Hasselt University

Centre for Environmental Sciences

Studying the cell wall of *Medicago sativa* stems in response to long-term cadmium exposure

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Doctoral thesis presented on the 21th November 2018 at Hasselt University

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[Sciences of today

are the misapprehensions of tomorrow]

Abstract

Environmental pollution with heavy metals is a major issue of the recent decade. Especially cadmium (Cd) is of great concern due to its persistence and high toxicity even in trace amounts. Plants have developed several mechanisms to counteract exposure to abiotic stress such as Cd and existing data suggest that the cell wall plays an important role in the defence strategy of plants during heavy metal exposure.

The experimental set-up of the present thesis particularly focused on long-term Cd exposure of *Medicago sativa* L. using realistic soil concentrations (10 mg Cd kg^{-1} soil dry weight (DW)), which makes the study relevant from an agricultural and societal point of view. Thereby, the influence on the cell wall structure, cell wall- and soluble proteins as well as plant metabolism in stems of *M. sativa* was investigated and results were complemented with targeted gene expression analyses.

Cadmium exposure altered the abundance of cell wall proteins involved in multiple physiological processes such as defence response and oxidation-reduction processes, but also carbohydrate metabolism and cell wall remodelling. Especially the abundance and enzymatic activity of pectin methylesterase increased in response to Cd exposure, which influenced the pectin pattern and promotes the creation of Cd binding sites as well as its sequestration in the cell wall. Observed changes in the polysaccharide composition support the hypothesis that Cd exposure initiates alterations in the cell wall structure, which establish the cell wall as a physical barrier against the external threat. Additionally, Cd exposure stimulated the phenylpropanoid pathway towards the biosynthesis of isoflavonoids rather than lignin and led to an accumulation of isoflavone conjugates.

Although a growth impairment of juvenile plants was observed, Cd exposure to 10 mg Cd kg^{-1} soil DW did neither affect the biomass production of mature *M. sativa* plants nor its physiological performance. During long-term exposure to realistic Cd concentrations *M. sativa* seemed to readjust its primary and

secondary metabolite profile and established a new metabolic steady-state, which conveyed acclimation to the applied stress. This said, *M. sativa* would be a considerable candidate for phytoremediation attempts to clean-up contaminated sites.

Samenvatting

Bodem degradatie en verontreiniging en het verlies aan productiviteit hierdoor veroorzaakt vormen een belangrijk maatschappelijk probleem. In een industriële omgeving wordt een groot deel van de verontreiniging veroorzaakt door zware metalen. Door zijn peristentie in het leefmilieu en de negatieve effecten van cadmium (Cd) op dierlijk en plantaardig leven zelfs bij zeer lage dosissen, spitst veel onderzoek zich toe op dit metaal. Planten hebben een gamma mechanismen ontwikkeld om abiotische stress, zoals groei op Cd-vervuilde grond, te weerstaan en studies tonen aan dat de cel wand hierin een bepalende rol heeft.

Om resultaten te bekomen die relevant zijn voor landbouw en samenleving werden experimenten gedaan die realistische omstandigheden zo veel mogelijk benaderen. Alfalfa (*Medicago sativa* L.) werd gedurende een volledig seizoen gegroeid op grond vervuild met 10 mg Cd per kg droge grond. Met stalen genomen van dit experiment werd een vergelijkende studie gedaan naar de impact van Cd op de structuur van de celwand, en dit gekoppeld met een analyse van de celwand eiwitten. Deze data werd in perspectief gebracht met informatie over de impact van Cd op de metabolieten en de expressie van genen waarvan geweten is dat ze betrokken zijn in processen gerelateerd aan de structuur en samenstelling van de celwand.

Groei op Cd-vervuilde grond leidde tot de veranderde accumulatie van eiwitten betrokken in het behoud en de modulatie van processen die zich afspelen in de celwand. De belangrijkste processen waarin veranderingen werden waargenomen zijn defensie en oxidatie-reductie maar ook celwand dynamisme en suiker metabolisme. Cadmium leidde tot een toename in de hoeveelheid en de enzymatische activiteit van pectin methylesterase, een toename die de structuur van de celwand kan beïnvloeden en leiden tot de creatie van Cd-bindingsplaatsen. Bovendien wordt de hypothese dat de celwand een physische barrière vormt tegen externe factoren ondersteund door gemeten veranderingen in de chemische samenstelling van de celwand. Blootstelling aan Cd beïnvloedt ook de productie en accumulatie van metabolieten van de phenylpropanoid

pathway met een toename in de accumulatie van isoflavones en hun conjugatie producten.

Hoewel de blootstelling aan Cd een zichtbaar negatieve impact had op de groei van jonge planten, was er op het einde van het experiment geen invloed op de productie van biomassa en de algemene physiologie van de plant. Dit wil zeggen dat onder de in deze thesis gebruikte omstandigheden, *M. sativa* in staat is om zijn primair en secundair metabolisme aan te passen en een nieuwe metabolische homeostasis te bereiken zonder verlies aan productiviteit. Dit vermogen tot acclimatisatie maakt dat alfalfa een kandidaat is voor de phytoremediatie van vervuilde gronden.

Acknowledgement

I will never forget the first time I arrived in Luxembourg. It was a cold, rainy day in October 2014 and it was already dark when I got to Belval. I stayed in the hotel just in front of the station. Out of the train, out of the station, into the hotel, check-in and into my bed, as I had a cold. I had no idea where I was and what to expect. Opening the curtains of my hotel room on the next morning was a real bummer. Still gray, still windy, still rainy, the most unpleasant weather one can imagine and on top of that, I was looking at some big industrial furnaces. Frankly, every single part of me wanted to go home. What was this place? A big construction site in the middle of nowhere? Would this be my future?

Indeed, it would become my future.

First of al,l I need to thank **Dr. Jean-François Hausman**, who gave me the opportunity to carry out a PhD at Luxembourg Institute of Science and Technology (then still CRP-Gabriel Lippmann) and I took this unique, challenging opportunity with gratitude. He strongly supported me during my last year and brought me back on track when I was rather lost and close to surrender. I cannot say thank you enough! I equally thank **Dr. Kjell Sergeant**, my day-to-day mentor, who supervised me during the last four years, corrected and criticised my work in the most professional way. His passion about proteins is admirable and his skills in analyzing MS data greatly contributed to my work and the final thesis. It will always remain a mystery to me how he can sequence a protein by simply looking at MS spectra and doing some fancy calculations in his head… Just like Jean-François, he supported me when I lacked motivation and brought me back on track, for which I am very grateful.

For the past four years, I had the pleasure to work in a professional and friendly environment, surrounded by extremely competent people, of whom I learned a lot. I take the opportunity to thank **Dr. Gea Guerriero** for sharing her knowledge and skills in the field of genomics with me. Her involvement greatly contributed to my project and she always impressed me with her professionality

and expertise. My sincere thanks go to my former PhD-fellows **Marc Behr** and **Bruno Printz**, who both contributed to my work by sharing their skills and knowledge with me, as well as to my other team members **Aude**, **Christelle**, **Laurent**, **Sylvain**, **Thomas**, **Valentin** and **Xuan**.

Dr. Jenny Renaut gave me the opportunity to work together with the platform, which provided a high-quality technical facility. In this regard, I express my gratitude to **Céline Leclerq**, **Sophie Charton**, **Johanna Ziebel** and **Audrey Lenouvel** for their amazing technical skills, accuracy and care. All of them highly contributed to my thesis by supporting me in the lab and by analyzing my samples. Furthermore, I thank **Sébastien Planchon**, a human Swiss knife. His technical expertise in the field of proteomics is extraordinary. He taught me all my practical knowledge in gel-based protein research, starting from extracting the proteins to evaluating the results. Moreover, whenever I bothered him with technical problems of any kind, he helped me to find a solution. We had some great professional and personal discussions and I always enjoyed working with him.

I am grateful to **Prof. Ann Cuypers**, my thesis promoter from the University of Hasselt, for her great support during the last four years. I really appreciated her critical revisions of my work. Her opinions and ideas meant a lot to me and were always a valuable contribution to my work. Furthermore, she gave me the opportunity to join her group for three month in total, where I was received with warmth. It was a delightful experience, during which I had the opportunity to work in a very friendly university atmosphere. I will always keep my stay in Hasselt in good memory, last but not least owing to the people I had the pleasure to meet there. I especially thank **Sophie Hendrix** and **Els Keunen** for their everyday support in the lab and for sharing some good ideas about my work, for nice chats and great laughs.

Furthermore, I thank **Sabine Lüthje** for welcoming me in her lab at the University of Hamburg for a short stay, which allowed me to gain some technical skills in the field of membrane protein extraction.

I also take the opportunity to thank the members of my jury **Prof. Jan Colpaert**, **Prof. Jaco Vangronsveld**, **Prof. Domenico Morabito**, **Prof.**

Nathalie Verbruggen and **Prof. Stanly Lutts** for their external scientific expertise concerning my thesis.

Moving to a new city, far away from my comfort zone, my family and friends was tough, not to say it would have been impossible without all the fabulous people that I have met in Luxembourg. **Annick** and **Jeanne** welcomed me in their home for the first three months, which made my start in Luxembourg a lot easier and evenings less lonely. **Jeanne** introduced me to the city, showed me the Luxembourgish countryside and we spent some great evenings together. She became and still is a very good friend. I have to thank **Chiara** and although I only got to know her for a few months, I owe her a lot. I took over her apartment, which became my little island during the past years and she introduced me to most of the people I had the pleasure to spend my time with in Luxembourg. I thank **Cristina**, **Mads** and **Fintan** for our cycling trips to work, our chats and all the other time we spent together. Especially **Cristina** became a very close friend. I will also never forget how I met **Dario**. He dared to approach a grumpy German girl on the train on her way home while she was reading a book, a story that still makes me giggle. I am thankful to my wee chicken **Catherin**, who supported me especially during my last year. Despite avoiding each other in the beginning, Catherine being afraid talking to me thinking that I would not understand her and me *de facto* not understanding her, she became a very good friend, up for a wee drink and a wee chat at all times. I thank **Marta** for just being Marta, always in a good mood and the key player in our little group, trying to bring everyone together for an evening out. Thanks to **Elisa** for her honesty and trust. Furthermore, I thank **Enrico** for his jolly character and the many laughs we had together. I also thank **Thomas** for his inspiring ease of life. He has lots of interesting opinions, which made me reflect my own.

I am most grateful for all my loyal friends in and around Berlin who have supported me over the last years and made my visits home such joyful events. Many of them I have known since my childhood and although life separated us geographically, it has never separated us personally. **Felix** and his wife **Vera**, **Andi** and **Marie**, **Elli**, **Rike** and **Jacqueline**. **Jacqueline** probably influenced me most thanks to our shared trip, during which I learned a lot about myself. I

experienced what it meant to depend on each other, to solve arguments in a democratic way, to search for reasonable compromises and during which I also decided to go for the PhD adventure. If I could survive South America I would also survive a PhD, right? Thanks, **Jacqueline** for sharing this memory with me. I am convinced that this adventurous journey gave me the self-confidence and courage to apply for a PhD outside of Germany.

For sure, I thank the friends that I made during my studies. As fate would have it, **Vicky** started her PhD in Namur at about the same time as I started mine in Luxembourg. Knowing a friend in reach, only a two-hour train ride away, made me feel less lonely. She was already one of my best friends at University, but she became even more so during my PhD. Furthermore, **Sarah** already living in Cologne by then, became a very good friend during my time in Luxembourg. Being located so "close" to each other brought Sarah and me closer together than living in the same city had done before and we paid each other a visit every now and then. Also, **Oli** found some days each summer to pass by and spent a few days with me. Generally, most of my friends came by to experience the Luxembourgish life style and I appreciated every single visit because it showed me that they cared and that they had not forgotten about me despite being rather far away.

However, most of this was rendered possible by the love and support of my family. My **grandparents** and **grandmother** followed my career development with interest and I am glad to still have them around. My **parents** gave me a roof over my head untill I was 25 because I had decided to go to University. After I finally moved out to continue my studies in Dresden, I still depended on their support, which they never denied me. When I moved to Luxembourg, equipped only with a backpack and a laptop, they did not hesitate a minute to jump into their car a few weeks later, drive all the 768 km from Teltow to Luxembourg and bring me some more necessities and personal items. To do so, my pitiful parents even needed to invest in a new car ;-). I am aware that I would not have come so far without them and I would like to express all my gratitude!

My biggest thanks go to **Boyfriend**. We most likely fell in love in IKEA, the most romantic place on earth. In the following months, we went out more than once for a casual drink, both too shy to realise what was actually happening. Since then, we have spent the most wonderful time together. Your never-ending support is amazing and thanks to you, I did not give up on the PhD. I hope that one day I can give you back what you gave and give to me. The last years have not been particularly easy for us. Therefore, I am looking forward to our future even more, curious what it will bring.

Last but not least, I need to thank **Luxembourg**. We have not been good friends from the start. I always found it too small and sleepy, the opposite of fun. I did not know what it meant to be on my own till I came here and suddenly was "forced" to spend much of my free time with me and myself. Surprisingly, this was not easy and I needed to learn to accept my own company without getting desperate or crazy. After a transition period, I managed to stay busy and to not turn into a couch potato. Instead, I started running and joined a swimming club. I am now more active than I was before. Who would have thought that I would ever run a half marathon? Not me in any case. Luxembourg has made me discover some new sides about me and probably contributed more than anything or anyone else to my metamorphosis into an adult human being. I also discovered Luxembourg anew. I suddenly saw all the advantages it has to live in a smaller city, I started to know my way around and I found my routines. For my personal development, it was one of my best choices to come to Luxembourg.

One question I always hear when I tell people that I am living in Luxembourg is "Wow Luxembourg! What is it like living in Luxembourg? " My answer is: "Well, it is different. It takes some time but you get used to it." And so I did.

At last I have to thank **Gerhard Menzel** for is extraordinary fast and accurate proof reading of the manuscript.

Thank you!

Thesis achievements

Published articles:

Gutsch A., Keunen E., Guerriero G., Renaut J., Cuypers A., Hausman, J-F., Sergeant, K. Long-term cadmium exposure influences the abundance of proteins determining for the cell wall structure in *Medicago sativa* stems. *Plant Biol. J.* (2018). doi: 10.1111/plb.12865

Gutsch A., Zouaghi S., Renaut J., Cuypers A., Hausman, J-F., Sergeant, K. Changes in the proteome of *Medicago sativa* leaves in response to long-term cadmium exposure using a cell wall-targeted approach. *Int. J. Mol. Sci.* (2018), 19, 2498. doi:10.3390/ijms19092498

Sergeant, K., Printz, B., Gutsch, A., Behr, M., Renaut, J., and Hausman, J.-F. Didehydrophenylalanine, an abundant modification in the beta subunit of plant polygalacturonases. *PLoS One* 12 (2017), e0171990. doi:10.1371/journal.pone.0171990.

Accepted for publication:

A. Gutsch, K. Sergeant and J. Renaut, Chapter 13.9.4. "Application of Bottom-up and Top-down proteomics in *Medicago spp*." Accepted for publication in "The Model Legume *Medicago truncatula*" Editor: Frans J. de Bruijn

Submitted articles:

Gutsch A., Sergeant, K., Keunen E., Prinsen E., Guerriero G., Renaut J., Hausman, J-F., Cuypers A.: Does long-term cadmium exposure influence the composition of pectic polysaccharides in the cell wall of *Medicago sativa* stems? *BMC Plant Biology (minor revisions received September 2018)*

Gutsch A., Sergeant K., Hendrix S., Guerriero G., Renaut J., Lutts S., Alseekh S., Fernie A.R., Hausman J-F., Vangronsveld J., Cuypers A.: Changing profile of primary and secondary metabolites facilitates acclimation of *Medicago sativa* after long-term Cd exposure. *Submitted to Frontiers in Plant Science*

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Supplemental Figure S3-1: PCA of primary metabolties in *M. sativa* leaves after long-term Cd exposure.

Abbreviations

2D DIGE	two-dimensional difference gel electrophoresis
4CL	4-coumarate ligase
AA	amino acid
ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACN	acetonitrile
ACO	ACC-oxidase
ACS	ACC-synthase
ADC	aginine decarboxylase
AmBic	ammonium bicarbonate
ANOVA	Analysis of variance
Ara	arabinose
Asn	asparagine
BABA	beta-aminobutyric acid
BSTFA	Bis(trimethylsilyl)trifluoroacetamide
C3H	coumarate 3-hydroxylase
C4H	cinnamate-4-hydroxylase
Ca	calcium
CaCl	calcium chloride
CAD	cinnamyl alcohol dehydrogenase
CCoAOMT	caffeoyl-CoA 3-O-methyltransferase
CCR	cinnamoyl CoA reductase
Cd	cadmium
cDNA	complementary DNA
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHI	chalcone isomerase
CHR	chalcone reductase
CHS	chalcone synthase
CID	collision-induced dissociation
CoA	coenzyme A
COMT	caffeate O-methyltransferase
Ctr	control
Cu	copper

CWP	cell wall pellet
CWR	cell wall residues
DAO	diamine oxidase
DNA	deoxyribonuceic acid
DTNB	5,5-dithiobis(2-nitro-benzoic acid
DTT	dithiothreitol
DUF	domain of unknown function
DW	dry weight
EDTA	Ethylendiamintetraacetat
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid
ESI	electrospray ionization
EST	expressed sequence tag
F5H	ferulate 5-hydroxylase
Fuc	fucose
FW	fresh weight
GABA	γ-aminobutyric acid
GACC	γ-glutamyl-ACC
Gal	galactose
GalA	galacturonic acid
GC-MS	gas chromatography coupled to mass spectrometry
Glc	glucose
GlcA	glucuronic acid
Gly	Glycine
GSH	glutathione
GSSG	glutathione disulphide
H_2O_2	hydrogen peroxide
HCT	hydroxycinnamoyl transferase
HG	homogalacturonan
His	histidine
HPLC-FLD	high-performance liquid chromatography coupled with a fluorescence detector
IAA	indole-3-acetic acid
ICAT	Isotope-Coded Affinity Tag
ICPL	Isotope-Coded Protein Label
IEF	isoelectric focusing
IFS	isoflavone synthase
Ile	Isoleucine

iTRAQ	isotope Tags for Relative and Absolute Quantification
JA-ACC	jasmonyl-ACC
K	potassium
kD	kilodalton
KOH	potassium hydroxide
LC	liquid chromatography
Leu	Leucine
LiCl	Lithium chloride
MACC	malonyl-ACC
MALDI	matrix assisted laser desorption ionisation
Man	mannose
MAPK	mitogen-activated protein kinases
Met	methionine
MIQE	minimum information for publication of qPCR experiments
MS	mass spectrometry
MW	molecular weight
NaOH	H sodium hydroxide
NCBI	national center for biotechnology information
NMR	nuclear magnetic resonance
NSAF	normalized spectral abundance factor
ORF	open reading frame
PA	polyamines
PAGE	polyacrylamide gel electrophoresis
PAL	phenylalanine ammonia-lyase
PCA	principle component analysis
PCR	polymerase chain reaction
Phe	phenylalanine
PME	pectin methylesterase
POX	peroxidase
ppb	parts per billion
ppm	parts per million
Pro	proline
PS	photosystem
Put	putrescine
RG	rhamnogalacturonan
Rha	rhamnose
RIN	RNA integrity number

RNA	ribunuceic acid
ROS	reactive oxygen species
RT-qPCR	quantitative real-time PCR
RuBisCO	ribulose-1,5 bisphosphate-carboxylase/-oxygenase
SAA	systemic acquired acclimation
SAM	S-adenosylmethionine
SAR	systemic acquired resistance
SDS	sodium dodecyl sulfate
SEM	Standard error of the means
Ser	serine
SILAC	Stable Isotopic Labelling with Amino Acids in Cell Culture
Spd	spermidine
Spd S	spermidine synthase
Spm	spermine
Spm S	spermine synthase
SuSy	sucrose synthase
TF	transcription factor
TFA	trifluoroacetic acid
Thr	threonine
TMT	Tandem Mass Tags
TNB	trinitrobenzol
TOF	time of flight
t_r	retention time
Tris	Trihydroxymethylaminomethane
Tyr	tyrosine
UHPLC-DAD	ultra-high performance liquid chromatography - diode array detector
UPLC-MS	ultra-performance liquid chromatography
Val	valine
Vh	volt hours
XGA	xylogalacturonan
Xyl	xylose
Zn	zinc

Introduction

1. CadWALL project – An integrated study on the effect of cadmium exposure on the plant cell wall

Plants have a central position in food, feed, fibre and fuel production to match the requirement for a sustainable agriculture in regards to the fast growing population, which will probably reach up to 9.1 billion human beings by 2050. However, environmental stresses of natural and anthropogenic origin limit plant growth significantly and affect its full valorisation potential (Ahuja et al., 2010). The plant cell wall forms a protective barrier around plant cells and is the primary target for external threats. Apart from modulating cell growth, shape and differentiation, the cell wall is furthermore important for food, feed, fibre and fuel production (Burton and Fincher, 2014). It is the most abundant carbon source in nature, giving an added value to the soil during decomposition and is a significant factor in human nutrition through its roughage supply. Cellulose microfibrils are the load-bearing structure of the plant cell wall, which are embedded in a polysaccharide matrix, composed of hemicellulose and pectin. Cell wall proteins contribute to its integrity. Due to the cation binding capacity of the cell wall, sequestration of harmful metal ions in the cell wall network is suggested to play an important role in the defence strategy of plants during heavy metal exposure. Especially the heavy metal cadmium (Cd) is of great concern due to its high toxicity even in trace amounts and the uptake by crops poses the main entry route into the human diet, representing a danger to human health. Therefore, an integrated study on the effects of environmentally realistic Cd exposure on cell wall properties and downstream metabolic processes is conducted using the crop species *Medicago sativa*. Increasing our knowledge on stress-induced effects in plants will help to improve the valorisation process of plants and will be an important key in respect to a sustainable future.

The present PhD thesis was prepared in the framework of the CadWALL project. The project was carried out in collaboration between the Environmental Biology group at Hasselt University (Belgium) and the Plant Biotechnology group at Luxembourg Institute of Science and Technology (Luxembourg). Due to an agreement for the support of bilateral projects between scientists based in Belgium and Luxembourg, the CadWALL project was co-financed by the Fonds voor Wetenschappelijk Onderzoek (FWO) and Luxembourg National Research Fund (FNR) (FNR/FWO project INTER/FWO/13/14). The scientific work undertaken during the project benefited from the provided cross-border research activity and gave an added value to the PhD thesis.

2. General perspective

2.1. What is abiotic stress?

Abiotic stress is the consequence of the exposure of a living organism to non-ideal environmental conditions, other than pathogens, to such extent that its physiology is negatively impacted below the optimum level (Cramer et al., 2011). This definition can be extended by distinguishing between eu-stress and dis-stress, whereby eu-stress is an activating and stimulating stress with a positive impact on plant development and dis-stress on the other hand has a negative influence on plants and causes damage (Lichtenthaler, 1998). In any case, stress should be considered as a dose-dependent matter. Stressors are of different natural appearance such as mechanical forces, extreme temperature, drought, water, but can also have anthropogenic origins such as chemicals. Plants face several stresses through one growing season either concurrently or at different time points. The natural adaptation of plants to certain stressors determines their natural distribution on a local and global scale and abiotic conditions are the main determinants for the agricultural production in a certain region. Climate changes and resulting climatic extremes endanger the natural distribution of plants with negative impacts on crop yield and agricultural productivity due to which the topic of abiotic stress gets increasing attention from a socio-economic point of view. In plants, abiotic stress reduces their over-all performance by affecting growth, development, photosynthesis (Chaves et al., 2009), induce oxidative stress in the cells and disrupting the redox balance

(De Azevedo Neto et al., 2006). Through interaction with the uptake of essential nutrients, exposure to heavy metals can result in nutrient deficiency, which has an effect on plant physiology (Tuna et al., 2007) and causes further secondary effects in a dose-dependent manner (Peralta-Videa et al., 2004; Siedlecka, 2014).

The plant response to stress can be differentiated in four phases (**Figure I-1**). Before the stress, plants are in a situation where they reach a physiological optimum. The presents of a stressor initiates 1) response phase: alarm phase; 2) restitution phase: stage of resistance; 3) end phase: stage of exhaustion (long-term stress) and eventually lead into the regeneration phase after the stressor is removed assuming that the caused damage had not been to severe (Lichtenthaler, 1998). After going through all these phases, the final plant response to stress can be either elastic (reversible) or plastic (irreversible).

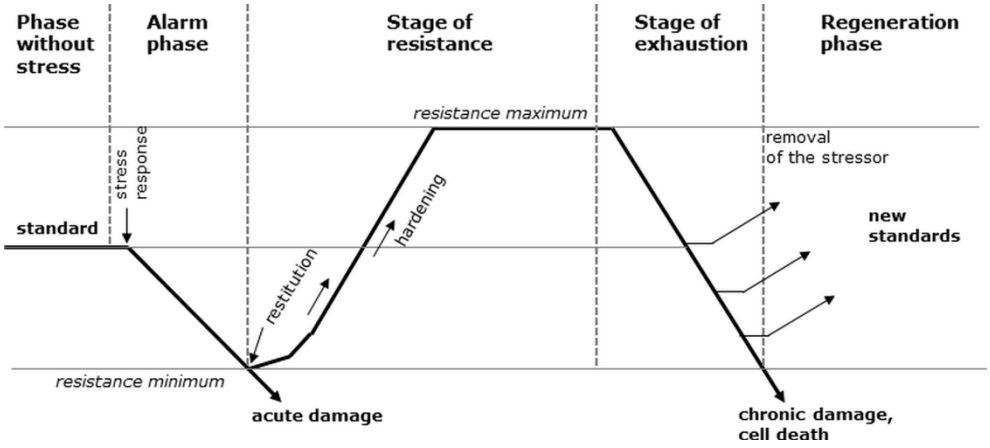

Figure I-1: General concept of stress syndrome responses of plants induced by stress exposure (adapted from Lichtenthaler (1998)).
Plants grow under physiological standard conditions. During stress exposure various responses and defence mechanisms will be put in place in order to cope with the stress. After removal of the stressor, a new physiological standard is established depending on the duration and the intensity of the applied stress.

Regardless of the exact stress, a suboptimal environment forces the plant to adjust its physiology by interlocking various mechanisms and eventually allowing the plant to survive by either stress avoidance or tolerance (Verslues et al., 2006). Most likely plants use a combination of the two (Touchette et al., 2009). Depending on the level and duration of the stress it can be defined as acute or

chronic, whereat acute stress is a severe and sudden event, which does not lead to an acclimatisation of the plant and requires a rapid response to counteract the provoked damage in order to anticipate plant death (**Figure I-1**). Opposing, chronical stress can be defined as a constantly present suboptimal event, which is light enough to not be lethal. Being exposed to chronic stress, the plant will enter a restitution stage during which a resistance to the stress will be reached, leading to a new homeostasis and regeneration (**Figure I-1**). Thereby, the evoked stress response in the plant differs fundamentally between the two forms, although a stressor is the same (Kovalchuk et al., 2007) and chronic subjection to sub-lethal stress can eventually lead to an acquired tolerance (Gratão et al., 2008) as an ability to survive unfavourable environmental conditions. Within this process, plants undergo multiple phases split into primary and secondary responses during which they develop a stress-specific profile. For example, short-term response appear on the level of primary metabolites, while long-term response rely on secondary metabolites (Kusano et al., 2011).

A stress is perceived through molecular responses, initiating a signalling transduction pathway to relay information about the received stress (**Figure I-2**). The initiated pathway eventually induces a physiological change, thereby the provoked physiological answer can be specific or a result of cross-talking between different signalling pathways. Membrane-localized receptor molecules such as sensory kinases sense for instant wounding, salt and osmotic stress and initiate complex downstream signalling networks (Osakabe et al., 2013). Calcium (Ca) is a ubiquitous second messenger during abiotic stress signalling. Changes in the cytosolic Ca profile appear during plant stress response depending on the stress, rate of stress development, previous exposure to stress conditions and tissue type. Those oscillations are detected and transduced by Ca-dependent protein kinases, which results in a molecular response (Chinnusamy et al., 2004). Another important key element in stress sensing are mitogen-activated protein kinases (MAPK) cascades as a cross-talk between different stress signals. These cascades are composed of at least three protein kinases, which activate each other sequentially through phosphorylation and eventually phosphorylate a specific target thereby modulating the activity of transcription factors, phospholipases, cytoskeletal proteins, microtubule-

associated proteins and the expression of specific sets of genes in response to environmental stimuli. MAPK cascades are involved in the response against pathogens, drought, salinity, cold, wounding, ozone, reactive oxygen species (ROS) and hormone stimuli (Danquah et al., 2014). Last but not least, hormonal signalling plays a role during plant stress signalling. For instance, abscisic acid mediates stress-responsive gene-expression and initiates the adaptation of physiological processes (Vishwakarma et al., 2017).

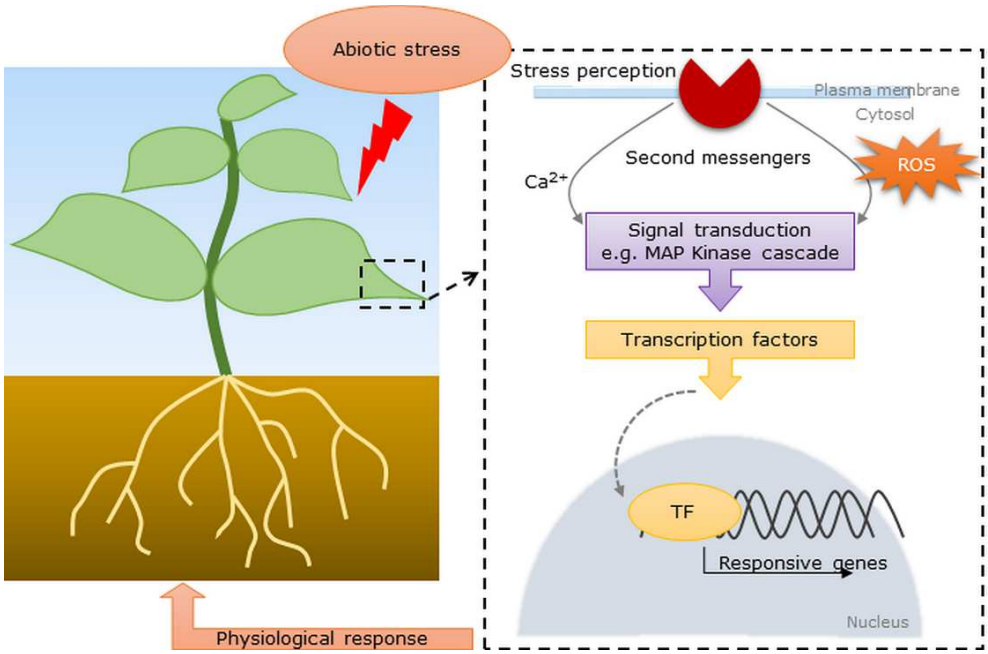

Figure I-2: Simplified signal transduction pathway of abiotic stress in plants.
Stress perception by membrane receptors is followed by the activation of a complex intracellular signalling cascade, involving second messengers, protein kinases, transcription factors (TF) etc., eventually initiating a physiological response.

Cadmium is one of the most studied environmental pollutants. Naturally the concentration of Cd in top soils is low although locally higher concentrations may occur. Anthropogenic activity, mines, metal smelters and refining industries emit Cd to the environment and the application of phosphate fertilizers are the major source of Cd in agricultural soil (Williams and David, 1974). Cadmium is a non-essential metal and toxic for all life forms. It is persistent in the environment and cannot be degraded into less harmful compounds. The first reports on negative human health effects appeared in the 1960 when people in Japan

suffered from a painful, osteoporosis-like bone disease (*itai-itai* disease), caused by a strong accumulation of Cd in their body. This incident raised the awareness of its poisonous nature and the danger it brings to the environment, especially in heavily industrialized areas. In average, European soils contain about 0.3 mg Cd per kg (Six and Smolders, 2014). However, the Cd emission in Europe has decreased by 73 % between 1990 and 2015 and only in a few European areas ambient air concentration still exceeds the target value[1]. Humans mainly take in Cd through their everyday diet. In bread and cereals Cd concentrations up to 35 ppb fresh weight were found. The concentration is much higher in animal products (pork kidney up to 1000 ppb) or some vegetables (spinach up to 120 ppb). According to the World Health Organization (WHO), the monthly intake of Cd should not exceed 25 µg per kg body weight[2]. Regardless, several hundred µg are consumed every day, while smoking of tobacco increases the daily intake dramatically (Pan et al., 2010).

The "fight or flight" response allows animals and humans to escape from harmful events in order to survive. Plants however are immobile and subjected to any kind of happening. Soil is their basis of life, providing essentials like water and nutrients, which are taken up via the root system from where it gets distributed throughout the plant. Plants are specifically impaired by growing on soil with an unbalanced nutrient profile, including an excess of Cd. In competition with essential elements such as potassium (K), Ca or copper (Cu), Cd enters the plant via metal transporters, embedded in the plasma membrane of root cells, thereby altering the uptake and distribution of essential nutrients. From there it arrives into the distribution flow and reaches other organs. Cadmium evokes parallel and/ or consecutive events that can be reason and result at the same time. Generally, Cd reduces plant growth, causes leaf chlorosis, interferes with the water balance and disrupts photosynthesis (Sanità Di Toppi and Gabbrielli, 1999). On a cellular level, Cd interferes with the redox status and enhances the production of reactive oxygen species (ROS) such as H_20_2, leading to oxidative stress (Cuypers et al., 2010). Free radicals in turn cause cross-links in the DNA,

[1] European Environment Agency, Air quality in Europe – 2017 report. https://www.eea.europa.eu//publications/air-quality-in-europe-2017

[2] Evaluation of the Joint FAO/WHO Expert Commitee on Food Additives (JECFA) http://apps.who.int/food-additives-contaminants-jecfa-database/chemical.aspx?chemID=1376

protein oxidation and degradation as well as fatty acid peroxidation, which changes the lipid composition of membranes. Furthermore, Cd deregulates enzymes and signalling components due to a chemical similarity with functional ions like Zn (Gallego et al., 2012). Finally, it also limits the valorisation potential of plants as it accumulates in them and hampers food/feed production but also combustion of plant biomass as Cd remains in the ashes. Returning those fly ashes to the ecosystem is a threat to the environment and counteracts the original idea to bring back minerals and nutrients to the soil through ash distribution.

Plants have developed several protective mechanisms to counteract Cd exposure and its evoked stress responses. Through the complexation with amino acids, organic acids and metal-binding peptides, Cd can be detoxified and sequestered in the vacuole to prevent further distribution. Additionally, Cd can be immobilized by binding it to the cell wall. As a general stress response, plants answer with an enhanced synthesis of stress proteins (Békésiová et al., 2008) and stimulated ethylene biosynthesis (Keunen et al., 2016). Furthermore, they enhance the antioxidant system to counteract the Cd-induced oxidative stress (Cuypers et al., 2010). Interestingly, some plant species are able to grow, survive and reproduce on soils with extremely high heavy metal concentrations, whereby most of these species behave as "excluders" characterized by a restricted metal uptake. To do so, toxic metals are retained and detoxified in the roots, thereby minimizing the translocation to other organs (Hall, 2002). Other species, however, show an opposite behaviour and actively take up large amounts of heavy metals from the soil, without showing signs of intoxification. Such plants tolerate high metal concentration, which are lethal to most other plants and are referred to as "hyperaccumulators" with *Thlaspi caerulescens* and *Arabidopsis halleri* as the most studied representatives (Verbruggen et al., 2009). They have a much greater ability in detoxifying and sequestering huge amounts of heavy metals in their aerials parts than non-hyperaccumulators and levels of metal take-up are typically one order of magnitude higher than those found in non-hyperaccumulating species. Those mechanisms of tolerance/hyperaccumulation do not rely on novel genes but moreover depend on the expression and regulation of genes, which are in common with non-

hyperaccumulating plants (Rascio and Navari-Izzo, 2011). Hyperaccumulators are of great interest for their possible application in phytoremediation of heavy metal contaminated sites. Their potential to remediate soil is an eco-friendly technique that can be used to gain back abandoned polluted areas and make them accessible again (Meyer and Verbruggen, 2012). This application is not limited to hyperaccumulators and also crop plants are investigated for their ability to take up and accumulate heavy metals from soils (Sekara et al., 2005). Therefore, a greater knowledge on metal uptake, plant defence networks and possible adaptation mechanisms are fundamental.

2.2. The cell wall of plants

An important feature of plant cells is their surrounding cell wall, which performs a number of essential functions. It provides shape to the many different cell types required for the formation of different tissues and organs. Cell walls form the interface between adjoining cells and thus are involved in intracellular communication. Constituting the cell surface, cell walls are the first interaction point between the plant cell and the environment. Therefore, they play a crucial role in biotic and abiotic stress responses. The fulfilment of its numerous functions is devoted to the cell walls' structure, which consequently is constantly changing during plant growth and development as well as during plant adaptation to various environmental conditions.

Primary cell walls are build-up during plant growth and need to be mechanically stable on one side but also extensible to allow cell expansion and plant growth. The main building blocks of the primary cell wall are cellulose, hemicellulose and pectin (**Figure I-3**). Cellulose is organized in microfibrils, which are composed of (1,4)-linked β-D-glucosyl residues. Their length is undefined and both, hydrophilic and hydrophobic properties can be displayed on the surface. These properties are an important determinant for the interaction of cellulose with other cell wall components (Cosgrove, 2014). Although it is known that membrane anchored cellulose synthase complexes polymerise UDP-glucose into linear β-1,4-glucan chains (Guerriero et al., 2010), the process of cellulose synthesis and more specifically its regulation and fine-tuning is poorly understood. Cellulose microfibrils are embedded in a complex matrix of hemicellulose and pectin. Hemicellulose is a rather heterogeneous group of

polysaccharides and their diversity was described in-depth by Scheller and Ulvskov (2010). Within the cell wall network, the main role of hemicellulose molecules is to bind to cellulose and form a flexible but strong structure. Hemicellulose backbones are characterised by β-1,4-linked glucose, mannose or xylose and hemicelluloses include xyloglucans, xylans, mannans and glucomannans as well as β-(1,3),(1,4)-glucans (Scheller and Ulvskov, 2010).

Pectins are by far the most complex polysaccharides of the cell wall and include homogalacturonan (HG), xylogalacturonan (XGA), apiogalacturonan, rhamnogalacturonans (RG) I and II (Cosgrove, 2005). A schematic overview of their basic structure is illustrated in **Figure I-4**, reproduced from Harholt et al. (2010). The backbones of pectins consist of unbranched galacturonic acid (GalA) linked at the O-1 and the O-4 position, which can be substituted at various positions with other sugar moieties such as xylose (XGA) and apiofuranose (apiogalacturonan). Besides these simple substitutions, also clusters of complex side chains build up by 12 types of glycosyl residues can be attached and form RGII (Harholt et al., 2010). Divergent from the other pectins, RGI has a backbone that does not only contain GalA but disaccharide (α-1,4-D-GalA-α-1,2-L-Rha) repeats. This backbone can be substituted with galactan, branched arabinan and arabinogalactan side chains. While RGII is highly conserved among vascular plants, the structure of RGI greatly varies (Harholt et al., 2010). The GalA backbone of pectin can be modified by methylesterification and/or acetylation, whereby the degree of methyl and acetyl esterification affects its physicochemical properties, notably the calcium-mediated complexation of HG molecules, which is hindered by methylesterification (Liners et al., 1992).

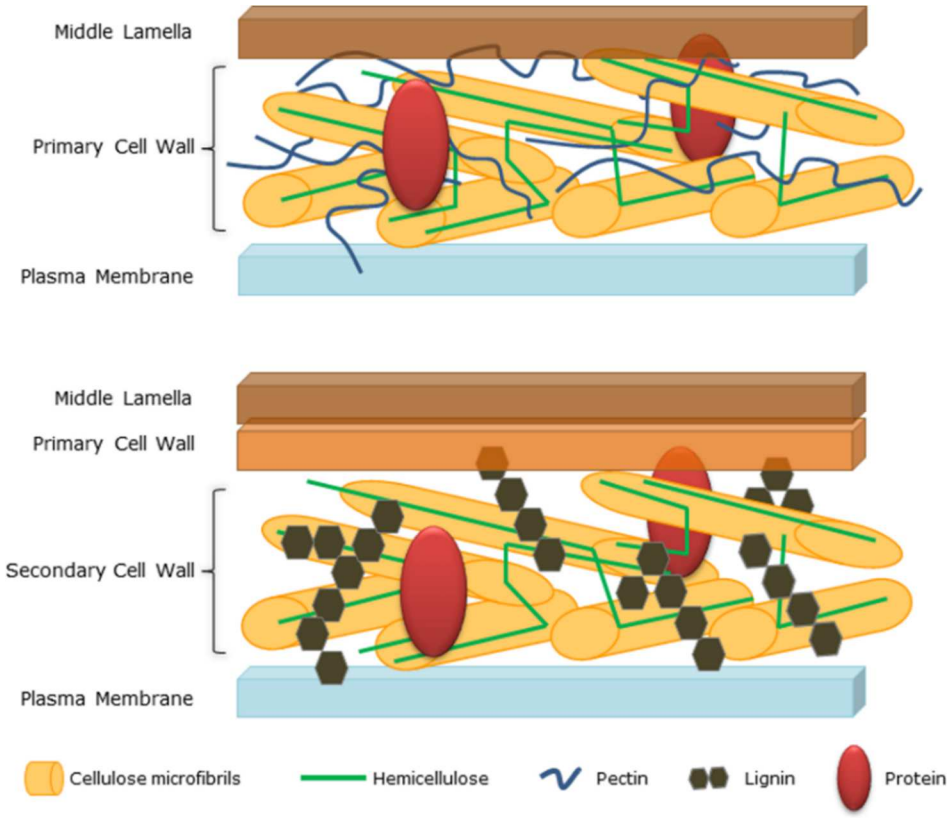

Figure I-3: Model of the primary and secondary plant cell wall.
The primary cell wall is located outside of the plasma membrane and mainly composes cellulose microfibrils, hemicellulose and pectin. The secondary cell wall is developed between the primary cell wall and the plasma membrane, providing strength and rigidity in tissues where growing has ceased. Lignin molecules are integrated, replacing pectin. Cell wall proteins are imbedded within the complex polysaccharide matrix of the primary and secondary cell wall. Adapted from Loix et al. (2017).

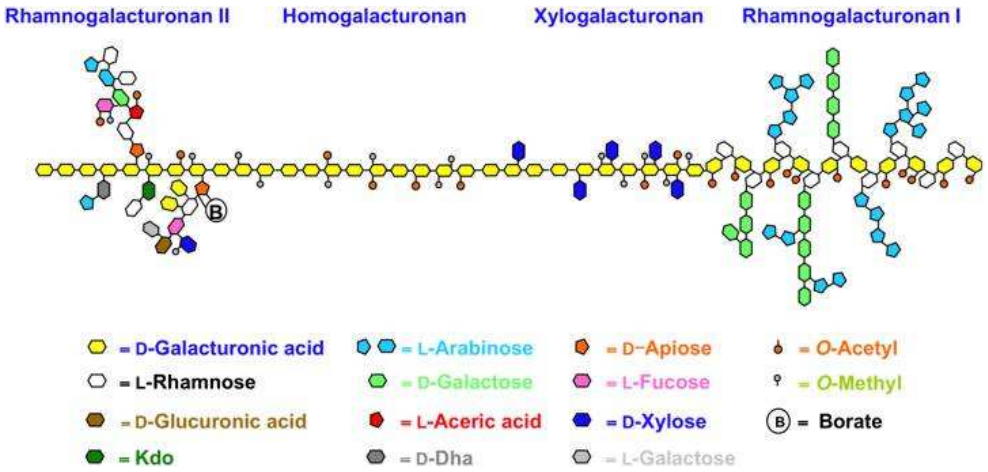

Figure I-4: Schematic structure of pectic polysaccharides:
Reproduced from Harholt et al. (2010)

Besides cellulose, which is synthesized by membrane-bound cellulose synthase complexes, matrix polysaccharides are synthesized in the Golgi apparatus from where they are transported in secretory vesicles for delivery to the plasma membrane. These vesicles fuse with the plasma membrane, releasing their cargo, which gets subsequently integrated into the cell wall (Lerouxel et al., 2006).

While plants mature, the secondary cell wall is developed between the primary cell wall and the plasma membrane, providing strength and rigidity in tissues where growing has ceased. Still, the structure of secondary cell walls can alter under certain circumstances whereby the process almost resembles this of the primary cell wall (Cosgrove and Jarvis, 2012). Likewise, it is composed of cellulose microfibrils but with a shift from pectin as second most abundant polymer to a higher quantity of hemicellulose. Lignin polymers are formed in secondary cell walls, enhancing mechanical strength, supporting wall thickening and provoke a general stiffness to plant cells (**Figure I-3**). Its water impermeability enables efficient water conduction within the vascular cells of stems. The main building blocks of lignin are the monolignols coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol (**Figure I-5**).

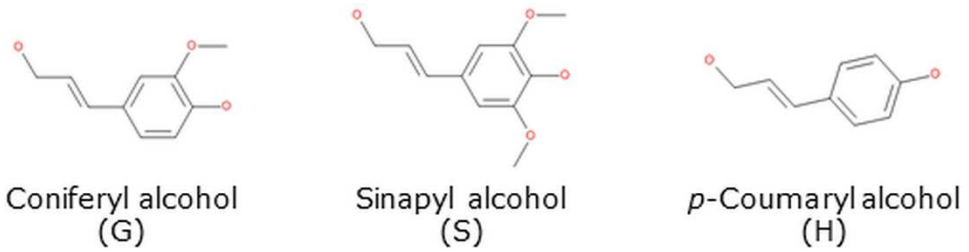

Figure I-5: Schematic structure of the three common monolignols.

They are translocated to the cell wall as monomers and are incorporated into the lignin polymers through oxidative radicalisation followed by radical coupling (Vanholme et al., 2010). Lignification negatively influences the degradability of plant material and secondary cell walls are less digestible as lignin limits the access to the cell walls' polysaccharides and their chemical/ enzymatic break down. From a plant physiological point of view, it protects the plant from multiple biotic stresses such as pathogen attacks. From an economic point of view, lignification limits the value of plant biomass in current industrial purposes as the removal of lignin is a costly process. However, lignification is responsible for the sustained use of wood in constructions.

Imbedded in the complex matrix of polysaccharides, cell wall proteins make up approximately ten percent of the cell wall mass. They are functioning in the communication with the surrounding matrix and maintaining signalling with the plasma membrane by sensing or releasing signalling molecules. Cell wall proteins are involved in oxidation-reduction processes, protein degradation and some interact with polysaccharides or other proteins via an interaction domain. Furthermore, structural proteins or those that act on polysaccharides such as hydrolases or expansin are found to be cell wall localized. Others are miscellaneous and for numerous cell wall-localized proteins no function has been assign yet (Jamet et al., 2006). All in all, the activity of cell wall-localized proteins modifies the cell wall in order to adapt it to the ambient circumstances so that the cell wall can fulfil its task. Different cell wall proteins can be found in altered abundance in different cell types, letting a cell-type specific function assume (Cassab, 1998). By far, *Arabidopsis thaliana* has the best documented cell wall proteome with 550 cell wall proteins presently identified, whereby a

well-accepted procedure identifies cell wall proteins when a signal peptide is recognized, directing the protein to the apoplast (Albenne et al., 2013). In recent years our knowledge on the cell wall proteome has grown thanks to new experimental strategies. Nevertheless, there is still a lack in the complete coverage of all cell wall proteins, leading to an absence or under-representation of certain protein families (Albenne et al., 2014).

To better understand the structure and dynamic nature of the plant cell wall it is essential to enlarge our knowledge on the cell wall proteome. They are an inevitable part of the cell wall, customizing its features in order to perform its tasks.

2.3. The cell wall as a defence strategy against cadmium stress?

The plant cell wall is the first structure that comes in touch with the external environment and hazardous elements such as Cd. The cell walls' capacity to retain Cd ions has been demonstrated (Ramos et al., 2002), being most effective in the roots (Zornoza et al., 2002). The nature of Cd binding is determined by the amount of available polysaccharide carboxyl groups. In higher plants, the pectic polysaccharide homogalacturonan (HG) is mainly responsible for Cd binding in the cell wall network, whereby a low methylesterification degree of HG supplies free carboxyl groups, which are able to bind Cd, promoting the formation of the egg-box structure (Krzesłowska, 2011). It has been demonstrated that Cd exposure alters the physical structure of the plant cell wall. It becomes thickened in places of Cd deposition whereby pectin was identified to provide the main binding sides (Vollenweider et al., 2006). Cadmium increases the abundance of negatively charged de-methylesterified HG (Douchiche et al., 2007), which can bind Cd and thereby promotes sequestration of Cd in the cell wall. The de-methylesterification is catalysed by pectin methylesterase, which is a known cell wall protein and its activity increases upon Cd exposure (Paynel et al., 2009). Obviously, pectin accounts for immobilization of Cd in the cell wall, preventing its further translocation through the plants and underpins is contribution to a defence strategy against this heavy metal.

In addition, lignification of the cell wall enhances during Cd exposure (Elobeid et al., 2012) and contributes in making the cell wall less permeable for Cd, thus limiting its entry into the cell. Lignification confers tolerance to plants on one side (Cheng et al., 2014; Van De Mortel et al., 2006) but also supports the process of xylogenesis, which restricts plant growth and makes the contribution of lignification paradoxical. Lignin is synthesised through oxidative polymerisation of monolignol molecules (Vanholme et al., 2010), which links the process directly to the redox status of the plant. Cadmium induces oxidative stress in plants, leading to the accumulation of H_2O_2, which functions as a signalling molecule triggering enzymatic activities (Cuypers et al., 2010, 2016). Higher activities of cell wall-localized peroxidases in response to Cd contributing to the lignification process of the cell wall are described in literature (Chaoui and El Ferjani, 2005). Lignin is synthesized via the phenylpropanoid pathway, of which phenylalanine ammonia lyase (PAL) catalysis the initial step. Its enhanced activity upon Cd exposure was shown (Kováčik and Klejdus, 2008; Pawlak-Sprada et al., 2011), emphasising its important role in the process of lignification during an active defence response. Furthermore, PAL activity is triggered by ethylene (Hyodo and Yang, 1971) and ethylene levels increase in response to Cd (Schellingen et al., 2014). Cadmium influences several steps upstream from lignification, all contributing to an effective defence response during Cd exposure.

Xyloglucan is another relevant polysaccharide in the cell wall that binds Cd in the cell wall and supports Cd tolerance acquisition of plants. Its cleavage is catalysed by the cell wall-localized xyloglucan endotransglucosylase/hydrolase and overexpression of the enzyme lowered the xyloglucan content in the cell wall resulting in lower Cd accumulation. Contemporaneously, Cd toxicity was alleviated (Han et al., 2014b).

Thus, the capacity of the cell wall to exclude or immobilize Cd results from alterations in the polysaccharide composition initiated upon Cd exposure. However, changes in the abundance of cell wall proteins are equally important during stress responses. Those proteins can be essential for the structural determination as illustrated in the previous examples, and also contribute to a limited entry of Cd into the cytoplasm. Multiple studies on changes in the

abundance of apoplastic proteins upon exposure to stress can be found in literature (Dani et al., 2005; Meng et al., 2016).

Clearly, the cell wall and its structure contribute to the defence response of plants during Cd exposure, allowing their survival and conferring tolerance to the constraint. Molecular studies of the plant cell wall will contribute to obtain insights into essential mechanisms that take place at the level of the cell wall during the defence against Cd and will help to characterise those molecules providing the highest protection.

2.4. *Medicago sativa* L.

The genus *Medicago* belongs to the legume family. From an agricultural point of view, legumes are highly important not only as forage for animals but also in the human diet. They are an excellent source of protein and dietary fibres, providing a variety of micronutrients and carbohydrates (Messina, 1999). Thanks to their root system, the cultivation of legumes gives an added value to the soil by ameliorating the soil structure. Many legume plants establish a symbiotic relationship with nitrogen-fixing bacteria, forming root nodules within the root system. The bacteria are fed sugars by the plant and in return serve as nitrogen source for the plants. Due to this unique symbiosis, legumes are often ploughed in the soil as green manure in crop rotation systems, where the fixed nitrogen gets released into the soil and serves as a nitrogen source for other plants. The use of crop rotation between legumes and non-legumes increases productivity, therefore, legumes are referred to as biofertilizer (Peoples et al., 2009; Vessey, 2003).

The globally important forage crop *Medicago sativa* (**Figure I-6**) is probably one of the oldest domesticated plants, which was and is mainly used for feeding livestock (Veronesi, 2010). It is easy to cultivate, grows under various climate conditions and thus can be cultivated in many regions, which coined it "Queen of Forages" with an annual production of over 50 million tons just in the USA. In Europe *M. sativa* is mainly produced in Italy, France and Spain using a surface of 90660 ha agricultural land[3]. As a legume it is capable of nitrogen fixation

[3] http://www.escaa.org/index/action/page/id/2

together with its symbiont *Rhizobium meliloti* and therefore gives an added value to the soil, limiting the need for fertilizer application. This makes *M. sativa* a frequently cultivated plant in organic, sustainable farming. The plant leaves have a high protein content, which fits the needs of the feed market while its less digestible stems can be used for industrial purposes such as bioethanol production (Samac et al., 2006; Sanderson et al., 2006). Its cultivation has a positive environmental balance when the energy requirements for input of its cultivation are related to the actual output. Therefore, *M. sativa* is a preferred species in sustainable practices (Kim and Dale, 2003). This economic and ecological importance makes *M. sativa* a highly interesting plant in research.

Figure I-6: Field of *M. sativa* in Belgium (summer 2018).

M. sativa is the centre of various agricultural research articles regarding its crop yield, quality and its fodder suitability (Douglas et al., 1995; Iannucci et al., 2002; Li et al., 2007) and a high interest exists in using *M. sativa* to clean up soil from heavy metal pollution. The process of phytoremediation is an ecological approach for the rehabilitation of contaminated soil in industrial, agricultural and urban areas using plants that can sustain hazardous metal exposure and the potential of *M. sativa* for heavy metal removal was demonstrated (Gardea-Torresdey et al., 1998; Singh et al., 2009). Onwards, the symbiosis between *Rhizobium meliloti* and *M. sativa* is in researchers' focus thereby using different approaches (Gage et al., 1996; Itzigsohn et al., 1993). As a rich source of secondary metabolites, it is studied for its bioactive compounds and their

application in industry and agriculture (Karimi et al., 2013; Rafińska et al., 2017). Also, *M. sativa* is used for genetic engineering attempts (Samac et al., 2004; Weeks et al., 2008) and gene expression studies are a useful tool to investigate nitrogen assimilation (Shi et al., 1997), metabolic pathways (McKhann and Hirsch, 1994; Xiang and Oliver, 1998) and the cell wall of *M. sativa* (Behr et al., 2015, 2018; Guerriero et al., 2014).

Medicago sativa has been used in numerous proteome studies addressing different scientific questions such as seed germination (Yacoubi et al., 2011), stem development (Printz et al., 2015b), nutrimental influence (Printz et al., 2016), stress response to heavy metals (Dai et al., 2016), salt-stress response (Rahman et al., 2015), temperature adaptation and water deficiency (Chen et al., 2015; Li et al., 2013; Rahman et al., 2016). Also targeted proteome studies are available specifically addressing the cell wall proteome (Verdonk et al., 2012; Watson et al., 2004) and a new optimized protocol for cell-wall protein isolation was published recently (Printz et al., 2015a).

3. Proteomics in *M. sativa* – a technical introduction

Adapted from: *A. Gutsch, K. Sergeant and J. Renaut, Chapter 13.9.4. "Application of Bottom-up and Top-down proteomics in Medicago spp." Accepted for publication in "The Model Legume Medicago truncatula" Editor: Frans J. de Bruijn*

Contribution: *A. Gutsch drafted the outline and the manuscript. K. Sergeant and J. Renaut participated in the manuscript refinement. J. Renaut furthermore provided the contact with the editor and was responsible for the submission.*

3.1. Proteomics in Plants

The proteome is the entire set of proteins present in a living organism at a certain time point under specific conditions. It overcomes the grey zone of genomics as proteins are the biological active molecules coded by genes. Gene expression does not necessarily infer that an active protein can be identified and quantitatively gene expression and protein abundance often do not correlate in a linear way. Furthermore, the proteome is larger than the genome as one gene can code for more than one protein due to alternative splicing. In addition, post-translational modification of proteins influences their functional properties, which separates them from their non-modified forms. Thus, a better picture of the actual functioning of an organism at a given time point under defined conditions will be obtained by characterizing the protein content, a study which is referred to as proteomics.

Tools for protein identification, identification of protein-protein interaction, and posttranslational modifications are available (Abdallah et al., 2012a). Protein identification and characterization approaches can be classified in two main routines: the bottom-up approach which is based on protein digestion, peptide identification, and finally protein assignment or the top-down approach wherein the initial step is based on the intact protein, including posttranslational modifications (Abdallah et al., 2012a; Oeljeklaus et al., 2009). While the quantification is done on the peptide level in the bottom-up approach, it is done on the protein level in the top-down approach.

The tool of proteomics has been applied in all aspects of plant research including plant development, response to environmental conditions (abiotic and biotic stress), and nutrimental influence to name some. Researchers try to understanding where and when proteins are present in an organism, their specific function, how they interact with each other and with the other molecules that are present in the organism at a given time point under certain conditions. Various reviews summarizing plant proteomics and provide an overview about used plants, tissues, investigated sub-proteomes, which scientific question was addressed and the used techniques (Jorrin Novo et al., 2015; Tan et al., 2017; Vanderschuren et al., 2013).

Initially proteomic studies in plants for instance in *A. thaliana* (Kamo et al., 1995) and rice (Komatsu et al., 1993) were based on 2D electrophoresis. Since then proteomics has been applied in model plants as well as in various crops, vegetables, fruits, weed and forest trees (Islam et al., 2003; Samyn et al., 2007; Sergeant et al., 2011; Tan et al., 2017). Although still frequently applied, classical 2D electrophoresis is no longer the only method of choice as gel-free liquid chromatography approaches are the current state-of-the-art and frequently used in plant proteome analysis (Matros et al., 2011). However, the classical 2D DIGE remains unrivalled when it comes to real protein complexity such as identification of protein modification and their quantification. This information can be of great biological importance in regard to the addressed scientific question and the data interpretation (Rabilloud, 2002).

3.2. Quantitative 2D gel electrophoresis in plant proteomics

Two-D electrophoresis is still the most commonly used method for proteome analysis in plants (Tan et al., 2017). This situation, in contrast to studies on animals and bacteria, is due to the difficulties using LC-MS based approaches on plants. First of all the model plant by excellence *A. thaliana* is without a societal and/or economic interest. While genome sequencing is straightforward in bacteria, the large genome size and polyploidy of most crop plants makes it less straight forward in plants. Furthermore, cross-species identification of proteins is difficult due to the large metabolic diversity in plants. Therefore, analysis of

huge datasets of LC-MS approaches is more error-prone when applied on plant proteome studies.

In 2D gel electrophoresis proteins are first separated based on their isoelectric point (isoelectric focusing) followed by a second separation based on their mass (polyacrylamide gel electrophoresis). The separated protein spots are visualized either by pre-separation labelling and fluorescence detection or by post-2D staining. First described in 1997, a more evolved variant of this approach makes quantitative protein analyses easier by labelling the samples with different dyes prior to separation (difference gel electrophoresis – DIGE) (Ünlü et al., 1997). Up to three different samples can be separated on one gel using Cy3, Cy5 and Cy2 as dyes which are covalently linked to the proteins. Thereby, two dyes are used for labelling two different samples while the third dye is used to label an internal standard, which is composed of an equal amount of all used samples (Arruda et al., 2011). By including a dye-swap in the experimental set-up, preferential protein labelling is avoided (Lilley and Friedman, 2004). Proteins of each sample will migrate according to their isoelectric point and size. Proteins common between samples will migrate to the same position. Afterwards the gel is scanned with different wavelengths unique for each dye. By comparing spot intensities, the relative abundance of proteins in each of the samples can be determined. Thereby, the internal standard serves as a reference for statistical evaluation of observed abundance changes, facilitating cross-gel quantification (**Figure I-7**). This gel-based approach was used in multiple proteome studies (Chen et al., 2016; Kieffer et al., 2009; Meisrimler et al., 2011; Semane et al., 2010) and since it is a top-down approach it allows to distinguish isoforms or post-translational modifications although identification depends on the MS analyses e.g. (Sergeant et al., 2017).

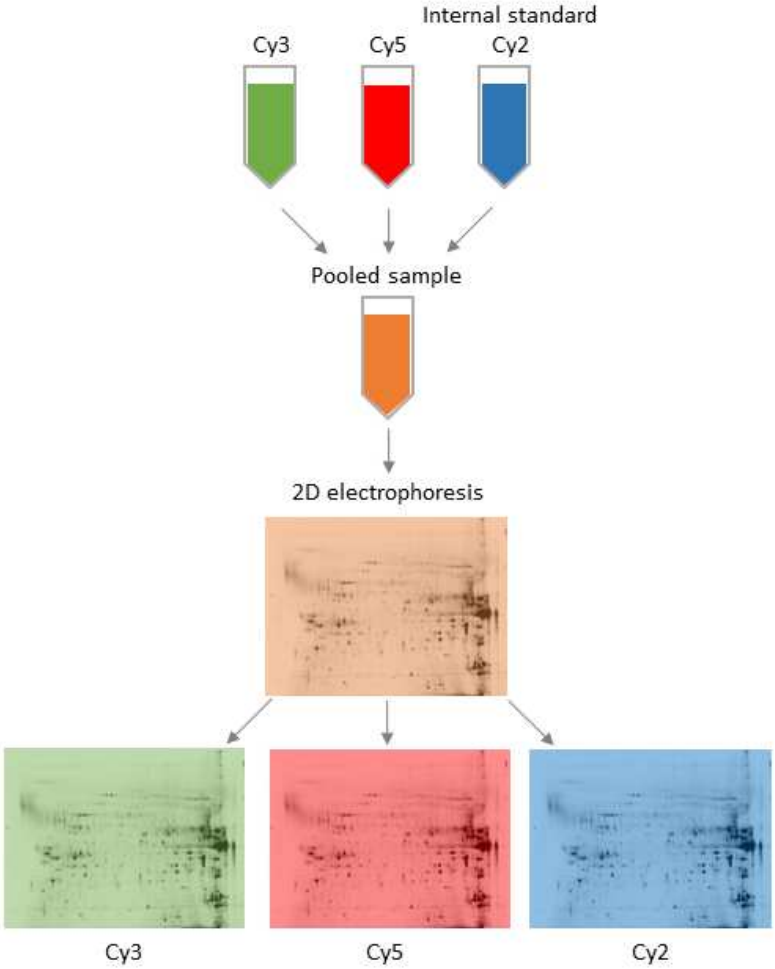

Figure I-7: 2D DIGE. Dyes are used to label different samples prior to electrophoresis.
Gel patterns are visualized by fluorescence with a wavelength unique for each label. Quantification is done by comparison of the florescence intensities. The use of a Cy2-labeled internal standard on all gels of an experiment facilitates the analysis of multiple gels.

Although being a commonly used technique in proteome research, 2D electrophoresis has limits in reproducibility and quantification. The aqueous conditions and the negative impact of charged detergents during gel electrophoresis limits its application to study certain groups of proteins such as hydrophobic or membrane-spanning proteins (Santoni et al., 2000). The complexity of biological samples generally exceeds the peak capacity of a 2D gel

and different proteins often migrate to the same position, which makes identification ambiguous, hindering accurate and specific quantification. Multiple protein identifications in the same spot are a great disadvantage of 2D electrophoresis and lower the number of significant identifications tremendously. Furthermore, detection of proteins with very low or very high molecular weights is difficult. Also, the separation of proteins with extreme pI values is limited (IPG strips typically ranging from pH 3 to 10). Both separation steps still suffer from an unequal and insufficient resolution for proteins (Arentz et al., 2015; Bae et al., 2003). Although the technical equipment is advancing, the total time for one experiment is long and the limited possibility for the automation of 2D experiments is a great bottleneck. Despite these disadvantages, 2D electrophoresis is still the only routinely used technique for the identification and characterization of intact proteins.

As an alternatively, gel-free top-town MS approaches can be used for targeted analysis, in which an intact protein is subjected for MS analysis and fragmented directly in the mass spectrometer. It is suitable for single protein identification or simple protein mixtures. As a top-down approach, it facilitates the identification of proteoforms and their quantitative abundance in the sample. The sample preparation is easy without the need of labelling. But the complexity of the proteome makes that current available top-down tools for separation lack resolution and/ or specificity. However, it has the potential to become the most powerful tool in proteome research, providing deeper insights into the complexity of proteomes and their proteoforms (Han et al., 2014a; Toby et al., 2016).

In the recent years new approaches have been developed to overcome 2D drawbacks and gel-free bottom-up proteomics gains more and more importance. It overcomes the limitations in running complex protein mixtures and allows high-throughput analysis.

3.2.1. Quantitative gel-based proteomics in *Medicago*

When going through literature, gel-based proteomics was used to study different topics in *Medicago* species for example in seeds of *M. truncatula* the heat-stable protein fraction was targeted in order investigate the abundance of proteins that

are specifically linked to desiccation tolerance (Boudet et al., 2006) and for profiling the heat-stable proteome during late maturation (Chatelain et al., 2012). Studies on the proteome of root nodules in correlation with phytohormones can be found (Prayitno et al., 2006; van Noorden et al., 2007) and gel-based proteomics was successfully applied when studying the stem proteome in response to iron deficiency (Rodríguez-Celma et al., 2016). Two-D electrophoresis was used to identify proteins, which are induced during root symbioses (Bestel-Corre et al., 2002) and furthermore, it was the method of choice in a comparative proteomic study between *M. truncatula* and *M. sativa* to investigate their salt stress response (Long et al., 2016).

M. truncatula is a widely used model plant for biological studies and available literature on gel-based proteome studies is much more abundant as for *M. sativa*. However thanks to its economic value, *M. sativa* gets into researchers focus and several proteome studies were done addressing multiple topics. For instance the proteome of *M. sativa* seeds was studied in the frame of seed vigor during salt stress using 2D electrophoresis (Yacoubi et al., 2011). Other studies addressed the changes in the stem proteome which occur during growth and in response to Cu nutrition (Printz et al., 2015b, 2016) and proteomic changes where investigated in the leaves of *M. sativa* when the plants were exposed to drought conditions (Aranjuelo et al., 2011). Furthermore, 2D electrophoresis was recently used to investigate how the cell wall proteome of *M. sativa* stems changes during mild, long-term Cd exposure (chapter 1).

All cited literature is only an extraction of available studies on *M. truncatula* and *M. sativa* and many more can be found for both species where a gel-based approach was used to answer various research questions. Although gel-free methods are state-of-the art in proteome science, the use of this approach is not without limits, of which some will be discussed below.

3.3. Gel-free quantitative proteomics in plants

Commonly used gel-free proteomics is a bottom-up strategy. Protein samples are digested and the resulting complex peptide mixtures are separated by liquid chromatography (LC) prior to mass spectrometry (MS). Mass spectrometric identification and quantification of these peptides allows to describe the

proteome composition in the initial sample. Since peptides are relatively uniform in physical and chemical composition, their separation by LC is much more uniform compared to the LC-separation of proteins, resulting in automation. This has accelerated the pace of proteome analysis making it a high throughput study. Thereby, the quantification can by either done by peptide labelling or by following a label-free approach.

3.3.1. Label-based gel-free quantification

Stable isotope labelling of proteins was the first gel-free approach to quantify proteins in different samples. Protein samples are digested and peptides labelled with different isotopic mass tags prior to LC-MS/MS. The relative protein quantity is calculated based on the intensity ratio of isotope-labelled peptide pairs (**Figure I-8**).

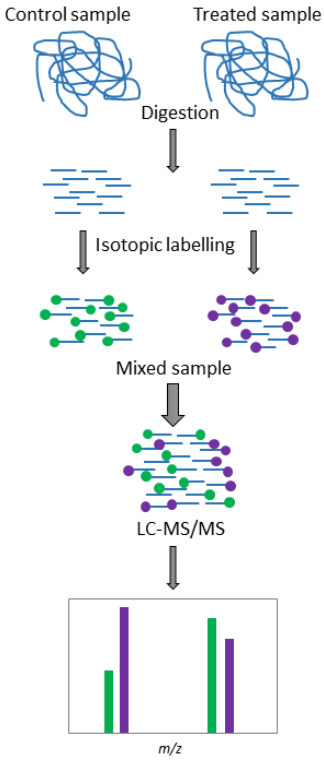

Figure I-8: Labelled gel-free quantitative proteomics.
Protein samples are digested and peptides are labelled with different isotopes. Samples are combined and analysed by LC-MS/MS. Relative protein quantification is based on the intensity ratios of isotope labelled peptide pairs.

Different labelling approaches have been developed such as $^{18}O/^{16}O$, Isotope-Coded Affinity Tag (ICAT), Isotope-Coded Protein Label (ICPL), isotope Tags for Relative and Absolute Quantification (iTRAQ), Tandem Mass Tags (TMT) and Stable Isotopic Labelling with Amino Acids in Cell Culture (SILAC), which have been successfully applied in different proteome studies (Islam et al., 2003; Nelson et al., 2006; Nogueira et al., 2012; Parker et al., 2012). Most common in plant proteomics is iTRAQ labelling and various studies in different species can be found in literature (Lan et al., 2010; Neilson et al., 2011; Qi Wang et al., 2014; Yang et al., 2013). During a study on *Brassica juncea* and its root-proteome changes induced by Cd exposure, iTRAQ quantification was compared with the 2D DIGE approach (Alvarez et al., 2009). Remarkably, nearly two-fold more quantitatively changed proteins were identified using iTRAQ (130 compared to 69 spots from 2D DIGE). Using 2D DIGE, multiple proteins were identified in a single spot, which excluded them from biological interpretation and diminished the number of proteins to 37 spots, in which only one individual protein was present. To some extent, the results from both methods overlapped but only a few of those overlapping identifications had a similar abundance change. Furthermore, using iTRAQ enabled the identification of proteins that are estimated to be of low abundance. Although both techniques are complementary, Alvarez and co-workers (Alvarez et al., 2009) revealed major weaknesses of gel-based proteomics such as low resolution of protein separation and underestimation of specific functional classes. On the other hand, DIGE enables the visualization of post-translational modifications and isoforms, which gives complementary information about the stress response. This information gain can for instance be seen in the 2D DIGE spots containing fructose bisphosphate aldolase-like protein. The protein was identified in three spots of which one had an altered abundance in the opposite direction (Alvarez et al., 2009). This observation likely corresponds to the differential phosphorylation of the protein due to exposure to abiotic stress (Hu et al., 2015).

Prior to labelling, each sample is manipulated a number of times (reduction, alkylation, protein digestion), and the used reagents can interfere with the labelling process, resulting in incomplete labelling and impacting quantification. Furthermore, technical variance can be introduced during each of these

preparation steps, affecting the results. Metabolic labelling can bypass those issues by introducing labels *in vivo*. Labels are incorporated during protein synthesis thus making it a useful tool for quantitative proteomics. SILAC for instance uses growth media containing labelled arginine or lysine, which are incorporated into proteins. This incorporation creates a known mass shift and thereby allows comparison of peptides between different samples. Since changes in the isotope composition do not induce a shift in LC-retention time, the quantification is based on the intensity ratio of isotope-labelled peptide pairs (Ong et al., 2002; Ong and Mann, 2006). Such an approach minimizes the introduction of errors during sample processing. However, SILAC is less suitable in plant proteomics as plants synthesize their amino acids instead of using an external source. Therefore, incorporation of labelled amino acids is low (e.g. only 80 % in *A. thaliana* cell culture (Gruhler et al., 2005)) and the application is restricted to cell cultures. Recently a protocol for SILAC *in vivo* was published, which opens new opportunities in plant research (Lewandowska et al., 2013). Although promising, this has for the moment hardly been applied. More efficient is N^{14}/ N^{15} labelling wherein incorporation reaches almost 100% and can be used in plants as well as in cell cultures (Engelsberger et al., 2006; Ippel et al., 2004). While for SILAC the introduced mass shift is known, it is unknown for N^{15}-labelling and sequence dependent, which makes it less straight forward to generate quantitative information. N^{15}-labelling is furthermore expensive and slow, requiring repeated passages to attain uniform labelling.

3.3.2. Label-free gel-free quantification

Label-based gel-free proteomics can be time consuming, complicated and expensive. Furthermore, labelling is done after several processing steps, which may introduce technical errors. Sample labelling can be incomplete leading to erroneous quantification and false data interpretation. One way to overcome these drawbacks is metabolic labelling such as SILAC although not without its own limitations. Label-free proteomics is a commonly used alternative approach in gel-free quantitative proteomics, overcoming these issues. Labelling prior to mass spectrometry is not needed, which significantly simplifies sample preparation and eliminates the possibility of technical errors. Each sample is individually subjected to LC-MS/MS for spectra acquisition.

Protein quantification can be done based on changes of the ion intensity or the peak area of the same peptide in the different samples. The chromatographic peaks of detected ions in LC-MS are correlated with the ion abundance in the sample and thus can be compared in different samples. By injecting calibrated concentrations of digested peptides, it was demonstrated that peak areas in LC-MS analysis increase with increasing concentration of the peptides. Furthermore, plotting the peptide concentration against the peak area showed a linear correlation (Chelius and Bondarenko, 2002). An alternative quantification method in label-free proteomics is spectral counting. The spectral counting approach compares the number of MS/MS spectra belonging to the same protein appearing in multiple MS/MS datasets. As an increased protein abundance results in the detection of an increased number of its peptides, an increasing number of MS/MS spectra are generated. To handle the effect of protein length on spectral counts a normalized spectral abundance factor (NSAF) was introduced, which is defined as number of spectral counts (SpC) identifying a protein, divided by protein length (L), divided by the sum of SpC/L for all proteins in the experiment. Therefore, an overestimation of the abundance of larger proteins is avoided (Zybailov et al., 2006) (**Figure I-9**).

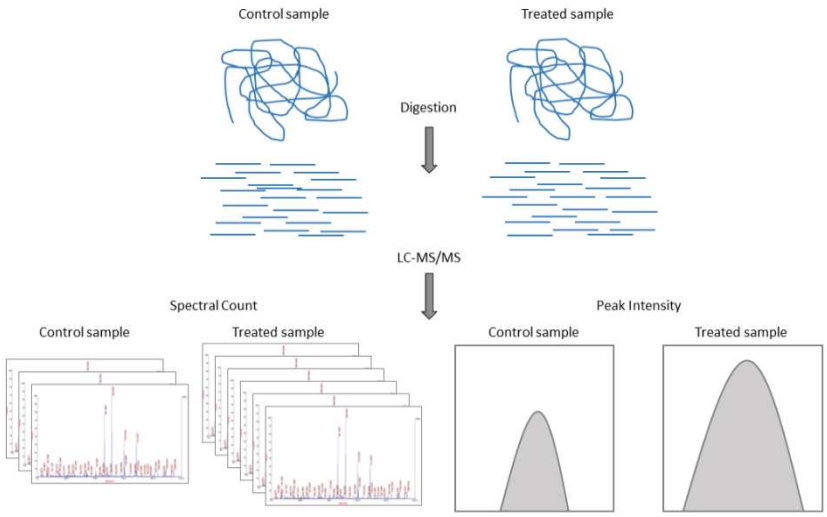

Figure I-9: Label-free quantitative proteomics.
Each sample is individually analysed by LC-MS/MS. Quantification is based on either comparing peak areas or spectral counts of identified peptides.

Accurate data processing is mandatory for reliable statistical evaluation of protein abundance changes. Therefore, eliminating background noise for peak detection, careful peak alignments to correctly match the corresponding peaks between multiple LC-MS runs and normalization is needed for final statistical analysis to determine the significance of changes in protein abundance. Protein identifications are done by database searches for which different software applications are available (Sandin et al., 2014).

Both quantification methods have been successfully applied in different studies (Shen et al., 2009; Zhu et al., 2010). When both methods are compared, protein ratios determined with peak intensities match those determined with spectral counting although the latter method is more sensitive in detecting protein abundance changes. Peak intensity as well as spectral counting have a good reproducibility between repetitions (Old et al., 2005). Nevertheless, some discrepancies in quantification results have been reported. Using spectral counting, the obtained fold changes in protein abundance are differently evaluated than in the peak intensity approach and applied programs for processing data attain peptide information differently, which leads to contradicting results (Katz et al., 2010). Researchers should bear in mind that the method of choice and the used software slightly impact their results and thus need to be considered when evaluating results.

3.3.3. Gel-free proteomics in *Medicago*

Gel-free quantitative proteome analysis can either be done label-based or label-free. Both methods were successfully applied in *Medicago* and will be the topic of the following paragraph.

A first application of iTRAQ-labelling on *Medicago* was done in *M. truncatula*. Authors coupled OFFGEL fractioning with iTRAQ-labelling, whereby during OFFGEL electrophoresis peptides and proteins where separated by their isoelectric point in a liquid-based system. The study aimed at optimizing the workflow for the analysis of microsomal proteins from roots (Abdallah et al., 2012b). As a model plant, *M. truncatula* was furthermore used in several studies in which label-free quantification was the method of choice to answer multiple scientific questions. Label-free protein identification was used for instance to

unravel changes in the root membrane proteome of *M. truncatula* in response to mycorrhizal symbiosis. By using a 1D fractioning prior to LC-MS/MS a total of 1226 proteins were identified in root membrane-enriched samples (Abdallah et al., 2014). Abdallah *et al.* used the spectral counting approach to quantify identified proteins and assigned 96 proteins as to be differently abundant in response to mycorrhization. Those findings largely overcome the number of differential expressed proteins found in previous proteome studies on myccorhization when using 2D analysis (Valot et al., 2005) and gives an added value to the field of membrane proteome research. With a focus on drought stress responses, 377 individual proteins were identified with LC-MS/MS in the root nodule of inoculated *M. truncatula*, *o*f which 90 proteins where shown to be differently abundant in response to drought stress based on their spectral count (Larrainzar et al., 2007). At that moment, it was the most comprehensive plant nodule proteome dataset and states the efficiency of LC-MS/MS in proteome research. As a result, new potential marker enzymes were identified which have an important role in drought stress response.

Gel-free proteome studies in *M. sativa* are scarce in literature. Anyhow, a recent study on the proteome of *M. sativa* was published in the background of hydrogen gas-induced Cd resistance (Dai et al., 2016). iTRAQ labelling was used for the label-based quantitative proteome analysis and authors assigned pathways and their associated proteins, which are specifically involved in H_2-driven Cd resistance. A label-free LC-MS/MS approach was applied to unravel quantitative changes in the cell wall proteome of *M. sativa* during long term Cd exposure. Several proteins determining the cell wall structure changed in abundance reinforcing the hypothesis that changes in the cell wall structure help to establish the call wall as physical defence barrier against Cd. The study will be presented in detail in chapter 2 of the present thesis.

3.4. Concluding remarks

Gel-free quantitative proteomics is a fast developing technique, which overcomes some disadvantages of commonly used gel-based approaches. It enables the analysis of certain protein groups that always have been problematic. The needed experimental time is short and streamlining of acquisition and data analysis has made high-throughput proteomics possible.

Several plant proteome studies can be found in literature using different gel-free approaches. To date, most of the available proteome studies on *M. sativa* used 2D gel electrophoresis as the method of choice and state-of-the-art gel-free studies are barely available. However, the status of *M. truncatula* as a model legume makes that numerous proteome studies using gel-free techniques are published. As a closely related species to *M. sativa*, these studies are highly interesting in the means of protocol development to make gel-free techniques applicable for *M. sativa*. However, to date 2D electrophoresis is still the only top-down approach enabling routine analyses of native proteins which makes is still a commonly used tool in proteomics.

4. Selecting the right database for protein identification

Protein identification by mass spectrometry relies on sequence databases and the more of the proteome of a species is available in the database the more efficient is the proteomic analysis. As a source for sequences, the National Center for Biotechnology Information (NCBI) provides the most comprehensive sequence collection that is freely available. Generally, sequenced and fully annotated genomes are available for a limited number of plant species including monocots and dicots showing a great proteome coverage. However, important crop plants remain underrepresented, making that most of the available data are from model species such as *Arabidopsis*, rice and poplar. These so-called model plants are important in research as tools for the detailed analysis of cellular and molecular processes enabled by the sequence availability. The focus on these species allowed the development of other resources (mutant seeds, genome maps, physical markers…) providing valuable biological insights and permitting functional assignments of genes and their coded proteins (for example *The Arabidopsis Information Resource* TAIR [https://www.arabidopsis.org/index.jsp]).

For the majority of plants, species-specific sequences databases are not available and protein identification is based on protein sequence similarities between different species, which will give the closest homolog as a result. Three possibilities can be successfully applied, of which the best working strategy

depends on the studied proteins. Firstly, a conserved sequence can be identified by a cross-species database search, whereby the use of a related species as a database entry for protein identification can give satisfying results as illustrated in **Figure I-10**. Most of the peptide masses/ sequences do not change and identification of the *M. sativa* protein can be done based on the *M. truncatula* entry.

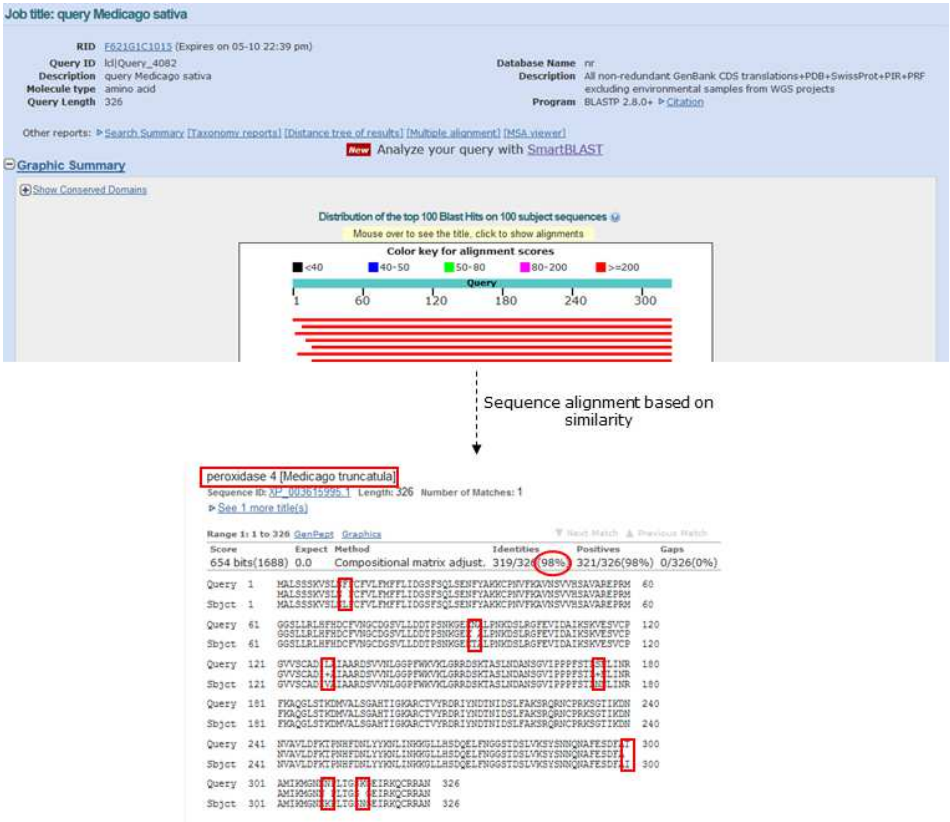

Figure I-10: Cross-species homology sequence search.
The BLAST algorithm provided by NCBI identifies similarities between biological sequences. It compares a query sequence to sequence databases and calculates the significance of the alignment. The submission of an unknown amino acid sequence of *M. sativa* gave a sequence identity 98 % to peroxidase 4 from *M. truncatula* with only a few mismatches.

However, cross-species homology search even between closely related species is not always evident (**Figure I-11**). The protein sequences of leghemoglobin from *M. sativa* (NCBI entry AAA32660) and *M. truncatula* (XP_003616494) are 90 % identical and 97 % of the amino acids are conserved. But only the first peptide

has the same sequence and mass (829.42 m/z), while the C-terminal peptide has indeed the same mass but the sequences are different. The three inner peptides would not be identified since the peptide mass is a primary determinant during database research. However, erroneous cross-species homology search becomes more pronounced with increasing phylogenetic distance (Champagne and Boutry, 2013).

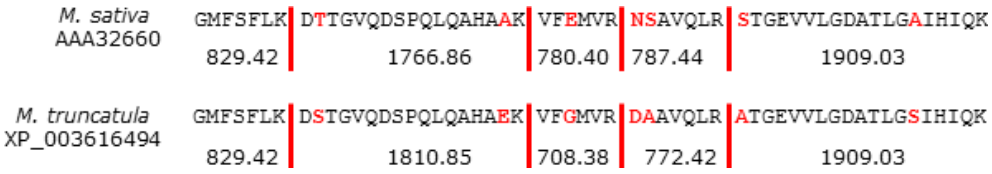

Figure I-11: Comparison of the leghemoglobin sequences from *M. sativa* and *M. truncatula*.
Amino acids that change are in red. Tryptic peptides are separated by red bars with their specific mass.

Secondly, the error tolerance can be broadened during cross-species database searches to ensure a possible identification but this results in an exponential increase in the number of false positives. The use of error tolerant searches is therefore scarce. Thirdly, a sequence can be determined *de novo* based on the MS/MS spectrum and the found sequence used in sequence alignment. To date the *de novo* sequencing software novor is the best available (Muth and Renard, 2017). All determined sequence possibilities, which were calculated based on their MS spectra are ranked by a score evaluating the quality of the matching between the computed peptide sequence and the input mass spectra (Ma, 2015). The best matching sequence gets the highest score. But the calculated scores ranges are rather narrow and sequences evaluated with almost the same score can be very variable, which makes the determination of the "correct" peptide sequence difficult. The results of *de novo* sequencing using the novor algorithm are exemplified in **Table I-1**. Although being equally scored (23.8 and 23.6), the *de novo* calculated peptide sequences for the MS/MS spectrum of a peptide with a mass of 842.48 Da are completely different (**ARGASLLR** and **KVGAFPPK** respectively). Both peptide sequences were calculated based on the same submitted mass spectra. The same can be seen for all other calculated scores and predicted sequences in the table.

Table I-1: Example for results of peptide sequencing obtained by de novo sequencing using the novor algorithm.

mz	pepMass (*de novo*)	err (*de novo*)	ppm	score	peptide
422.2572	842.5086	-0.0088	-10.4	23.8	**ARGASLLR**
422.2572	842.5014	-0.0016	-1.9	23.6	**KVGAFPPK**
422.2572	842.4974	0.0024	2.9	23.1	SKSGVPLR
422.2572	842.4974	0.0024	2.9	22.8	NLLGALSR
422.2572	842.4974	0.0024	2.9	21.3	NLGAKAAAK
422.2572	842.4875	0.0123	14.6	20.9	ARGHKFK

Although cross-species homology searches allow to efficiently apply proteomics on non-model species, the application of a non-specific database for protein identification can result in poor protein identifications when using a non-model plant such as oak (Galván et al., 2011), resulting in a significant amount of false positives or negatives. However, a fairly high number of proteins can be identified when a database of a phylogenetically close species is used, but this number decreased with decreasing ancestry of the query species from the species used in the experiment (Bräutigam et al., 2008).

Using collections of Expressed Sequence Tags (ESTs) can overcome this limitation and largely improve the quality of protein identification (Romero-Rodríguez et al., 2014). ESTs are short mRNA derived sequence reads, which present a snapshot of genes expressed in a given tissue at a given time point. These EST sequences are assembled into contigs that allow the reconstruction of longer transcripts to obtain a better protein coverage. With the availability of EST databases, proteome analysis is possible for any species and although significant protein identification highly depends on the quality of EST sequences, even low quality species-specific databases still provide better results regarding the number of identified proteins (Bräutigam et al., 2008), underlining their potential for proteomics application. Although nowadays the generation of contig databases is of low cost and takes little time, their utilization in proteome research is still scarce and the majority of studies use non-specific databases such as provided by NCBI (Champagne and Boutry, 2013). By combining results

from different databases as is commonly done in research, the number of identifications can be further maximized, thereby taken the ancestry of the studied species and the query species into account (Bräutigam et al., 2008). Taking this into consideration, fully annotated and properly curated databases from model plants can be transferred to closely related non-model plants.

Medicago sativa as a non-model plant is an economic and ecological important crop, which has become an interesting object in research. Illumina RNA-seq technology was used to develop a *de novo* transcriptome assembly for *M. sativa* using the subspecies *Medicago sativa ssp. sativa* and *Medicago sativa ssp. falcata*. The assembled database represents 112 626 unique transcript sequences and is publicly available (http://plantgrn.noble.org/AGED/index.jsp) (O'Rourke et al. 2015). Despite this, current studies one *M. sativa* still using the *M. truncatula* protein database (Dai et al., 2016).

4.1. Improving the protein identification quality for *Medicago sativa*

How useful the utilization of a species-specific contig database is and to which extent it improves protein identification was tested for the non-model plant *M. sativa* by using a LC-MS dataset from a quantitative cell wall- and soluble proteome study in *M. sativa* stems (chapter 2) prior to the preparation of the manuscript. In this study, cell wall proteins where sequentially extracted using three different buffers ($CaCl_2$, LiCl, EGTA) with increasing ionic strength to also extract the tightly bound cell wall proteins. The three different fraction were kept separately for the protein analysis. Furthermore, soluble proteins were extracted during the study and in total four different LC-MS/MS datasets where acquired, which were used for the here presented database comparison. Mass spectrometric spectra were analysed with Mascot-Daemon (version 2.4.2, Matrix Science) by searching against the alfalfa contig database downloaded from Samuel Roberts Noble website (675750 sequences; 304231702 residues, released on 3th November 2015) (O'Rourke et al. 2015) and a second search was performed against the Uniprot database restricted to *M. truncatula* (67808 sequences). In both searches the parameters were defined as following: 2 missed cleavages, mass accuracy precursor: 20 ppm, mass accuracy fragments:

± 0.5 Da, fixed modifications: carbamidomethyl (C), dynamic modifications: oxidation (M and P), acetyl (protein N-term), didehydro (F) and tryptophan to kynurenine. Proteins were considered as identified when at least two peptides passed the MASCOT-calculated score of ≥ 25 and the same peptides were identified in at least 80 % of the replicates. Mascot data were imported to PROGENESIS QI software for proteomics (NonLinear Dynamics) for quantitative analysis. Quantitative results were statistically evaluated by means of one-way ANOVA *p*-value ($p \leq 0.05$) as well as a fold-change of 1.5 to reveal proteins with significantly different abundance. Only identifications with at least two peptides of which one was unique for this identification were considered for the quantitative analysis.

A total number of 194 differently abundant proteins was identified when using the specific EST database for *M. sativa* while the *M. truncatula* database gave a total of 157 differently abundant protein identifications. The number of differently abundant proteins in each fraction is given in **Table I-2** and a comparable number of proteins was identified as being differently abundant for the different fractions. Thereby the number of identifications based on one unique peptide was much higher when using the contig database (**Table I-2**).

Protein identification based on homology relies on an accurate matching of spectra with the database, which is further determined by the database quality. The use of the species-specific database increases the confidence of the identified proteins by matching more MS/MS spectra that would not have been matched to a possible homolog in the non-specific database. Therefore, even with a unique peptide a protein can be significantly identified.

Table I-2: The number of obtained protein identifications with different abundance in *M. sativa* stems from each database search.

		Database	
	Fraction	*M. sativa*	*M. tr.*
# identified proteins	CaCl2	33	24
	EGTA	65	57
	LiCl	68	55
	Sol prot	28	21
# identifications with 1 unique peptide	CaCl2	5	2
	EGTA	21	13
	LiCl	25	10
	Sol prot	10	5

To investigate the differences of assigned peptides when using different databases and the resultant protein identifications, the LiCl fraction was chosen as an example and a total number of 41154 spectra acquired during a LC-MS/MS run were submitted to the Mascot server. From the submitted dataset 762 MS/MS spectra were matched to a peptide unique for a single protein when searched against the *M. sativa* contig database while a search against the *M. truncatula* database identified 550 unique peptides, which is almost 30 % less. Out of these, 496 peptides were identified in both databases which means that 293 peptides where uniquely identified when using the EST database (**Figure I-12**).

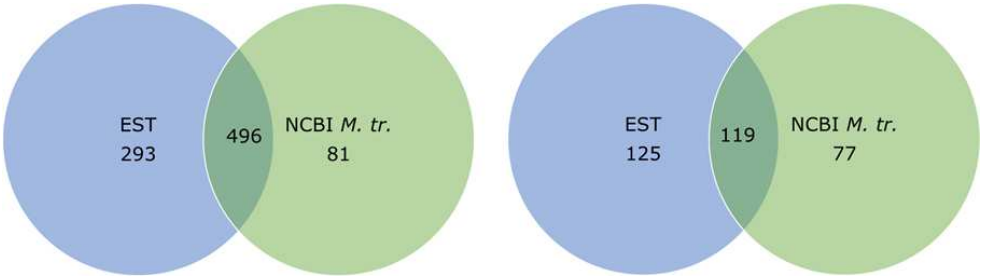

Figure I-12: Unique peptide matches for MS/MS spectra by two database searches in stems of *M. sativa*.
The number in the circle indicates the number of unique peptides matched by a certain database. The overlapping area of the two circles indicates the number of unique peptides matched by using both databases. Left Venn-diagram represents the total number of matched MS spectra. Right Venn-diagram represents matched MS-spectra with a Mascot score ≥ 25.

For each protein assignment by Mascot based on the identified peptides, a score is automatically calculated, evaluating the significance of the identification. During the further data processing, only peptides were considered for interpretation when the calculated Mascot score was 25 or higher. Applying this criterion, 244 unique peptides were identified within the *M. sativa* contig database and 196 within the *M. truncatula* database. One hundred nineteen peptides are in common between the databases, which leaves 125 peptides that were exclusively identified with the contig database. Based on these peptides, a protein identification list was established and the numbers of identified proteins are given in **Table I-2** and were discussed before.

Regarding the just described observations made in the LiCl fraction, the same observations were made for all other fraction when going through the data. To increase the number of identified proteins and to ensure confident identifications, it is advantageous to use a species-specific database if available. Yet, several peptides were identified exclusively with the Uniprot database when restricted to a phylogenetically close relative and it is therefore beneficial to combine results from different databases in order to maximise protein identifications.

The use of a species-specific database in this case is however not without problems. Typically, even curated contig databases contain numerous redundant or close-to-identical sequences. This issue is illustrated by the protein

glycerylaldehyde-3-phospahte dehydrogenase. It was identified in both the *M. truncatula* database and *M. sativa* nucleotide database. The *M. truncatula* database contains two entries (Uniprot identifier: G7KYC0 and G7J5Y4). Both entries are identified by unique peptides and are considered for quantification following the applied criteria for quantification as done in the above described study (**Table I-3**). Much more entries for the protein can be found when using the *M. sativa* database of which only a few are identified by a unique peptide (**Table I-4**). Applying the criteria that a protein must be identified by at least two peptides of which one peptide is unique, this protein is not considered for quantification, resulting in non-quantification.

Table I-3: Two different isoforms of Glycerylaldehyde-3-phosphate dehydrogenase identified with the *M. truncatula* Uniprot database and extraction of their matching peptides.

peptide	protein		unique	quantification
	G7KYC0	G7J5Y4		
K.GKLNGIALR.V	**X**	**X**	no	no
K.AAEGPLKGVLDVCDVPLVSVDFR.C		**X**	yes	yes
K.AAEGPLKGVLDVCDVPLVSVDFR.C		**X**	yes	yes
K.GKLNGIALR.V	**X**	**X**	no	no
K.VVSDRNPANLPWGELGIDLVIEGTGVFVDREGAGK.H	**X**		yes	yes

Table I-4: Eleven different isoforms of Glycerylaldehyde-3-phosphate dehydrogenase identified with the *M. sativa* specific contig database and extraction of their matching peptides.

peptide	contig											unique	quantification
	1	2	3	4	5	6	7	8	9	10	11		
K.GKLNGIALR.V	X	X	X	X	X	X	X	X	X	X	X	no	no
R.EGAGKHITAGAK.K			X				X					no	no
K.DTKTLLFGEK.E					X				X			no	no
R.VIDLIVHIASVA.-					X			X		X	X	no	no
K.ILEQIIK											X	yes	yes

4.2. The translation and annotation problem when working with a nucleotide database

Mass spectrometry data are commonly used for the identification of proteins whereby LC-MS is the state-of-the-art strategy for high-throughput proteomics. The protein sample is generally digested by trypsin, generated peptides are separated by LC followed by MS/MS for peptide identification. The generated MS/MS-spectra are submitted for a database search to assign them to a matching sequence. The algorithms subsequently assign the identified peptide sequences to proteins or protein families. Several algorithms and computer programs are available for automatic protein identification. While this works well for species with a properly annotated proteome database, the efficiency drops when no database entry with a high homology is available (**Figure I-11**). However, the ability to search nucleotide databases is a great advantage when analysing data from an organism, which genome is not yet fully annotated.

Species-specific nucleotide databases are used for proteomic research. Those databases contain short nucleotide sequence reads from 5’ and 3’ ends, which were obtained during deep-sequencing approaches and are assembled into much

longer sequences, called contigs, to obtain a better coverage of the entire coding sequence. The use of these databases is however not without flaws and some issues need to be considered. The nucleotide sequences burry frameshift errors, due to insertion and deletion or artefactual stop codons and will only partially match the sequence. A fair number of sequences derived from spliced RNA and random ligation of unrelated fragments is quite common, generating chimeric sequences. Although EST sequences are clustered into groups that are most likely derived from the same RNA, the databases contain a large degree of redundancy. Furthermore, the reading frame for translation is unknown and the nucleotide sequence needs to be translated in all six possible frames. This generates large quantities of random sequences, which to some degree matches with experimental data (Choudhary et al., 2001; Jongeneel, 2000). However, RNAseq and genome sequencing are now relatively cheap options to construct customized databases for genomic and proteomic analyses.

During three independent studies undertaken in *M. sativa*, *Craterostigma plantagineum* and *Cannabis sativa* using LC-MS data for protein identification inconstancies in the protein identifications were observed. By manually checking the data it was revealed that LC-MS based proteomics with a nucleotide database using parameters used for searching a protein database gave a lot of false positive results and about half of the first 20 identified contigs were not correct. A contig has six possible translation frames and it appeared that identified peptides based on MS/MS spectra were matched to different frames, which is a software inherent problem. For instance in a proteome study in *M. sativa* identified peptides were assigned to the protein FBT2_ARATH Probable folate-biopterin transporter, which corresponds to the sequence of the contig_56015 in the *M. sativa* database (http://plantgrn.noble.org/AGED/index.jsp) (O'Rourke et al. 2015). After translation of the contig sequence into its six reading frames and identification of the open reading frame (ORF), the ORF sequence was blasted against the NCBI database and indeed the annotation was correct. But the peptide sequences, on which the protein identification was based, were not present in that reading frame. All the peptides were found in a different ORF in another reading frame (**Figure I-13**). After blasting the sequence, it was identified as a ferritin

homologue. Consequently, the automatic protein assignment FBT2_ARATH Probable folate-biopterin transporter based on the identified peptides was wrong.

To avoid this problem, the parameters for a significant identification were optimized being aware that this generates more false negatives. Even then, all obtained identifications should always be handled with care and if feasible manual evaluation can avoid wrong data interpretation. But it furthermore reveals a more profound issue. How much of the sequence resulting in the construction of the contig_56015 correspond to FBT2_ARATH Probable folate-biopterin transporter and how much to ferritin? Since the gene expression analysis is based on the total content of sequences corresponding to the contig in the different conditions, this question is not trivial.

A way to tackle the problem of erroneous and low-quality nucleotide sequence databases is the integration of proteomics and genomics, which is referred to as proteogenomics. Proteogenomics describes the mapping of peptides identified by tandem mass spectrometry to its specific coding locus on the nucleotide sequence. Thereby MS/MS spectra are assigned under consideration of all possible ORFs encoded by a six frame translation of the nucleotide sequence. This mapping enables the discovery of novel coding sequences and refines the structural annotation of genes (Armengaud et al., 2014; Jaffe et al., 2004). Proteomic data can be used to improve the annotation of genomes and help to determine the structure of the corresponding genome, including boundaries and enumerations of its ORF. Additionally, proteomic data can help to verify unknowns that cannot be well established on the basis of homology.

4.3. Conclusion

The choice of a *M. sativa* specific database in proteomic analysis is important to improve the quantity and quality of the protein identifications. Results can further be maximised by combining results from different databases. As identifications are based on homology searches it is beneficial to choose a database from a phylogenetically closely related species. However, using an EST nucleotides database for protein identification holds some problems during the

computational identification process and should be taken into consideration when interpreting the acquired protein data.

Figure I-13: Translation of contig_56015 into two possible reading frames.
The protein-coding open reading frames (ORF) are marked in red. LC-MC generated peptide spectra were submitted to the *M. sativa* EST database for protein identification. Identified peptides are indicated by blue boxes. Peptides were assigned as FBT2_ARATH Probable folate-biopterin transporter although they are found in an alternative ORF coding for a ferritin.

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Objectives

Environmental pollution with heavy metals forms a significant problem. Notably cadmium (Cd) is of great concern due to its wide-spread appearance and persistence in the environment. Since its impact on human health was discovered, much attention has been payed to Cd and its harmful effects on living organisms. Cadmium is emitted into the atmosphere by anthropogenic activities and the application of phosphate fertilizers is by far the main source of Cd in agricultural soils.

Cadmium is hitchhiking its way into plants through metal transporters in competition with essential nutrients e.g. calcium, potassium or iron. As a toxic metal, it leads to disturbances on multiple morphological and physiological levels. Cadmium disturbs enzymatic activities (Dias et al., 2013), generates reactive oxygen species (Cuypers et al., 2010), disturbs the balance of essential nutrients (Yang et al., 1996) and interferes with photosynthesis (Azevedo et al., 2005). To tackle the stress, exposed plants activate their defence mechanisms related to Cd sequestration/detoxification and their antioxidative defence. Thereby, the plant cell wall is the first protective barrier between the cell and its surrounding environment. Alterations in its chemical characteristics in response to Cd allow an immobilization of Cd in the cell wall (Krzesłowska, 2011), preventing its further entry into the cell. Furthermore, Cd exposure induced remodelling of the cell wall in plants, which derives from a Cd-driven change in the enzymatic activity of cell wall proteins (Paynel et al., 2009). The Cd-induced accumulation of H_2O_2 triggers the activity of cell wall bound peroxidases and leads to an increased lignification of the cell wall through cross-linkage of monolignols (Passardi et al., 2004). To balance the disturbed cellular redox status, plants synthesise several anti-oxidative primary and secondary metabolites. To the latter belongs the diverse group of flavonoids. Their great capacity to scavenge oxidative radicals makes them essential during the response to oxidative stress (Michalak, 2006). As a phenolic compound, flavonoids derive from the same pathway as monolignols and the enhancement of the phenylpropanoid pathway during stress response in favour of flavonoid

synthesis counteracts the process of lignification (Pawlak-Sprada et al., 2011b, 2011a).

Clearly, all induced molecular responses are influencing each other, thereby intertwining like a clockwork and interconnecting the intimate relation between the cellular oxidative challenge and the cell wall as a defence barrier. Thus, an integrated study on the influence of Cd on the cell wall, cell wall proteins and plant metabolism will help to unravel the mystery of how the plant cell wall is involved during the defence against Cd and abiotic stress in general. Therefore, different –OMICS approaches were combined to shed light on the molecular mechanisms, which are put in place at the cell wall level but also in the cytosol in order to protect the plant from server damage. A particular focus in this study was given to long-term Cd exposure, as such a set-up reflects more realistic conditions and is closer to actual processes in the environment.

Based on the hypothesis that the plant cell wall states a dynamic defence barrier against Cd, the following research questions were addressed in the outline of this thesis:

1. How does long-term cadmium exposure influence the cell wall- and soluble proteome in *M. sativa* stems?
2. How does long-term cadmium exposure alter the cell wall composition of *M. sativa* stems?
3. Which metabolites are produced by *M. sativa* during long-term cadmium exposure? How do they contribute to the establishment of a new homeostasis, which enables *M. sativa* to grow under Cd stress?
4. To what extent does long-term cadmium exposure affect the mineral content and distribution in *M. sativa*? What are the alleviating effects of potassium and sodium on cadmium toxicity?

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Summary and Perspective

The introduction briefly reviewed some technical aspects of proteome research. Strategies for protein identification are classified in two main routines: the bottom-up approach which is based on peptide identification and finally protein assignment or the top-down approach which is initially based on the entire protein sequence including PTMs. As a top-down approach, 2D electrophoresis is the most commonly used technique in plant proteome research and labelling the samples with different dyes prior to separation (difference gel electrophoresis – DIGE) makes quantitative protein analyses more straightforward. However, gel-free proteomics as a bottom-up approach gains more and more importance, allowing automated high-throughput analyses of complex peptide mixtures. As it overcomes limitations of gel-based proteomics, gel-free protein identification is the current state-of-the-art method. Yet, 2D electrophoresis is still the only top-down approach which enables routine analysis of native proteins, visualisation/ identification of posttranslational modifications and the distinction of different protein isoforms, which makes it still a commonly used in tool in proteomics.

The quality and quantity of identified proteins strongly relies on the chosen database. Today, species-specific EST databases open proteome research to non-model plants such as *M. sativa* and facilitate the acquisition of comprehensive protein datasets. The combination of results from different databases can further enlarge the number of protein identifications. Thereby it is beneficial to use a database from a phylogenetical closely relates species like *M. truncatula* when working with *M sativa*.

The following chapter is divided into two parts and describes the quantitative analyses of cell wall - and soluble proteins in stems (Part 1) and leaves (Part 2) of *M. sativa* upon long-term mild Cd exposure. In the first part quantitative results obtained with 2D DIGE are combined with targeted gene expression analyses in *M. sativa* stems. The second part focuses on the leaves of *M. sativa* and how Cd influences the abundance of cell wall and soluble proteins. Thereby, the used extraction protocol and quantification approach was the same as for stems. Hereby, we demonstrate the applicability of the cell wall extraction

protocol on leaves. In both studies a strong emphasis is made on proteins, which are influencing the cell wall structure and how those structural alterations can be linked to the plant defence against Cd and the establishment of the cell wall as a physical barrier.

We followed the same approach in roots of *M. sativa* and again the same extraction protocol for cell wall proteins was used. However, the obtained results were not satisfying, which let us question the suitability of the applied protocol for roots as no discrimination between the three fractions was observed. Therefore, the general suitability on others samples should be thoroughly checked prior to application.

Chapter 1 – Part 1: Long-term cadmium exposure influences the abundance of proteins that impact the cell wall structure in *Medicago sativa* stems

Adapted from published article:

Gutsch A., Keunen E., Guerriero G., Renaut J., Cuypers A., Hausman, J-F., Sergeant, K.: Long-term cadmium exposure influences the abundance of proteins that impact the cell wall structure in *Medicago sativa* stems. *Plant Biol. J.* (2018). doi:10.1111/plb.12865.[4]

Contributions:

A. Gutsch, K. Sergeant, J-F. Hausman and A. Cuypers designed the experiment.

A. Gutsch performed the experimental work, analysed and interpreted the data and wrote the present manuscript.

G. Guerriero and E. Keunen contributed to the experimental set-up for qPCR.

K. Sergeant contributed to the protein identifications, data interpretation and critically revised the manuscript.

J. Renaut supplied and managed the technical equipment for the protein analysis.

All authors revised and approved the final version of the manuscript.

[4] Data obtained during this study contributed to the description of the didehydrophenylalanine in the beta subunit of plant polygalacturonases. Sergeant, K., Printz, B., Gutsch, A., Behr, M., Renaut, J., and Hausman, J.-F. (2017). Didehydrophenylalanine, an abundant modification in the beta subunit of plant polygalacturonases. *PLoS One* 12, e0171990.

ABSTRACT

Cadmium (Cd) is a non-essential, toxic heavy metal that poses serious threats to both the ecosystem and the health of humans. Plants employ various cellular and molecular mechanisms to minimize the impact of Cd toxicity and the cell walls function as defensive barrier during Cd exposure.

In this study, we adopted a quantitative gel-based proteomic approach (two-dimensional difference gel electrophoresis) to investigate changes in the abundance of cell wall- and soluble proteins in stems of *Medicago sativa* L. upon long-term exposure to Cd (at 10 mg Cd per kg soil as $CdSO_4$). Obtained protein data were complemented with targeted gene expression analyses.

Plants were affected by Cd exposure at an early growth stage but seemed to recover at a more mature plant stage as no difference in biomass was observed. The accumulation of Cd was highest in the roots followed by stems and leaves. Quantitative proteomics revealed a changed abundance for 179 cell wall proteins and 30 proteins in the soluble fraction upon long-term Cd exposure. These proteins are involved in cell wall remodelling, defence response, carbohydrate metabolism and promotion of the lignification process.

The data indicate that Cd exposure alters the cell wall proteome and underline the role of cell wall proteins in defence against Cd stress. The identified proteins are linked to alterations in the cell wall structure and lignification process in stems of *M. sativa*, underpinning the function of the cell wall as an effective barrier against Cd stress.

KEYWORDS: cadmium; cell wall proteins; gene expression; *Medicago sativa*; soluble proteins; 2D DIGE

1. Introduction

Anthropogenic influence led to environmental pollution with heavy metals of which cadmium (Cd) is one of the most common pollutants, released into the environment by mining or smelting activities and through the application of phosphate fertilizer. Its accumulation in crop plants introduces Cd into the human food chain, posing a threat to human health. Although being unessential, Cd can enter the plant via its roots and is translocated throughout the different tissues by a variety of unspecific transport systems (Clemens and Ma, 2016), thereby competing with essential nutrients (Zhang et al., 2014). Cadmium exposure impacts numerous physiological and biochemical processes in plants and leads to limited growth, chlorosis and oxidative stress induced by the production of reactive oxygen species (ROS) (Cuypers et al., 2010). Furthermore, plants suffer from water imbalance as an effect of stomatal closure (Perfus-Barbeoch et al., 2002) and the photosynthetic apparatus is affected (Sanità Di Toppi and Gabbrielli, 1999). Plants have evolved several mechanisms to minimize the induced toxic effects, including detoxification through chelation (Cobbett, 2000), compartmentation and sequestration in extracytoplasmatic compartments such as the cell wall (Krzesłowska, 2011).

The plant cell wall is a complex structure composed of polysaccharides and proteins, providing mechanical support and rigidity. At the interface between the in- and outside of the cell, it forms a protective barrier, which is important for the defence against biotic and abiotic threats to plant cells (Bradley et al., 1992; Brisson et al., 1994; Douchiche et al., 2010a). Cell wall-localized proteins function in intercellular communication and in the interaction between the cell and its environment. Therefore, the plant cell wall continously undergoes structural modifications in order to adapt to the plant's developmental stages and environmental conditions (Caffall and Mohnen, 2009; Loix et al., 2017). Plants exposed to Cd show concentration-dependent alterations in cell wall structure. Cell walls become thicker at sites of Cd deposition, with pectin as the main binding site for Cd (Vollenweider et al., 2006). Cadmium accumulation leads to an increased amount of low-methylesterified pectin in the outer part of the external tangential cell wall (Douchiche et al., 2007), which is mediated by the activity of pectin methylesterase (PME) and an increased activity of this

enzyme was reported during Cd exposure (Paynel et al., 2009). Low-methylesterified pectin can bind Cd in competition with calcium (Ca) and Cd-induced alterations seem to promote the barrier function of the cell wall (Douchiche et al., 2010a; Parrotta et al., 2015). Furthermore, Cd exposure enhances lignification mediated by cell wall-bound peroxidases (POX), leading to cell wall stiffening and limiting cell growth (Chaoui and El Ferjani, 2005).

Multiple molecular techniques have been applied to study abiotic stress responses in plants and the molecular mechanism behind it. Thereby, quantitative protein analyses are an essential tool to identify important players in plant stress responses such as Cd-exposure (Lopes Júnior et al., 2015; Villiers et al., 2011) and have been applied in different species and organs (Hossain et al., 2012; Kieffer et al., 2009; Semane et al., 2010). Most studies focus on short-term Cd-exposure in model plant species and not in economic relevant crops (Dupae et al., 2014; Lee et al., 2010). The current study focuses on the non-model plant *Medicago sativa* L., which is globally the most important forage legume. High in protein content, *M. sativa* meets the needs of the feed market. Its less digestible stems add up to more than 50 % of its biomass with a high yield in cell-wall material. It has a high economic value as the stems can be used for industrial applications such as bioethanol production. Since the composition and structure of cell walls are influenced by altered environmental conditions, this may have an impact on their valorization potential. Therefore, those alterations of the cell wall are of scientific but also of societal and economic interest. For this reason *M. sativa* is often used to study cell wall development and processes (Printz et al., 2016; Verdonk et al., 2012). Recently, an improved protocol to investigate the cell wall proteome was established (Printz et al., 2015). By expanding the knowledge about cell wall proteins and their role in plant defence against abiotic stress like Cd-exposure will enlarge our understanding of the plant cell wall.

In this study, *M. sativa* plants were grown on control and Cd-contaminated soil (10 mg kg^{-1} soil) with the aim to identify effects of this treatment on the proteome level and discover potential Cd-induced structural effects. Although current literature is dominated by studies on short-term exposure, long-term exposure experiments to a realistic Cd concentration as done in this study make

the data relevant for agricultural practices. Quantification of the stem cell wall and soluble proteome was performed with two-dimensional difference gel electrophoresis (2D DIGE), which enables separation of different protein isoforms and discrimination of modified proteins such as heterogeneous glycosylated cell wall proteins and other processed protein forms. Additionally, targeted gene expression analyses by quantitative real-time PCR (RT-qPCR) were used to complement and strengthen the proteomic data. Changes in protein patterns, their influence on cell wall structure and the role of the cell wall as a protective barrier against Cd exposure are discussed.

2. Material and Methods

2.1. Plant Material

Medicago sativa L. (cultivar Giulia) seeds were inoculated with *Sinorhizobium meliloti*. The potting mix was prepared in a single batch using 2:1 ratio of potting soil and sand. Half of the potting mix was supplemented with 10 mg Cd per kg soil (added as $CdSO_4$).Sowing was done in May 2015. For the two conditions, one being a control soil, without the addition of Cd, and the other being Cd-supplemented (10 mg Cd kg^{-1} soil), 12 times 12 pots were planted. Plants were kept in the greenhouse until the flowering stage was reached (in July) and subsequently cut as being agricultural practice. Neither temperature nor day-cycle was controlled during the study. After cutting, plants were kept outside to avoid the observed insect infestation during the first growth cycle. After a re-growing period till pre-flowering stage was reached, plants were put back into the greenhouse for one more week before the sampling was done on the 10^{th} of September. No fertilization was applied. The mineral composition of the two soils was analyzed in ten replicates and revealed no significant difference apart from the concentration in Cd (**Table S1-1**). Stems were separated from leaves and the first two and last two internodes removed to obtain a more homogeneous sample. Five replicates were sampled, with a pool of the stem material from 24 pots corresponding to one biological replicate. Samples were ground to a fine powder in liquid nitrogen using a mortar and a pestle and were kept at -80°C until further use for the quantitative protein

analysis and targeted gene expression by qPCR. Additionally, leaf, stem and root samples of each condition were taken to determine their Cd content.

2.2. Determination of Cd content by ICP-MS

Samples of approximately 250 mg fresh weight were oven-dried for 36 h at 60°C and mineralized in 7 mL nitric acid and 3 mL hydrogen peroxide using a Multiwave PRO microwave reaction system according to the manufacturer's instructions (Anton Paar GmbH). Subsequently, the sample volume was adjusted to 25 mL with MilliQ Water (Millipore) and Cd concentrations in the samples were determined by inductively coupled plasma-mass spectrometry (ICP-MS). An average concentration was calculated for the two conditions.

2.3. Extraction of cell wall proteins

The cell wall proteins were extracted according to (Printz et al., 2015). Four biological replicates were used. Briefly, cell walls were enriched from 7 g of ground *M. sativa* stems by using an increasing sucrose gradient (5 mM sodium (Na) acetate pH 4.6, 4°C supplemented respectively with 0.4 M, 0.6 M and 1 M sucrose). The final cell wall pellets were washed twice in 5 mM Na acetate (pH 4.6). To extract the cell wall proteins, 7.5 mL of extraction buffer C (5 mM Na acetate, 200 mM $CaCl_2$, pH 4.6, 4°C) was added to the cell wall fractions. Samples were placed on a rocking platform (30 min, 4°C), followed by centrifugation (10000 g, 15 min, 4°C). This step was repeated and supernatants were pooled (stored as $CaCl_2$ fraction, -20°C). Ten mL of extraction buffer E (5 mM Na acetate, 50 mM EGTA, pH 4.6, 4°C) was added to the pellets, which were shaken vigorously (37°C, 1 h) and centrifuged (10000 g, 15 min, 4°C). This extraction step was repeated twice and the supernatants pooled (stored as EGTA fraction, -20°C). Finally, the pellets were resuspended in 15 mL of extraction buffer L (5 mM Na acetate, 3 M LiCl, pH 4.6, 4°C), placed on a rocking platform overnight (4°C), centrifuged and the supernatants were stored as LiCl fraction at -20°C.

Protein extracts were concentrated with Amicon Ultra-15 10 K (Millipore) by centrifugation (4700 g, 4°C) till a volume of approximately 200 µl was reached. The concentrated extracts were washed and desalted using the ReadyPrep 2D Cleanup kit (Bio-Rad) according to the instruction manual. Samples were

solubilized in labeling buffer (7 M urea, 2 M thiourea, 2 % w/v CHAPS, 30 mM Tris) and protein concentrations were determined with the Bradford protein assay (Bio-Rad).

2.4. Extraction of soluble proteins

Extractions were done with trichloroacetic acid (TCA)/phenol- sodium dodecyl sulfate (SDS) (Wang et al., 2003). To 300 mg ground plant material 1 mL of ice-cold acetone containing 10 % v/v TCA and 0.07 % w/v dithiothreitol (DTT) was added, the suspension was briefly vortexed and left at -20°C overnight. Centrifugation (10000 g, 5 min, 4°C) was followed by two washing steps with ice-cold acetone (10000 g, 3 min, 4°C), and the final pellet was dried at ambient temperature overnight. Next, 800 µL of Tris-buffered phenol (Invitrogen, pH 7.5-7.8) and an equal volume of SDS-buffer (30 % w/v sucrose, 2 % w/v SDS, 0.1 M Tris-HCL, pH 8.0, 2 % 2-mercaptoethanol) were added to the pellets. After extensive vortexing (10 min) and centrifugation (10000 g, 3 min), 300 µL of the upper phenol phase was transferred to a new tube, 1.5 mL of pre-cooled 0.1 M ammonium acetate in methanol added and samples were kept at -20°C for 2 h. After centrifugation (10000 g, 5 min, 4°C) and two washing steps with pre-cooled 0.1 M ammonium acetate in methanol (10000 g, 3 min, 4°C) pellets were washed twice with 80 % acetone. The obtained protein pellets were dried at ambient temperature and solubilized in labeling buffer (see previous section). Again, the Bradford protein assay (Bio-Rad) was used to determine the protein concentration in the sample.

2.5. 2D DIGE and spot selection

Per condition and fraction ($CaCl_2$, EGTA, LiCl and soluble proteins), 50 µg of protein from four replicates were labeled with Cy3 or Cy5. The dyes were swapped between replicates to eliminate the effect of potential preferential labeling on the results. Given that each of the fractions has a highly characteristic protein profile, an internal standard was prepared separately for each of the four fractions. It was composed of 25 µg protein from each of the eight samples (four biological replicates for control and Cd plants respectively) and labeled with Cy2. Samples (one labeled with Cy3, one with Cy5 and the Cy2-labeled internal standard) were mixed and 9 µL Servalyte pH 3-10 (Serva

Electrophoresis GmbH) and 2.7 µL Destreak Reagent (GE Healthcare) were added. The volume was adjusted with lysis buffer (7 M urea, 2 M thiourea, 4 % w/v CHAPS) to 450 µL. The labelled samples were loaded onto an Immobiline™ DryStrip NL of 24 cm (GE Healthcare) during overnight rehydration, followed by isoelectric focusing (IEF) in a five step-program: (1) constant 100 V 4 h, (2) linear gradient up to 1000 V 4 h, (3) constant 1000 V 5 h, (4) linear gradient up to 10000 V 6 h, (5) constant 10000 V until a total of 80000 volt hours was reached. Equilibration of the IEF-strips was done in equilibration buffer (Serva Electrophoresis GmbH) supplemented with 6 M urea and 1 % w/v DTT for 15 min and subsequently in the same buffer supplemented with 6 M urea and 2.5 % w/v iodoacetamide for another 15 min. Strips were applied to 2D HPE™ Large Gel NF-12.5 % (Serva Electrophoresis GmbH), 2D electrophoresis was run on a HPE™ tower system according to the manufacturer's instructions and stopped after the front reached the bottom of the gel. Gels were placed into fixation buffer (15 % ethanol v/v, 1 % w/v citric acid) overnight prior to image acquisition. From each gel three pictures of the different dyes were acquired at different wavelengths [Cy2 488 nm, Cy3 532 nm, Cy5 642 nm (Typhoon FLA 9500 GE Healthcare). Quantitative image analysis was carried out using the SameSpots software (TotalLab). Since the same internal standard is run on each gel of a specific fraction, alignment and normalization with the image of the internal standard allows to compare spot volumes across different gels. The software uses an alignment-based approach and enables automatic statistical analysis for spot detection. When a treatment effect was reported (ANOVA *p*-value ≤ 0.05 and detected fold change ≥ 1.5), the spot was selected for protein identification (**Figure S1-1** and **S1-2**, also see **Table S1-2** for all spot volumes).

2.6. Protein digestion and MS/MS analysis

Selected spots were picked with an Ettan Spot Picker (GE Healthcare). A Freedom EVO II workstation (Tecan) was used for digestion. Briefly, gel plugs were washed with 50 mM ammonium bicarbonate solution in 50 % v/v methanol/MilliQ Water (Millipore) for 20 min and dehydrated for 20 min in 75 % acetonitrile (ACN). Proteins were digested with 8 µL of a solution containing 5 ng μL^{-1} trypsin (Trypsin Gold, Promega) in 20 mM ammonium bicarbonate

(overnight, 37°C). Digested peptides were extracted from the gel plugs with 50 % v/v ACN containing 0.1 % v/v trifluoroacetic acid (TFA), dried and resolubilized in 0.7 µL of 50 % v/v ACN containing 0.1 % v/v TFA. Peptides were spotted on a MALDI-TOF target and 0.7 µL of 7 mg mL^{-1} α-cyano-4-hydroxycinnamic acid in 50 % v/v ACN containing 0.1 % v/v TFA was added.

A MALDI mass spectrum was acquired using the Sciex 5800 TOF/TOF (Sciex). The ten most abundant peaks, excluding known contaminants, were automatically selected and fragmented. Both, MS and MS/MS were submitted to an in-house MASCOT server (Matrix Science, www.matrixscience.com) for database-dependent identifications against the NCBInr database limited to the taxonomy *Viridiplantae* (3334509 sequences). A second search was performed using the *M. sativa* sequences downloaded from the Samuel Roberts Noble website (http://plantgrn.noble.org/AGED/index.jsp) (675756 sequences, 304231576 residues) (O'Rourke et al., 2015). Parameters were a peptide mass tolerance of 100 ppm, a fragment mass tolerance of 0.5 Da, cysteine carbamidomethylation as fixed modification and methionine oxidation, double oxidation of tryptophan, tryptophan to kynurenine as variable modifications. Proteins were considered as identified when at least two peptides passed the MASCOT-calculated 0.05 threshold score of 40. When high-quality spectra were not matched to a protein, manual interpretation of the spectra was performed and/or the search parameters adjusted (semitryptic, single amino acid changes, post-translational modifications) to increase the sequence coverage of the identified protein. All identifications were manually validated and their subcellular locations determined using TargetP (Emanuelsson and Nielsen, 2000). The standard search parameters were used. In some cases predictions were corrected based on literature.

2.7. RNA extraction and cDNA synthesis

RNA was extracted from 100 mg finely ground stem tissue using the RNAqueouse™ Kit (Life Technologies) according the manufacturer's instructions. To increase the obtained RNA concentration, all samples were cleaned up by precipitating the RNA with 3 M sodium acetate and 100 % isopropanol. The obtained RNA pellet was washed with 70 % ethanol and resuspended in RNase-free water. The RNA concentration and purity were determined using a

NanoDrop® ND-1000 spectrophotometer (Thermo Fischer Scientific) ($A_{260/280}$ and $A_{260/230}$ ratios between 1.9 and 2.5). Equal amounts (1 µg) of the extracted RNA was DNase-treated (TURBO DNA-free™ Kit, Life Technologies) and reverse transcribed following the manufacturer's instructions of the PrimeScript™ RT Reagent Kit (Perfect Real Time, TAKARA BIO Inc.). The cDNA was diluted 10-fold in 1/10 Tris-EDTA (TE)-buffer (Sigma-Aldrich) and stored at -20°C.

2.8. Quantitative real-time PCR

The AGED database was used to obtain the coding sequences for the genes of interest. Specific primer pairs were designed with the Primer3Plus online tool (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and analyzed using the OligoAnalyzer 3.1 (https://eu.idtdna.com/calc/analyzer). Primer efficiency was measured using a dilution series of a pooled sample containing an equal amount of all cDNAs in the experiment (six dilution points) (see **Table S1-3** for primer sequences and efficiencies). Quantitative PCR reactions were performed in a 96 well plate with the 7500 Fast Real Time PCR System (Life Technologies). One amplification reaction contained 2 µl diluted cDNA (or RNase-free water as "no template"-control), forward and reverse primers (100 nM), Fast SYBR® Green Master Mix (Applied Biosystems) and RNase-free water to a final volume of 10 µl. The PCR-conditions were as follows: initial denaturation (20 s at 95°C), followed by 40 cycles of denaturation (3 s at 95°C), annealing and elongation (30 s at 60°C). At the end of the run a melting curve was generated to check the specificity of the amplification reaction. All details of the workflow according to the minimum information for publication of quantitative real time PCR experiments (MIQE) guidelines as described by Bustin *et al* (2009) (Bustin et al., 2009) are shown in **Table S1-4**.

Expression values are given as normalized relative expression values. Therefore, the relative expression of each gene was calculated as $2^{-\Delta Cq}$ and normalized by the average $2^{-\Delta Cq}$ -value of the three reference genes Cyclophilin, GAPD, PAB4 (**Table S1-3**), which were selected by the GrayNorm algorithm (Remans et al., 2014) out of ten tested reference genes (previously reported for *M. sativa* (Guerriero et al., 2014)). Data from the treated samples were expressed relatively to the data from the untreated samples (set at 1). To determine if the gene expression was significantly affected by Cd, a *t*-test was performed

(p-value ≤ 0.05) using Microsoft Excel. Hierarchical clustering of the normalized expression values from all five replicates was done with Clustal v3.0 using an uncentered Pearson correlation and complete linkage clustering (Eisen et al., 1999). The cluster was visualized as a heat map with Java TreeView (Saldanha, 2004).

3. Results

3.1. Plant growth

The biomass of the aerial plant parts for each replicate was determined at the end of the experiment. Long-term Cd exposure did not significantly impact plant growth (110.65 g ± 3.78 for control versus 120.32 g ± 4.67 for Cd-exposed). Nevertheless, a growth reduction due to Cd exposure was clearly visible at an early growth stage (**Figure 1-1**), but vanished at a more mature plant stage.

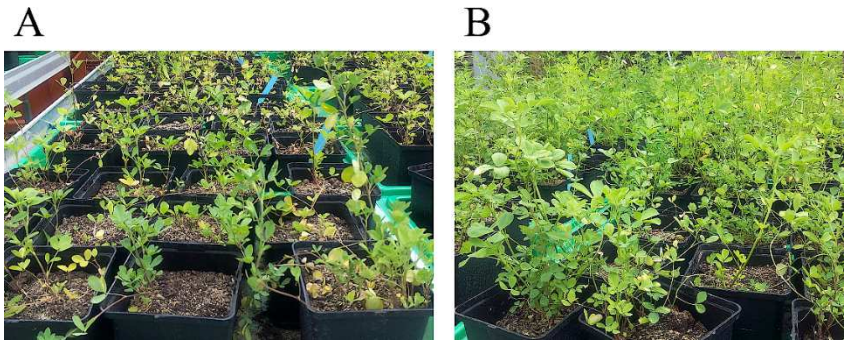

Figure 1-1: Pictures of *Medicago sativa* plants during their first growth period (summer 2015).
A) Plants growing on contaminated soil (10 mg Cd kg^{-1} soil). B) Plants growing on uncontaminated soil

3.2. Distribution of Cd in *Medicago sativa* leaves, stems and roots

The quantity of Cd in *M. sativa* leaves, stems and roots were assessed via ICP-MS (**Table 1-1**). Cadmium strongly accumulated in the roots and its quantity decreased in aerial plant parts. In stems only 24 % of the root Cd quantity was measured which decreased another 50 % in leaves. Changes in the amount of Cd in each tissue between the control and Cd-exposed plants are significant (p ≤ 0.05).

Table 1-1: Cadmium concentration (µg g^{-1} DW) in the different plant organs of *Medicago sativa* given as the mean ± standard error (SE) of 10 biological replicates.

	Leaves		***Stems***		***Roots***	
	AVG	**SE**	**AVG**	**SE**	**AVG**	**SE**
Ctr	0.31	0.06	0.24	0.01	2.36	0.74
Cd	21.54	5.16	40.09	8.22	169.91	72.13

AVG: average; SE: standard error; significance of difference in Cd quantity: leaves/stems $p = 1.03^{-5}$, stems/roots $p = 2.03^{-5}$, roots/leaves $p = 4.21^{-6.}$

3.3. Proteomic analysis

Four different protein fractions were analyzed: three different cell wall fractions ($CaCl_2$, EGTA, and LiCl) and a fraction containing soluble proteins.

3.3.1. Identification of cell wall proteins

Comparative DIGE analyses revealed a number of spots with a significant change in their abundance: changes in the fluorescence intensity of 107 spots from the CaCl2 fraction, 155 from the EGTA fraction and 87 spots from the LiCl fraction were detected (**Table S1-2**). After deletion of those spots wherein more than one protein was identified, 228 spots from the different cell wall fractions were considered for biological interpretation (**Table S1-5** and **Table S1-6 A, B, C**). From that dataset, 179 proteins (78.5 %) were predicted by TargetP to be secreted proteins targeted to the cell wall. Out of the remaining 49 proteins 34 are targeted to the chloroplast, 3 to mitochondria and 12 have no predicted subcellular target site and can be considered as intracellular proteins.

The highest number of proteins was identified in the EGTA fraction (94, of which 76 are secreted). Of the identified proteins 66 showed an increased abundance when plants were exposed to Cd. In the $CaCl_2$ and LiCl fractions, 73 and 61 proteins were identified respectively (44 and 59 respectively are predicted to be secreted). Of those, 45 and 25 proteins respectively increased in abundance as a result of Cd exposure. To gather information about the function of the identified proteins, they were clustered according to their predicted biological function using the Blast2Go software (**Figure 1-2** and **Table 1-2**). Strikingly, about 50 % of the higher-abundant proteins in each fraction have a designated role in plant defence.

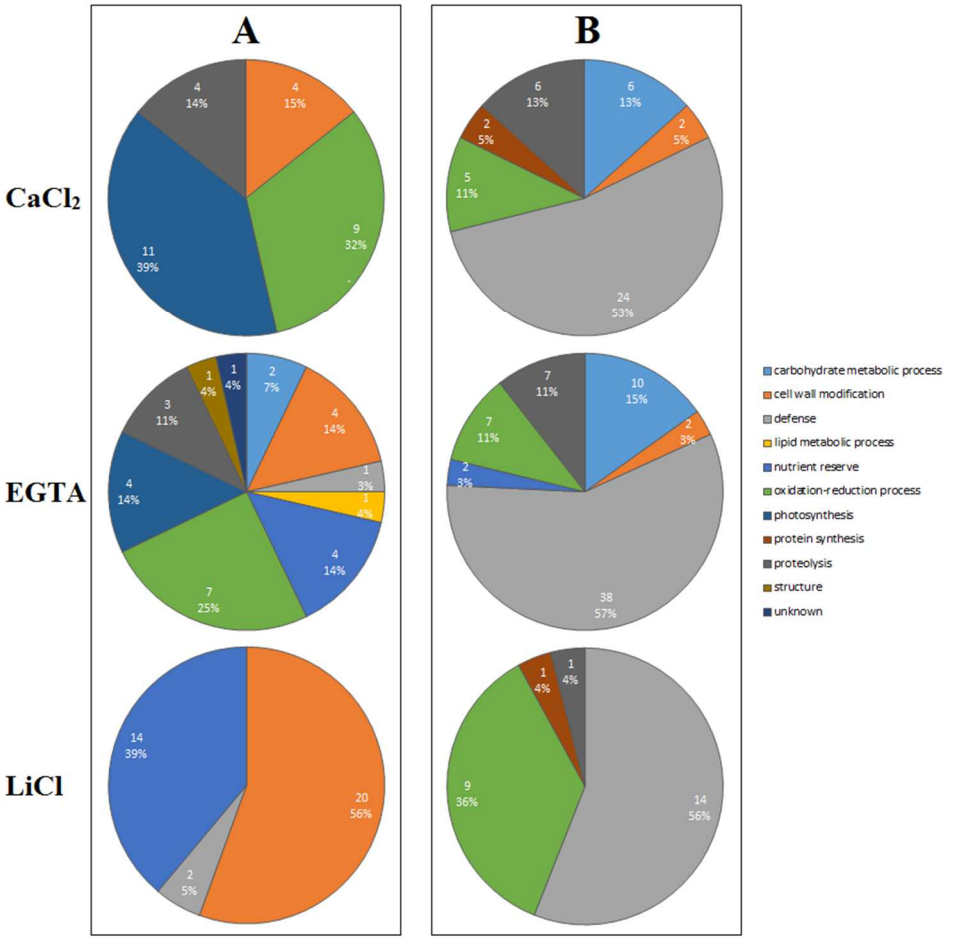

Figure 1-2: Functional classes of proteins with significant quantitative changes in the cell wall of *Medicago sativa* stems in response to Cd.
Plants were exposed to 10 mg of Cd kg^{-1} soil. Three different cell wall fractions were obtained. Quantitative analyses were performed based on 2D DIGE. Clustering into functional classes is based on their predicted biological function by using Blast2Go software. A) Functional classes of lower abundant proteins B) Functional classes of higher abundant proteins.

A large number of identified proteins are involved in carbohydrate metabolic processes, most prevalently glucan endo-1,3-β-glucosidase, but also glycoside hydrolase, family 17, α-galactosidase-like protein, which has a higher abundance in Cd-exposed plants.

For proteins identified in different spots, the intensity of these spots changed in the same direction with a comparable fold change, except for two proteins. In the EGTA fraction different spots containing β-like galactosidase (spots 1318 and 1433, **Table S1-5**) and β-xylosidase/alpha-L-arabinofuranosidase (spots 1620 and 1724, **Table S1-5**) showed an opposite change. The spectra corresponding to spots 1318 and 1433 were clearly different (**Figure S1-3**). An alignment of the identified sequences with the AGED-database resulted in contig 4097 (spot 1433) and the 40 amino acid longer contig 2997 (spot 1318). Although these contigs showed a different expression profile, no functional distinction between them is known. The identified isoforms of β-xylosidase/alpha-L-arabinofuranosidase had different sequences and matched with two different contigs from *M. sativa* (contig 15227 for spot 1620, contig 54428 for the spot 1724). Both of them have *Arabidopsis* homologs known to be differently expressed during pathogen infection (Huitema et al., 2003) and development (Hrubá et al., 2005). However, no functional difference between these proteins is known.

Proteins involved in cell wall remodeling are either of lower (fasciclin-like arabinogalactan protein, polygalacturonase non-catalytic protein, proline-rich extensin-like protein EPR1, dirigent protein 21-like) or higher abundance (pectinesterase, xyloglucan endotransglucosylase/hydrolase family protein, non-classical arabinogalactan protein 31-like, trichome birefringence-like protein) as a result of Cd exposure. Being involved in oxidation-reduction processes, a large number of peroxidases were identified with an increased abundance when plants are exposed to Cd. Designated to the same biological process, plastocyanin and plastocyanin-like domain protein have a lower abundance in stems of Cd-exposed plants. While the latter are predicted to be secreted, plastocyanin is targeted to the chloroplast. A large number of ferritins were found to be of lower abundance throughout the three cell wall fractions. We identified different ferritin isoforms and distinguished them from their N-terminal degradation

product phytosidirin (Laulhere et al., 1989). The relatively high density of intracellular ferritin-multimers might explain the enrichment of these protein complexes in the cell wall-enriched samples used in this study. Furthermore, proteins with a nutrient reserve function were identified. Auxin-binding protein ABP 19a and germin-like protein subfamily 3 member 1 were found to be of lower abundance due to Cd exposure while rhicadhesin receptor protein and bark storage protein increased in abundance. Some secreted proteins with a function in proteolysis were identified. KDEL-tailed cysteine endopeptidase CEP1 is of lower abundance in Cd-exposed plants while the abundance of papain family cysteine protease was higher.

During the MS-analyses numerous sequence variants, signal sequences, activation/inhibition sequences, and some post-translational modifications were identified (**Table S1-5**). The latter category includes the confirmation of an α-β didehydrophenylalanine as a potentially structure-determining modification in the β-subunit of polygalacturonase (Sergeant et al., 2017).

Table 1-2: Summary of identified proteins in the cell wall fractions ($CaCl_2$, EGTA, LiCl) of *Medicago sativa* stems that show a Cd-induced significant abundance change

Annotation	Species	Regulation	TargetP
carbohydrate metabolic process			
β-like galactosidase	*M. truncatula*	down/ up	S
β-xylosidase/alpha-L-arabinofuranosidase	*M. truncatula*	down/ up	S
α-galactosidase-like protein	*M. truncatula*	up	S
glucan endo-1,3-β-glucosidase	*M. truncatula*	up	S
Glycoside hydrolase, family 17	*M. truncatula*	up	/
cell wall modification			
fasciclin-like arabinogalactan protein	*M. truncatula*	down	S
polygalacturonase non-catalytic protein	*M. truncatula*	down	S
dirigent protein 21-like	*Cicer arietinum*	down	S
proline-rich extensin-like protein EPR1	*Cicer arietinum*	down	S
pectinesterase/pectinesterase inhibitor	*M. truncatula*	up	S
non-classical arabinogalactan protein 31-like	*Cicer arietinum*	up	S
trichome birefringence-like protein	*M. truncatula*	up	S
xyloglucan endotransglucosylase/hydrolase family protein	*M. truncatula*	up	S
defence			
Kunitz type trypsin inhibitor	*M. truncatula*	down	S
lectin	*M. sativa*	down	S
putative thaumatin-like protein	*M. truncatula var. truncatula*	down	/
allergen Pru protein, putative	*M. truncatula*	up	S
Chitinase (Class Ib) / Hevein	*M. truncatula*	up	S
Chitinase / Hevein / PR-4 / Wheatwin2	*M. truncatula*	up	S
chitinase	*M. sativa*	up	S
chitinase class III-1	*M. sativa*	up	S
class I chitinase	*M. sativa*	up	S
Defensin MtDef2.1	*M. truncatula*	up	S

legume lectin beta domain protein	*M. truncatula*	up	S
pathogenesis-related thaumatin family protein	*M. truncatula*	up	S
plant basic secretory protein (BSP) family protein	*M. truncatula*	up	S
calmodulin-like	*Brassica oleracea var. oleracea*	up	/
pathogenesis-related protein 1-like	*Brassica rapa*	up	S
thaumatin-like protein 1	*Cicer arietinum*	up	S
pre-hevein-like protein	*Pisum sativum*	up	/
calcium-dependent lipid-binding (CaLB domain) family protein	*M. truncatula*	up	M
CAP, cysteine-rich secretory protein, antigen 5	*M. truncatula*	up	S
LRR receptor-like kinase	*M. truncatula*	up	S
xyloglucanase-specific endoglucanase inhibitor protein	*M. truncatula*	up	S
lipid metabolic process			
glycerophosphoryl diester phosphodiesterase family protein	*M. truncatula*	down	S
oxidation-reduction process			
ferritin	*M. truncatula*	down	C
plastocyanin	*M. truncatula*	down	C
plastocyanin-like domain protein blue copper	*M. truncatula*	down	S
Plastocyanin-like domain early nodulin	*M. truncatula*	down	S
class III peroxidase	*M. truncatula*	up	S
lignin biosynthetic peroxidase	*M. truncatula*	up	S
peroxidase	*M. sativa*	up	S
peroxidase family protein	*M. truncatula*	up	S
peroxidase2	*M. sativa*	up	S
photosynthesis			
oxygen-evolving enhancer protein 2, chloroplastic	*Cicer arietinum*	down	C
Ribulose bisphosphate carboxylase small chain	*M. sativa*	down	C
oxygen-evolving enhancer protein 2-1	*M. truncatula*	down	C
photosystem II oxygen-evolving enhancer protein	*M. truncatula*	down	C
photosystem I reaction center subunit IV B, chloroplastic-like	*Solanum tuberosum*	down	C
protein synthesis			

60S ribosomal protein L18a-3	*M. truncatula*	up	/
eukaryotic translation initiation factor 5A	*Picrorhiza kurrooa*	up	/
proteolysis			
KDEL-tailed cysteine endopeptidase CEP1	*M. truncatula*	down	S
polyubiquitin	*Cicer arietinum*	down	/
eukaryotic aspartyl protease family protein	*M. truncatula*	up	C
papain family cysteine protease	*M. truncatula*	up	S
storage			
germin-like protein subfamily 3 member 1	*Cicer arietinum*	down	S
auxin-binding protein ABP19a	*M. truncatula*	down	S
bark storage-like protein	*M. truncatula*	up	/
rhicadhesin receptor	*M. truncatula*	up	S
structure			
phloem filament protein PP1	*M. truncatula*	down	S
unknown			
transmembrane protein, putative	*M. truncatula*	down	S

C: targeted to the chloroplast; M: targeted to the mitochondria; S: secreted; up: increased protein abundance; down: decreased protein abundance. Table S1-6 A-C.

3.3.2. Identification of soluble proteins

A significant change in abundance was detected for 52 spots in the soluble fraction (**Table S1-3**). These spots were picked, leading to the identification of 30 proteins. (**Table S1-5** and **Table S1-6 D**), which were clustered according to their predicted biological function using the Blast2Go software (**Figure 1-3** and **Table 1-3**).

Most proteins (26) showed a higher abundance in Cd-exposed plants and similar to the cell wall proteins, half of them (54 %) are involved in plant defence. Other proteins increasing in abundance are involved in metabolic processes (cobalamin-independent methionine synthase, sucrose synthase), carbohydrate metabolic processes (β-xylosidase/α-L-arabinofuranosidase), nutrient reserve (rhicadhesin receptor), oxidation-reduction processes (seed linoleate 9S-lipoxygenase) or protein synthesis (peptidyl-prolyl cis-trans isomerase, glycine-rich RNA-binding protein-like). Of all identified proteins, 43.3 % are predicted to be secreted, 16.7 % are targeted to the chloroplast, and 40 % are without a predicted target site (**Table S1-6 D**). Proteins identified in one or more of the cell wall fractions and in the soluble protein fraction had abundance changes in the same direction. These include ferritin and plastocyanin, involved in oxido-reduction processes and of lower abundance in Cd-exposed plants, as well as defence-related proteins which were more abundant in all extracted fractions from stems of Cd-exposed as compared to unexposed plants.

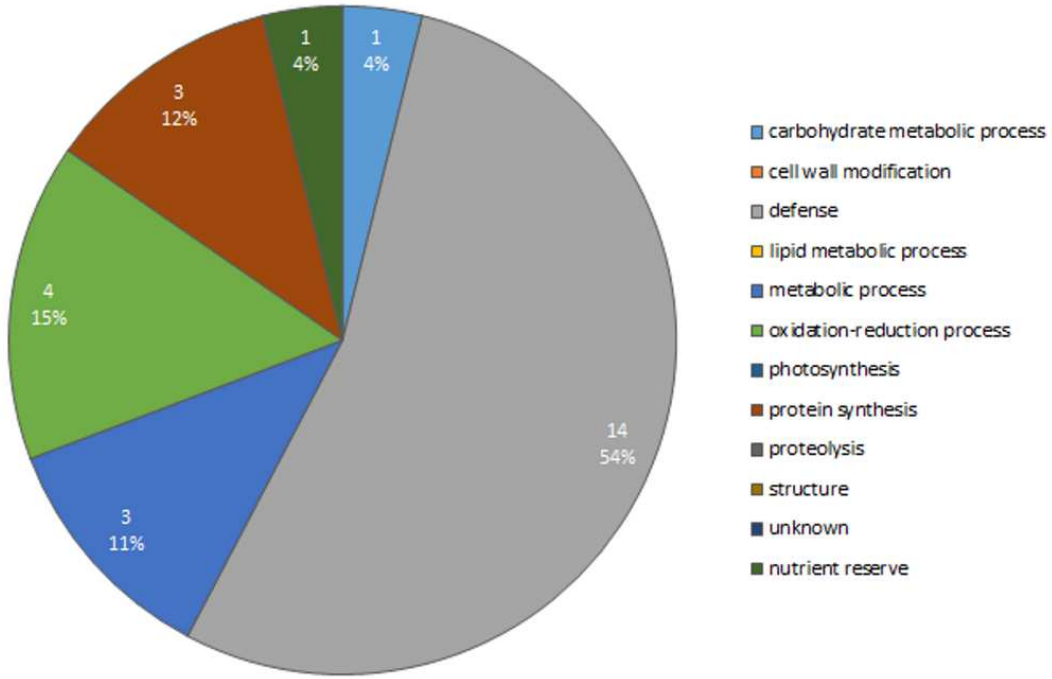

Figure 1-3: Functional classes of soluble proteins of *Medicago sativa* stems with higher abundance in response to Cd.
Plants were exposed to 10 mg of Cd kg^{-1} soil. Quantitative analyses were performed based on 2D DIGE. Clustering into functional classes is based on their predicted biological function by using Blast2Go software.

Table 1-3: Summary of identified soluble proteins of *Medicago sativa* stems that show a Cd-induced significant abundance change

Annotation	Species	Regulation	TargetP
carbohydrate metabolic process			
alpha-L-arabinofuranosidase/beta-D-xylosidase	*M. truncatula*	up	S
defence			
ABA-responsive protein	*M. truncatula*	up	/
epoxide hydrolase-like protein	*M. truncatula*	up	/
polyketide cyclase/dehydrase and lipid transporter	*M. truncatula*	up	/
Chitinase (Class Ib) / Hevein	*M. truncatula*	up	S
chitinase	*M. truncatula*	up	S
chitinase class III-1	*M. sativa*	up	S
class I chitinase	*M. sativa*	up	S
plant basic secretory protein (BSP) family protein	*M. truncatula*	up	S
pathogenesis-related protein 1-like	*Brassica rapa*	up	S
metabolic process			
cobalamin-independent methionine synthase	*M. truncatula*	up	/
Sucrose synthase	*M. sativa*	up	/
oxidation-reduction process			
ferritin	*M. truncatula*	down	C
plastocyanin	*M. truncatula*	down	C
seed linoleate 9S-lipoxygenase	*M. truncatula*	up	/
protein synthesis			
glycine-rich RNA-binding protein-like	*Arachis duranensis*	up	/
peptidyl-prolyl cis-trans isomerase	*M. truncatula*	up	C
Nutrient reserve			
rhicadhesin receptor	*M. truncatula*	up	S

C: targeted to the chloroplast; M: targeted to the mitochondria; S: secreted; up: increased protein abundance; down: decreased protein abundance. Table S1-6 D.

3.4. Expression of genes involved in cell wall structure and lignification

Based on our proteomic study, different genes related to cell wall structure and modification were selected for validation via RT-qPCR. Normalized gene expression values of all five replicates from unexposed and Cd-exposed plants (**Table S1-7**) are represented in a heat map (**Figure 1-4**). The expression of analysed genes in *M. sativa* stems cluster in two main branches. In the upper branch, all genes clearly showed a higher expression in Cd-exposed plants. These include genes encoding different POX isoforms, proteins functioning in carbohydrate metabolic processes (β-like galactosidase, glycoside hydrolase, β-xylosidase/α-L- arabinofuranosidase, endo-β-1,3-glucanase), pectinesterase/pectinesterase inhibitor and dirigent-like protein. With the exception of a β-like galactosidase isoform (contig 2998) all those expression changes were significant (p-value ≤ 0.05, **Table 1-4**).

The second branch is characterized by an unclear distinction between expression levels within stems of Cd-exposed and unexposed plants and a variable expression between the five replicates of the two conditions was observed, resulting in insignificant relative expression changes (**Figure 1-4**). Genes in that branch are related to cell wall modification and carbohydrate metabolism.

However, most of the observed significant expression changes were consistent with those observed for protein abundance.

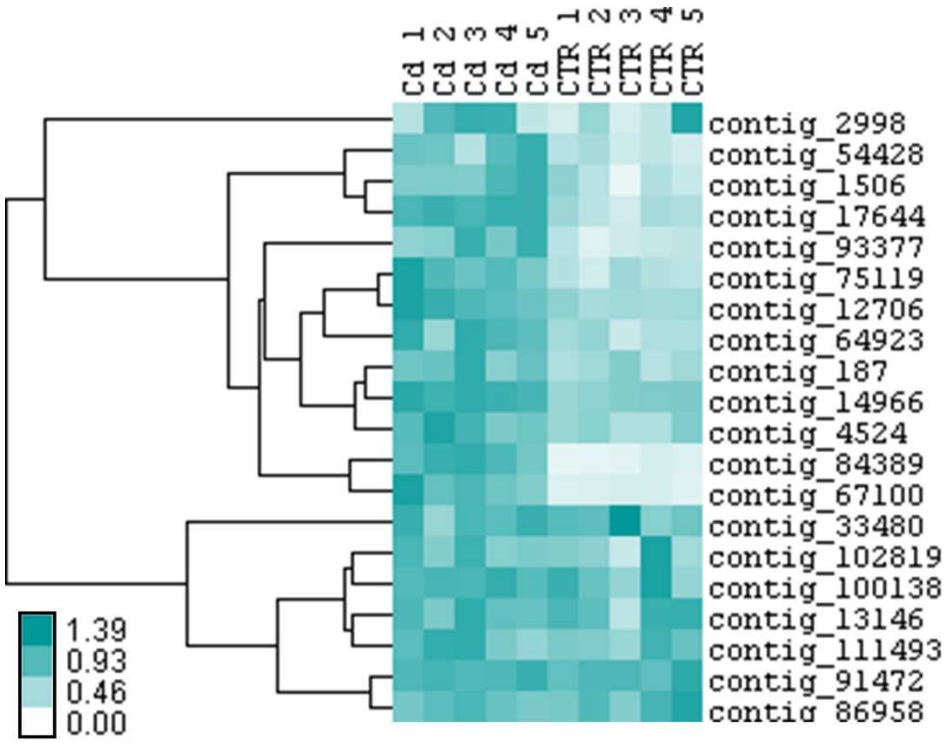

Figure 1-4: Heat map of expression levels from cell wall-related genes in *M. sativa* stems after long-term Cd exposure (10 mg kg^{-1} soil).
Hierarchical clustering (Pearson uncentered, complete linkage clustering) was done with normalized gene expression data from five biological replicates. Colour intensity is proportional to the actual expression value. Values are provided in Table S1-7. Contig numbers correspond to gene annotation in Table 1-4. Cd: Cd-exposed samples; CTR: control samples.

Table 1-4: Relative normalized gene expressions in stems of *Medicago sativa*.
Plant were exposed to 10 mg Cd kg^{-1} soil. Three reference genes were used for normalization. Expression levels were calculated relative to the non-exposed plants. Values are mean ± S.E from five biological replicates. Significant differences in gene expression (*p*-value ≤ 0.05) are indicated in green.

gene annotation	**contig_ID *M. sativa***	**gene ID *M. truncatula***	**rel. norm expression ± s.e.**	***p*-value**
beta-like galactosidase	2998	*Medtr1g018200.1*	1.507±0.325	0.323
beta-like galactosidase	1506	*Medtr2g039120.1*	2.272±0.229	0.004
α-L-arabinofuranosidase/ β-xylosidase	187	*Medtr2g008240.1*	1.656±0.157	0.007
α-L-arabinofuranosidase/ β-D-xylosidase	54428	*Medtr2g034720.1*	2.299±0.329	0.006
alpha-galactosidase-like protein	33480	*Medtr7g073650.1*	0.966±0.1	0.865
glucan endo-1,3-β-glucosidase	75119	*Medtr4g076470.1*	2.383±0.291	0.012
pectinesterase/pectinesterase inhibitor	93377	*Medtr7g050950.1*	2.854±0.364	0.001
non-classical arabinogalactan protein 31-like	91472		1.041±0.025	0.512
trichome birefringence-like protein	13146	*Medtr2g015720.1*	0.895±0.31	0.259
xyloglucan endotransglucosylase /hydrolase family protein	102819	*Medtr4g126920.1*	1.065±0.112	0.454
xyloglucanase-specific endoglucanase inhibitor protein	100138	*Medtr1g072420.1*	1.146±0.040	0.391
peroxidase family protein	12706	*Medtr2g029850.1*	1.953±0.151	0.0003
class III peroxidase	17644	*Medtr4g095450.1*	2.530±0.057	$2.45 \cdot e^{-6}$
lignin biosynthetic peroxidase	84389	*Medtr2g084020.1*	5.808±0.522	0.002
peroxidase family protein	64923	*Medtr6g043240.1*	2.083±0.235	0.005
class III peroxidase	14966	*Medtr5g074970.1*	1.669±0.043	$5.2 \cdot e^{-6}$
Glycoside hydrolase, family 17	67100	*Medtr2g034440.1*	4.567±0.494	$9.584 \cdot e^{-5}$
fasciclin-like arabinogalactan protein	86958	*Medtr4g059840.1*	0.920±0.053	0.512
polygalacturonase non-catalytic protein	111493	*Medtr8g064530.1*	1.156±0.143	0.405
dirigent protein 21-like	4524	*Medtr1g018200.1*	1.716±0.170	0.006

4. Discussion

Our data show the impact of long-term Cd exposure on the cell wall and soluble proteome in *M. sativa* stems. Especially proteins involved in cell wall structure and remodeling are highly interesting as they can be connected to structural changes in the cell wall induced by Cd. A brief overview is provided in **Figure 1-5**. Transcription levels from corresponding genes were further investigated to validate our protein data. Several proteins involved in photosynthesis were identified within the cell wall fraction. Although these proteins are not localized at the cell wall, they will be part of the discussion as their abundance is highly affected by Cd exposure, underlining the impairment of the photosynthesis due to Cd-exposure. Plants were grown for five months and the secondary shoot was sampled, as the cutting and re-growing of *M. sativa* is commonly used in agriculture. Cadmium-exposed plants exhibited severe effects during and after germination. Seeds grown on Cd-contaminated soil did not germinate in twice as much pots than grown on the control soil. Furthermore, Cd-exposed plants were inhibited in growth during the first weeks after germination (**Figure 1-1**). In a later more mature state, phenotypic differences evened out and at the end of our experiment (growth of secondary shoots) no significant difference in biomass was observed between control and Cd-treated *M. sativa* plants. This observation is in agreement with previous studies showing the strongest impact of heavy metals during germination and the initial stage of growth, while only slight growth inhibition was observed for several plant species in a more mature growth stage after attaining a new homeostasis on mildly polluted soils (Chaoui and El Ferjani, 2005; Chou et al., 2011; Peralta-Videa et al., 2004; Wang and Song, 2009). In a previous study undertaken in our laboratory on sunflowers exposed to a polymetallic constraint, this metal sensitivity of the initial growth stage was also observed (Printz et al., 2013). Although stress exposure passes through an alarm phase wherein rapid responses are intiatied to tackle the immediate threat and prevent damage (Lichtenthaler, 1996), a recent meta-study of proteome data indicates that long-term exposure of plants to low- to-moderate concentrations of heavy metals results in the establishment of a new homeostatic equilibrium (Dupae et al., 2014).

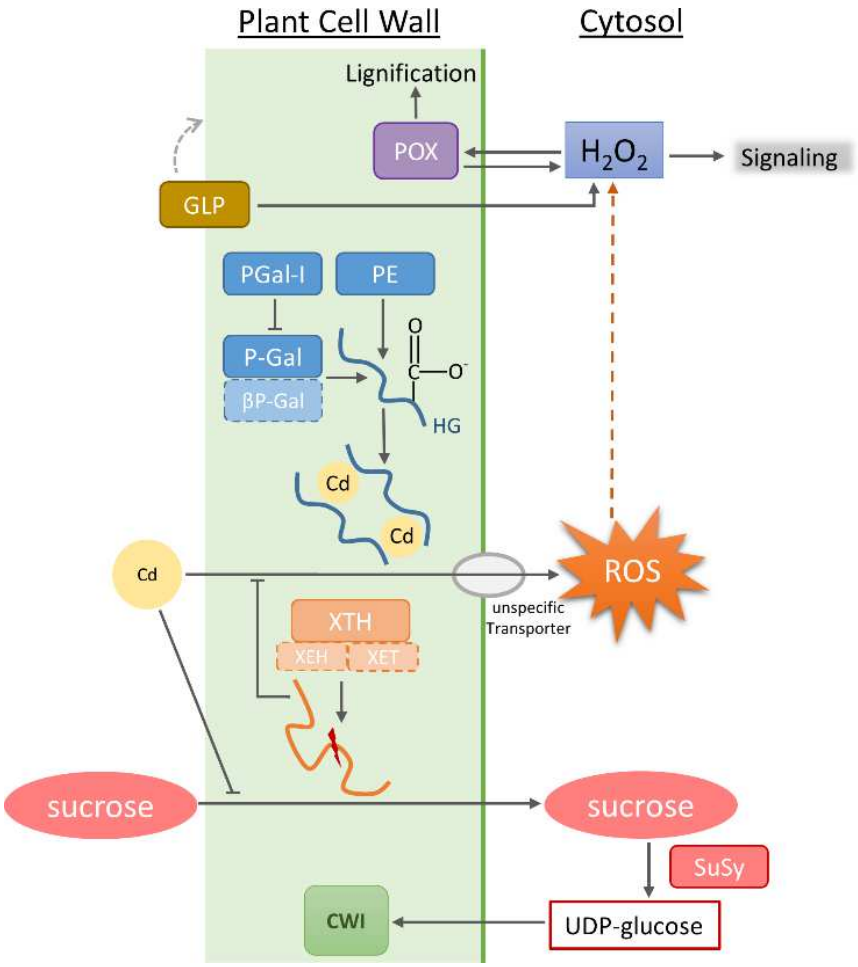

Figure 1-5: Effects of Cd on cell wall structure:
Cd might affect the cell wall structure in different ways. Cd influences lignification by acting on peroxidase (POX) activity. It further changes the activity of pectin methylesterase (PME), which leads to an enhanced content of low-methylesterified pectin and thereby creates binding sites for Cd within the cell wall. This is also supported by the activity of polygalacturonase (P-Gal) and polygalacturonase β-subunit (βP-Gal). The latter protein is negatively influenced by Cd. Cadmium directly affects the cell wall structure by changing the activity of xyloglucan endotransglucosylase/ hydrolase (XTH) and sucrose synthase (SuSy). By degrading sucrose, SuSy provides precursor molecules for cell wall synthesis. Intracellular, Cd indirectly triggers the production of reactive oxygen species (ROS), which subsequently activates POX activity and also operate as signaling molecules for down-stream processes. Germin-like proteins (GLP) induce ROS production and therefore influence ROS signaling and triggers POX activity.

4.1. Cd accumulation in the roots

In this study, roots had the highest Cd content in Cd-exposed plants and we observed a decreasing Cd content from roots > stems > leaves. The assessed Cd content in roots was four and seven times higher than in stems and leaves respectively (**Table 1-1**). Roots are directly exposed to Cd present in the soil. Ion transporters, which are embedded in the plasma membrane of root cells, preferentially take up nutrients from the surrounding soil, but do not discriminate between chemically similar metals and therefore also represent the main entry route for unessential metals such as Cd (Clemens, 2006; Song et al., 2016). Protective mechanisms occur primarily downstream of the up-take e.g. by trapping ions inside the cell through selective binding sites with a high affinity. Phytochelatins (PC) are cysteine-rich peptides synthesized in response to various metal ions with the ability to bind them. Among metals Cd is the most potent inducer of phytochelatin synthesis (Clemens et al., 1999; Grill et al., 1989). Phytochelatin - Cd complexes are sequestered in the vacuole, supporting the detoxification mechanisms of the cell (Cobbett, 2000) and it is assumed that vacuoles of root cells are the major site of metal accumulation. By retaining Cd in the roots and maintaining a low root-to-shoot translocation, physiological highly important organs such as leaves are protected from Cd-induced damage and essential process like photosynthesis can be maintained.

4.2. Cadmium affects the accumulation of proteins related to cell wall structure

Effects of Cd exposure are observed in the cell wall proteome and in the TCA/ phenol-extracted soluble proteome. The changes identified in the soluble proteome are consistent with those in the different cell wall fractions. Proteins uniquely identified in the soluble protein fraction include proteins involved in protein biosynthesis. Those proteins are of higher abundance, indicating that the plant is actively synthesizing proteins as a protective response to Cd stress. In the soluble protein fraction, a higher abundance was determined for sucrose synthase (SuSy). SuSy breaks down sucrose into fructose and UDP-glucose (Koch, 2004). The latter is used for cellulose biosynthesis and cell-wall bound SuSy isoforms might channel UDP-glucose directly into the cellulose synthase

complex (Brill et al., 2011). Cellulose is the main compound of the plant cell wall and during secondary cell wall synthesis a great amount of UDP-glucose molecules are required. The needed glucose molecules derive from photosynthesis and carbon fixation through the Calvin Cycle. Throughout all cell wall fractions, a Cd-induced lower abundance of photosynthetic proteins was observed in this study (**Figure 1-2**, **Table 1-2**), which suggests an impaired photosynthesis in *M. sativa* (Al-Hakimi, 2007; Wang et al., 2014). Impaired photosynthesis affects the carbon fixation and diminishes the available amount of glucose. The activity of SuSy could compensate for the missing glucose supply from the impaired photosynthesis and on account of this maintain the integrity of the cell wall during Cd stress.

Generally, proteins of the same functional classification have a change in abundance in the same direction. Even among members of larger protein families (chitinases, germins, POX), a similar directional change or trend was observed. Only in two cases (β-like galactosidase and β-xylosidase/α-L-arabinofuranosidase), spots that contained the same protein showed significant changes in different directions. Although MS analysis allowed the discrimination of different isoforms in the spots (**Table S1-5, Figure S1-3**), no functional specification of these isoforms was possible.

An important number of proteins with significant abundance changes are involved in cell wall modification and carbohydrate metabolic processes. A variety of these enzymes (including pectinesterase, β-like galactosidase, α-galactosidase and polygalacturonase non-catalytic protein) are responsible for structural modifications, degradation and changes within the pectin network during maturation and as response to stress (Ali et al., 2004; Caffall and Mohnen, 2009; Chrost et al., 2007; Dellapenna et al., 1990; Huang et al., 2016). Apart from polygalacturonase non-catalytic protein, all these proteins were more abundant in Cd-exposed *M. sativa* stems (**Table 1-2**), which corresponded to an increased expression of their encoding genes (**Table 1-4**). Cadmium induces changes in the pattern of the pectin homogalacturonane in plant cell walls (Douchiche et al., 2010b) and enhances PME activity (Paynel et al., 2009). The here observed higher abundance of pectinesterase supports elevated level of low-methylesterified homogalacturonane in the cell wall of Cd-

exposed *M. sativa* stems, creating binding sites for Cd and contributes to Cd deposition in the cell wall (Ramos et al., 2002), which prevents its further entry into the cell and translocation to the leaves. The function of the polygalacturonase non-catalytic protein remains ambiguous. Data indicate that it limits the access of hydrolases by tightly binding to pectin and that it prevents pectin release from the cell wall (Chun and Huber, 1997; Watson et al., 1994). Other studies found that overexpression of this protein results in a decreased pectin content and a diminished cell adhesion (Liu et al., 2014). In the current study, different isoforms of this protein showed a decreased abundance when plants were exposed to Cd, which might support the pectinesterase-catalyzed de-methylation of pectin.

The backbone of pectin is highly modified with different oligosaccharides and *O*-acetyl groups (Mohnen, 2008). As a response to Cd, we found β-xylosidase/α-L-arabinofuranosidase either of higher or lower abundance and a higher abundance of trichome birefringence-like protein (**Table 1-2**). The latter is an *O*-acetyltransferase, substituting cell wall polysaccharides (hemicellulose, pectin and lignin) with *O*-acetyl, which influences their properties and modifies the cell wall (Gille and Pauly, 2012). As a bifunctional hydrolase, β-xylosidase/α-L-arabinofuranosidase hydrolyzes terminal non-reduced L-Ara and D-Xyl and is involved in the reconstruction of the cell wall (Chavez Montes et al., 2008; Xiong et al., 2007). Two spots with a different direction of changes were identified, which can be ascribed to the bifunctional activity or to differences in substrate specificity of the different isoforms.

Furthermore an increased protein abundance of xyloglucan endotransglucosylase/hydrolase (XTH) due to Cd exposure was observed (**Table 1-2**). As XTH activity results in the re-construction of xyloglucan, it promotes cell wall loosening and cell expansion (Osato et al., 2006). Overexpression of this enzyme in Cd-exposed plants conferred Cd tolerance, correlating with the XTH-catalyzed declined xyloglucan content in the cell wall (Han et al., 2014). In another group of spots with an increased intensity after Cd stress, a cysteine-rich secretory protein (CAP) was identified. This small PR-1 protein has a cd05381 conserved domain with anti-fungal, as well as cell wall loosening activity. Homologous genes are down-regulated in salt- and Ca^{2+}-exposed plants

(Chan et al., 2008; Volkov et al., 2003), but it was highly increased in *Crambe abyssinica* exposed to arsenate (Paulose et al., 2010). Over-expression of a pepper homologue of this enzyme in tobacco plants resulted in an increased resistance to Cd and mercury exposure (Sarowar et al., 2005). Whether this is a result of its direct impact on the cell wall or an indirect impact on ROS generation is not known.

We found lignin biosynthetic peroxidase and other unspecific cell wall-linked peroxidases to be highly abundant in Cd-exposed *M. sativa* stems (**Table 1-2**). The corresponding gene transcripts were upregulated for all different isoforms and were more than five times higher in Cd-exposed plants for the lignin biosynthetic peroxidase (**Table 1-4**). Lignification results in cell wall stiffening and limits cell growth (Chaoui et al., 2004; Schützendübel et al., 2001). Cadmium is known to induce oxidative stress (Cuypers et al., 2011; Garnier et al., 2006) and H_2O_2 accumulation (Rodríguez-Serrano et al., 2006; Schützendübel et al., 2001). As a signaling molecule, H_2O_2 triggers secondary reactions such as an increased peroxidase activity which contributes to increased lignification. Lignification is further supported by dirigent-like proteins, which bind free radical monolignols species and coordinate their coupling during lignin biosynthesis (Davin and Lewis, 2000). A gene encoding a dirigent-like protein was induced by drought, salt and oxidative stress in *Saccharum officinarum*, highlighting their important role in plant resistance and defence (Jin-long et al., 2012) and its involvement in plant responses to metal stress was previously observed (Chen et al., 2016; Van De Mortel et al., 2006). Yet, we reported a Cd-mediated lower abundance of dirigent protein 21-like (**Table 1-2**), although the expression of the corresponding gene was significantly induced by Cd (**Table 1-4**). Gene expression and protein abundance do not necessarily correlate in a linear manner. Transcription and translation are controlled by different mechanisms which enhance or repress protein synthesis. Thereby, the degree of protein turn-over has probably the greatest influence on the transcript-protein correlation (Maier et al., 2009).

Overall, proteome and transcript data from stems of *M. sativa* support that Cd alters the cell wall structure and induces lignification as an important mechanism during plant defence. In the case of α-galactosidase-like protein, non-classical

arabinogalactan protein 31-like, trichome birefringence-like protein, xyloglucan endotransglucosylase/hydrolase family protein, xyloglucanase-specific endoglucanase inhibitor protein, fasciclin-like arabinogalactan protein and polygalacturonase non-catalytic protein, the protein abundance did not correspond with the determined transcript accumulation (**Table 1-4**, **Figure 1-4**). For those genes, expression changes due to Cd exposure were variable between replicates and therefore no significant changes in mRNA abundance was obtained. However, mRNA transcript levels and protein accumulation do not inevitably correlate due to the complexity of post-transcriptional mechanisms translating mRNA into proteins, fundamental differences of protein *in-vivo* half-lives as well as experimental limitations and errors. Reports about missing or poor proportional correlation of transcriptome and proteome data can be found in literature (Greenbaum et al., 2003). Therefore, transcriptomic and proteomic data should be seen as independent yet partly correlating information (Vélez-Bermúdez and Schmidt, 2014). Since 2D-PAGE has the potential to visualize all post transcriptional events, although in general without exact identification, correlations between 2D based proteome data and mRNA data is generally lower than when using a peptide-based proteome approach. The change in abundance of a specific proteoform, due to post transcriptional events, may therefore be poorly correlated or even reversed to transcriptional regulation. This observation underscores the complementarity of studying biology at the transcriptional and post transcriptional level.

4.3. Cadmium exposure results in the accumulation of stress response proteins

Exposure to Cd induces oxidative stress, which is consistent with an increased abundance of stress-related proteins in all fractions (**Figure 1-2**, **Figure 1-3**, **Table 1-2** and **Table 1-3**). The majority of these defence proteins are chitinase isoforms. Increased accumulations of chitinases in response to Cd were previously observed in different plants (Békésiová et al., 2008; Mészáros et al., 2014). In general, chitinases are ROS-regulated and thus the reported increased abundance of chitinases is not specific to Cd and rather reflects the general stress response of *M. sativa* (Hossain and Komatsu, 2013; Li et al., 2016; Metwally et al., 2003).

Germin-like proteins (GLP) in stem tissue of *M. sativa* show a decreased abundance when plants were exposed to Cd, even though GLP are involved in the response to abiotic stresses. Formally classified as storage proteins, GLPs are expressed in all plant tissues throughout all developmental stages and their association to the cell wall has been demonstrated (Christensen et al., 2004; Schweizer and Christoffel, 1999; Vallelian-Bindschedler et al., 1998). Many functions are assigned to GLPs: receptor, structure, enzyme activity (Bernier and Berna, 2001) including oxalate oxidase (OxO) and superoxide dismutase (SOD) activity (de los Reyes and McGrath, 2003; Gucciardo et al., 2007). Both reactions induce the generation of H_2O_2, a second messenger molecule during plant stress responses but also a trigger for peroxidase-induced lignification of the cell wall as discussed before. A stress-induced decreased GLP-SOD activity in the cell wall was observed before in cell cultures of *Barbula unguiculata* (Nakata et al., 2002). Lowering the abundance of GLPs in response to Cd exposure, will produce less ROS and prevents additional oxidative stress on top of the already induces oxidative burst by Cd.

5. Conclusion

Upon long-term exposure, the roots of *M. sativa* accumulated Cd, thereby restricting its mobility throughout the plant as further Cd-accumulation induces impairment of cellular, biochemical and physiological processes in the above-ground tissues. To test the hypothesis that the cell wall functions as an effective barrier for the entry of Cd, the present study was focused on the cell wall proteomes of the stems as the stems produce high amounts of cell wall material. Analysis of the cell wall and soluble proteome of the stem revealed a profound impact of Cd. Cadmium influences the abundance of cell wall proteins involved in multiple physiological processes such as plant defence response, oxidation-reduction processes, carbohydrate metabolism and cell wall remodeling. Transcriptome data of the study overall support these observations.

The identified proteins indicate that long-term Cd-exposure induces alterations in the structure of the cell wall and promotion of the lignification process. Currently, the determination of the cell wall composition and structure and the degree of lignification is ongoing. Although there is a negative impact of Cd

stress on the early stages of plant growth, the long-term exposure to Cd does not have an adverse impact on mature plants in terms of biomass production and the observed transcriptome and proteome changes do not have a significant impact on the produced biomass. This indicates that the mature plants established a new homeostasis and the induced protective mechanisms are more effective in mature than in juvenile plants.

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Chapter 1 – Part 2: Changes in the proteome of *Medicago sativa* leaves in response to long-term cadmium exposure using a cell wall-targeted approach

Adapted from published article:

Gutsch A., Zouaghi S., Renaut J., Cuypers A., Hausman, J-F., Sergeant, K.: Changes in the proteome of *Medicago sativa* leaves in response to long-term cadmium exposure using a cell wall-targeted approach. *Int. J. Mol. Sci.* (2018) 19, 2498. doi:10.3390/ijms19092498

Contributions:

A. Gutsch, K. Sergeant, J-F. Hausman and A. Cuypers designed the experiment.

S. Zouaghi performed all the experimental work and data analysis.

J. Renaut supplied and managed the technical equipment for the protein analysis.

A. Gutsch interpreted the data and wrote the present manuscript.

K. Sergeant contributed to the protein identifications, data interpretation and critically revised the manuscript.

All authors revised and approved the final version of the manuscript.

ABSTRACT

Accumulation of cadmium (Cd) states a serious problem for the environment and poses a threat to plants. Plants employ various cellular and molecular mechanisms to limit Cd toxicity and alterations of the cell wall structure were observed upon Cd exposure. This study focuses on changes in the cell wall protein-enriched subproteome of alfalfa (*Medicago sativa*) leaves during long-term Cd exposure. Plants grew on Cd-contaminated soil (10 mg kg^{-1} dry weight (DW)) for an entire season. A targeted approach was used to sequentially extract cell wall protein-enriched fractions from the leaves and quantitative analyses were conducted with two-dimensional difference gel electrophoresis (2D DIGE) followed by protein identification with matrix-assisted laser desorption/ionization (MALDI) time-of-flight/time of flight (TOF/TOF) mass spectrometry. In 212 spots that showed a significant change in intensity upon Cd exposure a single protein was identified. Of these, 163 proteins are predicted to be secreted and involved in various physiological processes. Proteins of other subcellular localization were mainly chloroplastic and decreased in response to Cd, which confirms the Cd-induced disturbance of the photosynthesis. The observed changes indicate an active defence response against a Cd-induced oxidative burst and a restructuring of the cell wall, which is , however, different to what is observed in *M. sativa* stems and will be discussed.

KEYWORDS: *Medicago sativa*, leaf cell wall proteome, cadmium, quantitative proteomics, 2D DIGE;

1. Introduction

Pollution of soil, water and air is one of the serious issues of recent decades. Amongst others, contamination with heavy metals is of great concern due to their stability in the ecosystem. Contaminated sites are inaccessible for humans in the context of urbanization, biomass- and food-production, which poses a major problem and exacerbates the already limited availability of soil. Cadmium (Cd) is one of the most common pollutants in the environment with a high degree of genotoxicity (Kovalchuk et al., 2001). Plants exposed to Cd suffer from an impairment of physiological and biochemical processes. They show limited growth and chlorosis and Cd leads to oxidative stress by generating reactive oxygen species (ROS) (Sanità Di Toppi and Gabbrielli, 1999). Cadmium interferes with photosynthesis by reducing the chlorophyll content, depressing the photosynthetic rate and induces direct damage to photosynthetic enzymes in a concentration- and time-dependent manner. Thereby, it was shown that Cd interferes more profoundly with the activity of photosystem II than photosystem I (Chugh and Sawhney, 1999; Wang et al., 2014). Cadmium can displace calcium (Ca) in photosystem II, thus inhibiting the formation of a functional complex and preventing photoactivation (Faller et al., 2005).

The plant cell wall is a dynamic cell-surrounding structure, which provides mechanical support and rigidity. It consists of cellulose, hemicellulose, pectin, as well as phenolic compounds. Proteins responsible for intercellular communication and interaction between the cell and the environment are imbedded in the cell wall. Those proteins make about 10 % of the cell wall mass and their tightly regulated enzymatic reactions can alter the cell wall structure and properties (Cassab, 1998; Fry et al., 1992), not only during plant development, but also during plant defence responses to biotic and abiotic stress (Bradley et al., 1992; Qin et al., 2003). Pectin methylesterase (PME), a cell wall protein, de-esterifies the pectic polysaccharide homogalacturonan (HG) creating binding sites for Ca^{2+}. Bound Ca mediates the bridging between two HG molecules to form a stable gel (egg-box structure) (Caffall and Mohnen, 2009; Krzesłowska, 2011). In the presence of Cd, PME showed an enhanced activity and the degree of low-methylesterified pectin in the cell wall increases concurrently with the deposition of Cd. By having the same charge, Cd^{2+} can bind pectin and displace Ca^{2+} as the

cross-linking ion in the egg-box structure (Douchiche et al., 2010b; Parrotta et al., 2015; Vollenweider et al., 2006). Additionally, Cd exposure has been shown to enhance lignification of the cell wall through an increased activity of cell wall-bound peroxidases, which causes cell wall stiffening and growth inhibition (Chaoui and El Ferjani, 2005; Paynel et al., 2009). Such Cd-induced alterations of the cell wall structure indicate that the cell wall is part of the defence mechanisms set-up by the plant and that those structural changes limit further translocation of Cd, thus, keeping cytosolic Cd concentrations low.

The plant cell wall proteome has been studied in different species including dicots and monocots. To date, the *Arabidopsis thaliana* cell wall proteome is the most comprehensive (Albenne et al., 2013). Yet, the leaf apoplastic proteome including cell wall proteins remains much less studied (Haslam et al., 2003) and information about cell wall proteins that change in abundance due to a treatment is underrepresented in the current scientific literature (Ndimba et al., 2003; Tran and Plaxton, 2008). However, comparative cell wall proteome studies in leaves already provided information on how the cell wall proteome changes when exposed to various stresses (Dani et al., 2005; Meng et al., 2016). To understand the mechanisms that take place in the cell wall during exposure to environmental constrains, it is important to unravel the cell wall proteome, its involvement in stress detection and response as well as its role in maintaining cell wall integrity.

Medicago sativa, commonly known as alfalfa, is an important forage legume and often used for research on cell wall development and stress adaptation (Printz et al., 2016; Verdonk et al., 2012). Contrary to most research, in the present study *M. sativa* plants were exposed to realistic Cd concentrations for a long-term period, which makes the here-obtained results relevant for agricultural practices. Relative quantitative changes of the cell wall protein-enriched subproteome from leaves were investigated using 2D DIGE, which not only enables relative quantification but also visualizes different protein isoforms and modified proteins caused by Cd exposure (Sergeant et al., 2017). A protocol for the enrichment of cell wall proteins was recently developed for *M. sativa* stems (Printz et al., 2015) and used in the current study on *M. sativa* leaves. The number of cytosolic contaminants in the different cell wall protein-enriched fractions remain low,

which facilitates an accurate understanding of the leaf cell wall proteome. Although *M. sativa* proteins can be identified based on homology with *M. truncatula* proteins, as performed in a recent study (Xiong et al., 2017), the combination of a search against the NCBI database and the *M. sativa* nucleotide database enlarges the number of identified proteins and the sequence coverage of the identified proteins, giving more comprehensive results. To our knowledge, this is the first study of the cell wall proteome of *M. sativa* leaves after long-term exposure to Cd.

2. Material and Methods

2.1. Plant material and treatment

M. sativa (cv Giulia) seeds were inoculated with *Sinorhizobium meliloti* and sown in May 2015 on Cd-polluted soil (10 mg kg^{-1} soil DW added as $CdSO_4$) and uncontaminated soil. The used soil was prepared as one batch (2/3 potting soil mixed with 1/3 sand (*w*/*w*)) before splitting in two conditions. For each condition 12 times 12 pots were planted. The plants were kept in the greenhouse until flowering stage was reached (July) and a first cut was done as during agricultural cultivation of *M. sativa*. Subsequently, plants were kept outside to avoid insect infestation. After a re-growing period till the pre-flowering stage was reached, plants were put back into the greenhouse for one more week before sampling was done on the 10th of September. No temperature or day cycle control was done during the experiment and no fertilizer was applied. A pool of leaves was sampled in four replicates for each condition and directly frozen in liquid nitrogen. All samples were kept at −80 °C until further use.

2.2. Cell wall protein enrichment

Cell wall protein-enriched fractions were obtained as described elsewhere (Gutsch et al., 2018) (chapter 1 - part 1) by using subsequently three different buffers with increasing ionic strength to extract also tightly bound proteins. Following the extraction, all three protein fractions ($CaCl_2$, ethylene glycol-*bis*(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid [EGTA], LiCl) were concentrated with Amicon Ultra 15 10 K (Millipore, Burlington, MA, USA) by centrifugation (4700× *g*, 4 °C) to an approximate volume of 200 µL. Subsequently, the ReadyPrep 2D Cleanup kit (Bio-Rad, Hercules, CA, USA) was used to desalt the

samples following the manufacturer's instruction. Cleaned samples were solubilized in labelling buffer (7 M urea, 2 M thiourea, 2 % *w*/*v* 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 30 mM Tris) and the protein concentrations determined using the Bradford method (Bradford reagent, Bio-Rad).

2.3. Quantitative protein analysis and identification

A 2D DIGE was undertaken to compare protein abundances between conditions in the three different fractions. Therefore, 50 µg of protein from each sample were labelled with either Cy3 or Cy5 and a dye swap was applied to avoid a possible effect of preferential labelling. An internal standard composed of 25 µg protein from each replicate (four biological replicates from control and cadmium, respectively) was labelled with Cy2 for each fraction ($CaCl_2$, EGTA, LiCl). Labelled samples were mixed, 9 µL Servalyte pH 3–10 (Serva Electrophoresis GmbH, Heidelberg, Germany) and 2.7 µL Destreak Reagent (GE Healthcare, Chicago, IL, USA) were added. The volumes were adjusted with lysis buffer (7 M urea, 2 M thiourea, 4 % *w*/*v* CHAPS) to 450 µL. Samples were loaded onto Immobiline™ DryStrip 3–10 NL, 24 cm (GE Healthcare, Chicago, IL, USA) overnight, followed by isoelectric focusing (IEF): (1) constant 100 V for 4 h; (2) linear gradient up to 1000 V for 4 h; (3) constant 1000 V for 5 h; (4) linear gradient up to 10,000 V for 6 h; and (5) constant 10,000 V until a total of 80,000 volt hours were reached. IEF-strips were equilibrated in equilibration buffer (Serva Electrophoresis GmbH) according to the manufacturer's instructions. 2D HPE™ Large Gels NF-12.5 % (Serva Electrophoresis GmbH) were used for the second dimension and were run on a HPE tower system following the manufacturer's instruction. After fixation (15 % ethanol *v*/*v*, 1 % *w*/*v* citric acid), three images from each of the gels were acquired using different wavelengths for the different labelling dyes (Cy2 488 nm, Cy3 532 nm, Cy5 642 nm) (Typhoon FLA 9500, GE Healthcare). SameSpots software v4.5 (TotalLab, Newcastle upon Tyne, UK) was used for the relative quantitative image analysis. Since the same internal standard is run on each gel of a fraction, alignment and normalisation with the internal standard allows comparison of the spots between repetitions. All statistical analyses were automatically done by the software. A spot was chosen for protein identification if a treatment effect was reported

(fold-change ≥ 1.2, ANOVA *p*-value ≤ 0.05, **Table S1-8** for spot volumes), if the spot was matched on all replicates and after manual validation.

Selected spots (**Figure 1-6** and **Figure S1-4**) were picked with an Ettan Spot Picker (GE Healthcare) and digested prior to MS/MS analyses as described before (Gutsch et al., 2018) (chapter 1 - part 1). Mass spectra were acquired with 5800 MALDI TOF/TOF (AB Sciex, Framingham, MA, USA). The ten most abundant peaks were automatically selected for fragmentation and spectra submitted to an in-house MASCOT server (Matrix Science, Available online: www.matrixscience.com) for database-dependent identifications. A first search was performed against the NCBInr database limited to *Viridiplantae* (3,334,509 sequences) and a second one against *M. sativa* sequences downloaded from the Samuel Roberts Noble website (plantgrn.noble.org/AGED (675,756 sequences, 304,231,576 residues)) (O'Rourke et al., 2015). The search parameters were as follows: mass tolerance 100 ppm, fragment mass tolerance 0.5 Da, cysteine carbamidomethylation as fixed modification, methionine oxidation, double oxidation of tryptophan, and tryptophan to kynurenine as variable modification. When at least two peptides passed the MASCOT-calculated 0.05 threshold score of 40, proteins were considered as identified. Additionally, if a high-quality spectrum was not matched to a protein, the interpretation was done manually and search parameters adjusted (semitryptic, single amino acid change, and post-translational modification) to increase the sequence coverage of identified proteins. After manual validation of the identifications, the subcellular location was predicted with the TargetP server using the standard search parameters (Available online: http://www.cbs.dtu.dk/services/TargetP) (Emanuelsson and Nielsen, 2000). Only proteins with a predicted signal peptide have been considered as cell wall proteins as done in current literature (Albenne et al., 2013; Duruflé et al., 2017). To validate the predicted subcellular location, a second location prediction was undertaken using DeepLoc (Almagro Armenteros et al., 2017). In some cases predictions were corrected after literature research. The Blast2Go software was used to gather information about the biological function of the identified proteins as well as the available literature.

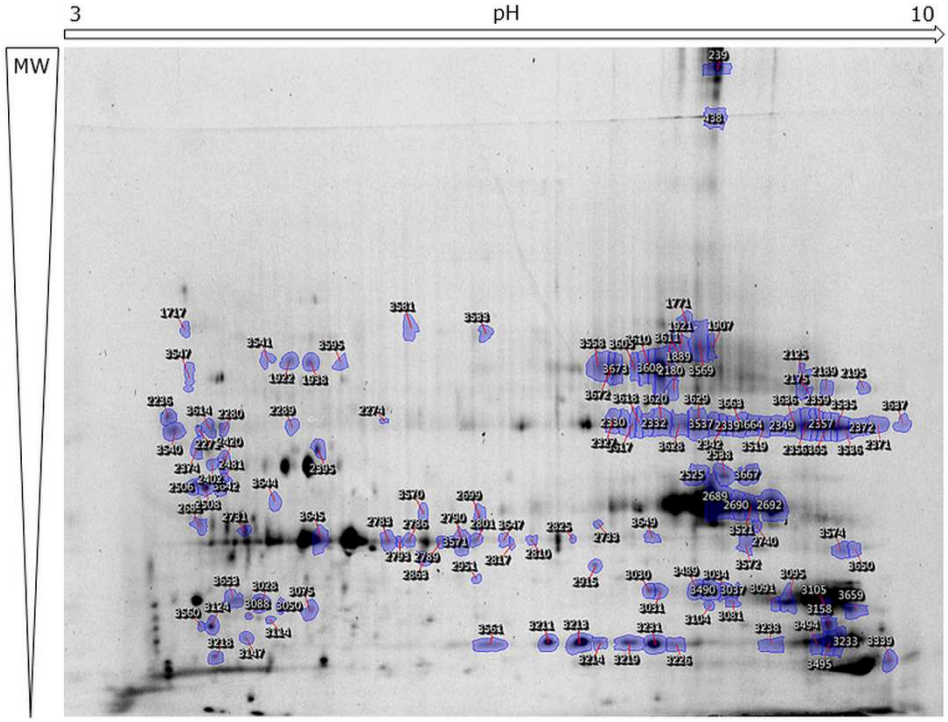

Figure 1-6: Illustration of 2D DIGE from the cell wall protein-enriched $CaCl_2$ fraction from leaves of *M. sativa*.
Extractions were performed in four replicates. Proteins were pre-labelled with different CyDye to enable relative quantitative protein analysis. Labelled samples were loaded onto ImmobilineTM DryStrip 3–10 NL, 24 cm (GE Healthcare) followed by migration on HPETM Large Gel NF-12.5 % (Serva Electrophoresis GmbH). Indicated spots were selected for picking based on statistical parameters calculated by the SameSpots software (TotalLab). Images of 2D DIGE from EGTA and LiCl fractions are provided in Figure S1-4. MW = molecular weight.

3. Results

Cell wall protein-enriched fractions were obtained by subsequently using three different buffers of increasing ionic strength containing $CaCl_2$, EGTA or LiCl, to extract proteins with various wall-binding affinities. Using a targeted extraction protocol, the contamination with cytosolic proteins is low. However, several proteins involved in photosynthesis were identified and quantitative changes in these proteins are consistent throughout the fractions and replicates. As photosynthetic proteins are highly abundant in leaves and as Cd affects photosynthesis, they are included in the results and discussion.

A principle component analysis (PCA) on the gel-based spot intensity data analysed with the SameSpots software (TotalLab) revealed a clear distinction between control and Cd-exposed samples in the three cell wall protein-enriched fractions (**Figure 1-7**).

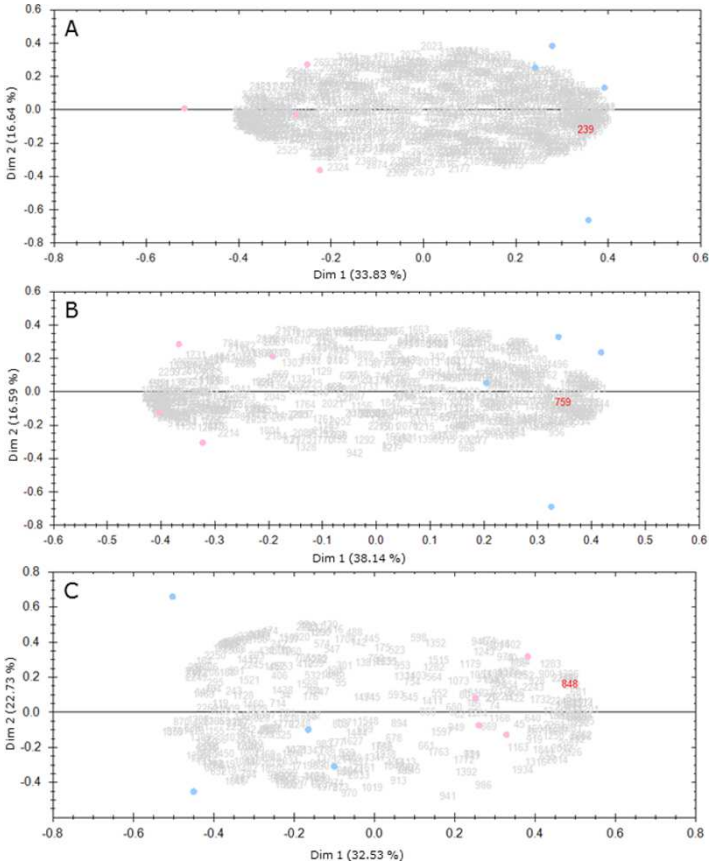

Figure 1-7: PCA analysis of the gel-based spot intensity data from the three cell wall protein-enriched fractions.
(**A**) $CaCl_2$; (**B**) EGTA; (**C**) LiCl. Statistical analysis was done with SameSpots software (TotalLab). Blue dots represent the four biological replicates of the control. Pink dots represent the four biological replicates of Cd-exposed samples. Grey and red numbers correspond to spot numbers considered for the statistical analysis.

After manual and statistical evaluation of all detected spots, 306 spots showed a significant abundance change in response to Cd exposure (fold-change ≥ 1.2, ANOVA *p*-value ≤ 0.05) and were picked for identification. All mass spectra (MS) and MS/MS data that resulted in the protein identifications using the MASCOT server are provided in **Table S1-9**. Out of the total number of significantly

changed spots, in 212 a single protein was identified and those were considered for biological interpretation. Based on the prediction for the subcellular localization with TargetP, 163 (76.9 %) of these proteins are predicted to be secreted. Thirty seven are targeted to the chloroplast and 12 do not have a predicted subcellular target site. These predictions are mostly coherent with those from DeepLoc (**Table S1-10**), whereby DeepLoc distinguished the different locations after a protein has entered the secretory pathway as the most important change compared to TargetP. However, ongoing research in our lab indicates that DeepLoc predictions are not always reliable and vacuolar proteins are designates as extracellular and vice versa. Therefore, we based ourselves on TargetP in the results and in the discussion. In the $CaCl_2$ fraction, 65 spots gave a significant identification of a single protein, of which 22 are of lower abundance and 43 are of higher abundance in response to Cd-exposure. Most proteins were identified in the EGTA fraction. Here, 93 proteins were found to increase (55 proteins) or decrease (38 proteins) in abundance. In the LiCl fraction a total number of 54 proteins were identified, of which 19 decreased and 35 increased in abundance. All proteins were clustered according to their predicted biological function to gain better insight on their physiological role and how this can be related to the plant's response during Cd exposure (**Figure 1-8**). A complete list of all spots containing a single protein identification and, therefore, considered for biological interpretation is provided in **Table S1-10**, including statistical values obtained by the SameSpots software (TotalLab, Newcastle upon Tyne, UK) and their biological function. This information is summarised in **Table 1-5**. In several spots the same nominal protein was identified, however, the observation that it was identified at a different pI and/or molecular weight indicates that this concerns proteoforms. An example of this is a C-terminal truncation of eight amino acids from chitinase (e.g., the spots EGTA 1225 and 1279) that is probably determining for its subcellular location. The observation of a semi-tryptic peptide, corresponding to a cleavage in the middle of the papain family cysteine protease active domain in the spot EGTA 1971 (**Table S1-9**) indicates that the degradation of this protein increases in Cd-exposed plants. In the spot LiCl 1250 the same protein was identified but this time with a semi-tryptic peptide corresponding to the start of the active domain, after removal of the N-terminal inhibitor domain. These observations are

confirmed by the position of the spots on the gel (**Figure S1-4**). Only by using a protein-based method, gel-based or gel-free, such proof of post-translational events can be obtained.

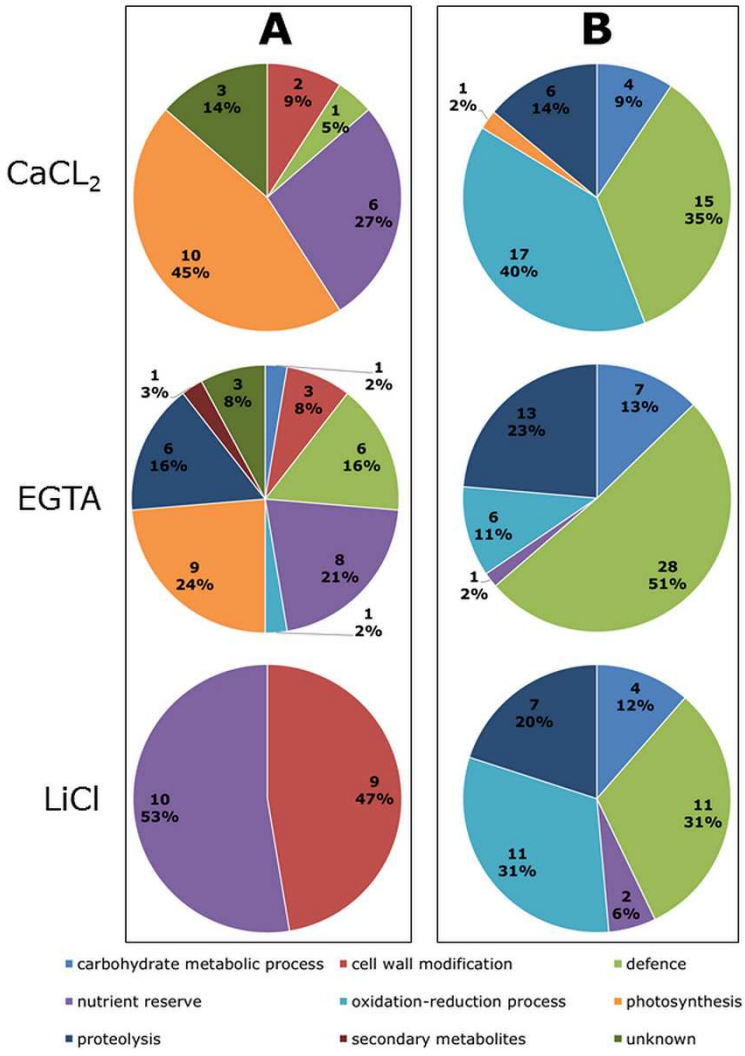

Figure 1-8: Functional classification of proteins present in cell wall protein-enriched fractions from *M. sativa* leaves after long-term exposure to Cd.
M. sativa plants were exposed to Cd (10 mg kg^{-1} soil DW) for an entire season. Quantitative analysis based on four replicates were done with 2D DIGE comparing Cd-exposed samples to control samples and identified proteins clustered according to their predicted function using Blast2Go. (**A**) Functional classes of lower abundant proteins; and (**B**) functional classes of higher abundant proteins.

A large part of the higher abundant proteins are involved in plant defence (**Figure 1-8**). Another class of proteins with higher abundance upon Cd exposure have a designated function in oxidation-reduction processes. This classification includes different peroxidase isoforms present in all three fractions, plus a plastocyanin-like domain protein identified in the EGTA fraction (**Table 1-5**). Likewise, some proteins involved in carbohydrate metabolic processes and proteolysis are found to be of higher abundance. A minor part has a nutrient reserve function (rhicadhesin receptor, auxin-binding protein ABP19a) or is involved in photosynthesis (photosystem I reaction centre subunit II) (**Table 1-5**).

The functions assigned to proteins with a decreasing abundance are more diverse in the $CaCl_2$ and EGTA fraction (**Figure 1-8**). Proteins identified in the LiCl fraction were only classified in cell wall modification (47 %) or nutrient reserve (53 %) (**Figure 1-8**). In those 19 spots only three different proteins were identified, namely auxin-binding protein ABP19a, stem 28 kDa glycoprotein, and polygalacturonase non-catalytic protein (**Table S1-10**). Most proteins of lower abundance in the $CaCl_2$ fraction are involved in photosynthesis (oxygen-evolving enhancer protein, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) small chain and PS II oxygen-evolving enhancer protein) (**Table S1-10**). Photosynthetic proteins are highly abundant in leaves. As expected, the highest percentage of proteins that are not predicted to be cell wall localized is found in the $CaCl_2$ fraction. It must be noted that no spot corresponding to RuBisCO large chain, the main component of the leaf proteome, is present. This protein makes up more than 50 % of the total leaf protein content and masking of lower abundant proteins by RubisCO large chain seems to be avoided using the here applied protocol for cell wall protein enrichment. Of the identified proteins, only the function of the plant/F18G18-200 protein, containing a DUF642 conserved domain, identified in the $CaCl_2$ and EGTA fraction remains unknown. The protein shows a decreased abundance in response to Cd and a secretion signal peptide was detected

Table 1-5: Summary of all identified proteins in cell wall protein-enriched fractions from *M. sativa* leaves, which changed significantly in abundance after long-term Cd exposure. The table is based on all identifications provided in Table S1-10. The targeted location was predicted with TargetP. C: chloroplast; S: secretory pathway; /: any other location.

Protein Identification	NCBI Identification*	Nr. of Spots wherein the protein was identified	TargetP
Lower Abundant in Cd-Exposed Plants			
Carbohydrate Metabolic Process			
Sedoheptulose-1,7-bisphosphatase	gi|357461143	1	C
Cell wall modification			
Pectinesterase/pectinesterase inhibitor	gi|357504799	3	S
Polygalacturonase non-catalytic protein	gi|922335979	10	S
Polygalacturonase-inhibiting protein 1	gi|374634428	1	/
Defence			
Cystatin	gi|74058377	1	/
Nod factor-binding lectin-nucleotide phosphohydrolase	gi|357508587	4	S
Pathogenesis-related thaumatin family protein	gi|922367846	1	S
CAP, cysteine-rich secretory protein, antigen 5	gi|357446161	1	S
Nutrient reserve			
Auxin-binding protein ABP19a	gi|357513969	11	S
Germin-like protein subfamily 3 member 1	gi|502156424	1	S
Stem 28 kDa glycoprotein	gi|357513539	12	S
Oxidation-reduction process			
1-cys peroxiredoxin PER1	gi|922395795	1	C
Photosynthesis			
Chlorophyll a-b binding protein 2	gi|3293555	1	C
Oxygen-evolving enhancer protein	gi|922331371	6	C
Ribose-5-phosphate isomerase A	gi|357512271	4	C
Ribulose bisphosphate carboxylase small chain	gi|3914601	5	C
Photosystem I reaction centre subunit IV A	gi|922402507	1	C
Photosystem II oxygen-evolving enhancer protein	gi|922336891	1	C
Proteolysis			
Eukaryotic aspartyl protease family protein	gi|922379288	6	S
Secondary metabolites			
Lactoylglutathione lyase-like protein	gi|922388614	1	/
Unknown			
Plant/F18G18-200 protein	gi|922395263	6	S
Higher abundant in Cd-exposed plants			
Carbohydrate metabolic process			
Glucan endo-1,3-β-glucosidase	gi|357474061	11	S
Glycoside hydrolase, family 17	gi|87240471	1	/
Glycoside hydrolase family 18	gi|357454031	3	S

protein			
Defence			
Allergen Pru protein, putative	gi|922401927	7	S
Chitinase (Class Ib)/Hevein	gi|922329699	6	S
Chitinase/Hevein/PR-4/Wheatwin2	gi|922347233	10	S
Chitinase	gi|357443753	4	S
Class I chitinase	gi|1800141	5	S
Disease resistance response protein	gi|922325015	2	S or/
Pathogenesis-related protein 1	gi|548592	1	S
Pathogenesis-related thaumatin family protein	gi|922338021	4	S
Plant basic secretory protein (BSP) family protein	gi|922407517	2	S
Pre-hevein-like protein	gi|7381205	1	/
Stromal 70 kDa heat shock-related protein	gi|821595433	1	C
CAP, cysteine-rich secretory protein, antigen 5	gi|357446161	7	S
Nutrient reserve			
Auxin-binding protein ABP19a	gi|357513969	1	S
Rhicadhesin receptor	gi|357511665	2	S
Oxidation-reduction process			
Anionic peroxidase swpb3 protein	gi|922380311	1	S
Class III peroxidase	gi|357476371	10	S
Peroxidase	gi|537317	7	S
Peroxidase family protein	gi|357448431	1	S
Peroxidase1b	gi|971560	3	S
Peroxidase2	gi|13992528	10	S
Plastocyanin-like domain protein	gi|922335020	2	S
Photosynthesis			
Photosystem I reaction centre subunit II	gi|357480841	1	C
Proteolysis			
Carboxyl-terminal peptidase	gi|922336331	2	S
Eukaryotic aspartyl protease family protein	gi|922327497	14	C
Papain family cysteine protease	gi|357437715	2	S
Polyubiquitin	gi|695063425	6	/
Subtilisin-like serine protease	gi|922333118	1	S

***** The given NCBI identification is representative, more than one identifier was assigned to the same protein. A complete protein identification list including all NCBI identification numbers is provided in Table S1-10.

Generally, if the same protein was identified in different spots, their changes in abundance were consistent with a comparable fold-change. However, spots containing auxin-binding protein ABP19a changed in different directions in the LiCl fraction (**Table S1-10**, spot 181 down and spot 1516 up) and were found to be less abundant in the $CaCl_2$ fraction. Although the abundance of distinct isoforms of this protein is differently influenced by Cd exposure, the spectra of these proteins are identical and no hint was found to explain the observed dissimilarity. On the other side, of all spots containing CAP (cysteine-rich

secretory protein, antigen 5) in the $CaCl_2$ fraction (**Table S1-10**, spot 3105 and spots 3028, 3030, 3031, 3034, 3037, 3081, and 3095), only spot 3105 has a decreased abundance. All of these spots contain a CAP most homologous to *Medicaco truncatula* CAP gi: 357446161. Nonetheless, only in spot 3028 the N-terminal peptide, after removal of the signal peptide, is QDSQADYVNAHNEAR, corresponding to contig 56,806 of *M. sativa*. In the other spots QDSQADYVNAHNDAR is identified as an N-terminal peptide corresponding to the contigs 111,668 and 1437 (**Table S1-9**). When calculating the pI of the translated contigs, the position on the gel confirms that spot 3028 is more acidic than the others (**Figure 1-6**).

A number of spots in the EGTA fraction contain eukaryotic aspartyl protease family proteins. Some of these spots are of lower intensity after Cd-exposure (spot 956, 961, 962, 964, 978, and 983), while others are of higher intensity (904, 911, 917, 948, 955, 2239, and 2240, **Table S1-10** and **Figure S1-4**). Based on the spectra and the matched peptide sequences (**Table S1-9**), it was found that aspartyl protease proteins with a decreasing abundance are predicted to be secreted, while those that are of increasing abundance have a chloroplast transit peptide. The distinction can also be observed on the gel images as the chloroplastic aspartyl proteases are acidic and thus cluster on the left side of the gel (**Figure S1-4**).

The secreted aspartyl proteases (EGTA fraction spot 956, 961, 962, 964, 978, and 983) can be divided into three groups according to their location on the gel: (1) 978, 983; (2) 956; and (3) 961, 962, 964 (**Figure S1-4**). The *M. sativa* contigs corresponding to those spots were blasted against the NCBI database and split into two different groups. Spots 978 and 983 (corresponding to contig 4015) show homology to the *M. truncatula* sequence XP_003594399.1, while the contigs corresponding to the remaining spots show the highest homology to the *M. truncatula* sequence XP_013459881.1. Those *M. truncatula* sequences only have a sequence identity of 37 %. However, both proteins carry the same conserved domain (pepsin_retropepsin_like superfamily) and no functional differences could be found in the literature.

The larger groups of spots with the same functional annotation were dissected, for instance, in 29 spots different chitinase proteins were identified. These can be divided in five groups based on the most homologous *M. truncatula* protein. This grouping is in agreement with the calculated pI and molecular weight of the different spots. In the spots 1279, 2259, 1225, and 2249 of the EGTA fraction, a class I chitinase (gi:1800141) was identified. Surprisingly, the identified protein lacks the eight C-terminal amino acids as shown by the identification of the peptide at *m/z* 2683.19 (**Table S1-9**). Similar observations have been conducted before (Neuhaus et al., 1994) and may relate to the fact that the protein is actually vacuolar and only removal of the C-terminal octopeptide allows the protein to be secreted (Stigliano et al., 2014). Of the chitinase-containing spots, those spots that are matched to the gi:357443753 (spot 1399, 1416, 1419, and 2508) have the highest fold change (**Table S1-10**). No clear functional differences between the chitinases could be found during this analysis.

In addition to sequence variants, signal sequences and the cleavage of activation/inhibition sequences, other post-translational modifications were also observed during MS analysis. Among these, α-β didehydrophenylalanine, as a potentially structure-determining modification, was identified in the β-subunit of polygalacturonase (Sergeant et al., 2017).

4. Discussion

The current study shows the impact of long-term Cd exposure on the *M. sativa* leaf subproteome, enriched in cell wall proteins. Identified proteins were clustered according to their biological function using Blast2Go (**Figure 1-8**) and those assignments as well as the number of proteins showing an increased or decreased abundance upon Cd exposure are comparable with results from a similar study on *M. sativa* stems (Gutsch et al., 2018) (chapter 1 – part 1). In both studies, results confirm that the enrichment obtained with the used protocol is better than that obtained with comparable protocols, but not 100 % and several non-apoplastic proteins, mainly chloroplast targeted, were identified most prominent in the $CaCl_2$ fraction (**Figure 1-8**) (Gutsch et al., 2018) (chapter 1 – part 1). Those proteins will also be discussed as they are highly

abundant in leaves and coherent, significant changes in abundance were observed between replicates and fractions in response to Cd exposure.

After planting, strong growth inhibition of Cd-exposed plants was observed at the end of the first growth cycle and coincided with the visual observation of leaf senescence. These phenotypical differences between Cd-exposed and control plants disappeared during the second growth cycle and no differences in appearance were observed at a later maturation state. Overall, long-term Cd exposure did not have any morphological impact on the leaves of *M. sativa*. Leaves from control and Cd-exposed plants had a rather heterogeneous appearance, due to which no leaf was representative for any of the conditions. Furthermore, to attain the amount of leaf material needed, four to five grams for each replicate, all leaves from young to old were sampled. The visual observation of limited impact of long-term Cd exposure is supported by the fact that the average plant biomass taken from five replicates at the end of the experiment was not significantly different between Cd-exposed (120.32 ± 4.67 g) and control plants (110.65 ± 3.78 g) (Gutsch et al., 2018) (chapter 1 – part 1). Although long-term experiments as presented here are rather scarce, the high sensitivity of initial growth stages followed by a low impact of the applied stress in adult plants was observed before for other plant species (Chaoui and El Ferjani, 2005; Peralta-Videa et al., 2004; Wang and Song, 2009). Obviously, plants do acquire a new steady-state when they are subjected to a constant severe but non-lethal stress (Gratão et al., 2008).

4.1. Cd-induced degradation of photosynthetic proteins

Throughout all three cell wall protein-enriched fractions a decreasing abundance of photosynthetic proteins was revealed (**Table 1-5**). Among others, RuBisCO small chain was less abundant in Cd-exposed plants as well as subunits of photosystem I and II. The Cd-induced decreased abundance of photosynthetic proteins was recently reported in *M. sativa* stems after long-term Cd exposure when analysing cell wall protein-enriched fractions using 2D DIGE (Gutsch et al., 2018) (chapter 1 – part 1). Likewise as in leaves, RuBisCO small chain, as well as a subunit of photosystem I, were less abundant in *M. sativa* stems when

exposed to Cd. The fact that Cd is disrupting photosynthesis and that it causes oxidative stress in plants by ROS production is well known (Sanità Di Toppi and Gabbrielli, 1999; Schützendübel and Polle, 2002). Reactive oxygen species are able to oxidise proteins, destining the oxidised protein for degradation, which goes together with an increasing protease activity (Romero-Puertas et al., 2002; Sandalio et al., 2001). Here, we observed that Cd induces a higher abundance of chloroplastic aspartyl protease isoforms in the EGTA fraction. The protein was identified before in leaf tissue of Cd-exposed *A. thaliana* (Semane et al., 2010) and its homology with cnd41-like proteins implies its function in senescence and nutrient recovery. Aspartyl proteases are implicated in degradation or processing of proteins and their occurrence during stress responses is established. They might have a crucial function in protein turn-over to prevent the accumulation of deactivated proteins, thereby increasing the pool of available amino acids, needed for the synthesis of defence-related proteins (Simões and Faro, 2004). They are involved in RuBisCO degradation and degradation of chloroplastic proteins (Bhalerao et al., 2003; Kato et al., 2004; Parrott et al., 2007). An increased abundance of this protease in *M. sativa* leaves correlates with the observed decreased abundance of photosynthetic proteins. Oxygen radicals, which appear during Cd exposure cause enhanced degradation of proteins involved in photosynthesis and impair the physiological processes in leaves. The increased abundance of a 70 kDa stromal heat shock protein ($CaCl_2$ spot 2402) likewise indicates that there is an increased need for protein refolding in the chloroplast.

A second group of spots in the EGTA fraction contains isoforms of aspartyl protease, which are of lower abundance in Cd-exposed plants. The proteins identified in these spots are predicted to be secreted (**Table S1-10**). Although no unambiguous function is established for secreted aspartyl proteases, they have been identified before, for instance in the pollen cell wall (Radlowski et al., 1996). Functional studies have implicated them in the defence against biotic stresses (Xia et al., 2004) and the *Arabidopsis* homolog AED1 was recently proposed to be part of a homeostatic feedback mechanism regulating the systemic acquired resistance (SAR) response (Breitenbach et al., 2014). This link between SAR, a salicylic acid-regulated process, and the here-observed

limited effect of long-term Cd exposure confirms previous studies, wherein salicylic acid application is shown to alleviate Cd-induced growth inhibition (Liu et al., 2016).

The only photosynthetic protein found with an increasing abundance is the photosystem I reaction centre subunit II ($CaCl_2$ fraction spot 3075, **Table S1-10**). The position of this spot is, however, at too low a pI and molecular weight, providing a further indication for increasing protein degradation in the chloroplast.

4.2. Cd influences the abundance of proteins related to the cell wall structure

Pectinesterase/pectinesterase inhibitor proteins are identified in three spots (**Table S1-10**, $CaCl_2$ fraction spot 2175 and EGTA fraction spot 989, 1007). The abundance of these proteins decreases in Cd-exposed plants. PMEs and their inhibitor are expressed as a single polypeptide and get subsequently processed by cleavage between the inhibitor and active domain. Our MS data do not confirm that the inhibitor domain is cut from the PME domain. However, the position on the 2D gel matches with what is predicted for the active protein and are at the same position as those found in *M. sativa* stems (Gutsch et al., 2018) (chapter 1 – part 1). In the latter study, cleavage between the inhibitor and the active domain was confirmed based on MS data, indicating that in leaves the inhibitor domain was also cleaved and thus the protein activated. However, the abundance of PME increased in *M. sativa* stems in response to long-term Cd exposure (Gutsch et al., 2018) (chapter 1 – part 1).

PME catalyses the demethylesterification of HG in plant cell walls. Those demethylated, acidic HG molecules form bridges between each other mediated by Ca^{2+} ions (egg-box structure) and confer rigidity to the cell wall (Caffall and Mohnen, 2009; Micheli, 2001). Cadmium ions have the same charge (Cd^{2+}) and can displace Ca^{2+} as cross linker in the pectin egg-box structure. During Cd exposure a higher abundance of PME was found in several studies and an enhanced activity demonstrated (Douchiche et al., 2010b; Paynel et al., 2009). Changes in HG pattern in response to Cd have been investigated together with a preferential allocation of Cd in the cell wall (Douchiche et al., 2010a; Ramos et

al., 2002; Vollenweider et al., 2006). Thus, the cell wall structure changes during Cd exposure and the sequestration of Cd in the cell wall protects the cytosol. In a recent study on the cell wall composition in *M. sativa* stems upon long-term Cd exposure, the most significant changes appeared in the pectin fraction towards a higher abundance of HG upon Cd exposure and an increased activity of PME in response to Cd was determined (chapter 2). This suggests a high demethylation degree, which creates binding sites for Cd and immobilize it in the cell wall. Opposite to what is observed in *M. sativa* stems (Gutsch et al., 2018) (chapter 1 – part 1), Cd exposure led to a decreasing abundance of PME in leaves, which would lead to a low demethylation degree of HG. The methylation degree of pectin has an influence on the cell wall structure and, furthermore, limits the accessibility for pectin-degrading enzymes, such as polygalacturonases (Wolf et al., 2009). In response to long-term Cd exposure, the structural changes in the pectin network of the leaf cell wall seem to be different from those anticipated in the stems of *M. sativa* in response to Cd (Gutsch et al., 2018) (chapter 1 – part 1) and an organ-specific influence of Cd on the cell wall can be assumed. The observed structural changes in the stems of *M. sativa* promote the creation of binding sites for Cd in the stem cell wall as a direct response to the applied stress (chapter 2). In this matter, the leaf cell wall plays probably a minor role in the retention of Cd but more data on the structural changes would be needed to draw a more comprehensive conclusion.

Polygalacturonase-inhibiting protein 1 and polygalacturonase non-catalytic protein or β-subunit are involved in pectin degradation. Conflicting data on the function of the latter has been obtained. Overexpression of the polygalacturonase non-catalytic β-subunit in rice resulted in a decreased pectin content and a higher susceptibility to abiotic stress due to lower cell adhesion (Liu et al., 2014). On the other hand, during fruit ripening in tomato, it limits the extent of pectin solubilisation and depolymerization (Watson et al., 1994). In the present study, a lower abundance occurred in the *M. sativa* leaf subproteome enriched in cell wall proteins (**Table 1-5**), as was previously reported in *M. sativa* stems (Gutsch et al., 2018) (chapter 1 – part 1). However, a quantitative change in the abundance of β-subunit of polygalacturonase is not necessarily correlated with a change in polygalacturonase activity (Tucker et al., 1981). The

functional linkage between these two polygalacturonase subunits remains to be solved as contradictory data exist. Furthermore, polygalacturonase inhibitor showed a decreasing abundance in our study. It can be speculated that this decreased abundance is a cross reaction to the lower abundance of polygalacturonase non-catalytic protein in order to keep the polygalacturonase activity in the cell wall stable. So far, polygalacturonase inhibitor has only been described during the defence against pathogen attacks by inhibiting the fungal polygalacturonase (De Lorenzo and Ferrari, 2002; Favaron et al., 1994). Those proteins have a specific binding site to interact with pectin in the plant cell wall, which is furthermore influenced by methylesterification patterns in the pectin network (Spadoni et al., 2006). Their function in the alteration of the plant cell wall structure in response to abiotic stress remains unclear.

Various peroxidase isoforms were highly abundant in the leaves of Cd-exposed *M. sativa* (**Table 1-5**). Previous studies on *M. sativa* stems (Gutsch et al., 2018) (chapter 1 –part 1)and poplar leaves gave similar results (Kieffer et al., 2009). Peroxidases are involved in oxidation-reduction and lignification processes in the cell wall (Loix et al., 2017). Using H_2O_2 molecules as a co-substrate, peroxidases catalyse the oxidation of monolignols, which then cross-link to form lignin (Passardi et al., 2004) and increasing peroxidase activity was positively correlated with the degree of cell wall lignification (McDougall, 1992). Cadmium enhances H_2O_2 accumulation in plants and increased peroxidase abundance and activity, leading to cell wall lignification, cell wall stiffening and growth impairment (Chaoui et al., 2004; Chaoui and El Ferjani, 2005; Radotic et al., 2000). Oxidative stress might also be responsible for the accumulation of carboxyl-terminal peptidase (**Table 1-5**). The protein contains two DUF domains (239 and 4409). The *Arabidopsis* homolog was found to be responsive to ROS and confers enhanced tolerance to oxidative stress (Luhua et al., 2008). The same protein was found to have a role in cell wall modification, influencing nutrient transport by modification of root endodermal barriers (Li et al., 2017).

4.3. Enhanced accumulation of defence proteins as a response to Cd

Different defence related proteins were identified in *M. sativa* leaves (**Table 1-5**). Their increased accumulation due to Cd exposure underline the strong defence response of the plant, which had been reported before when plants were exposed to heavy metals (Chen et al., 2016; Gutsch et al., 2018; Kieffer et al., 2009; Semane et al., 2010). Most prevalent, we identified different chitinase isoforms throughout all three fractions (**Table S1-10**), which is consistent with previous findings in *M. sativa* stems (Gutsch et al., 2018) (chapter 1 – part 1), where several chitinase isoforms increased in abundance upon long-term Cd-exposure. A study on different plant species exposed to different metals indicates that a metal-specific chitinase-expression profile may exist (Békésiová et al., 2008). Although such metal-specific functions of chitinases remain uncertain, an increased abundance of chitinases has been proposed as a marker for the induction of a SAR-response. Despite the small impact of long-term Cd exposure on biomass production (Gutsch et al., 2018) (chapter 1 – part 1), it appears to have a significant negative impact on the presence of proteins classified as having a nutrient reserve function (**Figure 1-8**), which was also reported in *M. sativa* stems (Gutsch et al., 2018) (chapter 1 – part 1). This may indicate that the plants are capable of maintaining growth under the conditions used in this study, but are not capable of establishing reserves. It must, however, be mentioned that multiple functions are attributed to these nutrient reserve proteins and that the observed decreased abundance may have other consequences (Bernier and Berna, 2001).

5. Conclusion

M. sativa plants were exposed to Cd (10 mg kg^{-1} soil DW) in a long-term experiment and the leaf cell wall protein-enriched subproteome was analysed. In total, 212 identified proteins changed significantly in response to Cd and a major part of these identified proteins is involved in defence responses, underlining the importance of the general defence machinery in response to Cd and linking the observations in this study with knowledge on the SAR response. Cell wall proteins related to oxidation-reduction processes are highly abundant in Cd-

exposed plants and might counteract the Cd-induced oxidative burst in the plant. Germin-like proteins, although classified as nutrient reserve, may also contribute. Interestingly, Cd provokes tissue-specific alterations in the pectin network of the cell wall in *M. sativa* leaves and stems (Gutsch et al., 2018) (chapter 1 – part 1). The leaf cell wall seems to be less involved in the assumed cell wall-promoted binding of Cd as a protective mechanism.

About 18 % of the identified proteins are targeted to the chloroplast and their relative abundance decreases upon Cd exposure concomitantly with an increase in chloroplastic, proteolytic proteins. Therefore, the increased protein degradation in the chloroplast confirms interference of Cd with the photosynthetic activity of plants. Nonetheless, Cd-exposed plants showed no difference in biomass production or in the growth at the moment of sampling in comparison to control plants, which suggests that the plants established a new metabolic steady-state during the long-term stress exposure. The important decrease in proteins with nutrient reserve function, however, indicates that the plants are weakened and may perform worse than control plants when exposed to secondary stresses.

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Highlights and Perspective Chapter 1

The heavy metal Cd accumulates in the upper soil due to anthropogenic influence. It enters into plants by their root system from where it gets distributed into other tissues, causing multiple toxic effects. By structural changes in response to Cd, it is assumed that the plant cell wall acts as a physical barrier against Cd. In the first part of chapter 1 we addressed how long-term Cd exposure changes the abundance of cell wall- and soluble proteins in stems of *M. sativa*. To do so, we applied a cell wall-targeted extraction protocol, which was developed in our group for *M. sativa* stems[5], combined with a global proteomics approach. Obtained data were complemented by targeted gene expression analysis. We used 2D DIGE for quantitative protein analysis as it is still the only top-down approach for native protein identification and commonly used in plant research. Our main results are:

- Long-term Cd exposure does not affect the plant biomass, although a high impact of Cd on the early stage of plant growth was observed
- Cd strongly accumulates in the roots and quantitatively decreases in the areal parts (root > stem > leaves)
- Cd has a profound impact on the cell wall proteome and influences the abundance of proteins, which are involved in multiple physiological processes such as defence response, oxidation-reduction process, carbohydrate metabolism and cell wall remodelling
- Data were overall supported by transcriptome data
- Identified proteins point to alterations in the cell wall structure and lignification of the cell wall

It is evident that Cd exposure alters the cell wall proteome, underlining the importance of cell wall proteins during stress response and in the defence against Cd stress. A number of the identified proteins can be linked to alterations in the cell wall structure and lignification, supporting the hypothesis

[5] Printz, B., Dos Santos Morais, R., Wienkoop, S., Sergeant, K., Lutts, S., Hausman, J.-F., et al. (2015). An improved protocol to study the plant cell wall proteome. Frontiers in Plant Science 6, art. 237.

of the cell wall as a protective barrier against Cd. In order to validate our results, it was important to investigate the cell wall composition and the lignin content in *M. sativa* stems. Chapter 2 refers to the same samples of *M. sativa* stems and focuses on the monosaccharide composition of the cell wall as well as the lignin content. Furthermore, targeted gene expression was used to investigate monolignol and ethylene biosynthetic pathways.

In the second part of chapter 1 we investigated the influence of long-term Cd exposure on the cell wall protein-enriched fractions of *M. sativa* leaves. Therefore, the same targeted extraction protocol was used in order test whether it can be applied to other organs. Our results confirm that the enrichment of cell wall proteins is fairly high. Protein identifications, assigned biological function as well as the number of proteins showing an increased or decreased abundance upon Cd exposure are comparable with results from *M. sativa* stems. Thereby, chloroplastic proteins are highly abundant in leaves and where the main non-cell wall located proteins. Their decreasing abundance confirmed the Cd-induced interference with photosynthesis. However, the PME abundance decreased in response to Cd, which is opposite to what was observed in *M. sativa* stems and a different alteration of the cell wall in stems and leaves is anticipated. Probably, the leaf cell wall is less involved in the retention of Cd in the pectic network of the cell wall.

In both studies presented in chapter 1, 2D DIGE was used for protein quantification. Using the obtained data in *M. sativa* stems and leaves, we were able to identify a fold-directing posttranslational modification on phenylalanine residues in the repeated tetrapeptide FxxY of the polygalacturonase beta-subunit. It was found that all identified phenylalanine residues in the sequence FxxY of this protein were modified to α,β-didehydro-Phe and the reproducible identification in different species augments its substantial fold-determining role[6].

[6] Sergeant, K., Printz, B., Gutsch, A., Behr, M., Renaut, J., and Hausman, J.-F. (2017). Didehydrophenylalanine, an abundant modification in the beta subunit of plant polygalacturonases. *PLoS One* 12, e0171990.

Chapter 2 - Does long-term cadmium exposure influence the composition of pectic polysaccharides in the cell wall of *Medicago sativa* stems?

Adapted from the submitted article:

Gutsch A., Sergeant, K., Keunen E., Prinsen E., Guerriero G., Renaut J., Hausman, J-F., Cuypers A.: Does long-term cadmium exposure influence the composition of pectic polysaccharides in the cell wall of *Medicago sativa* stems? *BMC Plant Biology* (*minor revisions received September 2018)*

Contributions:

A. Gutsch, K. Sergeant, J-F. Hausman and A. Cuypers designed the experiment.

A. Gutsch performed the experimental work, analysed and interpreted the data and wrote the present manuscript.

G. Guerriero and E. Keunen contributed to the experimental set-up for RT-qPCR.

K. Sergeant contributed to the protein identifications, data interpretation and critically revised the manuscript.

E. Prinsen performed the ACC determination and drafted the technical part of ACC determination.

J. Renaut supplied and managed the technical equipment for the protein analysis.

All authors revised and approved the final version of the manuscript.

ABSTRACT

The heavy metal cadmium (Cd) accumulates in the environment due to anthropogenic influences. It is unessential and harmful to all life forms. The plant cell wall forms a physical barrier against environmental stress and changes in the cell wall structure have been observed upon Cd exposure. In the current study, changes in the cell wall composition and structure of *Medicago sativa* stems were investigated after long-term exposure to Cd. Liquid chromatography coupled to mass spectrometry (LC-MS) for quantitative protein analysis was complemented with targeted gene expression analysis and combined with analyses of the cell wall composition. Several proteins determining the cell wall structure changed in abundance in response to Cd exposure. Structural changes of the cell wall mainly appeared in the composition of pectic polysaccharides. Data indicate an increased presence of demethylated homogalacturonan, which is able to bind and sequester Cd in the cell wall. The present work reveals structural changes in the cell wall of *M. sativa* stems, which occur in response to long-term Cd stress and underlines the function of the cell wall as a barrier involved in the plant's defence response to Cd.

KEYWORDS: long-term cadmium exposure, *Medicago sativa*, label-free protein quantification, gene expression, cell wall, lignin, pectin methylesterase

1. Introduction

Anthropogenic influence has led to an accumulation of cadmium (Cd) in the upper soil. Originating from fertilizer application, mining activity and sewage sludge, the concentration of Cd in the topsoil ranges from <0.01 to 14.1 ppm in Europe (Pan et al., 2010). Cadmium has a very high mobility and can enter plants via their root system, from where it is distributed to all plant parts and can cause multiple toxicity symptoms. Accumulation in the above-ground parts limits the economical valorisation of plant material but on the contrary, plants can also be used to remove Cd from the soil in the phytoremediation process.

The plant cell wall serves as a physical barrier against environmental threats such as Cd. The cell wall of plants is mainly composed of cellulose, hemicellulose and pectins. Cellulose is the main structural component and composed of β-1,4-linked glucose, forming crystalline microfibrils. Those microfibrils are embedded in a complex, heterogeneous polysaccharide matrix. Hemicelluloses bind to cellulose and include xyloglucans, glucomannans, xylans and mixed-linkage glucans, whereby their interaction with cellulose highly contribute to cell wall strengthening (Scheller and Ulvskov, 2010). Pectins are probably the most heterogeneous group of polysaccharides including homogalacturonan (HG), rhamnogalacturonans (RG) I and II, galactans, arabinans and more. The cell wall is dynamic and continuously undergoes structural changes to adapt to the plant's development and environmental conditions. Its structural components provide mechanical support and rigidity, which is furthermore maintained by the activity of its embedded cell wall proteins conferring optimal characteristics to the cell wall (Albenne et al., 2014; Cassab, 1998).

During Cd exposure, alterations in the cell wall structure occur and changes in the methylation pattern of HG were observed (Douchiche et al., 2010a; Vollenweider et al., 2006). Homogalacturonan is synthesized in the Golgi apparatus from where it gets transported to the cell wall in its methylated form and gets demethylated in the cell wall by pectin methylesterase (PME). An increased PME activity and an enhanced accumulation of *PME* transcripts following Cd exposure were shown in flax (Douchiche et al., 2010b; Paynel et al., 2009). Pectin methylesterase catalyses the de-methylesterification of HG,

thereby creating binding sites for Ca, which can be displaced by Cd due to a higher affinity of the latter (Dronnet et al., 1996). The possible sequestration of Cd in the cell wall prevents its further entry into the cell and is part of the plant's defence strategy against Cd stress (Krzesłowska, 2011). Additionally, Cd induces the activity of peroxidases enhancing cell wall lignification (Douchiche et al., 2010b; McDougall, 1992; Paynel et al., 2009), which is observed in different plant species (Chaoui and El Ferjani, 2005; Elobeid et al., 2012). By inducing the accumulation of reactive oxygen species (ROS), of which hydrogen peroxide (H_2O_2) acts as a signalling molecule and triggers secondary reactions such as peroxidase activity, Cd contributes to cell wall lignification followed by growth inhibition (Passardi et al., 2004; Rodríguez-Serrano et al., 2006; Sandalio et al., 2001). Lignin is build up by blocks of monolignols, namely *p*-coumaryl alcohol, sinapyl alcohol as well as coniferyl alcohol and a set of different enzymes is required for their biosynthesis (Vanholme et al., 2010) (**Figure 2-1**). In the initial step, phenylalanine (Phe) is converted into cinnamic acid by the activity of phenylalanine ammonia-lyase (PAL), whose activity is enhanced by the gaseous plant hormone ethylene (Hyodo and Yang, 1971). Ethylene is involved in multiple molecular, biological and physiological processes in the plant's life cycle and has a simple biosynthetic pathway (**Figure 2-1**). Exposure to heavy metals affects ethylene biosynthesis as well as signalling as reviewed by Keunen *et al*. (Keunen et al., 2016). In Cd-exposed *Arabidopsis thaliana* plants an increased concentration of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) was found and ethylene responsive genes were upregulated (Schellingen et al., 2014). Furthermore, inhibition of ethylene synthesis results in a decreased PAL activity and a lower lignin content in cucumber roots (Politycka, 1999).

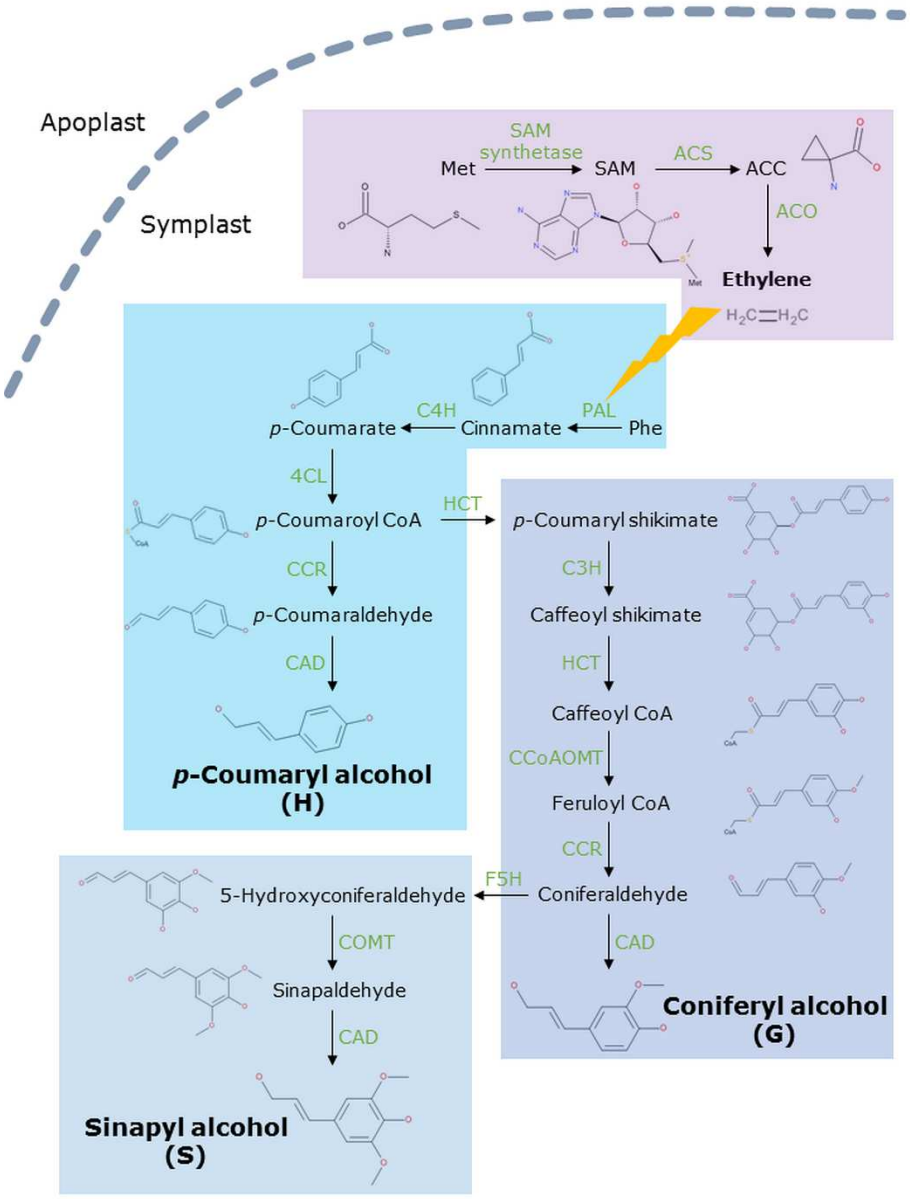

Figure 2-1: Monolignol and ethylene biosynthesis pathways.
Molecules and enzymes of the pathways are indicated. Met methionine; SAM *S*-adenosylmethionine; ACC 1-aminocyclopropane-1-carboxylic acid; ACS ACC-synthase, ACO ACC-oxidase; Phe phenylalanine, PAL phenylalanine ammonia lyase; C4H cinnamate-4-hydroxylase; C3H coumarate 3-hydroxylase; HCT hydroxycinnamoyl transferase; 4CL 4-coumarate ligase; CAD cinnamyl alcohol dehydrogenase; CCoAOMT caffeoyl-CoA 3-*O*-methyltransferase; CCR cinnamoyl CoA reductase; COMT caffeate *O*-methyltransferase; F5H ferulate 5-hydroxylase. Ethylene influences the activity of PAL (indicated as a lightning bold) and thereby affects monolignol biosynthesis.

Medicago sativa is an important forage legume worldwide and its stem tissue is often used in research to study processes taking place at the cell wall (Printz et al., 2016; Verdonk et al., 2012) as it represents more than 50% of the produced biomass and is rich in cell wall material. A previous gel-based study revealed changes in the cell wall proteome of *M. sativa* stems when plants are exposed to Cd (Gutsch et al., 2018) (chapter 1 – part 1). Several proteins involved in cell wall remodelling and carbohydrate metabolism were shown to be altered in their abundance which supports the hypothesis that Cd influences the cell wall structure and underlines its function as a defence barrier against Cd stress.

This study focuses on the cell wall monosaccharide composition and lignin content in *M. sativa* stems, when plants were exposed to a realistic Cd concentration in the soil for an entire season. Based on the hypothesis that the cell wall acts as a defence barrier against Cd, the present study complements a previous gel-based study by using a gel-free approach and different hypotheses on the changed cell wall structure upon Cd exposure are addressed by targeted analysis.

2. Material and Methods

2.1. Plant material and sampling

Medicago sativa L. seeds, cultivar Giulia, were inoculated with *Sinorhizobium meliloti* and sown on Cd-contaminated and control soil. The soil was prepared as one batch composed of 2/3 potting soil mixed with 1/3 sand (w/w). Half of the prepared soil was contaminated with Cd applied as $CdSO_4$ to a final concentration of 10 mg Cd per kg soil dry weight (DW). Plants were sown in May 2015 in 12 times 12 pots for each condition. The plants were kept in the greenhouse until the flowering stage was reached and subsequently cut similar to the agricultural practice. For the re-growing period, the plants were kept outside till the pre-flowering stage was reached followed by one more week in the greenhouse before the final sampling (10^{th} of September 2015). During the entire experiment no fertilizer was applied nor was the day-cycle or temperature controlled. The first and the last two internodes were removed from the stems and only the middle parts were sampled to obtain a more homogeneous sample. Stems were sampled in five biological replicates for each condition, with a pool

of stem material from 24 pots corresponding to one biological replicate. Samples were ground to a fine powder in liquid nitrogen and kept at -80°C till further use.

2.2. Label-free quantitative proteome study

2.2.1. Protein extraction and preparation

Cell wall and soluble protein extraction of five replicates from *M. sativa* stems were done as described before (Printz et al., 2015; Wang et al., 2003).

Digestion of proteins was performed using an Amicon Ultra-4 10K Centrifugal filter device (Millipore) (Abdallah et al., 2012). Cell wall and soluble proteins, 20 µg of each sample, were reduced with 10 mM DTT in 100 mM ammonium bicarbonate (AmBic) for 20 min and subsequently washed with 100 mM AmBic (30 min, 4700 g, 4°C). Reduced samples remained on top of the filter and were alkylated with 50 mM iodoacetamide dissolved in 100 mM AmBic for 30 mins in the dark. After two washing steps, samples were digested with 40 µL trypsin Gold (Promega), 5 ng ml^{-1} trypsin in 20 mM AmBic, at 40°C overnight. Afterwards, 100 µL H_2O was added on the filter, devices were centrifuged (40 min, 4700 g, 4°C) and peptides collected from the bottom of the tube. The peptides were dried under vacuum and solubilized in 40 µL of 5 % acetonitrile (ACN) and 0.01 % trifluoroacetic acid (TFA).

2.2.2. LC-MS/MS peptide separation and analysis

Peptides were analysed with a NanoLC-2D System (Eksigent) coupled to a TripleTOF 5600+ MS (Sciex). After desalting and enrichment on a C18 pre-column (PepMap™ 100, 5 µm, 5 mm x 300 µm I.D.,Thermo Scientific), the peptides were separated with a C18 reverse phase column (PepMap™ 100, 3 µm, 100 Å, 75 µm I.D. × 15 cm, Thermo Scientific) using a linear binary gradient (solvent A: 0.1 % formic acid; solvent B: 80 % ACN, 0.1 % formic acid) at a flow rate of 300 nL min^{-1}. Peptides were eluted from 5 % to 55 % solvent B over 40 min, afterwards eluent B increased to 100 % to wash the column and the column was re-equilibrated. The LC was coupled to the mass spectrometer with a NanoSpray III source. The CID fragmentations for MS/MS spectra acquisitions used the automatically adjusted system of rolling collision energy voltage. A full MS scan was performed (scan range: 300 – 1250 m/z,

accumulation time: 250 ms) and the 20 most intense precursors were selected for fragmentation. The CID spectra were analysed with Mascot-Daemon (version 2.4.2, Matrix Science) by searching against the alfalfa EST database downloaded from the Samuel Roberts Noble website (675750 sequences; 304231702 residues, released on 3rd of November 2015) (O'Rourke et al., 2015) using the following parameters: 2 missed cleavages, mass accuracy precursor: 20 ppm, mass accuracy fragments: ± 0.5 Da, fixed modifications: carbamidomethyl (C), dynamic modifications: Oxidation (M and P), Acetyl (protein N-term), Didehydro (F) and tryptophan to kynurenine. Proteins were considered as identified when at least two peptides passed the MASCOT-calculated score of ≥ 25 and the same peptides were identified in at least 80 % of the replicates. Mascot data were imported in PROGENESIS QI software for proteomics (NonLinear Dynamics) for quantitative analysis. Quantitative results were statistically evaluated by means of a one-way ANOVA *p*-value ($p \leq 0.05$) as well as a fold-change of 1.5 to reveal proteins with a significantly different abundance. In the quantitative analysis only unique peptides were considered. Proteins, for which a significant change was observed, were manually validated to avoid false positive identifications. The subcellular location was determined by the TargetP online tool (Emanuelsson and Nielsen, 2000) using standard parameters. A protein was considered as cell wall-targeted when TargetP predicted a secretion signal peptide or the subcellular localization was found based on literature. Identified cell wall proteins were categorised into functional classes following Duruflé et al. 2017 (Duruflé et al., 2017).

2.3. Real time quantitative PCR (qPCR)

The RNAqueouse™ Kit (Life Technologies) was used for RNA extraction from five biological replicates according to the manufacturer's instructions. The RNA was purified by precipitation with 3 M sodium acetate and 100 % isopropanol. The obtained RNA pellet was washed with 70 % ethanol and resuspended in RNase-free water. The RNA concentration and purity was determined using a NanoDrop® ND-1000 spectrophotometer (Thermo Fischer Scientific) ($A_{260/280}$ and $A_{260/230}$ ratio between 1.9 and 2.5). One µg of the extracted RNA was DNase treated (TURBO DNA-free™ Kit, Life Technologies) and reverse transcribed (PrimeScript™ RT Reagent Kit; Perfect Real Time, TAKARA Bio Inc.). The cDNA

was diluted 10-fold in 1/10 Tris-EDTA buffer (Sigma-Aldrich) and stored at -20°C.

The Alfalfa Gene Index and Expression Atlas Database (O'Rourke et al., 2015) was used to obtain the coding sequences for the genes of interest. Specific primer pairs were designed with the Primer3Plus online tool (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and analysed using the OligoAnalyzer 3.1 (https://eu.idtdna.com/calc/analyzer). Primer pairs for PAL and CAD were taken from literature (Guerriero et al., 2014). The primer efficiency was measured using a dilution series of a pooled sample containing an equal amount of all cDNAs in the experiment (six dilution points) (**Table S2-1**). All qPCR reactions were performed in a 96 well plate with the 7500 Fast Real Time PCR System (Life Technologies). One 10 µL reaction contained 2 µl diluted cDNA (or RNase-free water as "no template"-control), forward and reverse primers (300 nM), Fast SYBR® Green Master Mix (Applied Biosystems) and RNase-free water. The initial denaturation step (20 s at 95°C) was followed by 40 cycles of denaturation (3 s at 95°C), annealing and elongation (30 s at 60°C). All details according to Minimum Information for publication of Quantitative real-time PCR Experiments (Bustin et al., 2009) are shown in **Table S2-2**. At the end of each run, a melting curve was generated to check the specificity of the products. Gene expression was calculated according to the $2^{-\Delta Cq}$ method relative to the sample with the highest expression. Obtained data were normalised using the average $2^{-\Delta Cq}$ values of the three most stable reference genes which were selected by the GrayNorm algorithm (Remans et al., 2014) out of ten tested (Guerriero et al., 2014).

All values were expressed relative to the control samples. The significance was assessed with a *t*-test with *p*-value ≤ 0.05. Normalised relative expression data were clustered (uncentered Pearson correlation, complete linkage) (Eisen et al., 1999) and displayed as a heat map (Saldanha, 2004).

2.4. Isolation of cell wall material

A sufficient amount of powdered deep frozen plant material from five biological replicates was mixed with 40 mL 80 % methanol, sonicated for 10 min and shaken for 4 h at room temperature. The homogenates were subsequently

centrifuged (3700 g) and the pellet washed five times with 80 % ethanol by a vortexing/centrifugation cycle. The isolated cell wall residues (CWRs) were dried (45°C, 24 h) and served as material to analyse lignin, the cell wall composition and for the determination of the PME activity.

2.5. Sequential extraction of monosaccharides from the stem cell wall

Isolated CWRs (90 mg) were incubated in 1.5 mL 0.1 M acetate buffer (pH 5.0) for 20 min at 80°C followed by digestion with 10 µL α-amylase plus 10 µL amyloglucosidase shaken overnight at 37°C to remove the starch from the samples. The reaction was stopped by the addition of 6 mL 100 % cold ethanol. Samples were kept at -20°C for 3 h, subsequently washed in 100 % ethanol three times and dried at room temperature. From the isolated cell wall material, 15 mg were used for the sequential extraction, whereby the supernatant was kept after each extraction step and the remaining pellet was used for the next extraction step (**Figure 2-2**).

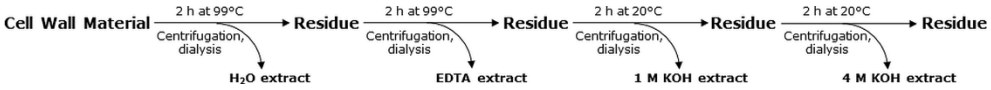

Figure 2-2: Workflow for the sequential extraction of monosaccharides from cell wall material.

The first extraction step was done three times with 1.5 mL water at 99°C, 1000 rpm for 2 h and contained water-soluble polysaccharides. The second extraction was done three times with 0.1 % EDTA (pH 7.5) at 99°C, 1000 rpm for 2 h to solubilise pectins. The third and fourth extractions were done with 1 M potassium hydroxide (KOH) and 4 M KOH respectively with addition of 10 mM sodium borohydride ($NaBH_4$). Both extraction steps were incubated for 2 h at 20°C, 1000 rpm and extracted mainly hemicelluloses. Prior to dialysis, the pH of 1 M and 4 M KOH fractions was neutralized with 75 µL and 300 µL of acetic acid respectively.

Float-A-Lyzer® G2 devices (Spectrum Laboratories) with a molecular weight cut-off of 0.5-1.0 kD were used to dialyze the extracts. The devices were washed with 10 % ethanol and H_2O prior to dialysis according to the manufacturer's

instructions. All dialysis steps were done under constant stirring. The 4 M KOH fraction was first dialyzed against a neutralized 1 M KOH solution (2 h) and subsequently transferred in a neutralized 0.25 M KOH solution together with the 1 M KOH fraction (2 h). Finally, all four fractions were dialyzed overnight against H_2O and freeze-dried (Alpha 1-4 LD plus, Christ). The isolated CWR, the four freeze-dried fractions and the final remaining pellet from the sequential extraction were hydrolysed at 99°C in 500 µL 2 M TFA for 2.5 h. Samples were cooled down on ice, centrifuged (10000 g, 3 min) and the supernatant was used for analysis in two-times and 100-times dilutions.

Quantitative results were obtained by Ionic Chromatography coupled to Pulsed Amperometric Detection (ICS 5000+, Thermo-Dionex). The voltage of the gold electrode was kept at 0.1 V for 0.4 s, reduced to -2 V within 0.02 s, increased to 0.6 V within 0.01 s, reduced to -0.1 V for 0.07 s. The AgCl electrode was used as a reference. Two chromatographic methods were applied to separate all monosaccharides. The eluents were prepared from ultra-pure water and sodium hydroxide (NaOH). For the first method, 5 µL were injected on a CarboPac PA20 column (3x150 mm + guard column 3x30 mm, Thermo-Dionex) at 30°C (0.5 mL min^{-1}). The gradient started with 12 mM NaOH for 10 min, increased to 300 mM within 2 min and was kept at 300 mM for 5 min. For the second method, 1 µL was injected on a CarboPac SA10-4µm column (2x250 mm + guard column 2x50 mm, Thermo-Dionex) at 45°C (0.38 mL min^{-1}). The gradient started with 1 mM NaOH for 5min, increased to 198 mM within 3 min and was kept at these conditions for 2 min.

2.6. PME activity assay

The PME activity assay was done as described before (Zhu et al., 2012). Any methanol that is released by the PME activity is converted into formaldehyde by alcohol oxidase. In a second reaction, formaldehyde forms a complex with purpald, resulting in a purple coloration. A standard curve was generated by using methanol at different concentrations. Ten mg of prepared CWR were suspended in 200 µL 1 M NaCl and shaken for 1 h at 4°C. Samples were subsequently centrifuged (13200 g, 10 min, 4°C) and the supernatant recovered for the PME activity assay. One reaction contained 100 µL pectin in PBS (0.64 mg mL^{-1}), 10 µL alcohol oxidase (0.01 U $µL^{-1}$) and 50 µL PME sample. The

reaction was incubated at 30°C for 10 min, subsequently 200 µL purpald (5 mg mL^{-1} in 0.5 N NaOH) was added and the mixture was incubated for 30 min at 30°C. Finally, 550 µL H_2O was added and the absorption measured at 550 nm. For both conditions, five biological replicates were analysed and measured in two technical replicates.

2.7. Extraction and characterization of lignin in stem cell wall

Five mg of CWR were digested with 2.6 mL of 25 % acetyl bromide in glacial acetic acid for 2 h at 50°C using a HACH LT200 system. Samples were cooled on ice and transferred into 10 mL of 2 M NaOH plus 12 mL glacial acetic acid. The reaction tube was rinsed with glacial acetic acid and 1.75 mL of 0.5 M hydroxylammonium chloride was added. Each volume was adjusted to 30 mL with glacial acetic acid, centrifuged (3000 g, 15 min) and lignin content was measured spectrophotometrically (280 nm, extinction coefficient ε = 22.9 g^{-1} L cm^{-1}).

Lignin was characterized in five biological replicates using the nitrobenzene oxidation method (Billa et al., 1996). Ten mg of CWR were digested with 2 mL 2 M NaOH plus 30 µL nitrobenzene at 165°C for one hour (HACH LT200 system). Samples were cooled on ice and 1.5 mL of the supernatant was collected after centrifugation (4700 g, 10min). Ten µL of vanillin-D3 (10mg mL^{-1} in 1,4-dioxan) were added as recovery standard. Samples were washed five times with ethyl acetate to remove the nitrobenzene. The sample pH was adjusted with 6 N HCl to a pH of 2 - 3 and the oxidation products of lignin were extracted with ethyl acetate. The extracts were cleaned by adding 500 µL of a saturated NaCl solution and dried with Na_2SO_4. The analysis by gas chromatography coupled to mass spectrometry (GC-MS) was performed after trimethylsilylation by the addition of Bis(trimethylsilyl)trifluoroacetamide (BSTFA) to 50 µL of dried extract and derivatization at 60°C for 30min. For the quantitative analysis a HP-5MS column (30 m x 0.25 mm, 0.25 µm, Agilent) was installed on a GC-MS system (7890B-5977A, Agilent). Injection was done at 250°C. The oven program started at 40°C for 5 min, increased to 230°C at 10°C min^{-1}, up to 320°C

(40°C min^{-1}) and hold for 10 min. Salicylic acid-D4 was used as internal standard.

2.8. Determination of compounds of the ethylene biosynthetic pathway

Extraction was done in 500 µL ice-cold 80 % methanol from 50 mg finely ground plant material. For quantification, D4-ACC (250 pmol, Olchemim Ltd.) and D3-Methionine (1000 pmol, Sigmar-Aldrich) was added. Half a milligram of OASIS HLB 0.3 µm solid phase bulk packing material (WATERS) was added to bind pigments. The packing material and cell debris were removed with centrifugation (14000 g, 4°C, 10 min). The ACC metabolites and precursors were analysed with ES^+ UPLC-MS/MS (ACQUITY TQD, WATERS) using a Waters Column ACQUITY UPLCr BEH Amide 1.7 µm Column. The eluents were 0.1 % formic acid in H_2O (A) and 0.1 % formic acid in ACN (B). The gradient was as following: gradient 0-2 min: 15.0 % A, 85.0 % B; 2-5.8 min: linear gradient to 35.0 % (A), 65.0 % B; 5.8-6.4 min: linear gradient to 80.0 % A, 20.0 % B; isocratic at 80.0 % A, 20.0 % B until 7 min. The flow rate was 0.4 mL min^{-1}. The partial loop injection mode was used with an injection volume of 6 µL. The specific transitions selected for multiple reaction monitoring (dwell time 0.034 sec. for each transition) were: 106.10 > 60.20 (cone: 14.0, collision energy 10.0) and 106.10 > 88.00 (cone 14.0, collision 8.0) for D4-ACC; 150.00 > 104.00 (cone 15.0, collision 15.0) for methionine; 153.00 > 107.00 (cone 14.0, collision 10.0) for D3-Methionine; 189.00 > 130.00 (cone 16.0, collision 12.0) for malonyl-ACC; 232.00 > 148.00 (cone 16.0, collision 12.0) for glutamyl-ACC; 295.00 > 148.00 (cone 16.0, collision 12.0) for jasmonyl-ACC and 399.40 > 250.00 (cone 16.0, collision 15.0) for SAM. The quantity of ACC was analysed by CI^- GC-MS/MS after derivatization using pentafluorobenzyl bromide following (Smets et al., 2003). Data are expressed in picomoles per gram fresh weight (pmol g^{-1} FW).

3. Results

3.1. Label-free quantitative proteome analysis

A LC-MS based quantitative protein analysis was carried out on cell wall proteins, extracted in three fractions, and soluble proteins. The used targeted approach results in a cell wall protein-enrichment in which contamination with cytosolic proteins is low. However, proteins involved in photosynthesis are found throughout the three cell wall protein fractions. Cadmium interferes with photosynthetic activity and a consistent impact on the abundance of chloroplastic proteins appeared in our data. Therefore, proteins involved in photosynthesis will be included in the results and discussion.

A total number of 166 significantly changed proteins were identified in the three cell wall fractions. Those proteins were categorized as proposed in (Duruflé et al., 2017) but keeping defence proteins and proteins involved in photosynthesis in separate categories (**Table S2-3**). Clearly more proteins were of higher abundance in response to Cd (116 proteins versus 50 proteins being of less abundance) (**Figure 2-3**).

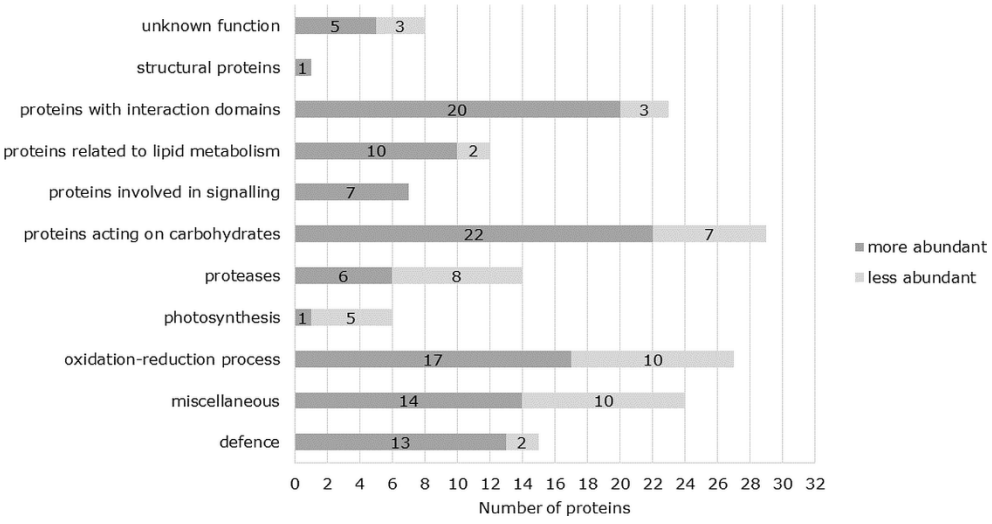

Figure 2-3: Functional classification of proteins from all three fractions ($CaCl_2$, EGTA, LiCl) that changed significantly in *M. sativa* stems exposed to Cd.
Quantification was done using the PROGENESIS QI software for proteomics (NonLinear Dynamics).

Looking at each cell wall protein fraction separately (**Table S2-3**), the highest number of proteins that changed significantly was found in the EGTA and LiCl fractions (65 and 68 respectively). While only five proteins were of less abundance due to Cd exposure in the EGTA fraction, 27 proteins in the LiCl fraction had a decreased abundance after Cd exposure. In the $CaCl_2$ fraction, 33 proteins showed a significant abundance change, of which 15 were more abundant. Although the used extraction approach is targeting cell wall protein, obtained extracts are an enrichment of cell wall proteins in which the contamination with proteins of other subcellular localisations is low. Among the proteins that changed significantly, the percentage of secreted, cell wall localized proteins was high in all three fractions: $CaCl_2$ fraction 66.67 %, EGTA 81.46 % and LiCl 77.94 %.

Strikingly, a high number of proteins that have an increased abundance after Cd exposure have a designated function in plant defence. These include chitinases and chitin-binding proteins (grouped as carbohydrate binding), pathogenesis-related thaumatin family proteins, allergen Pru proteins and Kunitz type trypsin inhibitor proteins (grouped as proteins with interaction domain). Only two defence-related proteins identified in the LiCl fraction were of lower abundance (*M. truncatula* homolog of drug resistance transporter-like ABC domain protein and LRR and NB-ARC domain disease resistance protein). Different peroxidases were identified in the category oxidation-reduction process and a higher abundance in response to Cd exposure was determined in the EGTA and LiCl fraction. Exceptionally, a lower abundance of peroxidase class III (contig 34984) was found in the LiCl fraction, as was determined likewise in the $CaCl_2$ fraction. Clustering of the identified peroxidases (Clustal Omega) and sequence comparison did not reveal a separation and no specific function was found for those peroxidases that are of lower abundance. Other proteins of the oxidation-reduction process which were less abundant are ferritin, multi-copper oxidase-like protein, basic blue-like protein, L-ascorbate oxidase, plastocyanin-like domain protein and early nodulin-like protein and were identified in the $CaCl_2$ and LiCl fractions. Most of the proteins classified as proteins acting on carbohydrates are involved in the structure of the plant cell wall. Most prevalent, glucan endo-1,3-beta-glucosidase was identified in all three fractions and

showed an increased abundance upon Cd exposure. Furthermore, alpha-galactosidase-like protein and expansin A10 in the $CaCl_2$ fraction and glycoside hydrolase family 18 protein and pectinacetylesterase family protein in the EGTA fraction had an increased abundance. Other proteins of higher abundance which are known to be involved in the assembly of the cell wall are dirigent-like proteins (categorized as miscellaneous), polygalacturonase inhibiting protein 1, pectinesterase/pectinesterase inhibitor, xyloglucanase-specific endoglucanase inhibitor protein (all categorized as proteins with interaction domains), extensin-like proteins (structural protein) and FASCICLIN-like arabinogalactan-proteins (categorized as involved in signalling). Most of them were found in the EGTA and LiCl fractions. In the latter fraction, several cell wall organizing proteins were also found to be of less abundance such as beta-galactosidase-like protein and polygalacturonase non-catalytic protein, but also proteins which are of higher abundance were identified such as alpha-glucosidase, pectinacetylesterase family protein, expansins and pectinesterase/pectinesterase inhibitor. Additionally, polygalacturonase inhibitor protein in the $CaCl_2$ fraction (contig 93293) was also identified to be less abundant.

In the soluble protein fraction a total number of 28 significantly changed proteins were identified of which 25 were more abundant and three proteins had a decreased abundance (see **Table S2-3** for all identifications). Of those proteins, 57.14 % had no predicted target site and can be considered as cytosolic. Six proteins were predicted to be secreted and another six proteins were chloroplastic. The majority of the identified proteins are miscellaneous, small proteins involved in translation, nucleotide binding or protein folding. Furthermore, a rhicadhesin receptor, a glutamine synthase and a sieve element occlusion protein were identified. Those proteins were increased due to Cd exposure and only a 60S ribosomal L4-like protein (contig 101331) was found to be less abundant. More abundant proteins are categorized as acting on carbohydrates (fructose-bisphosphate aldolase, glycoside hydrolase family 1 protein, glucosamine-6-phosphate isomerase/6-phosphogluconolactonase), defence (pathogenesis-related thaumatin family protein), oxidation-reduction process (nodulin-like protein, peroxidases), photosynthesis (thylakoid rhodanese-like) and proteases (eukaryotic aspartyl protease). In addition, a

peroxisomal NAD-malate dehydrogenase 2 and photosystem II subunit Q-2 were of less abundance due to Cd exposure.

3.2. Gene expression analysis

The expression of genes involved in monolignol, pectin and ethylene syntheses was investigated in *M. sativa* stems to reveal the impact of Cd exposure (see **Figure 2-1** for abbreviations of gene names). Five biological replicates were used in this experiment and relative normalised expression values were calculated as means with the standard error of the means (SEM) (**Table 2-1**). A heat map hierarchical clustering shows the expression of all genes and replicates assessed by qPCR (**Figure 2-4**). Two patterns of gene expression were observed. In the upper branch, a higher gene expression due to Cd exposure was measured for *ACS7*, *ACS1*, *ACO5*, *ERF1* and *ETR2*. With the exception of *ACS7*, those changes of expression are significant (**Table 2-1**). Furthermore, *4CL*, *C4H* and *COMT* are found in that upper branch but the expression difference between control plant and Cd-exposed plants is low. While *4CL* and *C4H* change significantly, expression of *COMT* does not (**Table 2-1**). Genes, which cluster in the lower branch have a lower expression in Cd-exposed plants but in general the expression values show a high variation between replicates in the same condition (**Figure 2-4**). Only the expression change of *ACO1* and *ACO4* is significant (**Table 2-1**). In a second group of the lower branch cluster *CAD*, *PAL* and *GAUT1*. The latter is a 1,4-galacturonyltransferase that synthesises HG (Sterling et al., 2006). While *GAUT1* is significantly higher expressed in Cd-exposed plants, great variations between replicates can be seen for *CAD* and *PAL*. Nevertheless, mean expression values of the three genes indicate a higher expression in response to Cd exposure (**Table 2-1**).

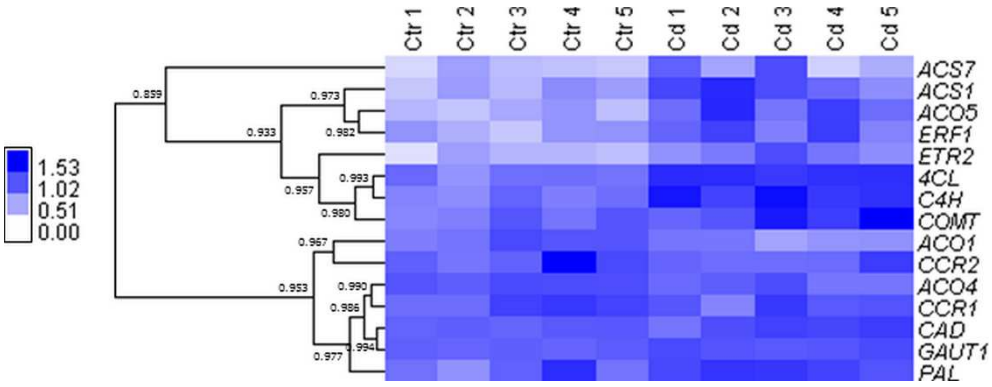

Figure 2-4: Heat map hierarchical clustering showing the expression of genes assessed by qPCR.
Values represent relative normalised expression values. The colour bar indicates the expression values as an increasing intensity gradient. Numbers refer to the Pearson correlation coefficient between the lengths of two branches. *ACS* (1-aminocyclopropane-1-carboxylic acid synthase), *ACO* (1-aminocyclopropane-1-carboxylic acid oxidase), *ERF* (ethylene responsive factor), *ETR* (ethylene-resistant), *4CL* (4-coumarate ligase), *C4H* (cinnamate-4-hydroxylase), *COMT* (caffeate O-methyltransferase), *CCR* (cinnamoyl-CoA reductase), *CAD* (cinnamyl alcohol dehydrogenase), *PAL* (phenylalanine ammonia lyase).

Table 2-1: Relative normalized gene expression in *M. sativa* stems.

gene annotation	contig_ID *M. sativa*	rel. norm expression (SEM)	*p*-value
Monolignol pathway			
PAL	(Guerriero et al., 2014)	1.269 (0.039)	0.241
CAD	(Guerriero et al., 2014)	1.091 (0.063)	0.241
C4H	1927	1.611 (0.079)	0.032
4CL	100732	1.525 (0.019)	0.008
COMT	95660	1.375 (0.133)	0.241
CCR1	66309	0.947 (0.071)	0.691
CCR2	16287	0.871 (0.051)	0.481
Pectin biosynthesis			
GAUT1	67060	1.101 (0.019)	0.002
Ethylene pathway			
ACS1	19914	1.930 (0.198)	0.004
ACS7	20122	1.749 (0.398)	0.115
ACO1	17355	0.733 (0.058)	0.015
ACO5	11971	2.182 (0.201)	$8.762*10^{-4}$
ACO4	7336	0.880 (0.045)	0.043
ETR2	62168	2.005 (0.186)	0.003
ERF1	35744	1.734 (0.164)	0.006

Normalised expression values are expressed relative to the control set at 1.00. Values are given as an average of five replicates with the standard error of the mean (SEM). A *t*-test was done to determine the significance ($p \leq 0.05$). Green: significantly upregulated by Cd; red: significantly downregulated by Cd.

3.3. Monosaccharide composition in the stem cell wall after long-term exposure to Cd

The total sugar composition of the cell wall from *M. sativa* stems was determined after TFA hydrolysis (**Figure 2-5**). This analysis excludes crystalline cellulose as it is TFA resistant. The most prominent monosaccharide was by far

xylose (Xyl), which represents almost 50 % in Cd-exposed (48.34 %) and control plants (47.46 %). The next high abundant monosaccharides were, in decreasing order, arabinose (Ara), galacturonic acid (GalA) and galactose (Gal). The lowest abundance was determined for fucose (Fuc), rhamnose (Rha), glucose (Glc), mannose (Man), and glucuronic acid (GlcA). No difference in the global monosaccharide composition between Cd-exposed and control plants was observed.

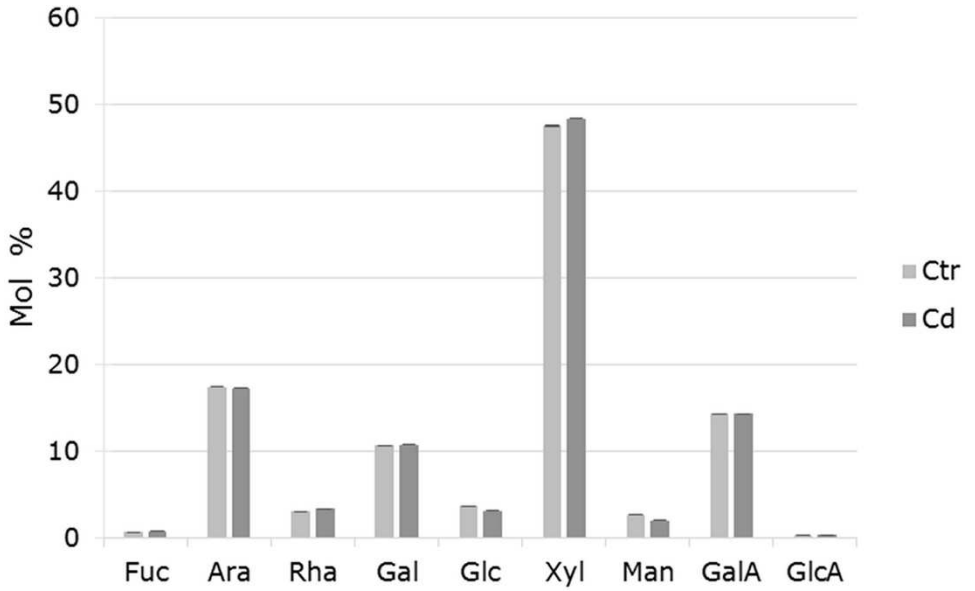

Figure 2-5: Monosaccharide composition of total cell wall material of stems from Cd-exposed and control *M. sativa* plants.
Given values are the average of five biological replicates. Error bars indicate the SEM.

The prepared total cell walls from Cd-exposed and control *M. sativa* stems were fractionated by sequential extraction (**Figure 2-2**). At the end of the sequential extraction, a final cell wall pellet (CWP) remained, which was also hydrolysed and the monosaccharide composition was analysed (**Table 2-2**).

Table 2-2: Monosaccharide composition of the different sequential extracts of *M. sativa* stems.
Values are given as an average from five biological replicates ± standart error. Significant changes were determined with a *t*-test (*p*-value ≤ 0.05) and are indicated with an asterisk.

		Average mol percentage (mol %)								
fraction		Fuc	Ara	Rha	Gal	Glc	Xyl	Man	GalA	GlcA
H_2O	Ctr	0.45 ±2.74^{-4}	36.63 ±1.46^{-2}	1.96 ±1.16^{-3}*	22.49 ±4.22^{-3}	12.30 ±7.37^{-3}	4.07 ±3.01^{-3}*	7.75 ±7.06^{-3}	13.70 ±1.2^{-2}	0.67 ±9.04^{-4}
	Cd	0.50 ±1.87^{-4}	34.56 ±8.14^{-3}	2.53 ±1.69^{-3}*	23.55 ±4.86^{-3}	10.54 ±3.06^{-3}	5.21 ±3.21^{-3}*	7.99 ±3.95^{-3}	14.61 ±7.07^{-3}	0.51 ±6.16^{-4}
EDTA	Ctr	0.81 ±2.13^{-4}	52.81 ±1.48^{-2}*	3.65 ±1.34^{-3}*	15.94 ±2.66^{-3}	1.96 ±7.97^{-4}*	4.77 ±2.91^{-3}*	4.16 ±1.38^{-3}	15.37 ±1.18^{-2}*	0.52 ±6.4^{-4}
	Cd	0.77 ±2.47^{-4}	44.67 ±6.82^{-3}*	4.26 ±2.2^{-3}*	15.77 ±3.23^{-3}	1.68 ±4.82^{-4}*	6.61 ±1.02^{-3}*	4.27 ±1.69^{-3}	21.30 ±6.99^{-3}*	0.68 ±1.71^{-3}
1M KOH	Ctr	0.23 ±2.29^{-4}	4.21 ±4.25^{-3}	1.18 ±1.18^{-3}	2.32 ±2.64^{-3}	2.37 ±2.95^{-3}	73.11 ±9.95^{-3}	1.10 ±8.91^{-4}	15.29 ±2.5^{-3}	0.20 ±9.69^{-5}
	Cd	0.25 ±3.2^{-4}	4.28 ±5.36^{-3}	1.49 ±2.05^{-3}	2.63 ±3.54^{-3}	2.05 ±2.61^{-3}	71.36 ±1.67^{-2}	1.69 ±4.05^{-3}	16.04 ±8.22^{-3}	0.21 ±3.74^{-4}
4M KOH	Ctr	1.76 ±7.38^{-3}	6.23 ±2.63^{-2}	0.44 ±1.53^{-3}*	5.60 ±5.12^{-3}*	10.74 ±6.21^{-2}	53.50 ±2.56^{-2}	11.67 ±3.92^{-2}	9.41 ±1.93^{-2}	0.65 ±5.6^{-3}
	Cd	1.65 ±3.41^{-4}	6.21 ±1.25^{-3}	0.70 ±7.72^{-4}*	6.30 ±5.68^{-4}*	15.01 ±7.45^{-3}	49.81 ±2.11^{-2}	11.78 ±2.09^{-2}	7.72 ±5.24^{-3}	0.82 ±2.15^{-3}
CWP	Ctr	0.54 ±1.58^{-4}	11.93 ±3.18^{-3}*	1.65 ±5.74^{-4}*	13.63 ±2.97^{-3}	20.50 ±3.78^{-3}	36.52 ±7.1^{-3}	3.62 ±1.06^{-3}*	11.17 ±2.17^{-3}	0.43 ±2.8^{-4}
	Cd	0.60 ±4.26^{-4}	17.00 ±1.76^{-2}*	2.14 ±1.18^{-3}*	14.54 ±1.44^{-2}	20.72 ±2.08^{-2}	30.58 ±4.8^{-2}	2.84 ±1.86^{-3}*	11.22 ±5.11^{-3}	0.37 ±5.98^{-4}

Ctr control, Fuc fucose, Ara arabinose, Rha rhamnose, Gal galactose, Glc glucose, Xyl xylose, Man mannose, GalA galacturonic acid, GlcA glucuronic acid; CWP cell wall pellet.

Almost all analysed monosaccharides were present in the H_2O fraction, mostly Ara, followed by Gal, GalA, Glc, Man, Xyl, Rha and traces of GlcA and Fuc. Slight significant differences between the cell walls of Cd-exposed and control plants appeared only for Rha and Xyl. Ara was the most abundant monomer in the EDTA fraction and of significantly lower abundance in Cd-exposed stem cell wall. Also Glc was of lower abundance. Furthermore a significant difference was observed for Rha, Xyl and GalA, which increased in Cd-exposed stems. While GalA represents the pectin backbone, Ara, Gal and Xyl are mainly components of the side chains. A slight shift in the side chain residues from Ara to Xyl in response to Cd-exposure was observed. The extracted amount of Ara significantly decreased about 8.14 % and Xyl slightly increased. In the KOH fractions, Xyl was found to be the most abundant. The 1 M KOH fraction contained mainly Xyl (more than 70 % in both conditions), followed by GalA. But no differences between Cd-exposed and control *M. sativa* plants appeared. Besides the great portion of Xyl, the 4 M KOH fraction contained all other analysed monosaccharides, to the least proportion Rha and GlcA. Some differences in the presence of monosaccharides (Gal, Glc, Xyl, GalA) appeared between exposed and non-exposed plants, of which only Gal significantly changed. Furthermore, a slight variation was detected for Rha which is significant. The final pellet which remained at the end of the sequential extraction was also hydrolysed with TFA. This fraction still contained high amounts of Xyl, Glc, Gal, Ara and GalA. Thereby, a significantly higher proportion of Ara and Rha was present in the remaining pellet of Cd-exposed plants. On the other hand, Man significantly decreased in Cd-exposed plants.

3.4. PME activity

The PME activity was assessed in five biological replicates of which each was measured in two technical replicates. Cadmium exposure significantly increased PME activity as was monitored by means of the methanol produced. Extracts from Cd-exposed plants resulted in the release of 48.05 % more methanol (**Figure 2-6**).

3.5. Analysis of lignification in stem after long-term exposure to Cd

The total lignin content was determined spectrophotometrically and compared between stems of Cd-exposed plants and plants grown on unpolluted soil after long-term exposure. No difference in the lignin concentration appeared. The composition of lignin in the CWR was assessed after nitrobenzene oxidation and covered the main lignin degradation products *p*-hydroxybenzaldehyde (H), vanillin (V) and syringaldehyde (S). The monolignol composition did not change between conditions. In fact, a high variability in the monomeric lignin composition was observed between replicates (**Table 2-3**).

Table 2-3: Lignin content and monomer composition of *M. sativa* stems.

	Lignin (% CWR)	**H (µmol g^{-1} CWR)**	**V (µmol g^{-1} CWR)**	**S (µmol g^{-1} CWR)**
Ctr	4.68	10.95 (3.01)	527.50 (275.10)	336.18 (145.77)
Cd	4.70	14.57 (2.61)	545.60 (172.55)	338.48 (103.22)

Given values are the average of five replicates with the standard error of the mean (SEM) in parentheses. Ctr control, CWR cell wall residues, H *p*-hydroxybenzaldehyde, V vanillin, S syringaldehyde.

3.6. Quantification of ethylene precursor molecules in response to long-term Cd exposure

To evaluate the effects of Cd on ethylene synthesis, concentrations of ACC and its conjugates were determined in Cd-exposed and unexposed plants (**Figure 2-7**). As the immediate precursor, the presence of ACC determines a rate-limiting step in ethylene biosynthesis and eventually influences the hormone concentration (Yang and Hoffman, 1984). Long-term Cd exposure did not affect the ACC content significantly but an enormous increase of conjugated ACC was observed (about 31.15 %), which is however not significant. Furthermore, *S*-adenosylmethionine (SAM) and methionine content were significantly increased about 46.39 % and 75.31 % respectively upon Cd exposure (**Figure 2-7**).

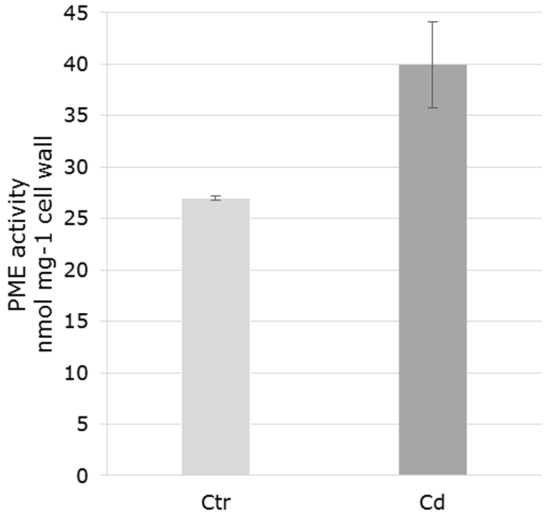

Figure 2-6: Effect of long-term Cd-exposure on pectin methylesterase (PME) activity in the cell wall of *M. sativa* stems.
Activity values represent mean values from five replicates, each measured in technical replicate. SEM is represented as error bars.

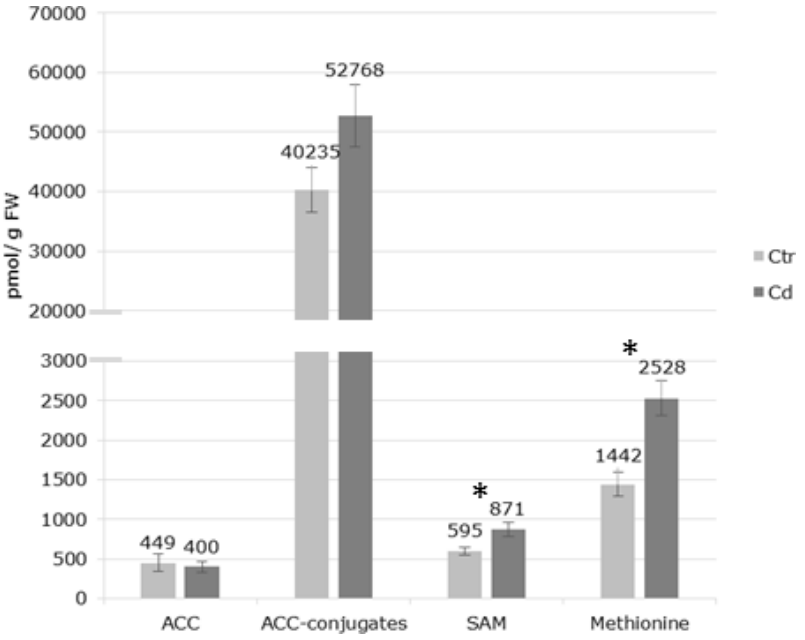

Figure 2-7: Content of precursor molecules of ethylene biosynthesis (pmol g^{-1} FW) in stems of *M. sativa* after long-term Cd exposure.
Data are given as means from five biological replicates. Error bars indicate the SEM. Significance was determined with a t-test ($p \leq 0.05$) and is indicated with an asterisk.

4. Discussion

For the current study, *M. sativa* plants were grown on Cd-contaminated soil (10 mg kg^{-1} soil DW) for an entire season (May till September). As it is agricultural practice, plants were cut and the secondary stem was sampled after a re-growing stage. Although strong growth inhibition occurred in the juvenile plant stage, no difference in biomass between control and Cd-exposed plants was measured by the time of sampling (Gutsch et al., 2018) (chapter 1 – part 1). The present study investigates how Cd exposure changes the composition and structure of the cell wall. Previous data which were based on a gel-based approach (Gutsch et al., 2018) (chapter 1 – part 1) are complemented with a gel-free approach using the same samples and different hypotheses on the changed cell wall structure upon Cd exposure are addressed by targeted analyses.

To target cell wall proteins, extraction buffers with increasing ionic strength were used successively to extract step by step also tightly bound proteins. Several proteins, which were found of higher abundance in the $CaCl_2$ and EGTA fraction, appeared in the LiCl fraction to be of lower abundance (**Table S2-3**). The LiCl buffer has the highest ionic strength and therefore extracts the more tightly bound cell wall proteins. Structural alterations in the cell wall might influence cell wall - protein interactions, thereby causing shifts of a protein in between the different fractions. This type of shifts in affinity due to changes in the cell wall can influence the interpretation during quantification. This observation could be a first indication for structural changes that appear in the cell wall in response to Cd. Changes in the composition and properties of cell wall polysaccharides can support the function of the cell wall as a physical barrier against Cd, thereby protecting the plant from severe Cd-induced damages. The possibility that the cell wall builds up a protective barrier against Cd is further discussed in the following paragraphs.

However, as was observed in a gel-based study on *M. sativa* stems (Gutsch et al., 2018) (chapter 1 – part 1), long-term Cd exposure led to a higher abundance of defence-related proteins such as different chitinases, pathogenesis-related thaumatin family proteins as well was glucan endo-1,3-

beta-glucosidase (**Table S2-3**) and reflect the general stress response of the plant which appears to be rather unspecific.

4.1. The effect of long-term Cd exposure on the cell wall composition in *M. sativa* stems

The plant cell wall is composed of cellulose as the main load-bearing polysaccharide, hemicelluloses and pectins. Cell wall proteins are embedded in this polysaccharide network, representing about 10 % of the cell wall. These proteins can act on the cell wall structure and customise the cell wall properties during development and environmental stresses.

In the total extraction of monosaccharides from isolated cell wall material of *M. sativa* stems, no significant differences between control and Cd-exposed plants appeared (**Figure 2-5**). Overall, under both growing conditions the main monosaccharide is Xyl. Already in 1987 Xyl was found to be the most prominent in mature *M. sativa* stems, enhancing their indigestibility by cross-linking with lignin (Albrecht et al., 1987).

The cell wall polysaccharides were furthermore sequentially extracted with H_2O, EDTA and KOH (1 M followed by an extraction with 4 M). Overall, results are comparable with those previously obtained from *M sativa* stems of the same developmental stage (Hatfield, 1992).

Pectic polysaccharides were extracted with the chelating agent EDTA. Galacturonic acid, the building block for HG, was the main pectic residue while glucuronic acid was present only in trace amounts (**Table 2-2**). A significant increase of GalA of about 38.58 % was found in response to Cd exposure. Together with an slight increased transcript level of *GAUT1*, a higher content of HG in the cell wall of Cd-exposed *M. sativa* can be assumed as GAUT1 catalyses the synthesis of HG (Sterling et al., 2006). In addition, an increased protein abundance of PME (**Table S2-3**) was determined. Pectin methylesterase cleaves off methylester groups from HG and PME activity decreases the ratio between high and low methylesterified pectin, therefore making metal-binding sites available and provoking mechanisms of metal sequestration (Zhu et al., 2012). Its activity is tightly regulated (Micheli, 2001). Here, a significant 48.04 %

higher PME activity was determined in stems of Cd-exposed plants (**Figure 2-6**), indicating an increased presence of demethylated HG. Demethylated HG can form complexes mediated by the binding of Ca (Caffall and Mohnen, 2009). Cadmium can displace Ca due to a higher affinity, thereby immobilizing Cd ions in the cell wall, preventing further entry into the plant (Krzesłowska, 2011). A change in the HG pattern and enhanced PME activity can thus be a way for *M. sativa* plant to respond to Cd exposure (Douchiche et al., 2010b; Paynel et al., 2009).

The major monosaccharide found in the EDTA fraction was Ara, followed by Gal, Xyl, Rha and Man in a decreasing order. The presence of Ara, Rha and Gal in the EGTA fraction indicates that RG is present in this fraction (Mc Neil et al., 1980). After Cu exposure, a decrease in Ara was recently observed in the cell walls of *Scopelophila cataractae* (Konno et al., 2010) and was also seen in the present study upon Cd-exposure. Simultaneously, an increased presence of Xyl was reported (**Table 2-2**), pointing to a shift in the side chains present in the cell wall matrix of Cd-exposed plants towards enhanced cell wall stiffening in stems of *M. sativa* (Willats et al., 1999). The co-extraction of Xyl derives from a covalent linkage of xyloglucan to pectin, most likely as side chains on RG (Popper and Fry, 2008). Side chains are known to be much more flexible than the HG components of pectic polysaccharides and therefore contribute to regulations of the porosity of the cell wall matrix in Cd-exposed plants. The nature of the attached side chains probably influence the accessibility of proteins acting on the cell wall (e.g. polygalacturonase) to their interaction sites (Dellapenna et al., 1990). The binding to pectin supports the interaction of xyloglucan with the cell wall components. Pectin-xyloglucan linkage is of structural importance by building up a strong three dimensional complex which limits enzyme accessibility. Furthermore, pectic moieties in such a complex have the capacity to cross-link within the cell wall, mediated by Ca-bridging (Morris et al., 1982). Cross-linkage of xyloglucan and strengthening of the cell wall matrix in response to Cd-exposure is further promoted by an increased abundance of xyloglucanase-specific endoglucanase inhibitor protein (**Table S2-3**), preventing cell wall loosening by constraining the cleavage of xyloglucan chains (Fry et al., 1992). The here made observations strongly support stiffening of the cell wall

upon long-term Cd-exposure derived from a change in the composition of the side chains present in the cell wall matrix towards a high degree of pectin-xyloglucan linkage in the cell wall of *M. sativa* stems.

However, different glycosyl hydrolase family proteins were of higher abundance (**Table S2-3**). These proteins support cell wall deconstruction, loosening and growth (Gilbert, 2010; Minic, 2008). Apparently, the growth during stressful conditions is a conflict between stiffening by cross-linking and loosening of the cell wall. While ROS and peroxidases support stiffening by cross-linkage of hemicellulose with lignin, expansins and glycosyl hydrolases support loosening (Tenhaken, 2015).

Most hemicelluloses were extracted with KOH of different concentrations. The most prevalent monosaccharide was xylose. Also present in both KOH fraction was GalA, which supports the hypothesis of the cross-linkage between pectin and xyloglucan (Popper and Fry, 2008). Other investigated monosaccharides were present only in a minor quantity. No significant quantitative changes were reported in the 1 M KOH fraction, while a slight but significant change appeared for Rha and Gal in the 4 M KOH fraction.

The cell wall composition and remodelling is involved in the responses to abiotic stress (Gall et al., 2015; Tenhaken, 2015). The here presented cell wall protein data reveal several proteins which significantly changed upon long-term Cd exposure with a function in cell wall structure. Those findings are in agreement with a previous study (Gutsch et al., 2018) (chapter 1 – part 1). Analysing the cell wall composition, the most significant changes appeared in the pectin fraction, where an increased abundance of HG was reported in Cd-exposed plants. A co-occurring higher abundance of PME and increased activity indicate that those HG molecules are demethylated and thus can bind Cd. Immobilizing Cd in the cell wall prevents its further distribution in the plant. These findings strongly support the hypothesis of the cell wall being a protective barrier against Cd exposure in *M. sativa* stems.

4.2. Long-term Cd exposure affects ethylene biosynthesis but not lignification and monolignol composition

Ethylene is a gaseous hormone which is involved in the response to multiple biotic and abiotic stresses (Ecker and Davis, 1987; Keunen et al., 2016; Lin et al., 2009) and its levels increased after metal exposure in different plants (Groppa et al., 2003; Schellingen et al., 2014). In the current study, long-term Cd exposure led to increasing content of methionine and SAM. Next, SAM is converted to ACC by the activity of ACC synthase (ACS). The transcript levels of both investigated gene isoforms *ACS1* and *ACS7* were upregulated in stems of Cd-exposed *M. sativa* plants, suggesting an enhanced production of ACC, which is the rate-limiting step in ethylene biosynthesis (Yang and Hoffman, 1984). While ACC content slightly decreased upon Cd exposure, the abundance of its conjugated forms increased, although this change is insignificant (p=0.086). The three known conjugates of ACC are γ-glutamyl-ACC (GACC), jasmonyl-ACC (JA-ACC) and malonyl-ACC (MACC) and conjugation might be a biochemical regulation of available ACC which affects ethylene levels (Van de Poel and Van Der Straeten, 2014). The synthesis of MACC positively correlates with ethylene production, suggesting that a self-regulated feedback control of ethylene synthesis is active which would stimulate the storage of ACC (Martin and Saftner, 1995). Possibly MACC can be converted back to ACC thereby stimulating the ethylene production (Van de Poel et al., 2014).

In a final step, ACC is oxidised to ethylene by ACC oxidase (ACO) and in the current study the decrease of ACC in Cd-exposed plants was accompanied by an upregulation of *ACO5* transcript levels and of the ethylene responsive genes *ETR2* and *ERF1*. Therefore, free ACC gets converted to ethylene, enhancing ethylene signalling in response to long-term Cd exposure.

Besides other mechanism (Keunen et al., 2016), ethylene positively affects the activity of PAL (Hyodo and Yang, 1971; Politycka, 1999), thereby augmenting the lignin content (Cass et al., 2015). The PAL enzyme catalyses the conversion of Phe to cinnamate, making the entry into monolignol biosynthesis. Those monolignols are the building blocks for lignin biosynthesis. The impact of Cd on

cell wall lignification and plant growth is well established (Elobeid et al., 2012; Finger-Teixeira et al., 2010; Rahoui et al., 2017) and changes in the lignin composition in the context of abiotic stress were observed (Moura et al., 2010). Nevertheless, after long-term Cd-exposure of *M. sativa* plants, lignin content of the cell wall and transcript level of *PAL* did not significantly change. Furthermore, the monolignol composition was not affected after long-term Cd exposure either (**Table 2-3**). Yet, a significantly changed accumulation of *C4H* and *4CL* transcripts were detected; both encoding enzymes of the monolignol biosynthetic pathway (**Table 2-1**). Cadmium-induced stimulation of PAL activity was shown to be dose and time dependent in roots of *Matricaria chamomilla*, which also applies for the content of soluble phenolics and their accumulation (Kováčik and Klejdus, 2008). In the current study, the applied Cd concentration was kept low to mimic realistic soil concentrations and could have been too low to significantly affect lignin biosynthesis and content, although the ethylene biosynthesis was induced accompanied by a variation of *PAL* transcript levels.

Lignification is driven by peroxidase activity. Quantitative LC-MS revealed an increased abundance of different peroxidase isoforms due to Cd-exposure as observed before (Gutsch et al., 2018) (chapter 1 – part 1). Peroxidases and their activity increase upon Cd exposure (Radotic et al., 2000) and therefore lead to increased lignification (Rahoui et al., 2017). For lignin formation, peroxidases require H_2O_2 as a substrate molecule. Furthermore, Cd induces oxidative stress in plant cells and enhances the generation of H_2O_2 (Cuypers et al., 2011). However, no difference in the lignification between stems of control and Cd-exposed plants was detected in the current study. Probably those peroxidases are more involved in H_2O_2 scavenging during long-term mild Cd-exposure instead of altering the lignin content (Sečenji et al., 2010; Tenhaken, 2015). During LC-MS analysis, a decreased abundance of some peroxidase isoforms was observed. After clustering all identified isoforms in a phylogenetic tree, no separation of the less abundant isoforms from the more abundant isoforms appeared (data not shown). Thus, the observed decrease of the identified peptides might be due to degradation fragments as Cd also induces proteolysis which is underlined by an increased abundance of proteases monitored in this study (**Table S2-3**).

5. Conclusion

Long-term Cd exposure led to an adaptation of *M. sativa* to the applied stress. Phenotypically, no difference was observed between Cd-exposed and control plants in their mature growing stage, which is reflected by a similar biomasses. During long-term Cd exposure the cell wall of *M. sativa* stems undergoes compositional changes in pectic polysaccharides. The increased abundance and activity of PME promotes demethylesterification of HG. Negatively charged HG molecules have a high affinity to bind Cd within their network and immobilise the metal ion in the cell wall. Furthermore, changes in the attached side chains in favour of Xyl influence the mechanical properties of the cell wall matrix. A higher abundance of xyloglucan promotes cross-linking and limits accessibility of cell wall-modifying enzymes. These observations confirm that the cell wall changes its structure driven by Cd stress and acts as a physical defence barrier. Although peroxidases are highly abundant in Cd-exposed plants, no increase in lignification was reported. Here, peroxidases are most likely accumulating as a response to Cd-induced oxidative stress in order to maintain the redox balance in *M. sativa*. The activity of PAL, making the entry into the monolignol synthesis, is not only induced by ethylene but further depends on substrate availability (Da Cunha, 1987) and the Phe content is a key-molecule in the process of cell wall lignification and cell wall stiffening. Understanding, how it is influenced by long-term Cd exposure in *M. sativa* stems would be valuable data to study the plant response to the applied stress.

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Highlights and Perspective Chapter 2

As previously shown in chapter 1, long-term Cd exposure influences the cell wall proteome of *M. sativa* stems, thereby changing the abundance of proteins involved in multiple physiological processes such as defence response, carbohydrate metabolism, oxidation-reduction process and cell wall remodelling. Our data suggest Cd-induced alterations of the cell wall structure as well as increased lignification, which foster the hypothesis of the cell wall acting as a physical barrier against Cd exposure.

To elaborate this, we investigated the monosaccharide composition and lignin content in the cell wall of *M. sativa* stems and results were presented in chapter 2. Besides, we studied the influence of Cd on ethylene biosynthesis and put the obtained results into context with cell wall remodelling. Main results indicate that:

- Long-term Cd exposure changes the composition of pectic polysaccharides in the cell wall of *M. sativa* stems
- Cd exposure increases the activity of pectin methylesterase, which catalyses the demethylation of homogalacturonan
- Side chains of cell wall polymers shift to a higher abundance of xyloglucan upon Cd exposure, which influences the mechanical properties of the cell wall matrix by promoting cross-linking
- Long-term Cd exposure enhances ethylene biosynthesis and signalling
- Cd induces the phenylpropanoid pathway in *M.sativa* stems but does not influence the lignin content or monolignol composition

Long-term Cd exposure clearly impacts the cell wall structure by altering the content of pectic polysaccharides and their side chain composition. These changes influence the mechanical properties of the cell wall matrix and limits the accessibility for cell wall proteins to their target sites. The increased abundance of PME (chapter 1) in response to Cd exposure and its increased enzymatic activity promotes the demethylation of homogalacturonane, which creates binding sites for Cd and would facilitate the immobilization of the heavy metal in

the cell wall, thereby preventing its further distribution throughout the plant. Our results corroborate Cd-driven changes in the cell wall structure to establish its function as a defence barrier against Cd stress. However, although we observed an induction of the phenylpropanoid pathway upon long-term Cd exposure and a higher abundance of peroxidases (chapter 1), which would promote lignification, neither the lignin content nor the monolignol composition was influenced. In this regard, we investigated the content of secondary metabolites as they also origin from the phenylpropanoid pathway. Additionally, the content of primary metabolites was determined in stems and leaves of *M. sativa* and data are supplemented with targeted gene expression analysis. The study is presented in chapter 3.

Chapter 3 - Changing profile of primary and secondary metabolites facilitates acclimation of *Medicago sativa* after long-term Cd exposure

Adapted from the submitted article:

Gutsch A., Sergeant K., Hendrix S., Guerriero G., Renaut J., Lutts S., Alseekh S., Fernie A.R., Hausman J-F., Vangronsveld J., Cuypers A.: Changing profile of primary and secondary metabolites enables acclimation of *Medicago sativa* after long-term Cd exposure. *Submitted to Frontiers in Plant Science*

Contributions:

A. Gutsch, K. Sergeant, J-F. Hausman and A. Cuypers designed the experiment.

A. Gutsch performed the experimental work, analysed and interpreted the data and wrote the present manuscript.

S. Hendrix contributed to the determination of glutathione content.

G. Guerriero contributed to the gene expression analysis.

J. Renaut supplied and managed the technical equipment.

S. Lutts contributed to the determination of polyamines in stems and drafted the method description.

A.R. Fernie and S. Alseekh contributed to the analysis of primary metabolites in leaves and drafted the method description.

All authors revised and approved the final version of the manuscript.

ABSTRACT

As a common pollutant cadmium (Cd) is one of the most toxic heavy metals accumulating in agricultural soil through the application of phosphate fertilizers. The uptake of Cd by plants is the main entry route into the human food chain, whilst in plants it elicits oxidative stress by unbalancing the cellular redox status. *Medicago sativa* was subjected to mild chronic Cd stress for five months. Targeted and untargeted metabolic analyses were performed on stem and leaf samples using different methods. The principal component analysis showed a clear separation between control and Cd-exposed plants. In total, 51 secondary metabolites were identified that changed significantly upon Cd exposure, of which the majority are flavonoid conjugates. Spermine, spermidine and putrescine levels increased upon Cd exposure and, in parallel, amino acid compositions altered with levels of asparagine, histidine and proline decreasing in stems but increasing in leaves after long-term Cd exposure. This suggests a tissue-specific metabolic stress response. Accumulated metabolites in leaves and stems function as antioxidants, tackling the Cd- induced oxidative stress and thereby preventing cellular damage. By an adequate adjustment of its metabolic composition, *M. sativa* reaches a new steady-state, which enables the plant to acclimate under chronical Cd stress.

KEYWORDS: *Medicago sativa*, cadmium, primary metabolites, secondary metabolites, flavonoids, environmental stress, acclimation

1. Introduction

Cadmium (Cd) is a nonessential heavy metal causing toxicity in plants when taken up via their root system. Numerous physiological and biochemical processes are impacted and plants show reduced growth, chlorosis, signs of water imbalance and impaired photosynthesis. In plant cells, Cd elicits oxidative stress as a result of a misbalanced production and neutralization of reactive oxygen species (ROS) (Perfus-Barbeoch et al., 2002; Sanità Di Toppi and Gabbrielli, 1999; Schützendübel and Polle, 2002; Yang et al., 1996). Enhanced ROS levels lead to DNA damage, lipid peroxidation and protein modification, which interferes with their activity (Romero-Puertas et al., 2002; Sandalio et al., 2001; Sharma et al., 2012). The mechanisms underlying Cd-induced oxidative stress can have different origins. Cadmium can interfere with the activity of various enzymes involved in antioxidative defence and physiological processes, thereby increasing the formation of ROS (Cuypers et al., 2011). Moreover, Cd depletes the pool of antioxidants such as glutathione (GSH), which disturbs the redox balance (Semane et al., 2007). Plants possess several anti-oxidative primary and secondary metabolites in order to counteract oxidative stress and to limit cellular damage.

Amino acids (AAs) accumulate in response to various stresses and altered levels of selected AAs in tobacco were reported following long-term exposure to zinc, indicating a possible role in plant stress adaptation (Pavlíková et al., 2014). Especially the accumulation of proline is commonly observed, highlighting its potential importance (Hayat et al., 2012; Szabados and Savouré, 2010). However, also increases of other AAs were reported in response to stress such as γ-aminobutyric acid (GABA), whose abundance increased under salt stress in *Glycine max* through the stimulation of diamine oxidases (Xing et al., 2007). That said, in response to long-term Cd exposure, both increases and decreases of specific AAs were observed in tomato leaves. Interestingly, the response differed between young and mature leaves and furthermore the accumulation of AAs deriving from diverse precursor molecules such as oxaloacetate and pyruvate also differed (Hédiji et al., 2010). Another group of primary metabolites are polyamines (PAs), which are small ubiquitous aliphatic amines with a known protective function during abiotic stress responses (Alcázar et al.,

2006; Hussain et al., 2011). The most abundant PAs are putrescine (Put), spermidine (Spd) and spermine (Spm) and their accumulation was observed in sunflower and wheat upon Cd exposure (Groppa et al., 2003). Especially Spd and Spm play important roles in the defence against Cd by alleviating its harmful effects and promoting seedling growth (Rady and Hemida, 2015). Exogenous application of Spd and Spm during Cd stress influences the antioxidant system, reduces ROS generation and prevents lipid oxidation (Groppa et al., 2007b; Zhao and Yang, 2008). Moreover, the synthesis of PAs is linked to the synthesis of the plant hormone ethylene by sharing the same precursor *S*-adenosylmethionine (SAM) (Bouchereau et al., 1999) (**Figure 3-1**).

Not only primary but also secondary metabolites may play a crucial role during multiple biochemical and physiological processes. Unlike primary metabolites which are ubiquitous, their synthesis is regulated by plant development and environmental cues. These compounds are involved in plant defence against biotic and abiotic stress. As such, phenolic compounds are a remarkably diverse group of secondary metabolites, which are synthesized via the phenylpropanoid pathway utilizing the AA phenylalanine (Phe) as their initial substrate. They include lignins, lignans and tannins but also flavones and isoflavones (Winkel-Shirley, 2001) (**Figure 3-1**). Phenolic compounds accumulate in response to multiple stresses, together with increased activities of enzymes involved in their metabolic pathways (Dixon and Paiva, 1995; Lavola et al., 2000). Moreover, considerable evidence exists in the literature for their antioxidant capacity during environmental stresses (Agati et al., 2012; Michalak, 2006). One such example is the increased abundance of flavones which occurs in response to heavy metals in different leguminous species (Pawlak-Sprada et al., 2011a; Skorzynska-Polit et al., 2004). Furthermore, they were reported to play a role in the resistance to aluminium toxicity in maize, which is consistent with a metal binding activity of flavonoids (Kidd et al., 2001).

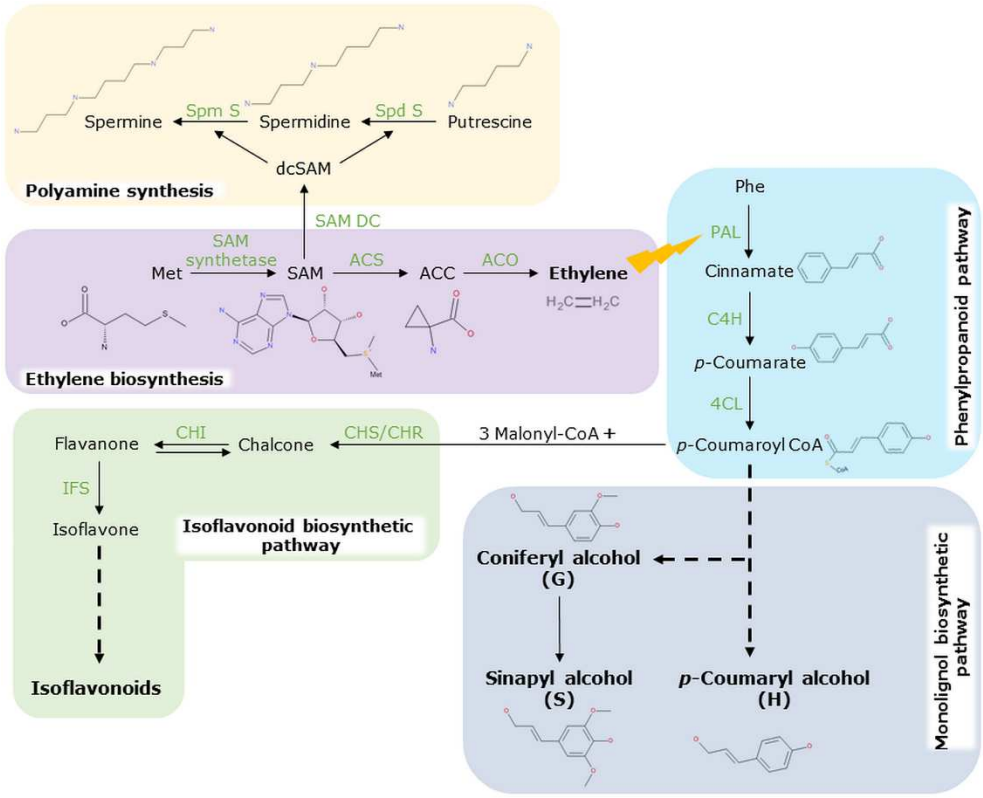

Figure 3-1: Schematic overview of the general phenylpropanoid pathway branching in the isoflavonoid and monolignol syntheses.
Ethylene stimulates the activity of phenylalanine ammonia lyase (indicated as lightning). The ethylene precursor *S*-adenosylmethionine (SAM) serves in its decarboxylated form (dcSAM) for the synthesis of spermidine and spermine by providing an aminopropyl group. Spm S spermine synthase; Spd S spermidine synthase; Met methionine; SAM DC *S*-adenosylmethionine decarboxylase; ACC 1-aminocyclopropane-1-carboxylic acid; ACS ACC synthase; ACO ACC oxidase; Phe phenylalanine; PAL phenylalanine ammonia lyase; C4H cinnamate-4-hydroxylase; 4CL 4-coumarate ligase; CHR chalcone reductase; CHS chalcone synthase; CHI chalcone isomerase; IFS isoflavone synthase. Broken arrows represent reactions, involving intermediate steps, which are not detailed in the figure.

Metabolomics is a fast-developing high-throughput approach that represents the step from targeted metabolite quantification to the generation of untargeted metabolite profiles. Depending on the question asked, either of these methods can be used to study the molecular phenotype of plants and metabolite profiling was applied in various monocot and dicot plant species (Schauer and Fernie, 2006). Metabolic profiling can be utilized in the process of gene annotation and the integration of genomics and metabolomics is beneficial for the

characterization of biological processes (Goossens et al., 2003; Hirai et al., 2004). Metabolites are often regarded as the biological active end-product compounds, resulting from the integration of gene expression and protein activities and as such better reflecting the end-phenotype than genes or proteins alone. It is estimated that the number of different metabolites in the plant kingdom ranges between 200,000 and 1,000,000, but for a single species it could be only a few thousands (approximately 5000 metabolites in *A. thaliana*) (Obata and Fernie, 2012). Different methods including gas chromatography-mass spectrometry, capillary electrophoresis, nuclear magnetic resonance spectroscopy and liquid chromatography (LC-MS) are commonly used for metabolic profiling (Fernie et al., 2004) but the immense variability in chemical structure and properties of metabolites makes it nearly impossible to identify them all using current techniques. However, LC-MS is able to separate metabolites based on their chemical properties and has the potential to identify a very wide variety of metabolites. Recent advances in ultra-performance liquid chromatography (UPLC-MS) have expended the sensitivity, resolution and throughput of this technique for metabolite analysis (Rogachev and Aharoni, 2012). To date, metabolomics is becoming increasingly important in plant research and is a frequently used approach to study plant stress responses in order to decipher which molecules are important during stress responses and tolerance acquisition (Nakabayashi and Saito, 2015; Rodziewicz et al., 2014; Sarry et al., 2006).

Medicago sativa L. is one of the most cultivated forage legumes in the world. Indeed, thanks to its high nutritional value it is often referred to as "the queen of forage". *M. sativa* is easy to grow and its nitrogen fixation by symbiotic rhizobia gives an added value to the soil. Besides its nutritional properties, *M. sativa* is rich in biologically active secondary metabolites, which confer antioxidant activities by scavenging free radicals and/ or act as metal chelators (Karimi et al., 2013). In the present study, targeted and untargeted metabolic approaches were used to investigate the involvement of primary and secondary metabolites in response to long-term Cd exposure. Subjecting the plant to an environmentally realistic Cd concentration makes the results relevant to agricultural practice. Data on metabolites are supplemented with targeted gene

expression data, which provide further insights in the metabolism of selected identified metabolites.

2. Material and Methods

2.1. Plant growth and sampling

Medicago sativa L. (cultivar Giulia) plants were exposed to 10 mg Cd kg^{-1} soil in a long-term experiment. Experimental conditions and sampling were previously described in detail (Gutsch et al., 2018) and metabolite analysis was undertaken using the same sample conditions. Briefly, plants were potted in 12 times 12 pots for each condition and grew for five months with an intermediate cutting as it is normal agricultural practice. Stems were sampled in five biological replicates for each condition, with a pool of stem tissue from 24 pots corresponding to one biological replicate. Samples were ground in liquid nitrogen and kept at -80°C till further use.

2.2. Measurement of glutathione in stem tissue

Glutathione (GSH) analysis was adopted from a previously published protocol (Queval and Noctor, 2007). All extraction steps were performed on ice. Five replicates of each condition were used for statistical analysis. Approximately 120 mg fresh weight (FW) of deep-frozen ground stem tissue were extracted with 200 mM HCl using mortar and pestle. After centrifugation (16000 g, 10 min, 4°C), 350 µL of the supernatant were transferred to a new tube and the pH of each extract was adjusted to 4.5 with 200 mM NaOH.

The GSH assay relies on the glutathione reductase (GR)-mediated reduction of 5,5-dithiobis(2-nitro-benzoic acid) (DTNB) using GSH to trinitrobenzol (TNB), which is monitored at 412 nm. Glutathione reductase (Sigma-Aldrich) was suspended in 200 mM NaH_2PO_4-EDTA to a concentration of 20 U mL^{-1} and kept in aliquots of 500 µL. For each measurement series a fresh aliquot was used. To measure only the oxidized glutathione disulphide (GSSG) content each extract was treated with 2-vinylpyridine to complex free GSH. Extracts were incubated at 20°C for 30 min and centrifuged (16000 g, 10 min, 4°C) to eliminate complexed GSH. Total GSH content (GSSG and GSH) was measured in the non-treated extract. Measurements were performed in triplicates in 96-well plates.

For the total GSH content 10 µL of each extract were pipetted into plate wells containing 200 mM NaH_2PO_4, 10 mM EDTA, 10 mM NADH and 12 mM DTNB. The reaction was started by adding GR and monitored at 412 nm. A GSH standard curve between 0 pmol – 1000 pmol was generated on the same plate. The GSSG was measured in a similar way using 40 µL of the treated extract and with a standard curve raging between 0 pmol – 400 pmol.

2.3. Quantification of primary metabolites

2.3.1. HPLC-FLD analysis of polyamines in stems

Polyamines were extracted from 200 mg ground stem tissues with 500 µL $HClO_4$ 4 % (v/v) containing 1,7-diaminoheptane (5 mg L^{-1}) as internal standard under vigorous vortexing. Samples were kept at 4°C for 1 h and subsequently centrifuged at 13,000 g at 4°C for 20 min. The pellet was re-extracted with 500 µL $HClO_4$ 4 % (v/v) and re-centrifuged. The two supernatants were used for free polyamine determination. For fluorescence detection, PAs were derivatized by dansylation (Lefèvre et al., 2001) and the dried extract was dissolved in 1 mL of methanol, filtered through 0.45 µm microfilters (Chromafil PES-45/15, Macherey-Nagel) and 5 µL of sample were injected on a Nucleodur C18 Pyramid column (125 x 4.6 mm internal diameter; 5 µm particle size) (Macherey-Nagel) maintained at 40°C. Analyses through high-performance liquid chromatography coupled with a fluorescence detector (HPLC-FLD) were performed on a Shimadzu HPLC system, equipped with a solvent delivery unit LC-20AT, a SIL-HTc autosampler and a RF-20A Fluorescence Detector (Shimadzu's-Hertogenbosch) with the excitation wavelength at 340 nm and the emission wavelength at 510 nm. The flow of the mobile phase was 1.0 mL min^{-1}. The mobile phase consisted of water (eluent A) and acetonitrile (ACN) (eluent B). The gradient program was as follows: 40 % B to 91 % B (20 min), 91 % B to 100 % B (2 min), 100 % B (8 min), 100 % B to 40 % B (1 min) and column equilibration at 40 % B during 4 min. Free PAs were quantified using six-points calibration curves with custom-made external standard solutions and internal standard (1,7-diaminoheptane), ranging from 3.125 to 100 µM and every ten injections, a check standard solution was used to confirm the calibration of the system. Internal standard gave information about the recovery of the extraction and derivatization during the evaluation of PAs content.

2.3.2. UHPLC-DAD analysis of amino acids in stems

Approximately 50 mg of ground stem tissue were freeze-dried prior to extraction. Extraction was done with 400 µL methanol containing 10 mM beta-aminobutyric acid (BABA) as internal standard under vigorous vortexing and subsequent shaking (1400 rpm, 15 min). Two hundred µL of chloroform were added, samples were shaken (1400 rpm, 15 min), 400 µL of H_2O were added and samples were vortexed and centrifuged (12000 g, 5 min). Six hundred µL of the supernatant were collected. A 50 µL sample aliquot was first dried using a vacuum centrifuge and resuspended in 50 µL H_2O prior to analysis. The derivatisation of samples and AA standards was done using the AccQ-Tag Ultra Derivatization Kit (Waters). Five µL of samples and 5 µL of the AA standards were mixed separately with 35 µL AccQ-Tag borate buffer. To these mixtures, 10 µL AccQTag reagent (Waters AccQ-Tag Ultra Derivatization Kit) were added, samples were vortexed, spun down and incubated for 10 min at 55°C. UHPLC-DAD (ultra-high performance liquid chromatography - diode array detector) was performed on an Acquity UPLC system (Waters) equipped with an Acquity tunable UV detector. The used column was Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm particle size, Waters). The flow rate was 0.7 mL min^{-1} and the column temperature was kept at 55°C. The injection volume was 3 µL and the detection wavelength was set at 260 nm. Two eluents were used: A) AccQ Tag eluent; B) ACN. The following gradient elution was used: 0-0.54 min, 0.1 % B; 6.5 min, 9.1 % B; 8.50 min, 21.2 % B; 8.90-9.50 min, 59.6 % B; 9.60-10.10 min, 0.1 % B.

2.3.3. GC-MS analysis of primary metabolites in leaves

Medicago sativa leaf tissue was ground with mortar and pestle in liquid nitrogen and extracted as described previously (Lisec et al., 2006). Briefly, 50 mg plant tissues were extracted by adding 700 µL 100 % methanol. Seven hundred µL from the extraction liquid were taken and transferred into a new eppendorf tube, flowed by adding 375 µL of $CHCl_3$ and 750 µL water. After centrifugation, 150 µl supernatant were dried under vacuum, and the residue was derivatized for 120 min at 37°C (in 40 µl of 20 mg ml^{-1} methoxyamine hydrochloride in pyridine) followed by a 30 min treatment at 37°C with 70 µl of MSTFA. The gas chromatography – mass spectrometry (GC-MS) system used was a gas

chromatograph coupled to a time-of-flight mass spectrometer (Leco Pegasus HT TOF-MS). An auto sampler Gerstel Multi Purpose system injected the samples. Helium was used as carrier gas at a constant flow rate of 2 mL s^{-1} and gas chromatography was performed on a 30 m DB-35 column. The injection temperature was 230°C and the transfer line and ion source were set to 250°C. The initial temperature of the oven (85°C) increased at a rate of 15°C min^{-1} up to a final temperature of 360°C. After a solvent delay of 180 s mass spectra were recorded at 20 scans s^{-1} with m/z 70-600 scanning range. Chromatograms and mass spectra were evaluated by using Chroma TOF v4.5 (Leco) and TagFinder v4.2 software (Lisec et al., 2006; Schauer et al., 2005).

2.4. Quantification of secondary metabolites

2.4.1. Extraction

Ground stem tissue samples (150 mg) from *M. sativa* were freeze-dried and extractions were done with 1790 µL of methanol:water (4:1, v/v). Ten µL of 4-methylumbelliferone (500 µg mL^{-1}) was added to the sample as extraction internal standard. Samples were homogenized using a vortex (30 s) and shaken for 4 h at room temperature to extract the metabolites. Samples were again vortexed (30 s) followed by centrifugation (20000 g for 30 min at 4 °C). The supernatant was collected and dried using a vacuum centrifuge. Dried extracts were solubilized in 1460 µL of methanol:water (5:95, v/v) and filtered through a syringe filter (0.2 µm, PTFE Millex-LG; Merck KGaA).

2.4.2. UHPLC-TripleTOF Analysis

Extracts were analysed with a Waters Acquity UPLC system (Milford, MA) coupled to a high resolution time of flight mass spectrometer (TripleTOF 5600+; AB Sciex). The separation of the 5 µL aliquot was performed in two technical replicates on a reverse-phase Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm particle size, Waters). In both ionisation modes, the eluents were 0.1 % formic acid in water (A) and 0.1 % formic acid in ACN (B). The gradient was as follows: 0 min, 1 % B; 4 min, 1 % B; 16 min, 5 % B; 35 min, 40 % B; 45 min, 100 % B; 50 min, 100 % B; 53 min, 1 % B; 60 min, 1 % B. The flow rate was of 0.5 mL min^{-1} and the column temperature was 50°C. Compounds were ionized with an electrospray ionization (ESI) source using the following parameter values for the positive and negative mode: source temperature,

650°C; ion spray voltage of 4.5 and – 4.5 kV, respectively; curtain gas (nitrogen) of 30; nebulizer gas (air) of 55; turbine gas (air) of 50. Precursor charge state selection was set at 1. For information dependent acquisition (IDA in high sensitivity mode), survey scans were acquired in 175 ms and the 10 most abundant product ion scans were collected if exceeding a threshold of 100 counts per sec. The total cycle time was fixed at 2.25 s. Four time bins were summed for each MS1 scan at a pulser frequency value of 16.4 kHz. A sweeping collision energy setting of 15 eV in positive and – 15 eV in negative mode was applied to all precursor ions for collision-induced dissociation. The declustering potential was set at 60 eV and – 60 eV in positive and negative mode, respectively. Dynamic exclusion was set for 8 s after 2 occurrences. For MS1, full HR-MS spectra between 100 and 1300 mass-to-charge ratio (m/z) were recorded. MS2 scans were recorded between 25 and 1300 m/z.

2.4.3. Data Processing

Data were processed and analysed using Progenesis QI (v2.3, Nonlinear Dynamics). Each UPLC-MS run was imported as an ion-intensity map and runs were aligned in the retention time direction. A corporate run representing the compounds in all samples was used for peak picking and was compared to all the runs, to ensure that the same ions are detected in every run. Data were normalized according to total ion intensity. Only features with MS2 data, and both fold-change ≥ 1.5 and *p*-value ≤ 0.05 in two technical replicates were considered for identification. Metabolites were identified by their accurate masses using an in-house database as well as CHEBI 3-star (https://www.ebi.ac.uk/chebi), MassBank, PubChem and the NIST MS/MS database. The output was manually reviewed with PeakView (v1.2.0.3, AB SCIEX), Metlin (https://metlin.scripps.edu/index.php) and PubChem databases (https://pubchem.ncbi.nlm.nih.gov) as well as literature data for structure elucidation.

2.5. Gene expression analyses

The RNAqueous™ Kit (Life Technologies) was used for RNA extraction from five biological replicates according to the manufacturer's instructions. The RNA was purified by precipitation with 3 M sodium acetate and 100 % isopropanol,

subsequently washed with 70 % ethanol and resuspended in RNase-free water. The RNA was quantified with a NanoDrop® ND-1000 spectrophotometer (Thermo Fischer Scientific) ($A_{260/280}$ and $A_{260/230}$ ratio between 1.9 and 2.5) and a Bioanalyzer (Agilent Life Science). The RNAs displayed a RIN value between 8.7 and 9.0. Reverse transcription was carried out with the ProtoScript II Reverse Transcriptase (NEB) following the manufacturer's instruction. Sequences for the genes of interest were obtained by searching the Alfalfa Gene Index and Expression Atlas Database (http://plantgrn.noble.org/AGED/index.jsp). Specific primer pairs were designed with the Primer3Plus online tool (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and validated using the OligoAnalyzer 3.1 (https://eu.idtdna.com/calc/analyzer) (**Table S3-1**). qPCR runs were performed in 384 well plates with the Taykon SYBR Green low ROX (Eurogentec) on a ViiA7 Real-Time PCR system (Applied Biosystems). Minimum information for publication of quantitative real-time PCR experiments (Bustin et al., 2009) is detailed in **Table S3-2**. At the end of each run, a melting curve was generated to check the specificity of the products. Relative gene expression was determined using qBase^{PLUS} software v2.5 (Biogazelle). Reference genes were selected according to literature (Guerriero et al., 2014) and the two most stable ones were used for data normalisation (**Table S3-1**).

3. Results

3.1. Glutathione

No significant changes in total GSH (control: 400.74 nmol g^{-1} FW; Cd: 389.30 nmol g^{-1} FW) nor in reduced GSH (control: 284.58 nmol g^{-1} FW; Cd: 273.52 nmol g^{-1} FW) were observed in response to Cd exposure. In addition, the measured average concentration of the oxidised form GSSG remained the same in control (58.08 nmol g^{-1} FW) and Cd-exposed *M. sativa* stems (57.89 nmol g^{-1} FW).

3.2. Amino acids and Polyamines

The effect of long-term Cd exposure on the content of AAs in *M. sativa* stems was determined using UHPLC-HR-MS. In order to visualize sample grouping and derived AA differences in four biological replicates of control and Cd-exposed plants, a PCA was performed (**Figure 3-2**) using the data of 31 quantified amino

acids (**Table S3-3**). The plot shows that both conditions are clearly discriminated from each other along the first and second dimension. Control plants are separated from the Cd-exposed plants along the first dimension, which explained 52.93 % of the total observed variability.

The total average free AA content decreased about 1.5 fold in Cd-exposed *M. sativa* stems (control: 64.82 µmol g^{-1} DW; Cd: 43.91 µmol g^{-1} DW). Amino acids, which changed significantly ($p \leq 0.05$) in response to Cd exposure are given in **Figure 3-3**. The most abundant AA in control samples was Asn (26.793 µmol g^{-1} DW). Its concentration was about three fold lower in Cd-exposed *M. sativa* stems. Furthermore, the concentration of OH-Pro and His decreased upon Cd exposure. Thereby, OH-Pro had the highest fold-change (7.9 fold). The content of all other AAs with significant changes such as L-Ser, L-Gly, L-Thr, GABA, L-Tyr, L-Met, L-Val, L-Ile, L-Leu and L-Phe increased in response to Cd exposure.

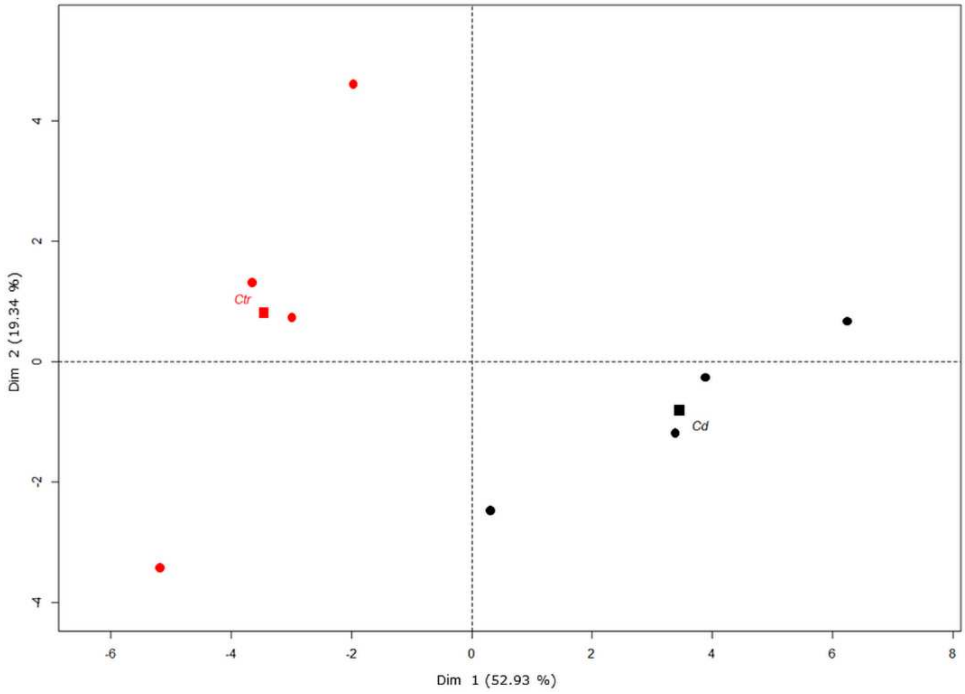

Figure 3-2: PCA of AA content in *M. sativa* stems.
M. *sativa* plants were exposed to Cd (10 mg kg^{-1} soil dry weight) in a long-term experimental set-up. Amino acids were measured in four biological replicates (Table S3-3). The baric centre of the two conditions is indicated with a square.

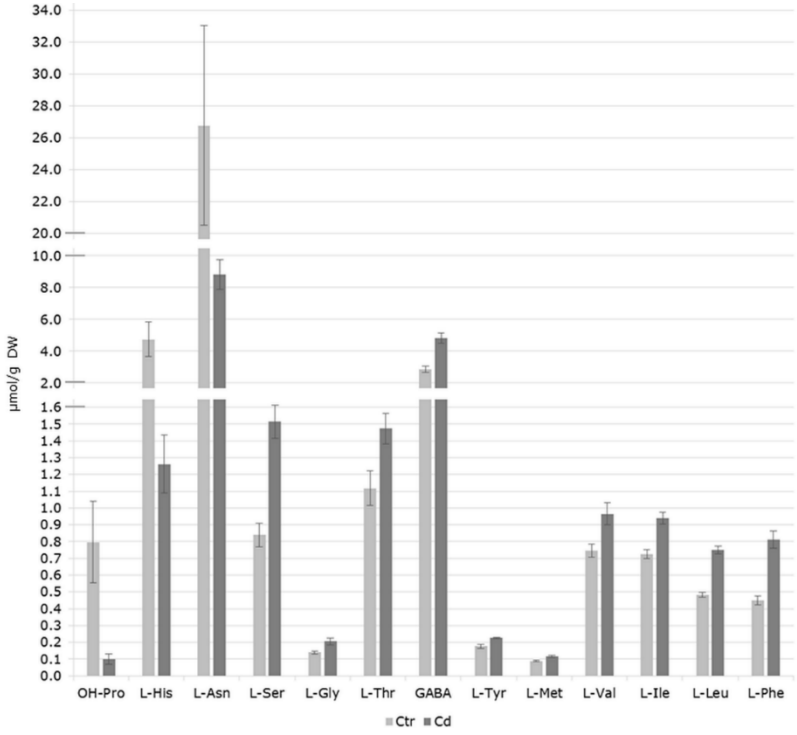

Figure 3-3: Significantly changed free AAs in response to long-term Cd-exposure in *M. sativa* stems ($p \leq 0.05$).
The AA content was measured in four biological replicates. Significance was determined with a t-test with $p \leq 0.05$. Standard errors are indicated by error bars. The actual values for all AAs are provided in Additional file 2. DW dry weight.

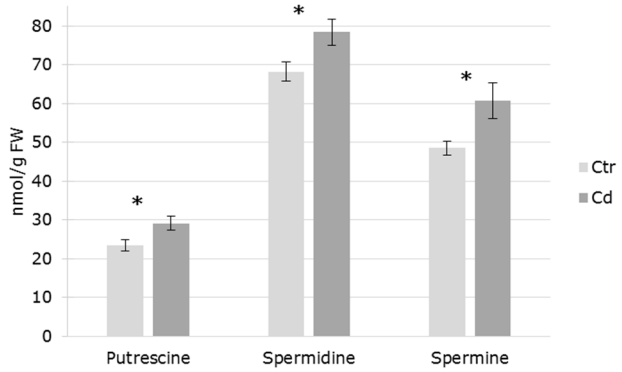

Figure 3-4: Polyamine content in *M. sativa* stems in response to long-term Cd exposure.
The average content is calculated from five biological replicates. Standard errors are indicated by error bars. Significance was determined with a *t*-test ($p \leq 0.05$) and is indicated by an asterisk.

In a separate analysis the polyamine content was determined. Long-term Cd exposure lead to increased contents of Put, Spd and Spm significantly (p = 0.04 for all) in *M. sativa* stems. Putrescine increased about 24.6 %, Spd about 15.1 % and Spm about 25.0 % (**Figure 3-4**).

Supplementary, data on the content of primary metabolites in *M. sativa* leaves upon long-term Cd exposure were obtained, which showed distinct metabolic profiles between control and Cd-exposed samples (**Figure S3-1**). The full result list is provided as supplementary material (**Table S3-4**).

3.3. Identified secondary metabolites

In order to better understand the metabolic changes associated with long-term Cd exposure in *M. sativa* stems, metabolic profiling was performed using UPLC-TripleTOF in negative and positive mode, which revealed a clear distinction of the metabolic patterns from Cd-exposed and control plants along the first and second dimension using a PCA (**Figure 3-5**). In negative mode, 1362 metabolite features were detected that significantly changed upon Cd exposure (with p-value ≤ 0.05 and fold-change ≥ 1.5) in both technical replicates and in total 25 metabolites were putatively identified (**Table 3-1**). In positive mode, 769 metabolite features significantly changed in response to Cd (with p-value ≤ 0.05 and fold-change ≥ 1.5) in both technical replicates and a total number of 26 metabolites were putatively identified (**Table 3-2**).

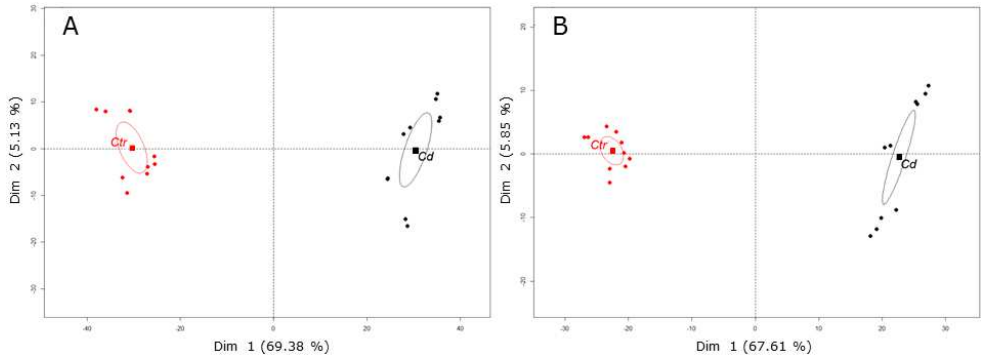

Figure 3-5: PCA of metabolic compounds in *M. sativa* stems after long-term Cd exposure.
Metabolites were measured in two technical replicates from five biological replicates. A) negative mode. B) positive mode. The baric centre of samples from control and Cd-exposed plants is indicated with a square.

Table 3-1: List of differentially abundant compounds in *M. sativa* stems after long-term Cd exposure.
Data were obtained by UPLC–TTOF in negative ESI mode, with MS/MS experiments. The relative abundance of each peak in stems of control and Cd-exposed plants is based on the selected ion current and is presented as the average normalised value of two technical replicates from five biological replicates. tr, retention time; nd not detected.

$t_{r\ (min)}$	Relative abundance		Fold	[M-H]	Error (ppm)	Molecular Formula	MSMS [M-H]	Putative identity	Reference
	Cadmium-treated	Control							
2.23	446	281	1,59	164,0719	1,22	C9H11NO2	147,0434 103,0539- 164,0693 72,0108	L-Phe*	
2.50	1	89	89,00	274,0932 [M-H2O-H]	-0,11	C11H19NO8	89,0280 274,0929 100,0413 159,8914 232,0821	*N*-acetyl-hexosamine derivative	
15.37	21	1	39,40	669,1687 [M+FA-H]	2,18	C28H32O16	299,0537 284,0294 461,1077	Trihydroxy-methoxy (iso)flavone di-hexoside	(de Rijke et al., 2006; Lin et al., 2000; Staszków et al., 2011)
18.18	284	30	9,36	299,0768	-1,47	C13H16O8	137,0227 109,0266 135,0047 153,0146 91,0181	Hydroxybenzoyl-hexoside	(Farag et al., 2008)

19.07	464	69	6,69	605,1163	2,50	C27H26O16	113,0234 253,0478 351,0579 85,0294 193,0316	Dihydroxy(iso)flavone di-hexuronide	(de Rijke et al., 2006; Staszków et al., 2011)
19.41	21	1	24,86	299,0773	0,20	C13H16O8	137,0198 135,0049 100,9291	Hydroxybenzoyl-hexoside	(de Rijke et al., 2006; Farag et al., 2008)
19.49	171	15	11,37	605,1164	2,63	C27H26O16	113,0211 253,0500 85,0285 351,0572 193,0314	Dihydroxy(iso)flavone di-hexuronide	(de Rijke et al., 2006; Staszków et al., 2011)
19.64	63	6	10,26	461,1093	0,78	C22H22O11	284,0273 299,0545 255,0303 135,0046	Trihydroxy methoxy-(iso)flavone hexoside	(de Rijke et al., 2006; Lin et al., 2000; Staszków et al., 2011)
20.06	228	50	4,58	429,0824	-0,75	C21H18O10	253,0489 85,0297 113,0224 135,0058 117,0322	Dihydroxy(iso)flavone - hexuronide	(de Rijke et al., 2006; Staszków et al., 2011)
20.41	50	7	6,88	429,0823	-0,98	C21H18O10	253,0481 117,0344 135,0079 85,0281 280,7946	Dihydroxy(iso)flavone-hexuronide	(de Rijke et al., 2006; Staszków et al., 2011)

21.83	52	123	2,35	447,0931	-0,42	C21H20O11	284,0327 447,1017	Tetrahydroxy(iso)flavone-hexoside	(Staszków et al., 2011; Wojakowska et al., 2013)
23.22	26	nd	treated only	283,0610	-0,71	C16H12O5	268,0387 147,9081 211,0346 283,0632	Dihydroxy-methoxy(iso)flavone	(Farag et al., 2007; Lin et al., 2000; Staszków et al., 2011)
23.80	59	2	33,23	283,0608	-1,41	C16H12O5	268,0340 211,0382 239,0319 195,0406	Dihydroxy-methoxy(iso)flavone	(de Rijke et al., 2006; Prasain et al., 2003)
23.96	68	10	6,59	253,0504	-0,91	C15H10O4	253,0470 117,0329 135,0080 91,0203 133,0290	Dihydroxy(iso)flavone	(de Rijke et al., 2006)
24.03	28	1	19.01	313,0712	-1,79	C17H14O6	255,0286 298,0491 2830201 227,0312 171,0404	Dihydroxy- dimethoxy-(iso)flavone	(de Rijke et al., 2006)
25.11	151	68	2,22	263,1285	-1,44	C15H20O4	153,0898 219,1360 204,1122 136,0514	Abscisic acid*	

26.22	29	2	18,56	267,0663	0,07	C16H12O4	252,0402 223,0395 195,0422 267,0628 251,0305	Hydroxy-methoxy(iso)flavone	(Prasain et al., 2003; Staszków et al., 2011)
26.8	588	88	6,69	267,0664	0,45	C16H12O4	252,0406 223,0371 195,0419	Hydroxy-methoxy(iso)flavone	
27.73	39	nd	treated only	299.0920	-1.62	C17H16O5	135,0072 91,0170 299,0911 284,0303 269,0444 256,0339	Unknown compound	
28.34	129	17	7,65	473,1464 [M+FA-H]	2,28	C24H26O10	254,0571 209,0582 211,0394 225,0548 269,0838	Hydroxy-methoxy-pterocarpan malonate hexoside derivative	(Sumner et al., 1996)
30.19	12	1	10.12	297.0763	-1.85	C17H14O5	239,0363 282,0544 183,0049 195,0418 254,0552 211,0394	Dimethoxy-hydroxy(iso)flavone	(Staszków et al., 2011)
34.76	38	3	14,57	313,0715	-0,83	C17H14O6	255,0277 298,0473	Dihydroxy-dimethoxy(iso)flavone	(de Rijke et al., 2006; Staszków

							227,0329 270,0526 183,0420			et al., 2011)
36.49	71	3	22,64	509,1244	0,41	C30H22O8	237,0909 263,0704 135,0058 373,1054 399,0872	Hydroxylated dimer	chalcone	(de Rijke et al., 2006)

*Identity of the compound was confirmed with an authentic standard.

Table 3-2: List of differentially abundant compounds in *M. sativa* stems after long-term Cd exposure.
Data were obtained by UPLC–TTOF in positive ESI mode, with MS/MS experiments. The relative abundance of each peak in stems of control and Cd-exposed plants is based on the selected ion current and is presented as the average normalised value of two technical replicates from five biological replicates. Abbreviations: t_r, retention time; nd, not detected.

$t_{r\ (min)}$	Relative abundance		Fold	[M+H]	Error (ppm)	Molecular Formula	MSMS [M+H]	Putative identity	Reference
	Cadmium-treated	Control							
2.5	1	114	113.29	276,107	-2,82	C11H19NO8	138,0521 96,0424 144,0625 126,0524 84,0413	*N*-acetyl-hexosamine derivative	
16.62	82	312	3,92	271,0593	-2,95	C15H10O5	215,0675 149,0229 57,0700 43,0562	Trihydroxy(iso)flavone conjugated	(de Rijke et al., 2006; Staszków et al., 2011)
18.17	288	16	17,84	241,0853	-2,57	C15H12O3	107,0438 131,0451 147,0454 123,0414 77,0363	Unknown compound	
19.04	380	65	5,83	607,1284	-1,58	C27H26O16	255,0602	Dihydroxy(iso)flavone-di-hexuronide	(de Rijke et al., 2006; Staszków et al., 2011)

19.46	139	15	9.17	607.01278	-2.57	C27H26O16	255.0560	Dihydroxy(iso)flavone di-hexuronide	(de Rijke et al., 2006; Staszków et al., 2011)
19.65	36	3	12,82	463,1224	-2,35	C22H22O11	301,0698 241,0524 269,0445 213,0523 145,0240	Trihydroxy-methoxy-(iso)flavone hexoside	(de Rijke et al., 2006; Lin et al., 2000; Staszków et al., 2011)
20.07	281	70	3,99	431,0957	-3,64	C21H18O10	255,0646 137,0214 145,0236 119,0462	Dihydroxy(iso)flavone hexuronide	(de Rijke et al., 2006; Staszków et al., 2011)
20.37	58	10	6,03	431,0959	-3,18	C21H18O10	255,0629 137,0224 145,0267 119,0485	Dihydroxy(iso)flavone hexuronide	(de Rijke et al., 2006; Staszków et al., 2011)
21.13	76	nd	treated only	489,1382	-1,9	C24H26O12	241,0828 131,0497 147,0428 213,0866 107,0483	Unknown compound	(de Rijke et al., 2006)
21.82	24	53	2,22	449,1063	-3,43	C21H20O11	287,0529 153,0133 269,0445 259,0586	Tetrahydroxy(iso)flavone-hexoside	(Staszków et al., 2011; Wojakowska et al., 2013)

23.07	104	4	23,42	563,1382	-2,36	C26H26O14	315,0839 299,0534 254,0531	Dihydroxy- dimethoxy-(iso)flavone malonate-hexoside derivative	(de Rijke et al., 2006; Staszków et al., 2011)
23.22	70	1	61,76	533,1279	-2,01	C25H24O13	285,0756 270,0515 253,0510 225,0545 242,0553	Dihydroxy methoxy-(iso)flavone malonate-hexoside derivative	(de Rijke et al., 2006; Staszków et al., 2011)
23.94	61	17	3,51	255,0640	-4,67	C15H10O4	255,0638 137,0209 145,0263 119,0462 93,0307	Dihydroxy(iso)flavone	(de Rijke et al., 2006)
24.02	86	2	37,18	563,1376	-3,43	C26H26O14	315,0834 300,0593 283,0562 255,0645	Dihydroxy- dimethoxy-(iso)flavone malonate-hexoside derivative	(de Rijke et al., 2006; Staszków et al., 2011)
24.78	70	1	90,39	563,1376	-3,43	C26H26O14	315,0868 207,0628 175,0371 300,0625 147,0419	Dihydroxy- dimethoxy-(iso)flavone malonate-hexoside derivative	(de Rijke et al., 2006; Staszków et al., 2011)
25.19	32	9	3.75	299.0907	-2.34	C17H14O5	299,0955 284,0663 93,0674 256,0754	Dimethoxy-hydroxy(iso)flavone	(Staszków et al., 2011)

							166,0265		
26.18	74	7	9,93	517,1324	-3,19	C25H24O12	269,0809 254,0608 237,0551 213,0890 107,0488	Hydroxy-methoxy(iso)flavone malonyl hexose	(Farag et al., 2007; Staszków et al., 2011; Sumner et al., 1996)
26.79	1999	287	6,97	517,1324	-3,19	C25H24O12	269,0804 254,0569 213,0902 237,0544 226,0615	Hydroxy-methoxy-(iso)flavone malonate-hexoside derivative	(Staszków et al., 2011; Sumner et al., 1996)
26.90	593	50	11,85	547,1433	-2,41	C26H26O13	299,0914 284,0680 256,0706 239,0703	Dimethoxy-hydroxy(iso)flavone malonate-hexoside derivative	(Staszków et al., 2011)
27.55	46	nd	treated only	533,1272	-3,26	C25H24O13	151,0376 123,0401 285,0806	Unknown compound	
28.35	611	100	6.10	519,1478	-3,70	C25H26O12	137,0575 271,0961 161,0581 123,0421 201,1603	Hydroxy methoxy-pterocarpan malonate-hexoside derivative	(Farag et al., 2007; Staszków et al., 2011; Sumner et al., 1996)
28.50	32	nd	treated	329,1011	-2,70	C18H16O6	329,1008 313,0722	Unknown compound	

			only				285,0746 295,0591 267,0649		
30.18	387	105	3,69	299,0904	-3,34	C17H14O5	299,0915 284,0663 256,0721 132,0539 227,0699	Dimethoxy-hydroxy(iso)flavone	(de Rijke et al., 2006; Staszków et al., 2011)
31.13	73	19	3,86	271,0954	-4,02	C16H14O4	137,0573 109,0621 79,0524 123,0413 161,0578	Pterocarpan	(Farag et al., 2007; Staszków et al., 2011)
36.48	23	nd	treated-only	511,1372	-3,01	C30H22O8	137,0214 375,1088 439,2446 265,0972	Hydroxylated chalcone dimer	(de Rijke et al., 2006)

3.4. Gene expression analysis

Normalized relative gene expression quantities were measured from genes involved in polyamine metabolism, flavone synthesis and the glutathione redox cycle (**Table 3-3**). For genes involved in polyamine synthesis, most of the targeted genes showed an increased expression upon Cd exposure. Among these, the changes in expression of *arginine decarboxylase* (*ADC*) and *S-adenosylmethionine decarboxylase* (*SAM DC*) were insignificant. In addition, *diamine oxidase* (*DAO*) had a significantly decreased expression in Cd-exposed *M. sativa* stems. All assessed expression levels of genes involved in flavone synthesis were significantly higher in response to Cd exposure, apart from *chalcone synthase* (*CHS*). The expression of genes involved in the biosynthesis of GSH were stimulated such as *homoglutathione synthase* and *glutathione synthase 2*, however, levels of genes involved in the turnover between oxidized and reduced GSH did not show significant changes in response to long-term Cd exposure (*glutathione reductase 1* and *2)* with the exception of *monodehydroascorbate reductase*, for which a significant increased expression change was determined.

4. Discussion

The objective of this study was to determine the effect of long-term Cd exposure (10 mg kg^{-1} soil DW) on the profile of primary and secondary metabolites. Plant growth was negatively influenced by Cd in the juvenile plant stage but plants phenotypically recovered when they were more mature and consequently no difference in the total biomass produced was observed at the end of the experiment (Gutsch et al., 2018) (chapter 1 part 1). In agreement with these results, previous studies undertaken in rice, *M. sativa* and *Trifolium repens* L reported a pronounced impact of heavy metal exposure during germination and early plant growth phases, while the impact was only minor in later growth stages (Chou et al., 2011; Peralta-Videa et al., 2004; Wang and Song, 2009). This observation might result from the establishment of a new steady-state, which permits acclimation and allows *M. sativa* to grow under Cd stress. By studying the metabolic profile further insights can be gained about the key processes and molecules involved in such an acclimation.

Table 3-3: Changes of normalized relative gene expression quantities in *M. sativa* stems upon Cd exposure.
Normalized expression values are given relative to the control set at 1.00. Values are given as an average of five replicates. A *t*-test was performed to determine the significance ($p \leq 0.05$). Green significantly up-regulated; red significantly down-regulated.

gene annotation	contig ID *M. sativa*	rel. norm. gene expression	*p*-value
Polyamine synthesis			
arginine decarboxylase	46745	1.123	0.548
diamine oxidase	14769	0.800	0.042
glutamate decarboxylase	103359	1.685	0.019
SAM decarboxylase	9152	1.141	0.459
spermidine synthase	5341	1.213	0.042
spermine synthase	92526	1.341	0.032
Flavone synthesis			
chalcone isomerase	60809	1.809	0.019
chalcone reductase	59796	2.753	0.019
chalcone synthase	99463	1.247	0.371
isoflavone synthase	10252	2.699	0.019
Glutathione synthesis and redox cycle			
homoglutathione synthetase	(Cui et al., 2012)	1.355	0.01
γ-glutamylcysteine synthetase	(Cui et al., 2012)	0.959	0.361
glutathione reductase 1	(Cui et al., 2012)	1.105	0.071
glutathione reductase 2	(Cui et al., 2012)	1.043	0.681
monodehydroascorbate reductase	(Cui et al., 2012)	1.177	0.048
glutathione synthetase 2	10444	1.384	0.004

4.1. Cadmium induces changes in the abundance of amino acids and amino acid-derived molecules

Amino acids can be classified according to their side chain into charged, polar uncharged and hydrophobic. After long-term Cd exposure, several AA with polar, uncharged side chains (L-Ser, L-Thr and L-Tyr) significantly increased, while L-Asn and L-His significantly decreased about 3-fold and 3.8-fold respectively (**Figure 3-3)**. Asparagine (Asn) was the most abundant AA in *M. sativa* stems (Ctr: 26.79 µmol g^{-1} DW; Cd: 8.83 µmol g^{-1} DW) observed in this study. Asparagine was identified as the dominant amino acid in xylem sap of *M. sativa* and represents the primary transport form of nitrogen (Kim et al., 1993). A decrease can be a reflection of an interference with nitrogen fixation caused by Cd exposure (Marino et al., 2013). Nevertheless, Asn accumulation under Cd stress was observed in roots and shoots of *Lupinus albus* (Costa and Spitz, 1997) and in tomato leaves (Hédiji et al., 2010). Asparagine, similar to His, is a metal chelator, forming complexes with metal ions and thus contributes to detoxification (Bottari and Festa, 1996; Krämer et al., 1996), thereby conferring tolerance to the plant. Roots are the main organ of Cd accumulation in *M. sativa* after long-term Cd exposure (Gutsch et al., 2018) (chapter 1 part 1) and detoxification is primarily important at the root-cell level rather than in stems and an elevated requirement of complexing molecules such as Asn and His can be assumed. Nevertheless, the quantification of AAs and primary metabolites in *M. sativa* leaves revealed higher contents of Asn and His upon long-term Cd exposure (**Table S3-4**). Accordingly, accumulation of Pro was observed in leaves of *M. sativa* after long-term Cd exposure accompanied by an increased tyramine content (**Table S3-4**), an aromatic monoamine which is believed to be involved in the regulation of Pro accumulation (Aziz et al., 1998) under stress condition and which was reported to accumulate in response to Cd stress (Ghabriche et al., 2017). However, contrary to leaves Pro levels decreased in stems (Ctr: 9.46 µmol g^{-1} DW; Cd: 6.07 µmol g^{-1} DW; *p*-value = 0.152; **Table S3-3**) as well as the level of hydroxyproline (**Figure 3-3**). Proline is a highly important AA during plant stress responses and its accumulation under heavy metal stress was reported in various plant species (Costa and Spitz, 1997; Dobra et al., 2010; Schat et al., 1997). Exogenously applied, proline alleviates

stress symptoms and maintains nutrient uptake (Ali et al., 2008). It can complex with Cd (Sharma et al., 1998) and furthermore possesses ROS scavenging potential by directly reacting with H_2O_2 and $^{\bullet}OH$ to form stable radical adducts of proline and hydroxyproline (Liang et al., 2013), thereby reducing ROS levels (Rejeb et al., 2014). The here made observations in *M. sativa* stems are in accordance with previous observations made in *Noccaea caerulescens* and in tomato leaves upon long-term Cd exposure, were decreases in Pro were reported over time (Hédiji et al., 2010; Zemanová et al., 2013).

Obviously, leaves and stems of *M. sativa* showed distinct AA profiles after long-term exposure to Cd and differences in AA levels in different organs can play a significant role during adaptation to Cd stress (Zemanová et al., 2014). A study on two related *Asteraceae* species revealed distinctive AA profiles in roots, stems and leaves. While the total content of free AAs was highest in the stems in the hyperaccumulating species, free AAs strongly accumulated in the leaves of the non-tolerant species exposed to Cd (Zhu et al., 2018) suggesting a different strategy in the defence response to Cd. In the present study, *M. sativa* accumulated Asn, His and Pro in leaves rather than in stems. The leaf tissue consists of many metabolically very active cells, while this is less the case for stems. Asparagine, His and Pro have a high potential for metal chelation and ROS scavenging and their accumulation in leaves can help to protect and maintain photosynthesis. The protection of stem tissue, however, probably underlies different mechanisms and long-term Cd exposure significantly increased the content of the polyamines Put, Spd and Spm (**Figure 3-4**) in *M. sativa* stems as well as the expression levels of the genes *SpdS* and *SpmS* that are involved in their biosynthesis (**Table 3-3**). By contrast, the Put level in *M. sativa* leaves decreased in response to Cd (**Table S3-4**) but the change was not significant. Polyamines are involved in plant responses to multiple biotic and abiotic stresses (Gupta et al., 2013) such as Cd exposure (Nahar et al., 2016). Alterations in polyamine levels in response to different Cd concentrations were monitored over time in *Helianthus annuus*, showing a concentration- and time-dependent accumulation of Put, Spd and Spm (Groppa et al., 2007a). By applying exogenous polyamines, the toxic effects of Cd were alleviated (Zhao and Yang, 2008), decreasing the production of ROS, preventing lipid

peroxidation (Yang et al., 2013) and permitting stress tolerance. During plant stress responses to heavy metals PAs are important as antioxidants and signalling molecules (Hussain et al., 2011). Their accumulation in *M. sativa* stems upon long-term Cd exposure might attenuate oxidative stress, preventing ROS-induced cellular damage and finally allowing *M. sativa* to tolerate the imposed stress. Moreover, PAs are important for cell membrane- and cell wall stabilization (Geuns et al., 1997) and therefore their accumulation in stems may help to maintain cell integrity during Cd exposure.

4.2. Cd increases the abundance of isoflavones

The metabolic profile in stems of *M. sativa* plants exposed to Cd was determined via UHPLC-HRMS. Almost all of the identified metabolites belong to the flavone family and accumulate in response to Cd exposure with only a few exceptions being of less abundance (*N*-acetyl-hexosamine derivate, tetrahydroxy(iso)flavone-hexoside, trihydroxy(iso)flavone conjugate). However, all identified (iso) flavones were conjugated with a hexoside. Those that are accumulating in response to Cd exposure are hydroxylated or dihydroxylated (**Table 3-1** and **Table 3-2**). A higher abundance of isoflavone glycoconjugates malonylated at different positions of the sugar moiety was observed upon Cd exposure in roots of lupine (*Lupinus luteus*) (Pawlak-Sprada et al., 2011b). Naturally, flavonoids and isoflavonoids are glycoconjugated (Staszków et al., 2011) and this glycosylation can be crucial for their bioactivities (Liu et al., 2002). Glycosylation increases solubility as well as stability of (iso)flavones and promotes their uptake into the vacuole and facilitates their transport through membranes (Zhao and Dixon, 2010). The results in the present study together with the study from Pawlak-Sprada et al. (2011b) underpin the importance of isoflavone glycosylation during Cd stress, permitting an effective antioxidant function by providing binding sites for trace metals (Pietta, 2000). Such metal complexes are stored in the vacuole for detoxification (Martinoia et al., 2007) and it is thought that glycoconjugation might be essential in this process given that it promotes vacuolar uptake. Furthermore, isoflavones are able to scavenge ROS and prevent oxidative stress, with hydroxylated and dihydroxylated flavones effectively contributing to this function. Taken together, cumulative evidence suggests that the chemical structure of isoflavones determines their

bioactivity and antioxidative efficiency (Montesinos et al., 1995; Tsuchiya, 2010).

Flavonoids and isoflavonoids derive from the phenylpropanoid pathway, whereby isoflavones are nearly exclusively produced in *Fabaceae* such as *M. sativa*. The precursor molecule for this pathway is Phe, which is converted by phenylalanine ammonia lyase (PAL) into cinnamic acid. In two consecutive steps catalysed by cinnamate-4-hydroxylase (C4H) and 4-coumarate ligase (4CL), 4-coumaroyl-CoA is formed, branching off either into the monolignol biosynthetic pathway or into the flavonoid biosynthetic pathway (**Figure 3-1**). Stress stimulates the phenylpropanoid pathway (Dixon and Paiva, 1995) and increases lignification of the cell wall (Kováčik and Klejdus, 2008; Rahoui et al., 2017). However, when the lignin content was analysed in stems of *M. sativa* after long-term Cd exposure, neither changes in the content nor in the composition were observed (Gutsch et al., submitted) (chapter 2). Nevertheless, the expression of the biosynthesis genes *C4H* and *4CL* significantly increased and an increasing trend in the gene expression of *PAL* was detected upon Cd exposure (Gutsch et al., submitted) (chapter 2). It is, however, noteworthy that the activity of PAL does not necessarily correlate with the accumulation of its RNA transcript. Moreover, the activity is clearly driven by the availability of its substrate (Da Cunha, 1987) and an increased Phe level in the stems of Cd-exposed *M. sativa* was assessed by two independent methods (**Table 3-1, Figure 3-3**). Furthermore, the activity of PAL is positively influenced by ethylene and as was recently shown *(*Gutsch et al., submitted) (chapter 2) ethylene biosynthesis is stimulated in *M. sativa* stems by Cd exposure. The accumulation of isoflavones in the present study was accompanied by an increased expression of genes of their biosynthetic pathway (**Table 3-3**), suggesting an enhancement of the phenylpropanoid pathway in favour of secondary metabolite synthesis rather than lignin synthesis. This is in accordance with previous observations made in lupine seedlings under Cd stress (Pawlak-Sprada et al., 2011a, 2011b).

In their capacity as ROS scavengers, isoflavones limit the oxidative stress in *M. sativa* and contribute to Cd detoxification, which renders them essential during the acclimation process and in the establishment of the new steady-state, which allows *M. sativa* to grow under Cd stress.

4.3. Abscisic acid supports tolerance acquisition to Cd

Abscisic acid (ABA) is essential during the plant life cycle, controlling multiple physiological processes such as seed dormancy and development and promoting stomatal closure under conditions in which plants water loss needs to be controlled. Its increased synthesis during stress is well known and therefore ABA is often considered as a plant stress hormone (Vishwakarma et al., 2017). In the present study, an elevated level of ABA in stems of *M. sativa* upon long-term Cd exposure was observed (**Table 3-1**). Studies on *Brassica napus* L and *Solanum photeinocarpum* demonstrated increases in biomass production when ABA was applied, alleviating the growth inhibiting effects of Cd (Meng et al., 2009; Wang et al., 2016). Abscisic acid, furthermore, positively affects the chlorophyll content in Cd-stressed plants (Hsu and Kao, 2003; Meng et al., 2009) and its application under Cd stress decreases the plant transpiration rate (Hsu and Kao, 2003). Transpiration is the motor for Cd translocation from roots to shoots (Salt et al., 1995) and a low transpiration enforces Cd accumulation in the roots, thereby lowering the concentration in the aerial parts as was observed in *M. sativa* upon long-term Cd exposure (Gutsch et al., 2018) (chapter 1 - part 1). Under conditions which include a decreased transpiration rate and lowered Cd content following ABA application, plants also show an increasing tolerance against Cd (Hsu and Kao, 2003). Abscisic acid is important during systemic acquired acclimation (SAA) of plants and its accumulation is initiated by elevated ROS levels. Eventually, ABA signalling initiates stomatal closure, which plays a crucial role in the process of SAA (Mittler and Blumwald, 2015). ROS homeostasis and stomatal closure are modulated by the flavone content, which in turn is increased by ethylene (Watkins et al., 2017). Cadmium initiates ethylene biosynthesis and signalling in stems of *M. sativa* as previously reported (Gutsch et al., submitted) (chapter 2) by using the same sample material and increasing levels of isoflavones in response to Cd were assessed in the present study (**Table 3-1, Table 3-2).** Therefore, acclimation of *M. sativa* in response to long-term Cd exposure must be a tightly regulated interplay between ROS accumulation, antioxidant activity of isoflavones as well as ethylene and ABA signalling.

5. Conclusion

A combined approach of targeted and untargeted metabolic analysis was used to determine the effect of long-term Cd exposure on primary and secondary metabolites in *M. sativa*. Control and Cd-exposed plants can be discriminated by their AA pattern, whereby differences in the accumulation of several AA appeared between stems and leaves. Asparagine, His and Pro increased in leaves; on the other hand, PAs (Put, Spm and Spd) increased in stems upon Cd exposure but Put level decreased in leaves. This suggests differences in the cellular responses to Cd stress in different tissues of *M. sativa*, which differ in their metabolic activity. The increases of AAs with a high potential in detoxification is more important in leaves, which have a high metabolic activity. Stems, as a less active tissue rely on polyamines as a protecting mechanism. Additionally, Cd stimulated the phenylpropanoid pathway in favour of flavonoid/isoflavonoid synthesis rather than lignin and flavonoid conjugates increased under long-term mild Cd exposure. Flavonoids and isoflavones have a designated function in ROS scavenging, thereby reducing oxidative stress and cell damage. However, the important antioxidant GSH plays a minor role during long-term Cd exposure and it was demonstrated in previous studies that GSH synthesis often does not correlate with plant stress tolerance (de Knecht et al., 1994; Delhaize et al., 1989). Additionally, ABA was highly abundant in Cd-exposed plants. Our results emphasise the importance of primary and secondary metabolites for acclimation potential during long-term Cd exposure and their distinct accumulation in different plant tissues. The establishment of a new metabolic homeostasis enables *M. sativa* to perform well under inept conditions as no differences in phenotype or biomass production was observed.

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Highlights and Perspective Chapter 3

So far, it was demonstrated that long-term Cd exposure influenced the cell wall proteome in stems and leaves of *M. sativa*. Several proteins, which are determining for the cell wall structure changed in their abundance. Thereby, the abundance of PME increased in stem tissue due to the applied stress and it is evident from literature that the protein plays a crucial role during alterations in the cell wall in response to environmental stress (chapter 1). In fact, an increased activity of PME was demonstrated in *M. sativa* stems and its enzymatic activity catalysis the demethylation of homogalacturonan, thereby creating binding sites for Cd in the pectic network, which would promote the immobilization of Cd in the cell wall and consequently restrict its sequestration throughout the plant (chapter 2).

The determination of the cell wall composition in stems revealed a Cd-induced shift in the constitution of side chains towards xylose, which supports cell wall stiffening and limits the access of cell wall proteins to their target sites. This furthermore supports the hypothesis of a changing cell wall towards the establishment as a physical barrier in response to Cd exposure. However, although long-term Cd exposure stimulates the phenylpropanoid pathway, no changes in the lignin content and composition of the cell wall appeared (chapter 2).

The most remarkable observation was the recovery of exposed plant during the long-term exposure to Cd. While plant growth was fairly restricted in juvenile plants, no phenotypical difference between control and exposed plants was observed in mature plants and the produced plant biomass was the same at the end of the experiment. Obviously *M. sativa* reached a new steady-state, which enables the plant to perform well under this unsuitable condition.

This said, the following question arose. Do changes in the metabolic profile convey acclimation of *M. sativa* and which molecules are important during this process? To tackle the question, targeted and untargeted metabolomic approaches were applied to decipher changes in the metabolic profile of

M. sativa in response to long-term Cd exposure and results were presented in chapter 3. Main results showed:

- Increasing content of isoflavone conjugates in stems of *M. sativa* upon Cd exposure
- Distinct AA pattern between control and Cd-exposed stems of *M. sativa*
- Important stress responsive AAs such as Asn, His and Pro decreased in stems, but showed inverse results in leaves of *M. sativa*, where Asn, His and Pro content increased
- A tissues specific response to environmental stress is suggested where AAs play a minor role in stems but are more important in leaves
- Increased content of Phe in stems due to long-term Cd exposure, which is the key molecule into the phenylpropanoid pathway

In chapter 3 an increased content of isoflavones in *M. sativa* stems was determined. Theses secondary metabolites origin from the phenylpropanoid pathway and the stimulation of the pathway in response to long-term Cd exposure was discussed in chapter 2. In this regard, the observed Cd-induced stimulation favours the biosynthesis of secondary metabolite rather than lignin biosynthesis and a high importance of isoflavones for the acclimation of *M. sativa* during long-term Cd exposure can be assumed.

However, when determining the nutrient content and distribution in *M. sativa* plants in response to long-term Cd exposure, it was observed that K and Na had an increasing content in roots, stems and leaves, while the content of all other investigated nutrients increased in the roots but decreased in the aerial plant part. This observation brought up the question, if the selective accumulation of certain nutrients such as K and Na in aerial plant parts is of protective gain against Cd exposure. In this regard, an experimental set-up was designed, to investigate the possible protective role of K and Na in *M. sativa* during long-term Cd exposure and preliminary results will be presented in chapter 4.

Chapter 4 – Are potassium and sodium beneficial for the acclimation of *Medicago sativa* to cadmium exposure?

1. Introduction

Pollution of the ecosphere is a serious issue of major public concern. When it comes to soil pollution, an estimated number of 2.5 million potentially polluted sites in Europe are assumed, with less than half of this number currently identified. Waste disposal and treatment (38.1 %) as well as industrial and commercial activities (34 %) are the key sources of soil contamination. Although the chemical nature of pollutants is diverse, their stability in the ecosystem makes heavy metals a major problem. They are responsible for 34.8 % of the affected sites (http://www.eea.europa.eu/data-and-maps/indicators/progress-in-management-of-contaminated-sites-3/assessment). Among heavy metals, cadmium (Cd) is one of the most frequent pollutants. Although being an unessential metal for plant and animal metabolism, it can enter the plant via the roots and is translocated throughout the plant by a variety of transport systems (Clemens et al., 2002). Thereby, Cd competes with the uptake and translocation of essential nutrients like potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), copper (Cu) and zinc (Zn) for the same transmembrane carriers, thus influences the global nutrient content of plants (Nazar, 2012). Alterations in the nutrient content in different plants have been reported in response to Cd exposure (Yang et al., 1996; Zhang et al., 2002).

Cadmium inhibits plant growth and can be lethal in a dose-dependent manner. Symptoms of toxicity upon Cd exposure are due to multiple interactions at the cellular and molecular level. Cadmium stimulates the formation of reactive oxygen species (Cuypers et al., 2010), alters the cell wall structure (Loix et al., 2017), disrupts photosynthesis (Ekmekçi et al., 2008; Wang et al., 2014) and the plant water status (Perfus-Barbeoch et al., 2002). Furthermore, it interferes with protein activity (Kieffer et al., 2009; Romero-Puertas et al., 2002) and

elicits DNA damage (Gichner et al., 2004). Through the application of different substances, those symptoms of toxicity can be alleviated. For instance, the plant hormone salicylic acid has beneficial effects on the overall plant performance when applied to Cd-exposed *Phaseolus vulgaris* and *Cannabis sativa* (Semida et al., 2015; Shi et al., 2009). Furthermore, through the exogenous application of other plant growth regulators such as jasmonic acid, gibberellic acid and abscisic acid (ABA) Cd-induced toxicity was reduced in seedlings of *Brassica napus* L. (Meng et al., 2009) and it is demonstrated that ABA restricts Cd uptake and upregulates antioxidant enzymes (Han et al., 2016). Additionally, the presence of other substances can have a beneficial effect on plants exposed to heavy metals. For instance, the alleviating effects of silicon are discussed in literature (Adrees et al., 2015) and it was demonstrated that silicon supply mitigates Cd toxicity in peanut seedlings, which correlates with a reduced Cd content in the shoot, an altered Cd distribution in leaves and a stimulation of antioxidative enzymes (Shi et al., 2010). Potassium as an essential nutrient for plants has ameliorating effects when plants are subjected to various abiotic stresses (Cakmak, 2005), whereby the supplementation with K during Cd exposure alleviates reduced growth, photosynthesis rate and nutrient uptake in soybean (Shamsi et al., 2010) and improves physiological and biochemical processes in Cd-stressed *Vicia faba* (Siddiqui et al., 2012). Interestingly, although soil salinity caused by elevated levels of NaCl is generally considered as an abiotic stressor (De Azevedo Neto et al., 2006), low concentrations in a range of 50 mM counteract Cd toxicity and reduces the Cd content inside the plant (Han et al., 2012; Zhang et al., 2013). Besides the influence on the plant physiology through the application of certain nutrients or compounds, also the soil quality can play a significant role during the alleviation of heavy metal toxicity. Liming as a classical alkalinizing treatment can be used as a tool to manipulate the soil properties and influences the mobility of essential nutrients as well as immobilizes toxic heavy metals such as Cd in the soil (Bolan et al., 2003). Another promising approach is the addition of biochar on contaminated soils, which reduces the phytoavailability of heavy metals and significantly increases plant biomass production as it improves the fertility of the soil (Paz-Ferreiro et al., 2014).

In a recent study, *Medicago sativa* was exposed to long-term Cd stress (10 mg kg^{-1} soil) during which the plants were cut and regrown as it is agricultural practice. Thereby, a strong growth inhibition of Cd-exposed plants was observed at the end of the first growth cycle but plants fully recovered during the second growth cycle and no difference in biomass production was observed between Cd-exposed and control plants at the time of sampling (Gutsch et al., 2018) (chapter 1). In this regard, an initial study was conducted on the mineral composition of different plant parts upon Cd exposure (roots, stems, leaves) in order to investigate the effect on the distribution of nutrimental elements. In addition, supplementation of monovalent ions (K, Na, Li) was evaluated on the extent of Cd by measuring the leaf fluorescence and analysing the leaf proteome to investigate the impact at the metabolic level.

2. Material and Methods

2.1. Plant growth and sampling

Medicago sativa L. seeds, cultivar Giulia, were inoculated with *Sinorhizobium meliloti* and sown on Cd-contaminated and control soil, respectively. A detailed description of the growing and sampling conditions is given in Gutsch *et al*. 2018 (chapter 1). Following the sampling of the second cut, Hoagland solution (Phyto Technology Laboratories, LLC™) was prepared, diluted ten times and supplemented with 30 mM Na, 30 mM K and 30 mM Li, respectively. Furthermore, a mixture of 10 mM Na, K and Li was prepared. Thirty six pots of Cd-exposed and of control plants were divided in four conditions and watered with 20 mL of the appropriate supplemented Hoagland solution. Additionally, the same number of pots with plants growing on Cd-polluted soil and control soil were watered with 1/10 Hoagland solution alone. Sampling was done after six weeks and slit up into leaves, stems, small roots and big roots. All samples were directly frozen in liquid nitrogen and kept at -80°C till further use.

2.2. Leaf water content

Leaves of *M. sativa* were sampled in five replicates and the biomasses measured prior to drying (76°C, three days). Afterwards, the dry weight was determined and the water content was calculated (**Equation 1**).

$$L = 100 \times \frac{Mf - Md}{Mf}$$

Equation 1: Calculation of the water content. M_f = fresh weight; M_d = dry weight; L= water content.

2.3. Photochemical efficiency analysis

The fluorescence from photosystem (PS) II was recorded at wavelength around 650 nm with a Plant Efficiency Analyser (Hansatech). Thereby the light intensity was set at 80 %. In preparation of the measurements, leave-clip devices were installed on ten randomly picked leaves from each condition 30 min prior to the measurements to allow dark adaptation. Leaves were homogenously illuminated with emitting diodes to reach the maximal fluorescence. The fluorescence signal is received by a sensor head, recorded and displayed on the device. To draw conclusions on the plants' photochemical efficiency for each condition, the F_v/F_m parameter was calculated, which is widely used to indicate the maximum quantum efficiency of PS II and considered to be a sensitive indicator for plant photosynthetic performance. Thereby, F_v represents the variable fluorescence and F_m the maximum fluorescence.

2.4. Mineral content analyses by ICP-MS

Approximately 250 mg of plant organs (leaves, stems, roots) were oven-dried for 36 h at 60°C and mineralized in 7 mL nitric acid and 3 mL hydrogen peroxide. A Multiwave PRO microwave reaction system was used according to the manufacturer's instructions (Anton Paar GmbH). After mineralization, the sample volume was adjusted to 25 mL with MilliQ Water (Millipore). The mineral content was determined by inductively coupled plasma-mass spectrometry (ICP-MS). The concentration of eleven elements [manganese (Mn), iron (Fe), cobalt (Co), copper (Cu), zinc (Zn), molybdenum (Mo), sodium (Na), magnesium (Mg), potassium (K), calcium (Ca) and cadmium (Cd)] from ten biological replicates were analysed. For statistical analyses, outliers were removed by using the interquartile range (IQR) approach (removal of values above or below 1.5 IQR) and an average concentration from all nutrients was calculated for the two conditions. Significant concentration differences between both conditions were analysed by a *t*-test (p-value ≤ 0.05).

2.5. Label-free protein quantification

2.5.1. Protein extraction and digestion

The soluble proteins from five replicates of *M. sativa* leaves were extracted with TCA/phenol-SDS as described before (Wang et al., 2003) and solubilised in lysis buffer (7 M urea, 2 M thiourea, 0.5 % CHAPS). The protein concentration was determined with the RCDC protein assay (Biorad) according to the manufacturer's instruction. In preparation of the in-gel digestion prior to LC-MS, 20 µg protein from each sample were run on a Criterion™ precast 1-D gel (Bio-Rad) following manufacturer's instruction for 10 min with 200 V. Each band was cut into cubes and split into two technical replicates. Gel bands were reduced with 100 mM ammonium bicarbonate (AmBic) supplemented with 10 mM dithiothreitol (56°C, 30 mins), followed by alkylation with 100 mM AmBic supplemented with 55 mM iodoacetamide (room temperature ,20 min in the dark). The destaining of the gel was done with 50 mM AmBic in 50 % methanol (37°C, 20 min) and the solution was subsequently replaced by 200 mM AmBic (37°C, 20 min). Proteins were digested with trypsin (5 ng μl^{-1} in 20 mM AmBic) over night (37°C). The peptides were extracted with a solution containing 50 % acetonitrile (ACN) and 0.1 % trifluoroacetic acid (TFA) (37°C, 10 min) and the step repeated. Peptides were dried in a vacuum centrifuge and kept at -20°C till further use.

2.5.2. Peptide separation and quantification

Label-free protein quantification was done with liquid chromatography coupled to mass spectrometry (LC-MS) using a NanoLC-2D system (Eksigent) coupled to a tripleTOF 5600+ MS (Sciex). After desalting and enrichment on a C18 pre-column (PepMap™ 100, 5 µm, 5 mm x 300 µm I.D., Thermo Scientific), the peptides were separated with a C18 reverse phase column using the following binary gradient: solvent A: 0.1 % formic acid (FA), solvent B: 80 % ACN, 0.1 % FA; flow rate of 300 nL min^{-1}. Peptides were eluted from the column with solvent B at 5 % to 55 % for 40 min. The concentration of solvent B was subsequently increased up to 100 % to wash the column prior to re-equilibration. The LC was coupled to the mass spectrometer with a NanoSpray III source. Collision-induced dissociation (CID) fragmentations for MS/MS spectra acquisition used the automatically adjusted system of rolling collision energy voltage. A full MS

scan was performed with the scan range of 300 – 1250 m/z (250 ms accumulation time) and the 20 most intense precursors were selected for fragmentation. Acquired CID spectra were submitted to Mascot-Daemon (v2.4.2, Matrix Science) and searched against the alfalfa EST database downloaded from the Samuel Robert Noble website (675750 sequences, 304231702 residues, released on the 3rd of November 2015) (O'Rourke et al., 2015) for identification using the following parameters: two missed cleavages; mass accuracy precursor: 20 ppm; mass accuracy fragments: ± 0.5 Da; fixed modification: carbamidomethyl (C); dynamic modifications: oxidation (M and P), acetyl (protein N-term), didehydro (F) and tryptophan to kynurenine. Proteins were considered as identified when at least two peptides passed the Mascot-calculated score of ≥ 25, present in at least 80 % of the replicates. Mascot data were imported into PROGENESIS QI software for proteomics (NonLinear Dynamics) for quantitative analysis. A changes in abundance was considered as significant with a *p*-value of ≤ 0.05 and a fold-change of ≥ 1.5. Only unique peptides were considered.

3. Results

3.1. Mineral content

The mineral composition of roots, stems and leaves was analysed using ICP-MS and changes in the profile of control and Cd-exposed plants were revealed in all organs. All considered minerals accumulated in the roots upon Cd exposure possibly as a result of translocation blockage to the aerial part. Interestingly, an accumulation of the monovalent cations K and sodium (Na) was observed in stems and leaves of *M. sativa* in addition to their accumulation in the roots (**Figure 4-1**). Regarding their physiological importance and based on previous studies (Cakmak, 2015; Zhang et al., 2013) on the alleviative effects of K and Na supplementation on Cd toxicity in plants, it can be hypothesised that the increasing content of K and Na in *M. sativa* is a direct response to Cd put in place by the plant as a protecting mechanism to alleviate Cd toxicity. To our best knowledge, the specific accumulation of these two monovalent ions in response to Cd has not been observed before and could be a proof for the beneficial effects of K and Na in plants without explicit supplementation.

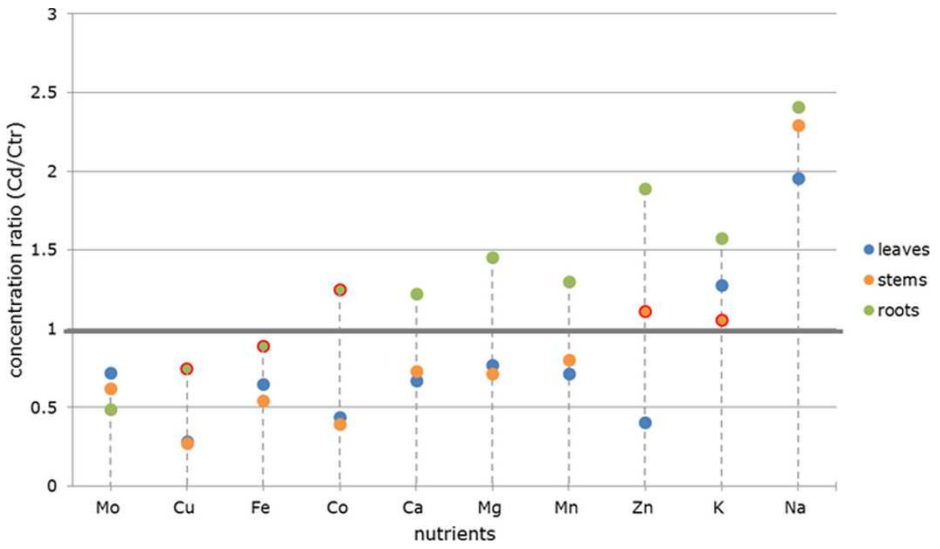

Figure 4-1: Concentration ratios of nutrients between Cd-exposed and control *M. sativa* plants.
Plants were exposed to 10 mg of Cd kg^{-1} soil. Mineral content was determined by ICP-MS. Concentration differences were considered as significant with a *p*-value ≤ 0.05. Insignificant changes are indicated (○). Total values are provided in Table S4-1.

In order to determine how monovalent cations affect *M. sativa* during the exposure to Cd, the experiment was repeated and after the second cut they were watered with 1/10 diluted Hoagland solution to facilitate proper regrowth of the plants as it can be assumed that the soil was exhausted after such a long-lasting growing period. The diluted Hoagland solution was supplemented with the monovalent cations K, Na, lithium (Li) as well as a mixture of these three, respectively, whereby Li is not an essential nutrient but also a monovalent ion. The different solutions were applied on Cd-exposed and control plants. During plant growth, leaf fluorescence was measured to monitor photochemical efficiency under each condition. Leaves, stems and roots were sampled to determine the mineral composition. Leaves are the site of carbon assimilation and primary metabolism, which is essential for the plants and photosynthesis is largely affected by Cd. As an exceptional accumulation of K and Na was observed in leaves and stems of *M. sativa* during the first experiment upon long-term Cd exposure (**Figure 4-1**), the soluble proteome of the leaves was studied in representation of the aerial organs.

3.2. Water content and photochemical efficiency

Significant increase in the leaf water content was determined in Cd-exposed plants without supplementation in comparison to the respective control plants. The supplementation with K, Na, Li or a combination of all ions induced changes in the leaf water content of control and Cd-exposed plants and differences between control and Cd-exposed plants became insignificant. When comparing only the control condition, the leaf water content in *M. sativa* increased significantly in ctr (+Na) and ctr (+Li). No significant changes in the water content were observed among the Cd-exposed plants (**Figure 4-2**).

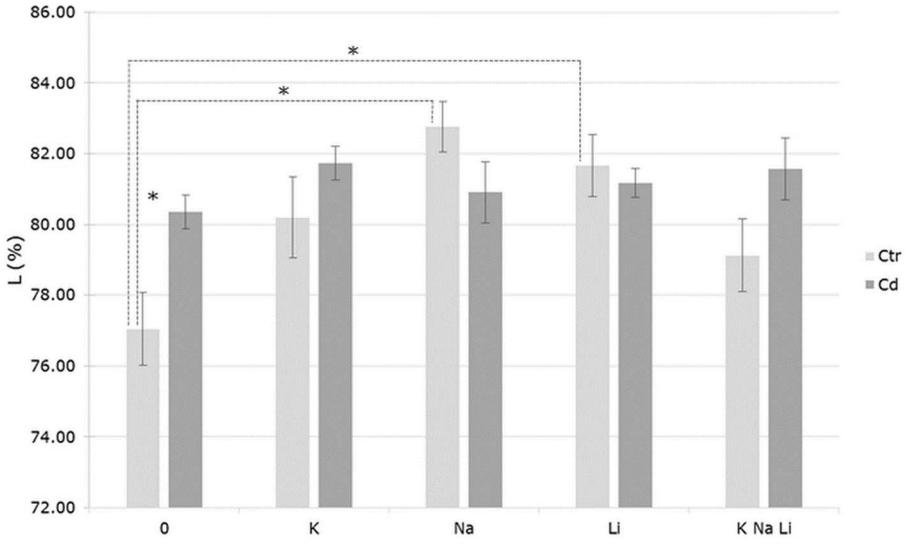

Figure 4-2: Effect of monovalent ions on leaf water content (*L*) from *M. sativa*. Control and Cd-exposed plants were watered once with 20 mL 30 mM of K, Na and Li respectively. Additionally, a solution containing 10 mM of K, Na and Li each was applied. The leaf water content was determined as *L* (%) = 100 x $((M_f-M_d)/M_f)$. Significant changes ($p \leq 0.05$) are indicated (*). Error bars indicate the standard error of the mean (n = 5). M_f = fresh weight; M_d = dry weight.

Cadmium exposure alone significantly reduced the photochemical efficiency in leaves of *M. sativa*. Through the addition of K and Na this effect was alleviated and the efficiency reached the value of the unexposed plants. Also Li and the mix of all ions had an alleviating effect on the efficiency but values did not reach those of control plants and the impact of Cd remained still significantly different when the mix was applied (**Figure 4-3**). The addition of monovalent ions has no impact on the photochemical efficiency in control plants.

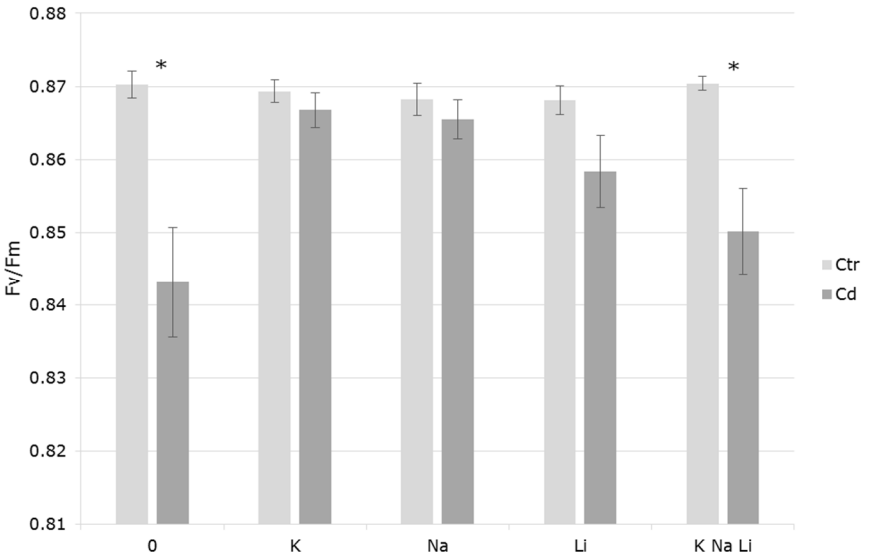

Figure 4-3: Photochemical efficiency (F_v/F_m) of *M. sativa* leaves.
Control and Cd-exposed plants were watered once with 20 mL 30 mM of K, Na and Li respectively. Additionally, a solution containing 10 mM of K, Na and Li each was applied. Significant changes (p = ≤ 0.05) are indicated (*). Error bars indicate the standard error of the mean (n = 10).

3.3. Quantitative protein analysis

Soluble proteins in leaves of *M. sativa* were quantified label free with LC-MS. The addition of K, Na, Li and a mix of the three ions provoked changes in protein abundances in control and Cd-exposed plants. In leaves of control plants 50 (+ K), 58 (+ Na), 28 (+Li) and 46 (+ mix) proteins changed significantly in abundance and in Cd-exposed plants 69 (+K), 44 (+ Na), 32 (+ Li) and 9 (+ mix) (**Figure 4-4**). The addition of monovalent ions, individually or as a mix, induced the accumulation of multiple proteins involved in transcriptional as well as translational processes (classified as miscellaneous) in control as well as in Cd-exposed plants (**Table S4-2**). Furthermore, K, Na and Li induced the accumulation of photosynthetic proteins in control and Cd-exposed plants, which was also observed in control plants when the ion mix was applied. Other proteins that accumulated under all conditions were assigned to defence, oxidation-reduction processes, proteolysis, proteins acting on carbohydrates, proteins possibly involved in signalling, proteins related to lipid metabolism, proteins with interaction domain, proteins of unknown function and transport

activity (**Figure 4-4**, **Table S4-2**). The latter had an increased abundance in leaves of control plants after the addition of K, Na and the mix in which a K^+ transporter-like protein (contig_82176) was identified under all three conditions. Additionally, a magnesium transporter (contig_55579) accumulated in leaves of control plants + Na (**Table S4-2**).

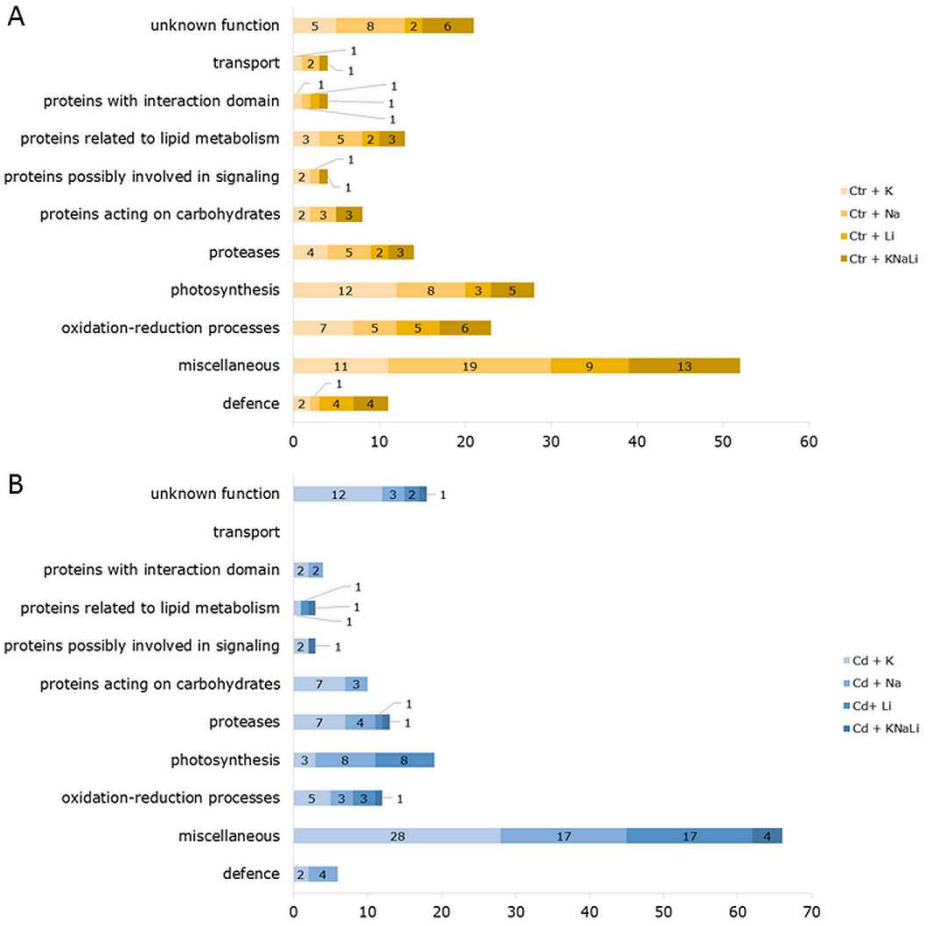

Figure 4-4: Functional classification of significantly changed soluble proteins in leaves of *M. sativa* after supplementation of 30 mM K, 30 mM Na, 30 mM Li and a mix (10 mM K, Na, Li each).
A) control **B)** Cd-exposed. Quantification was done with the PROGENESIS QI software for proteomics (NonLinear Dynamics). A protein was reported as changed significant in abundance with a fold-change of ≥ 1.5 and *p*-value ≤ 0.05.

4. Discussion

In the frame of a study on the effect of long-term Cd exposure on *M. sativa* (chapter 1 to 4), the content and distribution of nutrients between roots, stems and leaves was investigated. All considered nutrients accumulated in the roots when plants are exposed to Cd (**Figure 4-1**) and a concurrent decreasing content in stems and leaves was determined for most of them, except Na and K, which accumulated more in all investigated organs upon Cd exposure as compared to control conditions (**Figure 4-1**). The Na concentration increased more than twice in Cd-exposed plants, whereas the difference for K was less pronounced and insignificant in stems. During the experiment no Na or K was applied to the plants and the observed selective accumulation of both monovalent ions might be a result of an increased uptake and translocation from roots to shoots actively set up by the plant. For their physiological optimum, plants maintain a high cytosolic K/Na ratio. However, they have a certain level of plasticity regarding this ratio within the limits of a physiological, still optimal frame, which allow plants to adapt to environmental conditions and is facilitated through the activity of selective or unselective transport systems (Maathuis and Amtmann, 1999). In the present study, changes in the cellular K and Na levels were observed and a modulation of the K/Na ratio in *M. sativa* can be assumed in response to Cd, towards a new physiological optimum that is required under the applied stress. In this regard, the maintenance of physiological processes is guaranteed and it can be assumed that such an adjustment is part of the defence mechanisms of *M. sativa* in order to counteract Cd toxicity.

However, to investigate the beneficial effects of K and Na, *M. sativa* plants were watered with K and Na supplementation. Furthermore, Li was added as another monovalent ion to determine if especially K and Na are beneficial or if the general accumulation of monovalent ions alleviated Cd toxicity.

The addition of K, Na or Li alone to control plants as well as the application of a mixture did not change the photochemical efficiency of *M. sativa* and the F_v/F_m ratio reached about 0.87 (**Figure 4-3**). This parameter is commonly used to indicate the maximum quantum efficiency of photosystem II and can reach a value of 0.86 for a healthy, non-stressed plant with an optimal photosynthetic

performance (Rohacek, 2002). The here measured photochemical efficiency demonstrate that the addition of monovalent ions to control plants did not cause any disturbance on the overall plant performance. Even in Cd-exposed *M. sativa* plants the F_v/F_m ratio still reached 0.84, which represents an optimal photosynthetic performance, comparable with those obtained in healthy, non-stressed plants (Björkman B., 1987). However, the addition of either of the three monovalent ions to Cd-exposed plants or the application of the mix increased the photochemical efficiency (**Figure 4-3**) and values matched those of control plants when Na or K was added. In fact, *M. sativa* plants did not show any signs of toxicity after long-term exposure with a comparable biomass production for control and exposed plants (Gutsch et al., 2018) (chapter1) and plants established a new metabolic steady-state, which conveyed acclimation to long-term Cd exposure (chapter 3). The here observed F_v/F_m values are an indication for the healthy state of the plants, regardless of the applied Cd stress.

The water status in *M. sativa* leaves was assessed as another physiological parameter, which is determined by three different processes: water uptake, water transport and water loss. Heavy metal stress such as Cd disturbs the plant water status through the interference with all of these process (Barceló and Poschenrieder, 1990). In *M. sativa* leaves a significant increase in water content was reported after long-term Cd exposure (**Figure 4-2**), which might be an effect of Cd-induced stomatal closure (Perfus-Barbeoch et al., 2002). Moreover, the application of K, Na, Li and a mixture of all ions increased the relative water content in leaves of control and Cd-exposed plants. However, changes are not significant (**Figure 4-2**). Alteration in the plant water content upon Cd exposure depends on Cd concentration, whereby excess concentrations led to decreased water content and transpiration rate in *Helianthus annuus* L. when plants were exposed to different metals applied in different concentrations for 48 h (Kastori et al., 1992). While lower concentrations such as 100 ppm Cd increased the leaf water content, higher concentrations (500 ppm Cd) decreased the water content in soil-cultured *Brassica juncea* L., which most likely results from a limited water uptake due to reduced root growth caused by 500 ppm Cd (Singh and Tewari, 2003).

Especially K ions have an important role in physiological processes (photosynthesis, protein synthesis, enzyme activation) as well as biotic and abiotic stress resistance. Potassium contributes to cell membrane stability, osmotic adjustment and play a crucial role in stomatal regulation (Cakmak, 2005; Wang et al., 2013). Potassium attenuates ROS production (Ahmad et al., 2016) and the necessity of sufficient K supply for photosynthesis was demonstrated in tomato plants (Behboudian and Anderson, 1990). In the present study it was observed that K addition increased the photochemical efficiency of Cd-exposed plants (**Figure 4-3**). Furthermore, K addition of control plants induced the accumulation of several proteins involved in photosynthesis and various proteins of the transcriptional mechanism, pointing to a high cellular activity promoting increased plant performance (**Figure 4-4**, **Table S4-2**). Supplementation with K might counteract the negative impact of Cd by rehabilitating metabolic processes, reducing Cd-induced oxidative stress and contributing to the maintenance of photosynthesis by preventing the degradation of chlorophyll and aminolevulinic acid and maintain photosynthetic electron transport (Siddiqui et al., 2012). This physiological effect is supported by the fact that the application of K and Cd resulted in the accumulation of proteins related to transcriptional and translational process, as was also observed in control plants grown under K addition (**Table S4-2**) and points to a higher activity of the cells. However, K did not induce the accumulation of photosynthetic proteins in Cd-exposed plants.

In addition, a positive impact of Na on Cd-exposed *M. sativa* plants was observed (**Figure 4-3**). Sodium is the predominant toxic ion during salinity stress of plants and its uptake disrupts the physiologically optimal K:Na ratio, which inactivates proteins and affects metabolic processes (Hauser and Horie, 2010). However, up to a certain dose abiotic stress can be beneficial for plants ("eu-stress") by activating metabolism and stimulating physiological activity without doing any damage to the plants (Lichtenthaler, 1996) and low doses of NaCl positively affect plant growth and performance. In combination with another abiotic stressor such as Cd, the addition of 50 mM NaCl increased the biomass of *Kosteletzkya virginica* and limited the translocation of Cd into the aerial parts (Han et al., 2012). The addition of 25 mM NaCl to Cd-exposed *A.*

thaliana decreased the accumulation of ROS, improved the plasma membrane integrity and increased the accumulation of metal-chelating compounds (Xu et al., 2010). Besides improving the photochemical efficiency of Cd-exposed *M. sativa* (**Figure 4-3**), Na supplementation increased the water content in leaves of control and Cd-exposed plants (**Figure 4-2**). Furthermore, Na addition to Cd-exposed *M. sativa* increased the accumulation of photosynthetic proteins in the leaves, which most likely contributes to the alleviation of Cd effects on photosynthesis (**Figure 4-4**). Likewise to K, Na increased the abundance of proteins involved in transcriptional and translational processes (**Table S4-2**). Consequently, alleviation of Cd stress by Na supplementation in *M. sativa* underlies a different physiological mechanism than the alleviation through K and it can be suggested that Na increased the overall plant performance by causing a mild stress, which in turn stimulated physiological activity and metabolism. On the other hand, Na can functionally replace K. Sodium can fulfil the biophysical functions of K in plant metabolism, whereby they probably not only substitute but also complement each other (Subbarao et al., 2003). After long-term exposure to Cd an increased content of K and Na was observed in *M. sativa* (**Figure 4-1**). If both ions could adopt each other's functions, their beneficial effects are more intensified resulting in a maintenance of photosynthesis and therefore primary metabolism. However, functional replacement of K by Na in photosynthesis is contradictory (Subbarao et al., 2003).

As for low NaCl concentration, Li-induced toxic effects strongly depend on concentration and plant species (Hawrylak-Nowak et al., 2012; Kalinowska et al., 2013), whereby low Li concentrations can boost plant growth and increase water absorbance, while it has inhibiting effects at higher concentrations. Here, a positive effect on the photochemical efficiency was observed when Li was added to Cd-exposed plants (**Figure 4-3**). However, results on photosynthesis are conflicting. No change in chlorophyll a and b content appeared in sunflowers when plants were subjected to 5-50 mg $(dm^3)^{-1}$, while these parameters decreased in maize when exposed to the same conditions (Hawrylak-Nowak et al., 2012). Likewise, as for the addition of Na, an accumulation of photosynthetic proteins was observed in Cd-exposed *M. sativa* leaves when Li was applied (**Figure 4-4**), which could explain the restoration of the photosynthesis. As for

mild salt stress, an observed increased plant performance is a result of the mild stress independent from a specific physiological role (Lichtenthaler, 1996).

Most described transporters for cellular K uptake are highly selective but these entry routes are known to contribute as well to the uptake of Na (Maathuis and Amtmann, 1999). Also non-selective monovalent cation transporters might play a role in Na and K uptake (Schachtman and Liu, 1999). Although Li is a non-essential and non-physiological element, it is taken up by the plant from its environment. Thereby, similar physicochemical properties of Li to K and Na probably promote the uptake through the same transport systems (Shahzad et al., 2016). In the present study, the treatment with K and Na ions induced the accumulation of a potassium transporter (contig_82176) in leaves of control plants (**Table S4-2**), which might be a result of an increased uptake through the root cells and increase K/ Na levels in the plant.

5. Conclusion

The alleviation of Cd toxicity through K, Na and Li underlies different mechanisms. Potassium is needed for multiple physiological processes in plant cells and a sufficient supply increases the overall plant performance. Sodium and Li are stressors, however, in low doses they provoke a beneficial stress response in plant cells and increase cell activity and metabolism, which results in a positive effect on the overall plant performance. During long-term mild Cd exposure an accumulation of K and Na in *M. sativa* plants was observed, which might be a direct defence response against the provoked stress. The adjustment of the cellular K/Na ratio probably counteracts the Cd stress and has beneficial effects on the physiological performance, which contributes to the acclimation of the plant when exposed to long-term Cd stress. The present results are preliminary and further studies on stems and roots are needed to obtain a comprehensive picture on the effected of K, Na and Li addition on Cd-exposed *M. sativa* plants covering gene expression analysis, protein abundance and the mineral distribution.

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General Discussion and Perspective

1. Scientific background

Plants play a central role in human life as they are key resource for food, feed, fibre and fuel production. They are essential elements for the development of a bio-based economy and sustainable agriculture in a future perspective. However important, plant growth and valorisation potential is significantly affected by environmental stresses of natural and anthropogenic origin. Especially environmental pollution with heavy metals, notably Cd, is of great concern due to its longevity and high toxicity even in minimal amounts. Apart from affecting plant production, Cd poses a serious risk to human health (Sigel et al., 2013), whereby the human diet is the main source of Cd intake, which is facilitated by Cd emission into agricultural soils and its uptake and accumulation by crops (Pan et al., 2010). In the European Union 1,170,000 potentially contaminated sites polluted with different kinds of hazardous substances were identified in 2014 (Van Liedekerke et al., 2014) and 17.3 billion Euro of remediation costs are annually estimated. From that, about 137,000 km^2 of agricultural land is affected, of which 2.56 % show concentrations that would require remediation due to safety considerations (Tóth et al., 2016). Moreover, contaminated sites are not available to their full extent for an economic, ecologic and societal use, limiting the valorisation of the soil. Bearing in mind that the world population is continuously growing, the demands for food production as well as living space are rising and strategies need to be developed in order to gain back the abandoned land. The increased knowledge on how plants are affected when growing on polluted sites will be beneficial to improve their role to fulfil these demands and to pave the way into a green, sustainable future.

The exposure of plants to environmental stressors such as Cd provokes a complex stress response in the plant during which the plant cell wall is believed to play a significant role. Cell wall-embedded proteins maintain the communication of the cell with its environment and the cell wall constitutes a physical barrier against external threats. Its composition, structural characteristics, the abundance and activity of cell wall proteins as well as the

content of lignin and other phenolic compounds are affected in response to Cd exposure as reported in current literature (Douchiche et al., 2007; Gall et al., 2015; Gutsch et al., 2018; Paynel et al., 2009). Thereby, the Cd-induced creation of functional groups in the cell wall confers the ability to bind Cd in its network, which limits the translocation of Cd into the cytosol (Parrotta et al., 2015). Both, the primary and the secondary cell wall utilize different protection mechanisms, whereby negatively charged pectin can efficiently sequester Cd in the primary cell wall while lignin, present in the secondary cell wall, strengthens the cell wall and decreases the permeability for Cd, creating a solid barrier. Cadmium-induced cellular responses such as oxidative stress should not be considered minor as they strongly influence the cell wall, for example the H_2O_2-driven stimulation of cell wall-located peroxidases, which increases the degree of lignification (Loix et al., 2017). As literature provides a strong body of evidence underlining the involvement of the cell wall in response to Cd, it is an important structure to study when discussing the effect of environmental stress on plants, with the aim to identify those molecules that provide the highest protection against Cd and the molecular mechanisms behind these mechanisms.

Stress responses in plants strongly differ in a time- and concentration-dependent manner. During short-term exposure, they only enter in the alarm phase, where a rapid response of the plant can be observed. During long-term exposure on the other side, plants enter from the alarm phase into a stage of resistance, which eventually leads into a regeneration phase and adaptation of the plant to the applied stress (Lichtenthaler, 1998). Therefore, the influence of different durations of exposure on the response of the plant cannot be neglected and needs to be considered when defining the scope and aim of an experiment (Dupae et al., 2014). When transferring laboratory research to reality, it is unlikely to observe acute heavy metal stress as a sudden event in the environment. Soil pollution in moderate concentrations is permanent and in general plants have to cope with the stress from germination on. In this regard, investigating the impact of heavy metal stress and particularly Cd on crop plants, a long-term exposure makes the obtained results much more relevant for agriculture and will be of higher societal and economic importance.

Medicago sativa is a world-wide important crop plant in agriculture as it requires low input of nitrogen, has perennial lifecycle and is used as green fertilizer. Its leaves have a high nutritive value and serve as cattle feed, while its less digestible stems are used for biofuel production. Its high economic value makes it an interesting plant to study.

The current thesis aims at investigating the influence of long-term Cd exposure on the cell wall- and soluble proteome, to study Cd-driven structural as well as compositional changes in the cell wall and to unravel changes of the metabolome in stems of *M. sativa* caused by Cd exposure. By puzzling these results together, a more complete picture has been obtained about the mechanisms that are put in place as a direct defence response to Cd exposure, which eventually convey acclimation.

In this regard, gel-based (2D DIGE) and gel-free (LC-MS) proteomic approaches were adopted to investigate quantitative changes in the cell wall- and soluble proteome in stems of *M. sativa* upon long-term Cd exposure (chapter 1 and 2). In a following step, we focused on the cell wall- composition and structure in relation to observed changes in the cell wall proteome and how this contributes during the defence against Cd (chapter 2). Since metabolites are the biological active compounds in the plant and better reflect the end-phenotype, they play a crucial role during plant adaptation to environmental stress. Here, targeted and untargeted metabolomics approaches were combined with the ambition to characterize the metabolome in *M. sativa* stems after long-term Cd exposure and to draw conclusions on the importance of primary and secondary metabolites during stress response (chapter 3). During the assessment of the nutrient composition in *M. sativa* after long-term Cd exposure, we observed an accumulation of potassium and sodium in all investigated organs, which brought up the question if a promoted uptake of certain nutrients and their accumulation is part of the defence mechanism against long-term Cd exposure (chapter 4).

2. The cell wall as a physical barrier?

Plants are immobile and therefore have to face multiple stressful situations during their life span. The quality of the soil, as their base of life providing essential nutrient and water, influences the well-being of plants directly if the

nutrient profile is unbalanced or if it is contaminated with heavy metals such as Cd. Just like essential nutrients, Cd is taken up through the root system from where it gets distributed throughout the plant by different transport mechanisms in competition with nutrimental elements such as K, Ca or Cu (Clemens and Ma, 2016; Zhang et al., 2014). Cadmium negatively affects the plant metabolism at multiple levels, thereby reducing the total biomass production, disrupting the water status and interfering with photosynthesis (Perfus-Barbeoch et al., 2002; Sanità Di Toppi and Gabbrielli, 1999). Furthermore, it disturbs the nutrient balance (Nazar, 2012), leading to a retention of most nutrimental elements in the roots of *M. sativa* (chapter 4, Figure 4-1) (**Figure D-1**).

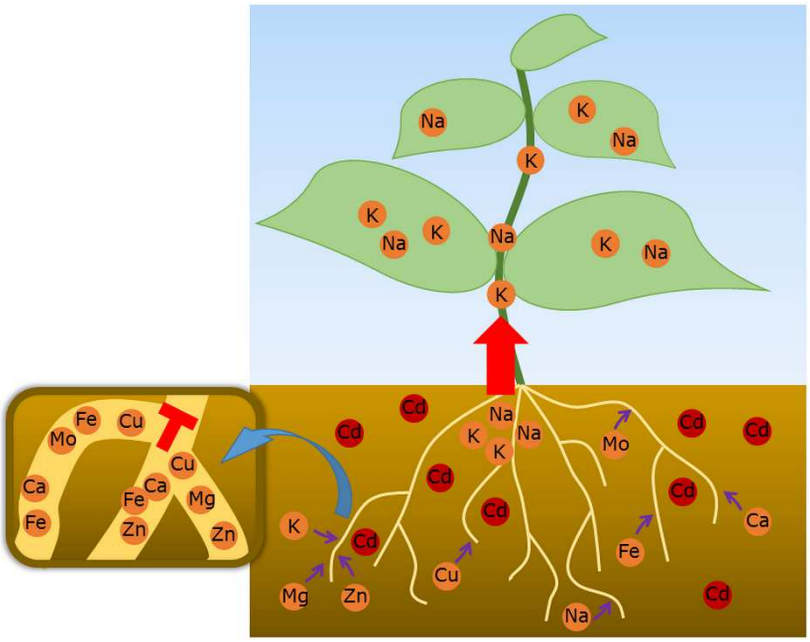

Figure D-1: Influence of Cd on the nutrimental uptake by *M. sativa*.
Cadmium interferes with the uptake of nutrients from the soil, leading to a retention of most nutrimental elements in the roots. Exceptionally, the translocation of K and Na to the areal parts is maintained upon Cd exposure and an accumulation of both elements in roots, shoots and leaves is observed. Illustration is based on chapter 4, figure 4-1 (see also Table S4-1 for absolute values).

In plant cells, Cd unbalances the redox status by enhancing the production of reactive oxygen species (ROS), which leads to oxidative stress (Cuypers et al., 2010) and causes several secondary effects such as cross-linking of DNA, protein oxidation and degradation, fatty acid peroxidation as well as enzymatic-

and signalling deregulation by replacing functional ions (Gallego et al., 2012; Jacobson et al., 2017). This being said, plants developed different protective mechanisms to tackle the induced stress.

The plant cell wall undergoes structural changes when plants are exposed to heavy metals and it is hypothesized that the cell wall acts as a physical barrier against external threats (Loix et al., 2017; Parrotta et al., 2015). The cell wall proteome is of high importance as cell wall proteins, among other functions, determine the cell wall structure (Jamet et al., 2006). Picking up this hypothesis, *M. sativa* was subjected to a realistic Cd concentration (10 mg kg^{-1} soil DW) for a long-term period (5 month) and quantitative changes in the cell wall proteome were studied in stems by applying a gel-based (2D DIGE) and a gel-free approach (LC-MS). A targeted protocol was used to obtain cell wall protein-enriched extracts, in which the abundance of proteins with other subcellular locations is kept low (chapter 2, Table 1-2). Long-term Cd exposure altered the abundance of proteins that are involved in multiple physiological processes such as defence response, oxidation-reduction processes, carbohydrate metabolism and cell wall remodelling (chapter 1, Figure1-2). A higher abundance was determined for pectin methylesterase (PME), β-like galactosidase and α-galactosidase (chapter 1, Table 1-2), which are all responsible for structural changes of the cell well as well as changes within the pectin network during plant development and in response to stress. Pectin methylesterase catalyses the demethylation of homogalacturonane (HG) and the here reported higher abundance of PME in the cell wall of *M. sativa* stems in response to long-term Cd exposure was accompanied by its increased enzymatic activity (chapter 2, Figure 2-6). The pectin composition and the degree of HG methylesterification are determining for biochemical and biomechanical properties of the cell wall, which are crucial during the response to abiotic stress (Levesque-Tremblay et al., 2015) and a high demethylesterified HG profile is reported due to Cd exposure (Douchiche et al., 2010a, 2010b). The PME-mediated demethylation of HG creates binding sites for Ca ions, through which pectin assembles to a strong gel-like network. Cadmium can displace those Ca ions within the HG network (Krzesłowska, 2011) due to a higher affinity. In this way Cd gets immobilized in the cell wall, which prevents its further distribution throughout the plant. In

support to the here obtained data on the abundance and enzymatic activity of PME, an elevated content of HG molecules was determined in stems of Cd-exposed *M. sativa* (chapter 2, Table 2-2). These results suggest a higher abundance of demethylesterified pectin in the cell wall network, thereby increasing the availability of metal binding sites and provoking mechanisms of metal sequestration in the plant cell wall (Ramos et al., 2002; Wójcik et al., 2005). The here investigated changes in the pectic composition of the cell wall of *M. sativa* stems and the enhanced activity of PME were put in place as a direct response to long-term Cd exposure. These observations highlight the involvement of the cell wall structure in the defence response against Cd exposure, whereby the immobilization of the heavy metal within the cell wall in promoted (**Figure D-2**).

Furthermore, structural changes of the cell wall appeared in the composition of the side chains of pectin, whereby Cd induced a shift from arabinose to xylose visible in the EGTA fraction, which extracted mainly pectic polysaccharides (chapter 2, Table 2-2). This gave indication to an increase of covalently linked xyloglucan to pectin. Side chains regulate the porosity of the cell wall matrix and influences the accessibility of proteins to their target sites within the cell wall network (Dellapenna et al., 1990). In this regard, pectin-xyloglucan builds up a strong, three dimensional complex (Morris et al., 1982), which limits the access for enzymes to cell wall polymers and their interaction sites, strengthens the cell wall and promotes cell wall stiffening in response to long-term Cd exposure. In accordance, xyloglucanase-specific endoglucanase inhibitor protein increases and prevents cleavage of xyloglucan (chapter 2, Table S2-1). Thus, changes of the cell wall structure as well as its mechanical properties appear in direct response to long-term Cd exposure in stems of *M. sativa* and probably help to establish the cell wall as a physical barrier against this heavy metal (**Figure D-2**).

3. Stimulation of the phenylpropanoid pathway in favour of isoflavonoid synthesis

Lignification of the cell wall is commonly observed in response to Cd exposure, making the cell wall less permeable for Cd and limiting its entry into the cell

(Chaoui and El Ferjani, 2005; Elobeid et al., 2012). Lignin is synthesised through oxidative polymerization of monolignol molecules, which is mediated by cell-wall located peroxidases (McDougall, 1992; Vanholme et al., 2010). Various peroxidase isoforms were found to be of higher abundance in *M. sativa* stems due to long-term Cd exposure using a gel-based and a gel-free quantification method (chapter 1, Table 1-2; chapter 2 Table S2-1), suggesting an increased lignification in response to Cd stress. Peroxidase activity, furthermore, directly links the lignification process to the redox status of the cell as its activity is triggered by H_2O_2 (Passardi et al., 2004) and plants challenge an increased generation of ROS in response to Cd (Cuypers et al., 2010). However, when assessing the lignin content and composition, no differences appeared between control and Cd-exposed plants (chapter 2, Table 2-3).

Monolignol molecules as the modules of lignin are synthesised via the phenylpropanoid pathway, into which phenylalanine (Phe) is the entry molecule that is converted into cinnamate by phenylalanine ammonia lyase (PAL) during the initial step (chapter 2, Figure 2-1). When investigating transcript levels of different genes of the phenylpropanoid pathway, an increased expression of *cinnemate-4-hydroxylase* and *4-coumarate ligase* was detected in Cd-exposed *M. sativa* stems (chapter 2, Table 2-1) and results indicate a Cd-driven stimulation of this pathway. Also, *PAL* gene expression increased but this was not reported as significant. The activity of PAL depends on the availability of Phe as its substrate (Da Cunha, 1987) but it is also positively influenced by ethylene (Hyodo and Yang, 1971). In *M. sativa* stems, Phe accumulated upon long-term Cd exposure (chapter 3, Figure 3-3 and Table 3-1) and furthermore, when investigating the influence of Cd on ethylene biosynthesis, a higher content of conjugated 1-aminocyclopropane-1-carboxylic acid (ACC) was determined in Cd-exposed plants (chapter 2, Figure 2-7). ACC is the precursor of ethylene. The formation of ACC conjugates could regulate the ACC pool and consequently the ethylene production through a self-regulating feedback control and it is suggested that conjugated ACC converts back to ACC thereby stimulating ethylene production through developmental or environmental excitations (Martin and Saftner, 1995; Van de Poel et al., 2014). In support, transcript levels of genes involved in the biosynthesis of ACC and genes responsive to ethylene

increased upon Cd exposure (chapter 2, Table 2-1). Therefore, the obtained results strongly indicate the stimulation of the phenylpropanoid pathway by Cd (**Figure D-2**).

Other phenolic compounds deriving from the phenylpropanoid pathway are flavones and isoflavones (**Figure D-2**) and their accumulation in response to various stresses is reported in literature, accompanied by an increased activity of enzymes of their biosynthetic pathway (Dixon and Paiva, 1995; Lavola et al., 2000). In this regard, the expression levels of genes involved in isoflavone biosynthesis, namely *chalcone reductase*, *chalcone synthase* (*CHS*), *chalcone isomerase* and *isoflavone synthase* were investigated in *M. sativa* stems after long-term Cd exposure. Their expression was significantly induced by Cd, with the exception of *CHS*, having indeed a slightly higher but was not significant expression in response to Cd (chapter 3, Table 3-3). Ultra-performance liquid chromatography was used to determine the content of secondary metabolites and results showed a higher abundance of different isoflavone conjugates in stem tissue, when *M. sativa* was exposed to Cd (chapter 3, Table 3-1 and 3-2). Thereby, the observed glycoconjugation might be crucial for their bioactivity and is of importance during the defence response against Cd (Liu et al., 2002; Staszków et al., 2011).

In conclusion, long-term Cd exposure stimulates the phenylpropanoid pathway, thereby promoting isoflavone biosynthesis instead of monolignol biosynthesis (**Figure D-2**). In accordance to the here made observation, the Cd-induced activation of the phenylpropanoid pathway promoting an increased isoflavonoid content was reported in roots of *Lupinus luteus* seedlings, whereby lignin content decreased contemporaneous (Pawlak-Sprada et al., 2011). Isoflavones provide binding sites for trace metals, thereby contributing to the cellular detoxification by complexing Cd (Martinoia et al., 2007). In addition, they are efficient ROS scavengers counteracting the Cd-induced oxidative burst (Pietta, 2000). In that matter, the chemical structure of isoflavones determines their bioactivity and efficiency, whereby the observed glycosylation is highly essential to fulfil their full function (chapter 3, Table 3-1 and 3-2) (Montesinos et al., 1995; Tsuchiya, 2010). By reaching a new metabolic steady-state, *M. sativa* acclimates to Cd exposure and overcomes initial phytotoxic signs of growth

impairment as well as leaf chlorosis and at the end of the experiment neither phenotypical differences nor differences in the produced biomass were observed. That *M. sativa* plants are doing well under Cd exposure is furthermore reflected in their photochemical efficiency, which is about 0.84 and thus equal to healthy, unstressed plants (chapter 4, Figure 4-3)

The importance of the phenylpropanoid pathway during acclimation processes was previously demonstrated in *Brassica napus* during acclimation to cold, where the inhibition of PAL activity led to abrogation of cold-induced growth effects, decreased photochemical efficiency and decreased leaf tolerance to extracellular ice formation (Solecka and Kacperska, 2003). The term acclimation is defined as a rather dynamic process during which metabolic pathways adjust in response to a disrupted homeostasis initiated by environmental stress (Shulaev et al., 2008). Thereby, the accumulation of secondary metabolites supports the survival of the plant in its environment and initiates particular physiological or phenotypical responses, which provide advantages to the plant in order to adapt and survive certain conditions. Furthermore, distinct accumulation patterns of different metabolites can be observed over time, which makes the process of acclimation rather dynamic (Hectors et al., 2014). Interestingly, findings in wild-type *A. thaliana* revealed an early stress response, which is characterized by major changes in the level of primary metabolites, while phenolic compounds such as flavonoids accumulated in a later response. It is therefore postulated that the primary metabolism undergoes a reprogramming during early stress towards an efficient diversion of resources into the production of secondary metabolites (Kusano et al., 2011).

However, every stress condition provokes a unique acclimation response in the plant, tailored according to its specific needs. The subjection to multiple stresses at once requires also a unique acclimation process, which makes clear scientific statements difficult (Mittler, 2006).

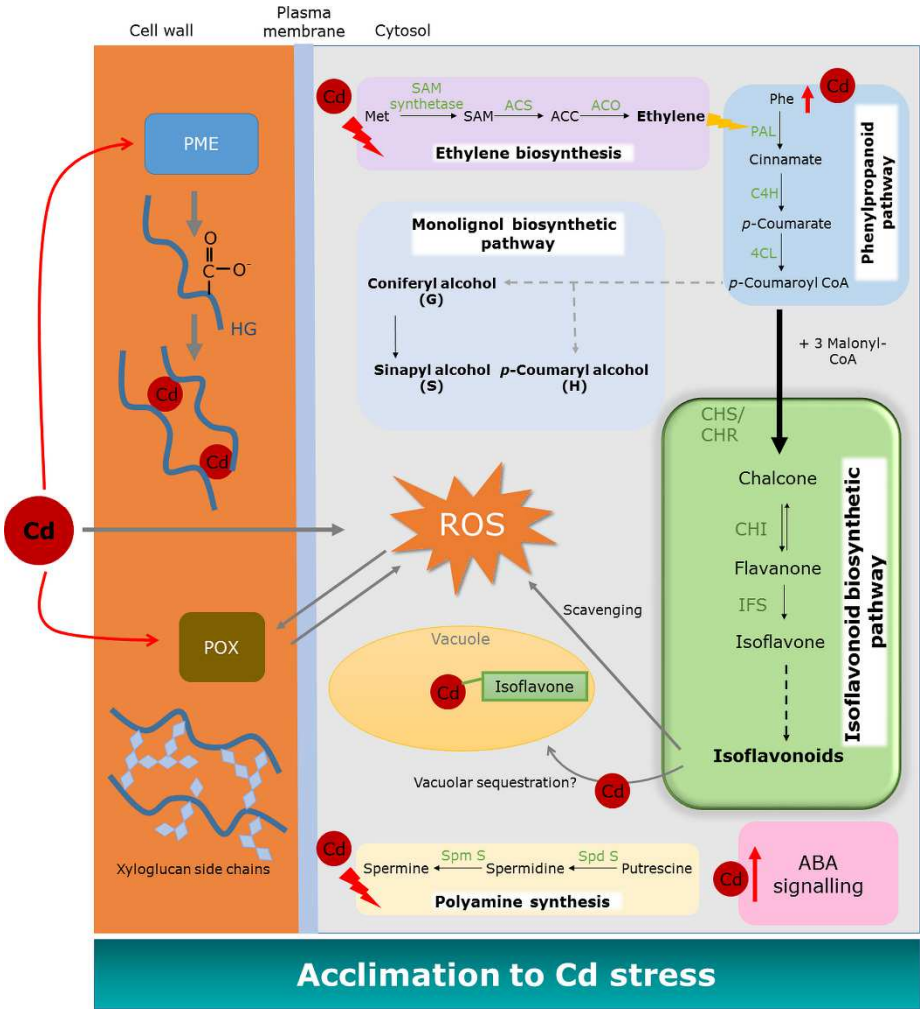

Figure D-2: Influence of long-term Cd exposure on the cell wall structure and metabolites in *M. sativa* stems.
Cd exposure disturbs the cellular oxidative balance and enhances the production of reactive oxygen species (ROS). Cd exposure changes the abundance and activity of pectin methylesterase (PME). PME activity catalyses the demethylation of homogalacturonan (HG), which creates binding sites for Cd in the cell wall. Attached pectin side chains shift to xyloglucan, building up a strong complex, which limits the access for enzymes to their target site in the polysaccharide network, strengthens the cell wall and promotes cell wall stiffening. Cd exposure increases the abundance of peroxidases (POX), whereby POX activity and ROS are influencing each other. It stimulates ethylene biosynthesis, which stimulates the phenylpropanoid pathway in favour of isoflavonoid biosynthesis. Broken arrows represent reactions, involving intermediate steps, which are not detailed in the figure. Lightning indicates stimulation. Red, straight arrows symbolise accumulation.

4. Perspective

4.1. The CadWALL project in retrospect

For the scope of the thesis a long-term experiment was chosen, during which *M. sativa* plants were exposed to Cd for an entire season including an intermediate cutting and regrowth of the plants before sampling. Hereby, the applied Cd concentration of 10 mg kg^{-1} soil DW was fairly low and reflected realistic soil concentrations. Thus, the provoked biochemical and biomechanical responses of the plant are comparable with environmental conditions and obtained results are relevant from an economic and agricultural point of view.

During the experiment we observed a strong impact of Cd exposure on juvenile plants, resulting in limited growth and leaf chlorosis. However, after the first cut the Cd-exposed plants recovered from the stress and at the end of the experiment no difference in growth was observed between exposed and control plants. The produced total biomass was similar for both conditions. Obviously, mature *M. sativa* plants are less affected by Cd exposure than juvenile plants and during the duration of the experiment *M. sativa* reached a new metabolic steady-state, which conveyed acclimation to the applied stress.

It is clear that it would have been beneficial to observe this acclimation process by setting up a time-lapse experiment. By monitoring physiological parameters such as the photochemical efficiency for the duration of the experiment, the process of acclimation can be followed and the window of the turning point when plants start to adapt can be narrowed down. Tissue samples can then be taken in reasonable intervals for molecular biological studies such as gene expression analysis. Thereby, targeted gene expression analysis of genes known to play an important role during plant stress response will give insights into the process of rapid stress responses in the alarm phase, entering the stage of resistance and finally reaching the regeneration phase. The identification of the molecular key players, which are important or characteristic in these different stress response stages will allow us to draw a more comprehensive picture on the stress response of *M sativa* to Cd in the means of alterations of the cell wall structure and the metabolite profile in a time-dependent manner. However, at present

such a comprehensive set-up would have gone partly beyond the scope of the current thesis.

As a future approach, it would also be interesting to undertake such an experiment on contaminated land. Are our observations made on a small scale transferable to a bigger, agricultural scale? How does *M. sativa* cope with multiple stresses at the same time (more than one contaminant, weather conditions, temperature …)? For instance, in the proteome results we observed a decrease in proteins with nutrient reserve function. Will this impact the winter survival of the plants when growing on contaminated soil? The gained knowledge will help to evaluate the use of *M. sativa* in a future perspective as will be discussed in a following paragraph.

As mentioned before, the observed structural changes in the cell wall of *M. sativa* in response to long-term Cd exposure imply Cd deposition in the cell wall network. The actual deposition of Cd in the cell wall can be visualized through different methods, whereby quantification and visualization are achieved for instance with synchrotron-based micro-X-ray fluorescence imaging, which was successfully applied in *Noccaea (Thlaspi) praecox* and *Lotus japonicus*. The cell wall in both species was identified as a sink for Cd (Koren et al., 2013; Nayuki et al., 2014). Another approach is energy-dispersive X-ray microanalysis, which was done in *Thlaspi caerulescens* and demonstrated Cd localization in the cell wall of its roots (Wójcik et al., 2005). The method was also used in *A. thaliana* to examine the subcellular localization of Cd in roots and leaves and Cd-baring granular deposits were observed in the cell wall (Van Belleghem et al., 2007). In the latter study, realistic Cd concentrations were used in order to obtain physiological and ecological relevant data and thus are comparable to our study.

These quoted studies underline the ability of the cell wall to retain Cd as a mechanism of detoxification and emphasis the role of the cell wall during Cd exposure. Therefore, together with the occurring structural changes in the cell wall of *M. sativa* stems upon Cd exposure, visualizing Cd in the framework of the present thesis would have proven the assumption of Cd retention in the cell wall and should have been considered. However, the deposition of Cd in the cell wall could also have been indirectly demonstrated by using the isolated cell wall

residues of *M. sativa* stems for ICP-MS analysis. Through the comparison of the Cd content in those cell wall residues between control and Cd-exposed plants conclusions could have been made on the quantities specifically present in the cell wall. Unfortunately, there was insufficient time for the repetition of the experiment.

4.2. *Medicago sativa* – a key crop into a green, sustainable future?

The pollution of soil with heavy metals limits their use for economical and agricultural purposes. Hence, their remediation is inevitable to regain the needed surface for food production and living space. Phytoremediation is a plant-based bioremediation technology and defined as the use of plants and their associated microbiota for *in situ* treatment of contaminated soil and ground water. This method can be applied to organic and inorganic contaminants in the soil at a low cost when compared to conventional physico-chemical techniques and different processes of phytoremediation are described in literature (Raskin et al., 1997; Vangronsveld et al., 2009) (**Figure D-3**).

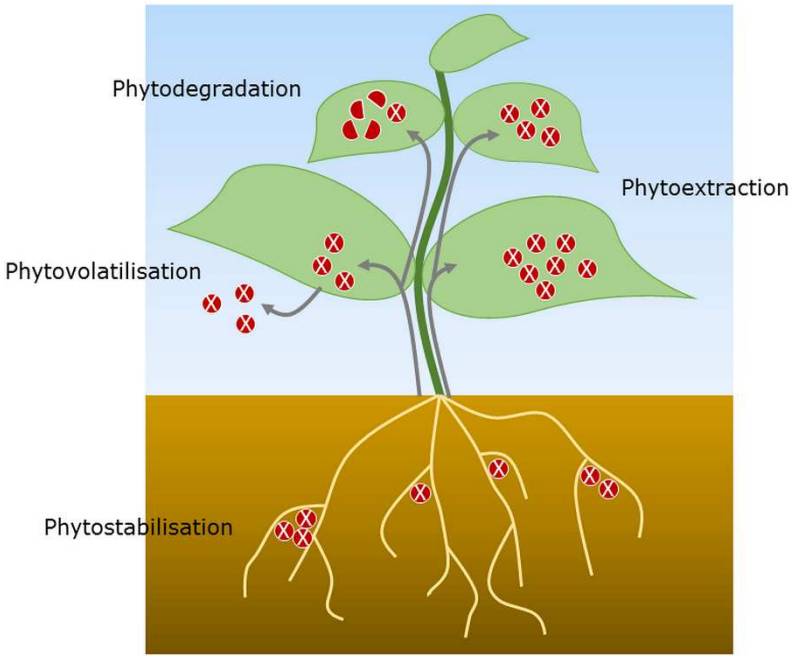

Figure D-3: Phytoremediation processes of heavy metal-polluted soil.

One of them is the phytostabilisation where remediation depends on the ability of the roots to limit the mobility and bioavailability of contaminants in the soil. Grown plants prevent soil erosion and hinder spreading of contaminated soil throughout the area. Furthermore, the water percolation through the soil is decreased and contaminated leachate limited. As a drawback the contaminant remains in the soil. The approach of phytoextraction is based on the removal of the contaminant from the soil, whereby plants absorb and accumulate toxic metals from the soil into their biomass. The method suites best for surficial pollution in a relatively low concentration to prevent phytotoxicity (Ghosh and Singh, 2005). Lately, biochar got into the focus to face the heavy metal pollution of soil. Biochar is a commonly used soil amendment made of organic waste matter via pyrolysis and, when worked into the soil, it influences the biogeochemical properties of the soil and positively impacts plant productivity. Additionally, biochar has the potential to immobilize heavy metals in the soil, in particular Cd (Uchimiya et al., 2010). Through a joint strategy of biochar application for phytoremediation purposes, the efficiency of soil restoration can be optimized in order to tackle soil heavy metal pollution (Houben et al., 2013; Lu et al., 2015).

Within the framework of the thesis it was shown that long-term Cd exposure to realistic Cd concentrations in the soil does not affect the biomass production of mature *M. sativa* plants (chapter 1) as well as their photochemical performance (chapter 4, Figure 4-3).This makes *M. sativa* a considerable candidate for phytoremediation attempts in respect to low soil Cd concentrations (Peralta-Videa et al., 2004) and its potential for phytoremediation was proven in a hydroponic solution experiment (Singh et al., 2009). The symbiosis with Rhizobia makes it an efficient biological system as it improves the overall plant performance in the presence of Cd. Nodulation increases the biomass production, the uptake of Cd into the roots and its translocation to the shoots, resulting in a higher potential of phytoextraction (Ghnaya et al., 2015). Furthermore, as a legume it is often co-planted with other crops to increase crop yield, due to their ability of nitrogen fixation, which serves as a nutrient source for the main plant. This characteristic can be used during phytoremediation by co-cultivating *M. sativa* with another phytoextracting plant. Doing so, the

biomass production of the latter can be improved, which positively correlates with its phytoremediation potential by increasing the absorption of heavy metals from the soil (Xiong et al., 2018).

The feasibility of phytoextraction through high biomass producing crops becomes economically interesting if the biomass can be subsequently valorised and a combination of phytoremediation in combination with energy production is proposed (Van Ginneken et al., 2007). In this context, the biomass of *M. sativa* can be utilized in biogas production where it gives a yield of 0.43-0.65 m^3 kg^{-1} dry mass (Bond and Templeton, 2011). Biogas utilization reduced the use of wood and coal and it states a sustainable, renewable energy source. This said, the cultivation of *M. sativa* on contaminated sites would promote the reuse and valorisation of abandoned land. Hereby, agricultural land will not get occupied and allows a coexistence of energy and food crops, limiting the competition for farmland.

However, the utilization of contaminated plant biomass is questionable. Depending on the metal concentration, harvested plant biomass from phytoextraction might be considered as hazardous waste and needs proper disposal. Regardless, its use during anaerobic digestion for biogas production is still acceptable (Tian et al., 2012), whereby the presence of heavy metals, like Cd, in a certain concentration range can enhance the biogas production (Jain et al., 1992; Zhang et al., 2013). The experiments presented here indicate that *M. sativa* could serve the purpose of phytoremediation of Cd-polluted soil in combination with biogas production, which opens the possibility of its use for a bio-based energy production in a future perspective reusing abandoned land.

4.3. Critical amendment to chapter 2

In the spirit of the initial statement "*Sciences of today are the misapprehensions of tomorrow*", it is important to reflect the interpretation and discussion of the data made in chapter 2 of the present thesis. After a peer revision of the submitted manuscript, which correspond to chapter 2, and the addition of a new experiment, new insights were gained and led to a new interpretation and discussion of the cell wall structure upon Cd exposure.

The main comment of the reviewer on this manuscript was that the actual methylation degree of the cell wall was initially not determined. A definite conclusion about the methylation degree should not be drawn only from the PME activity. One could for example imagine that pectin remains highly methylated despite the measured increased enzymatic PME activity due to a lower accessibility of the enzyme to its target sites caused by structural alterations of the cell wall in response to Cd. As a compensatory mechanism, the measured enzymatic activity of PME, corresponding to a higher abundance of the protein (chapter 1, part 1, Table 1-2), could increase. The standard method for this is to determine the released methanol from the cell wall by saponification, which is very similar to the already described method to determine the PME activity. Saponification was done as described before (Klavons and Bennett, 1986) with slight modifications and to determine the methanol present in the extract, the protocol used for the PME activity assay was applied (chapter 2, 2.6. PME activity assay). The obtained results revealed that the released methanol from the cell wall after saponification was similar for control and Cd-exposed samples. Despite an increased PME activity (chapter 2, Figure 2-6), Cd exposure did not alter the methylation degree of the cell wall (**Figure D-4**) as was anticipated in chapter 2.

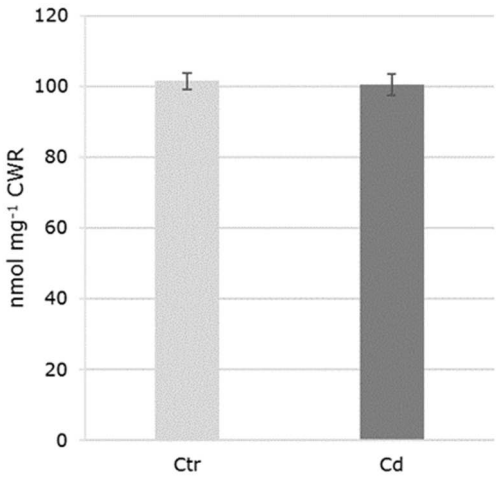

Figure D-4: Released methanol (nmol mg-1 CWR) from cell wall residues of *M. sativa* stems after saponification.
Saponification was done with 1 M KOH. Values represent mean values from five biological replicates, each measured in two technical replicates. Error bars represent the SEM.

In consequence of this new experiment, it was required to go back to the data on the chemical composition of the cell wall and to do a reinterpretation. This was furthermore because the reviewer pointed out that it is not common to extract xyloglucan in the pectin fraction as suggested in chapter 2 and the original manuscript. The co-extraction of xylose (Xyl) and galacturonic acid in the EDTA fraction most likely represents the presence of xylogalacturonan (XGA) in the cell wall of *M. sativa* stems. Xylogalacturonan is a commonly found pectic polysaccharide in the cell wall of legumes (Huisman et al., 2001; Shiga and Lajolo, 2006) and data presented in chapter 2 suggest a higher abundance of XGA in response to Cd exposure (chapter 2, Table 2-2). The galacturonic acid backbone backbone of XGA can be partially methylated, whereby the attached Xyl residues limit the accessibility for PME to its target sites and would stabilise a high methylation degree of pectin (Willats et al., 2001; Yu and Mort, 1996). Xylogalacturonan in the cell wall certainly affects the gel-properties and Cd-binding capacities of the pectin network. The limited ability of the cell wall to bind Cd excludes the heavy metal from the apoplast and restricts its uptake into the symplast. Studies suggest that heavy metal tolerance is determined by the degree of pectin methylation, whereby tolerant populations were found to have a higher degree of pectin methylation (Brunner and Sperisen, 2013; Colzi et al., 2012).

Probably, the cell wall of *M. sativa* stems is composed of XGA-rich domains, which are protected from the PME activity and domains with less XGA. The higher abundance and activity of PME could compensate for the XGA-rich domains and a particular methylation degree of the cell wall is maintained. Against the initial hypothesis that the cell wall undergoes structural changes to support the immobilization of Cd in the pectin network, the exclusion of Cd from the apoplast in combination with a local binding of Cd to pectin limits the entry of the heavy metal into the cytosol and might be an important factor for tolerance acquisition.

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