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Exploring the role of plant hormones in root developmental responses to metal-stress

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Content

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Abstract

Plants possess a high level of plasticity, especially in the root system. This enables the plants to adjust their growth and development according to a changing environment in order to survive. In light of the second green revolution the responses of the root have received more attention in order to increase biomass production with minimal input of scarce resources like water and fertilizers. Also during stress conditions, altered root system architectures have been found and were named Stress-Induced Morphogenic Responses (SIMR). In this research project, the link between metal stress and the resulting SIMR of the root is investigated. Metals with different properties are studied to increase the probability of finding stress-specific responses. For this reason cadmium (Cd), copper (Cu) and zinc (Zn) were chosen: Cu and Zn are essential to the plant, while Cd is a non-essential element, and Cd and Zn are non-redox active, whereas Cu is redox active in plants. Previous research of the group Environmental Biology (CMK, Hasselt University) had reported metal-specific effects of these metals on the root system architecture of wild-type *Arabidopsis thaliana*. Both Cd and Cu were found to increase the number of outgrowing lateral roots, but Cd inhibited the elongation of these lateral root more severely than Cu. Zn caused stronger negative effects on both lateral root outgrowth and elongation. Given the involvement of phytohormones in growth and development of the plant, and on reports of altered phytohormone concentrations during stress (including metal stress), this research project hypothesizes that phytohormones form a link between metal stress and specific root developmental responses.

To explore the involvement of phytohormones in these metal-specific SIMRs of the roots, the effects of metal exposure was investigated in different mutants of *A. thaliana.* The mutants have a disrupted hormone signalling pathway: *abi4-1* has a dysfunctional abscisic acid (ABA) signalling pathway and *ein2-1* is mutated in a key component of the ethylene signalling. The effects of the three metals on the root system of the mutants was examined and compared to reactions in the wild-type. The *abi4-1* mutant was more sensitive to moderate Cd exposure and less sensitive to excess Zn. The response to the Cu treatment did not result in a different root system architecture than that of the wild-type. The root system of *ein2-1* seemed to be more sensitive to Cu and Zn than the root system of the wild-type. The gene expression of marker genes of hormonal signalling pathways was examined in wild-type *A. thaliana,* exposed to Cd, Cu and Zn. The marker genes were selected among endpoints of hormonal signalling that have been shown to be transcriptionally induced. Cd, Cu and Zn were found to alter the gene expression of marker genes of every hormone. Except for Cu, which did not alter the expression level of any of the tested ethylene responsive genes.

The results are indicative of the involvement of ABA-signalling through ABI4 in Cd-specific responses and ethylene-signalling through EIN2 in Cu-specific responses of lateral root elongation. ABI4 and EIN2 are both suspected to be involved in the Zn-specific responses. The Zn-specific effects on lateral root number are probably ABI4-mediated, while ethylene-signalling through EIN2 takes place in the effects of Zn on lateral root elongation.

The nature of hormone involvement in the metal-specific root responses however remains unclear and needs to be further investigated.

Abstract - Nederlands

Planten bezitten een hoge plasticiteit in de ontwikkeling, die prominent aanwezig is in het wortelsysteem. Hierdoor kunnen planten hun groei en ontwikkeling aanpassen aan een veranderende omgeving, en dus kunnen overleven in deze nieuwe omgeving. In het kader van de tweede groene revolutie hebben deze wortelresponsen meer aandacht gekregen om de biomassaproductie te kunnen verhogen met een minimale input van schaarse middelen zoals water en meststoffen. Het gewijzigde wortelsysteem werd benoemd als 'Stress-Induced Morphogenic Responses' (SIMR), of stress geïnduceerde morfogene responsen van de wortels. In dit onderzoek werd de link tussen metaalstress en het resulterende SIMR van het wortel systeem onderzocht. Om de kans te verhogen dat in deze studie metaal-specifieke SIMRs werden gevonden, werden metalen met verschillende eigenschappen onderzocht. Hierom werden cadmium (Cd), koper (Cu) en zink (Zn) gekozen: Cu en Zn zijn essentieel voor de plant, terwijl Cd een niet-essentieel element is, en Cd en Zn zijn niet-redoxactief, terwijl Cu redoxactief is in de plant. Vorig onderzoek in de groep Milieubiologie (CMK, Universiteit Hasselt) had reeds metaal specifieke effecten van deze metalen op de wortels van wild-type Arabidopsis thaliana gerapporteerd. Zowel Cd als Cu verhoogde het aantal laterale wortels, maar Cd inhibeerde the elongatie van deze laterale wortels ernstiger dan Cu. Zn veroorzaakte meer negatieve effecten op laterale uitgroei en elongatie. Door de betrokkenheid van fytohormonen in de groei en ontwikkeling van de plant enerzijds en meldingen van een veranderde concentratie van fytohormonen onder stress (inclusief metaalstress) anderzijds, werd in dit onderzoek de hypothese gesteld dat fytohormonen een link vormen tussen metaalstress en specifieke wortel ontwikkelingsresponsen. Om de betrokkenheid van fytohormonen in deze metaal-specifieke SIMRs van de wortels te bestuderen werden de effecten van de metaalblootstelling in verschillende mutanten van A. thaliana onderzocht. De mutanten misten een component van een hormoon signaaltransductie pathway: abi4-1 is disfunctioneel in de signaaltransductie van abscisinezuur (ABA) en ein2-1 bevat een mutatie in een sleutelcomponent van de ethyleen signaaltransductie. De effecten van de drie metalen op het wortelsysteem van de mutanten werden bestudeerd en vergeleken met reactie in het wild-type. De abi4-1 mutanten was sensitiever voor gematigde Cd concentraties en minder gevoelig op overtollige Zn blootstelling. De respons op de Cu behandeling resulteerde niet in een gewijzigde wortelarchitectuur ten opzichte van het wild-type. Het wortelsysteem van ein2-1 leek sensitiever voor Cu en Zn dan het wortelsysteem van het wild-type. De genexpressie van merkergenen van de signaaltransductie pathways van de hormonen werd onderzocht in blootgestelde wild-type A. thaliana. De merkergenen werden geselecteerd onder de eindpunten van hormonale signaleringen waarvan geweten is dat hun transcriptie geïnduceerd wordt. Cd, Cu en Zn beïnvloedde de genexpressie van de merkergenen van elk hormoon. Enkel Cu kon de expressie van ethyleenresponsieve genen niet beïnvloeden. De resultaten indiceren de betrokkenheid van ABA-signalering via ABI4 in Cd specifieke responsen en ethyleen-signalering via EIN2 in Cu specifieke responsen van de laterale wortel elongatie. Er wordt gedacht dat ABI4 en EIN2 beide betrokken zijn in de Zn specifieke respons. De Zn specifieke effecten op het aantal laterale wortels zijn waarschijnlijk gemedieerd door ABI4, terwijl EIN2 tussenkomt in de effecten op laterale wortelelongatie. De precieze manier waarop de hormonen de metaal specifieke responsen veroorzaken wordt verder onderzocht.

List of abbreviations

A. thaliana	*Arabidopsis thaliana*
ABA	Abscisic acid
ABI4	ABA Insensitive 4
ACC	1-aminocyclopropane-1-carboxylic acid
BLAST	Basic Local Alignment Search Tool
Cd	Cadmium
cDNA	Complementary Deoxyribonucleic acid
$CdSO_4$	Cadmium sulphate
CMK	Centre for Environmental Sciences
COI1	Coronatine Insensitve 1
Cu	Copper
$CuSO_4$	Copper sulphate
DZ	Differentiation zone
EIN2	Ethylene Insensitive 2
EZ	Elongation zone
gDNA	Genomic deoxyribonucleic acid
IAA	Indole-3-acetic acid
K_2SO_4	Potassium sulphate
KOH	Potassium hydroxide
LOX	Lipoxygenase
LRP	Lateral Root Primordium
MES	2-(N-morpholino)ethanesulfonic acid
MZ	Meristematic zone
NASC	Nottingham Arabidopsis Stock Centre
PCR	Polymerase chain reaction
PR	Primary Root
QC	Quiescent Centre
qPCR	Quantitative PCR
qRT-PCR	Quantitative reverse transcription PCR
RAM	Root Apical Meristem
RG	Reference Gene
SIMR	Stress-Induced Morphogenic Responses
TAIR	The Arabidopsis Information Resource
TE buffer	Tris-HCl Na_2-EDTA buffer
TIR1	The Transport Inhibitor Response 1
VAPs	Vertical Agar Plates
Zn	Zinc
$ZnSO_4$	Zinc sulphate

1 Introduction

The global world population exceeded 7 billion in 2011. With a current growing rate of ± 1.10% per year, the population will probably reach 8 billion in 2025 (http://www.worldometers.info/world-population/). Consequently, our ever growing population gives rise to a growing industry and pollution. This all contributes to the relative decrease in agricultural area, **making the growing demands for food supply and biomass production for energy difficult to be met.**[1] Since many are already struggling with famine, the International Food Policy Research Institute has launched the 2020 Vision Initiative. Its primary goal is to reach sustainable food security for the whole population by 2020 and to cut the number of chronically undernourished people by 50% by 2015 (http://www.ifpri.org/book-753/ourwork/program/2020-vision-food-agriculture-and-environment).

About 50-60 years ago the green revolution[2] was initiated in order to increase biomass production. It brought about a steady rise in food production as a result of better agricultural practices and the intensive use of fertilizers and pesticides. However, in the near future, **agriculture will face water deficits and shortage of fertilizers** (due to the limited reserve of phosphorus).[3] Consequently a **new approach of optimizing plant growth and a well-considered use of existing land, including marginal land containing stress factors or lacking nutrients, is necessary.**

The Second Green Revolution[3] focuses on **increasing plant efficiency** while using a minimal input of scarce resources like water and fertilizers. Special attention is paid to plant root systems because they are responsible for several vital functions such as (1) nutrient and water uptake, (2) anchorage of the plant in the substrate (3) and interaction with symbiotic organisms. [3–5]

1.1 Stress-Induced Morphogenic Responses of the roots

Plants possess a **high developmental plasticity, particularly in the root system,** which enables them to **adjust their growth to changing environments.**[6–8] This is essential for the survival of these sessile organisms, since they are not able to escape from adverse environmental conditions. The roots can sense the physical and chemical heterogeneity of the soil, thus when faced with stress they will adjust their growth and development accordingly.[9,10] These Stress-Induced Morphogenic Responses (SIMRs)[11,12] of the roots have been studied for a variety of biotic and abiotic stresses like drought, salinity, sub-optimal temperatures and nutrient deficiencies.[6,8,9,12–14] Many studies have reported a reduced primary root elongation and increased lateral root density for different stresses. Therefore Potters et al. (2009)[11] postulated that various stresses all result in a similarly altered root architecture system.

The applied aim of these studies was to optimize plant growth when nutrients are scarce, or when plants are faced with drought or salt stress, since large agricultural areas are facing these problems or will face them in the near future. However, large areas that are contaminated with excess metals, like cadmium (Cd), copper (Cu) and zinc (Zn), exist as well, due to agricultural and industrial practices.[12] Metal contamination is a very common and widespread type of pollution that forms a major threat for general public health by direct exposure as well as accumulation in the food chain. **When plants are exposed to excess metals, their growth is negatively influenced.**[6,8,12] Whereas a substantial number of studies have been conducted to understand the root responses to nutrient limitation or salt stress,[3,5,13,14] the effects of metal pollution on plant

growth and the underlying mechanisms remain poorly understood. Given that excess metals are heterogeneously distributed in the soil, the positioning of the roots is an important determinant of contaminant uptake.[12] **Therefore the root system architecture is an important factor when plant growth needs to be optimized on contaminated soils.** Thus, understanding the root responses to metals can be considered as a major priority in order to enable future optimization of plant growth on metal contaminated soils. The contaminated soils can be used for the purpose of either (1) safe biomass production or (2) phytoremediation. **This would contribute to sustainable use of otherwise unused areas, that are preferably used for food production, and take the pressure of agricultural fields.** Because of the heterogeneous metal distribution, optimal root responses for safe biomass production should include efficient avoidance mechanisms and minimal systemic growth inhibition by the excess metals in order for the plants to grow and capture nutrients within the most favourable (least contaminated) soil patches. As such, **root system optimization with minimal contaminant uptake would lead to safe biomass production, e.g. energy biomass, on contaminated fields**. For the **purpose of phytoextraction**, on the other hand, **root growth needs to be stimulated in contaminated areas**.

Previous studies in the Environmental Biology research group showed that when roots were exposed to Cd, Cu or Zn, different SIMRs were induced.[12] These findings contradict the hypothesis by Potters et al. (2009)[11] that different stresses trigger similar morphological outcomes. However, the description of the root system architecture in the findings of Potters et al. were limited to primary root growth and lateral root densities,[11] indeed leading to similarities that could be found between many stress conditions. By the inclusion of the lateral root growth rate as a component of the root system architecture a more detailed examination of the SIMRs can be formed. So far lateral root outgrowth has received little attention, although it is a major determinant of the root system architecture.[12] **However, the effect of excess metals on lateral root elongation and root growth redistribution has not been studied thus far.**

1.2 Metal stress induced by Cd, Cu or Zn

Previous research of the group Environmental Biology (CMK) reported metal-specific effects of Cd, Cu and Zn on root system architecture of the model plant *Arabidopsis thaliana*.[12] Cd, Cu and Zn were chosen because they possess different chemical properties and since they are common pollutants. By comparing metals with different properties, metal-specific stress responses are explored. Cd is a non-essential element and it is non-redox active, while Cu is essential and redox active and Zn is essential but non-redox active in plants. Because Cd is a non-essential element, it is toxic at very low concentrations, whereas essential metals as Cu and Zn will exert toxic effects when they are present in excess.

The results of the previous study showed that Cd and Cu both caused an increased number of outgrowing lateral roots, but Cd inhibited the elongation of these lateral roots more severely than Cu. Excess Zn caused a switch-off effect, with a strong negative effect on both lateral root outgrowth and elongation.[12] Since these findings refute the hypothesis of Potters et al. (2009)[11] that different stresses yield similar responses, the question **what could form the molecular basis of the metal-stress specific root responses** is raised. In order to comprehend the stress-

dependent character of the root responses the current knowledge of the physiological and molecular aspects of root development under abiotic stress needs to be expanded.

To organize their root development, plants make use of phytohormones.[5,15–20] Several studies have reported altered phytohormone levels under stress conditions[5,21–23] and phytohormones have also been found to be involved in SIMRs of roots under salt stress.[14,24] Therefore, the hypothesis of this research project is that **phytohormones form the intermediary link between metals and a stress-dependent altered root system architecture**.

1.3 Development of the root systems

Root structure can be described in a radial and an apical-basal polarity (Figure 1). At the centre of the root lays the **vascular bundle**, consisting of phloem and xylem, the conductive tissues of the plant. The vascular bundle is surrounded by **pericycle cells** that can give rise to lateral root primordia. Next layers are the **endodermis**, which forms a selective barrier for ions and the **cortex** that provides protection and mechanical support. The **epidermis** encloses the other tissues and contains the trichoblast lineage, which gives rise to root hairs.[25] Growth of the root takes place using stem cells originating from the Root Apical Meristem (**RAM**). The RAM contains a set of stem cells surrounding the Quiescent Centre (**QC**), a group of non-mitotically active cells. This QC plays a major role in organizing the meristem and is essential for the specification of the stem cell niche and maintenance of the undifferentiated state of the surrounding stem cells. Stem cells located on the lateral sides and above (shootwards) of the QC differentiate into vascular, endodermal, cortical, epidermal and lateral root cap (**LRC**) cells, whereas stem cells under the QC produce the columella root cap. [5,25] When the primary root grows, the stem cell niche at the root tip, protected by the root cap, is pushed further into the soil by the elongating cells. New cells that are produced by the stem cells in the RAM will later elongate and differentiate, leading to different zones in the root: meristematic (MZ), elongation (EZ) and differentiation zone (DZ) (Figure 1.B).[5] The boundaries of the zones are defined by the location of the cells in the corresponding developmental stage, thus when the zones are observed in a growing root, their boundaries along the root axis will adjust.[5,25] For the development of lateral roots, a lateral root founder cell located in the pericycle lineage becomes primed by an auxin pulse. Once a minimal threshold distance between the founder cell and the root tip is reached, cell division of the founder cell is activated to form a Lateral Root Primordium (**LRP**). The emergence of the LRP root through the parent root epidermis is thought to arise mostly by cell expansion. Once the lateral root is emerged, its apical meristem is activated and the lateral root begins to grow (Figure 1.C).[5] **In summary, both primary and lateral roots grow due to meristematic activity and cell elongation. the formation of lateral roots is determined at three levels : (1) the priming of the founder cell in the basal MZ[26], (2) the activation of LRP formation in DZ and (3) the activation of the meristem.**

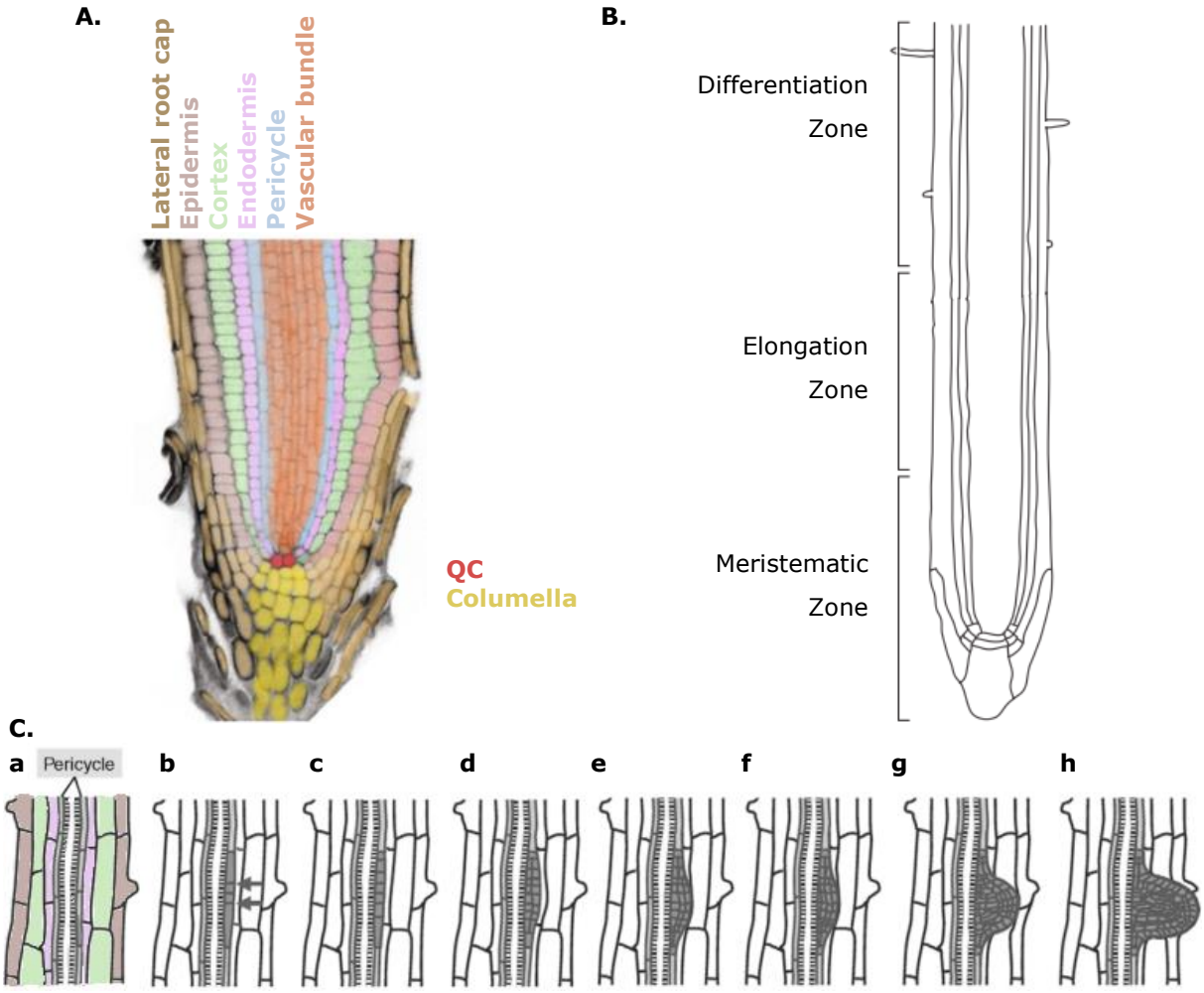

Figure 1: Root histology and development

A. Different tissues of the *A. thaliana* root apex, each differently coloured. From most outer layer to inside core: lateral root cap, epidermis, cortex, endodermis, pericycle, vascular bundle and the quiescent centre (QC) and columella in the tip. (Adapted from Sanz et al. (2012)[25])

B. Distinct development zones in the *A. thaliana* root consisting of the meristematic, elongation and differentiation zone with growing lateral roots. (Adapted from Petricka et al. (2012)[5])

C. Lateral root development in *A. thaliana*: (Adapted from Fukaki et al. (2007)[18] and Petricka et al. (2012)[5])

- a Before lateral root initiation
- b Lateral root initiation : anticlinal division of pericycle cells
- c Divided cells are radially expanded = **Stage I**
- d Outer and inner cell layers are formed by periclinal divisions = **Stage II**
- e Dome shape of the LRP is apparent (three-layered) = **Stage III**
- f In **stage IV** the LRP becomes four-layered as a result of periclinal divisions. During **stage V**, cells undergo anticlinal divisions, resulting in a LRP that begins to push through the cortex of the primary root.
- g In **Stage VI**, the LRP starts to resemble mature root tip (epidermal, cortex and endodermal cell layers) = **Stage VII**
- h Lateral root meristem is established

1.4 Involvement of phytohormones in root development under normal conditions

Phytohormones are the most important **endogenous modulators** of root system development.[27] They act at very low, sub-micromolar concentrations and their biosynthesis is not restricted to specialized tissues. The influence they exert on the development of a tissue is triggered by transcriptional reprogramming of affected cells.[28] The phytohormone levels change over the course of normal development, thereby altering intrinsic patterns of growth and development.[29] Furthermore it is known that phytohormone levels are modulated in response to environmental signals, therefore they can be considered **key components of response pathways**.[29] The overlapping hormone signalling pathways, of developmental and response pathways, **can provide the complexity that is needed for regulating the initiation of lateral root development,** considering that the root system is constantly integrating all information from different sources.[5,27,29] The interaction between phytohormones, referred to as crosstalk, can be divided into direct and indirect crosstalk, and co-regulation (Figure 2).[28]

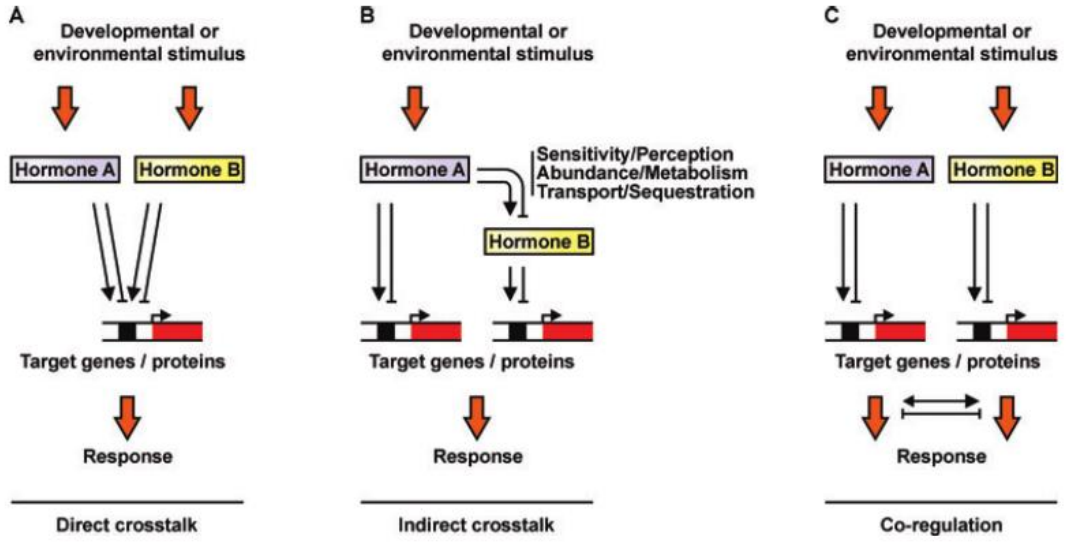

Figure 2: Three different modes of phytohormone interactions (Adapted from Hoffmann et al. (2011) [28])

A. Direct crosstalk: a developmental/environmental stimulus affects the homeostasis of two or more phytohormones. The hormones will in turn act on defined target genes, triggering one response.
B. Indirect crosstalk: a stimulus affects the homeostasis of one phytohormone, which will in turn act on a defined target gene as well as the sensitivity/perception, abundance/metabolism and transport/sequestration of another hormone. The effect on homeostasis can either be positive or negative and this will also act on a defined target gene.
C. Co-regulation: a given stimulus can activate two or more signalling pathways, which control separate outcomes. The effect can be additive, synergistic, antagonistic or they might not even interact at all. This will lead to a combined response.

This research project is focused on the actions of auxin, ethylene, jasmonates and abscisic acid (ABA). These phytohormones are part of signalling networks that are involved in plant development and stress responses.[28,30]

Auxin, under its most common form indole-3-acetic acid (**IAA**), is one of the most studied phytohormones. It plays an important role in many aspects of plant growth and development, including lateral root formation, embryogenesis, maintenance of apical dominance, shoot organ formation, vascular formation and adventitious root formation. [17–19,22,28] **It has been proven to**

be a key regulator of lateral root formation, particularly during lateral root initiation and primordium development.[17–19,31] Strong evidence suggests that the founder cell selection and its division involves auxin. The exact site of lateral root initiation is probably more a consequence of auxin transport than of *de novo* auxin synthesis.[17,27] Both acropetal and basipetal auxin transport are required for LR formation.[17,19] Overproduction of auxin or application of exogenous auxin results in an increased number of initiation events, [4,13,17,27,29] whereas auxin-resistant mutants display a decreased lateral root number.[4,18,29,32] Using transgenic lines carrying the DR5 auxin responsive promoter coupled to the GUS or GFP reporter gene, the involvement of auxin redistribution was apparent when *A. thaliana* was exposed to excess salt, Cd or Cu.[22,24]

Ethylene (C_2H_4) is a **gaseous plant hormone** that regulates plant growth, development and responsiveness to a variety of stresses.[33,34] Known actions of ethylene are **root growth inhibition and root hair elongation**.[35] Studies found that when ethylene was administered as its precursor 1-aminocyclopropane-1-carboxylic acid (**ACC**), it **reduces cell elongation** in a concentration-dependent manner. Cells with a ceased elongation cannot recover, however when ACC was removed newly formed cells showed no harm from the treatment.[34] There is evidence of a **cross-talk between ethylene and auxin**, because several auxin signalling mutants of *A. thaliana* were also ethylene-insensitive, and because the expression of ACC-synthase is strongly auxin-inducible.[27] High concentrations of **ethylene (or ACC) inhibit lateral root initiation** by modifying auxin transport and/or bio-synthesis in specific cells in the treated roots.[19,34] Whereas, low concentrations of ethylene promote lateral root initiation by **increasing auxin synthesis**.[19]

Jasmonates, including jasmonic acid and its bioactive derivatives, inhibit root growth, regulate pollen development, defend against pests/pathogens and respond to mechanical wounding.[20,35,36] Jasmonates are **oxylipins** that are synthesized by the oxygenation of α-linolenic acid by the action of lipoxygenases (**LOXs**).[30,37] **It has been shown that all jasmonate mediated responses analysed so far required the jasmonate receptor COI1**. [20,30] Both **jasmonates and ethylene** stunt root growth and both are required for plant defence responses to necrotrophic pathogens.[30] They display an interesting **crosstalk** in which ethylene can exert its inhibitory effect on root growth not only via the normal pathway of ethylene-receptors, but also by the jasmonates responsive COI1 pathway. However, the actions of ethylene in the COI1 pathway did not present themselves when the plants were placed in the dark.[35] Furthermore, it is suggested that ethylene antagonizes the jasmonates-induced inhibition of germination.[35] There is also growing evidence for substantial **indirect crosstalk between auxin and jasmonates**. Auxin formation in *A. thaliana* is enhanced by jasmonate-mediated induction of auxin biosynthesis genes and auxin has also been reported to induce jasmonate production.[28] In the *A. thaliana* root, indirect crosstalk between auxin and jasmonates is involved in the initiation of lateral roots. Jasmonate-mediated induction of IAA synthesis can either lead to the initiation of lateral roots, but because of the dose-dependent effect of IAA it might as well result in growth inhibition.[17,28] Jasmonates can modulate root development through an auxin-dependent and - independent manner. This was shown when jasmonate-treated *tir*-mutants (auxin resistant) showed inhibition of the root growth.[20]

ABA plays a major role in stress responses, but it also has essential non-stress-related regulatory functions.[16] It is able to repress both lateral root initiation and meristem activation after lateral root emergence and it controls seed dormancy and drought response.[15,16,27,38] **ABA-auxin**

coregulational crosstalk exerts itself in lateral root formation. ABA-induced inhibition of lateral root formation could not be rescued by auxin, suggesting that there is an ABA-sensitive, auxin-independent checkpoint involved.[15,16,19,29] Also the *ABI3* gene, encoding a transcription factor necessary for ABA signalling, is auxin-inducible in LRP.[16,19,29] Evidence suggests that ABA-induced root growth inhibition requires, at least in part, a functioning **ethylene** signalling network.[16,24] Signalling **crosstalk between ABA and jasmonates is thought to balance a compromise** between plant growth and defense.[28] This is reflected in the regulation of seed germination, early seedling growth and the regulation of resistance to different pathogens.[28,35,39]

The hormonal influences on root development are apparent. As a consequence it is interesting to investigate which phytohormones and what influences take part in the metal response.

1.5 Exploring the link between the development of stress-specific root architectures and phytohormones

In this study the underlying molecular parameters involved in the metal-specific root growth responses to Cd, Cu and Zn will be determined in the model plant *Arabidopsis thaliana.* This objective will be met by examining both **(1) the root architecture** and **(2) the gene expression** of several marker genes of hormonal signalling pathways.

A. thaliana is used as a model species, because it allows an efficient combination of genetic and molecular analyses since **molecular data on root growth under normal conditions is available** and because **mutants** are available. [40] Information obtained for *A. thaliana* may then be used for **validation in other species**, e.g. *A. thaliana* is closely related to crop species from the *Brassicaceae* family.[40]

(1) Root architecture will be analysed in a **reverse genetics approach**, in which the effect of a gene knock-out is studied in available mutants. The effect of homogeneous exposure to Cd, Cu and Zn exposure on the root developmental responses will be studied in these mutants and compared to the responses of wild-type *A. thaliana*. The mutants used are missing a key component of the auxin, ethylene, jasmonates or ABA signalling pathway. Using a mutation in a key protein of the signalling pathway decreases the chance of the pathway being completed via a parallel signalling route. For this study *tir1-1* (auxin), *ein2-1* (ethylene), *coi1-1* (jasmonates), *abi4-1* (ABA) genotypes were selected.

The Transport Inhibitor Response 1 (TIR1) protein is an auxin-signalling F-box protein that is required for the degradation of negative regulators of the auxin response.[32] Mutations in this protein result in the accumulation of Aux/IAA proteins, through which the normal auxin response is repressed.[18,32] *Tir1* mutants are auxin-resistant and therefore they display a variety of growth defect like reduced lateral root formation[19,20,28,32,41,42]

Mutations in the **Ethylene Insensitive 2 (EIN2)** locus result in an insensitive phenotype to both exogenous and endogenous ethylene. *Ein2* mutants have a reduced germination rate and a decreased root growth rate. [5,19,33,34,43]

As mentioned earlier, all jasmonates mediated responses, analysed so far, required COI1.[20,30] Mutants in **Coronatine Insensitive 1 (COI1)** are unresponsive to jasmonates and their growth phenotype is resistant to the inhibition by JA. They show various degrees of male sterility and increased susceptibility to pathogens/pests/fungi.[5,19,20,30,35,41,44]

ABA Insensitive 4 (ABI4) is a transcription factor involved in the response to ABA. *Abi4* mutants have a decreased sensitivity to the inhibitory actions of ABA on germination and lateral root formation, resulting in an increased number of lateral roots.[5,15,16,19,24,39,45]

Although these mutants may already show growth defects compared to wild-type plants under normal conditions, a genotype*treatment interaction will reveal the involvement of a particular signalling pathway in the metal-induced responses.

(2) The **gene expression of marker genes of hormonal signalling pathways** was examined in wild-type *A. thaliana* exposed to excess Cd, Cu and Zn, by **quantitative reverse transcription PCR** (qRT-PCR). The marker genes were selected among endpoints of hormonal signalling that have been shown to be transcriptionally induced. A difference in gene expression of certain phytohormone responsive genes may then be indicative of the involvement of this phytohormone in the altering of the root system architecture.

2 Materials and Methods

2.1 Plant materials and growth conditions

Arabidopsis thaliana ecotype Col-0 and mutants *abi4-1*, *ein2-1, coi1-1 and tir1-1* were obtained from NASC (Nottingham Arabidopsis Stock Centre). The genotype of mutant plants had been verified by allele specific PCR before starting the experiments (see below). Seeds were surface-sterilized in 0.1% (w/v) NaOCl and 0.1% (v/v) Tween 80 for 1 min and washed four times with distilled water over 20 min. After sterilization they were cultivated on 12x12 vertical agar plates containing modified 50x diluted Gamborg's B5 medium (see below). Twenty seeds were sown on germination plates, which were afterwards closed with parafilm. To allow air exchange, a gap was made in the parafilm on both sides of the plate. Plates were incubated at 4°C in the dark for 2 days before being transferred to the culture room to avoid heterogeneous germination. Conditions in the culture room were 21°C and 12h light per day (150 µmol m^{-2} s^{-1} at leaf level). A certain number of days after incubation (dependent on the experiment) plants were transferred to treatment plates at 5 plants per plate. The medium in these treatment plates was supplemented with different concentrations of $CdSO_4$, $CuSO_4$ or $ZnSO_4$. The top cm of the solid medium was removed to ensure plants were solely exposed to the metals via the roots. Plates were closed with parafilm, two gaps were made and they were placed back into the culture room.

2.2 Verification of mutated genotypes

A small leaf sample of every mutant plant used in seed production, and a mixed sample of several wild-type *A. thaliana* plants was added into 20 µl dilution buffer (Direct Plant PCR kit, Thermo Scientific, Lafayette, USA). The plant material was disrupted using a sterile pipet. Next, 0.5µl of the sample was used as DNA template in a 50µl reaction mix containing 10µl 5x Phusion High-Fidelity buffer, 1µl 10 mM dNTPs, 2.5µl forward primer (10µM), 2.5µl reverse primer (10µM), 0.5µl Phusion hot Start II DNA polymerase and 33µl RNase free water. The primer sequences listed in Table 1.

Table 1: Primer sequences for identification of the mutants (in 5' to 3' direction)

Mutant	Forward primer	Reverse primer
abi4-1	GAGATCCGAGAGCCACGTAA	CCACCGAACCAGCTAGAGAG
coi1-1	CAAGGAATGGAGGACGAAGA	TTGATTCACTTCCGGGACTC
tir1-1	GGTGTGCAAGTCATGGTACG	CGCAAAATCTTGACCAAACC

Optimal annealing temperature was first examined by applying a temperature gradient from 61°C to 69.2°C. PCR was then performed according to the manufacturers protocol (Thermo Scientific, Lafayette, USA; Appendix 1), with the standard annealing temperature of 67°C for the *abi4-1* and *tir1-1*primers and 61°C for the *coi1-1* primer pair. 10µl of the PCR product was verified for single amplicons after agarose gelelectrophoresis (1% agarose). The remaining PCR product was send to Macrogen Europe (Amsterdam, Netherlands) for purification and sequencing. Sequencing data were investigated for presence of the known point mutations indicative of the mutant genotype. Seeds were harvested from plants with confirmed genotypes only, to be used in the experiments. It was chosen to investigate the *ein2-1* and *abi4-1* in extenso. The mutants *tir1-1* and *coi1-1* have been successfully genotyped and seeds were harvested for future experiments. The mutation in *ein2-1*

plants that delivered the seed stock for this experiment was specified prior to this study, using 5'-GCGGAAGCTCAAATATGGAA-3' forward primer and 5'-GCTTGTAGGAGCAGCTTTGG-3' reverse primer and sequencing of the PCR product (Kerim Schellingen, Hasselt University, personal communication).

2.3 Preparation of vertical agar plates

Growth medium in the vertical agar plates consisted of a 50 times diluted Gamborg's B5 medium, with adjusted $CuSO_4$ concentration to avoid Cu deficiency. Detailed composition of the medium is shown in Table 2.

Table 2: Composition of 50x diluted Gamborg's B5 medium

Compound	Final Concentration (µM)	Compound	Final Concentration (µM)
$(NH_4)_2SO_4$	20	$CuSO_4.5H_2O$	0.100
$MgSO_4$	20	$ZnSO_4.7H_2O$	0.139
$CaCl_2$	20	$Na_2MoO4 \cdot 2H_2O$	0.021
$NaH_2PO_4.2H_2O$	20	$CoSO_4.H_2O$	0.0021
KNO_3	490	KI	0.090
H_3BO_3	0.970	$FeNO_3.9H_2O$	2
$MnSO_4.H_2O$	1.183		

All plates contained 0.5g l^{-1} 2-(N-morpholino)ethanesulfonic acid (MES) buffer, 10 g l^{-1} plant tissue culture agar and were adjusted to pH 5.8 with KOH. Medium destined for germination plates was enriched with 5g l^{-1} sucrose. Vertical agar plates were prepared by adding 40 ml autoclaved agar medium at 60°C to 12x12 square petri dishes. Plates were left open in the laminar airflow until the medium had solidified. In treatment plates 400 µl of 100x metal solution ($CdSO_4$, $CuSO_4$ or $ZnSO_4$) was added to 40 ml medium. All plates were supplemented with K_2SO_4 to establish a constant sulphate concentration in every plate. Treatment plates were made in triplicate.

2.4 Root architecture analysis

Five 7-day-old seedlings with a root length of approximately 2 cm were transferred to treatment plates. Treatments for Cd and Cu exposure were 0µM, 2µM, 5µM, 7.5µM and 10µM as sulphate salts. Each plate received a total SO_4^{2-} concentration of 10µM by K_2SO_4 supplementation. Treatments for Zn were 0µM, 20µM, 50µM, 75µM and 100µM, each supplemented to 100µM SO_4^{2-}. After transfer to treatment plates, the position of the primary root tip was marked and plates were scanned at 300 dpi (Epson scanner) daily. If tangled, roots were separated using a sterile toothpick to improve the quality of the scan for analysis. Shoots were cut and placed flat on the agar at the end of the experiment to image shoot surface area. After completing the experiment all data was extracted from the scans using Optimas Image Analysis Software by measuring **(1) primary root growth** per day, utilizing the daily markings, **(2) lateral root length**, **(3) coordinates of the emergence point** on the primary axis of all the laterals, coordinates of the **base of the root** and **root tip at the day of transfer** and **(4) total shoot area** of each plant. Using Microsoft Excel 2010, *ein2-1* and *abi4-1* mutants were compared to wild-type *A. thaliana* in separate experiments. *Ein2-1* mutants were monitored for 6 days, *abi4-1* mutants were monitored for 7 days.

2.5 Gene expression analysis

Samples

A. thaliana Col-0 seedlings were transferred 7 days after incubation to treatment plates containing 75µM K_2SO_4 (control plates), 5µM $CdSO_4$, 5µM $CuSO_4$ or 75µM $ZnSO_4$ (Final SO_4^{2-} concentration in Cd and Cu plates was supplemented to 75µM using K_2SO_4). Roots were harvested after 1, 2 and 3 days of exposure at 6 biological samples per condition (making a total of 72 samples), with each sample consisting of 20 root systems.

RNA extraction

RNA was extracted using the Nucleospin RNA XS kit (Machery-Nagel, Düren, Germany; Appendix 2), with an on-column DNase reaction. The elution step was executed in duplicate using 12 µl RNase free water. To evaluate the efficiency of DNA removal in the RNA samples, a qPCR was run with the first elutes of every sample together with a dilution series of cDNA and gDNA for comparison. The on-column DNase activity turned out insufficient, therefore an additional DNA removal was performed.

Additional DNase reaction

To clear the remainders of genomic DNA in the samples and additional DNase reaction was performed using the Turbo DNA-Free kit according to the manufacturers protocol (Life Technologies Europe, Gent, Belgium; Appendix 3), which also included a DNase inactivation reagent.

Purification and concentration of RNA samples

RNA in the DNase treated and DNase inactivated samples was precipitated by adding 1/10 volume sodium-acetate (3M), 0.1µl glycogen and 2.5 volumes 100% (v/v) ethanol, washed using 400 µl 70% (v/v) ethanol, and resuspended in 8.5µl RNase free water. RNA concentrations and purity (A260/A280; A260/A230) were measured using the NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA). All samples were diluted with RNase free water to obtain a final RNA concentration that corresponds with an RNA input in the next reverse transcription step of 100ng in the samples with 1 day of exposure and 200ng in the samples with 2 and 3 days of exposure.

Constructing cDNA with reverse transcription

For the construction of cDNA the Primescript RT reagent kit TaKaRa (10µl reaction) was used. The reaction contained 2.0µl 5x PrimeScript Buffer, 0.5µl PrimeScript RT Enzyme Mix I, 0.5µl Oligo dT Primers (50µM) and 0.5µl Random 6-mers (100µM) and 6.5µl RNA sample. Reverse Transcription-PCR (RT-PCR) was performed according to the manufacturers protocol (Clontech, Mountain View, USA; Appendix 4).

Primer design

New primers were designed for the *POX, ARF3, ABI5, ABI3, ABI8, NAC1, DBP, AIR3, NAC2, AIR12* and *IAA19* genes. Gene sequences were obtained from the TAIR10-gene database (www.arabidopsis.org). Primers pairs were preferably chosen so that one primer tides over an exon-exon boundary and that both primer annealing sites are present in every splice variant the gene.

Primer pairs were searched using the web-based program, Primer3 - version 3.0.0 (http://biotools.umassmed.edu/primer3/primer3web_input.htm). Using the 'Overlap Junction List' option, primers containing an exon-exon boundary were specifically targeted. Custom settings were used for 'Primer Tm' (Min 59 – Opt 60 – Max 61), 'Primer GC%' (Min 40 – Max 60) and 'Product Size Ranges' (80-120). It was verified that the selected primers were only compatible with the corresponding gene and its splice variants by blasting the primer sequences to the TAIR transcriptome and genome. Finally, primers were checked for self- and hetero-dimerization using the web-based program, OligoAnalyzer 3.1 (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) Primer sequences of the measured genes are listed in Table 3.

Real-time PCR

A tenfold dilution of the samples was made by adding 10x diluted TE Buffer (1mM Tris-HCl, pH 8.0, 0.1 mM Na_2-EDTA). In order to compare the gene expression of the cDNA samples to a dilution series, a pooled sample was made, containing 5µl of every cDNA sample. The pooled sample was repeatedly 2x diluted until a 128x dilution was reached. Gene expression was measured by real-time PCR (qPCR) using the Fast SYBRgreen dye (Life Technologies Technologies Europa, Gent, Belgium).

The reaction contained 5µl 2x Fast SYBRgreen, 0.3µl forward primer (10µM), 0.3µl reverse primer (10µM), 2.4µl RNase free H_2O and 2µl cDNA sample. Primer efficiencies were calculated based on the Ct-values of the dilution series, according to Pfaffl et al. (2004)[46]:

$$Primer\ efficiency = 10^{-\frac{1}{slope}} \qquad using\ slope\ of\ C_t\ [log(dilution)]$$

Analysis

The qPCR output was analysed using the $2^{-\Delta Ct}$ method with normalization[46]. The normalization factor was comprised of four reference genes. Reference genes were evaluated using the GeNorm[47] (version 3.5) software. Dissociation curves were checked to be indicative for single amplicons.

Table 3: Genes measured by qRT-PCR, their locus in the *A.* thaliana genome, primer sequences and characteristics.

The primer sequences are presented in the 5' to 3' direction. Product size (bp) of the cDNA product is given for each primer pair. Primer efficiency was calculated based on the Ct-values of the dilution series (optimal efficiency should be between 80-110%). Genes that were chosen as reference genes are indicated by RG in their annotation information.

Gene	Locus	Forward primer	Reverse primer	Product Size (bp)	Primer Efficiency	Annotation
SAND	AT2G28390	AACTCTATGCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC	61	82%	RG : SAND family protein [48]
YLS8	AT5G08290	TTACTGTTTCGGTTGTTCTCCATTT	CACTGAATCATGTTCGAAGCAAGT	61	84%	RG : Mitosis protein YLS8 [48]
UBIQ10	AT4G05320	GGCCTTGTATAATCCCTGATGAATAAG	AAAGAGATAACAGGAACGGAAACATAGT	61	96%	RG : Ubiquitin [48]
EF-1α	AT5G60390	TGAGCACGCTCTTCTTGCTTTCA	GGTGGTGGCATCCATCTTGTTACA	76	77%	RG : Elongation Factor 1α [48]
LOX1	AT1G55020	TTGGCTAAGGCTTTTGTCGG	GTGGCAATCACAAACGGTTC	101	73%	Lipoxygenase[12,49,50]
LOX2	AT3G45140	TTTGCTCGCCAGACACTTG	GGGATCACCATAAACGGCC	102	70%	Lipoxygenase[12,49,50]
LOX3	AT1G17420	ACGTTGTCGTACTGGTCGCC	GTCTCGTGGCACATACATAGGTAATG	91	75%	Lipoxygenase[12,49,50]
LOX5	AT3G22400	GGCAAAACCGGCCGTAAAT	GGCAAAACCGGCCGTAAAT	91	96%	Lipoxygenase[12,49,50]
VSP2	AT5G24770	GGCGTGACCTACTGGAAGCA	CGAGACTCTTCCTCACCTTTGACTT	91	79%	JA responsive[30]
MYC2	AT1G32640	GGCGTTGATGGATTTGGAGTT	ACCCATCTTCACCGTCGCTT	91	90%	JA responsive TF, ABA responsive [30]
ERS1	AT2G40940	TAGAAAACGTGGCGGATCAGG	TGCTCCATAAGCTGGTCACGA	92	94%	Ethylene response sensor 1[35]
ERS2	AT1G04310	AGTCTCAACGCTTGCCAAAACAT	CAACTGAGACGCTTTTCACCAAAC	93	100%	Ethylene response sensor 2[35]
ETR2	AT3G23150	TTCGAACCGGGCAGTTACAC	AATGGCGGTAAGGCAATCG	91	77%	Ethylene response 2[35]
ERF1	AT3G23240	TCCTCGGCGATTCTCAATTTT	CAACCGGAGAACAACCATCCT	91	116%	Ethylene response factor 1[35]
POX	AT5G22140	CTAATATCCCTGAGATGAAACAAGG	TCTTCTTTTTCCCACCTGACAT	99	81%	9-LOX responsive[49]
NAC1	AT1G56010	TCTCTGAGCTCTCCAAAGGAA	TGCAGAGGCTGTCTCATCAA	114	74%	Early auxin responsive[42,51]
NAC2	AT5G39610	CCCCAAACAGCTAAGAACGA	CCATTCGGTTAATGTGTGGA	106	81%	Auxin responsive[42,51]
IAA19	AT3G15540	TGTGGCCTTGAAAGATGGTGA	TGCATGACTCTAGAAACATCCCC	99	119%	Auxin, ABA responsive[27,31,52]
DBP	AT2G45820	GTTCGGCCGATAGAGATGTG	TCTTTTGTGCCCTGTTCTCA	114	72%	Auxin responsive[42,53]
AIR12	AT3G07390	ACAGCAGTCTCGTCGAAGGT	GGAACCTTAACCGTCGTGAA	103	80%	Auxin, ABA responsive[16,54]
ABI5	AT2G36270	CGCGAGTCTGCTGCTAGATC	CCTCTCCAACTCCGCCAATG	121	80%	ABA responsive[16,39,55]
ABI8	AT3G08550	GCTCGGGTTCAAGATCACCT	GACAGCAGCCTCCTCCAATT	96	99%	ABA responsive[16,39,55]

2.6 Statistics

All statistical analysis were conducted in R [56]. Suspected outliers in the data were statistically verified using the Grubbs test and a web-based tool (http://www.graphpad.com/quickcalcs/Grubbs1.cfm).

In order to determine whether a parametric or non-parametric test should be used, normality and homoscedasticity were tested. The data matrix was also transformed to a square-root, inverse, exponential and logarithmic data matrices. The 'Shapiro test' was performed on each matrix to find which matrices had a normal distribution. The homoscedasticity variances of every matrix were tested using the 'Bartlett test'. Only if both tests resulted in a p-value>0.05, parametrical test could be conducted. Parametrical statistical analysis of the original data matrix was preferred, however if the original data matrix did not meet the criteria, another data matrix that did fulfil the criteria was used in parametric testing. Root architecture assays were analysed using parametrical 'two-way ANOVA' (genotype and treatment variables), while for gene expression data 'one-way ANOVA' (treatment variable) was used. Parametrical two-by-two comparisons were done using the 'Tukey test' HSD (Honestly Significant Difference). Only when none of the data matrices could be tested parametrically, a non-parametric test was performed using the original data. The homoscedasticity variances were non-parametrically tested by the 'Fligner test', again the desired p-value exceeds 0.05. Non-parametric ANOVA (two-way for root architecture analysis and one-way for gene expression experiments) were made using 'Kruskal-Wallis test' and the two-by-two comparisons by the 'Pairwise Wilcoxon rank sum'.

3 Results and discussion

3.1 Verification of mutant alleles

As mentioned in 'Material and Methods' mutations in *abi4-1, coi1-1* and *tir1-1* mutants were checked. The mutation in *ein2-1* mutants (C to T substitution at NT position 3448, leading to stop codon) was previously verified in the research group and was therefore not repeated. Using the chromatogram, the nature of the mutation was examined. Homozygous mutations were needed in *abi4-1* and *tir1-1* mutants, while the mutation in *coi1-1* had to be heterozygote, because of the male sterility in homozygous plants (Figure 3). These heterozygous plants then segregate ¼ homozygous seeds that can be used for testing root growth responses.

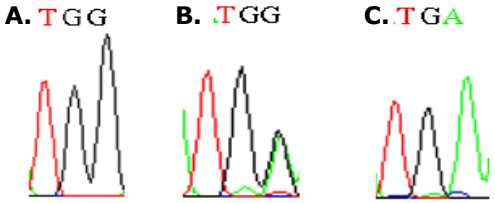

Figure 3: Chromatogram fragment of the *coi1-1* mutant

A. Homozygous wild-type: no mutation. **B.** Heterozygous G to A mutation: wanted heterozygous mutation. **C.** Homozygous G to A conversion: sterile phenotype

Abi4-1 mutants were checked for single G deletion at NT position 469 of the coding sequence, leading to a frameshift. Mutation in *tir1-1* consists of a G to A substitution at NT position 440 of the genomic sequence. *Coi1-1* mutations contained a tryptophan to stop conversion as a result of a G to A substitution at NT position 2523 of the genomic sequence.

3.2 Root architecture analysis of *abi4-1* and *ein2-1* mutants

In this experiment *abi4-1* mutants were exposed to 0µM, 2µM, 5µM, 7.5µM and 10µM Cd or Cu, and to 0µM, 20µM, 50µM, 75µM and 100µM Zn for 7 days. The *ein2-1* mutants were 6 days exposed to the same concentrations, but this experiment did not include the highest concentrations (10µM, 100 µM). The term upper zone refers to the pre-existing first ± 2cm of the root system, while the lower zone refers to the part of the root system that was grown after the transfer of the seedlings to the treatment plate. For each parameter the sample size 'n' will be given as an interval since some plants with an abnormal growth were discarded from the analysis, with a maximal sample size per concentration of 15 plants. A selection of data is presented in this section, grouping all graphs that supported the same hypothesis, the remaining data can be found in appendix. Some technical malfunctions in the newly adopted culture room caused the lights to be permanently on for 3 days – instead of the normal 12h/12h light/dark cycle - during the *ein2-1* experiment. This apparently caused abnormal root systems in the form of curly root growth and overlapping, tangled lateral roots. Therefore a substantial number of root systems were difficult to analyse. The presented data are based on a limited number of root systems which were analysable and selected to be representative for the treatment. Because of the remaining small sample size, the statistical analyses in this experiments may not be representable for the biological effects and are therefore not presented. Graphs of *ein2-1* will be evaluated for their indicational value. No such problems were encountered during the experiment using the *abi4-1* mutants, and a full statistical analysis is presented.

First assessment : comparison with previously described metal-specific phenotypes

Prior to a detailed examination, some indicative findings are compared with previous results on metal specific effects on root growth to check whether the data are in accordance. The experiment with *abi4-1* showed that the increasing exposure is translated to a decreased growth of the primary root in wild-type plants of this experiment (Figure 4.A) as well as in the mutants (Figure 5). The graphs in Figure 4.A also confirm that a homogeneous set of seedlings with the same root length was selected for transfer, as the primary root lengths of the upper zone are the same. The total lateral root length (cm) was also found to be inhibited by the exposure, with the Cu treated plants at 5-10µM exposure showing less strong inhibition than Cd and Zn treated plants at higher concentrations. (Figure 4.B) In the experiment with *ein2-1 t*he effects that were suspected in these parameters are visible to some extent, however they are not as apparent as they were in the experiment conducted with *abi4-1,* likely as a consequence to the technical problems during this experiment.

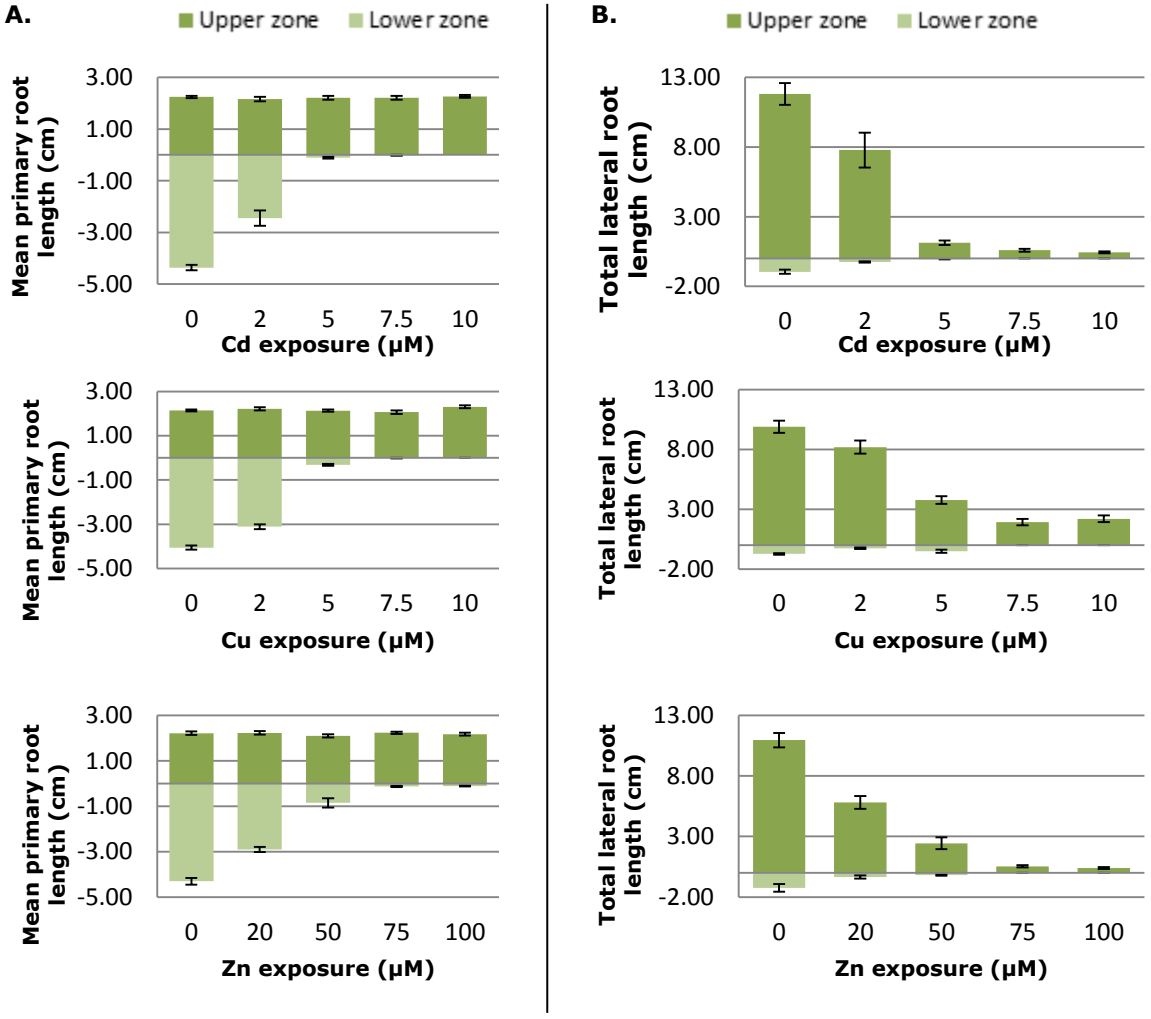

Figure 4: Indicative findings to check with previous results

Mean primary root length (**A.**) and total lateral root length (**B.**) of wild-type *A. thaliana* exposed to Cd, Cu or Zn. The effects on mean primary root length are found in the lower zone, while in the total lateral root length the effects are found in the upper zone. No statistics are presented since this selection of graphs was only used to verify whether the data were in accordance with previous results.

abi4-1 mutants are more sensitive to moderate Cd exposure

The effects on the **mean primary root length** of WT and *abi4-1* (Figure 5) show that the primary root growth decreases significantly with an increasing Cd exposure. ***Abi4-1* seems to be slightly more sensitive to 2µM Cd than the wild-type**. Although not significantly different here, this altered sensitivity was also observed in other parameters (see below and relative primary growth rate, Appendix 5).

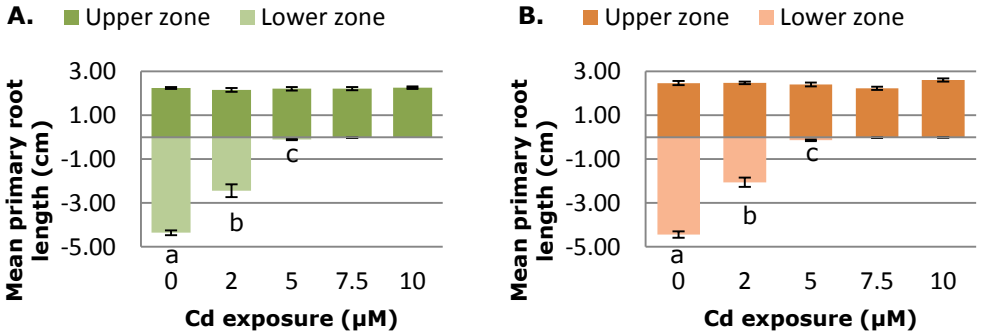

Figure 5: Mean primary root length under Cd exposure (focused on lower zone)

Mean primary root length (cm) of (**A.**) wild-type *A. thaliana* and (**B.**) *abi4-1* mutants, after 7 days of Cd exposure (0µM – 10µM). Different letters indicate statistically significant differences within a genotype and zone ($p < 0.05$), after Kruskal-Wallis test and Pairwise Wilcoxon rank sum test in two-way ANOVA (n = 11-15). No significant differences were found between the effect on WT and *abi4-1* exposed to the same concentration.

The **total lateral root length** (cm) also showed a decrease with increasing Cd exposure (Figure 6). This parameter **is significantly decreased in *abi4-1* when exposed to 2µM Cd**, while the wild-type did not display a significant decrease here. However, when the total lateral root length of the wild-type and *abi4-1*, both exposed to 2µM Cd, were compared, no significant interaction effect was found. In this parameter, the focus lies upon the upper zone. Many lateral roots in the lower zone are still in the developing stage. These are still very small and can therefore not be correctly analysed on macroscopic scale, while the roots in the upper zone have already emerged from the epidermis of the primary root.

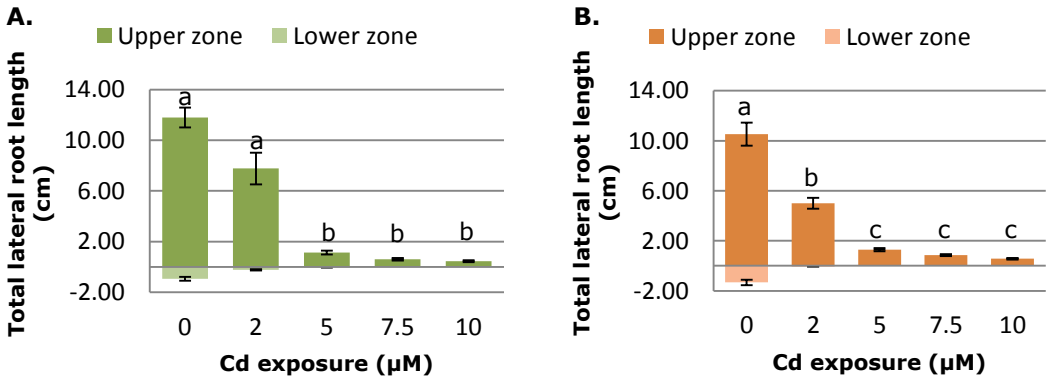

Figure 6: Total lateral root length under Cd exposure (focused on upper zone)

Total lateral root length (cm) of (**A.**) wild-type *A. thaliana* and (**B.**) *abi4-1* mutants, after 7 days of Cd exposure (0µM – 10µM). Different letters indicate statistically significant differences within a genotype and zone ($p < 0.05$), after Kruskal-Wallis test and Pairwise Wilcoxon rank sum test in two-way ANOVA (n = 11-15). No significant differences were found between the effect on WT and *abi4-1* exposed to the same concentration.

The total lateral root length is the result of mean lateral root length and mean lateral root number. The mean lateral root length (Figure 7.A, B) has a significant drop from 0µM to 2µM Cd in both wild-type and *abi4-1,* **with a significant genotype*treatment interaction difference between wild-type and *abi4-1* at 2µM Cd exposure**. Figure 7.C shows that Cd seems to have a stimulating effect on the mean number of lateral root in the wild-type, which changes into a significant decrease after 5µM Cd. The pattern that Cd exposure brought about on the lateral root number in wild-type plants does also reoccur in *abi4-1*, but the trend of stimulation was less prominent. The significant decrease in lateral length and no significant effect on lateral root number of *abi4-1* exposed to 2µM Cd, translates into the significant decrease of mean total lateral length of *abi4-1* at 2µM Cd. When the effects between wild-type and *abi4-1* on mean lateral root number were compared, no significant difference was found. Thus, although the mean total lateral length could only find an indication of *abi4-1* reacting differently to 2µM Cd, the mean lateral root length could prove this to be a significant interaction effect.

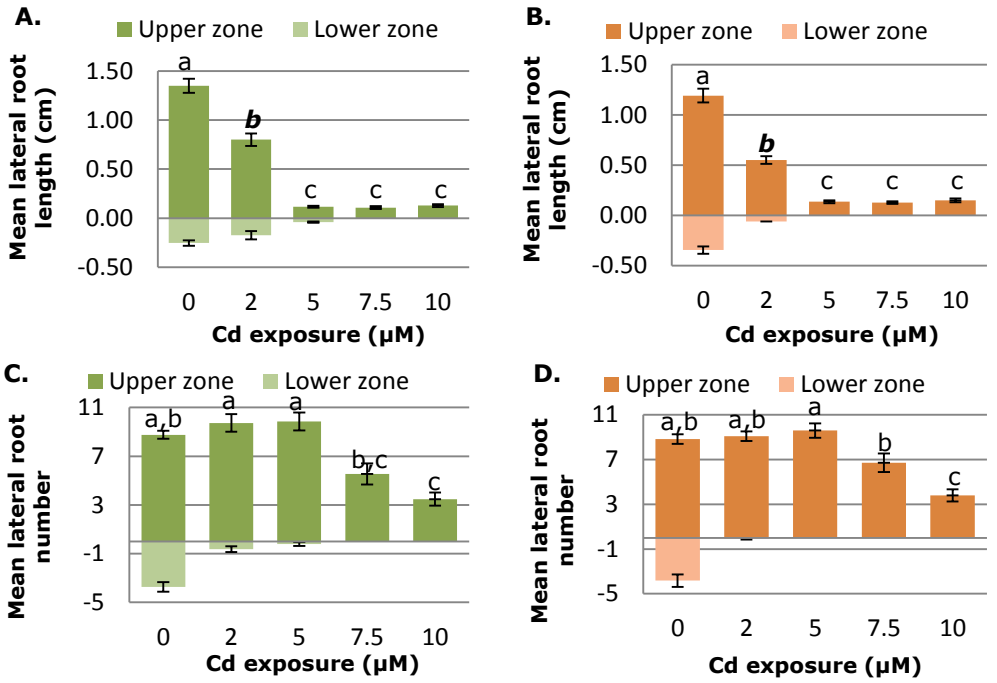

Figure 7: Dividing total lateral root length into its components: lateral length and number

Mean lateral root length (cm) of (**A.**) wild-type *A. thaliana* and (**B.**) *abi4-1* mutants and mean lateral root number of (**C.**) wild-type and (**B.**) *abi4-1*, after 7 days of Cd exposure (0µM – 10µM). Different letters indicate statistically significant differences within a genotype and zone ($p < 0.05$), after Kruskal-Wallis test and Pairwise Wilcoxon rank sum test in two-way ANOVA for the mean lateral root length (**A.** and **B.**) and for mean lateral root number after Parametric ANOVA and Tukey (n = 11-15). Letters in ***bold***, ***italic*** represent a significant difference ($p<0.05$) between wild-type and *abi4-1* in that concentration (genotype*treatment interaction effect).This parameter was also focused on the upper zone.

In the parameters 'longest lateral length' and the 'mean length of laterals of all plants' (Appendix 5), *abi4-1* seems to have a more severely decreased length than the wild-type at 2µM Cu, however this was not a statistical significant difference (using Kruskal-Wallis test and Pairwise Wilcoxon rank sum test). The same trend was found in the mean shoot area (Appendix 5), also in this parameter no statistical difference was found (using parametric two way ANOVA and Tukey test).

The results indicate the involvement of ABA-signalling in the Cd- specific root response. The lateral root elongation was more affected by Cd in the *abi4-1* mutant than in the wild-type, but no distinct differences between the genotypes were found in the lateral root number. **Therefore, it can be concluded that ABI4 is involved in the Cd-specific alterations of the lateral root length, but not in effects on lateral root number.** Since the mean shoot area shows the same phenotype, it could be that the shoot area is decreased because of lower nutrient intake with the smaller root system. Alternatively, stress effects in the shoot may interfere with photosynthesis, leading to decreased growth that is stronger in *abi4* mutants.[12,36]

abi4-1 mutants are less sensitive to excess Zn

The same parameters as in the previous section were examined under Zn exposure. The **mean primary root length** under Zn stress also showed a significant reduction with increasing Zn exposure, but no significant differences were found between wild-type *A. thaliana* and *abi4-1* (Appendix 5). The relative growth rates of the primary root (Appendix 5), suggested no differences in the response of the two genotypes, but in the kinetic primary root length it seems that *abi4-1* has a slightly altered response at 50µM Zn exposure (Figure 8).

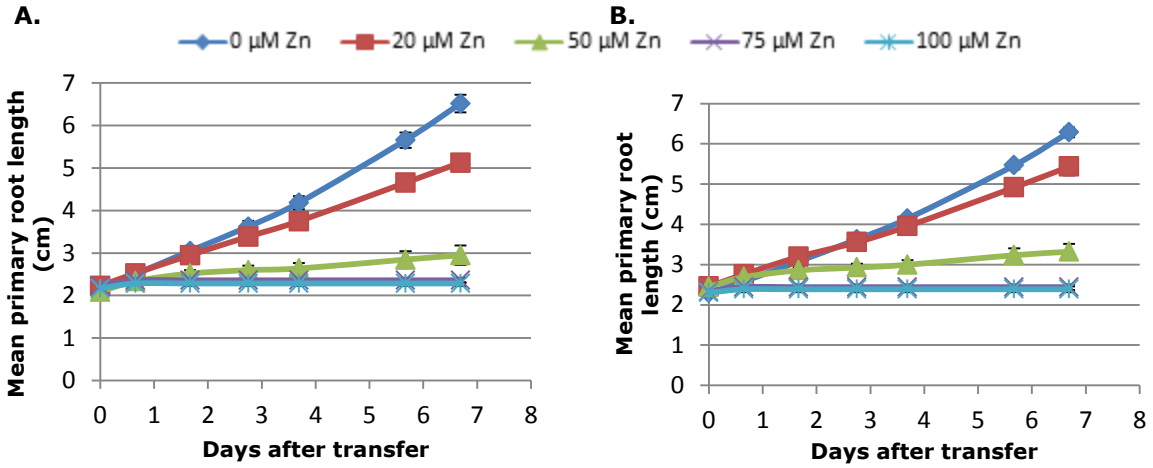

Figure 8: Kinetic primary root length under Zn exposure (Total root)

Mean primary root length (cm) of (**A.**) wild-type *A. thaliana* and (**B.**) *abi4-1* during 7 days of Cd exposure (0µM-10µM). No statistical analysis was performed on this parameter, as the data only serve an indicational purpose. (n = 11-15)

The total lateral root length is clearly inhibited by the 50µM Zn treatment (Figure 9.A, B). However the decrease is less prominent in *abi4-1* than in the wild-type, suggesting that the mutant is less sensitive to the effect of excess Zn. Comparing the **total lateral root length** between the genotypes shows that ***abi4-1* was significantly less affected by 50µM than the wild-type was**. In order to explain this effect, the components of the total lateral root length were again examined separately (Figure 9). The mean lateral root length also showed the significant decrease with increasing Zn exposure, however this response did not differ between the genotypes (Figure 9.C, D). **The less sensitive phenotype of *abi4-1* is prominent in the lateral root number** (Figure 9.E, F). The effects of excess Zn induced a significant drop in lateral root number from 20µM to 50µM in the wild-type, but not in *abi4-1.* Also at 75 and 100µM Zn, *abi4-1* seems to have more lateral roots than wild- types.

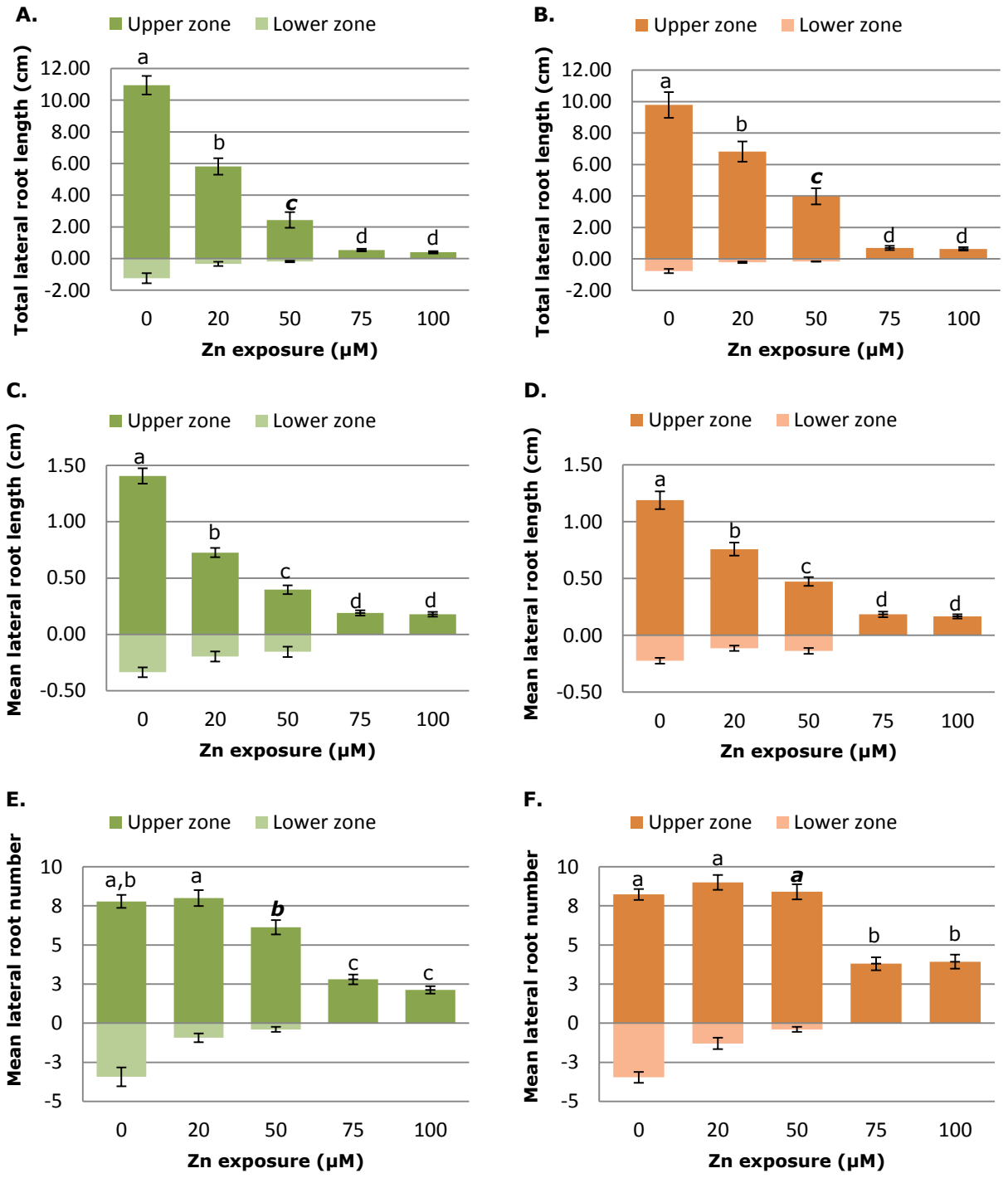

Figure 9: Total lateral root length and its components: lateral length and number under Zn exposure

Focused on upper zone : total lateral root length (cm) of (**A.**) wild-type *A. thaliana* and (**B.**) *abi4-1* mutants; mean lateral root length (cm) of (**C.**) wild-type and (**D.**) *abi4-1* and mean lateral root number of (**E.**) wild-type and (**F.**) *abi4-1*,after 7 days of Zn exposure (0µM – 100µM). Different letters indicate statistically significant differences within a genotype and zone ($p < 0.05$), after Kruskal-Wallis test and Pairwise Wilcoxon rank sum test in two-way ANOVA for total and mean lateral root length (**A.-D.**) and for mean lateral root number after Parametric ANOVA and Tukey (n = 10-15). No statistics are presented in the lower zone, as these data are not relevant. Letters in ***bold***, ***italic*** represent a significant difference ($p<0.05$) between wild-type and *abi4-1* in that concentration.

The parameters 'longest lateral length' and 'mean length of laterals of all plants' did not found a significant difference between *abi4-1* and the wild-type (using relatively Parametric two-way ANOVA and Tukey, and Kruskal-Wallis test and Pairwise Wilcoxon rank sum test). The 'mean shoot area' showed no different response between the genotypes.

The previous reported[12] effect of Zn decreasing the lateral root number in wild-type A. *thaliana* was also found in this experiment. Interestingly, the decrease was significantly less severe in *abi4-1* than it was in the wild-type. **Consequently ABI4 is most likely involved in this Zn-specific decrease in lateral root number.** Since the lateral length of *abi4-1* and the wild-type were similarly affected by Zn, this response is not thought to be ABI4-mediated.

Roots of abi4-1 did not show an altered response to Cu exposure

All the previously discussed parameters of **root responses showed no difference** between the response of wild-type and *abi4-1* plants to Cu exposure (Appendix 5. **In conclusion, ABI4 is probably not involved in Cu-specific root responses.**

The mean shoot area decreased under Cu exposure. **At 5µM Cu exposure, *abi4-1* had a significant lower shoot area than the wild-type**, indicating that *abi4-1* might be more sensitive to 5µM Cu for this parameter.

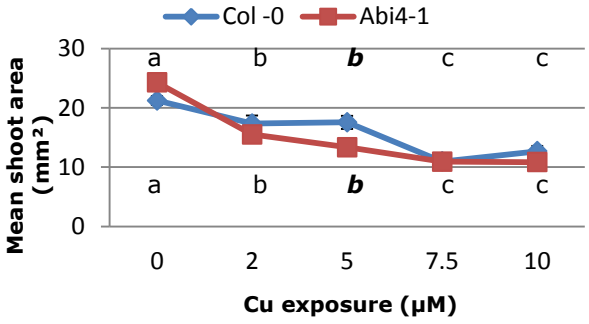

Figure 10: Mean shoot area under Cu exposure

Mean shoot area (mm²) of wild-type *A. thaliana* and *abi4-1* mutants, after 7 days of Cu exposure (0µM – 10µM). Different letters indicate statistically significant differences within a genotype and zone ($p < 0.05$), after Kruskal-Wallis test and Pairwise Wilcoxon rank sum test in two-way ANOVA (n = 11-15). Letters presented above the curves correspond to Col-0 (wild-type) and letters below the curves correspond to *abi4-1*. Letters in ***bold***, ***italic*** represent a significant difference ($p<0.05$) between wild-type and *abi4-1* in that concentration.

Because the involvement of phytohormones differ in the shoot and root, it is possible that ABI4 is involved in the response of the shoot to Cu stress. However, for more certainty on this hypothesis, a different approach and more research is needed, that was not provided in the framework of this report.

Ein2-1 mutants are more sensitive to Cu stress

As expected, the **primary root length** decreased with increasing Cu exposure (Figure 11 A,B). ***Ein2-1* mutants seem to be more sensitive to Cu stress than the wild-type at 5µM Cu**. This is observed in primary root growth and total lateral root length (Figure 11 C, D).

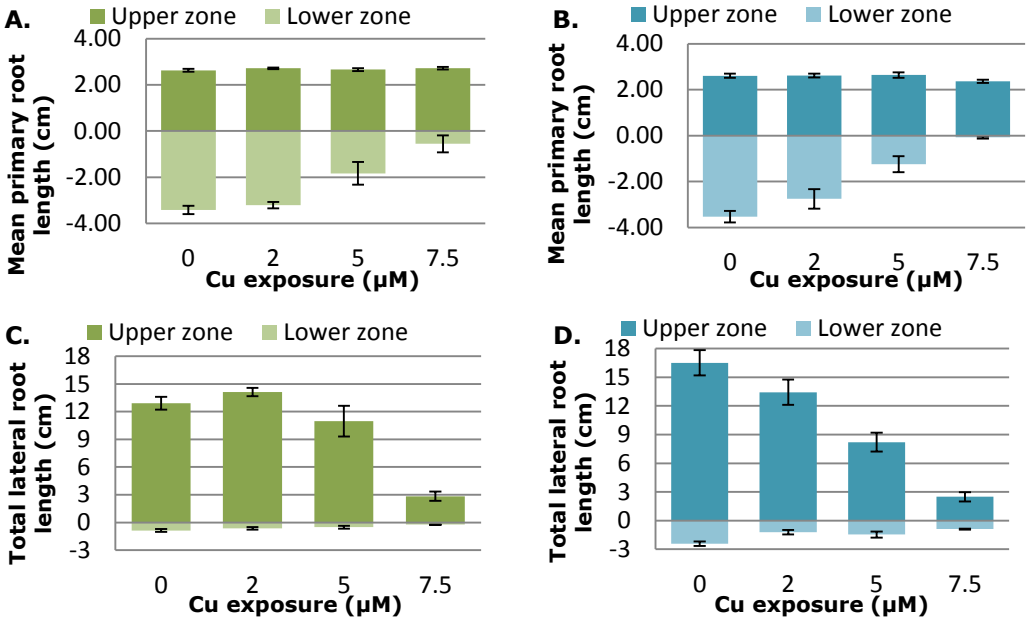

Figure 11: Primary root length and Total lateral root length under Cu exposure

Primary root length (cm; focused on lower zone) of (**A.**) wild-type *A. thaliana* and (**B.**) *ein2-1* mutants and the total lateral root length (cm; focused on upper zone) of (**A.**) wild-type and (**B.**) *ein2-1* mutants, after 6 days of Cu exposure (0µM – 7.5µM). No statistics are presented, as the data only serve an indicational purpose.

When total lateral root length is divided into its components 'mean lateral root length' and 'mean lateral root number', this trend is found again (Figure 12). The **mean lateral root length** is inhibited by the Cu exposure and ***ein2-1* seems to be more sensitive to 5µM Cu than the wild-type**. There also seems to be a different response in the lower zone of *ein2-1.* Overall the mean lateral root length of *ein2-1* in the lower zone is slightly longer than that of the wild-type, with the most pronounced difference in 5µM Cu. The mean lateral root number seems to be similarly effected in the wild-type and in *ein2-1.* The parameters 'longest lateral length' and 'mean length of all laterals' also presented a more sensitive reaction of *ein2-1* to 5µM Cu (Appendix 6).

The results point to an involvement of EIN2 and the ethylene signalling pathway in the root responses to Cu exposure. This involvement is found in the inhibitory effects of Cu on the lateral root elongation.

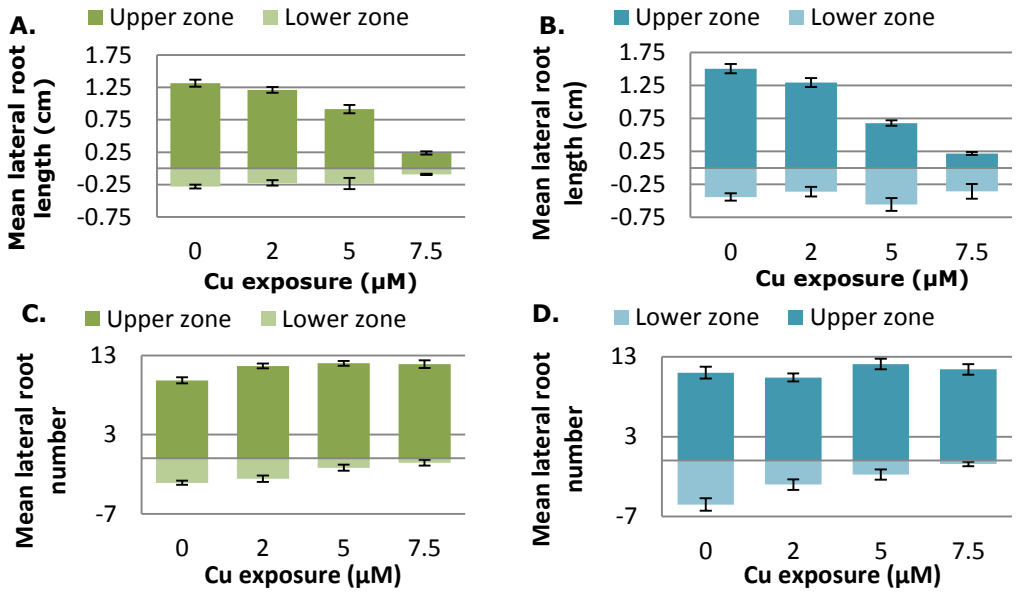

Figure 12: Dividing total lateral root length into its components: lateral length and number

Mean lateral root length (cm) of (**A.**) wild-type *A. thaliana* and (**B.**) *ein2-1* mutants and mean lateral root number of (**C.**) wild-type and (**D.**) *ein2-1*, after 6 days of Cu exposure (0µM – 7.5µM). No statistics are presented, as the data only serve an indicational purpose.

The sensitive phenotype of *ein2-1* mutants was also apparent in the effects of Cu exposure on the mean shoot area. The shoot area decreases with increasing Cu exposure, however the shoot area in the wild-type seems to be stimulated by 2µM Cu. (Figure 13) Possibly, the shoot was decreased as an effect of the decreased root system, but it might be possible that EIN2 is involved in Cu- specific shoot responses as well.

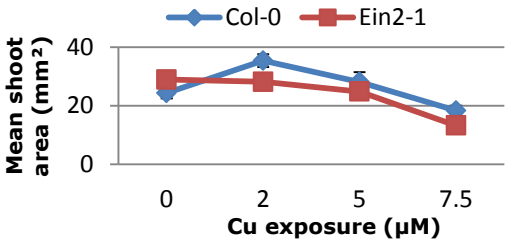

Figure 13: Mean shoot area under Cu exposure

The mean shoot area (mm²) of wild-type *A. thaliana* and *ein2-1* mutants, after 6 days of Cu exposure (0µM – 7.5µM). No statistics are presented as this graphs is only used to form an indication.

Ein2-1 mutants are sensitive to excess Zn

The primary root length is in both genotypes inhibited by Zn exposure. **The primary root length of wild-type *A. thaliana* and *ein2-1* mutants show a distinctive difference in the response of the genotypes to 50µM Zn exposure** (Figure 14)**.** This distinction is also prominent in the relative kinetic primary root growth over 6 days (Appendix 6). Although the growth patterns found are irregular and standard errors are large (possibly due to the abnormal growth conditions caused by a period of permanent illumination), the difference in response between the genotypes to 50µM Zn is rather large.

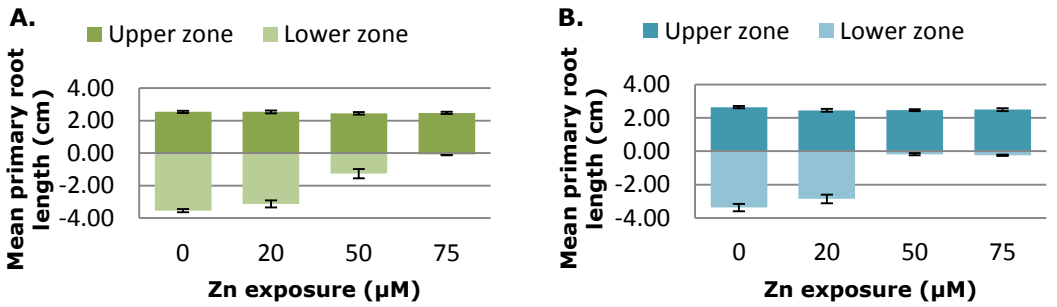

Figure 14: Mean primary root length (cm; focused on lower zone) under Zn exposure

Primary root length (cm) of (**A.**) wild-type *A. thaliana* and (**B.**) *ein2-1* mutants, after 6 days of Zn exposure (0µM – 75µM). No statistics are presented, as the data only serve an indicational purpose.

The **total lateral root length of *ein2-1* also displayed an apparent altered response to 50µM Zn**, in the endpoint analysis as well as in the kinetic data (Figure 15).

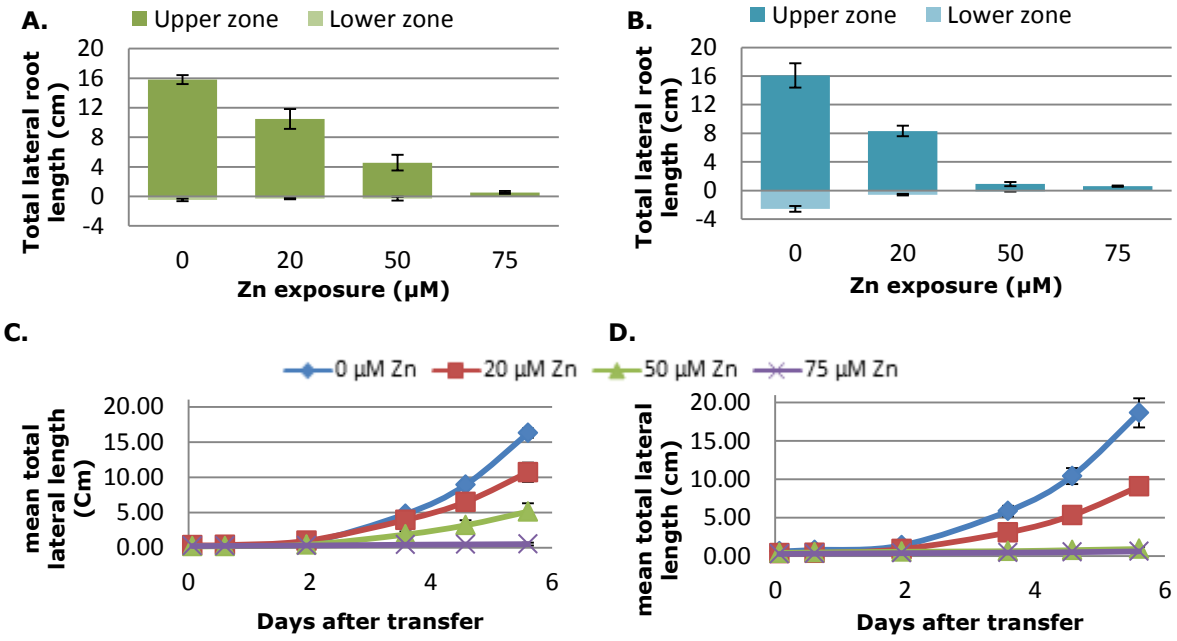

Figure 15: Endpoint and kinetic total lateral length under Zn exposure (upper zone)

Total lateral length (cm) of (**A.**) wild-type *A. thaliana* and (**B.**) *ein2-1* mutants, after 6 days of exposure to Zn (0µM – 75µM) and the kinetic data of (**C.**) wild-type (**D.**) *ein2-1* during these 6 days of exposure. No statistics are presented as the data only serve an indicational purpose.

The components of the total lateral length give the same results: **the mean lateral root length and number are much lower in *ein2-1* than in the wild-type at 50µM Zn exposure** (Figure 16). Interestingly, the mean lateral root number at 75µM seems to be less effected in *ein2-1* than in the wild-type. All the observations were made in both endpoint and kinetic analyses of the different parameters. Parameters 'longest lateral length' and 'mean length of laterals of all plants' contributed to the observation of a more sensitive response of *ein2-1* to 50µM Zn (Appendix 6). Analysis of the mean

shoot area of wild-type *A. thaliana* and *ein2-1* mutants after 6 days of Zn exposure, did not show any differences between the genotypes.

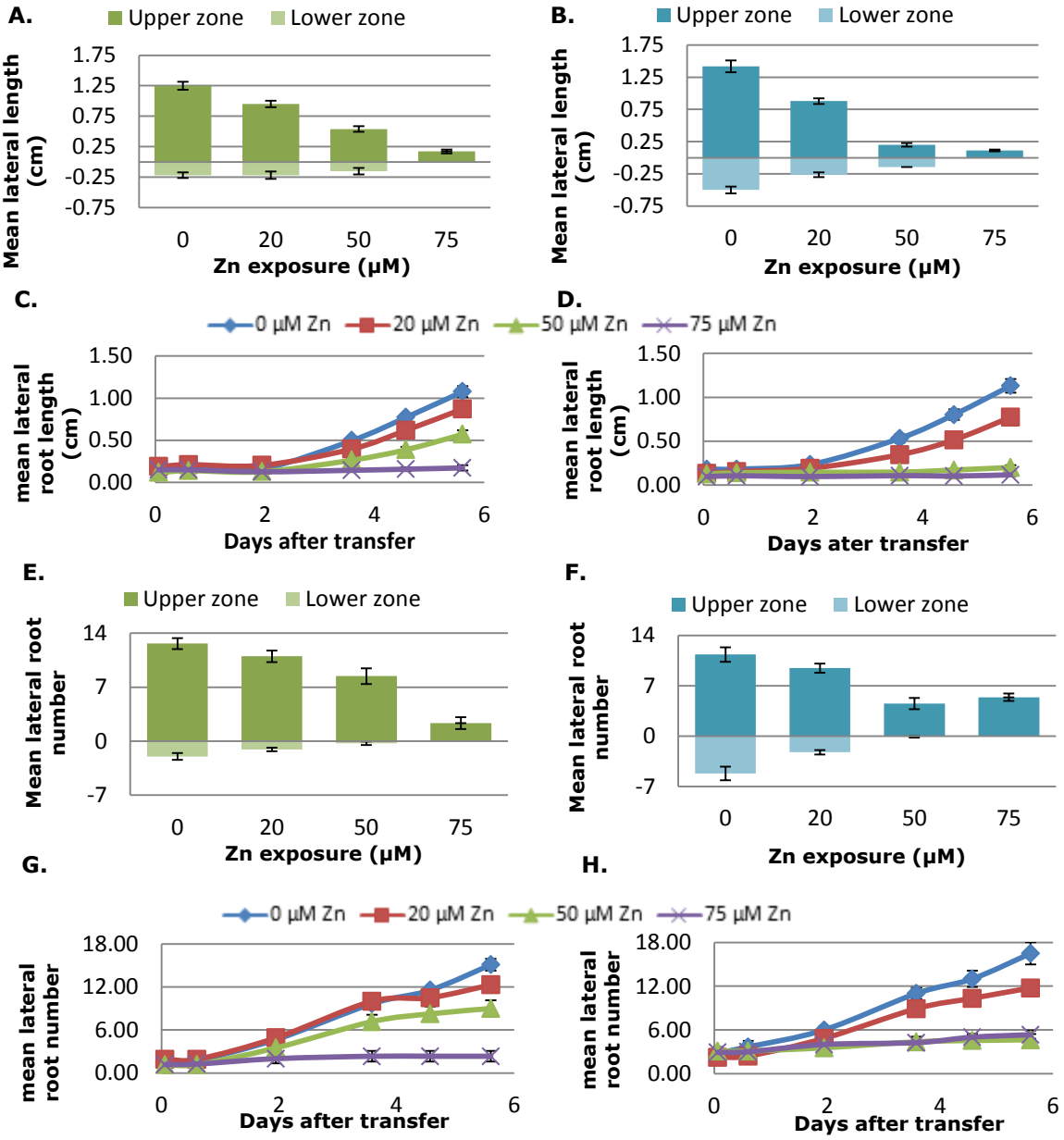

Figure 16: Endpoint and kinetic lateral root length and number under Zn exposure (upper zone)

Mean lateral root length (cm) of (**A.**) wild-type *A. thaliana* and (**B.**) *ein2-1* mutants, after 6 days of Zn exposure (0µM – 75µM) and kinetic date of (**C.**) wild-type and (**D.**) *ein2-1* during these 6 days of Zn exposure. Mean lateral root number of (**E.**) wild-type and (**F.**) *ein2-1*, after 6 days of Zn exposure and kinetic data of (**G.**) wild-type and (**H.**) *ein2-1* during these 6 days of Zn exposure. Graphs serve as indications, thus no statistics are presented.

In conclusion the ethylene signalling pathway is probably involved in the Zn-specific root responses. Because a lot of different parameters all found that the root length differed in the *ein2-1* mutant compared to the wild-type, the involvement of EIN2 in the Zn-induced effect on lateral root elongation is probable. The effect on the lateral number however, is not as clear. Both endpoint and kinetic

analysis of the lateral root number show a more sensitive response of *ein2-1* to 50µM but less sensitive to 75µM, in comparison to the wild-type. This shift in reaction cannot be reasoned and can possibly be the results of the abnormal growth resulting from the technical defects. Consequently; the found effects of ethylene being involved in the effects of Zn on the lateral root number are interpreted with caution. This finding will for now be excluded from the conclusion of this report, but will be kept in mind for a next experiment.

Roots of ein2-1 mutants had no altered response to Cd

All the previously discussed parameters of **root responses showed no difference** in the response of wild-type and *ein2-1* plants to Cd exposure (Appendix 6). Interestingly, the shoot area of *ein2-1* plants seemed to be less effected by Cd exposure (Figure 17).

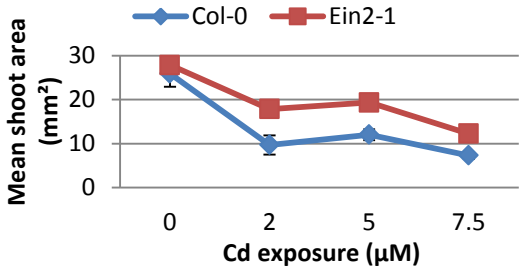

Figure 17: Mean shoot area under Cd exposure

Mean shoot area (mm²) of wild-type *A. thaliana* and *ein2-1* mutants after 6 days of Cd exposure (0µM – 7.5 µM). No statistics are presented since the data only serve an indicational purpose.

This could imply that EIN2 is involved in the Cd-induced shoot responses, but is not involved in Cd-induced root responses.

Discussion on VAPs experiments

All results have to be confirmed by a repetition of the experiments, to investigate whether the same conclusion can be formed. The experiment of *ein2-1* has to be repeated as the results may have been influenced by the technical problems. Nevertheless, interesting differences between wild-type and *ein2-1* mutants were observed. The primary root growth was similarly inhibited by each metal, therefore this parameter is not likely to be Cd, Cu or Zn specific. On the other hand, the same effect could still have a different molecular basis, as observed for the involvement of LOX1 in Cd but not in Cu-induced primary root growth inhibition.[12] It should be kept in mind that the findings in the mutants are not solely resulting of the actions of ABA and ethylene signalling respectively, since a major network of hormonal crosstalk is also at work in these stress responses. Future test with *tir1-1* and *coi1-1* mutants may point to an involvement of auxin and jasmonic acid signalling as well. In all the different sections the results of the metal-specific effects on the mean shoot area were given as well. These data just give a first idea about the relation between roots and shoot responses but they cannot be used to draw any conclusions, for now. A correlation between the root and shoot effects may be investigated in both wild-types and mutants.

3.3 Gene expression analysis in wild-type *A. thaliana*

In this experiment the gene expression of marker genes (Table 3) of hormonal signalling pathways in wild-type *A. thaliana* exposed to Cd, Cu and Zn was examined. Wild-type plants were exposed for 1, 2 or 3 days to 5µM Cd, 5µM Cu or 75 µM Zn. The effect of the treatment was examined per exposure time (see below). Data are bundled according to their annotation (Table 3). Because optimizations of this experiment were made, not every treatment group consisted of 6 samples. The number of samples of the control group, Cd, Cu and Zn respectively after 1 day of exposure were 4, 2, 4, 3; after 2 days of exposure were 5, 6, 4, 5; and after 3 days of exposure were 6, 6, 6, 5. Each sample contained 20 root systems. Although the number of samples was not consistent throughout the treatments, the results give a good first indication of the phytohormones involved. Also the data from this experiment give an idea of the effect size in order to calculate the number of samples that is needed for a next experiment with a high statistical power.

Optimization of gene expression on small root systems

Using small root systems for RNA extractions, the number of handlings that can cause loss of RNA should be limited. The on-column DNase treatment was shown to be insufficient when it was performed according to the manufacturer's manual. Because the additional DNase treatment is very labour-intensive in this sort of experiment (large amount of samples), but most importantly because a small volume of the sample is lost in the removal of the DNase inhibitor, an optimization of the on-column DNase treatment is suggested. Since the samples of the roots are already very small and preferably a large number of genes are tested, it is best to minimize the volume loss. By increasing the volume of the DNase enzyme and increasing the treatment period, the on-column DNase reaction could eventually be sufficient. The normalization was performed using four reference genes. The used reference genes all had an average expression stability (M-value) below the cut-off value of M<1.5, proposed by Vandesompele et al. (2002)[47]. The V-value (Pairwise variation) was larger than 0.15, suggesting that the use of additional reference genes may give more optimal results. However in this experiment no extra reference genes could be tested because the samples lacked sufficient volume.

Results of the genes involved in jasmonate signalling

The relative expression levels of jasmonate-responsive genes are given in Figure 18, except for the *LOX2* gene, because it has a very low expression level in the roots, expression of this gene in most samples could not be detected. The expression of the *LOX1* gene was upregulated for all treatments after 1 day of exposure, but after 2 and 3 days only Cd caused upregulation. The *LOX3* gene was upregulated by Cd and Cu after 1 day of exposure and only by Cd after 2 days. The only response of the *LOX5* gene was a downregulation by Cd after 3 days of exposure and of the *VSP2* gene an upregulation by Cu after 1 day. Cu and Zn treatments induced an upregulation of the *MYC2* gene after 3 days. The *POX* gene was upregulated by every exposure period of Cd treatment and by 1 day of excess Zn exposure.

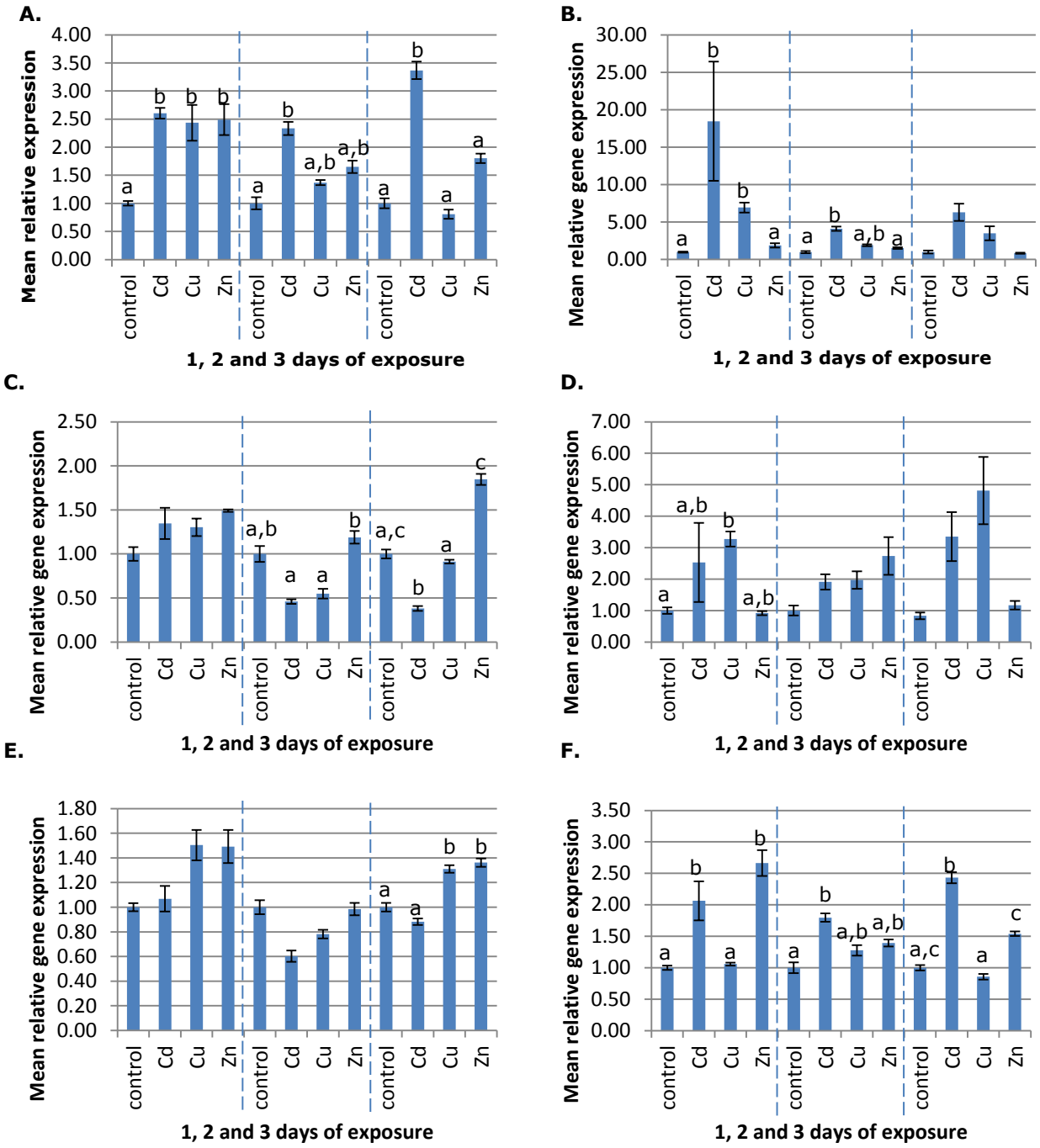

Figure 18: Gene expression of jasmonate responsive genes

Mean relative expression level (normalized to the expression of four reference genes) of the (**A.**) *LOX1* (**B.**) *LOX3* (**C.**) *LOX5* (**D.**) *VSP2* (**E.**) *MYC2* (**F.**) *POX* gene in wild-type *A. thaliana* after 1, 2 and 3 days of exposure to 5µM Cd, 5µM Cu or 75µM Zn. Different letters indicate statistically significant differences within the exposure period ($p<0.05$), after parametric one-way ANOVA and Tukey (number of samples: 0, 2, 4, 3, - 5, 6, 4, 5, - 6, 6, 6, 5, each containing 20 root systems). Days on which no letters are presented did not find any significant differences. If the original data were not normally distributed the parametric one-way ANOVA and Tukey were performed on a transformed data matrix: logarithmic matrix for 1 day exposed *LOX1, LOX3, VSP2, POX* and for 3 days exposed *VSP2*, and a square root matrix for 2 days exposed *LOX3.*

Results of the genes involved in ethylene signalling

In the gene expression of *ERS1, ERS2* and *ETR2,* no effect was detected after 1 day of exposure and after both 2 and 3 days Cd and Zn treatments resulted in an upregulation for every gene. The *ERF1* gene was only upregulated by Cd, in every exposure period. (Figure 19)

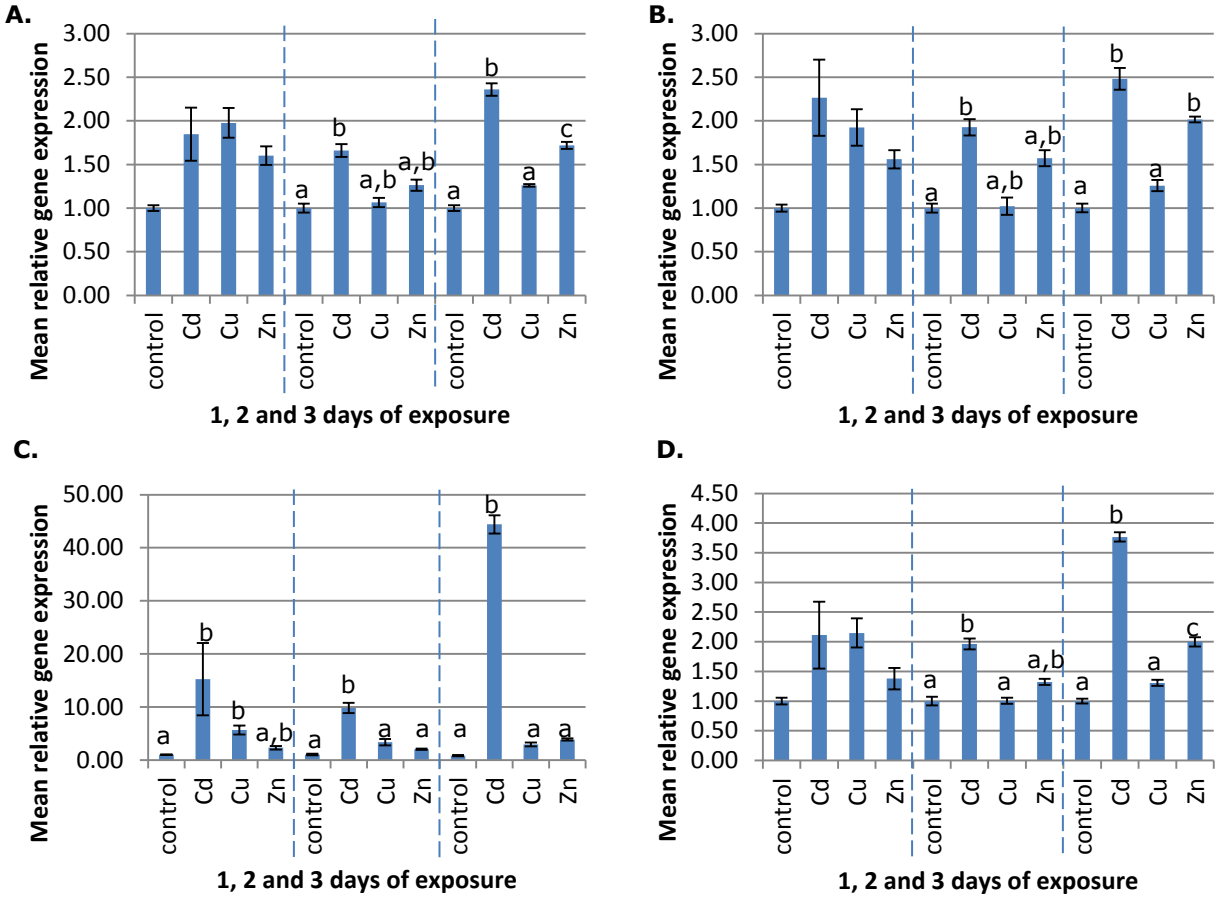

Figure 19: Gene expression of ethylene responsive genes

Mean relative expression level (normalized to the expression of four reference genes) of the (**A.**) *ERS1* (**B.**) *ERS2* (**C.**) *ERF1* (**D.**) *ETR2* gene. in wild-type *A. thaliana* after 1, 2 and 3 days of exposure to 5µM Cd, 5µM Cu or 75µM Zn. Different letters indicate statistically significant differences within the exposure period ($p<0.05$), after parametric one-way ANOVA and Tukey (number of samples: 0, 2, 4, 3, - 5, 6, 4, 5, - 6, 6, 6, 5, each containing 20 root systems). Days on which no letters are presented did not find any significant differences. If the original data were not normally distributed the parametric one-way ANOVA and Tukey were performed on a transformed data matrix: logarithmic matrix for 3 days exposed *ERS1, ERS2* and for *ERF1* (1 and 2 days exposed), and a square root matrix for 3 days exposed *ERF1.*

Results of the genes involved in auxin signalling

The *NAC1* gene was uregulated by Zn treatment after 3 days of exposure. Cd and Zn both caused for every exposure period an upregulation in the *NAC2* gene. The *DBP* gene was downregulated by Cd treatment after 2 and 3 days. The *IAA19* and *AIR12* were both upregulated by Cd for each exposure period (except 1 day *AIR12* had no effects). (Figure 20)

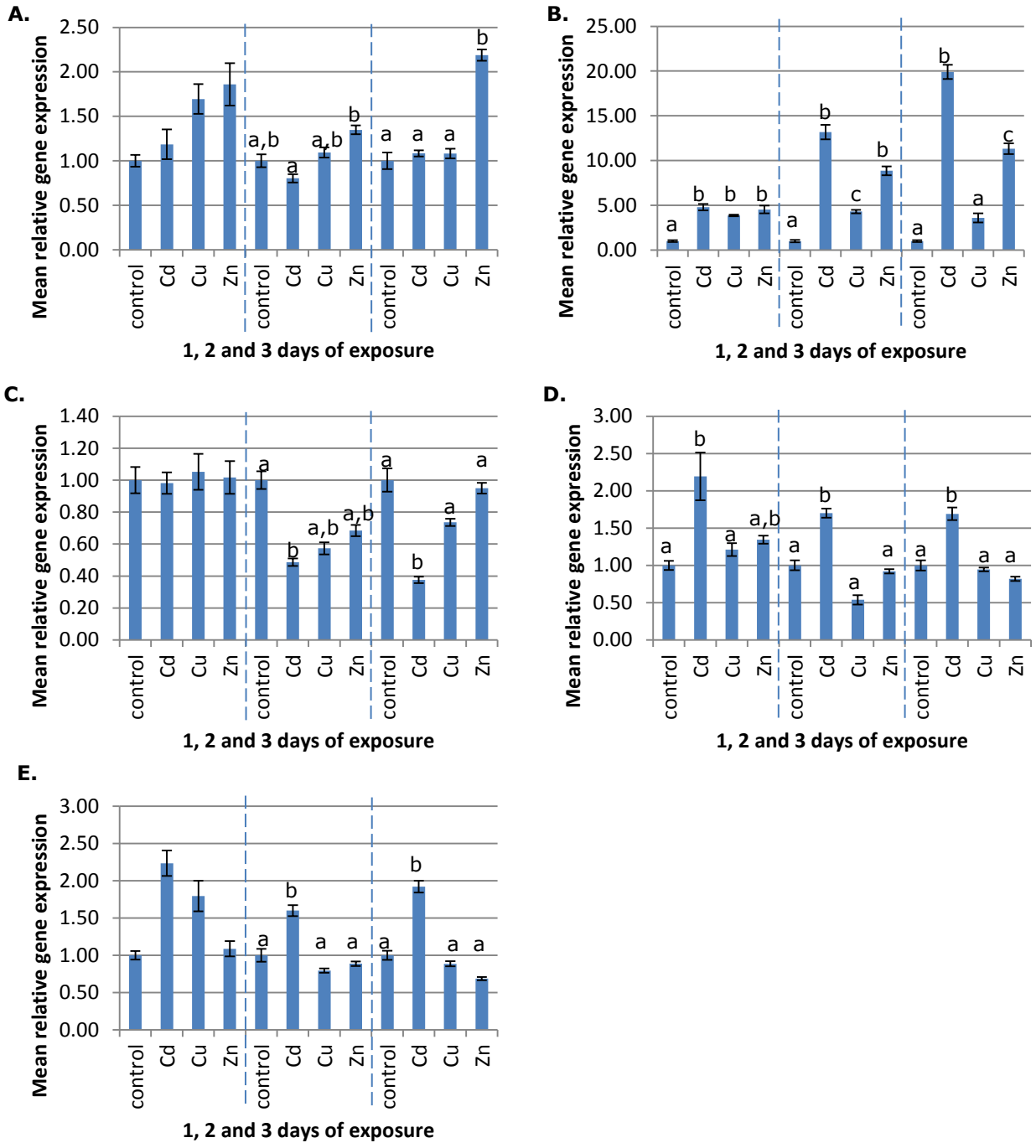

Figure 20: Gene expression of auxin responsive genes

Mean relative expression level (normalized to the expression of four reference genes) of the (**A.**) *NAC1* (**B.**) *NAC2* (**C.**) *DBP* (**D.**) *IAA19* (**E.**) *AIR12* gene in wild-type A. thaliana after 1, 2 and 3 days of exposure to 5µM Cd, 5µM Cu or 75µM Zn. Different letters indicate statistically significant differences within the exposure period ($p<0.05$), after parametric one-way ANOVA and Tukey (number of samples: 0, 2, 4, 3, - 5, 6, 4, 5, - 6, 6, 6, 5, each containing 20 root systems). Days on which no letters are presented did not find any significant differences. If the original data were not normally distributed the parametric one-way ANOVA and Tukey were performed on a transformed data matrix: logarithmic matrix for 3 days exposed *DBP, IAA19* and *AIR12*. The statistics of 2 days exposed *NAC2* expression were found by non-parametric Kruskal-Wallis test and Pairwise Wilcoxon rank sum test.

Results of the genes involved in ABA signalling

The expression of the *ABI5* gene was upregulated by 1 and 3 days of Cd exposure. No changes were detected in the expression of the *ABI8* gene.

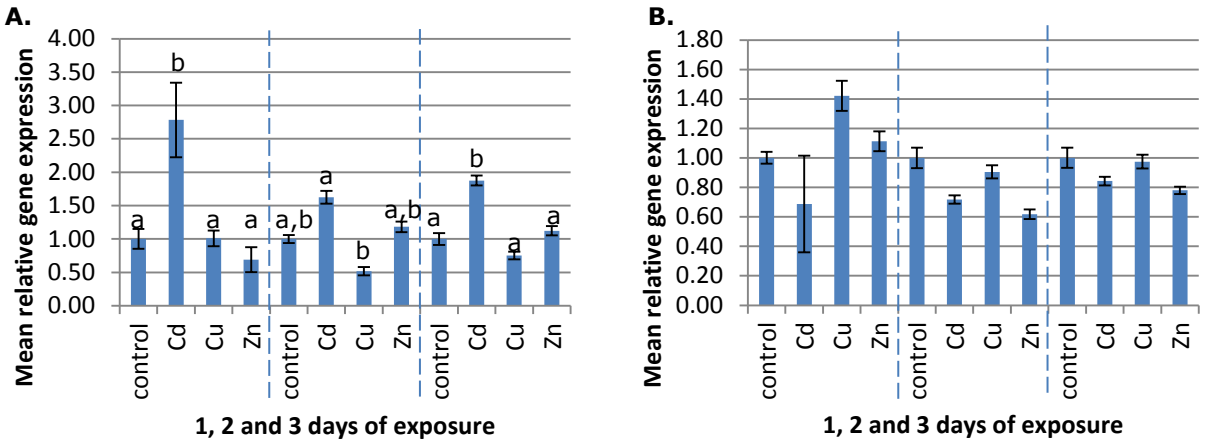

Figure 21: Gene expression of ABA responsive genes

Mean relative expression level (normalized to the expression of four reference genes) of the (**A.**) *ABI5* (**B.**) *ABI8* gene in wild-type A. thaliana after 1, 2 and 3 days of exposure to 5µM Cd, 5µM Cu or 75µM Zn. Different letters indicate statistically significant differences within the exposure period ($p<0.05$), after parametric one-way ANOVA and Tukey (number of samples: 0, 2, 4, 3, - 5, 6, 4, 5, - 6, 6, 6, 5, each containing 20 root systems). Days on which no letters are presented did not find any significant differences. All significant differences were found using untransformed data.

Discussion on gene-expression experiment

The effects of metal exposure on the expression of marker genes of the phytohormones signalling pathways in wild-type root systems give a good first impression on the involvement of the different phytohormones involved in metal-specific root responses. The results of the experiment are placed together with the observations of the VAPs experiments and are summarised in Table 4.

Table 4: Summary of the gene-expression and VAPs experiments

	Auxin	ABA	Jasmonates[1]	Ethylene	*abi4-1*	*ein2-1*
Cd	↑*NAC2* ↓*DBP* ↑*IAA19* ↑*AIR12*	↑ABI5	↑*LOX1* ↑*LOX3* ↓*LOX5* ↑*POX*	↑*ERS1* ↑*ERS2* ↑*ERF1* ↑*ETR2*	↓ Lateral root elongation	
Cu	↑*NAC2*		↑*LOX1* ↑*LOX5* ↑*VSP2* ↑*MYC2*			↓ Lateral root elongation
Zn	↑*NAC1* ↑*NAC2*		↑*LOX1* ↑*POX* ↑*MYC2*	↑*ERS1* ↑*ERS2* ↑*ETR2*	↑ Lateral root number	↓ Lateral root elongation

[1] *LOX1, LOX5* and *POX* are 9-LOX responsive, *LOX2* and *LOX*3 are 13-LOX responsive.

The results of the gene expression analysis in wild-type *A. thaliana* show that the different metals induce several genes that are responsive to different phytohormones. Interestingly Cu had no effect on the tested ethylene responsive genes, but did induce a different response in the ethylene-insensitive mutant than in the wild-type. Zn was the only metal that induced altered root systems (compared to the wild-type) in both the studied mutants, while Cd only affected *abi4-1* and Cu *ein2-1*. The effects of Cd on the root system of *abi4-1* and of Cu and Zn on the root system of *ein2-1* were excreted in the elongation, rather than lateral root number. The effects on lateral root number only different from the wild-type in Zn-stressed *abi4-1.* As in the VAPs experiments, a repetition of the experiment is needed and will also have to be performed on mutants to allow a more detailed examination and interpretation.

Because the expression of auxin and jasmonates-responsive genes was affected in the wild-type, experiments with *tir1-1* and *coi1-1* can possibly fill the blanks of the parameters involved in Cd, Cu and Zn-specific altered root systems.

4 Conclusion

The effects of Cd, Cu and Zn were studied on the root system. Cu and Zn are essential elements, while Cd is non-essential. Because Cd is non-essential it is toxic even at very low levels. The studied responses of the roots can be divided into primary root growth, lateral root growth and number of lateral roots. The primary root growth was similarly inhibited by each metal, therefore this parameter is not likely to be Cd, Cu or Zn specific. The results leading to the formed hypothesis are summarized in Figure 22.

From the results of the Cd-specific root responses it can be concluded that ABA-signalling through ABI4 is involved in the metal-specific lateral root elongation response. The lateral root elongation was affected more by Cd in the *abi4-1* mutant than it was in the wild-type. Also, ABA-responsive genes were upregulated in wild-type plants after Cd treatment. The results of the VAPs experiments indicate that EIN2 is not involved in Cd-specific root responses, although Cd exposure did induce the expression of ethylene responsive genes. Possibly ethylene is involved in shoot specific responses to Cd, as the mean shoot area of the *ein2-1* mutant were also found to be lower than of the wild-type. No alterations in the Cd-specific effect on lateral root number were found. Since Cd treatment could alter the gene expression of auxin and jasmonates responsive genes, further test with *tir1-1* and *coi1-1* could maybe identify the pathway involved in Cd-specific effects on lateral root number.

Also for Cu, only specific effects on the lateral root elongation were found and further testing with *tir1-1* and *coi1-1* are needed for more information. **The ethylene pathway was found to be involved in Cu- specific responses in lateral root elongation.** The lateral roots of *ein2-1* were shorter that the lateral roots of the wild-type under Cu stress. Cu treatments did not alter the gene-expression of ethylene responsive genes in the wild-type. For more insights in the involvement of ethylene in Cu-specific lateral root growth alteration, a larger number of ethylene responsive genes needs to be tested in wild-type, as well as in *ein2-1* mutants.

Stress by excess Zn was found to be ABA-mediated for the Zn-specific effects on lateral root number and ethylene-mediated for the Zn-specific altered lateral root growth. The number of lateral roots in *abi4-1* mutants was significantly higher than in wild-type plants under Zn stress. This leads to the hypothesis that the Zn-specific 'switch off' effect is ABA-mediated. Zn-specific effects on lateral root elongation involve the ethylene signalling pathway, since the lateral lengths in *ein2-1* mutants were shorter under Zn treatment than in the wild-type. In the gene-expression experiment on the wild-type Zn was found to upregulate both ABA and ethylene responsive genes.

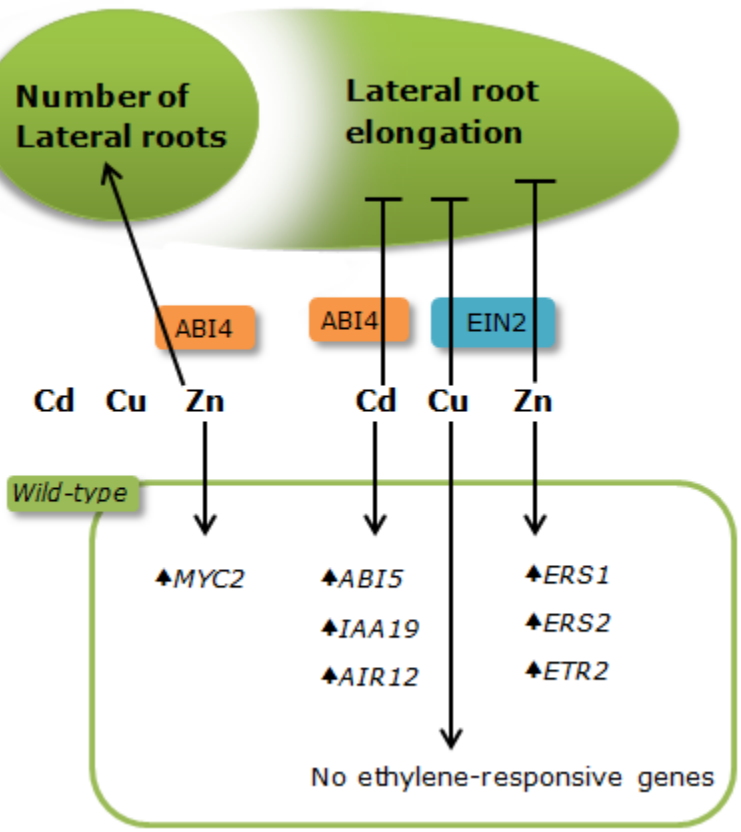

Figure 22: Schematic representation of results supporting the formed hypotheses

Future perspectives

As already mentioned in the discussion, the findings have to be confirmed by repetition. A number of improvements were made over the course of the experiments, facilitating future experiments. However the on-column DNase treatment in the gene expression experiment needs to be adjusted. In the used set-up, the gene-expression was measured on a homogenous root sample. However, *in vivo* the expression of different genes is spatiotemporal dependent. If the gene expression analysis can be further optimized for the already small samples, maybe it will become possible to measure the expression in distinct regions of the root to provide an interpretation that corresponds more to the expression in the root. A correlation between the root and shoot responses can also be made, as already mentioned in the discussion.

When the metal specific responses of the root are better understood, a next step is to investigate the responses in to a heterogeneous metal exposure. This experimental set-up resembles the effect in the field more, but will also be an interesting asset to compare the findings with the homogenous exposure. Ultimately, predictions of how the root system can be manipulated by phytohormones to grow optimally in contaminated soil can be tested. For testing in the field, for phytoremediation purposes the root system has to colonize the contaminated patches. However, this colonization has to be in balance with the plants tolerance and survival. In order to avoid using genetically modified organisms on field application, the results can be reproduced using bacteria producing phytohormones. The results can be extrapolated to a crop species like *Brassica napus,* because it is closely related to *A. thaliana*.

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Appendix

Appendix 1 - Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo scientific)

Product Information

Thermo Scientific Phusion Hot Start II High-Fidelity DNA Polymerase

F-549S/L, 100 U/500 U

- Use Phusion DNA Polymerase at 0.5–1.0 U per 50 µl reaction volume. Do not exceed 2 U/50 µl. (See 4.1)
- Use 15–30 s/kb for extension. Do not exceed 1 min/kb. (See 5.4)
- Use 98°C for denaturation. (See 5.1 & 5.2)
- The annealing rules are different from many common DNA polymerases (such as *Taq* DNA polymerases). Read Section 5.3 carefully.
- Use 200 µM of each dNTP. Do not use dUTP. (See 4.3)
- Note: Phusion DNA Polymerases produce blunt end DNA products.

IMPORTANT NOTES

Store at -20°C

Thermo SCIENTIFIC

1. Introduction

Thermo Scientific Phusion Hot Start II High-Fidelity DNA Polymerase offers superior performance for all PCR applications. A unique processivity-enhancing domain makes this *Pyrococcus*-like proofreading enzyme extremely processive, accurate and rapid. The error rate of Phusion® Hot Start II DNA Polymerase is equal to that of Phusion DNA Polymerase (4.4×10^{-7} in Phusion HF-buffer) when determined with a modified *lacI*-based method[1]. It is approximately 50-fold lower than that of *Thermus aquaticus* DNA polymerase and 6-fold lower than that of *Pyrococcus furiosus* DNA polymerase. Phusion Hot Start II High-Fidelity DNA Polymerase is capable of amplifying long amplicons such as the 7.5 kb genomic and 20 kb λ DNA used in Thermo Scientific's quality control assays.

Phusion Hot Start II DNA Polymerase combines the DNA polymerase and a reversibly bound, specific Affibody® protein[2,3], which inhibits the DNA polymerase activity at ambient temperatures, thus preventing the amplification of non-specific products. In addition, the Affibody ligand inhibits the 3'→5' exonuclease activity of the polymerase, preventing degradation of primers and template DNA during reaction setup. At polymerization temperatures, the Affibody molecule is released, rendering the polymerase fully active. Phusion Hot Start II DNA Polymerase does not require any separate activation step in the PCR protocol.

Phusion Hot Start II DNA Polymerase possesses the following activities: 5'→3' DNA polymerase activity and 3'→5' exonuclease activity. It generates blunt ends in the amplification products.

2. Package information

F-549S	100 U (2 U/µl) Material provided: Phusion Hot Start II DNA Polymerase 100 U (50 µl), 5x Phusion HF Buffer (2 x 1.5 ml), 5x Phusion GC Buffer (1.5 ml), DMSO (500 µl) and 50 mM $MgCl_2$ solution (1.5 ml).	
F-549L	500 U (2 U/µl) Material provided: Phusion Hot Start II DNA Polymerase 500 U (250 µl), 5x Phusion HF Buffer (6 x 1.5 ml), 5x Phusion GC Buffer (2 x 1.5 ml), DMSO (500 µl) and 50 mM $MgCl_2$ solution (2 x 1.5 ml).	**Reaction buffer:** 5x Phusion HF Buffer and 5x Phusion GC Buffer both contain 7.5 mM $MgCl_2$.

Material safety data sheet (MSDS) is available at www.thermoscientific.com/fermentas.

3. Guidelines for using Phusion Hot Start II DNA Polymerase

Carefully mix and centrifuge all tubes before opening to ensure homogeneity and improve recovery. When using Phusion Hot Start II DNA Polymerase, it is not necessary to perform the PCR setup on ice. Prepare a master mix for the appropriate number of samples to be amplified. The DNA polymerase should be pipetted carefully and gently as the high glycerol content (50 %) in the storage buffer may otherwise lead to pipetting errors.

Protocols optimized for Phusion® DNA Polymerase can be applied to Phusion Hot Start II DNA Polymerase reactions. Due to the high salt concentration in the reaction buffer, Phusion Hot Start II DNA Polymerase tends to work better at elevated denaturation and annealing temperatures. Please pay special attention to the conditions listed below when running your reactions. Following the guidelines will ensure optimal enzyme performance.

Table 1. Pipetting instructions (add items in this order).

Component	50 µl react.	20 µl react.	Final conc.
H_2O	add to 50 µl	add to 20 µl	
5x Phusion HF Buffer*	10 µl	4 µl	1x
10 mM dNTPs	1 µl	0.4 µl	200 µM each
primer A**	x µl	x µl	0.5 µM
primer B**	x µl	x µl	0.5 µM
template DNA	x µl	x µl	
(DMSO***, optional)	(1.5 µl)	(0.6 µl)	(3 %)
Phusion Hot Start II DNA Polymerase (2 U/µl)	0.5 µl	0.2 µl	0.02 U/µl

* Optionally 5x Phusion GC Buffer can be used. See section 4.2. for details.
** The recommendation for final primer concentration is 0.5 µM, but it can be varied in a range of 0.2–1.0 µM if needed.
*** Addition of DMSO is recommended for GC-rich amplicons. DMSO is not recommended for amplicons with very low GC % or amplicons that are >20 kb.

Table 2. Cycling instructions.

Cycle step	2-step protocol		3-step protocol		Cycles
	Temp.	Time	Temp.	Time	
Initial denaturation	98°C	30 s	98°C	30 s	1
Denaturation	98°C	5–10 s	98°C	5–10 s	25–35
Annealing (see 5.3)	–	–	X°C	10–30 s	
Extension (see 5.4)	72°C	15–30 s/kb	72°C	15–30 s/kb	
Final extension	72°C	5–10 min	72°C	5–10 min	1
	4°C	hold	4°C	hold	

4. Notes about reaction components

4.1 Enzyme

The optimal amount of enzyme depends on the amount of template and the length of the PCR product. Usually 1 unit of Phusion Hot Start II DNA Polymerase per 50 µl reaction volume gives good results, but the optimal amount can range from 0.5 to 2 units per 50 µl reaction depending on the amplicon length and difficulty. **Do not exceed 2 U/50 µl (0.04 U/µl), especially for amplicons that are > 5 kb.**

When cloning fragments amplified with Phusion Hot Start II DNA Polymerase, blunt end cloning is recommended. If TA cloning is required, it can be performed by adding A overhangs to the blunt PCR product with Thermo Scientific *Taq* DNA Polymerase, for example. However, before adding the overhangs it is very important to remove all Phusion Hot Start II DNA Polymerase by purifying the PCR product carefully. Any remaining Phusion Hot Start II DNA Polymerase will degrade the A overhangs, creating blunt ends again. A detailed protocol for TA cloning of PCR fragments amplified with any of the Phusion DNA Polymerases can be found on website: www.thermoscientific.com/pcrcloning.

4.2 Buffers

Two buffers are provided with the enzyme: 5x Phusion HF Buffer (F-518) and 5x Phusion GC Buffer (F-519). The error rate of Phusion Hot Start II DNA Polymerase in HF Buffer (4.4×10^{-7}) is lower than that in GC Buffer (9.5×10^{-7}). Therefore, HF Buffer should be used as the default buffer for high-fidelity amplification. However, GC Buffer can improve the performance of Phusion Hot Start II DNA Polymerase on some difficult or long templates, such as GC-rich templates or those with complex secondary structures. For applications such as microarray or DHPLC, where the DNA templates need to be free of detergents, detergent-free reaction buffers (F-520, F-521) are available for Phusion DNA Polymerases.

4.3 Mg^{2+} and dNTP

The concentration of Mg^{2+} is critical since Phusion Hot Start II DNA Polymerase is a magnesium-dependent enzyme. Excessive Mg^{2+} stabilizes the DNA double strand and prevents complete denaturation of DNA. Excess Mg^{2+} can also stabilize spurious annealing of primers to incorrect template sites and decrease specificity. Conversely, inadequate Mg^{2+} may lead to lower product yield. The optimal Mg^{2+} concentration also depends on the dNTP concentration, the specific template DNA and the sample buffer composition. In general, the optimal Mg^{2+} concentration is 0.5 to 1 mM over the total dNTP concentration for standard PCR. If the primers and/or template contain chelators such as EDTA or EGTA, the apparent Mg^{2+} optimum may be shifted to higher concentrations. If further optimization is needed, increase Mg^{2+} concentration in 0.2 mM steps.

High quality dNTPs should be used for optimal performance with Phusion Hot Start II DNA Polymerase. The polymerase cannot read dUTP-derivatives or dITP in the template strand so the use of these analogues or primers containing them is not recommended. Due to the high processivity of Phusion Hot Start II DNA Polymerase there is no advantage of increasing dNTP concentrations. For optimal results always use 200 µM of each dNTP.

4.4 Template

General guidelines for low complexity DNA (e.g. plasmid, lambda or BAC DNA) are 1 pg–10 ng per 50 µl reaction volume. For high complexity genomic DNA, the amount of DNA template should be 50–250 ng per 50 µl reaction volume. If cDNA synthesis reaction mixture is used as a source of template, the volume of the template should not exceed 10 % of the final PCR reaction volume.

4.5 PCR additives

The recommended reaction conditions for GC-rich templates include 3 % DMSO as a PCR additive, which aids in the denaturing

Appendix 2 - Nucleospin RNA XS kit (Machery-Nagel)

5.2 RNA purification from tissue

Before starting the preparation:

- Check if TCEP, Carrier RNA, rDNase, and Wash Buffer RA3 were prepared according to section 3.

1 Supply sample

Provide tissue sample such as a biopsy in a microcentrifuge tube (not provided).

For appropriate sample amounts see section 2.2.

2 Lyse and homogenize tissue

Add **200 µL Buffer RA1** and **4 µL TCEP** to the tissue sample and vortex vigorously (2 x 5 s).

+ 200 µL RA1
+ 4 µL TCEP

Disruption with a rotor-stator homogenizer or with a shaker and steel balls are recommended methods for the homogenization of tissue samples. For further comments on homogenization methods see section 2.3.

If multiple samples are processed, the preparation of a master-premix is recommended (e.g., 2.2 mL Buffer RA1 and 44 µL TCEP for 10 preparations). Use 204 µL of the premix.

3 Add Carrier RNA

Add **5 µL Carrier RNA working solution** (20 ng) to the lysate. Mix by vortexing (2 x 5 s). Spin down briefly (approx. 1 s 1000 x *g*) to clear the lid.

+ 5 µL Carrier RNA

Mix

For preparation of Carrier RNA working solution see section 3.

4 Filtrate lysate

Reduce viscosity and clear the lysate by filtration through **NucleoSpin® Filter** (violet ring): Place the NucleoSpin® Filter (violet ring) in a Collection Tube (2 mL; provided), apply the mixture, and centrifuge for **30 s** at **11,000 x *g***.

11,000 x *g*
30 s

In case of visible pellet formation (depending on sample amount and nature), transfer supernatant without any formed pellet to a new 1.5 mL microcentrifuge tube (not included).

5 Adjust RNA binding condition

Discard the NucleoSpin® Filter (violet ring), add **200 µL ethanol (70 %)** to the homogenized lysate and mix by pipetting up and down (5 times).

Alternatively, transfer flow-through into a new 1.5 mL microcentrifuge tube (not provided), add **200 µL ethanol** (70 %), and mix by vortexing (2 x 5 s). Spin down briefly (approx. 1 s 1000 x *g*) to clear the lid. Pipette lysate up and down two times before loading the lysate.

+ 200 µL 70 % EtOH

Mix

After addition of ethanol a stringy precipitate may become visible which will not affect the RNA isolation. Be sure to disaggregate any precipitate by mixing and load all of the precipitate on the column as described in step 6. Do not centrifuge the ethanolic lysate before loading it onto the column in order to avoid pelleting the precipitate.

6 Bind RNA

For each preparation, take one **NucleoSpin® RNA XS Column** (light blue ring) placed in a Collection Tube and load the lysate to the column. Centrifuge for **30 s** at **11,000 x *g***. Place the column in a new Collection Tube (2 mL).

Load lysate

11,000 x *g*
30 s

The maximum loading capacity of NucleoSpin® RNA XS Columns is 600 µL. Repeat the procedure if larger volumes are to be processed.

7 Desalt silica membrane

Add **100 µL MDB** (Membrane Desalting Buffer) and centrifuge at **11,000 x *g*** for **30 s** to dry the membrane. It is not necessary to use a fresh Collection Tube after this centrifugation step.

+ 100 µL MDB

Salt removal will make the following rDNase digest much more effective. If the column outlet has come into contact with the flow-through for any reason, discard the flow-through and centrifuge again for 30 s at 11,000 x g.

11,000 x *g*
30 s

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8 Digest DNA

Prepare **rDNase reaction mixture** in a sterile microcentrifuge tube (not provided): for each isolation, add **3 µL** reconstituted rDNase (also see section 3) to **27 µL Reaction Buffer for rDNase**. Mix by flicking the tube.

+ 25 µL rDNase reaction mixture

Apply **25 µL rDNase reaction mixture** directly onto the center of the silica membrane of the column. Close the lid. Incubate at **room temperature** for **15 min**.

RT
15 min

It is not necessary to use a new Collection Tube after the incubation step.

---

9 Wash and dry silica membrane

+ 100 µL RA2

1st wash

Add **100 µL Buffer RA2** to the NucleoSpin® RNA XS Column. Incubate for **2 min** at **RT**. Centrifuge for **30 s** at **11,000 x *g***.

RT
2 min

11,000 x *g*
30 s

Place the column into a new Collection Tube (2 mL).

Buffer RA2 will inactivate the rDNase.

+ 400 µL RA3

2nd wash

Add **400 µL Buffer RA3** to the NucleoSpin® RNA XS Column. Centrifuge for **30 s** at **11,000 x *g***. Discard flow-through and place the column back into the Collection Tube.

11,000 x *g*
30 s

3rd wash

Add **200 µL Buffer RA3** to the NucleoSpin® RNA XS Column. Centrifuge for **2 min** at **11,000 x *g*** to dry the membrane. Place the column into a nuclease-free Collection Tube (1.5 mL; supplied).

+ 200 µL RA3

If for any reason the liquid level in the Collection Tube has reached the NucleoSpin® RNA XS Column after centrifugation, discard flow-through and centrifuge again.

11,000 x *g*
2 min

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10 Elute highly pure RNA

Elute the RNA in **10 µL H_2O** (RNase-free; supplied) and centrifuge at **11,000 x *g*** for **30 s**.

+ 10 µL RNase-free H_2O

If higher RNA concentrations or higher elution volumes are desired, elution volume may be varied in the range of 5–30 µL.

11,000 x *g*
30 s

For further details on alternative elution procedures see section 2.4.

Appendix 3 - Turbo DNA-Free kit (Life Technologies Europe)

- Label 0.2 mL tubes in an Isofreeze with the corresponding sample numbers.
    - Calculate the volume of RNA sample needed for 1 µg RNA input.
      [Optional: when RNA concentrations greatly differ between individual samples, you can dilute all your samples to the same concentration (e.g. 250 ng/µl)]
- Subtract the volume of RNA needed for 1 µg from 11 µL; this volume corresponds to the volume of RNase-free water to be added.
- For each sample, add the correct volume of RNase-free water to the 0.2 mL tubes.
- For each sample, add the correct volume of RNA to the water.
- Store samples in the Isofreeze at 4°C.
- Prepare Turbo DNA-free mastermix on ice, mix (flick) and short spin.

	per sample (prepare 10% extra)
10X TURBO DNase Buffer (1/10th volume)	1.1 µL
TURBO DNase	0.25 µL
	1.35 µL

- Add 1.35 µL mastermix per RNA sample and vortex 2 seconds.
- Incubate samples during 25 minutes at 37°C.
- Vortex DNase Inactivation Reagent to resuspend.
- Add 2 µL DNase Inactivation Reagent per sample (1/10th volume but a minimum of 2 µL needs to be added) and vortex 10 seconds.
- Incubate samples during 2 minutes at room temperature and vortex them twice during this period to ensure the functioning of the Inactivation Reagent.
- Spin samples for 1.5 minute at 10 000 g and store them in the Isofreeze.
- Transfer 5 µL of supernatant containing RNA to a fresh 0.2 mL tube.
- Store samples in the Isofreeze at 4°C.

Appendix 4 - Primescript RT reagent kit TaKaRa (10µl reaction) (Clontech)

- Thaw kit products on ice (stored at -20°C).
- Mix (flick) and short spin the products once thawed.
- Prepare RT mastermix on ice

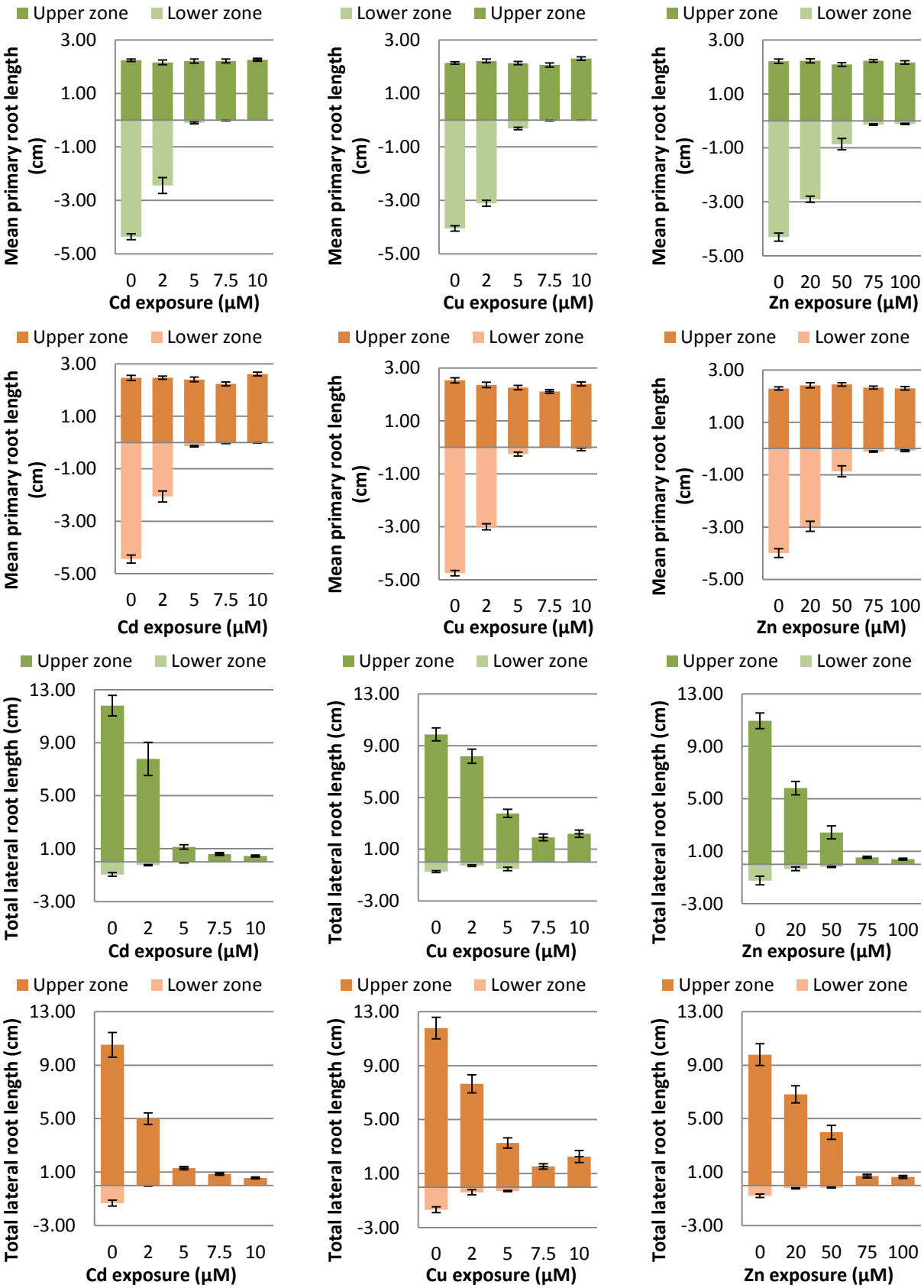
		per sample (prepare 10% extra)
5X PrimeScript Buffer (for Realtime)		2.0 µL
PrimeScript RT Enzyme Mix I		0.5 µL
Oligo dT Primer (50 µM)	0.5 µL	
Random 6 mers (100 µM)	0.5 µL	
Nuclease-free water		1.5 µL
Total volume per reaction		**5 µL**

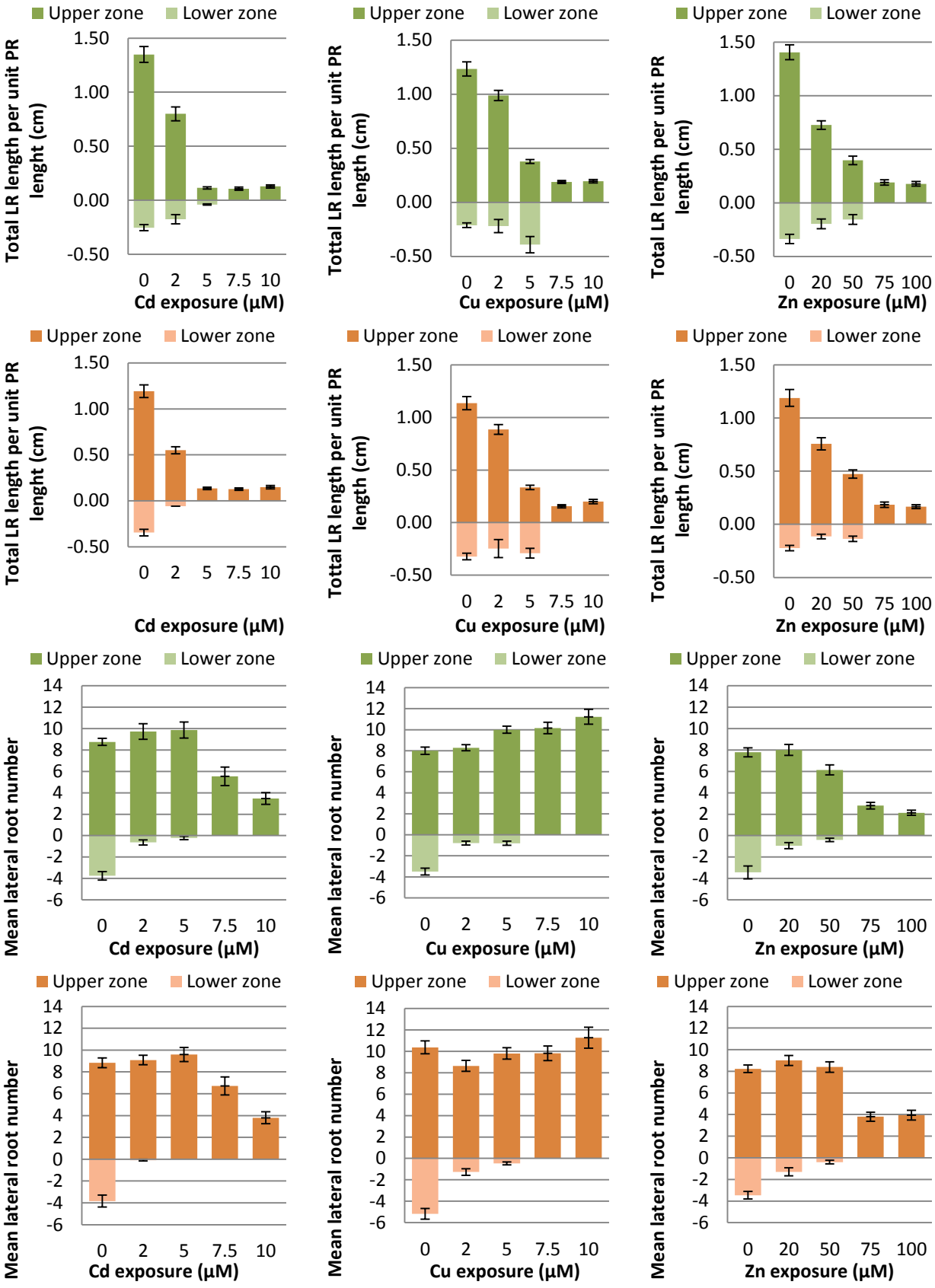
- Mix (flick) and short spin mastermix and keep on ice.
- Add 5 µL mastermix to each RNA sample and pipet up and down twice to mix
- Mix (flick) and short spin samples and start the PCR run

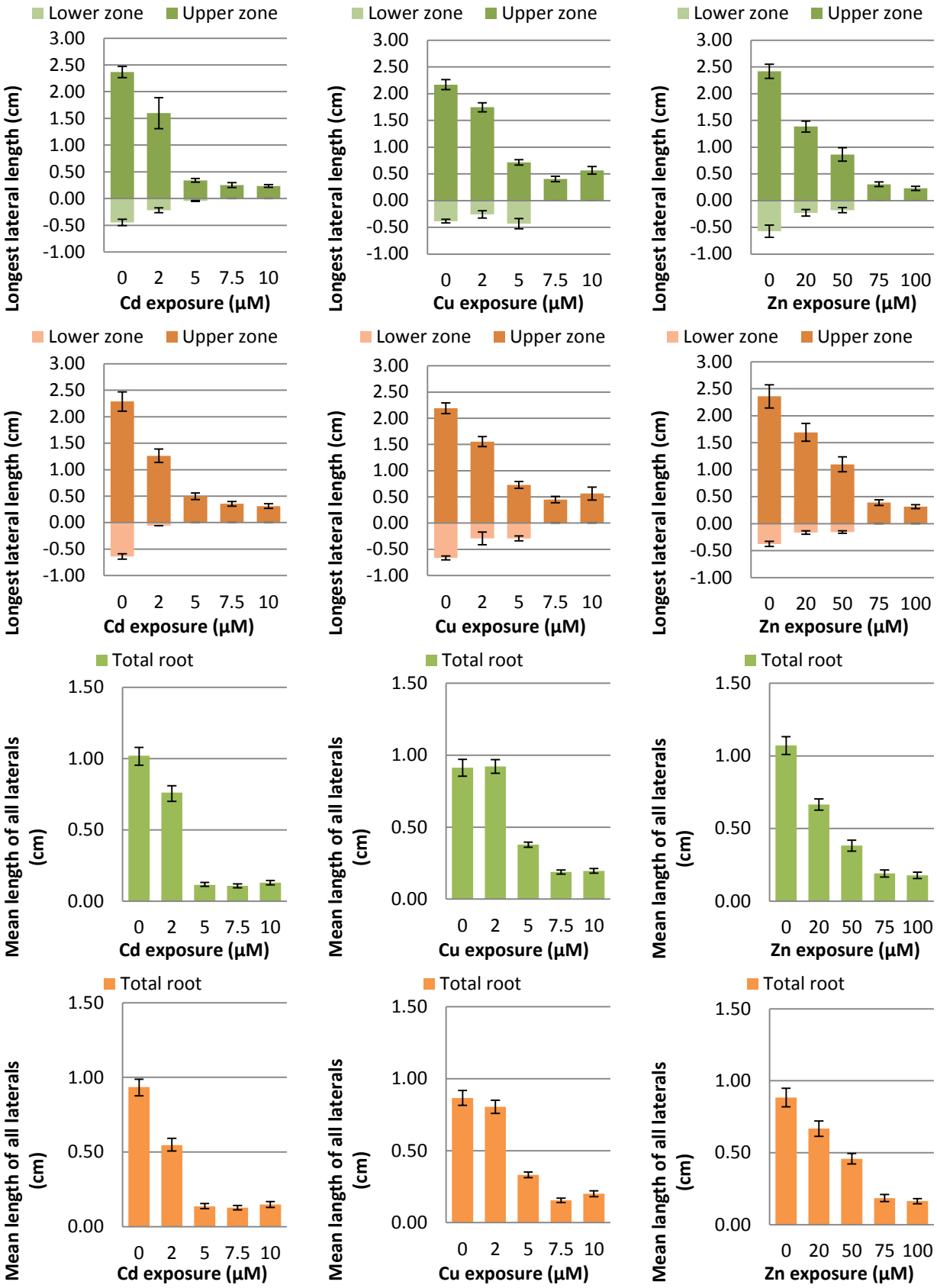
	Step 1	**Step 2**	**Step 3**
Temperature	37°C	85°C	4°C
Time	15 min	5 sec	∞

- Short spin cDNA samples after the PCR run
- Dilute 5 µL 10x in a 0.5 mL tube:
  5 µL cDNA + 45 µL 1/10 TE buffer.
- Store diluted samples to use in real-time PCR and undiluted samples for potential primer testing both at -20°C

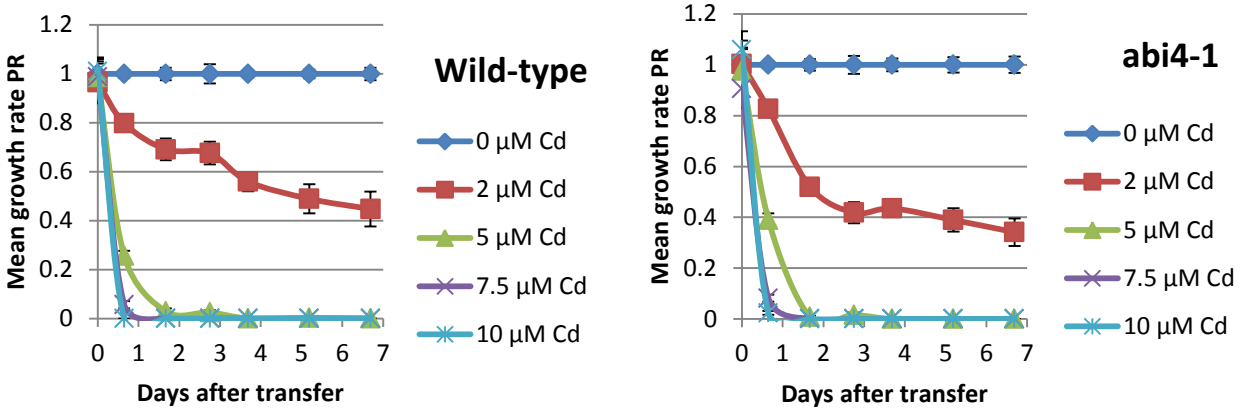
Appendix 5 – Results of the VAPs experiment with *abi4-1* (orange) and wild-type (green)

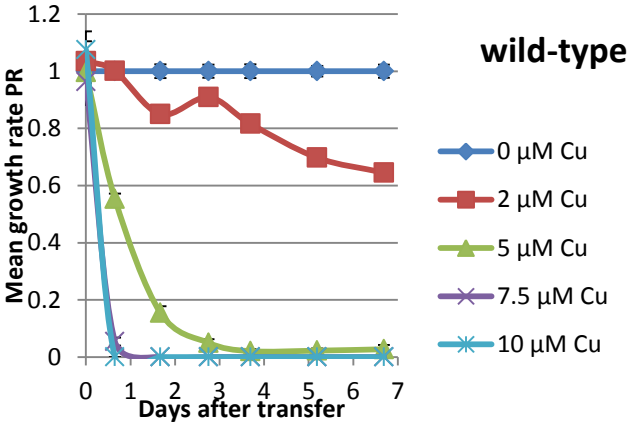


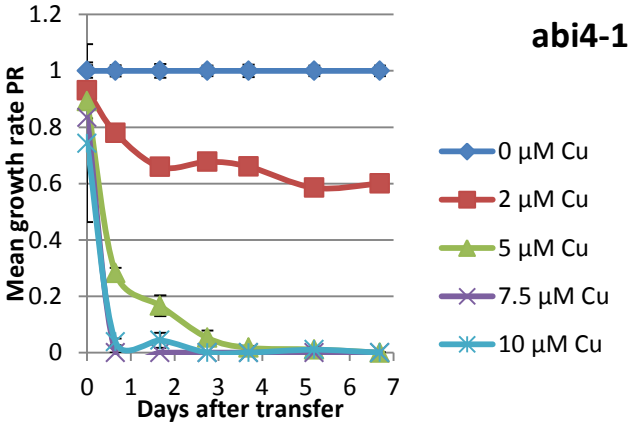

Upper zone
Lower zone
Total LR length per unit PR lenght (cm)
Tottal LR length per unit PR length (cm)
Total LR length per unit PR length (cm)
1.50
1.00
0.50
0.00
-0.50
0 2 5 7.5 10
0 20 50 75 100
Cd exposure (µM)
Cu exposure (µM)
Zn exposure (µM)
Mean lateral root number
14
12
10
8
6
4
2
0
-2
-4
-6

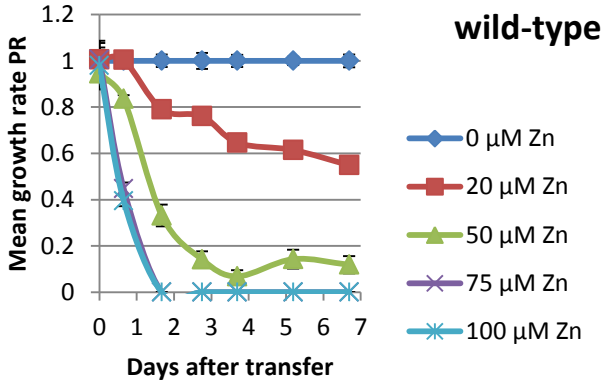

Lower zone
Upper zone
Longest lateral length (cm)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
0 2 5 7.5 10
Cd exposure (µM)
Cu exposure (µM)
0 20 50 75 100
Zn exposure (µM)
Total root
Mean length of all laterals (cm)
1.50
1.00
0.50
0.00

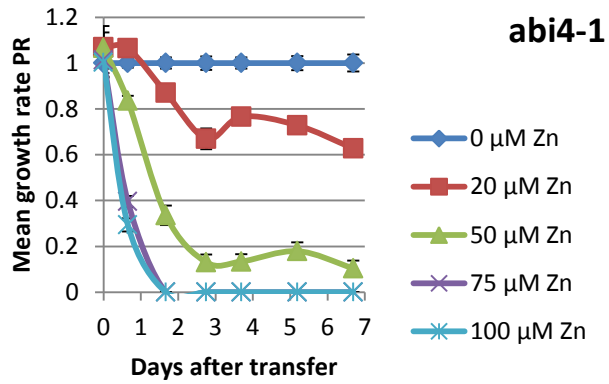
Results of the kinetic analyses : PR = Primary root

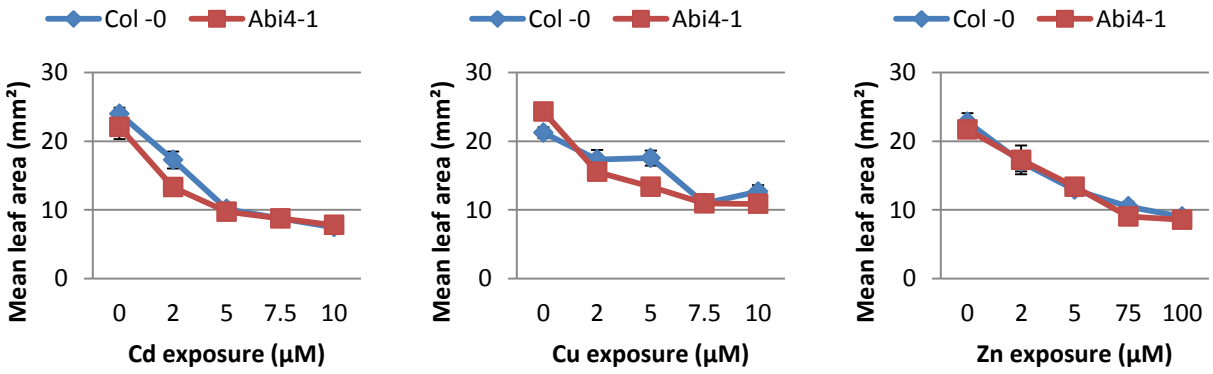




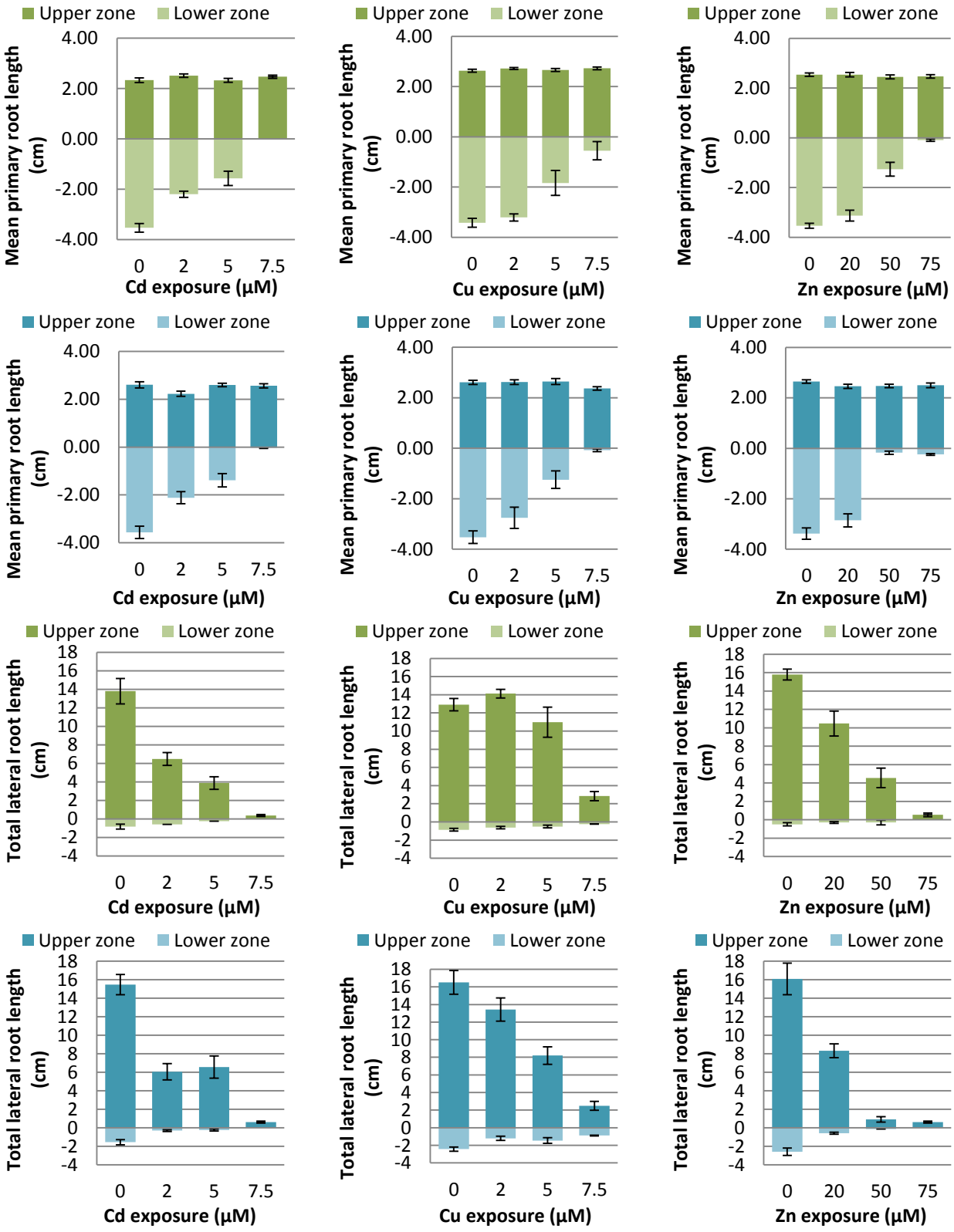


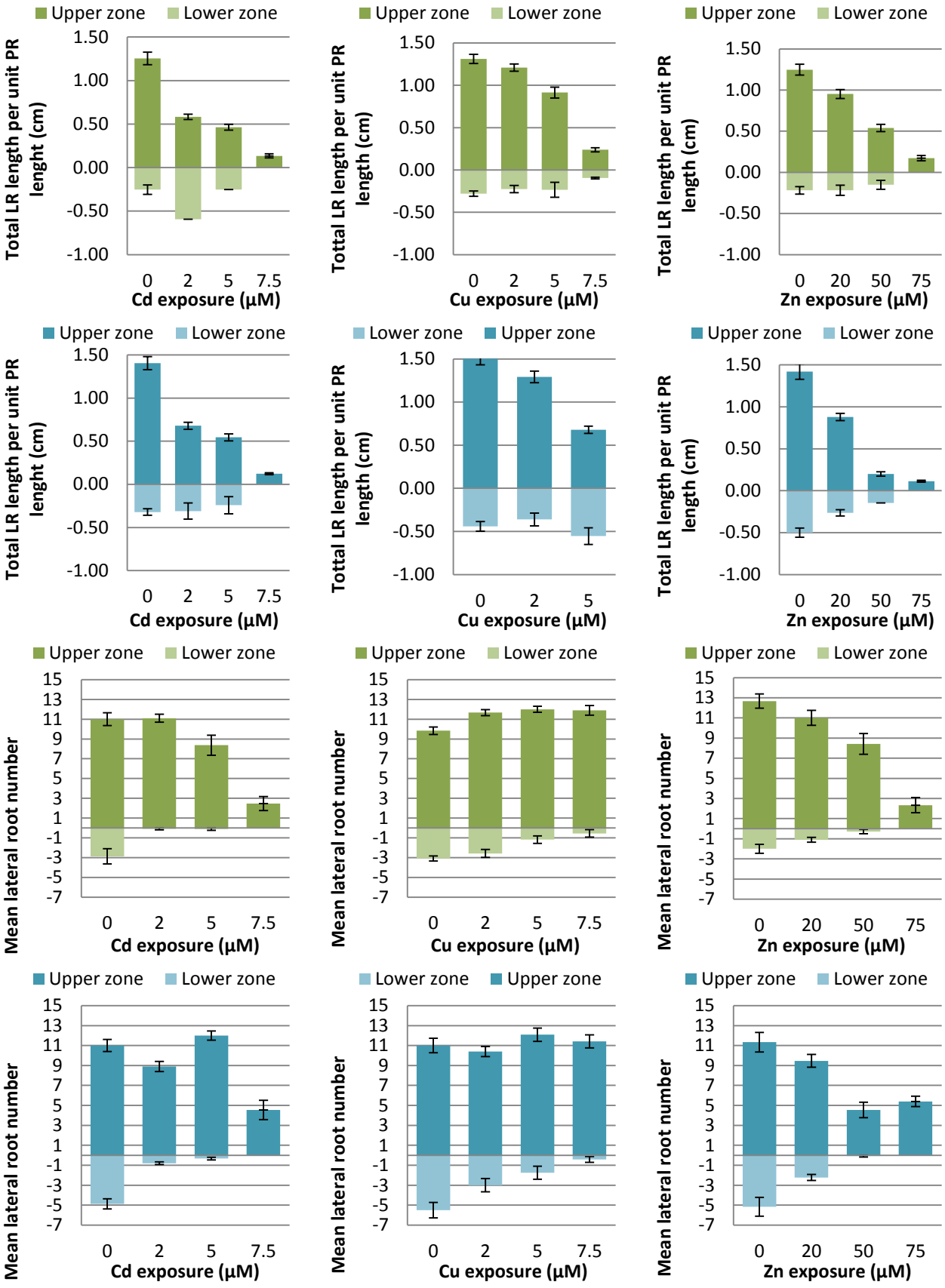




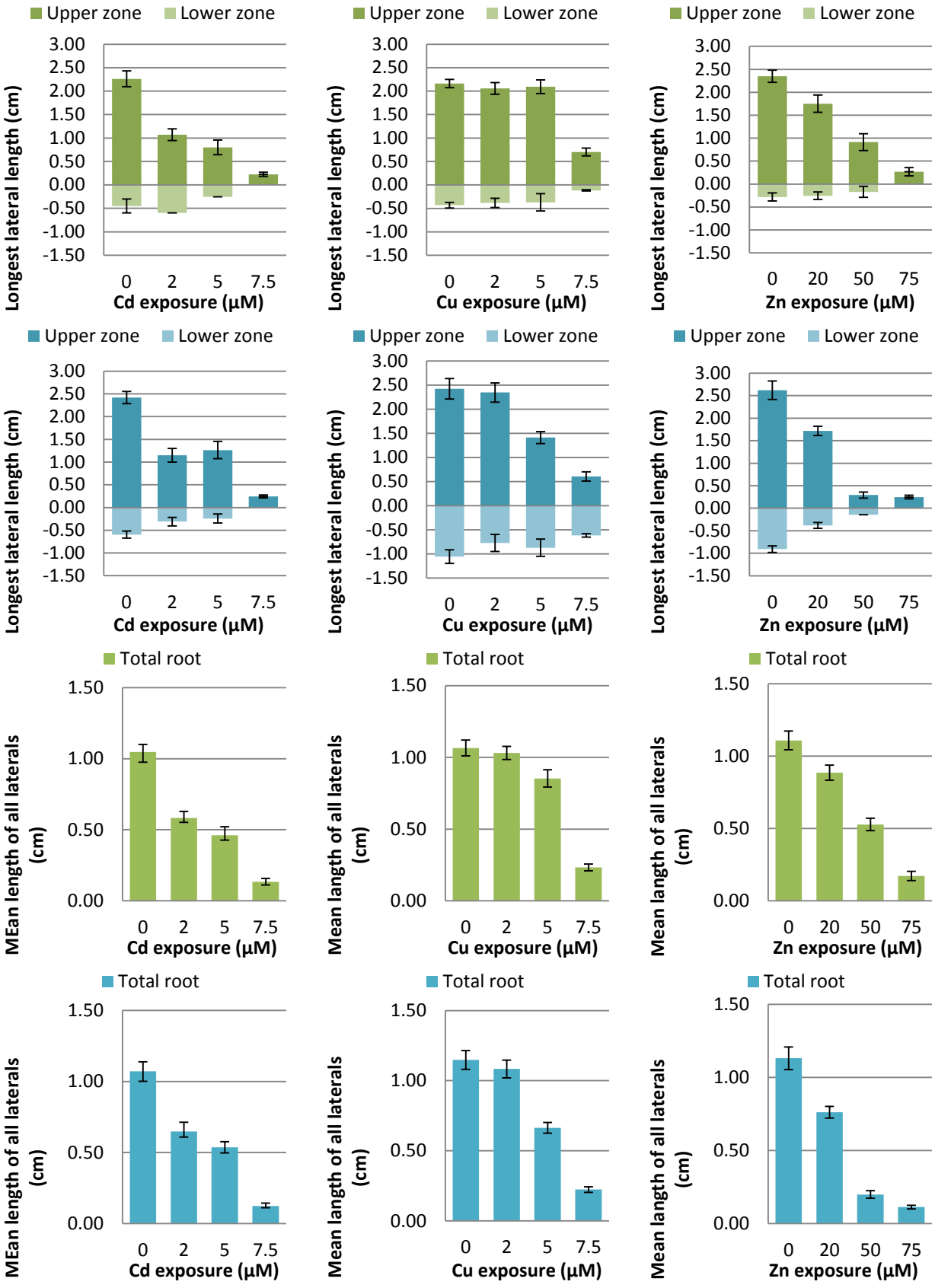


Appendix 6 – Results of the VAPs experiment with *ein2-1* (blue) and wild-type (green)

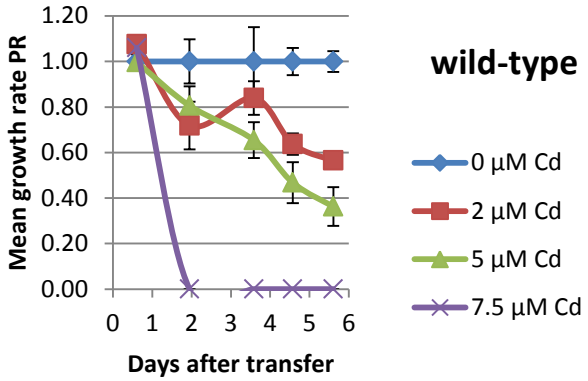


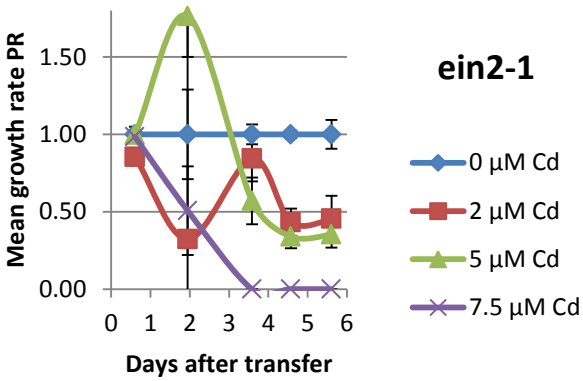

Upper zone
Lower zone
Total LR length per unit PR lenght (cm)
Cd exposure (µM)
Total LR length per unit PR length (cm)
Cu exposure (µM)
Zn exposure (µM)
Mean lateral root number
0
2
5
7.5
20
50
75

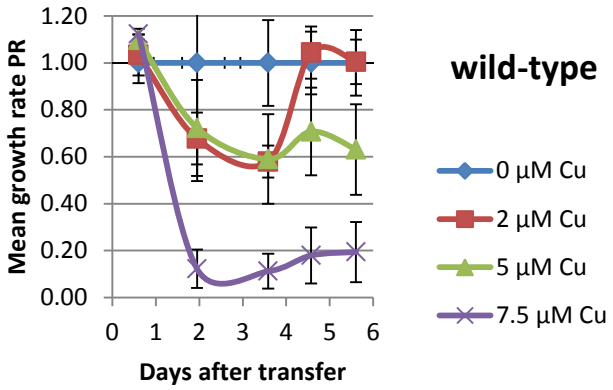
x

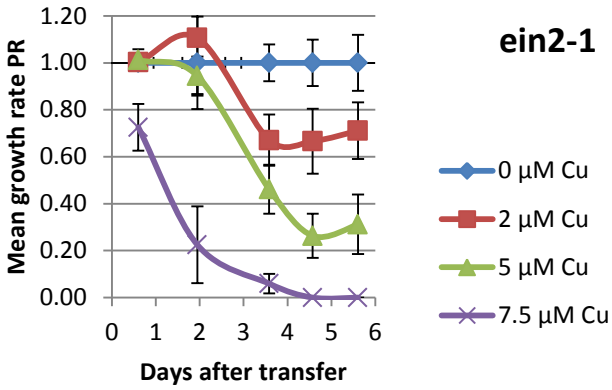

Upper zone
Lower zone
Longest lateral length (cm)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
0
2
5
7.5
Cd exposure (µM)
Upper zone
Lower zone
Longest lateral length (cm)
0
2
5
7.5
Cu exposure (µM)
Upper zone
Lower zone
Longest lateral length (cm)
0
20
50
75
Zn exposure (µM)
Upper zone
Lower zone
Longest lateral length (cm)
0
2
5
7.5
Cd exposure (µM)
Upper zone
Lower zone
Longest lateral length (cm)
0
2
5
7.5
Cu exposure (µM)
Upper zone
Lower zone
Longest lateral length (cm)
0
20
50
75
Zn exposure (µM)
Total root
MEan length of all laterals (cm)
1.50
1.00
0.50
0.00
0
2
5
7.5
Cd exposure (µM)
Total root
Mean langth of all laterals (cm)
0
2
5
7.5
Cu exposure (µM)
Total root
Mean langth of all laterals (cm)
0
20
50
75
Zn exposure (µM)
Total root
MEan length of all laterals (cm)
0
2
5
7.5
Cd exposure (µM)
Total root
Mean langth of all laterals (cm)
0
2
5
7.5
Cu exposure (µM)
Total root
Mean langth of all laterals (cm)
0
20
50
75
Zn exposure (µM)

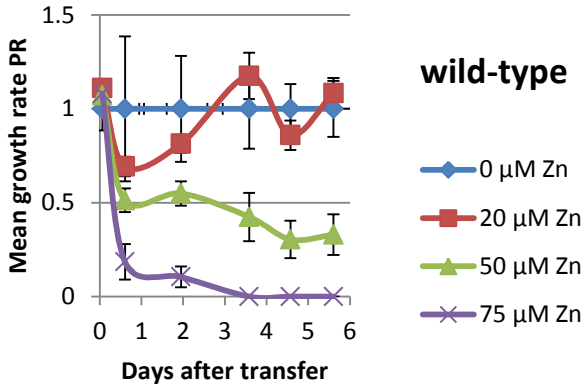
Results of the kinetic analyses : PR = Primary root

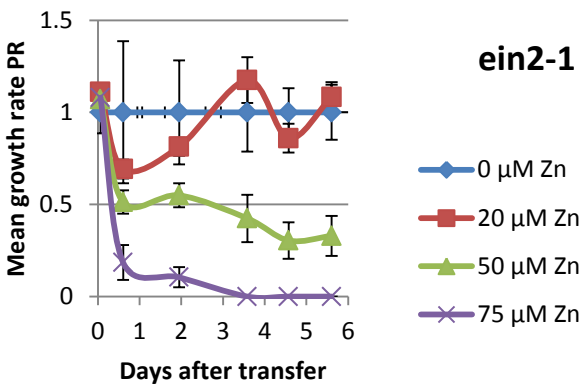


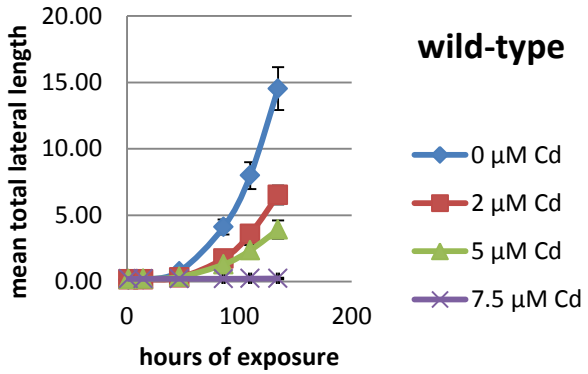


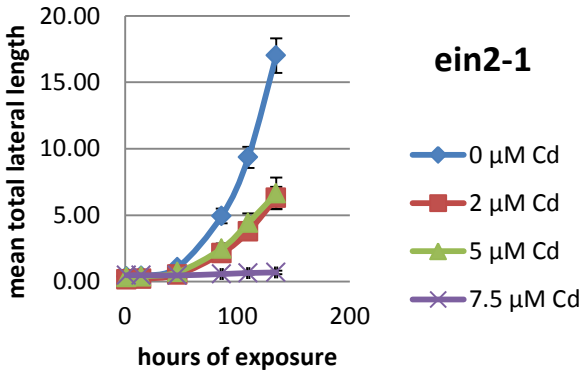


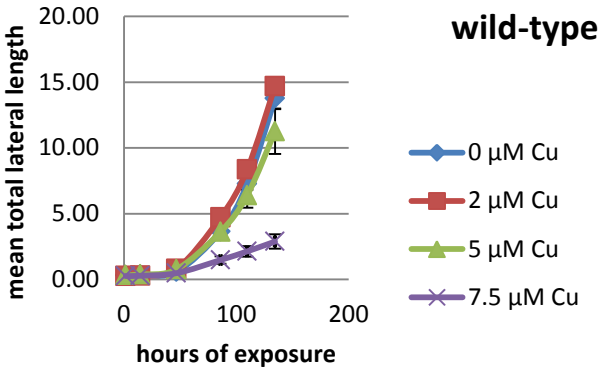


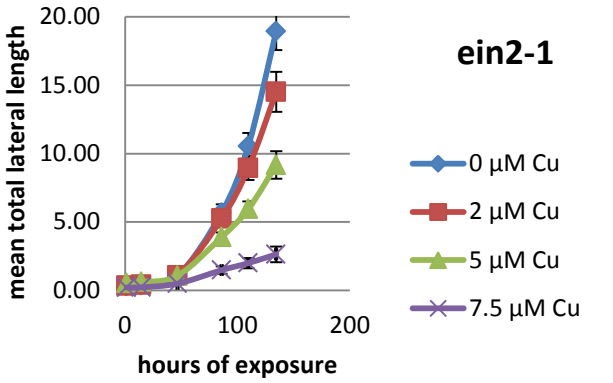


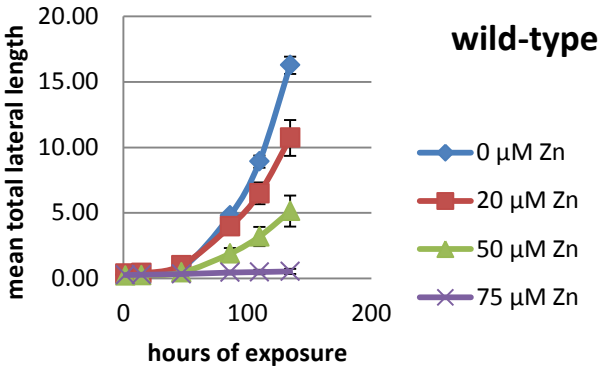


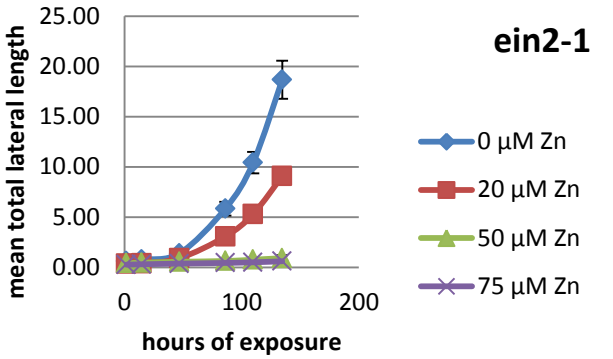


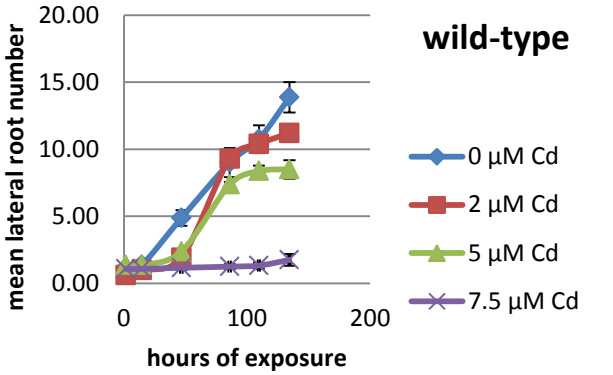


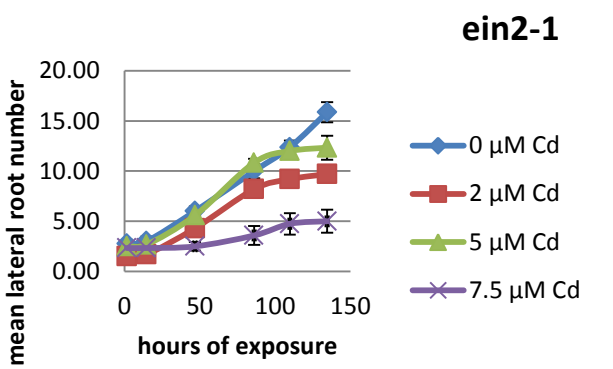

wild-type
mean total lateral length
20.00
15.00
10.00
5.00
0.00
0
100
200
hours of exposure
0 µM Cu
2 µM Cu
5 µM Cu
7.5 µM Cu

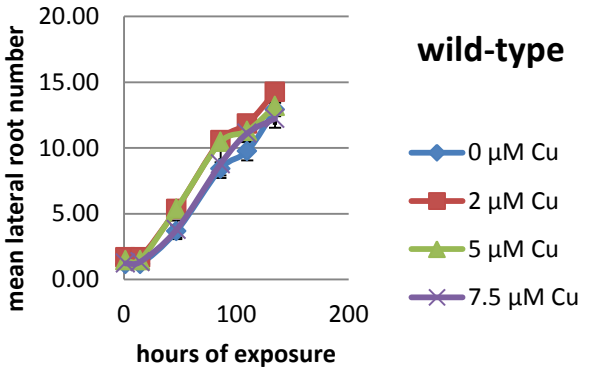

ein2-1
mean total lateral length
20.00
15.00
10.00
5.00
0.00
0
100
200
hours of exposure
0 µM Cu
2 µM Cu
5 µM Cu
7.5 µM Cu

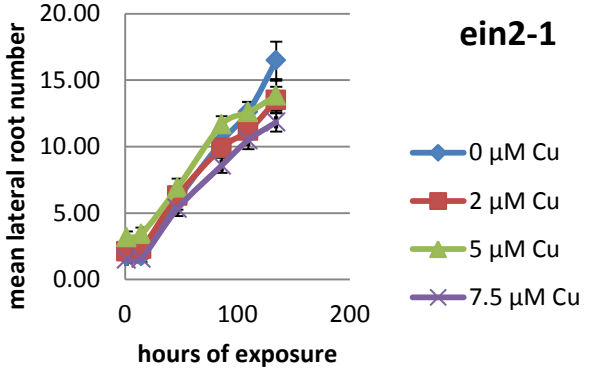

wild-type
mean total lateral length
20.00
15.00
10.00
5.00
0.00
0
100
200
hours of exposure
0 µM Zn
20 µM Zn
50 µM Zn
75 µM Zn

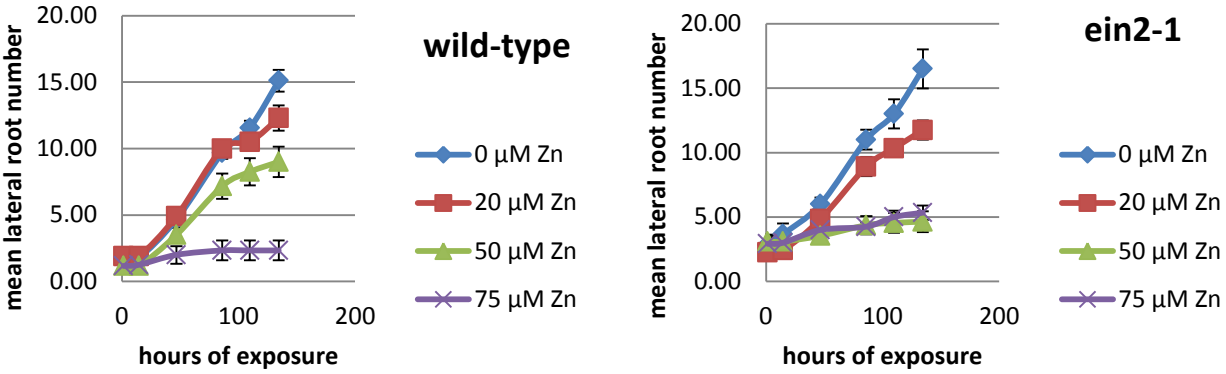

ein2-1
mean total lateral length
25.00
20.00
15.00
10.00
5.00
0.00
0
100
200
hours of exposure
0 µM Zn
20 µM Zn
50 µM Zn
75 µM Zn

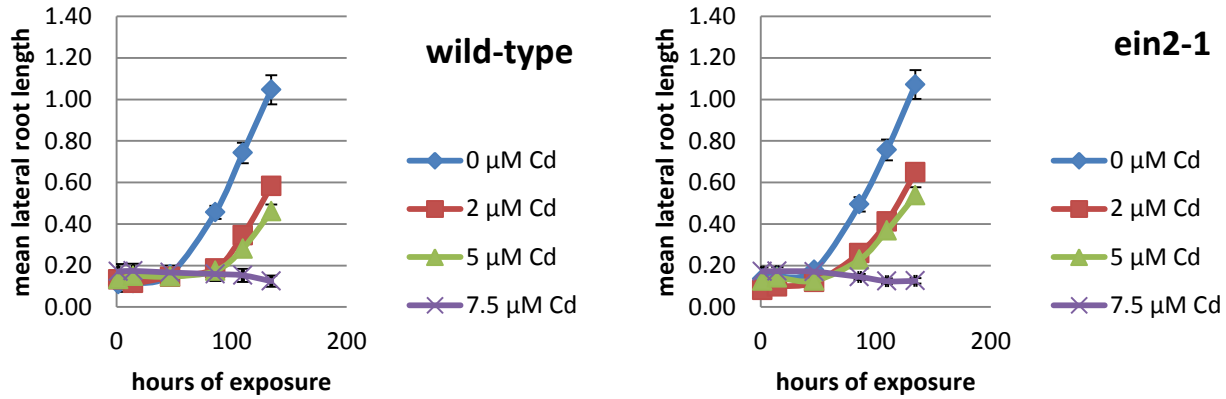

wild-type
mean lateral root number
20.00
15.00
10.00
5.00
0.00
0
100
200
hours of exposure
0 µM Cd
2 µM Cd
5 µM Cd
7.5 µM Cd

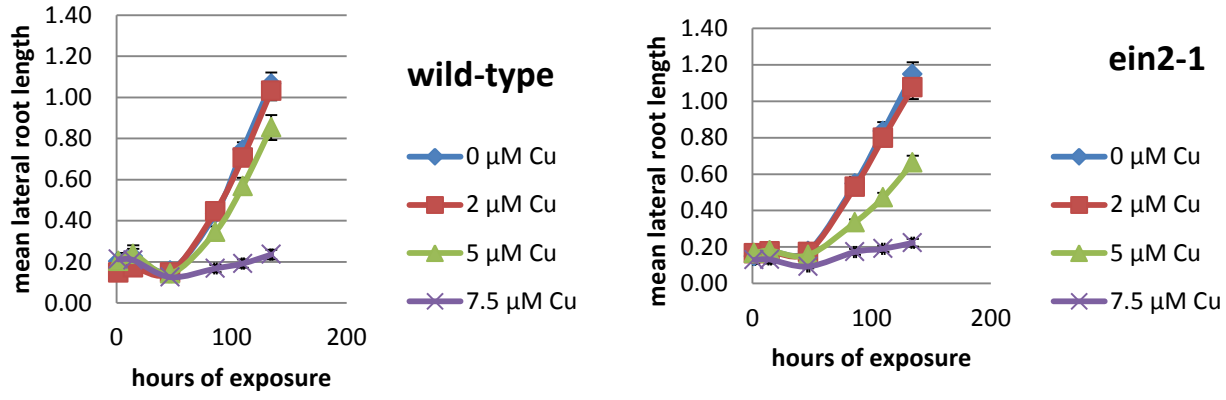

ein2-1
mean lateral root number
20.00
15.00
10.00
5.00
0.00
0
50
100
150
hours of exposure
0 µM Cd
2 µM Cd
5 µM Cd
7.5 µM Cd

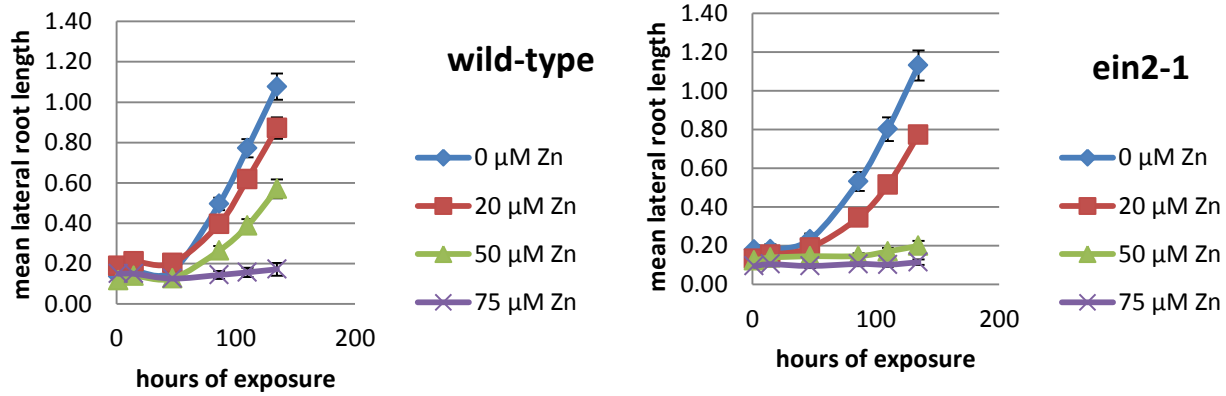

wild-type
mean lateral root number
20.00
15.00
10.00
5.00
0.00
0
100
200
hours of exposure
0 µM Cu
2 µM Cu
5 µM Cu
7.5 µM Cu


ein2-1
mean lateral root number
20.00
15.00
10.00
5.00
0.00
0
100
200
hours of exposure
0 µM Cu
2 µM Cu
5 µM Cu
7.5 µM Cu


wild-type
mean lateral root number
20.00
15.00
10.00
5.00
0.00
0
100
200
hours of exposure
0 µM Zn
20 µM Zn
50 µM Zn
75 µM Zn
ein2-1
mean lateral root number
20.00
15.00
10.00
5.00
0.00
0
100
200
hours of exposure
0 µM Zn
20 µM Zn
50 µM Zn
75 µM Zn


wild-type
mean lateral root length
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
100
200
hours of exposure
0 µM Cd
2 µM Cd
5 µM Cd
7.5 µM Cd
ein2-1
mean lateral root length
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
100
200
hours of exposure
0 µM Cd
2 µM Cd
5 µM Cd
7.5 µM Cd


wild-type
mean lateral root length
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
100
200
hours of exposure
0 µM Cu
2 µM Cu
5 µM Cu
7.5 µM Cu
ein2-1
mean lateral root length
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
100
200
hours of exposure
0 µM Cu
2 µM Cu
5 µM Cu
7.5 µM Cu


wild-type
mean lateral root length
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
100
200
hours of exposure
0 µM Zn
20 µM Zn
50 µM Zn
75 µM Zn
ein2-1
mean lateral root length
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
100
200
hours of exposure
0 µM Zn
20 µM Zn
50 µM Zn
75 µM Zn