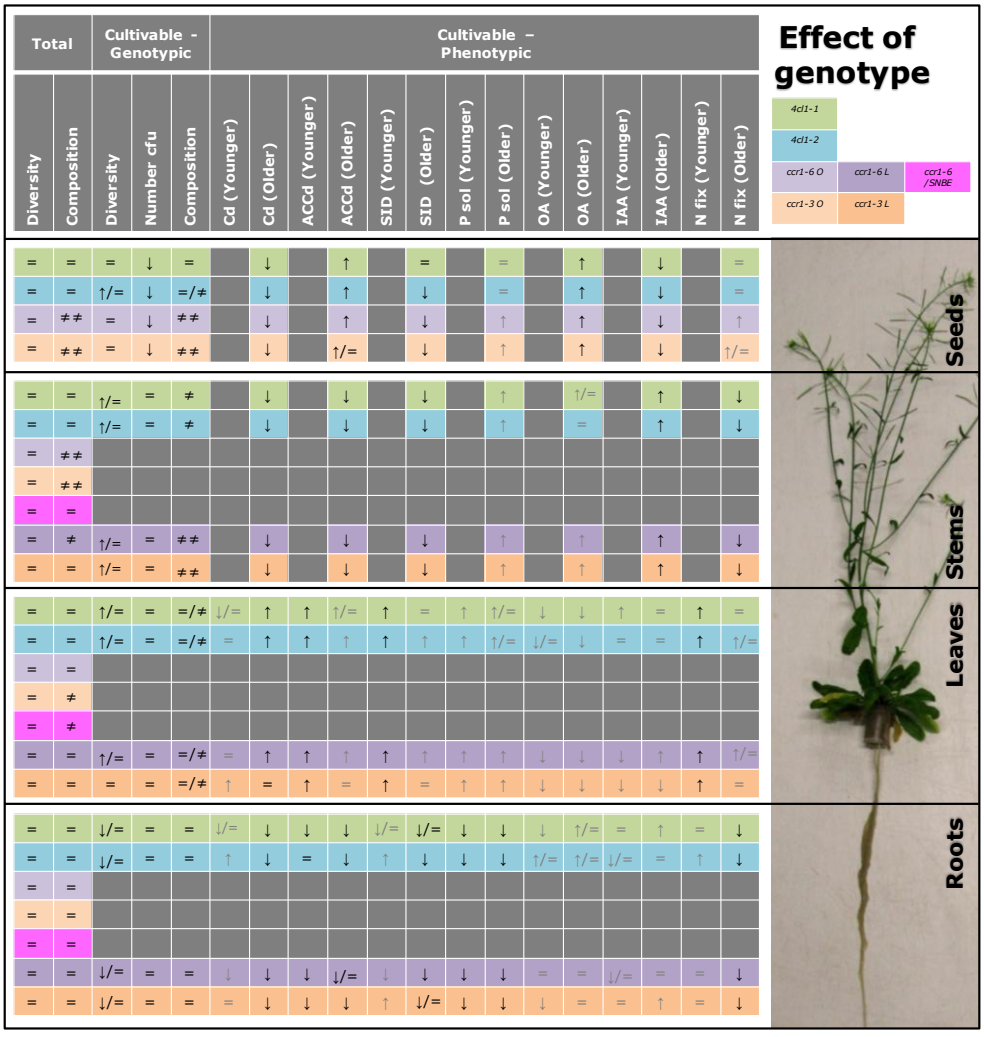


Figure 4.6.1: Summary of the effects of the lignin-reduced genotype related to the culture-independent (total) and dependant (cultivable) communities of roots, leaves, stems and seeds of *4cl1* and *ccr1* mutants in comparison to WT plants. Abbreviations: O: *ccr1* mutants with a similar age in comparison to WT, L: *ccr1* mutants with a similar development in comparison to WT, *ccr1-6*/SNBE: *ccr1-6* mutant wherein the *CCR1* expression is restored in the vessels while the plant still lacks *CCR1* expression in other cell types. Cd: Cd tolerance to 0.4mM Cd, ACCd: ACC deaminase capacity, SID: siderophore production capacity, P sol: phosphate solubilisation capacity, OA: organic acid production capacity, IAA: indole-3-acetic acid production capacity, N fix: nitrogen fixation capacity. Results which are not discussed in the text are displayed in grey.

4.6.1 Effects of the lignin-reduced genotype

Several effects of the lignin-reduced genotypes on both the culture-independent and cultivable bacterial communities are summarised in figure 4.6.1. In table 4.6.1 the 3 most represented genera in the non-Cd-exposed plant genotypes are presented.

The **diversity** of the **cultivable** fraction of the community did not differ in function of the genotype in seeds, though tended to be lower in lignin-reduced mutant roots while increasing trends were observed in leaves and stems. However, the **total** endophytic communities were not affected in their diversity due to changes resulting from the T-DNA knockout of *4CL1* or *CCR1* (Fig. 4.6.1).

Concerning the cultivable bacterial communities: the numbers of isolated **colony forming units** (cfu) were only clearly different in the seeds. Seeds of lignin-reduced mutants displayed lower numbers of cfu in comparison to the WT plants (Fig. 4.6.1).

Roots

Both in the **culture-dependent and independent communities of roots**, **no prominent effects of the lignin-reduced genotype** were observed (Fig. 4.6.1). *Bacillus* was only isolated (cultivable fraction) from roots of older plants of all lignin-reduced mutants and was one of the 3 most observed genera in *4cl1* mutants (Table 4.6.1). Furthermore, higher relative abundances (cultivable) of *Microbacterium* and *Pseudomonas* strains were observed respectively in roots of younger and older WT plants. In roots of younger plants (cultivable fraction), *Acidovorax* strains were only detected in *4cl1* mutants (Table 4.6.1). However, in all the selected plant genotypes culture-independent communities of roots of older plants, *Pseudomonas* and *Acidovorax* strains were observed (Table 4.6.1). Therefore, the differences in the cultivable fractions may also be connected with the cultivability of these strains.

The isolated root endophytes (cultivable fraction) from lignin-reduced mutants displayed lower relative abundances of *in vitro* siderophore production, P solubilisation, N fixation and ACC deaminase capacity. Also lower relative abundances of Cd-tolerant strains were observed in the lignin-reduced mutant root communities (Fig. 4.6.1). However, the effect in Cd-tolerance cannot be explained as a result of Cd exposure since these plants were not exposed.

Subsection 4.6

Table 4.6.1: Three most represented genera (indicated with 1, 2 and 3) in the non-exposed total and cultivable communities from roots (of younger and older plants), leaves (of younger and older plants), stem and seeds of WT, *4cl1-1*, *4cl1-2*, *ccr1-6*, *ccr1-3* and *ccr1-6*/SNBE plants. Genera represented with prevalence below 5% are not displayed, /: no additional genera were present with a percentage above 5%. Abbreviations: O: *ccr1* mutants with a similar age in comparison to WT, L: *ccr1* mutants with a similar development in comparison to WT, *ccr1-6*/SNBE: *ccr1-6* mutant wherein the *CCR1* expression is restored in the vessels while the plant still lacks *CCR1* expression in other cell types.

				WT	4Cl1-1	4Cl1-2	ccr1-6 O	ccr1-3 O	ccr1-6/SNBE	ccr1-6 L	ccr1-3 L
Non-exposed	Stem	Cultivable	1	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*				*Sediminibacterium*	*Spingomonas*
			2	/	*Variovorax*	*Rhizobium*				*Staphylococcus*	*Rhizobium*
			3	/	*Pelomonas*	*Pedobacter*				*Fuviicola*	*Pedobacter*
		Total	1	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Alcaligenes*	*Alcaligenes*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*
			2	*Alcaligenes*	*Alcaligenes*	*Alcaligenes*	*Pseudomonas*	*Pseudomonas*	*Alcaligenes*	*Alcaligenes*	*Alcaligenes*
			3	/	/	*Uc Burkholderiales*	/	*Acidovorax*	/	*Acidovorax*	/
	Seed	Cultivable	1	*Rhizobium*	*Rhizobium*	*Paenibacillus*	*Microbacterium*	*Microbacterium*			
			2	/	*Staphylococcus*	*Rhizobium Rhodococcus Staphylococcus*	/	*Bacillus*			
			3	/	/	/	/	/			
		Total	1	*Rhizobium*	*Rhizobium*	*Uc Rhizobiales*	*Pseudomonas*	*Pseudomonas*			
			2	*Deinococcus*	*Pseudomonas*	*Rhizobium*	*Leifsonia*	*Chryseobacterium*			
			3	*Uc Alphaproteobacteria*	*Uc Rhizobiales*	/	*Microbacterium*	*Microbacterium TM7*			

				WT	4Cl1-1	4Cl1-2	ccr1-6 O	ccr1-3 O	ccr1-6/SNBE	ccr1-6 L	ccr1-3 L
Non-exposed	Root (Younger)	Cultivable	1	*Pelomonas*	*Pelomonas*	*Pelomonas*				*Pelomonas*	*Pelomonas*
			2	*Microbacterium*	*Bacillus*	*Bacillus*				*Microbacterium*	*Hydrocarboniphaga Nevskia*
			3	*Bacillus*	*Acidovorax*	*Acidovorax*				/	/
	Root (Older)	Cultivable	1	*Pelomonas*	*Pelomonas*	*Pelomonas*				*Pelomonas*	*Pelomonas*
			2	*Pseudomonas*	*Sphingomonas*	*Sinorhizobium*				*Elizabethkingia*	*Mycobacterium*
			3	/	*Bacillus*	*Bacillus*				/	*Sinorhizobium*
		Total	1	*Acidovorax*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Acidovorax*	*Acidovorax*
			2	*Pseudomonas*	*Acidovorax*	*Acidovorax*	*Uc Oxalobacteraceae*	*Serratia*	*Truepera*	*Uc Oxalobacteraceae*	*Pseudomonas*
			3	*Uc Oxalobacteraceae*	*Uc Bacteroidetes*	*Uc Oxalobacteraceae*	*Acidovorax*	*Acidovorax*	*Acidovorax*	*Uc Comamonadaceae*	*Uc Oxalobacteraceae*
	Leaf (Younger)	Cultivable	1	*Pelomonas*	*Brevundimonas*	*Pseudomonas*				*Pelomonas*	*Pseudomonas*
			2	*Pseudomonas*	*Pelomonas*	*Bacillus*				*Bacillus*	*Pelomonas*
			3	/	*Arthrobacter*	*Brevundimonas*				*Pseudomonas*	*Bacillus sp.*
	Leaf (Older)	Cultivable	1	*Microbacterium*	*Elizabethkingia*	*Deinococcus*				*Deinococcus*	*Pelomonas*
			2	*Sphingomonas*	*Microbacterium*	*Brevundimonas*				*Pelomonas*	*Variovorax*
			3	*Pseudomonas*	*Exiguobacterium Sphingopyxis*	*Rhizobium*				*Micrococcus*	*Sphingomonas*
		Total	1	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*
			2	*Acidovorax*	*Microbacterium*	*Stenotrophomonas*	*Uc Oxalobacteraceae*	/	/	*Arthrobacter*	*Acidovorax*
			3	*Rhizobium*	/	/	*Acidovorax*	/	/	*Uc Enterobacteriaceae*	/

Leaves

Pelomonas was observed in high relative abundances in the **cultivable community** of **leaves of younger plants.** Furthermore, *Pseudomonas* strains were also highly represented in the leaves of younger plants (Table 4.6.1). The high relative abundance of *Pelomonas* and *Pseudomonas* made all cultivable communities from all plant genotypes resemble each other. Nevertheless, some differences were observed. *Bacillus* strains were only detected in the leaves of younger lignin-reduced mutants and were part of the 3 most represented genera in *4cl1-2*, *ccr1-6* and *ccr1-3* mutants (Table 4.6.1) and *Brevundimonas* was part of the 3 most represented genera in leaves of younger *4cl1* mutants but was not observed in leaves of younger plants of other plant types (Table 4.6.1).

The **cultivable communities** of **leaves** of **older plants** appeared **rather different**. This was mainly due to high relative abundances of *Pelomonas* in *ccr1-3* and *Microbacterium* in WT plants while the other plant genotypes displayed a diverse distribution of genera in their endophytic community of leaves of older plants (Table 4.6.1). However, in the **leaves of older plants no effects** of the lignin-reduced genotype were detected on the **total community**. Total communities of these leaves of older plants were dominated by OTUs belonging to the genus *Pseudomonas (*as observed in leaves of younger plants cultivable communities) (Table 4.6.1). Suggesting that the observed effects on the cultivable community might be biased by the cultivability of strains.

Although no clear effects were observed based on the genera composition, the **phenotypic traits** of the **cultivable bacterial** communities tended to be **affected by the lignin-reduced genotype** (Fig. 4.6.1). Lignin-reduced mutant leaves of older plants included higher relative abundances of Cd-tolerant endophytes while leaves of younger plants included higher relative abundances of endophytes with ACC deaminase capacity, siderophore production and N fixation capacity in comparison to the WT community. *In vitro* IAA producing endophytes were less represented in leaves of younger *ccr1* mutants in comparison to the WT community (Fig. 4.6.1).

Stems and seeds

The total endophytic communities in the **stems** of ***ccr1-6* and *ccr1-3* mutants with a similar age** as WT plants (indicated with O) were **different** from the

WT communities (Fig 4.6.1). In plants with a similar stem length (indicated with L) a minor difference was observed only for the *ccr1-6* in comparison to WT plants (Fig 4.6.1). Furthermore, the *4cl1* and *ccr1-6*/SNBE mutants have similar development as WT plants and their total community was also similar to that of WT plants. Therefore, it is possible that the **delayed development** of the *ccr1* mutant stems affects the bacterial communities more than other lignin-related changes. This may be elucidated in future experiments in case a WT condition with a stem length similar to the *ccr1* O mutants (WT plants sown at a later point in time) is included. *Pseudomonas* and *Alcaligenes* OTUs dominated the total communities in the stems of all genotypes: in the *ccr1* O mutants, which differed from total communities of WT plants, *Alcaligenes* dominated the community while *Pseudomonas* dominated the total communities of all other examined conditions (Table 4.6.1).

In contrast, the **cultivable fraction** of the stem communities of all lignin-reduced mutants was **different** from the WT community. As observed for the total communities the WT and *4cl1* stem cultivable communities were dominated by *Pseudomonas* (Table 4.6.1). However, the occurrence of these *Pseudomonas* strains tended to be higher in the WT stem community wherein almost no other genera were observed while the cultivable community of *4cl1* stems included a number of other genera. Most notably *Brevundimonas* strains, which were exclusively observed in *4cl1* mutants (as observed in the cultivable fraction of leaves of younger plants), although this genus did not belong to the 3 most observed genera. No *Pseudomonas* strains were observed in the cultivable stem community of *ccr1* mutants and other genera such as *Sphingomonas* were detected in the *ccr1-3* mutant (Table 4.6.1). Furthermore, no *Alcaligenes* strains, as observed in the total community, were detected in the cultivable fraction (Table 4.6.1).

These differently selected genera from the stems showed also different **phenotypic traits** concerning growth promotion capacity and Cd tolerance (Fig 4.6.1). However, the selection appeared not at random and **lignin-reduced mutants exhibited a similar selection**. For all lignin-reduced mutants, the relative abundances of endophytes with the capacity to produce IAA were higher in comparison with the WT stem community while endophytes with Cd tolerance, ACC deaminase capacity, SID production and N fixation capacity were relatively

less numerous in the selected lignin-reduced mutant stem communities (Fig 4.6.1).

Both the **total and cultivable communities of seeds** of WT, *4cl1-1* and *4cl1-2* harboured the genus *Rhizobium*. The genus *Rhizobium* was accompanied by unclassified Rhizobiales in the total communities of these seeds. The cultivable communities of seeds of *ccr1-6* and *ccr1-3* mutants were dominated by *Microbacterium* strains (Table 4.6.1). *Microbacterium* OTUs were also present in high relative abundances in the culture-independent communities although here the most observed OTUs belonged to the genus *Pseudomonas* (Table 4.6.1). Therefore, it can be concluded that the total and cultivable seed communities of ***4cl1*** **mutants** were **similar** to the WT communities while the communities of ***ccr1*** were highly **different** from communities of WT seeds. However, in the production of the seeds the delayed development of the *ccr1* seeds cannot be ruled out and may influence the results. Moreover, although the seeds were produced by plants grown for several generations in identical conditions, the origin of the initial seed stocks was different. The eventual effects of vertical transmission of endophytes in these *ccr1* seeds should be examined.
Also in the seeds different (*in vitro* examined) **growth promotion capacities were selected in lignin-reduced genotypes** as compared to WT seeds (Fig 4.6.1). Higher relative abundances of endophytes with ACC deaminase capacity and OA production capacity were found in the lignin-reduced mutants while endophytes with Cd tolerance, SID and IAA producing capacity were less numerous in the lignin-reduced mutants (Fig 4.6.1).

Furthermore, the cultivable communities of **stems and seeds**, and selected strains of other plant compartments, were examined for their use of ferulic acid and *p*-coumaric acid as a sole carbon source. However, no clear conclusion could be drawn since the outcome included a high percentage of undetermined strains. Nevertheless this information was of high importance to select strains for inoculation experiments.

4.6.2 Effects of exposure to cadmium

A summary of the effects of Cd exposure on the endophytic communities is presented in figure 4.6.2. Table 4.6.2 presents the 3 most represented genera in the Cd-exposed plant genotypes.

In all examined plant compartments, the **diversity** of both the total and cultivable endophytic communities was **not significantly affected by Cd exposure** of the plants.

Roots

The **cultivable endophytic communities** from **roots of younger and older Cd-exposed plants** were dominated by *Pelomonas* strains and their presence even tended to increase in comparison to the non-exposed plant genotypes (Table 4.6.2). Therefore, the communities of all plant genotypes appeared **similar** under Cd-exposed conditions (Fig 4.6.2). In the **culture-independent** assay, only ***4cl1-1*** root endophytic communities showed a **Cd-effect** (Fig 4.6.2). All total root endophytic communities of Cd-exposed plants contained many *Pseudomonas* and unclassified Oxalobacteraceae OTUs. Although in the *4cl1-1* mutant community of Cd-exposed plants, *Pseudomonas* OTUs decreased and unclassified Oxalobacteraceae increased and became the most observed genus. This Cd effect was not observed for the other Cd-exposed plant genotypes (Table 4.6.2).

The cultivable communities of roots were unchanged at their genera composition, though the **phenotypic** traits of the endophytes concerning IAA production showed some differences between lignin-reduced mutants and WT plants after Cd exposure. In communities of roots of older plants the IAA producing capacity increased in WT plants while the IAA producing capacity decreased proportionally in the communities of the lignin-reduced mutants after Cd exposure of the plants (Fig 4.6.2). For other traits, the effects of Cd on the root endophytic communities of WT plants and lignin-reduced mutants were similar or allelic variants of the lignin-reduced mutants did not show clear patterns and conclusions were difficult to be drawn (Fig 4.6.2).

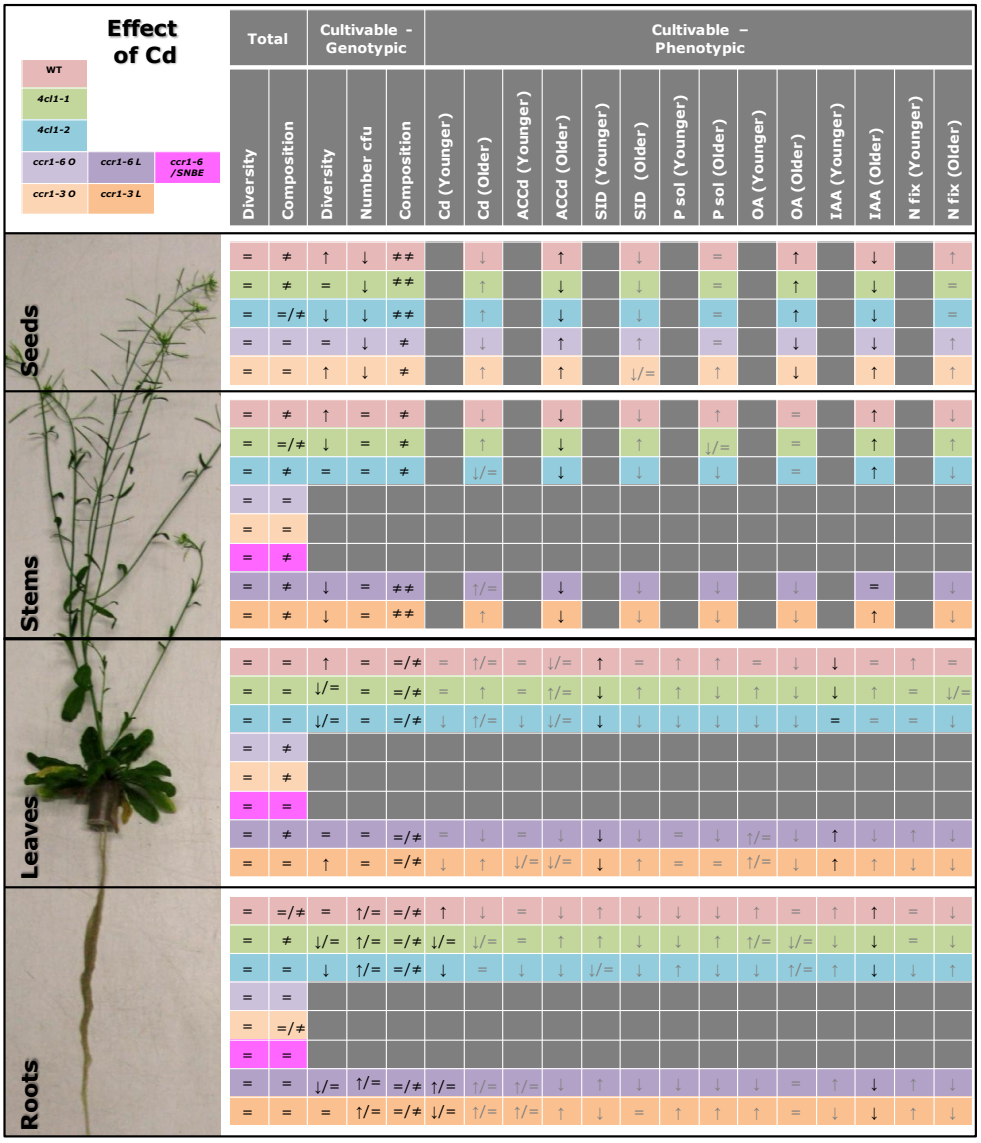


Figure 4.6.2: Summary of the effects of Cd exposure related to the culture-independent (total) and dependant (cultivable) communities of roots, leaves, stems and seeds of WT plants and *4cl1* and *ccr1* mutants. Abbreviations: O: *ccr1* mutants with a similar age compared to WT, L: *ccr1* mutants with a similar development compared to WT, *ccr1*-6/SNBE: *ccr1-6* mutant wherein the *CCR1* expression is restored in the vessels while the plant still lacks *CCR1* expression in other cell types. Cd: Cd tolerance to 0.4mM Cd, ACCd: ACC deaminase capacity, SID: siderophore production capacity, P sol: phosphate solubilization capacity, OA: organic acid production capacity, IAA: indole-3-acetic acid production capacity, N fix: nitrogen fixation capacity.
Data which is not discussed in the text is displayed in grey.

Leaves

Only the **total leaf** communities of *ccr1* mutants with a similar age in comparison to WT plants (O) (hence with delayed development) and the *ccr1-6* L community were affected by Cd exposure of the plants (Fig 4.6.2). Other total leaf communities (WT, *4cl1-1*, *4cl1-2*, *ccr1-6*/SNBE and *ccr1-3* L) **did not change after Cd exposure** of the plants. Almost all leaf communities of Cd-exposed plants contained a high amount of OTUs identified as *Pseudomonas* and *Acidovorax*. The most obvious difference were the increases in *Acidovorax* after Cd exposure of plants of almost all plant genotypes (Table 4.6.2).

In contrast, the **cultivable fraction** of the leaf communities tended to be **differently affected by Cd exposure** of the plants. The cultivable communities of leaves of **younger** lignin-reduced mutants showed increases in *Pelomonas* strains and contained many *Pseudomonas* strains while in the WT community the relative abundance of *Pelomonas* did not increase after Cd exposure and high relative abundances of *Bacillus* and *Sphingobium* strains were found (Table 4.6.2).

In the cultivable communities of **leaves of older plants**, the most obvious Cd effect was observed in WT plants where *Microbacterium* strains were decreased in relative abundance although in lignin-reduced mutants no general conclusions about genera could be drawn (Table 4.6.2). No cultivable *Acidovorax* strains were observed in communities of leaves of Cd-exposed younger or older plants as observed in high relative abundances in the total communities of Cd-exposed plants.

Concerning the **phenotypic** traits, SID production tended to increase in the endophytic communities of leaves of younger Cd-exposed WT plants whereas it decreased proportionally in the lignin-reduced mutants. Another difference was observed for the IAA production capacity, which was proportionally more increased in the communities of leaves of younger *ccr1* mutants though not in WT plants or in *4cl1* mutants. Other plant growth promoting traits tended to be similar to WT plants (Fig 4.6.2).

Table 4.6.2: Three most represented genera (indicated with 1, 2 and 3) in the total and cultivable communities from roots (of younger and older plants), leaves (of younger and older plants), stem and seeds of Cd-exposed WT, *4cl1-1*, *4cl1-2*, *ccr1-6*, *ccr1-3* and *ccr1-6*/SNBE plants. Genera represented with prevalence below 5% are not displayed, /: no additional genera were present with a percentage above 5%. Abbreviations: O: *ccr1* mutants with a similar age in comparison to WT, L: *ccr1* mutants with a similar development in comparison to WT, *ccr1-6*/SNBE: *ccr1-6* mutant wherein the *CCR1* expression is restored in the vessels while the plant still lacks *CCR1* expression in other cell types.

				WT Cd	*4Cl1-1* Cd	*4Cl1-2* Cd	*ccr1-6 O* Cd	*ccr1-3* O Cd	*ccr1-6*/ SNBE Cd	*ccr1-6* L Cd	*ccr1-3* L Cd
Cd-exposed	Stem	Cultivable	1	*Sphingomonas*	*Pseudomonas*	*Pseudomonas* *Brevundimonas*				*Mezorhizobium* *Kaistia*	*Sphingomonas*
			2	*Kaistia*	*Staphylococcus*	*Pedobacter*				/	/
			3	* *Several genera*	*Deinococcus*	*Pimelobacter*				/	/
		Total	1	*Alcaligenes*	*Alcaligenes*	*Alcaligenes*	*Alcaligenes*	*Alcaligenes*	*Alcaligenes*	*Alcaligenes*	*Alcaligenes*
			2	*Uc* *Phyllobacteriaceae*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*
			3	/	/	*Dysgonomonas*	/	*Aquabacterium*	/	*Dysgonomonas*	/
	Seed	Cultivable	1	*Stenotrophomonas*	*Microbacterium*	*Microbacterium*	*Microbacterium*	*Bacillus*			
			2	*Microbacterium*	*Paenibacillus*	*Bacillus*	*Staphylococcus*	*Microbacterium*			
			3	*Bacillus*	/	*Paenibacillus*	*Methylobacterium*	*Sphingomonas*			
		Total	1	*Pseudomonas*	*Pseudomonas*	*Rhizobium*	*Microbacterium*	*Pseudomonas*			
			2	*Nocardioides*	*Microbacterium*	*Microbacterium*	*Deinococcus*	*Deinococcus*			
			3	*TM7*	*Uc* *Microbacteriaceae*	/	*Alcaligenes*	*Chryseobacterium*			

				WT Cd	*4Cl1-1* Cd	*4Cl1-2* Cd	*ccr1-6* O Cd	*ccr1-3* O Cd	*ccr1-6*/SNBE Cd	*ccr1-6* L Cd	*ccr1-3* L Cd
Cd-exposed	Root (Younger)	Cultivable	1	*Pelomonas*	*Pelomonas*	*Pelomonas*				*Pelomonas*	*Pelomonas*
			2	*Blastomonas*	*Blastomonas*	*Blastomonas*				*Pseudacidovorax*	*Microbacterium*
			3	*Microbacterium*	*Microbacterium*	*Rhodococcus Elizabethkingia*				/	*Pseudacidovorax*
	Root (Older)	Cultivable	1	*Pelomonas*	*Pelomonas*	*Pelomonas*				*Pelomonas*	*Pelomonas*
			2	*Acidovorax*	*Bacillus*	*Staphylococcus*				*Elizabethkingia Blastomonas*	*Variovorax*
			3	/	*Acidovorax Kaistia*	*Bacillus Phenylobacterium conjunctum*				/	*Bacillus*
		Total	1	*Pseudomonas*	*Uc Oxalobacteraceae*	*Pseudomonas*	*Acidovorax*	*Pseudomonas*	*Pseudomonas*	*Acidovorax*	*Acidovorax*
			2	*Alcaligenes*	*Pseudomonas*	*Uc Oxalobacteraceae*	*Pseudomonas*	*Serratia*	*Curvibacter*	*Uc Oxalobacteraceae*	*Pseudomonas*
			3	*Uc Oxalobacteraceae*	*Alcaligenes*	*Alcaligenes*	*Uc Oxalobacteraceae*	*Uc Oxalobacteraceae*	*Uc Oxalobacteraceae*	*Pseudomonas*	*Uc Oxalobacteraceae*
	Leaf (Younger)	Cultivable	1	*Pelomonas*	*Pelomonas*	*Pelomonas*				*Pelomonas*	*Pelomonas*
			2	*Bacillus*	*Pseudomonas*	*Brevundimonas Rheinheimera*				*Pseudomonas*	*Brevundimonas*
			3	*Sphingobium*	*Staphylococcus*	*Pseudomonas*				*Microbacterium*	*Pseudomonas*
	Leaf (Older)	Cultivable	1	*Brevundimonas Pelomonas*	*Deinococcus*	*Exiguobacterium*				*Pelomonas*	*Rhizobium*
			2	*Microbacterium Fluviicola*	*Pseudomonas*	*Pelomonas*				*Brevundimonas*	*Microbacterium*
			3	*Deinococcus*	*Microbacterium*	*Microbacterium*				*Pedobacter*	*Variovorax*
		Total	1	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*	*Acidovorax*	*Pseudomonas*
			2	*Acidovorax*	*Acidovorax*	*Acidovorax*	*Acidovorax*	*Acidovorax*	*Acidovorax*	*Stenotrophomonas*	*Acidovorax*
			3	*Uc Pseudomonadaceae*	/	/	*Rhizobium*	/	/	*Uc Comamonadaceae*	/

Stems and seeds

All **total communities of stems of plants** grown under conditions with a **similar development** were **affected by Cd exposure** of the plants (WT, *4cl1-2*, *ccr1-6*/SNBE, *ccr1-6 L*, *ccr1-3 L* (p=0.1) and a trend in *4cl1-1*). *Alcaligenes* dominated the total communities of the stems of all exposed plants (Table 4.6.2). However, in stems of *ccr1-6* O and *ccr1-3* O (plants of *ccr1* with a similar age but with a retardation in growth in comparison to WT plants), *Alcaligenes* strains were already highly abundant, which might explain the lack of change in *ccr1* mutants after Cd exposure of the plants.

However, no cultivable *Alcaligenes* strains were isolated from Cd-exposed plants.

All **cultivable stem communities changed after Cd exposure of the plants**. The most different cultivable communities were observed in ***ccr1* mutants**, which even showed a big difference from the Cd-exposed WT plants (*e.g.* dominance of *Sphingomonas* in *ccr1-3)* (Fig 4.6.2).

The most prominent effects of Cd on the investigated **phenotypic traits** in the cultivable communities were observed in the IAA production. In all plant genotypes (except *ccr1-6*), Cd exposure led to increases of IAA producing strains. No endophytes with ACC deaminase capacity were found anymore in *ccr1* stem communities after Cd exposure of plants, hence it strongly decreased in comparison to the non-exposed plants. The relative abundance of ACC deaminase producing endophytes also decreased in stems of WT plants and *4cl1* mutants exposed to Cd. (Fig 4.6.2)

In the **cultivable communities** of **seeds** of WT plants and *4cl1* mutants, the relative abundance of *Microbacterium* strains increased after Cd exposure of the plants (Table 4.6.2). However, in the *ccr1* mutants, the relative abundance of *Microbacterium* strains decreased, although it was still one of the 3 most observed genera (Table 4.6.2). In seeds of Cd-exposed *ccr1-3* mutants, *Sphingomonas* strains were observed in a similar relative abundance as in stems of *ccr1-3* non-exposed mutants. In the seeds of WT plants, many *Stenotrophomonas* strains were isolated after Cd exposure of the parental plants. This was not the case for lignin-reduced mutants (Table 4.6.2).

Total endophytic communities of seeds within the **Cd-exposed plants** included a diverse collection of several genera though the most observed genera

were not necessarily identical to the ones that could be isolated, indicating a bias of the cultivability. The total seed communities were **affected by Cd in WT plants and *4cl1* mutants** though **not in *ccr1* mutants** (Fig 4.6.2). However, the difference in development of the *ccr1* mutants and seeds may be a possible confounder in the study of the *ccr1* communities that cannot be ruled out.
The relative abundances of **phenotypic** traits of seed endophytic communities of lignin-reduced mutants were differently affected after Cd exposure of the parental plants in comparison to the effects on the WT community. Less organic acids producing strains were observed in *ccr1* seed communities after Cd exposure of the parental plants while no changes after Cd exposure were observed in WT plants or *4cl1* mutants. Furthermore, ACC deaminase capacity was lower in WT and *ccr1* seed communities after Cd exposure of the parental plants while decreases were observed in the seed endophytic community of the *4cl1* mutants (Fig 4.6.2). The relative abundance of IAA producing bacteria was affected in a similar decreasing way as observed in WT plants and lignin-reduced mutants communities of Cd-exposed plants.

4.6.3 General

To summarise all above-mentioned effects: in **roots and leaves no or slight changes** in the **total and cultivable endophytic communities** were observed due to the lignin-reduced genotype mutation. However, both in the **stems and seeds**, especially the *ccr1* mutants showed **strong effects** on both the **cultivable and total communities**.

Cd exposure did **not affect** the **total and cultivable root** endophytic communities. Under some conditions (*e.g. ccr1* O mutants and *ccr1-6* L mutant) the **total leaf** communities were affected by Cd. However, conclusions are difficult to draw since the endophytic leaf communities of *ccr1-3* L mutants were not affected. All **cultivable endophytic communities** of leaves tended to be **affected** by Cd exposure of the plants. **The total endophytic communities of stems** of all plant genotypes with a similar development were **affected** after Cd exposure of the plants whereas ***ccr1-6* O and *ccr1-3* O** communities **were not changed**. Also different effects of Cd exposure of the plants were observed on the **cultivable communities** of stems and the communities of *ccr1* mutants

changed the most. **The total seed endophytic communities** of **WT plants and *4cl1* mutants were affected** by Cd exposure of the plants though the *ccr1* communities were not. The same tendency was observed for the **cultivable** fraction of the communities where ***ccr1* mutants were less affected** after Cd exposure of the plants.

Moreover, in all plant compartments, non-exposed lignin-reduced mutants tended to select differently for some **growth promoting traits and Cd tolerance** of the cultivable endophytes (*e.g.* lower relative abundances of bacteria with growth promotion in lignin-reduced mutant roots). Also after Cd exposure of the plants, differences in plant growth promoting capacities and Cd tolerance were observed for the cultivable endophytes of roots, leaves, stems and seeds, although to a minor extent. In future experiments, the total endophytic community may be explored for several of these features using a function-based metagenomic analysis (Kaul *et al.* 2016) to gain more information about the relative abundances of endophytes possessing these traits.

Features inherent to the lignin-reduced mutants can affect the endophytic communities. For instance, cell walls are important during plant colonisation by bacteria (Compant *et al.* 2010, Bulgarelli *et al.* 2012), xylem vessels may be collapsed in the *ccr1* mutants (Mir Derikvand *et al*. 2008), different phenolic intermediates of the monolignol biosynthesis pathway and derivatives thereof may be used as a C-source (Beckers *et al*. 2016b) or can have antimicrobial action (Rice-Evans *et al.* 1996) and flavonoid production may be affected. All these features might affect the endophytic communities (Weston and Mathesius *et al.* 2013).

The observed differences in the total endophytic communities of the stems may also originate from the **differences in development** of the different genotypes since the differences in composition of the communities were smaller or even not present in case plants at a similar developmental stage were studied. More evidence for this hypothesis may be obtained by including WT plants with a delayed development (WT plants which are sown at a later moment than other

conditions) in future studies. Also the observed changes in the *ccr1* mutant seed communities may be due to the observed developmental delay (section 3.2). Nevertheless, the developmental effect during seed production is difficult to rule out. Moreover, seeds of *ccr1* mutants also appear heavier and less seeds per plant are produced, which might have an effect on the endophytic communities. A better knowledge concerning the composition of these seeds (*e.g.* water content, composition of the endosperm and seed coat) may help to elucidate some of these effects. Moreover, vertical transmission of seed endophytes needs to be examined in detail (Truyens *et al*. 2015) because the seed stocks of the different genotypes have other origins.

In our study, we also aimed to restore growth, which was inhibited by both the *ccr1* mutation and Cd exposure. For this purpose, promising bacterial strains were selected to perform *in vivo* growth promotion assays. Almost all selected strains were **able to promote growth** of the leaves by generation of volatiles. Furthermore, one *Agrobacterium* strain promoted root growth in *ccr1-3* in non-exposed conditions and one *Microbacterium* strain did the same for *ccr1-6.* In other plant genotypes only some trends were observed. After Cd exposure, one *Sphingomonas* and one *Brevundimonas* strain for *ccr1-6,* another *Sphingomonas* strain for WT plants and *ccr1-6* mutants and one *Rhizobium* strain for *ccr1-3* revealed promising results in function of the attenuation of the negative effects of Cd. These preliminary results should be further explored in new inoculation experiments using more realistic conditions (*e.g.* plants grown on sand as a substrate) including more comprehensive parameters such as dry weight and several stress parameters. Furthermore, the endophytic origin of these effects should be explored by re-isolation of the inoculated strains.
In future experiments, results concerning endophytic communities and plant growth promotion should be examined with economically important crops (*e.g.* poplar) under field conditions.

SECTION 5
CONCLUSIONS AND PERSPECTIVES

Production of renewable energy from biomass is a good alternative to meet the increasing energy demand. However, the production of energy crops should not compete with food and feed production to ensure food supply for a growing population (Chen and Fu 2016). This food versus fuel competition can be reduced by making use of second-generation biofuels, *e.g.* bioethanol, derived from lignocellulosic energy crops (poplar, switchgrass, *Miscanthus* sp. and others) (Naik *et al.* 2010, Van Acker *et al.* 2014). These crops can be grown on marginal lands such as nutrient poor or contaminated lands (*e.g.* cadmium (Cd) contaminated soils) (López-Bellido *et al.* 2014, Schroder *et al*. 2008). However, the recalcitrance of the lignin polymers in the lignocellulosic biomass is challenging because of their negative impact on the enzymatic conversion of biomass into fermentable sugars and ultimately biofuel. In this respect, genetically modified plants with lower lignin content or more easily cleavable lignins can partially overcome the problem of conversion efficiency (Marriott *et al.* 2016).

In our study, we examined effects of mutants down-regulated for genes involved in the monolignol biosynthesis pathway on both (i) plants (growth, stress responses and lignification) and (ii) their associated microbiomes since endophytes play a role in plant health and growth. Furthermore, the effects on the plant traits and endophytic communities of the lignin-reduced mutants exposed to toxic amounts of Cd were examined. *A. thaliana* was used as a model species for woody plants (Nieminen *et al*. 2014); T-DNA knockout mutants for *4-COUMARATE:CoA LIGASE 1 (4CL1)* and *CINNAMOYL-COA-REDUCTASE 1* (*CCR1*) of the monolignol production pathway were chosen for our study.

5.1 The effects of the lignin-reduced genotype

The most prominent effects of the lignin modification were observed in **stems** of the lignin-reduced mutants (Fig 5.1). This was not surprising since the stem is the most lignified tissue (Boerjan *et al*. 2003) in *Arabidopsis*. For all examined lignin-reduced mutants the **lignin concentration** was reduced (Fig 5.1) and

compositional differences were observed such as lower H+G+S retrieval after thioacidolysis (Fig 5.1), indicating a more condensed lignin (Leplé *et al*. 2007), and a higher S/G ratio and H concentration for the *4cl1* mutant (Fig 5.1), supporting the outcomes of many other studies (Lee *et al*. 1997, Van Acker *et al*. 2013, Mir Derikvand *et al*. 2008, Smith *et al*. 2013, and others). In addition, the *ccr1* mutants showed a **retardation in stem development** as demonstrated by their growth phase and fresh weight (Fig 5.1). Furthermore, the inhibited growth of *ccr1* mutants was also observed for leaves, flowers, siliques and seeds (subsection 3.2). This is an additional support of effects of *CCR* down-regulation that were previously reported (Ralph *et al*. 1998, Leplé *et al.* 2007, Patten *et al*. 2005, Van Acker *et al*. 2013, Van Acker *et al*. 2014, O'Connell *et al*. 2002, Mir Derikvand *et al*. 2008, Dauwe *et al*. 2007, Jones *et al*. 2001, Goujon *et al*. 2003, Chabannes *et al*. 2001, Prashant *et al*. 2011, Ruel *et al*. 2009).

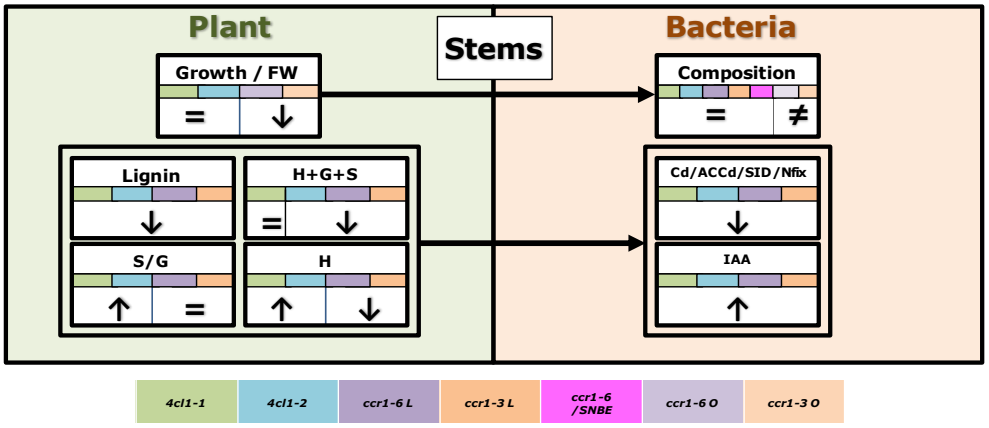


Figure 5.1: Presentation of the plant traits and subsequent effects on the endophytic community of stems of the lignin-reduced mutants in comparison to WT plants. Abbreviations: FW: fresh weight, Lignin: lignin concentration, H+G+S: monolignol measurement after thioacidolysis, S/G: syringyl/guaiacyl ratio, H: *p*-hydroxyphenyl concentration, Cd: cadmium tolerance, ACCd: ACC deaminase capacity, SID: siderophore production capacity, Nfix: nitrogen fixation, Psol: phosphate solubilisation, OA: organic acid production capacity, IAA: indole acetic acid production capacity, O: *ccr1* mutants with a similar age in comparison to WT plants, L: *ccr1* mutants with similar stem length in comparison to WT plants, *ccr1-6*/SNBE: complemented *ccr1-6* mutant with restored lignin levels in the vessels.

These changes at the plant level due to the genetic modification also affected the **endophytic communities** of the lignin-reduced mutants (predominantly) in

the stems. At first sight, the total bacterial endophytic community composition was most **different for the developmentally delayed *ccr1*** mutants (plants with a similar age in comparison to WT plants (O)) (Fig 5.1) and no effects on the diversity of the communities were observed (subsection 4.2). In *ccr1* mutants with a **similar stem length** (L) as WT plants we observed limited effects of the genetic modification on the endophytic communities. Furthermore, all lignin-reduced mutants revealed clear differences from WT plants concerning the selection of **phenotypic traits** of their associated bacterial community (Fig 5.1). For instance, in all examined lignin-reduced mutants, IAA producing capacity seemed much more important than for WT communities. Furthermore, lower relative abundancies of endophytes showing Cd tolerance, N-fixation, ACC deaminase capacity and siderophore production were observed in comparison to the communities of WT plants.

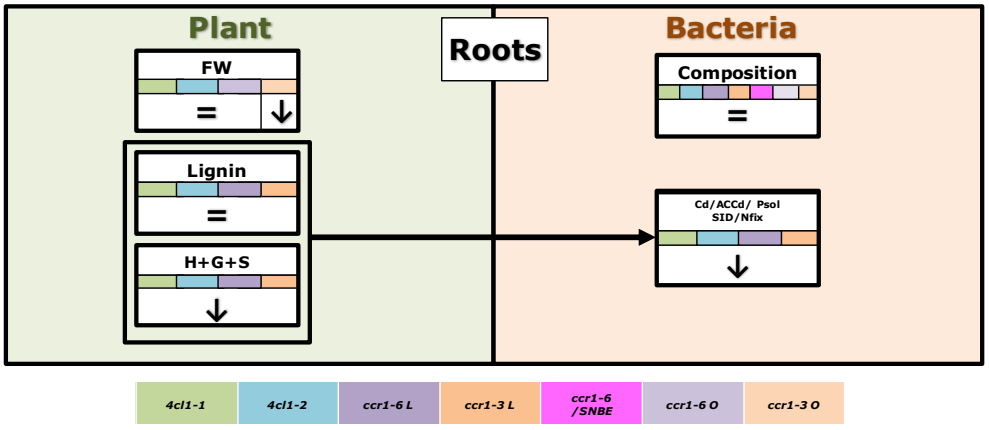


Figure 5.2: Presentation of the plant traits and subsequent effects on the endophytic community from roots of the lignin-reduced mutants in comparison to WT plants. Abbreviations: FW: fresh weight, Lignin: lignin concentration H+G+S: monolignol measurement after thioacidolysis, Cd: cadmium tolerance, ACCd: ACC deaminase capacity, SID: siderophore production capacity, Nfix: nitrogen fixation, Psol: phosphate solubilisation, OA: organic acid production capacity, O: *ccr1* mutants with a similar age in comparison to WT plants, L: *ccr1* mutants with similar stem length in comparison to WT plants, *ccr1-6*/SNBE: complemented *ccr1-6* mutant with restored lignin levels in the vessels.

In comparison to the stems, the effects of the genetic modification of the *4CL1* and *CCR1* genes on the **roots** were limited for both the growth and lignin concentration (Fig 5.2). The root **growth**, as determined by fresh weight, was lower for the *ccr1-3* mutant, although not for the other mutants (Fig 5.2). The

lignin concentration in the roots of lignin-modified plants was **not affected** (Fig 5.2) though the **lignin composition was different** as observed by lower H+G+S products after thioacidolysis (Fig 5.2), indicating a more condensed lignin. Our study is the first report to investigate the lignin levels in the roots of lignin-reduced mutants.

These limited effects at the root level were also found in the bacterial communities: **no effects on the endophytic composition** were detected on the total communities of roots of the lignin-reduced mutants in comparison to the WT root community (Fig 5.2). Moreover, also in the cultivable fractions of the root endophytes only slight differences were observed (subsection 4.3). However, **the phenotypic** traits of the selected root endophytes in the communities of all lignin modified plant genotypes were **affected** in a similar way (Fig 5.2). Lower relative abundancies of Cd tolerant, ACC deaminase producing, siderophore producing, P solubilising, and N fixating endophytes were detected in the lignin-reduced mutant root communities.

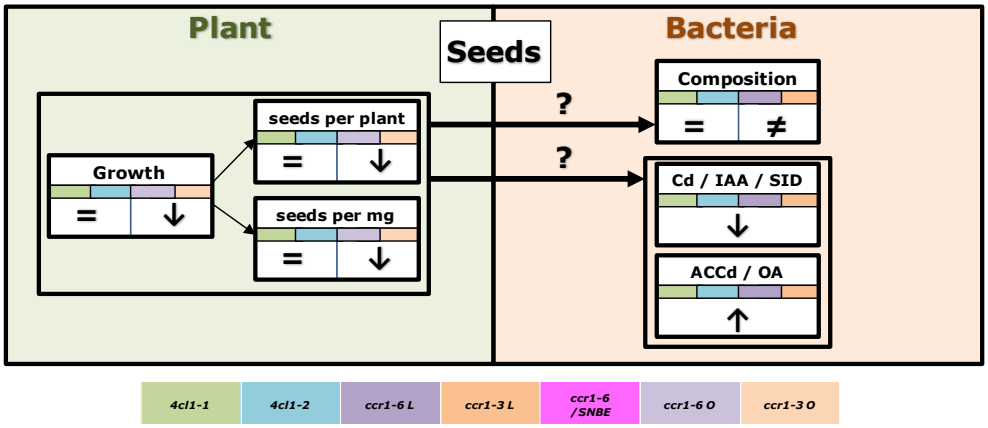


Figure 5.3: Presentation of the plant traits and subsequent effects on the endophytic community from seeds of the lignin-reduced mutants in comparison to WT plants. Abbreviations: Cd: cadmium tolerance, ACCd: ACC deaminase capacity, SID: siderophore production capacity, OA: organic acid production capacity, IAA: indole acetic acid production capacity, O: *ccr1* mutants with a similar age in comparison to WT plants, L: *ccr1* mutants with similar stem length in comparison to WT plants, *ccr1-6*/SNBE: complemented *ccr1-6* mutant with restored lignin levels in the vessels.

The most pronounced effects were observed on the endophytic communities of the seeds. The **total communities of seeds of the *ccr1* mutants were highly different** from the WT seed communities (Fig 5.3). However, the seeds of *ccr1* mutants have a different appearance and the developmental differences of *ccr1* mutants are difficult to circumvent. More research is required to comprehend the composition of the seed coat, the endosperm and other seed traits such as water content. Further, also in the seeds **all lignin-reduced mutants** selected in a same manner for **phenotypic traits** of the endophytic communities (Fig 5.3). Lower percentages of Cd tolerant and IAA producing endophytes, and higher percentages of ACC deaminase and organic acid producing strains were detected in the lignin-reduced mutants.

To summarize, the bacterial community composition in the stems was **most affected by the developmental stage**. In the seeds, the slower development of *ccr1* mutants may also affect the endophytic community composition though more research on seed traits is needed to better understand the effect. However, the **down-regulation of the monoligonol biosynthesis genes and subsequent reduction of the lignin concentration and alteration of the lignin composition clearly have effects** on the plant traits, resulting in a **different selection** of the **the endophytic community** based on their **phenotypic traits** which was the case **in all examined plant parts (roots, leaves, stems and seeds)**. These results are in line with those of Beckers *et al*. (2016b) wherein a selection of a specific phenotypic trait, the degradation capacity of ferulic acid, occurred in *CCR* down-regulated poplars. The selection of the endophytic community traits might be connected with the differences in lignin concentration and composition.

The observed changes in the endophytic community might be related to the fact that the genetic lignin modification can lead to (i) differences in permeability and thus **penetrability of the cell walls** of the lignin-reduced mutants for microorganisms (Miedes *et al*. 2014). Ions or other substances and endophytes may pass the cell wall barrier more easily. Indeed, higher concentrations of elements (non-exposed plants: Mg, K and Cu ($p<0.1$), Ca, Mn ($p<0.05$)) were observed in leaves of the *ccr1* mutants (Table 3.2.4 subsection 3.2) and could not be explained by increased up-regulation of metallothionein genes

(subsection 3.3). (ii) the endophytic community traits can also be affected by the **accumulation of different phenolic acids** in the lignin-reduced mutants which can have antimicrobial action (Rice-Evans *et al.* 1996) or can be used as alternative C-sources (Beckers *et al*. 2016b). (iii) another effect of the genetic modification of genes involved in the monoligninol biosynthesis pathway can be the **collapsing of the vascular system** (Mir Derikvand *et al.* 2008) which can affect the translocation of endophytes. However, this cannot explain the observed selection of phenotypic traits since all lignin-reduced mutants, including the *4cl1* mutants that have a normal vasculature, were affected in a similar manner. The selection of microbial functions in the community should be further examined using metagenomics and/or metaproteomics based on the total community (Kaul *et al*. 2016).

Furthermore, the **growth retardation of *the ccr1* mutants might be an obstacle in future applications** that would make use of *CCR* down-regulated plants (*e.g.* poplar) for bioenergy production. In our study, we found 2 promising **endophytic strains** (*Agrobacterium sp.* B8 in *ccr1-3* and *Microbacterium sp*. B15 in *ccr1-6*), which can improve root growth in direct contact with the endophytes, and 14 strains that enhanced **leaf growth of** *ccr1* mutants by release of volatile organic compounds (subsection 4.5). However, the growth improvement obtained in the roots was not sufficient and should be further optimised (Truyens *et al.* 2013). The use of consortia might contribute to this (Thijs *et al*. 2016).

Moreover, we should keep in mind the differences between our used hydroponics system using *A. thaliana* grown in cultivation rooms and field grown economically valuable plant species such as poplar. The hydroponic system has the advantage to allow the control of temperature, light conditions, humidity, pH of the growth medium and water supply. Moreover, a homogenous nutrient distribution in which all necessary macro and micro nutrients are bioavailable in sufficient concentrations for a good plant growth is guaranteed (Smeets *et al.* 2008). Though, the obtained results may be rather fundamental since *A. thaliana* is a plant that forms a ground rosette and some developmental processes characteristic to wood formation in trees may not be entirely identical

in this model organism (*e.g. A. thaliana* does not form parenchyma rays)(Nieminen *et al.* 2004). However, in field conditions in which more economically valuable species could be used, the parameters such as temperature, humidity, sunlight, pH of the soil and water availability may vary from day to day and seasonal effects are possible (Trupiano *et al.* 2014). Moreover, plants in field conditions may encounter other challenges such as erosion or wind (FAO 2008). Nutrients may be heterogeneously distributed (Walch-Liu *et al.* 2006) and shortages of nutrients can be possible. Another factor influencing the plants health may be competition of these plants with other plants (*e.g.* weeds) or soil living organisms. On the other hand, field grown plants may benefit from the presence of mycorrhiza, which cannot colonise our model plant *A. thaliana* (Vance 2008). Moreover, a high bacterial diversity is present in soils (Thijs *et al.* 2016) which can be advantageous since this gives a broad start inoculum whereof plants can select their endophytes though in case of inoculation experiments these native soil bacteria can also compete with these from the inoculum. Moreover, in field conditions plant roots will be anchored in the soil, which is not the case in our hydroponic system in which roots are suspended in an aqueous solution. By consequence a higher lignification may be needed in field conditions and more energy can be spent in this root system. Another difference between field grown economically valuable plants and *A. thaliana* in controlled conditions may be the duration of the experiment. Trees in field conditions can be grown for years whereas *A. thaliana* has a short regeneration time (Koornneef and Meinke 2010).

5.2 The effects of cadmium exposure

Lignin-reduced mutants were affected in another way by Cd exposure than WT plants. The **lignin concentration** of **stems** did not change for all studied plant genotypes (including WT) after Cd exposure of the plants (Fig 5.4), which is a promising result since additional lignification is unwanted for future applications. A slight difference in **%H** was observed in stems as it decreased after Cd exposure in the WT plants but remained unchanged in lignin-reduced mutants (Fig 5.4). On the other hand, after Cd exposure of the plants, the **lignin concentration** increased in WT **roots** while it decreased in **all lignin-reduced**

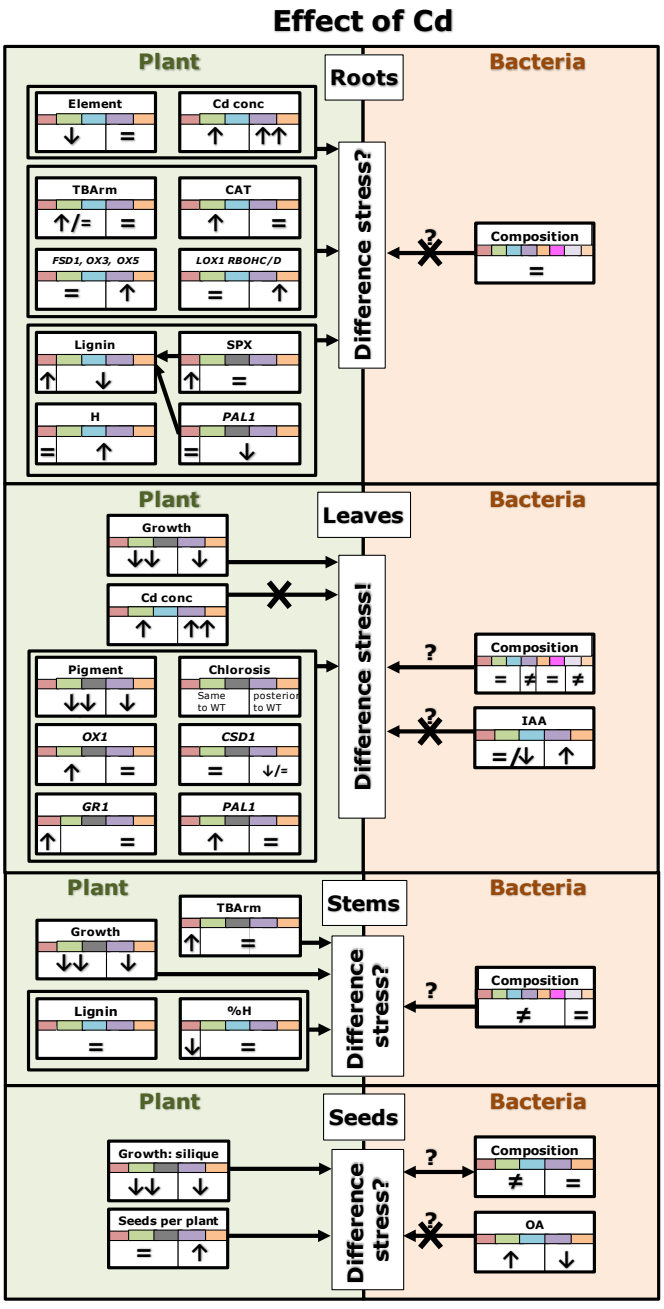


Figure 5.4: Presentation of effects after Cd exposure (Cd-exposed genotype in comparison to the non-exposed plant genotype) on the plant characteristics and subsequent on the endophytic community of roots, leaves, stems and seeds. Abbreviations: Cd conc: Cd concentration, Element: elemental concentration, TBArm: measure of lipid peroxidation, pigment: pigment concentrations, CAT: catalase capacity, *FSD1*: Fe super oxide dismutase gene expression, *CSD1*: Cu/Zn super oxide dismutase 1 gene expression, *OX1, OX3* and *OX5*: oxidative stress marker gene expression, *LOX1*: lipoxigenase 1 gene expression, *RBOHC* end *D*: respiratory burst oxidase homolog gene expression, %H: percentage of *p*-hydroxyphenyl monoligonols according to H+G+S, Lignin: lignin concentration, SPX: syringaldazine peroxidase capacity, *PAL1*: phenylalanine ammonia-lyase 1 gene expression, Nfix: nitrogen fixation, OA: organic acid production capacity, IAA: indole acetic acid production capacity, O: *ccr1* mutants with a similar age compared to WT plants, L: *ccr1* mutants with similar stem length compared to WT plants, *ccr1-6*/SNBE: complemented *ccr1-6* mutant with restored lignin levels in the vessels.

mutants (except for *ccr1-6* where it remained unchanged) (Fig 5.4). Furthermore, Cd exposure of WT plants lead to increased **SPX capacity** but not for the lignin-reduced mutants (Fig 5.4) and ***PAL1* expression** was unchanged in WT plants but lignin-reduced mutants displayed decreased gene expression corresponding to an enzyme that is involved in lignification (Fig 5.4). The H concentration in roots of WT plants did not change after Cd exposure while the H concentration increased for the lignin-reduced mutants (Fig 5.4). These results suggest that **Cd affects lignin concentration and/or composition differently in the lignin-reduced mutants**.

Further, several parameters responded in a different way to Cd exposure in ***ccr1* mutants** in comparison to WT plants. The **elemental concentration** (*e.g.* Ca, Zn) of the **roots** was lower after Cd exposure of WT plants though this was not the case for *ccr1* mutants (Fig 5.4). Additionally, the *ccr1* mutants did not show increased **lipid peroxidation** in roots after Cd exposure while increases were observed for WT plants (Fig 5.4), suggesting that the *ccr1* mutants might be less sensitive to Cd. Lipid peroxidation levels in stems of WT were significantly increased though not in lignin-reduced mutants. Further, ***ccr1* mutant leaves** revealed **a retardation in chlorosis** in comparison to WT leaves (Fig 5.4) and **pigment concentrations** (chlorophyll a, b and total carotenes) were less inhibited after Cd exposure of the plants for *ccr1* leaves in comparison to WT leaves (Fig 5.4). Also stress-related parameters revealed differences in the stress response of *ccr1* leaves. For example, ***OX1*** expression increased in WT plants while it was unchanged in *ccr1* leaves, ***GR1*** increased in WT plants but not in lignin-reduced mutants and ***CSD1*** expression tended to decrease in *ccr1* mutants but not in WT (Fig 5.4). Furthermore, ***PAL1*** was increased for WT leaves but not for *ccr1* leaves (Fig 5.4). Suggesting again that *ccr1* mutants should be less sensitive to Cd exposure.

In addition, **leaf, stem, flower, silique and seed** production were **less inhibited** after Cd exposure of *ccr1* mutants in comparison to WT plants (Fig 5.4). This was accompanied by **increased concentrations of Cd-ions** in **roots and leaves** of the *ccr1* mutants in comparison to WT plants (Fig 5.4). The

increased Cd concentration might be a result of more permeable cell wall barriers in the lignin-reduced mutants.

To summarize**, although *ccr1* mutants were taking up more Cd than WT plants, they showed a lower sensitivity towards Cd exposure, especially at the level of the leaves.**

We hypothesized that Cd exposure could influence the bacterial communities of the lignin-reduced mutants differently from WT plants and might eventually allow to explain differences in sensitivity to Cd of *ccr1* mutants. All communities (L) of developmentally similar stems, including those of WT stems, were changed upon Cd exposure while those of *ccr1* mutants with a similar age to WT plants (developmentally delayed in comparison to WT plants (O)) were unaffected (Fig 5.4). In the seed compartment, the *ccr1* mutants showed no change due to Cd exposure while other communities (WT and *4cl1* mutants) were affected upon Cd exposure (Fig 5.4). However, the difference in development of the *ccr1* mutants, which cannot be ruled out during seed production, may influence this observation for the *ccr1* seed communities. The differences in composition of *ccr1* seeds (*e.g.* endosperm, water content, accumulation of phenolic compounds) must be examined before further conclusions can be drawn. Moreover, in leaves, Cd-induced changes were observed in *ccr1* (O) mutants and *ccr1-6* (L) mutants though not in *ccr1-3* (L) mutants (Fig 5.4). Since only differences in bacterial communities were established in developmentally delayed plants and differences in Cd sensitivity and Cd exposure were observed in all *ccr1* conditions, this may not explain the observations concerning the lower sensitivity of *ccr1* mutants to Cd exposure. Furthermore, in the root compartment no differences were observed after Cd exposure or were not established in both allelic mutants of the same condition (similar development or similar age to WT) (Fig 5.4). We observed some differences in the proportions of phenotypic traits of the communities such as an increased percentage of IAA producing bacteria in the leaves of younger *ccr1* mutants and decreased percentages of organic acid production in *ccr1* seed communities (Fig 5.4). However, based on the phenotypic traits, the **bacterial communities did not seem decisive in determining the sensitivity**

towards Cd of these *ccr1* mutants. Nevertheless, the cultivability of strains may bias the observation (Ellis *et al*. 2003) and the total community phenotypic traits (Kaul *et al*. 2016) should be studied to allow further conclusions.

A possible explanation for the lower sensitivity of *ccr1* mutants to Cd can be the accumulation of **phenolic** intermediates of the monolignol biosynthesis pathway and derivatives thereof (Vanholme *et al*. 2012a, Xue *et al*. 2015) (*e.g.* cinnamic acid, *p*-coumaric acid, ferulic acid and sinapinic acid). These compounds indeed posses at least one carboxyl group that can **scavenge Cd** and thus can lower Cd toxicity. Moreover, they may enhance the uptake and translocation of metals (Anjum *et al*. 2015) in the *ccr1* mutants without increasing toxicity. Furthermore, these same phenolic compounds have **antioxidative** actions (Rice-Evans *et al*. 1996), which can even lower the Cd toxicity in *ccr1* mutants. An enhanced total antioxidative capacity was reported by van der Rest *et al*. (2006) in non-exposed *CCR* down-regulated tomato plants. The role of these phenolic acids in the Cd sensitivity should be elucidated in future experiments.

Further, we identified some endophytic strains that can **enhance** root **growth** of Cd-exposed plants (subsection 4.5). *Sphingomonas sp.* B6 in WT plants and *ccr1* mutants*, Brevundimonas sp.* B7 in *ccr1-6* mutants, *Sphingomonas sp.* B11 in WT plants and *ccr1-6* mutants and *Rhizobium sp.* B12 in *ccr1-3* mutants lowered the growth inhibition that was caused by Cd. However, differences were limited and inoculation of consortia might be a good strategy to improve root growth even more in Cd-exposed conditions.

5.3 Conclusion and perspectives

It can be concluded that in **non-exposed** plants the endophytic communities of stems and seeds of *ccr1* mutants are distinct from the WT communities. Although, we have evidence that these changes may be a result of the developmental retardation of these plants rather than effects related to the lignin-reduction itself. However, in root, leaf, stem and seed communities all lignin-reduced mutants selected the same, though different from WT plants, endophytic phenotypic traits. These changes can be linked to the lower lignin concentration (as demonstrated for stems) or different composition of lignin (as

demonstrated for stems and roots) though also the phenolic compounds (Vanholme *et al.* 2012a), differences in permeability of the cell walls (not observed in roots in our study) and the collapsing vascular system (Mir Derikvand et al. 2008) (not true for *4cl1* mutants) may be responsible for this observation.

In future experiments, the effect of the developmental retardation of *ccr1* mutants can be further explained by growing WT plants at a later moment, resulting in similar developed plants in comparison to the retarded *ccr1* mutants, and establish the developmental effect on the bacterial endophytic community. Moreover, the effects on the seed communities may be unravelled by examination of the composition of the seed coat, the endosperm and other seed traits such as water content. In addition, the effects on the phenotypic traits of the communities can be further examined using metagenomics and/or metaproteomics based on the total community.

It can be concluded that in **Cd-exposed** conditions the lignin-reduced mutants and especially *ccr1* mutants may be less sensitive to Cd toxicity while higher concentrations of Cd in roots and Cd and other elements (*e.g.* Ca, K, S, Zn, Cu, Na, Fe) in roots and leaves were found. Differences in reaction of the total endophytic communities were observed for *ccr1* mutants in comparison to WT plants though no prominent effects on the bacterial communities phenotypic traits were observed. A higher permeability of the cell wall, for which evidence of a lower induction of lignification due to Cd in roots and differences in lignin composition (H concentration) due to Cd exposure was found in our study, and presence of metal chelating phenolic compounds (*e.g.* ferulic acid) can explain these observations. Hence, the *CCR* down-regulated plants showed some promising results to grow them on marginal soils contaminated with Cd since (i) no additional lignification of the stems was observed and (ii) they seemed less sensitive to Cd toxicity.

In future experiments, the higher concentrations of elements in the lignin-reduced mutants can be further examined by exploring the gene expression of several metal transporters (*e.g. NRAMP6*). Further, the estimation of the total

antioxidant capacity in the lignin-reduced mutants with and without Cd exposure can be conducted by making use of the FRAP (Ferric Reducing Antioxidant Power) assay (Penarrieta *et al.* 2008, Kerchev and Ivanov 2008) and for the phenolic compounds the antioxidant capacity can be further explored using Folin-Ciocalteau colorimetric method (Singleton and Rossi 1965). Also further understanding the presence of various phenolic compounds and the abundances thereof can deliver further insight in the possibility of the hypothesis concerning the phenolic compounds.

Moreover, in our study we found evidence that both the **negative effects** of the **genetic modification** (*ccr1* mutation) and **Cd-exposure** can be counteracted by making use of **endophytic strains**. However, only small effects were established and the inoculation process and use of consortia should be further optimised to achieve better growth promoting results.
Our work was performed using *A. thaliana* as a model species for woody plants (Nieminen et al. 2014). Consequently, these results should be validated using an economically valuable species like poplar.

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