GENEESKUNDE
master in de biomedische wetenschappen: milieu en gezondheid

Masterproef
Molecular mechanisms of metal-specific root growth responses in Arabidopsis thaliana

Promotor :
dr. Tony REMANS
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An Loos

Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting milieu en gezondheid

universiteit hasselt

KNOWLEDGE IN ACTION

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List of abbreviations

ABA	Abcisic acid
ASC	Ascorbate
APX	Ascorbate peroxidise
CAT	Catalase
Cd	Cadmium
$CdSO_4$	Cadmium sulphate
Ct	Cycle Threshold
Cu	Copper
$CuSO_4$	Copper sulphate
(c)DNA	(Complementary) Deoxyribonucleic acid
dH_2O	Distilled water
Fe	Ferric
$FeNO_3.9H_2O$	Ferric nitrate nonahydrate
GSH	Glutathione
JA	Jasmonate
KOH	Potassium hydroxide
K_2SO_4	Potassium sulphate
LOX	Lipoxygenase
MES	2-[N-Morpholino]ethanesulfonic acid
Mn	Manganese
Ni	Nickel
NTC	No template control
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SE	Standard error
SIMR	Stress-induced morphogenic response
SOD	Superoxide dismutase
UV	Ultra violet
Zn	Zinc
$ZnSO_4$	Zinc sulphate

Abstract (English)

Background   Understanding more about root development under stress conditions is essential for future crop improvement, which may be aimed at safe biomass production or clean up of polluted soils. Sublethal stress-exposed roots exhibit stress-induced morphogenic responses (SIMRs). However, for the metals cadmium (Cd), copper (Cu) and zinc (Zn) in *Arabidopsis thaliana* seedlings, metal-specific growth responses were found instead of the general SIMR. It is hypothesised that *LOX*-genes are involved in these metal-specific growth adaptations.

Objectives   The aim of this study is to participate in unravelling the molecular mechanisms underlying these metal-specific growth responses in *Arabidopsis thaliana* and to understand more about local and systemic effects on root development in a more relevant context of heterogeneous exposure.

Methodology   The phenotypic change under metal-stress conditions of *lox*-mutants (*lox1-1, lox3A, lox5-1)* compared to wild-type was investigated in split-root systems. Furthermore, the expression of genes involved in plant hormone synthesis or metabolism was examined in metal-exposed wild-type roots using quantitative reverse transcription PCR.

Results   Phenotypic comparison of *lox*-mutants with wild-type after growth in split-root vertical agar plates revealed that Cd, Cu and Zn in the lower zone stimulate lateral root elongation and number in the upper control zone, which can be due to the elevation of the inhibition on lateral root elongation by *LOX3* and *LOX5.* An incomplete primary root avoidance was observed in the Cd- and Zn-containing lower zone. The incomplete avoidance under Cd-exposure may be due to 9-LOX-signalling. *LOX*-genes did not seem to be involved in primary root growth under Cu-exposure, which may be due to the fact that Cu can induce reactive oxygen species directly. The systemic Zn-effect in wild-types is not present in *lox3*. The gene expression data revealed an significant increase in *AAO1*-expression under Cu- and Zn-exposure.

Conclusion   *LOX*-genes are involved in the metal-specific growth responses and have a preference for the metal-free zone under heterogeneous exposure.

Abstract (Nederlands)

Achtergrond   Het beter begrijpen van de wortelontwikkeling onder stress omstandigheden is essentieel voor toekomstige gewasverbetering. Wortels die blootgesteld zijn aan een reeks van subletale stresscondities vertonen stressgeïnduceerde morfogenische responsen (SGMRs). Echter, voor de metalen cadmium (Cd), koper (Cu) en zink (Zn) in *Arabidopsis thaliana* zaailingen, zijn er metaalspecifieke groeiresponsen gevonden in plaats van een algemene SGMR. Er wordt verondersteld dat *LOX*-genen hierin betrokken zijn.

Objectieven   Het doel van deze studie is om deel te nemen in het ontrafelen van de moleculaire mechanismen die ten grondslag liggen aan deze metaalspecifieke groeiresponsen in *Arabidopsis thaliana* en om meer te begrijpen over lokale en systemische effecten op de wortelontwikkeling in een meer relevante context van heterogene blootstelling.

Methodologie   De fenotypische verandering onder metaalstress van *lox*-mutanten (*lox1-1, lox3D, lox5-1)* vergeleken met het wildtype werd onderzocht. Splitwortel systemen werden gebruikt om te onderzoeken of *LOX*-genen betrokken zijn in de locale en/of systemische inhibitorische effecten van Cd, Cu en Zn. Bovendien wordt de genexpressie van genen betrokken bij planthormoonsynthese of metabolisme onderzocht in wortels van metaalblootgestelde wildtype planten met behulp van kwantitatieve reverse transcriptie PCR.

Resultaten   Fenotypische vergelijking van *lox*-mutanten met wildtype na groei in splitwortel verticale agarplaten toonde aan dat Cd, Cu en Zn in de onderste zone de elongatie en het aantal van de zijwortels stimuleren in de bovenste controle zone, die mogelijk te wijten zijn aan de opheffing van de inhibitie op de elongatie van de zijwortels door *LOX3* en *LOX5*. Een onvolledige primaire wortelvermijding werd waargenomen in de Cd- en Zn-bevattende onderste zone, die te wijten kan zijn aan 9-LOX-signalering. *LOX*-genen lijken niet betrokken te zijn in de primaire wortelgroei onder Cu-blootstelling, wat te wijten kan zijn aan het feit dat Cu RZV direct kan induceren. Het systemische Zn-effect aanwezig in het wildtype is niet teruggevonden in *lox3*. De genexpressie data toonde een significante stijging van *AAO1*-genexpressie onder Cu- en Zn-blootstelling.

Conclusie   *LOX*-genen zijn betrokken in de metaalspecifieke groeiresponsen en hebben een voorkeur voor de metaalvrije zone onder heterogene blootstelling.

1. Introduction

Major challenges for the near future are providing food security and biofuels to the rapidly growing world population. Alternative ways of increasing plant production, other than by increasing the classic use of fertilizers and/or pesticides, need to be aimed for because of the environmental impact and the decreased mineral resources such as phosphorus. Furthermore, there is not sufficient agricultural land available to meet the demands for both food and biomass for energy of the rapidly growing world population[1]. This pressure on agricultural land can be relieved by using contaminated soils (e.g. metal contamination) for the cultivation of energy crops. This ensures that more agricultural land can be used for food production. However, plants do not grow well on polluted soils because the contaminants typically induce stress symptoms and responses[2]. Since roots are in direct contact with soil pollution, understanding more about root development under stress conditions is essential for future crop improvement that can lead to stress-resistant plants that survive better in these contaminated regions. Additionally, this can also be combined with phytoremediation[3]. So growth improvement of plants on contaminated soils may be aimed at either safe biomass production or clean up of polluted soils, which in turn increases agricultural land.

1.1 Root growth responses under stress conditions

The first experience of the stress factor in contaminated soils is on the root level and the root system architecture is also a factor determining the fitness of the plant in these soils. The root architecture shows a high developmental plasticity in response to the local soil conditions. It is hypothesized that this is one of the many defence responses that plants have to cope with unfavourable conditions as they cannot flee away or defend themselves like animals do[4-6].

Since roots contain membrane transporters for metals, the uptake of excess metals is inevitable. Normally these transporters are selective for essential metals such as Fe, Zn, Mn, Ni, and Cu, however non-essential metals such as Cd and As are also taken up by these transporters. Once the metals are taken up they can cause a cellular

redox imbalance in plants through: (1) interaction with functional groups on proteins; (2) displacement of essential elements; and (3) increase in ROS-production. This cellular redox imbalance can cause oxidative damage which in turn can inhibit plant growth. In order to cope with these metal-induced oxidative challenges, plants developed other protective responses besides root growth adaptations such as the sequestration of heavy metals by phytochelatins and metallothioneins and the scavenging of reactive oxygen species (ROS) by antioxidants[7,8].

An optimum morphological response would be when metal uptake would be avoided by avoiding root growth in contaminated zones (at least for safe biomass production). So far it is known that roots, which are exposed to a range of sublethal stresses, exhibit stress-induced morphogenic responses (SIMRs). A SIMR is a combination of growth inhibition and activation responses, resulting in a reorientation of growth rather than a general growth inhibition. This response has been described as an inhibition of root elongation and an increase in lateral root number. It is thought that SIMRs are characteristic of a range of abiotic stresses, such as temperature, water scarceness and excess metals[4,5]. However, the SIMRs have been described for juvenile plants and the effect of different stresses on lateral root elongation, a major factor determining root architecture, has not been described.

Previously, for the metals cadmium (Cd), copper (Cu) and zinc (Zn) in *Arabidopsis thaliana* plants, metal-specific growth responses were found instead of the general SIMR[9] (figure 1). At a concentration where all the three metals caused a similar primary root growth inhibition, differences were found in lateral root growth. Both Cd and Cu induced an increase in lateral root density, where in Cd-exposed plants the lateral roots were more inhibited than in Cu-exposed plants. Zn, however, caused a decrease in density and elongation of the lateral roots. Remans et al. (2012) also found that the inhibitory effect of Cd and Cu on the lateral roots was local, while it was systemic in Zn-exposed *A. thaliana* plants. After these findings, the question rises what molecular mechanisms are underlying these metal-specific growth responses in *Arabidopsis thaliana*.
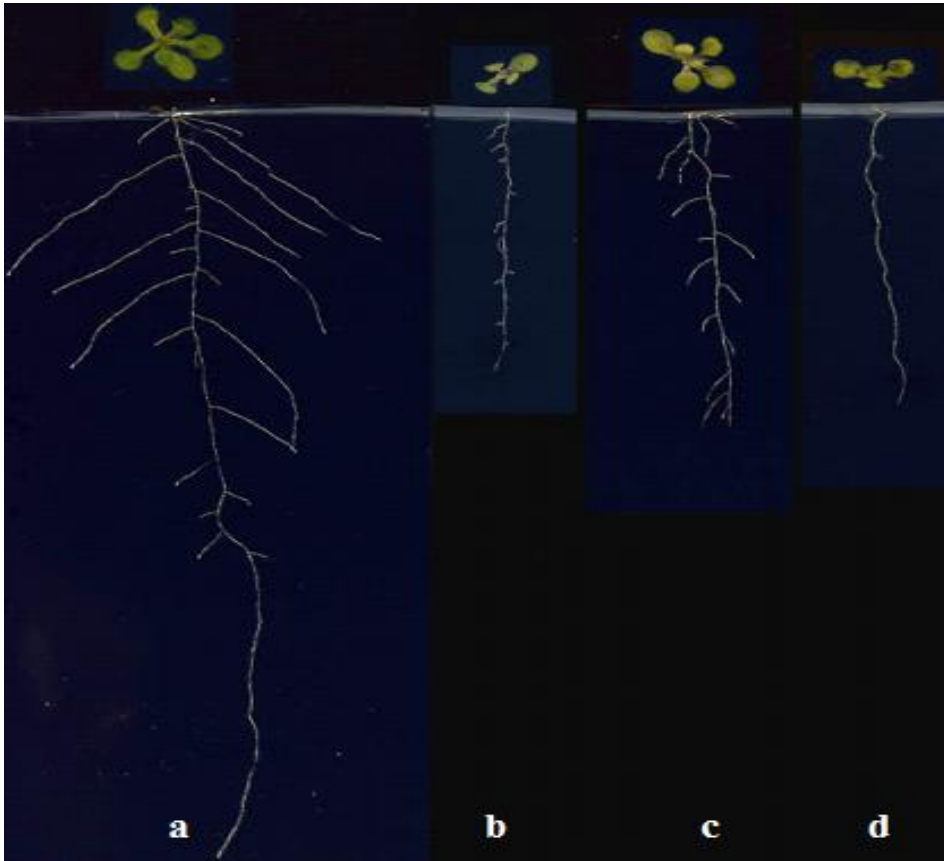
Figure 1: Metal-specific root adaptations after eight days of exposure of seven days old *Arabidopsis thaliana* seedlings to (b) 5 µM CdSO4, (c) 10 µM CuSO4 or (d) 75 µM ZnSO4 compared to (a) control plants of the same age. At these metal concentrations, a similar primary root growth inhibition was observed, but the lateral root growth differed. Cd and Cu both stimulated lateral root density, but lateral root growth was less inhibited by Cu then by Cd and Zn. Zn caused a decrease in density and elongation of the lateral roots (from Remans et al., 2012).

1.2 Molecular mechanisms underlying root growth responses

Plants adapt to the external stimuli in changing environments by regulating their growth and development[10-12]. Plant growth regulators mediate these adaptive growth responses. They inlcude plant hormones such as …abscisic acid (ABA), ethylene, cytokinine, auxin, jasmonates (JAs), etc[10,13]. Besides phytohormones, reactive oxygen species (ROS) have also been associated with morphogenic stress responses[14]. However, these two signalling pathways should not be seen separately since they can also affect each other. Crosstalk has been found between auxin and ROS signalling[5]. Additionally, crosstalk between phytohormones have also been demonstrated, namely ethylene-auxin interactions in root elongation in *Arabidopsis thaliana*[15].

Phytohormones have been found to regulate morphological responses to a variety of stress conditions. For example, salt stress induced SIMR in *A. thaliana* that was mediated by auxin, ABA and an ethylene signalling factor[16,17]. In cold stress, ABA synthesis was induced and this improved the cold tolerance of plants[18]. The adaptive responses of plants exposed to drought and ozone stress are controlled by ABA and ethylene[19].

Besides phytohormones, reactive oxygen species (ROS) have also been associated with a SIMR phenotype. ROS are produced in various biotic and abiotic stresses such as heat stress[20], UV-radiation stress[21], heavy metal stress[22], pathogen attacks[23], etc. Excess ROS may cause membrane lipid peroxidation and damage to DNA and proteins. However, there are also ROS-scavenging systems present in the plant that prevent oxidative damage in general, such as superoxide dismutases (SODs), catalases (CATs), ascorbate peroxidises (APXs), ascorbate (ASC) and glutathione (GSH). This ROS-scavenging system can be seen as a protection mechanism against various stresses[4,5]. Although ROS can induce oxidative damage, they also act as important signalling molecules that have been associated with plant growth and development. Pasternak et al. (2005) found strong similarities in SIMR-phenotypes in *A. thaliana* seedlings exposed to excess copper, paraquat, salicylic acid and a hydrogen peroxide analogue. The genes that are subject of the present study, lipoxygenase *(LOX)*-genes, encode proteins that can cause lipid peroxidation under stress conditions, but they are also at the origin of oxylipin signalling molecules[24].

1.3 Lipoxygenase (*LOX*)-genes

In *A. thaliana* there are six *LOX*-genes (*LOX1-LOX6*) (table 1). *LOX1*, *LOX3* and *LOX5* are expressed in the developing lateral roots[24]. *LOX*-genes encode for lipoxygenases. There are two types of lipoxygenases, namely 9-lipoxygenase (9-LOX) and 13-lipoxygenase (13-LOX). They differ in oxygenation of fatty acids at carbon atom 9 or 13. Based on the amino acid sequences, *LOX1* and *LOX5* are proposed to encode for 9-LOXs and *LOX3* for a 13-LOX[25].

Lipoxygenases catalyze the oxygenation of polyunsaturated fatty acids (e.g. linoleic acid and linolenic acid) into reactive hydroperoxides. This process is called hydroperoxidation of lipids. Hydroperoxides are further catalyzed to form oxylipins. LOX-derived oxylipins (e.g. jasmonates) have been shown to act as regulators of root growth[24]. It is also known that lipoxygenase gene expression is influenced by exposure to metals[26]. According to this information, the involvement of *LOX*-genes in metal-specific growth responses is investigated.

Table 1. Genes encoding lipoxygenases in *Arabidopsis thaliana*[27]

Locus	Annotation	Nomenclature	A*	B*	C*
AT1G55020	lipoxygenase 1	*LOX1*	859	98044.4	5.2049
AT3G45140	lipoxygenase 2	*LOX2*	896	102044.7	5.3177
AT1G17420	lipoxygenase 3	*LOX3*	919	103725.1	8.0117
AT1G67560	lipoxygenase family protein	*LOX4*	917	104514.6	8.0035
AT3G22400	lipoxygenase 5	*LOX5*	886	101058.8	6.6033
AT1G72520	lipoxygenase, putative	*LOX6*	926	104813.1	7.5213

*A, amino acids; B, molecular weight; C, isoelectric point.

1.4 Aim of the study

So far it is known that there are metal-specific growth responses observed instead of a general SIMR in response to the metals Cd, Cu and Zn in *Arabidopsis thaliana* plants. Furthermore, the inhibitory effect of Cd and Cu on the lateral roots is local, while it is systemic in Zn-exposed *A. thaliana* plants[9].

The aim of this study is to participate in unravelling the molecular mechanisms underlying these metal-specific growth responses in *Arabidopsis thaliana* and to understand more about local and systemic effects on root development in a more relevant context of heterogeneous exposure. In order to identify these molecular mechanisms, root growth will be examined in metal-exposed *Arabidopsis thaliana* plants. Two parallel approaches will be performed.

Above, links between *LOX*-genes, metals and root growth were presented, leading to the hypothesis that *LOX*-genes are involved in metal-specific growth adaptations. By

means of reverse genetics, the phenotypic change under metal-stress conditions of *lox*-mutants compared to wildtype was investigated. This will give us more information on the involvement of *LOX*-genes and oxylipin signalling. Split-root systems will be used to examine if *LOX*-genes are involved in the local and/or systemic inhibitory effects of Cd, Cu and Zn. Besides *LOX*-genes, other genes involved in plant hormone synthesis and metabolism may be implicated in the metal-specific growth responses. The expression of these genes was examined in roots of metal-exposed wild-type plants.

All the experiments were performed with the model organism *Arabidopsis thaliana,* which remains a very good model to study the basic elements of root growth and environmental adaptation because of the high existing molecular information on intrinsic root development and availability of mutants. The easy cultivation and the absence of an ethical objection against the use of this plant also facilitates the research[1].

2. Materials and methods

2.1 Preparation of vertical agar plates

Growth medium was based on a 50 times diluted Gamborg's B5-medium except for $CuSO_4$ which was supplemented to a final concentration of 100 nM to avoid copper deficiency[28]. The growth medium was made by adding 20 mL/L of 50 times concentrated macronutrients (supplement 1), 1 mL/L of 1000 times concentrated micronutrients (supplement 2), 1 mL/L of 1000 times concentrated $FeNO_3.9H_2O$ (supplement 3), 0.5 g/L MES hydrate (2-[N-Morpholino]ethanesulfonic acid hydrate) (Sigma, St. Louis, USA), 5 g/L D-sucrose (Fluka, St. Louis, USA) (germination plates only) and 10 g/L plant tissue culture agar (Lab-M, Bury, UK). After adjusting the solution to pH 5.7-5.8 with KOH (Merck, Darmstadt, Germany), agar medium was autoclaved at 121°C for 10 minutes and kept overnight in an oven of 65°C. Forty mL of the agar medium at 65°C was added to 12x12 square Petri dishes (Greiner Bio-one, Wemmel, Belgium). The vertical agar plates were left open under the laminar airflow no longer then until the medium had solidified. This was done to prevent the accumulation of excess water due to evaporation and condensation during the experiments. If the vertical agar plates were not used directly, they were stored in their plastic bags at room temperature for maximum one week.

For the treatment plates, 1 cm of agar was removed at the top to form an air gap for the shoots and a small section (1 mm) of agar was removed (split root plates only) in such a way that an upper zone of 2 cm x 12 cm ($24cm^2$ = 6.7 mL) was separated from the lower zone of 9 cm x 12 cm (108 cm^2 = 30 mL) (supplement 4). $ZnSO_4$, $CdSO_4$, $CuSO_4$ and K_2SO_4 (all Merck, Darmstadt, Germany) solutions were filter sterilized (0.2 µm). For split root plates, 100 times concentrated sterilized solutions of $ZnSO_4$, $CdSO_4$, $CuSO_4$ and K_2SO_4 were spread out on the plates (67 µL to the top zone and 300 µL to the bottom zone). To complement growth media to the same concentrations of SO_4^{2-}, K_2SO_4 was added to the different concentrations of metals. Sulphate can influence root growth or plant stress responses[17], but the additional potassium added to the medium that already contained 500 µM potassium, however, has little effect on root growth.

2.2 Plant material and growth conditions

Arabidopsis thaliana Col-0 seeds were obtained from Lehle seeds (Round Rock, Texas, USA) and the *Arabidopsis thaliana lox1-1, lox3A, lox5-1* seeds from the NASC (Nottingham Arabidopsis Stock Centre). Seeds had been verified by genotyping before (data not shown). The seeds were transferred to a 90 mm filter paper (Whatman No. 542, hardened ashless) that was folded into a cone-shaped bag and sealed with a paper clip. The seeds were sterilised by means of 0.1% (v/v) NaOCl (Sigma-Aldrich, St. Louis, USA) with 0.1% (v/v) Tween 80 (Fluka, Steinheim, Germany) for 1 minute. The seeds were rinsed four times with a small volume of sterilized distilled water (dH$_2$0) and soaked four times five minutes in a larger volume of sterile distilled water so that no odor of chlorine remained. The seeds were spread out and dried on the filter paper in a laminar air-flow. The dried sterile seeds can then be sown or they can be stored in a tightly closed Petri dish for one month or more.

Twenty sterile *Arabidopsis thaliana* seeds were sown on germination plates in a straight line at 1 cm below the top. The plates were sealed with Parafilm and in two locations at the bottom (left and right) of the plates 2 cm gaps were made to allow air exchange. For one or two days, plates were incubated at 4°C in the dark which ensures uniform germination. Plates were placed vertically, slightly leaning backwards, in a climate chamber (18°C/22°C ± 1°C, alternately twelve hours light and twelve hours dark, light intensity of about 100 µmol/m^{-2}/s (delivered by blue, red and far red light LEDs)). Genotypes were placed alternately so that each genotype had the same conditions in the climate chamber. After seven days of incubation, a homogenous set of seedlings (± 2.5 cm) were transferred to the split-root plates (four plants per plate) or treatment plates for gene expression (8 plants per plate). Shoots were placed in the air gap at 1 cm below the top, and (split-root system) the roots contacted both zones with the primary root tip (apex) in the lower zone of the split-root system (supplement 4). Finally, plates were sealed with Parafilm containing two gaps at the bottom to allow air exchange and incubated in a climate chamber as above.

At the end of the experiments, the biological material of mutants was destroyed by means of autoclaving (121°C) or by removing the shoots from the roots by means of a scissor. This ensures that the genetically modified plants can not spread in the environment.

2.3 Imaging and harvesting

After transferring the seedlings to the treatment or split root plates, each day the primary root tip was marked on the plate. At the end of the experiment all plates were scanned with a Canoscan 4400F (Canon) or an Epson Perfection V330 photo scan at 300 dpi to allow root analysis and kinetic analysis of the primary roots. For seedlings destined for gene expression analysis whole roots of 24h exposed plants were harvested by means of a scissor and a forceps. All eppendorfs (each containing ± twenty four plants) were frozen in liquid nitrogen and stored in a freezer (-80°C).

2.4 Root analysis

Root parameters ((kinetic) primary root length, mean lateral root length, mean lateral root number, lateral root density, total lateral root length, % of total lateral root length in zone, last lateral to apex and lateral root length per unit primary root length) were analysed using the program Optimas 6.1. Mean, standard errors and graphs were determined using Microsoft excel 2007.

2.5 Gene expression determination

2.5.1 RNA-extraction

Tissue samples were destroyed by a mechanical lysis. About ten glass beads were added to each sample followed by a shredding step at an amplitude of 90 for 1 minute using the MM 2000 Mixer Mill (Retsch, Aartselaar, Belgium). Lysis buffer (300 µL) was added to each sample for a chemical lysis and all samples were vortexed in order to facilitate the lysis process. The lysis buffer contained RLT buffer (Qiagen,

Maryland, USA) and β-mercapto-ethanol (Sigma-Aldrich, St. Louis, USA) (1µL/1mL RLT). Hereafter, 200 µL phenol-chloroform pH 4.5 (Ambion, Texas, USA) was added to each sample in order to separate RNA from the DNA and the proteins. This resulted in an organic (containing proteins) and an aqueous phase (containing RNA). Samples were flicked and homogenized for 5 minutes at room temperature. After 5 minutes of centrifugation at maximum speed (13 400 rpm) (4°C), the aqueous phase was transferred to a new eppendorf containing 200 µl chloroform (VWR Prolabo, Leuven, Belgium) and again centrifuged at maximum speed for 2 minutes at 4°C. This washing step with chloroform was repeated once more. The aqueous phase was transferred to an empty eppendorf and centrifuged for 3 minutes at maximum speed (4°C) to know with certainty whether the organic phase was no longer present. The next step is the RNA-precipitation. Two volumes of ice-cold 100% Ethanol (Sigma-Aldrich, St. Louis, USA) and 1/10 volume of 3M Sodiumacetate pH 5.2 (Fermentas, St. Leon-Rot, Germany) was added to each sample. After the storage in the freezer (-80°C) for 30 minutes, samples were centrifuged for 15 minutes at maximum speed (4°C) and all liquid was removed from the pellet. RNA was washed with 200 µl ice-cold 80% Ethanol (Sigma-Aldrich, St. Louis, USA) followed by a centrifugation at maximum speed for 2 minutes (4°C). All liquid was removed of the pellet. The RNA-washing step with 80% Ethanol was repeated once more. Finally, samples were first dried in an oven (37°C) and then the pellet was resuspended in 15 µl RNA-free water. The concentration and purity of RNA was tested by means of NanoDrop ND-1000 Spectrophotometer (Isogen Life Science, De Meern, Netherlands). RNA-samples were subsequently stored in the freezer (-80°C).

2.5.2   cDNA-synthesis

Reverse Transcriptase-PCR or RT-PCR converts the extracted RNA to cDNA. First, remaining contaminant DNA was removed from the RNA-containing samples using the TURBO DNA-free kit (Applied Biosystems, Foster City, USA). Equal amounts of RNA (100 ng) were dissolved in RNase-free water to an end solution of 13.2 µL in 0.2 mL eppendorfs and stored in an Isofreeze at 4°C. To each sample, 1.5 µL 10X TURBO DNase buffer and 0.25 µL TURBO DNase was added. After an incubation of 25 minutes at 37°C, 2 µL DNase Inactivation Reagent was added. All eppendorfs

were incubated at room temperature for 2 minutes and twice vortexed during the incubation. After a centrifugation of 1.5 minutes at 10 000 g, 13.2 µL of the supernatant was transferred to a new eppendorf.

cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (Ambion, Texas, USA). To each sample, 6.8 µL Reverse Transcriptase mastermix was added containing 2.0 µL 10X RT buffer, 0.8 µL 25X dNTP Mix (100 mM), 2.0 µL 10X RT Random Primers, 1.0 µL MultiScribe Rverse Transcriptase and 1.0 µL Nuclease-free water. After proper mixing, reverse transcription was accomplished using the following incubations:

Stage 1: 10 minutes at 25°C (annealing primers to complete transcriptome)
Stage 2: 120 minutes at 37°C (reverse transcriptase activation)
Stage 3: 5 minutes at 85°C (reverse transcriptase inactivation)
Stage 4: Storing samples at 4°C

2.5.3 Quantitative PCR

To the obtained cDNA (20 µL/sample), 80 µl of 1/10 TE buffer was added. A pooled sample was prepared for a calibration curve to determine the primer efficiency for each investigated gene. A no template control (NTC) was included for each investigated primer set to determine possible genomic contamination and/or primer-dimers. In a 96-well plate, 8 µL mastermix and 2 µL cDNA (or water for NTC) was added to each well. The mastermix contained 2.4 µL RNase-free water, 5 µL 2x FAST SYBR GREEN reaction mix (Applied Biosystems, Foster city, USA), 0.3 µl or 300 nM of each primer (reverse and forward). The 96-well plates (Applied Biosystems, Foster city, USA) were covered with an adhesive film (Applied biosystems, Foster city, USA) and centrifuged for 30 seconds at 3000 rpm. Finally, the plates were analysed with the 7500 Fast Realtime PCR-system (Applied biosystems, Foster city, USA). Reaction volume was set to 10 µL and the next program was started:

Step 1: 5 minutes at 95°C (activation Taq polymerase)

Step 2 (repeated 45 times): 4 seconds at 95°C (denaturation), 40 seconds at 60°C (annealing primers and extension)

Step 3: melting curve generation: increase of temperature from 60° to 95° over 15 minutes.

2.5.4 Analysis

The 7500 Fast Realtime PCR-program monitored the amount of fluorescence in each of the 45 PCR cycles. The exponential phase was used to determine the initial concentration of RNA because a pure doubling of PCR product takes place in this phase. The exponential phase was log transformed to a linear phase. A threshold was selected that did not contain background fluorescence and that passed through the linear phase. For each investigated gene, cycle threshold (Ct-)values were determined according to the chosen threshold and a melting curve was established to detect the specificity of PCR-amplification (unspecific amplifications are e.g. primer dimers and/or genomic DNA). Relative expression values of all genes were calculated by means of the $E^{-\Delta Ct}$ method[29] with E = actual primer efficiency. A set of candidate reference genes (table 2) were analyzed by geNorm[29] and the three most stable reference genes were used to calculate a normalization factor as the geometric mean of the expression levels. This factor normalizes for technical errors during the entire experiment, such as differences in RNA sample quality, RNA input quantity and enzymatic efficiency in reverse transcription. The genes of interest are represented in table 3.

Table 2: List of candidate reference genes in *Arabidopsis thaliana* and their primer sequences[30].

Locus	Annotation	Forward primer	Reverse primer
AT2G28390	*SAND family*	AACTCTATGCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC
AT3G18780	*ACT2*	CTTGCACCAAGCAGCATGAA	CCGATCCAGACACTGTACTTCCTT
AT4G34270	*TIP41-like*	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA
AT5G08290	*Mitosis protein YLS8*	TTACTGTTTCGGTTGTTCTCCATTT	CACTGAATCATGTTCGAAGCAAGT
AT5G15710	*F-box protein*	TTTCGGCTGAGAGGTTCGAGT	GATTCCAAGACGTAAAGCAGATCAA
AT5G25760	*UBC*	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC
AT4G05320	*UBQ10*	GGCCTTGTATAATCCCTGATGAATAAG	AAAGAGATAACAGGAACGGAAACATAGT
AT5G60390	*EF-1α*	TGAGCACGCTCTTCTTGCTTTCA	GGTGGTGGCATCCATCTTGTTACA

Table 3: Genes of interest and their primer sequences.

Locus	Annotation	Forward primer	Reverse primer
AT1G55020	*LOX1*	TTGGCTAAGGCTTTTGTCGG	GTGGCAATCACAAACGGTTC
AT1G17420	*LOX3*	ACGTTGTCGTACTGGTCGCC	GTCTCGTGGCACATACATAGGTAATG
AT3G22400	*LOX5*	GGCAAAACCGGCCGTAAAT	CGTCCCTTGGCACGTATATGTT
AT5G20960	*AAO1* (auxin synthesis)	GGAGGAAGACATCCGATGAA	CGGTTAACCCTGCATCAAGT
AT3G44310	*NIT1* (auxin synthesis)	TTGCTGGGAGAATAGGATGC	TTGCCATTCTTTCGAACCAT
AT2G19500	*CKX2* (cytokinin degradation)	GACCACCAATTCCACCATTC	TTAAGAAGACGGCGGAGAAA
AT2G36750	*ZOG1* (cytokinin degradation)	TGGAGGAATTGATGGGTGAT	CTCCTTCTTCCACAGCCTTG
AT3G23630	*IPT7* (cytokinin synthesis)	GCCGGTGGATCAAACTCTTA	AACGTCGACCCAAATGAAAC

2.7 Statistics

Normality was tested by means of a Shapiro-Wilk test. A log transformation was performed when normality was not met. The homogeneity of variances was tested with a Fligner-Killeen test. Reverse genetics data were statistical analyzed with a an one way or a two way Anova for normally distributed data, while Kruskal Wallis rank sum test was used for data that was not normally distributed. A multiple comparison was performed after Anova with a Tuky's test and a Wilcoxon rank sum test combined with Bonferroni was used after a Kruskal Wallis rank sum test. Gene expression data were statistical analyzed by an one way Anova followed by a Dunnet test or a non-paramtric Kruskal Wallis rank sum test followed by a Wilcoxon rank sum test. All statistical analyses were performed with a significance level of 0.05.

3. Results

In this study, the phenotypic change of root architecture under metal-stress conditions was investigated, and the involvement of lipoxygenase genes assessed in a reverse genetics approach using *lox*-mutants (*lox1-1, lox3A, lox5-1*). Split-root systems were used to reveal local and systemic effects on root development in a more relevant context of heterogeneous exposure. Furthermore, the expression of genes involved in plant hormone synthesis or metabolism was examined in the roots of metal-exposed wildtype *Arabidopsis thaliana* seedlings.

3.1 Effects of *LOX*-genes on root growth in metal-exposed *Arabidopsis thaliana* seedlings in a more relevant context of heterogeneous exposure

Links between *LOX*-genes, metals and root growth were presented in the introduction, leading to the hypothesis that *LOX*-genes are involved in metal-specific root growth responses. The phenotypic change of *lox*-mutants under metal-stress conditions was compared with the wild-type after growth in split-root vertical agar plates. Concerning the conditions for comparison in which wild-type plants were homogeneously exposed to metals, most morphological responses to Cd, Cu and Zn were consistent with previous conclusions on metal specific effects[9]. Cu increased the number of lateral roots, whereas Zn had a strong inhibitory effect (figure 2). However the increase in lateral root number (figure 2) and lateral root density after exposure to Cd was not found (supplement 6). Normally, at certain levels of exposure Cd increases lateral root density, but under high exposure this stimulating effect is lost and a decrease is possible. The growth systems were also different than in previous experiments (deviating growth conditions concerning light intensity and quality).
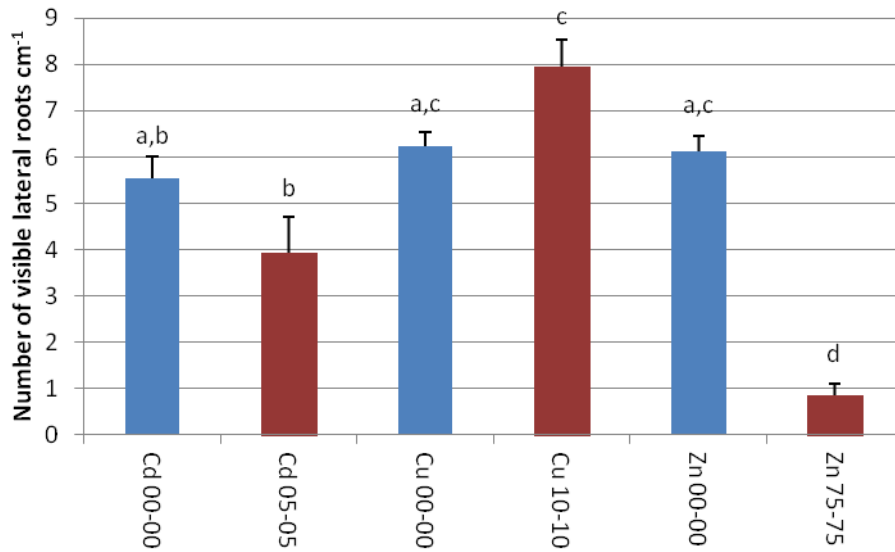
Figure 2: Number of visible lateral roots cm^{-1} of *Arabidopsis thaliana* seedlings (Col-0) in the upper zone of split-root vertical agar plates after eight days of homogeneous exposure to 5 µM Cd, 10 µM Cu and 75 µM Zn. Data are means of 9 to 16 independent replicates. Different letters indicate significant differences at P<0.05. Data were statistically analyzed with a two way Anova followed by a Tukey's test.

Knowledge in homogeneous exposure has already been gained, now more information is gathered concerning heterogenous exposure and *lox* mutants. With most of the responses of wild-type plants in homogeneous exposure confirmed, the phenotypic changes in wild-type plants under heterogeneous exposure were investigated and the involvement of lipoxygenase *(LOX)*-genes could be derived from deviating results in the *lox* mutants. Only a selection of the data that answers the specific research questions are represented. Full results of all conditions and parameters are provided in the supplement.

The research questions concern local and systemic effects that have an influence on the capacity for avoidance and colonization. When a primary root comes in contact with metals, two questions can be asked: (1) is there an avoidance of the metal containing zone and (2) is there a systemic effect on the control zone where the colonization should continue as much as possible (systemic inhibition/no effect/stimulation)?

3.1.1 What is the effect of the control upper zone on the avoidance capacity in the lower zone containing Cd, Cu or Zn?

Growth of primary roots of plants that are homogeneously exposed to the metals are strongly inhibited at the metal concentrations used (5 µM Cd, 10 µM Cu, 75 µM Zn). Here we compare the growth of the primary root in the lower zone between plants that are homogeneously exposed and those that are only exposed by their root tip.

An incomplete avoidance of the primary root under Cd and Zn exposure was observed for Col-0. *Lox1* and *lox5* showed a complete avoidance after exposure to Cd, however, the complete avoidance in *lox3* is less clear under Cd-exposure (figure 3a). *LOX1* and *LOX5* are 9-LOXs, while *LOX3* is a 13-LOX. It is possible that 9-LOX signalling prevents the complete avoidance of the primary root under Cd-exposure. Wild-types and all mutants show the same avoidance capacity under Zn-exposure, but at least in the case of *lox3* the primary root growth seems less sensitive in homogenous exposure to 75 µM Zn (figure 3c). Furthermore, *lox*-mutants appear not to be involved in primary root growth after exposure to Cu (figure 3b). Thus *lox*-mutants differ in primary root length under Cd- and Zn-exposure, while under Cu-exposure no differences are observed. This may indicate that Cd and Zn can induce an effect on primary root growth by producing ROS via *LOX*-genes, while Cu can induce ROS directly because its redox-active, and thus may not need LOX-activity to trigger downstream effects on growth. Quantification of lateral roots in the lower zone containing Cd, Cu or Zn is of little relevance because of the rather limited growth and thus small size of the primary root axis.
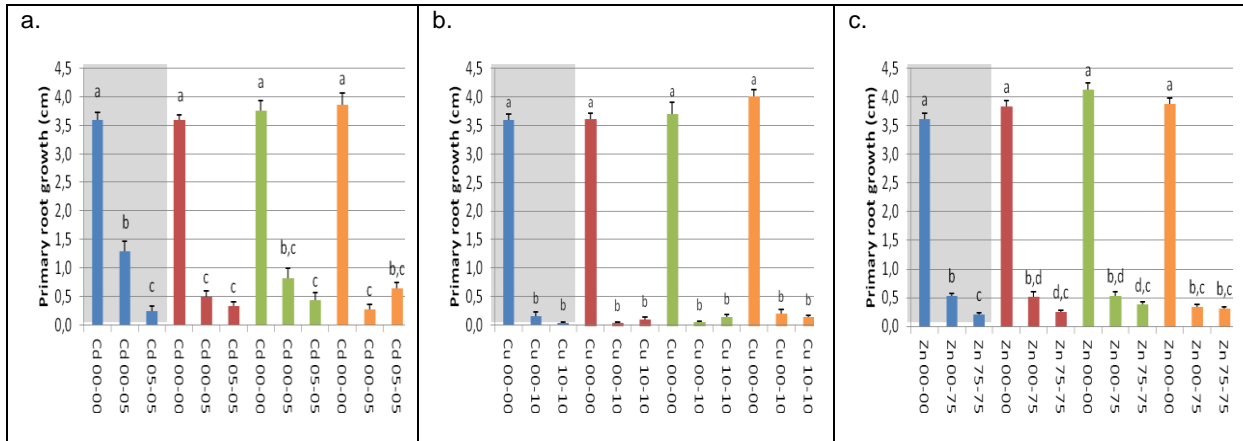
Figure 3: Primary root growth (cm) in the lower zone of the split root vertical agar plate of *Arabidopsis thaliana* seedlings (red) *lox1*, (green) *lox3* and (orange) *lox5* compared to (blue) wild-type after eight days of heterogeneous (lower zone) and homogeneous exposure to (a) 5 µM Cd, (b) 10 µM Cu and (c) 75 µM Zn. Data are means of 9 to 16 independent replicates. Different letters indicate significant differences at P<0.05. Data were statistically analyzed with a two way Anova followed by a Tukey's test or a non-parametric Kruskal wallis rank sum test followed by a Wilcoxon rank sum test combined with Bonferroni test.

The kinetic analysis of primary root growth also indicates that the avoidance by the primary root of the Cd-containing lower zone was improved in *lox*-mutants in comparison with the wild-type (Cd 0-5 vs. Cd 5-5; figure 4). The incomplete avoidance of the wild-type in the Zn-containing lower zone (at least in the first four days) was improved in *lox*-mutants from the day after transfer (Zn 0-75 vs. Zn 75-75; figure 4). Furthermore, no differences in kinetic primary root growth was observed in *lox*-mutants compared to wild-type. *Lox*-mutants appear not to be involved in primary root growth after exposure to Cu (figure 4).
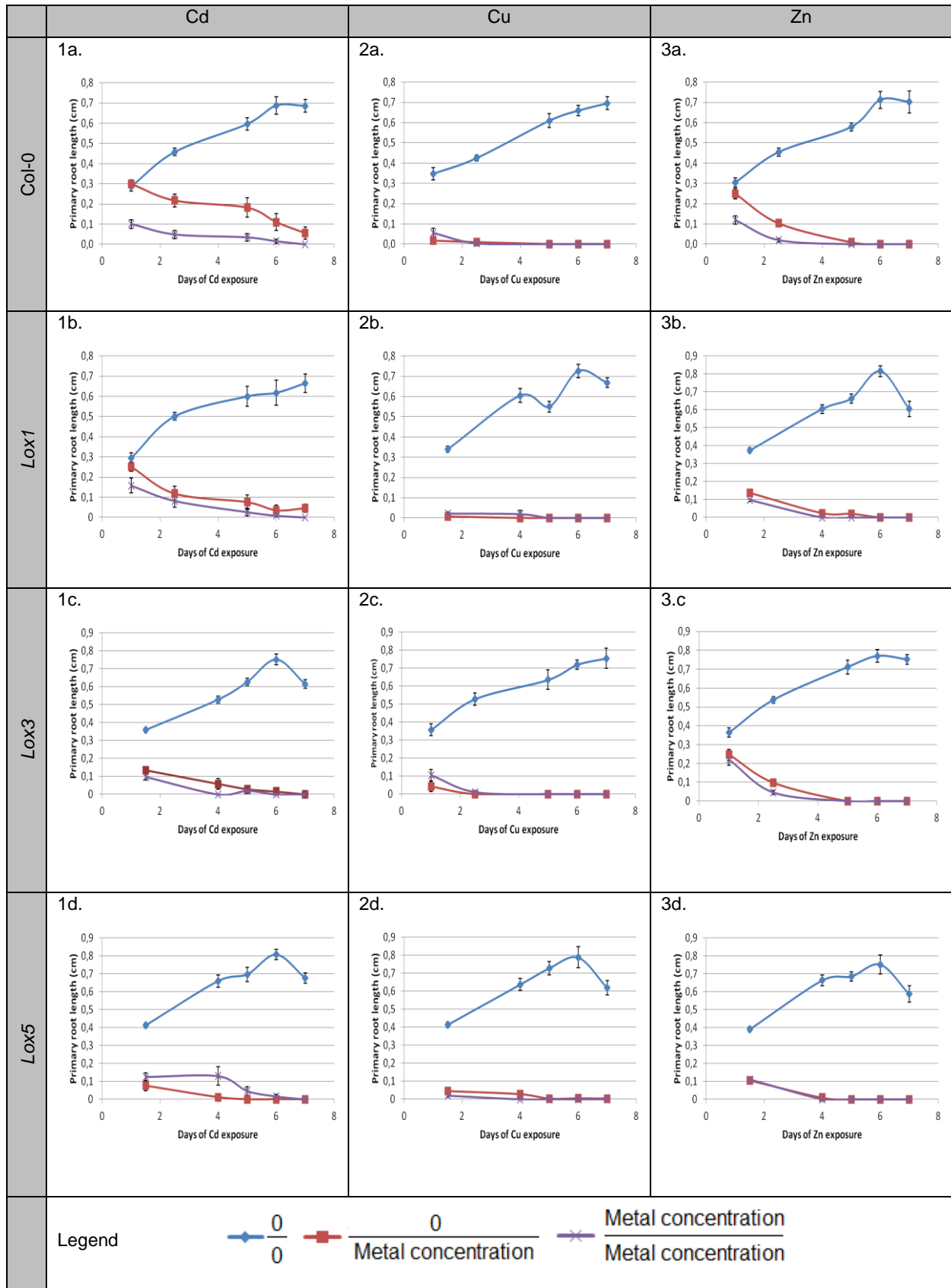
Figure 4: Kinetics of primary root length during seven days after transfer of *Arabidopsis thaliana* seedlings (a) Col-0, (b) *lox1*, (c) *lox3* and (d) *lox5* to heterogeneous (lower zone) and homogeneous exposure to (1) 5 µM Cd, (2) 10 µM Cu and (3) 75 µM Zn. Data are means of 10 to 16 independent replicates.

3.1.2 What is the effect of primary root tip exposure to Cd, Cu and Zn in the lower zone on the proceeding of the colonization capacity in the upper zone containing no metals?

When the primary root tip is exposed to metals, systemic effects may influence the colonization capacity by the lateral roots above. A stimulation of the total lateral root length for Col-0 was observed under Cd-, Cu- and Zn-exposure in wild-type plants (figure 5). This was due to the stimulation of both lateral root elongation and lateral root number (figure 6,7). *Lox3* en *lox5*, however, have longer lateral roots than wild-type plants at the beginning and no additional stimulation of elongation is observed in the upper zone when Cd, Cu or Zn are administrated in the lower zone (figure 6). Since the total lateral root length in wild-types exposed to metals at the root tip, equals that of *lox3* and *lox5* mutants under control conditions, it may be that *LOX3* and *LOX5* are down regulated under metal exposure so that the inhibition on lateral root elongation by *LOX3* and *LOX5* is lifted. The remaining small stimulation of the total lateral root length of *lox3* and *lox5* when their root tip is exposed to metals, is due to the stimulation of lateral root number (figure 7). However, for *lox3* under Zn-exposure no additional stimulation of total lateral root length was observed by the metal exposure of the root tip (figure 5c). *Lox1* behaves the same as Col-0 except under Cd-exposure where the stimulation of the total lateral root length only is determined by lateral root number.
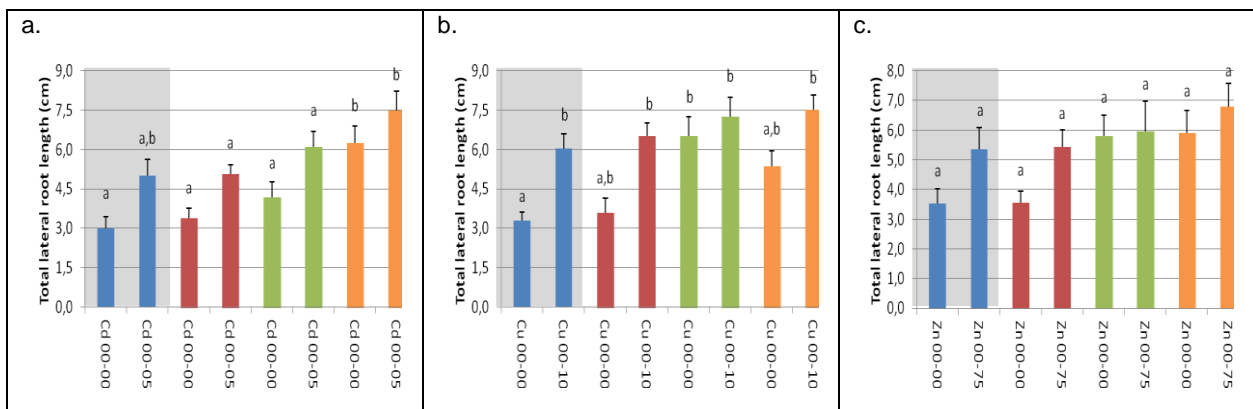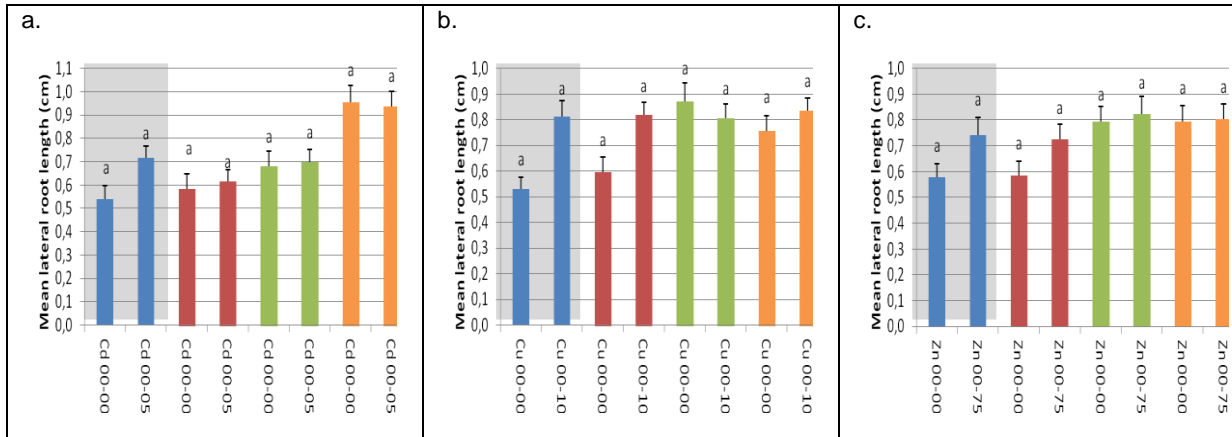

Figure 5: Total lateral root length (cm) in the upper zone of the split-root vertical agar plates of *Arabidopsis thaliana* seedlings (red) *lox1*, (green) *lox3* and (orange) *lox5* compared to (blue) wild-type after eight days of heterogeneous exposure to (a) 5 µM Cd, (b) 10 µM Cu and (c) 75 µM Zn in the lower zone. Data are means of 9 to 16 independent replicates. Different letters indicate significant differences at P<0.05. Data were statistically analyzed with a two way Anova followed by a Tukey's test or a non-parametric Kruskal wallis rank sum test followed by a Wilcoxon rank sum test combined with Bonferroni test.
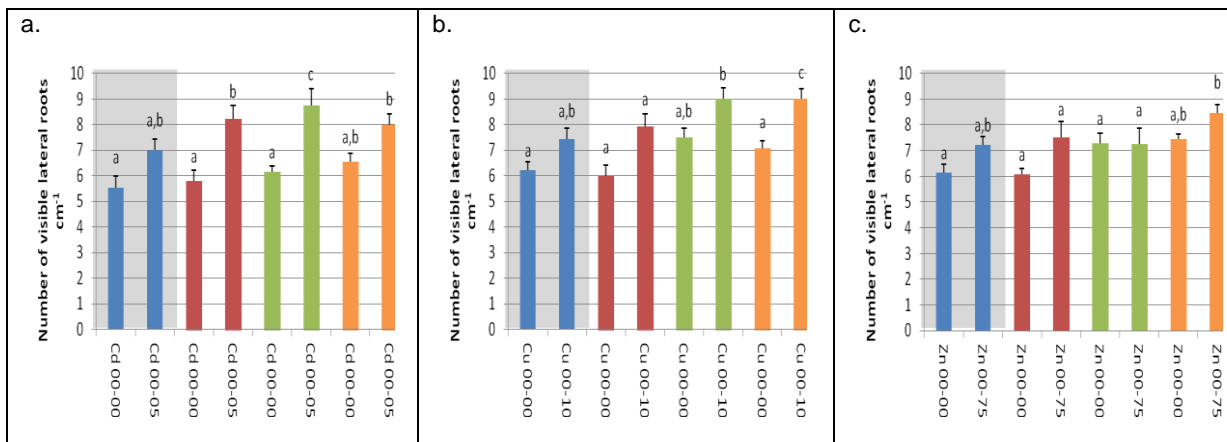
Figure 6: Mean lateral root length (cm) in the upper zone of the split-root vertical agar plates of *Arabidopsis thaliana* seedlings (red) *lox1*, (green) *lox3* and (orange) *lox5* compared to (blue) wild-type after eight days of heterogeneous exposure to (a) 5 µM Cd, (b) 10 µM Cu and (c) 75 µM Zn in the lower zone. Data are means of 9 to 16 independent replicates. Different letters indicate significant differences at P<0.05. Data were statistically analyzed with a two way Anova followed by a Tukey's test or a non-parametric Kruskal wallis rank sum test followed by a Wilcoxon rank sum test combined with Bonferroni test.

Figure 7: Number of visible lateral roots cm^{-1} in the upper zone of the split-root vertical agar plates of *Arabidopsis thaliana* seedlings (red) *lox1*, (green) *lox3* and (orange) *lox5* compared to (blue) wild-type after eight days of heterogeneous exposure to (a) 5 µM Cd, (b) 10 µM Cu and (c) 75 µM Zn in the lower zone. Data are means of 9 to 16 independent replicates. Different letters indicate significant differences at P<0.05. Data were statistically analyzed with a two way Anova followed by a Tukey's test or a non-parametric Kruskal wallis rank sum test followed by a Wilcoxon rank sum test combined with Bonferroni test.

3.1.3 What is the effect of exposure to Cd, Cu and Zn in the upper zone on the colonization capacity in the lower zone containing no metals?

Primary root growth under Zn-exposure shows an incomplete colonization of the metal-free zone, whereas this colonization is less inhibited in *lox3* and *lox5* (figure 8).
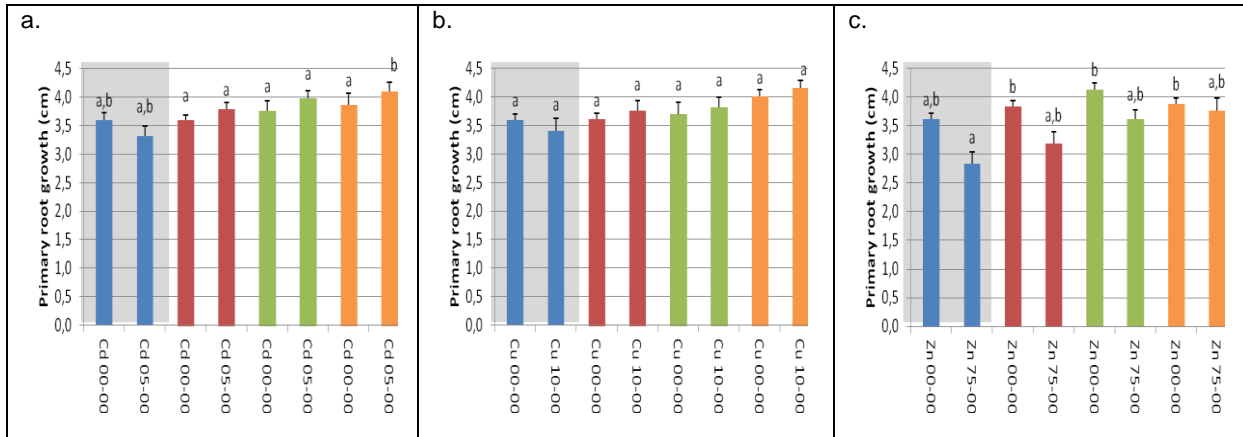
Figure 8: Primary root growth (cm) in the lower zone of the split root vertical agar plate of *Arabidopsis thaliana* seedlings (red) *lox1*, (green) *lox3* and (orange) *lox5* compared to (blue) wild-type after eight days of heterogeneous exposure to (a) 5 µM Cd, (b) 10 µM Cu and (c) 75 µM Zn in the upper zone. Data are means of 9 to 16 independent replicates. Different letters indicate significant differences at P<0.05. Data were statistically analyzed with a two way Anova followed by a Tukey's test or a non-parametric Kruskal wallis rank sum test followed by a Wilcoxon rank sum test combined with Bonferroni test.

The kinetic analysis of primary root growth indicates that *lox*-mutants show a stimulated colonization of a lower control zone when the rest of the plant above experiences Cd- or Zn-exposure (Cd 5-0 vs Cd 0-0; Zn 75-0 vs Zn 0-0; figure 9), except *lox1* under Zn-exposure, which shows a reduced colonization of the metal-free zone as is seen in the wild-type. Meanwhile *lox*-mutants appear not to be involved in primary root growth after Cu exposure (figure 9).
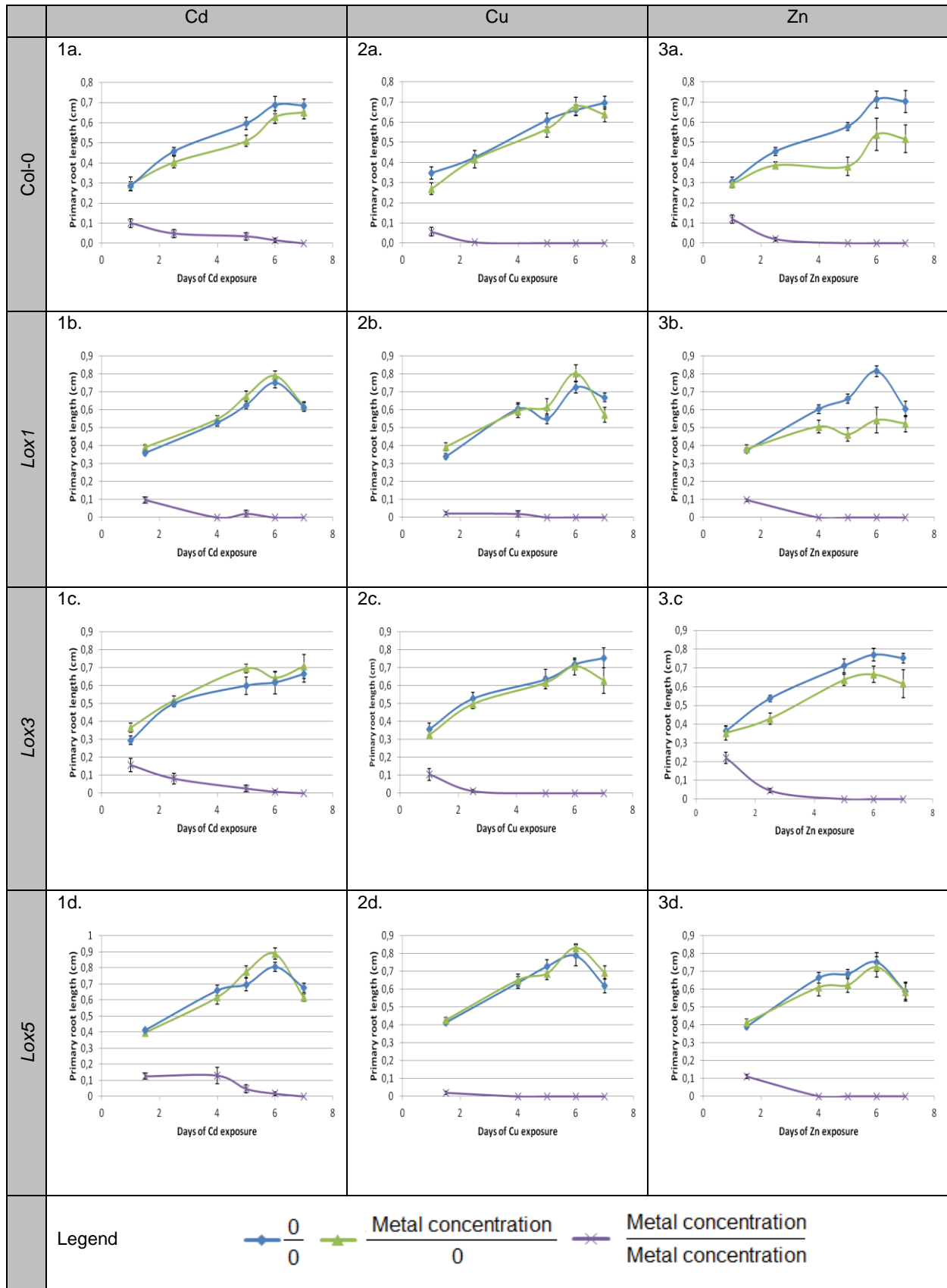
Figure 9: Kinetics of primary root length during seven days after transfer of *Arabidopsis thaliana* seedlings (a) Col-0, (b) *lox1*, (c) *lox3* and (d) *lox5* to heterogeneous (upper zone) and homogeneous exposure to (1) 5 µM Cd, (2) 10 µM Cu and (3) 75 µM Zn. Data are means of 10 to 16 independent replicates.

Cd stimulates total lateral root length in wild-type plants (fig. 10a) by stimulating lateral root elongation (fig. 11a), while Cu and Zn have a opposite effect on total lateral root length (fig. 10b,c) by inhibiting lateral root elongation (only Cu, fig. 11b) and lateral root number (Zn, fig. 12c). The inhibition effect of Zn on total lateral root length is also found in *lox1* and *lox5*. In *lox3* this inhibition is less clear since lateral root number was less inhibited than in *lox1* and *lox5*. A lateral root number inhibition was also found for *lox5* under Zn-exposure but total lateral root length decreased because lateral root elongation was inhibited. When lateral root number was corrected for the difference in primary root length (lateral root density), still no inhibition was found in *lox3* (figure 13). In general *lox*-mutants have a better colonization of the metal-free zone than wild-type plants, as is seen for percentage total lateral root length in zone (Percentage in lower zone: Wild-type vs *lox1*: 5-0 Cd, 58-73%; 10-0 Cu, 67%-75%; 75-0 Zn, 74%-79%. Wild-type vs *lox3*: 5-0 Cd, 58%-73%; 10-0 Cu, 67%-73%; 75-0 Zn, 26%-16%. Wild-type vs *lox5*: 5-0 Cd, 58%-69%; 10-0 Cu, 67%-76%; 75-0 Zn, 74%-79%.) (supplement 6).
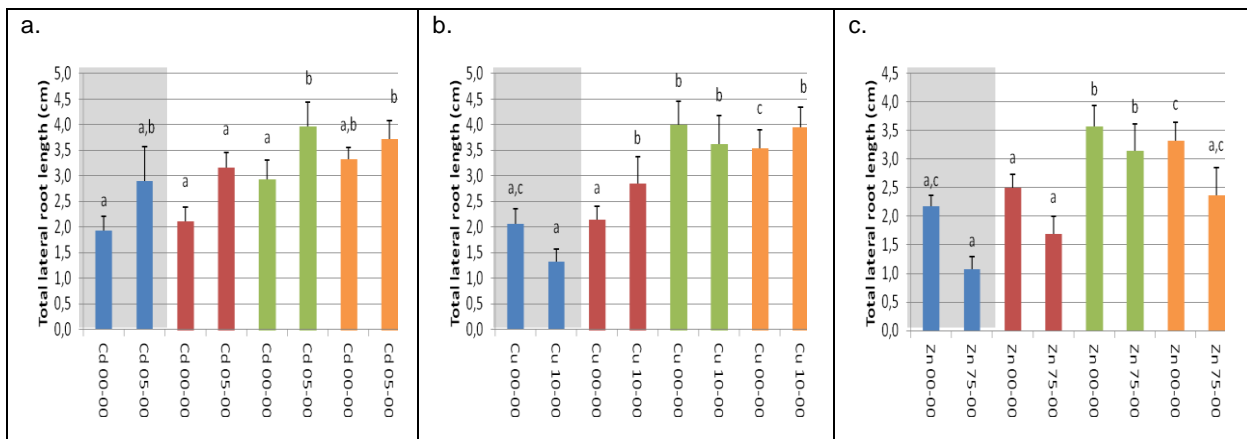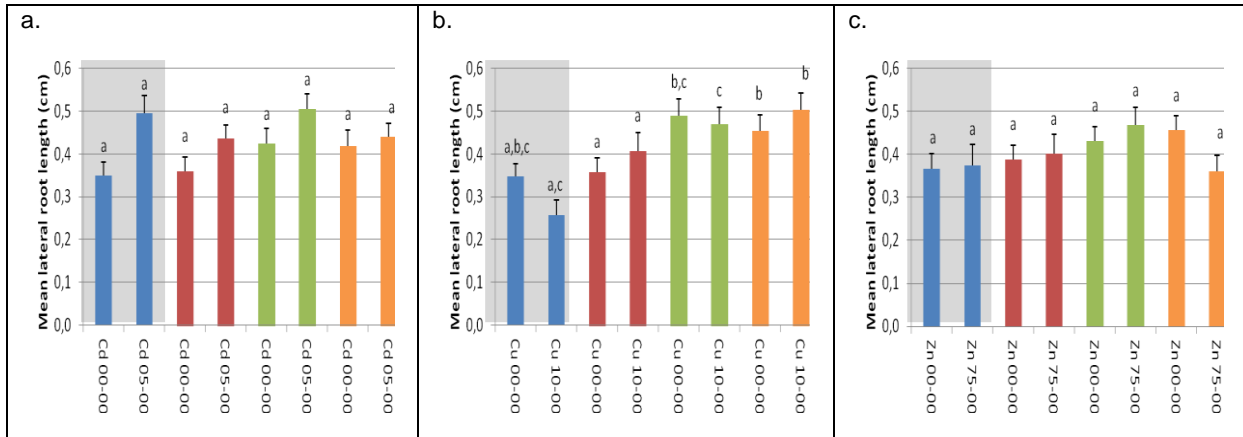

Figure 10: Total lateral root length (cm) in the lower zone of the split-root vertical agar plates of *Arabidopsis thaliana* seedlings (red) *lox1*, (green) *lox3* and (orange) *lox5* compared to (blue) wild-type after eight days of heterogeneous exposure to (a) 5 µM Cd, (b) 10 µM Cu and (c) 75 µM Zn in the upper zone. Data are means of 9 to 16 independent replicates. Different letters indicate significant differences at P<0.05. Data were statistically analyzed with a two way Anova followed by a Tukey's test or a non-parametric Kruskal wallis rank sum test followed by a Wilcoxon rank sum test combined with Bonferroni test.

Figure 11: Mean lateral root length (cm) in the lower zone of the split-root vertical agar plates of *Arabidopsis thaliana* seedlings (red) *lox1*, (green) *lox3* and (orange) *lox5* compared to (blue) wild-type after eight days of heterogeneous exposure to (a) 5 µM Cd, (b) 10 µM Cu and (c) 75 µM Zn in the upper zone. Data are means of 9 to 16 independent replicates. Different letters indicate significant differences at $P<0.05$. Data were statistically analyzed with a two way Anova followed by a Tukey's test or a non-parametric Kruskal wallis rank sum test followed by a Wilcoxon rank sum test combined with Bonferroni test.
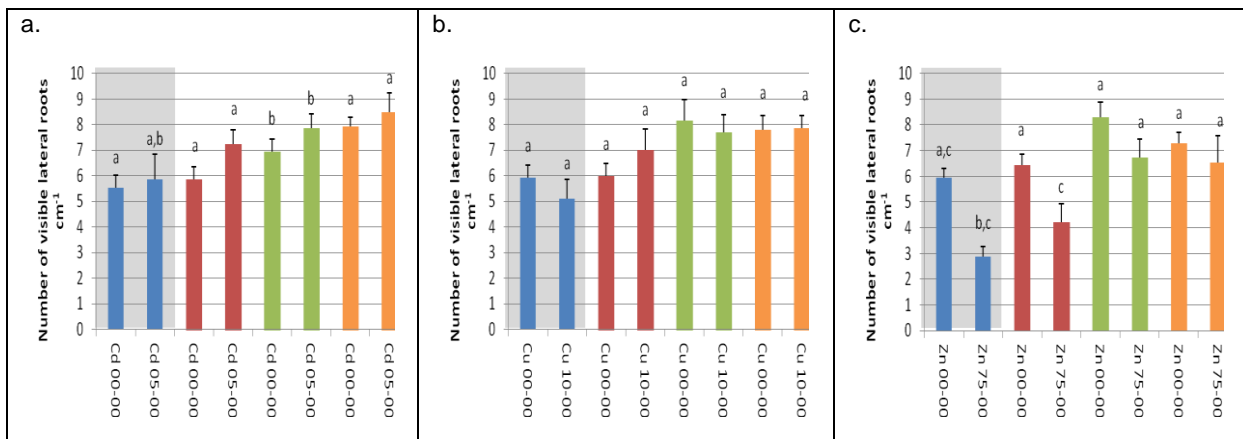

Figure 12: Number of visible lateral roots cm^{-1} in the lower zone of the split-root vertical agar plates of *Arabidopsis thaliana* seedlings (red) *lox1*, (green) *lox3* and (orange) *lox5* compared to (blue) wild-type after eight days of heterogeneous exposure to (a) 5 µM Cd, (b) 10 µM Cu and (c) 75 µM Zn in the upper zone. Data are means of 9 to 16 independent replicates. Different letters indicate significant differences at $P<0.05$. Data were statistically analyzed with a two way Anova followed by a Tukey's test or a non-parametric Kruskal wallis rank sum test followed by a Wilcoxon rank sum test combined with Bonferroni test.

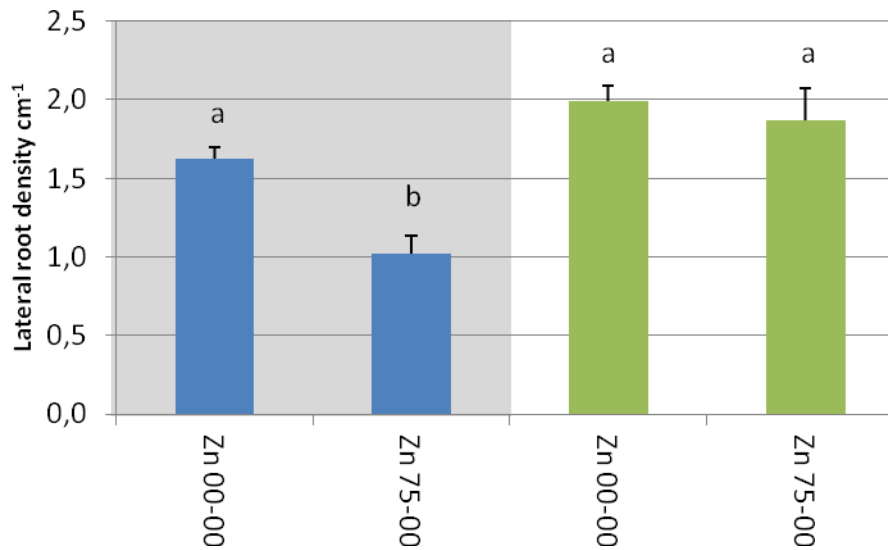Figure 13: Lateral root density cm^{-1} in the lower zone of the split-root vertical agar plates of *Arabidopsis thaliana* seedlings (green) *lox3* compared to (blue) wild-type after eight days of heterogeneous exposure to 75 µM Zn in the upper zone. Data are means of 9 to 16 independent replicates. Different letters indicate significant differences at P<0.05. Data were statistically analyzed with a two way Anova followed by a Tukey's test or a non-parametric Kruskal wallis rank sum test followed by a Wilcoxon rank sum test combined with Bonferroni test.

3.1.4 What is the effect of the lower control zone on the maintenance of the avoidance capacity in the upper zone containing Cd, Cu or Zn ?

No loss in total lateral root avoidance has been observed under Cu- and Zn-exposure, while under Cd-exposure a small loss of total lateral root avoidance can be seen (figure 14). This small loss in total lateral root avoidance is also seen for the *lox*-mutants. The small loss of total lateral root avoidance is caused by less suppression on lateral root elongation (figure 15). Cu induced a stronger total lateral root avoidance by suppressing lateral root elongation, while Zn already has a strong inhibition by suppressing lateral root elongation and number (figure 15,16).
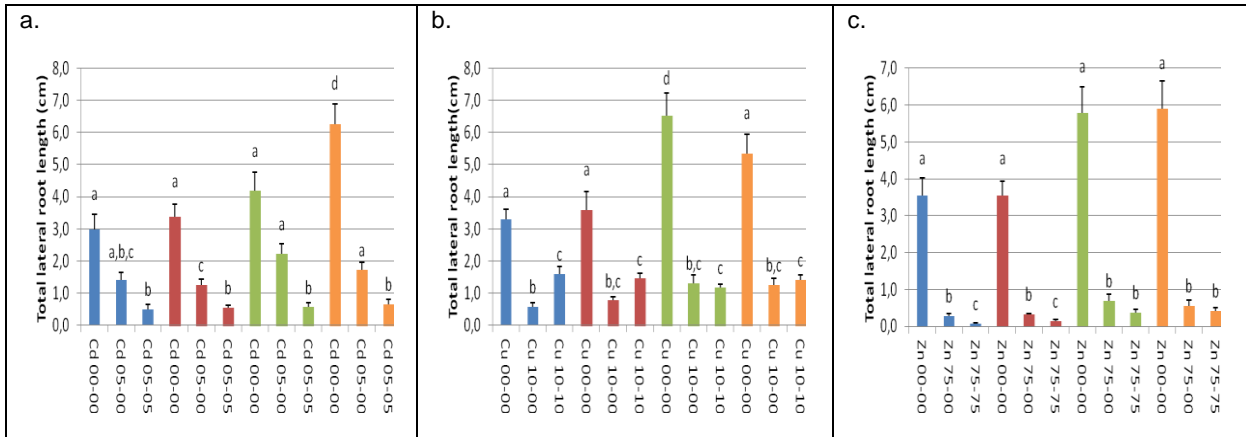
Figure 14: Total lateral root length (cm) in the upper zone of the split-root vertical agar plates of *Arabidopsis thaliana* seedlings (red) *lox1*, (green) *lox3* and (orange) *lox5* compared to (blue) wild-type after eight days of heterogeneous (upper zone) and homogeneous exposure to (a) 5 μM Cd, (b) 10 μM Cu and (c) 75 μM Zn. Data are means of 9 to 16 independent replicates. Different letters indicate significant differences at P<0.05. Data were statistically analyzed with a two way Anova followed by a Tukey's test or a non-parametric Kruskal wallis rank sum test followed by a Wilcoxon rank sum test combined with Bonferroni test.
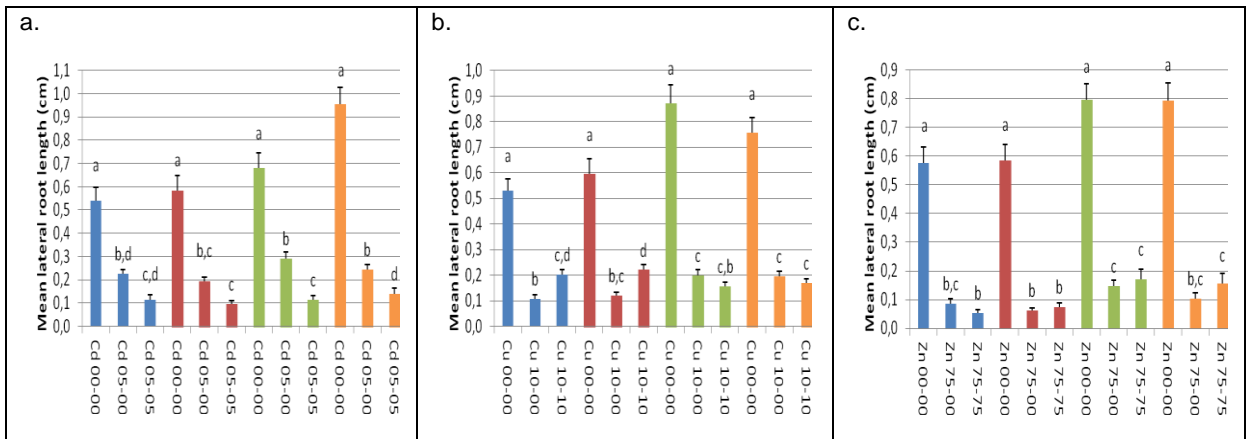

Figure 15: Mean lateral root length (cm) in the upper zone of the split-root vertical agar plates of *Arabidopsis thaliana* seedlings (red) *lox1*, (green) *lox3* and (orange) *lox5* compared to (blue) wild-type after eight days of heterogeneous (upper zone) and homogeneous exposure to (a) 5 μM Cd, (b) 10 μM Cu and (c) 75 μM Zn. Data are means of 9 to 16 independent replicates. Different letters indicate significant differences at P<0.05. Data were statistically analyzed with a two way Anova followed by a Tukey's test or a non-parametric Kruskal wallis rank sum test followed by a Wilcoxon rank sum test combined with Bonferroni test.
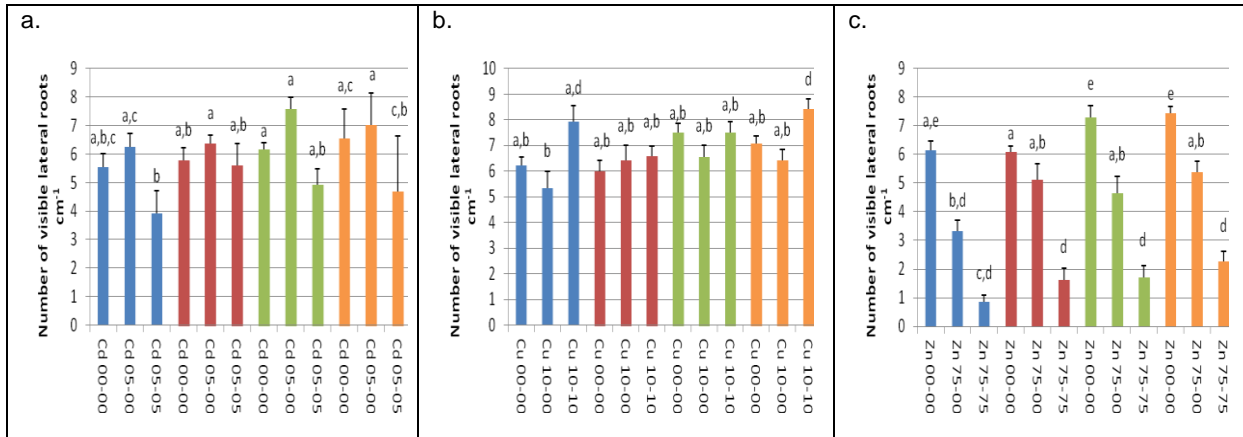
Figure 16: Number of visible lateral roots cm^{-1} in the upper zone of the split-root vertical agar plates of *Arabidopsis thaliana* seedlings (red) *lox1*, (green) *lox3* and (orange) *lox5* compared to (blue) wild-type after eight days of heterogeneous (upper zone) and homogeneous exposure to (a) 5 µM Cd, (b) 10 µM Cu and (c) 75 µM Zn. Data are means of 9 to 16 independent replicates. Different letters indicate significant differences at P<0.05. Data were statistically analyzed with a two way Anova followed by a Tukey's test or a non-parametric Kruskal wallis rank sum test followed by a Wilcoxon rank sum test combined with Bonferroni test.

3.2 Gene expression analysis to reveal the involvement of plant hormones in metal-specific growth responses in *Arabidopsis thaliana*

The gene expression of a number of genes involved in plant hormone levels (table 3) was investigated in the primary roots (± 2.5 cm) of 24 hours metal-exposed *Arabidopsis thaliana* seedlings. Root architecture is determined in the lateral root primordia that are developing in the primary root axis, and in the lateral root tips. Previously, differences were found in number of lateral roots under Cd-, Cu- and Zn-exposure[9], and here, it is explored whether gene expression measurements could give insight in the mechanisms behind these metal-specific effects (new candidate genes for reverse genetics). Gene expression in lateral root tips and in lateral root primordia define root architecture, but measuring gene expression on large root systems that have lateral roots would suffer from a dilution effect in the gene expression profiles due the RNA-extraction from the large amount of irrelevant plant tissue between the lateral root tips and the primary root axis. Therefore, gene expression should be measured in lateral root tips only (effect on lateral root elongation) or in the primary root axis (developing lateral root primordia: effect on lateral root density). Here, we choose to measure gene expression in the primary root axis containing the newly developing primordia. Plants were exposed to metals no

longer than 24 hours and changes in gene expression were determined. The actual morphological responses to Cd, Cu and Zn were verified and confirmed in a number of plants that was left to grow for seven days (data not shown).

3.2.1 Reference genes

Reliable gene expression analysis depends on normalization of the data with reliable internal control genes or reference genes. A set of candidate reference genes (table 2) were analyzed by geNorm[29]. GeNorm calculates M values on the basis of relative quantities ($E^{-\Delta ct}$) of candidate reference genes and compares them. The highest M value (lowest expression stability) is eliminated and the whole process is repeated until only two genes are left. The relative quantities were expressed as $E^{-\Delta ct}$ (E= actual primer efficiency) instead of $2^{-\Delta ct}$ since the actual primer efficiency deviated from two.

A total of eight candidate reference genes were tested, but four of these were not implemented in the geNorm analysis. Mitosis protein YLS8 and F-box protein were excluded on account of low expression levels leading to the inability to determine the efficiency of these primers using our cDNA samples. Poor linearity was found in the efficiency curve of ACT2 (R^2=0.81) and TIP41-like (R^2=0.80). The optimal pair of reference genes with the highest expression stability was UBC and UBQ10 (M=0.96669), whereas SAND family (M=2.6629) had the lowest expression stability (figure 17). Still, the V-value was above the proposed cut-off of 0.15 [29], but this may be due to the heterogeneous sample panel derived from three metal exposures. Since the pairwise variation increases with the inclusion of a fourth reference gene (V3/4), it was therefore decided to use three reference genes with the lowest M value for standardization (figure 18).
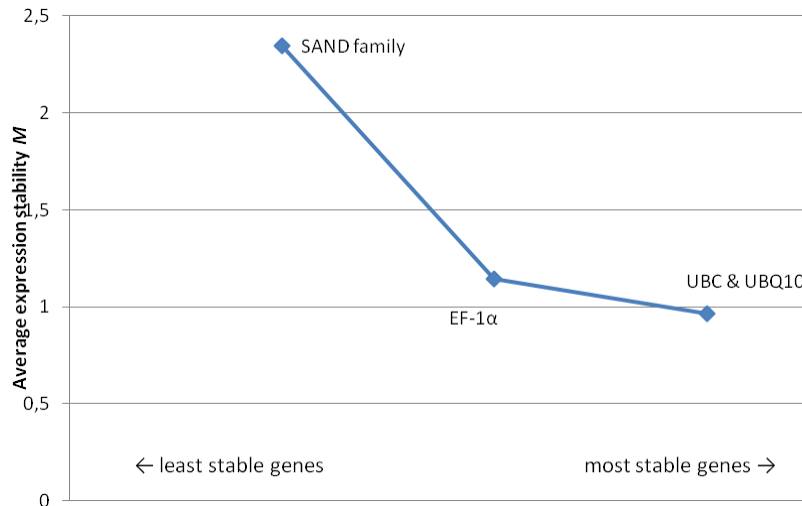
Figure 17: Evaluation of four selected candidate reference genes in seven days old *Arabidopsis thaliana* seedlings exposed to 5 µM Cd, 10 µM Cu and 75 µM Zn during 24 hours. The ranking of the candidate reference genes is a result of a stepwise exclusion of reference genes with the highest *M* value.
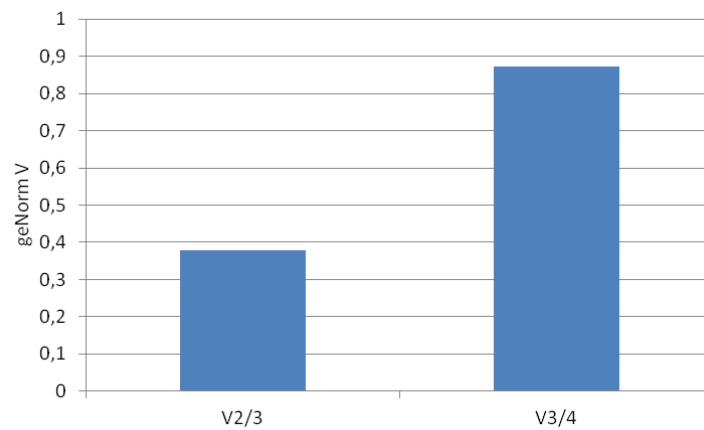

Figure 18: Determination of the optimal number of reference genes.

3.2.2 Genes of interest

A detectable expression was observed for four genes of interests (*LOX1*, *LOX5*, *NIT1* and *AAO1*). The failure to detect reliable gene expression levels of the other measured genes of interests (*LOX3*, *CKX2, ZOG1 and IPT7*) may be due to their relatively low expression levels and the low quantity of fresh tissue to extract RNA from.

No significant differences were found in the gene expression of *LOX5*, *LOX1* and *NIT1*, however a trend can be observed (figure 19). *LOX5* showed a downward trend

under Cd-exposure, while under Cu it shows the opposite. Zn did not influence *LOX5* gene expression. *LOX1* exhibited a downward trend under all three metals, where in Cu-exposed plants the expression of *LOX1* was higher than in Cd- en Zn-conditions. *NIT1*, which is involved in auxin synthesis, showed an upward trend under Cu-exposure. The expression of *AAO1,* which is also involved in auxin synthesis*,* exhibited an upward trend after exposure to Cd and a significant increase in Cu- and Zn-conditions.
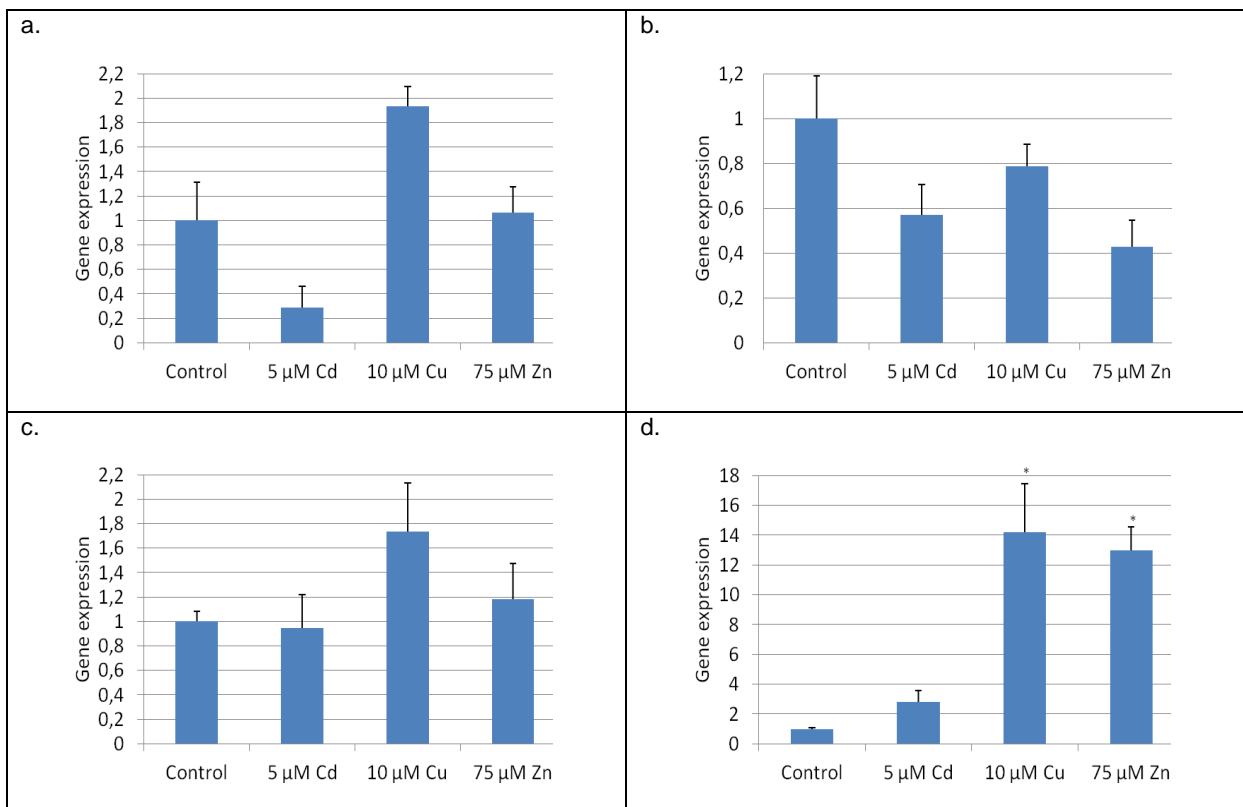

Figure 19: Gene expression of (a) *LOX5*, (b) *LOX1*, (c) *NIT1* and (d) *AAO1* in seven days old *Arabidopsis thaliana* seedlings exposed to 5 µM Cd, 10 µM Cu and 75 µM Zn during 24 hours. Data are means of 3 to 4 independent replicates (significance level: * : P<0.05). Data are statistical analyzed by a non-parametric Kruskal Wallis rank sum test followed by a Wilcoxon rank sum test or an one way Anova followed by a Dunnet test.

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4. Discussion and conclusions

Understanding more about root development under stress conditions is essential for future crop improvement that may be aimed at either safe biomass production or clean up of polluted soils (phytoremediation), which in turn increases agricultural land. So far it is known that there are metal-specific growth adaptations observed instead of a general stress-induced morphogenic response (SIMR) in response to the metals Cd, Cu and Zn in *Arabidopsis thaliana* seedlings[4,5,9]. The aims of this study were (1) to understand more about local and systemic effects on root development in a more relevant context of heterogeneous exposure, (2) to investigate whether *LOX*-genes are involved in the metal-specific growth responses in *Arabidopsis thaliana* seedlings, and (3) to explore if gene expression measurements can be used to detect new candidate genes for reverse genetics.

4.1 Effects of *LOX*-genes on root growth in metal-exposed *Arabidopsis thaliana* seedlings in a more relevant context of heterogeneous exposure

The hypothesis that *LOX*-genes are involved in the metal-specific growth adaptations was examined by means reverse genetics. Split-root systems were used to investigate if *LOX*-genes are involved in the local or systemic effects. Two questions can be asked: when roots are in contact with metals (1) to what extend is there an avoidance of the metal containing zone and (2) is there a systemic effect on the control zone where the colonization should continue as much as possible (systemic inhibition/no effect/stimulation)?

4.1.1 Avoidance capacity of the wild-type under metal exposure

Wild-types under heterogeneous metal exposure showed an incomplete avoidance of the primary root in the Cd- and Zn-containing lower zone. A small loss of lateral root avoidance was also observed in the Cd containing upper zone, which is due to decreased suppression of lateral root elongation. Cu induced a stronger total lateral

root avoidance by suppressing lateral root elongation, while Zn already has a strong inhibition by suppressing both lateral root elongation and number.

4.1.2 Colonization capacity of the wildtype under metal exposure

A systemic effect in the control lower zone was observed for the primary root under Zn-exposure. Wild-types showed an increased lateral root colonization in the control upper zone under Cd-, Cu- and Zn-exposure, which was due to the stimulation of lateral root elongation and number. This was also seen in the control lower zone under Cd-exposure, except it was only stimulated by lateral root elongation. Cu and Zn have an opposite effect on total lateral root length by inhibiting lateral root elongation (only Cu) and lateral root number (Cu and Zn). Zn clearly has a systemic effect on the control zone.

4.1.3 Effects of *LOX*-genes on root growth under metal exposure

Phenotypic comparison of *lox*-mutants with wild-type under metal-stress conditions after growth in split-root vertical agar plates revealed that *LOX3* en *LOX5* possibly inhibit lateral root elongation since their mutants contain longer lateral roots in the control conditions compared to wild-type and no additional stimulation was observed when metals were administrated in the lower zone. Vellosilo et al (2007) showed that a mutation of *LOX1* and *LOX5* resulted in an increase in lateral root number compared to wild-type. Under the growth conditions used in this study, *lox3* and *lox5* showed an increase in lateral root number compared to wild-type under control conditions, while for *lox1* this increase was less clear. It can be hypothesized that the stimulation of lateral root elongation by Cd, Cu and Zn in the upper control zone in wild-types can be due to the elevation of the inhibition on lateral root elongation by *LOX3* and *LOX5*. It can be speculated that *LOX3* and *LOX5* are "brakes" on root development an that this inhibition can be lifted when its needed (e.g. stimulation of root development in the top control zone). In agreement, we also observed a decrease in *LOX5* expression under Cd-exposure, which may enable increased lateral root density. *LOX1* and *LOX5* prevent the complete avoidance of the primary

root under Cd-exposure. This prevention of complete avoidance under Cd-exposure may be due to 9-LOX signalling. *LOX*-genes did not seem to be involved in root growth under Cu-exposure, which may be due to the fact that Cu can induce ROS directly because its redox-active and Cd and Zn can induce an effect on primary root growth by producing ROS via *LOX* genes. *LOX3* seems to be involved in the Zn-systemic-effect since the mutant exhibits no systemic effect of Zn on the colonization capacity in the lower control zone under Zn-exposure. Maybe *LOX3* is involved in Zn-transport/distribution? In general, *lox*-mutants seem to have a preference for the metal-free zone compared to wild-type.

4.2 Involvement of plant hormones in metal-specific growth responses in *Arabidopsis thaliana*

The gene expression analysis of genes involved in plant hormone synthesis and metabolism (*LOX1*, *LOX5*, *AAO1*, *NIT1*) examined in the roots of 24 hour metal-exposed wildtype plants using quantitative reverse transcription PCR revealed that different expression levels were present under metal exposure, except for *LOX5* that was not influenced by Zn and *NIT1* was not influenced by Cd. A significant increase in *AAO1* gene expression has been observed under Cu- and Zn-exposure.

4.3 Future research

For future research, a number of improvements are necessary. Primer efficiencies in the gene expression analysis were not optimal what can result in a poor amplification of the products what eventually can lead to an distorted picture. The poor primer efficiencies could be the result of impure RNA-samples (presence of inhibitors) and low RNA yields. In a next experiment, an improved RNA-extraction protocol and more starting material are required to obtain more RNA and purer samples. The gene expression experiment implemented above provides an indication how the genes of interest behave under metal exposure, but additional experiments on different tissues (lateral root tips), and also different molecular levels (transcriptomics, proteomics, metabolomics) are necessary to confirm that their products also behave the same.

The actual function on root growth can then be examined by reverse genetics for those genes where differences are found. In this study a significant increase in *AAO1* gene expression has been observed under Cu and Zn exposure. *AAO1* may be involved in metal-specific growth responses since it is involved in auxin synthesis[31], which is known to have an effect on root development [5].

For the reverse genetic experiment, the statistical analyses should normally be performed with three separate equal wild-type groups to be compared with each of the mutants instead of one. The reverse genetic experiment above is used as a screening experiment that provides an indication of the involvement of *LOX*-genes in metal-specific growth adaptations in a relevant context of heterogeneous exposure.

According to the obtained results above, it can be concluded that *LOX*-genes are involved in the metal-specific growth responses. Furthermore, it is hypothesized that *LOX3* is involved in regulating Zn-transport and distribution since the systemic Zn-effect observed in wild-types was not present in *lox3*. Additionally, *lox*-mutants seem to have a preference for the metal-free zone compared to wild-type. Therefore, further investigation of selected hypothesis concerning *LOX*-genes need to be combined with metal determinations in order to link their responses to metal uptake.

In the distant future, an important question is whether the molecular mechanism found in the model organism *Arabidopsis thaliana* are conserved in more agricultural relevant species, such as *Brassica napus*. Finally, it is also worth to mention that crop improvement per se is not enough to meet the demands of the rapidly growing world population, since the population each year continues to increase and the usable land threatens to reach its maximum capacity.

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Supplement

Supplement 1

Preparation of 50 times concentrated B5/50 macronutrients.

Macronutrients		B5/50	Final concentration B5/50	50x B5/50 macronutrients*
	MW	mg/l	mM	mg/l
(NH4)2SO4	132.14	2.68	0.02	134
(Sigma-Aldrich, St. Louis, USA)				
MgSO4	120.37	2.44	0.02	122.09
(Sigma-Aldrich, St. Louis, USA)				
CaCl2	110.98	2.26	0.02	113.24
(Sigma-Aldrich, St. Louis, USA)				
NaH2PO4.2H2O	156.01	3.39	0.02	169.5
(Sigma-Aldrich, St. Louis, USA)				
KNO3	101.10	50	0.49	2500
(Merck, Darmstadt, Germany)				

*Prepare in distilled water and store in fridge. If precipitation occurs: redissolve or discard.

Supplement 2

Preparation of 1000 times concentrated B5/50 micronutrients.

Micronutrients		B5/50	Final concentration B5/50	1000x B5/50 micronutrients*
	MW	mg/l	mM	mg/l
H3BO3	61.83	0.06	0.970	60
(Merck, Darmstadt, Germany)				
MnSO4.H2O	169.02	0.2	1.183	200
(Merck, Darmstadt, Germany)				
CuSO4.H2O	249.69	0.025	0.100	25
(Merck, Darmstadt, Germany)				
ZnSO4.H2O	287.56	0.04	0.139	40
(Merck, Darmstadt, Germany)				
Na2MoO4.2H2O	241.95	0.005	0.021	5
(Merck, Darmstadt, Germany)				
CoSO4.H2O	155	0.00032	0.0021	0.32
(Sigma, St. Louis, USA)				
KI	166	0.015	0.090	15
(Merck, Darmstadt, Germany)				

*Prepare in distilled water and store in the fridge. If precipitation occurs: redissolve or discard

Supplement 3

Preparation of 1000 times concentrated B5/50 Fe.

|		B5/50	Final concentration B5/50	1000x B5/50 Fe*
	MW	mg/l	mM	mg/l
FeNO3.9H2O	404	0.808	0.002	80.8
(Merck, Darmstadt, Germany)				

*Prepare in distilled water (mg/100mL) and store in the fridge. If precipitation occurs: redissolve or discard.

Supplement 4
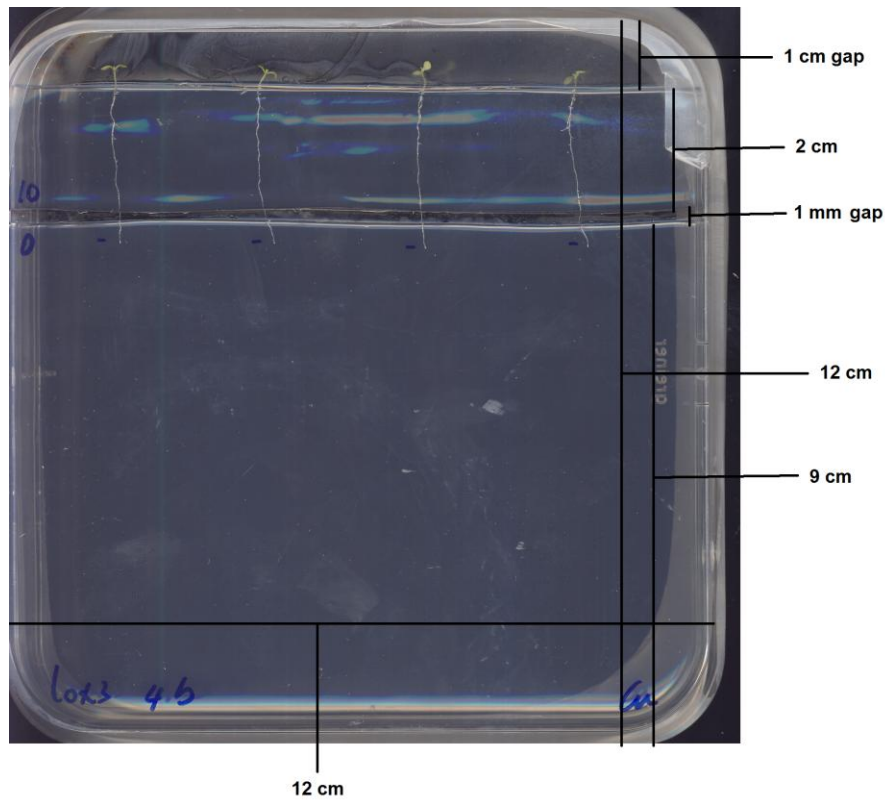

Model of a vertical agar plate (VAP) with a split-root system. An upper zone of 2 cm x 12 cm ($24cm^2$ = 6.7 mL) was separated from the lower zone of 9 cm x 12 cm ($108 cm^2$ = 30 mL) by means of a small air gap (1 mm). Agar (1cm) was removed at the top to form an air gap for the shoots. The roots contacted both zones with the primary root tip in the lower zone of the split-root system. VAPs without a split-root system do not contain a 1 mm air gap in the middle.
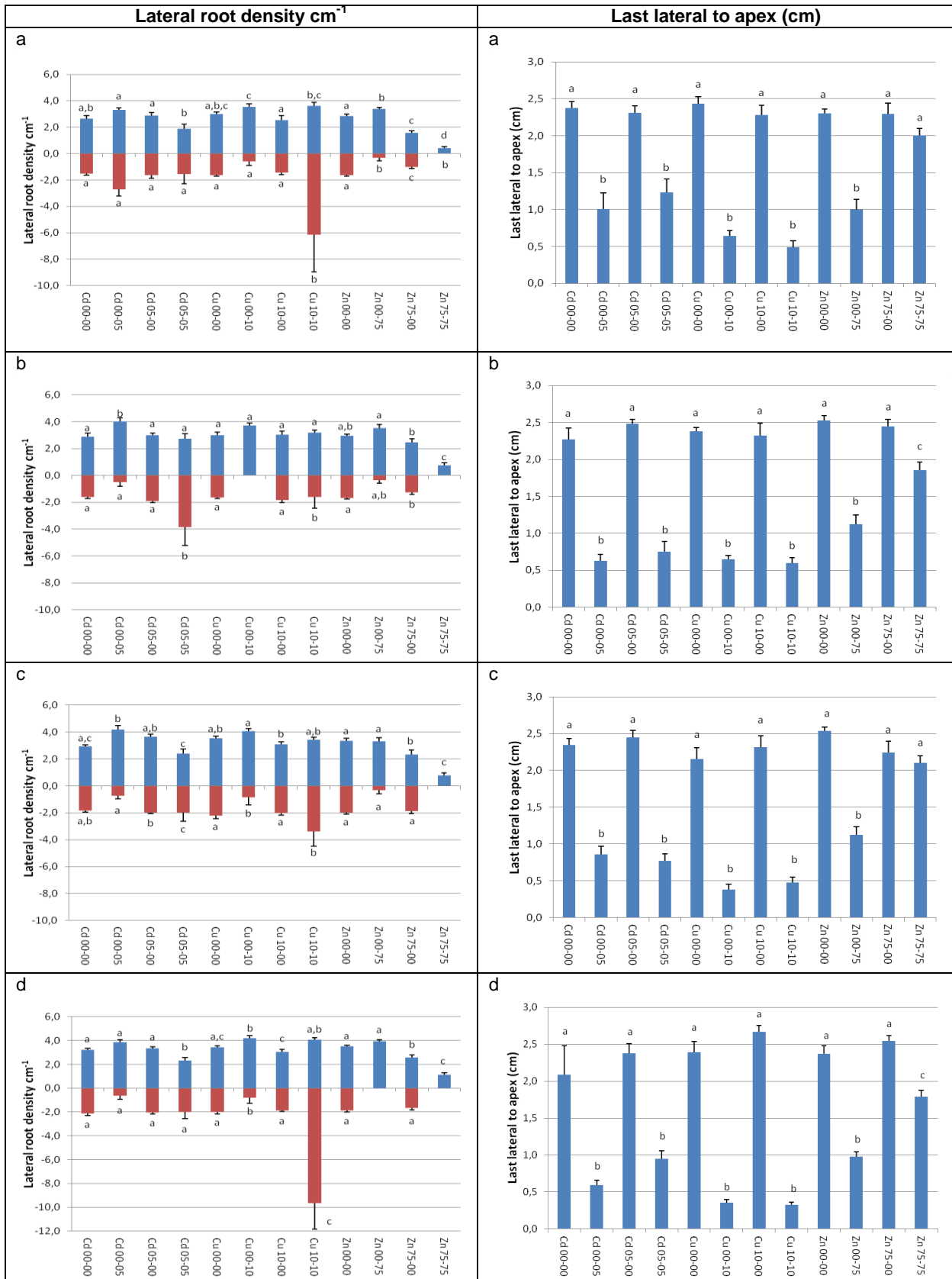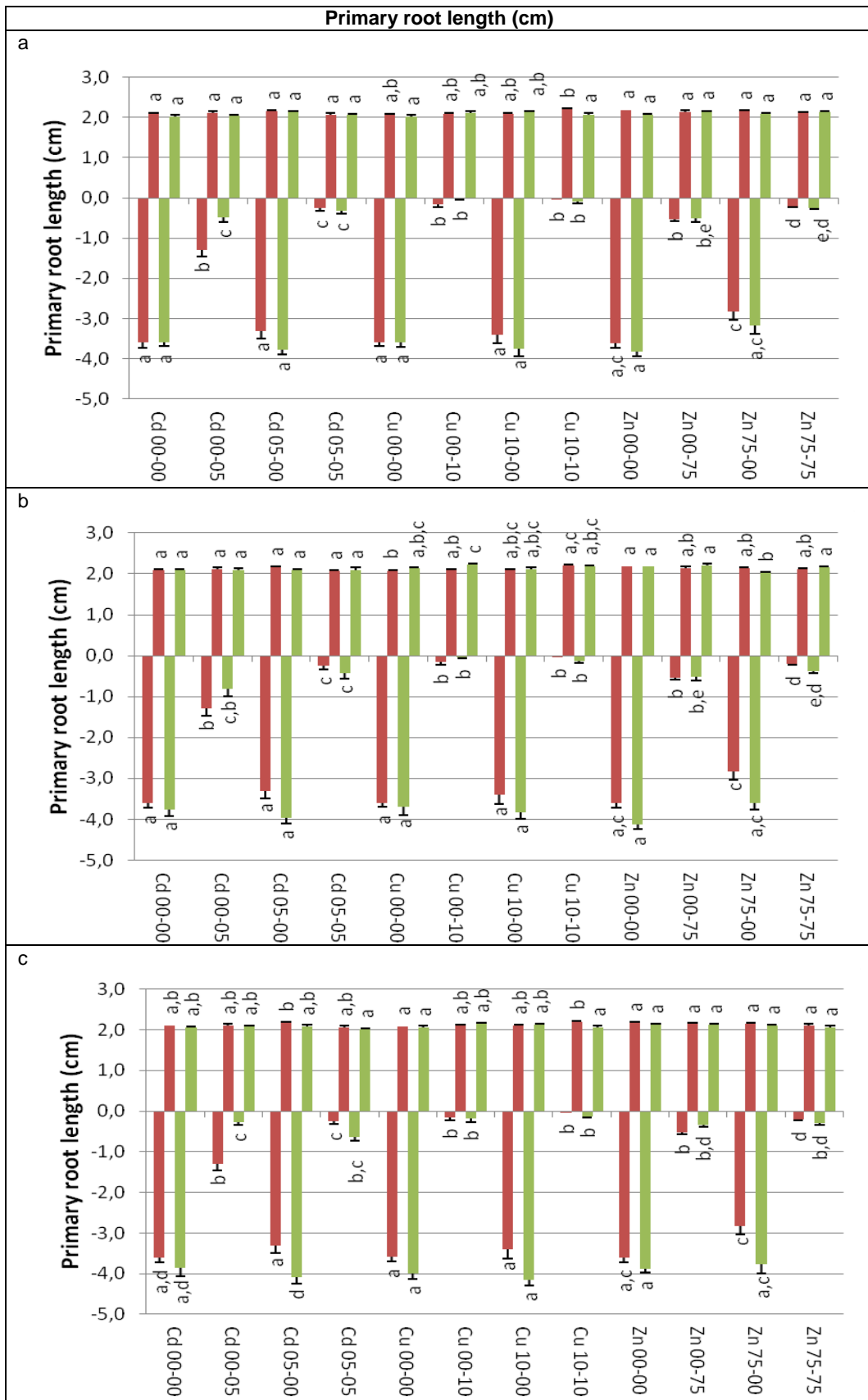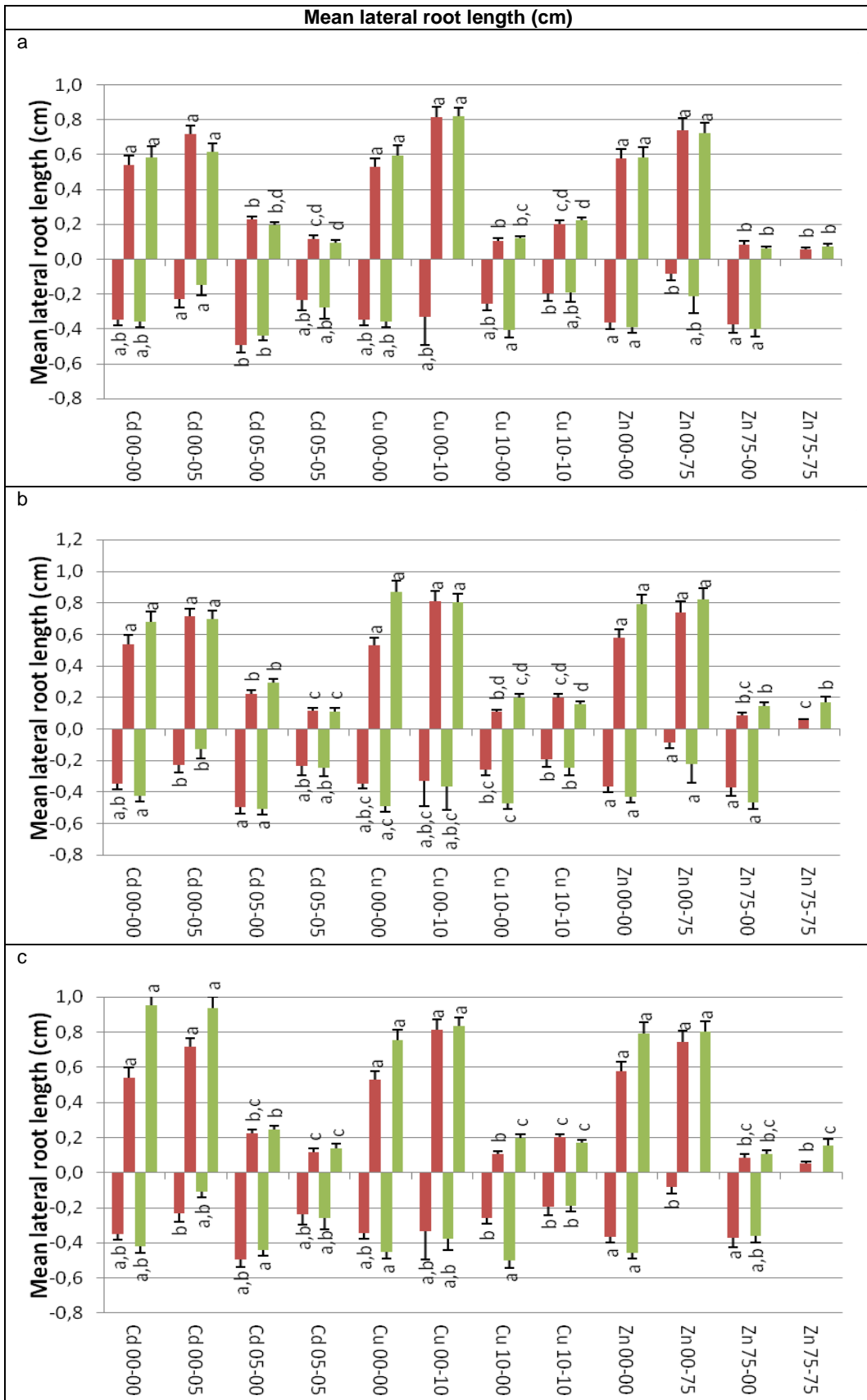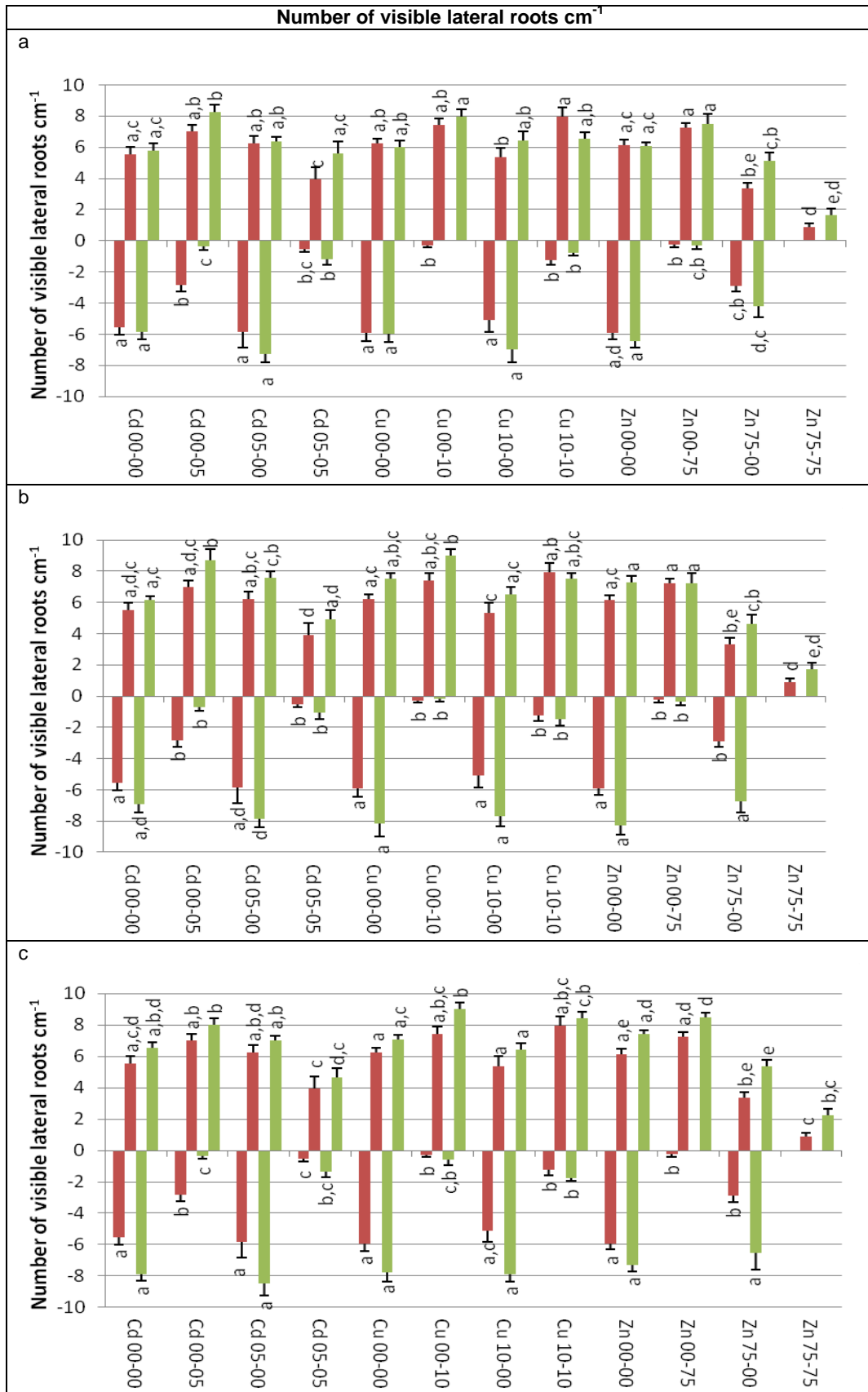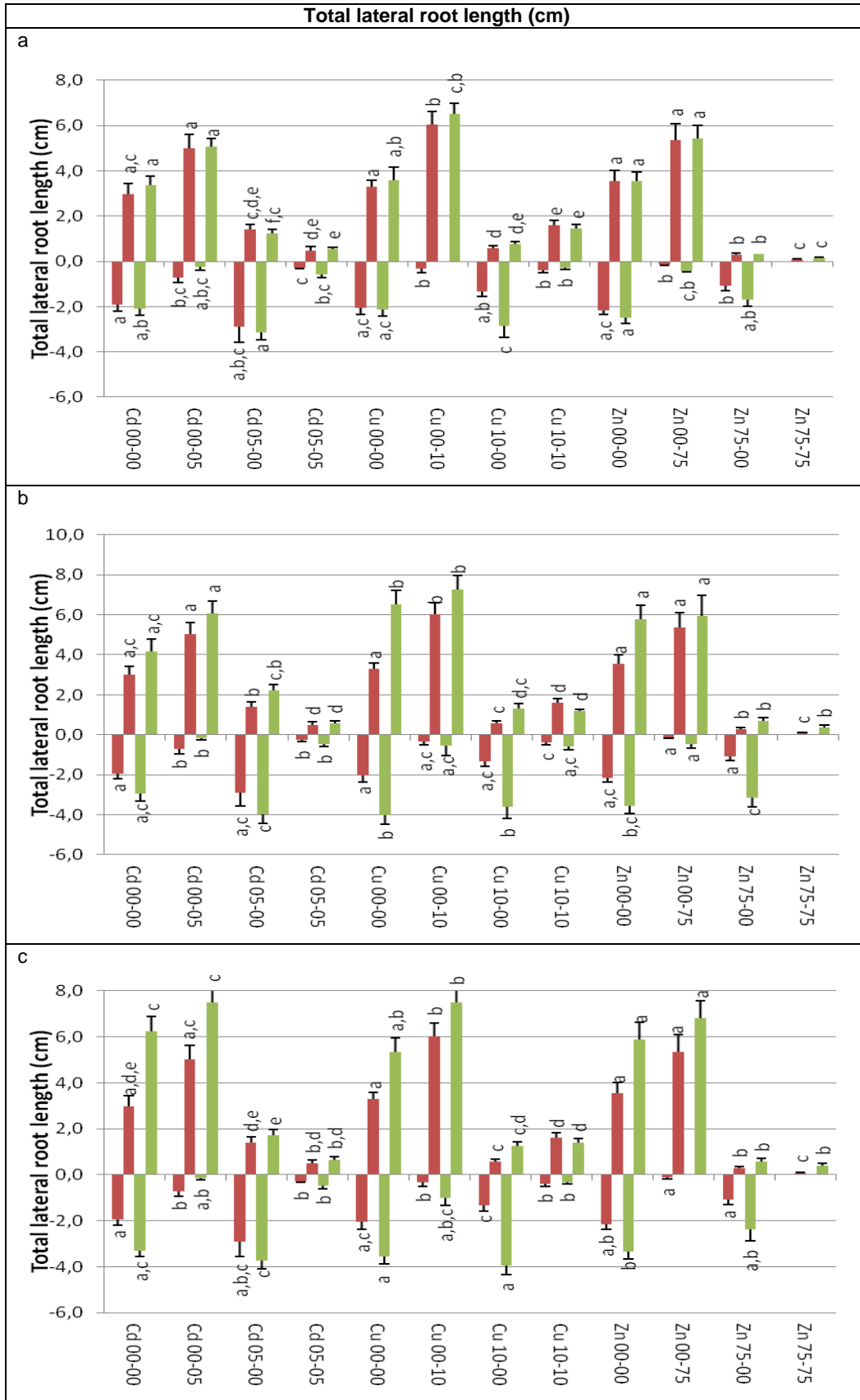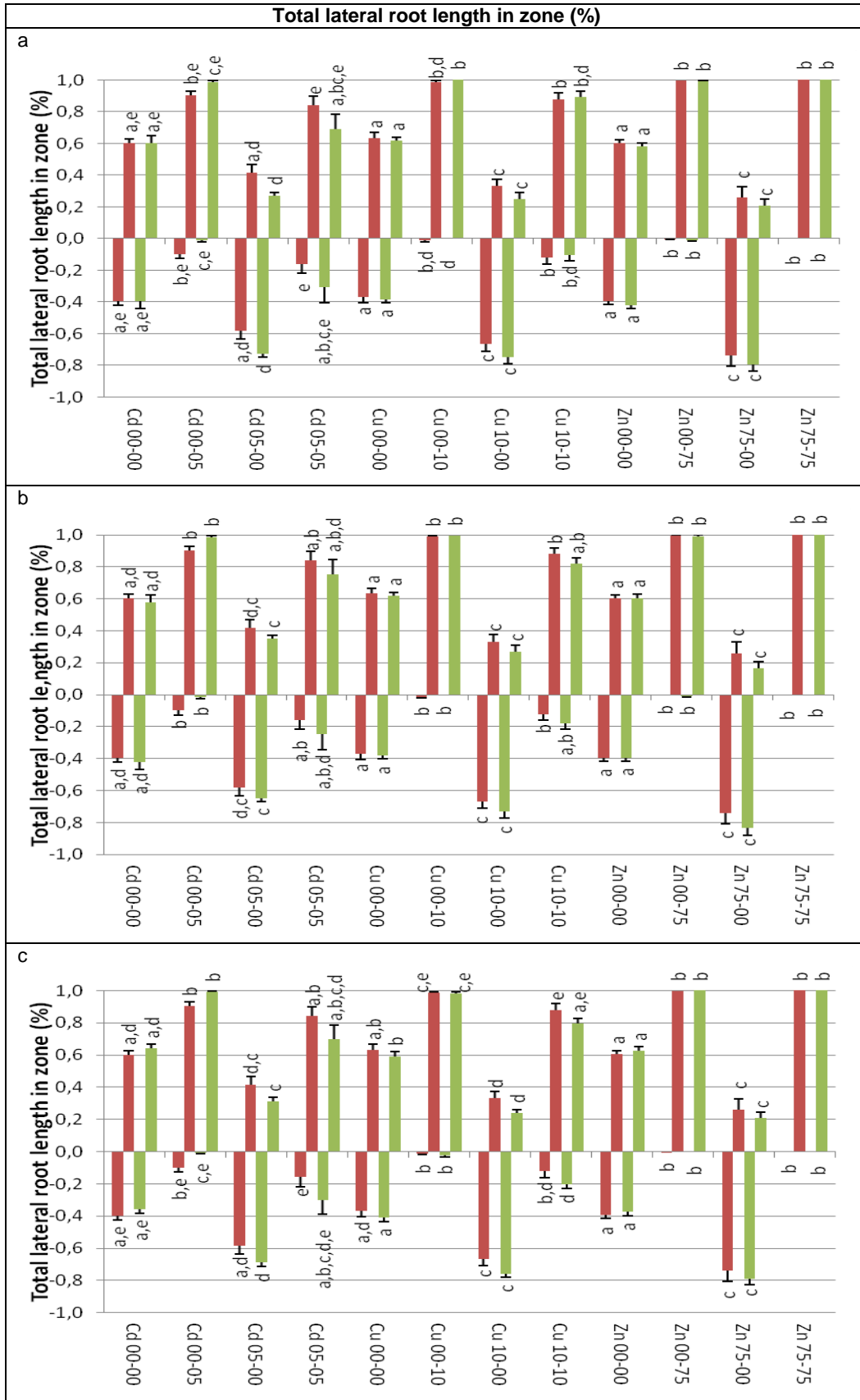
Supplement 5
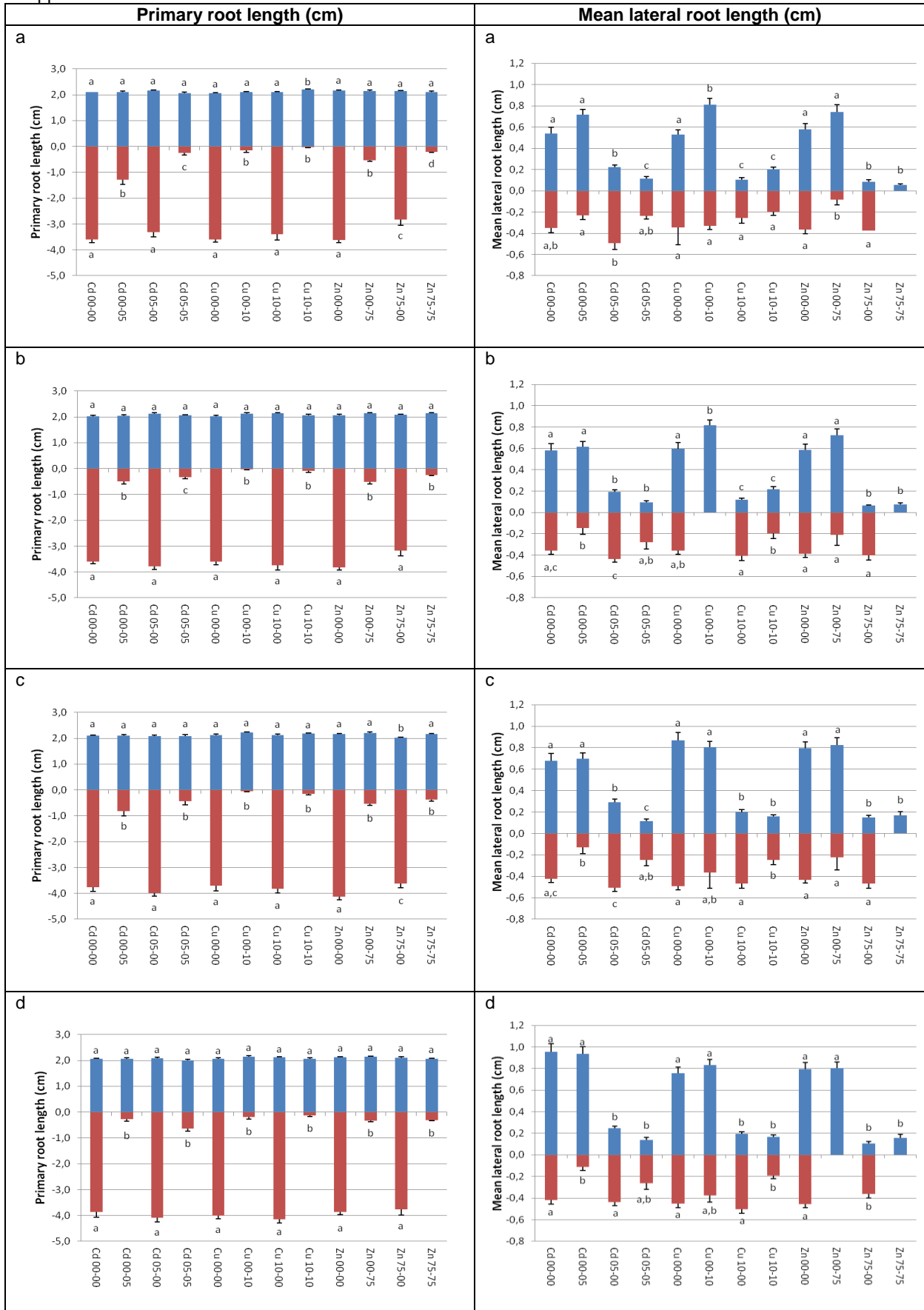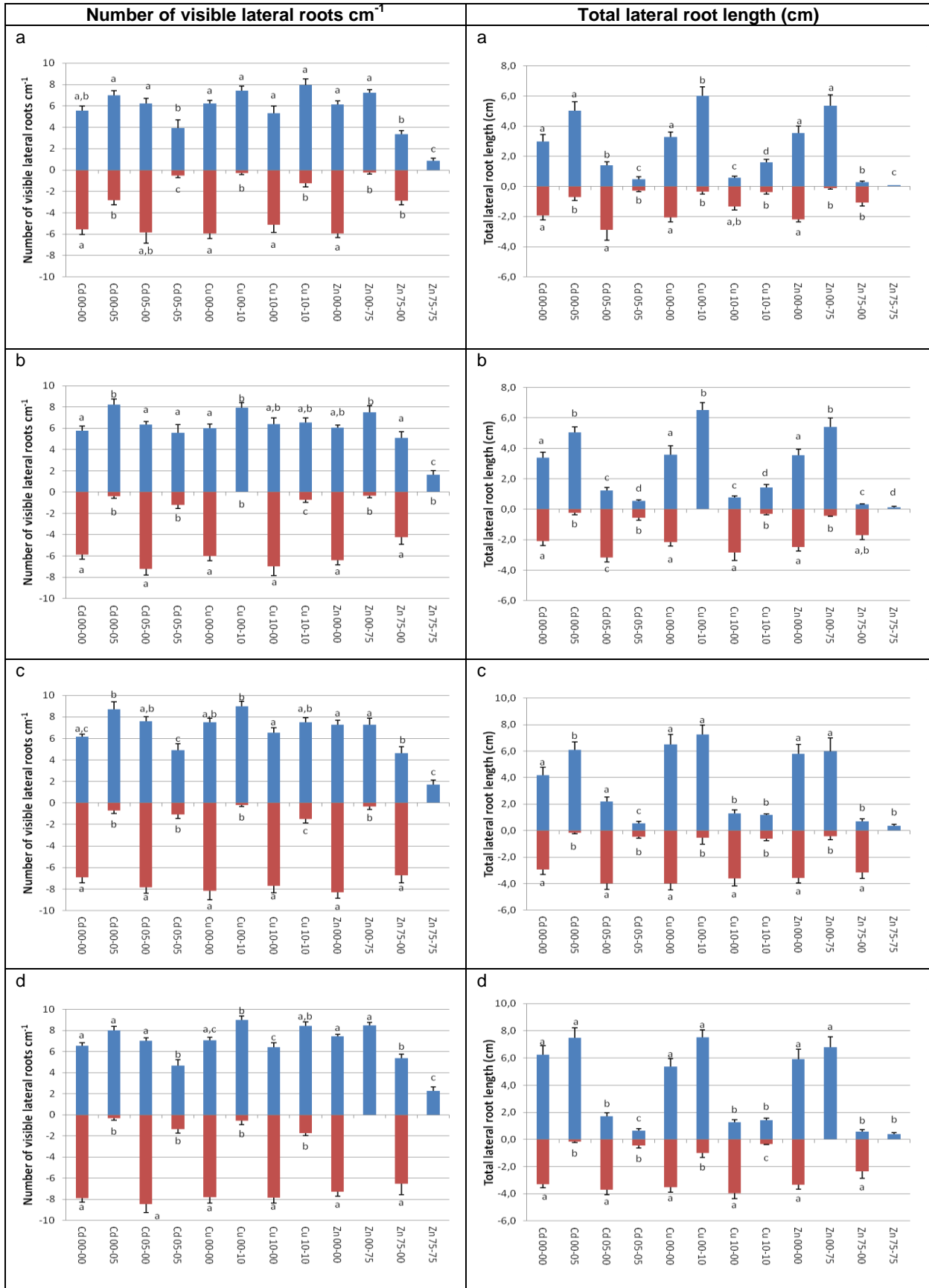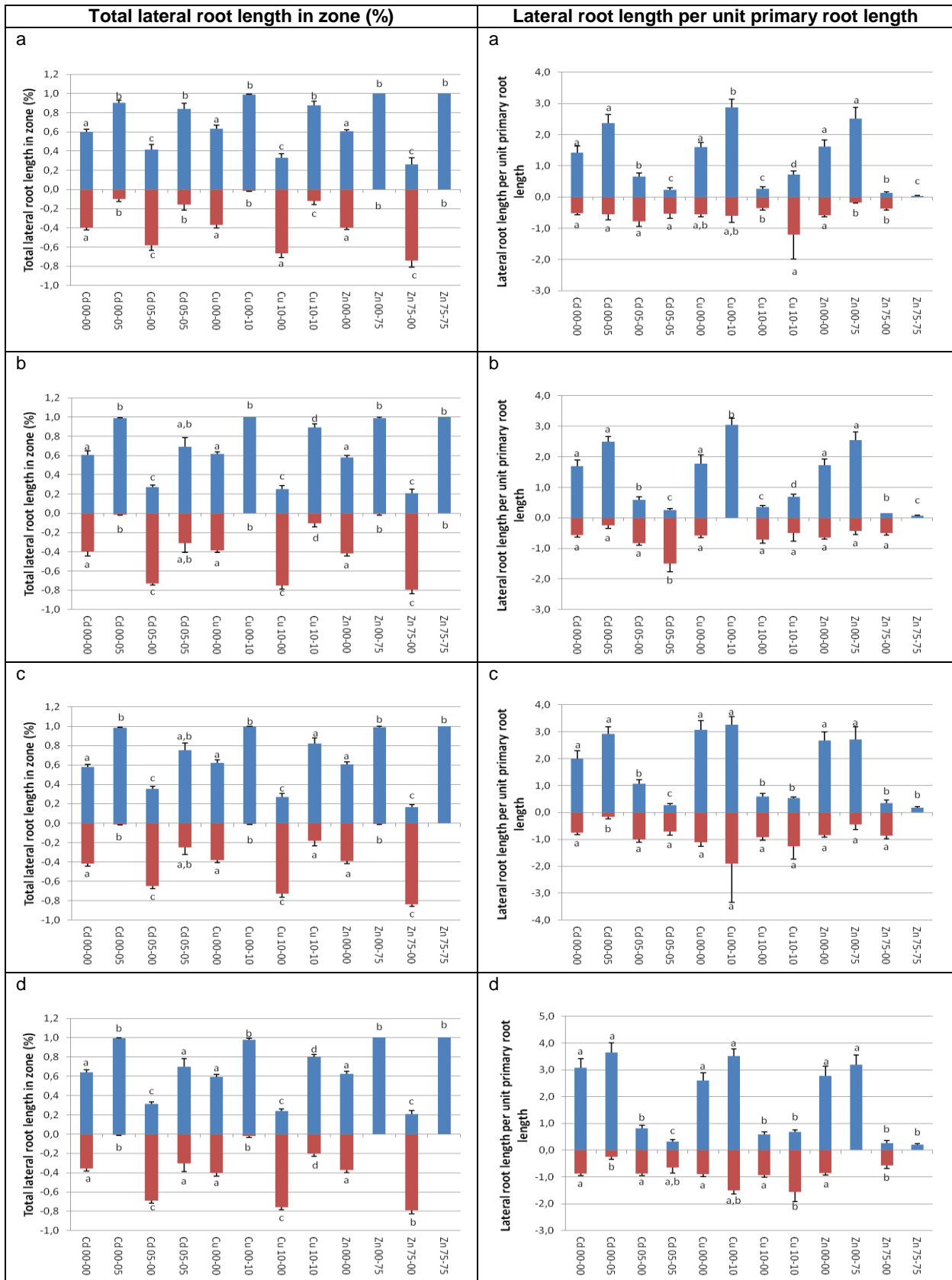
Root parameters of *Arabidopsis thaliana* seedlings with the genotypes wildtype (a), *lox1* (b), *lox3* (c) and *lox5* (d) after eight days of heterogeneous and homogeneous exposure to (a) 5 µM Cd, (b) 10 µM Cu and (c) 75 µM Zn. Data are means of 9 to 16 independent replicates and were statistically analyzed with an one way Anova (followed by a Tukey's test) or non-parametric Kruskal wallis rank sum test (followed by a Wilcoxon rank sum test combined with Bonferroni test). Different letters indicate significant differences at P<0.05.

Supplement 6

Primary root length (cm)

Mean lateral root length (cm)

Number of visible lateral roots cm⁻¹

Total lateral root length (cm)

Total lateral root length in zone (%)

Lateral root length per unit primary root length
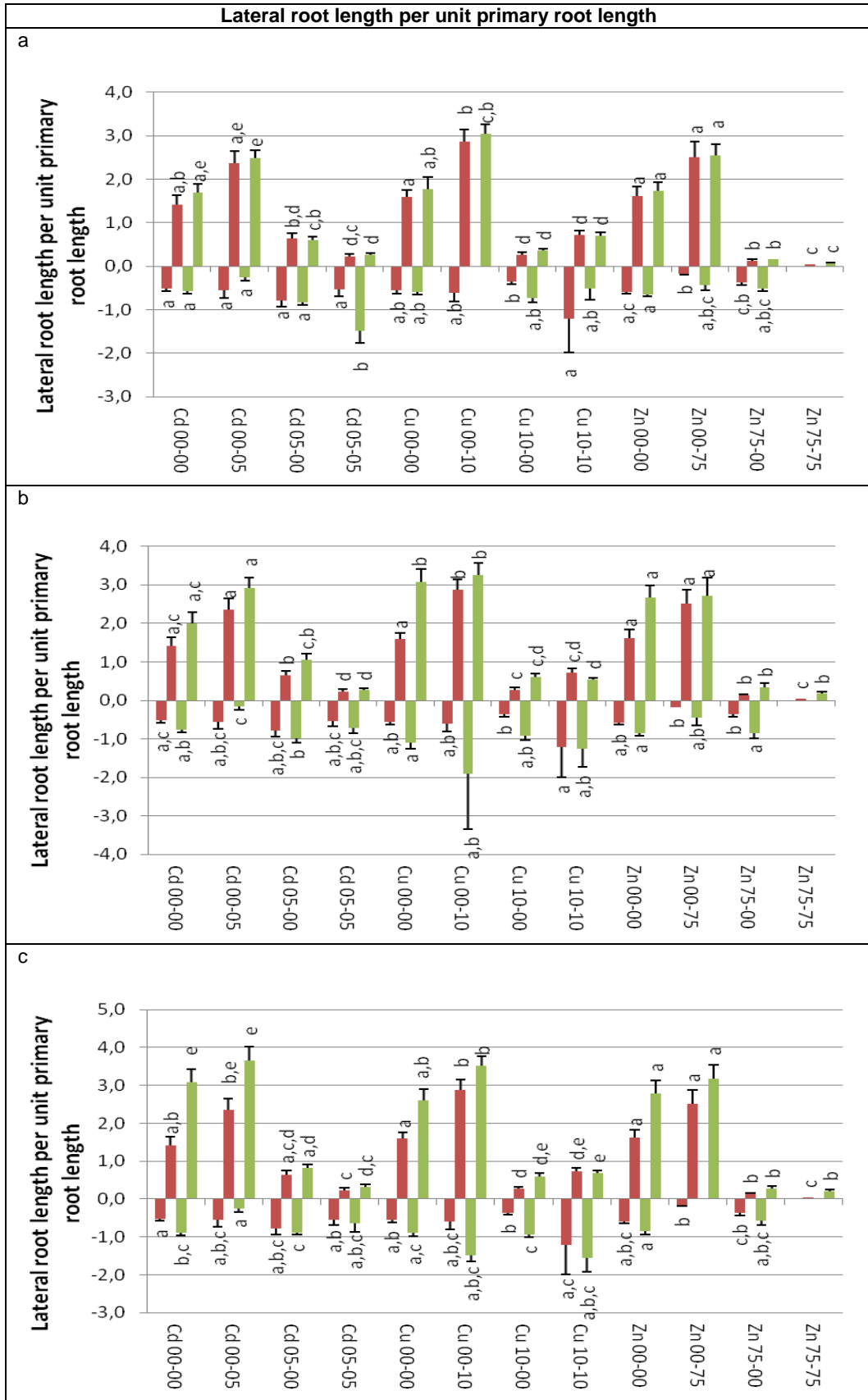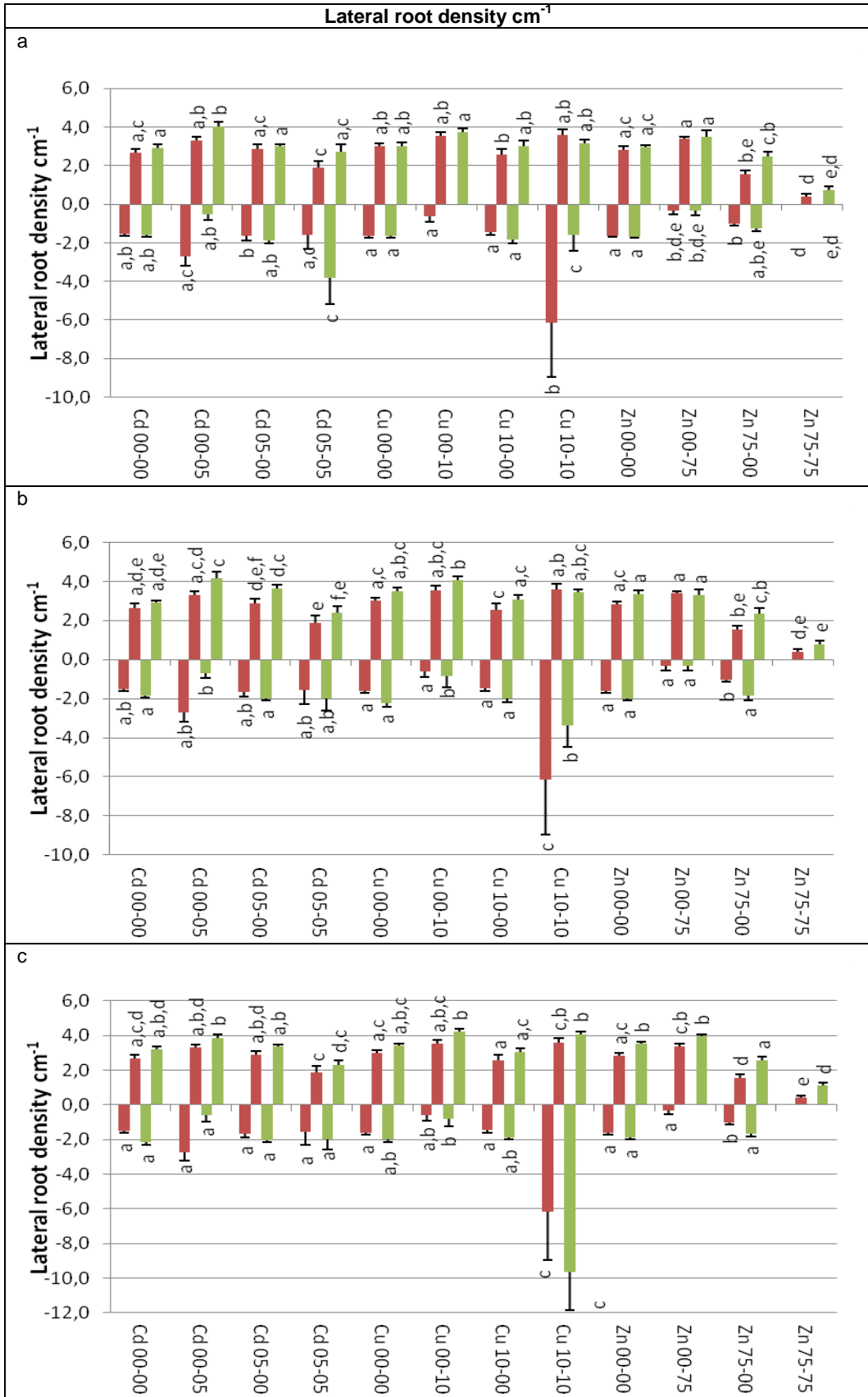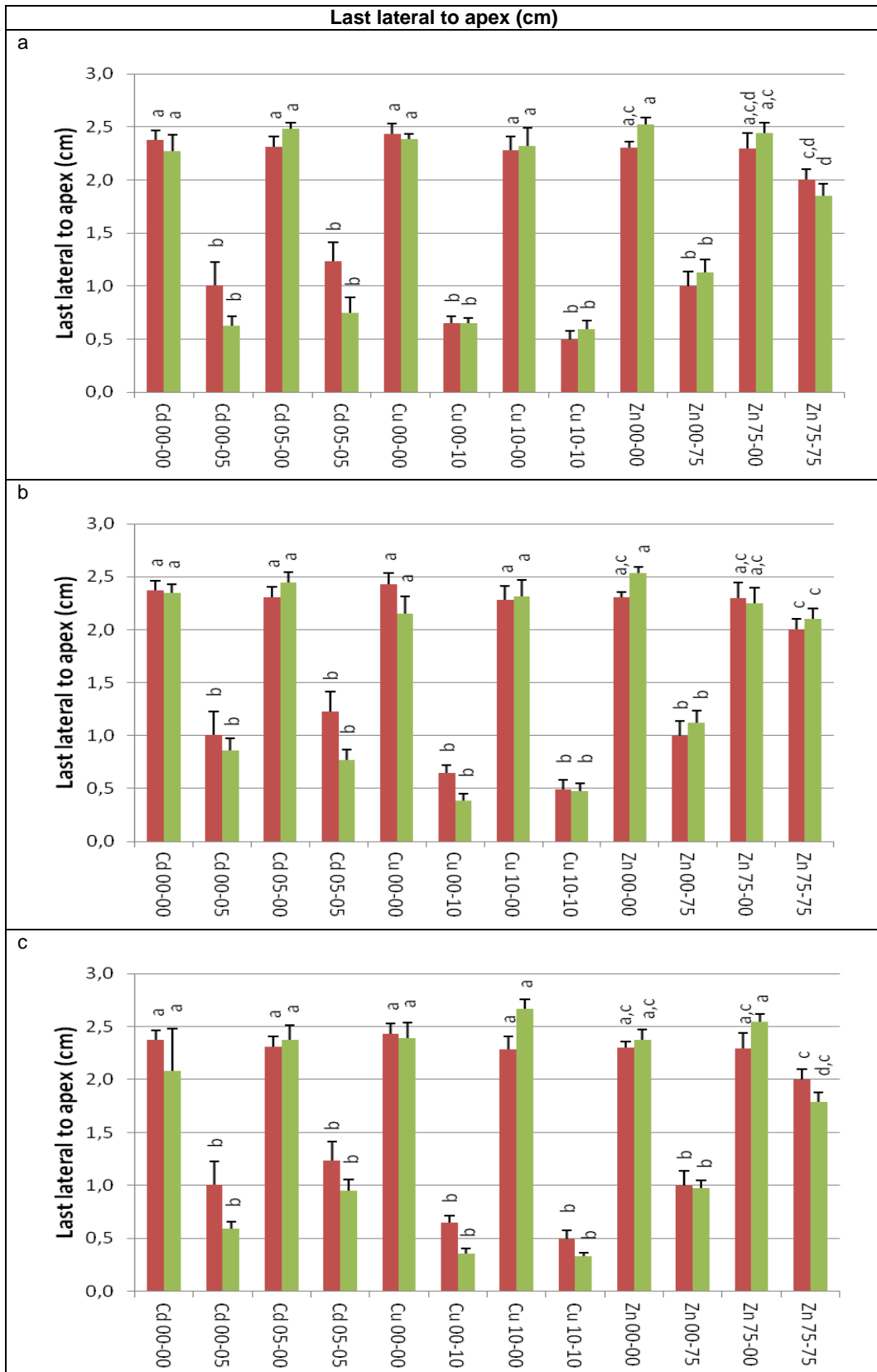
Lateral root density cm^{-1}

Root parameters of *Arabidopsis thaliana* seedlings (a) *lox1*, (b) *lox3* and (c) *lox5* compared to wildtype (indicated in red) after eight days of heterogeneous and homogeneous exposure to (a) 5 µM Cd, (b) 10 µM Cu and (c) 75 µM Zn. Data are means of 9 to 16 independent replicates and were statistically analyzed with an two way Anova (followed by a Tukey's test) or non-parametric Kruskal wallis rank sum test (followed by a Wilcoxon rank sum test combined with Bonferroni test). Different letters indicate significant differences at P<0.05.

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