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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen: milieu en gezondheid

Masterproef

A forward genetics screen towards identifying mechanisms and genes involved in lateral root inhibition by excess zinc

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

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ACS	Aminocyclopropane-1-carboxylate synthase
BS	Brassinosteroids
CK	Cytokinin
CWR	Cell wall remodelling
EMS	Ethyl methanesulphonate
ET	Ethylene
FC	Founder cell
GA	Gibberellic acid
GECN	Genetically effective cell number
IAA	Indole-3-acetic acid
JA	Jasmonic acid
LR	Lateral root
LRI	Lateral root initiation
LRP	Lateral root primordia
LZ	Lower zone
PA	Polyamines
RH	Root hair
ROS	Reactive oxygen species
RSA	Root system architecture
SHY	Short hypocotyl
SIMR	Stress induced morphogenic response
SL	Strigolactones
TRP	Tryptophan
UZ	Upper zone

ABSTRACT (ENGLISH)

Background The detrimental effect of abiotic environmental stresses on plant growth, survival and reproduction reduces available arable land alongside the ongoing competition between feed, food and bio-fuels. The root system architecture (RSA) is known to adapt to its environment aiding survival by exploiting nutrient rich soil patches and avoiding contaminated patches. Further understanding of this redirected root growth could hand a solution by minimizing or maximizing contaminant uptake by manipulating exposure. One distinct example of contaminant avoidance is displayed by excess zinc, inhibiting primary root growth as well as lateral root initiation.

Objectives The aim of this study is to initiate the research towards the mechanisms underlying the inhibitory effect caused by excess zinc and to establish whether it relies on excessive toxicity and cell death or whether gene products are involved. A genetic screen for causal genes will be established.

Methodology Excess zinc causes a distinct phenotype, such that it can be used in a forward genetics screen to uncover the underlying genes and gene products. Subjecting a population of *Arabidopsis thaliana* seeds to the mutagen EMS, the mutations involved in the zinc inhibition effects are screened for. To uncover if genes and their products cause the effect, an auxin complementation experiment is conducted, applying exogenic auxin in an attempt to overwrite the inhibition effect of the excess zinc.

Results The auxin complementation experiment indicated that the inhibitory effect exerted by excess zinc can be bypassed by phytohormones, hinting towards the cause lying with genes and their gene products rather than with excessive toxicity and cell death. The *diz* mutants displaying desired deviating phenotypes in the forward genetics screen and their confirmation in the next generation of these mutants further assisted the idea of the involvement of genes in the this inhibitory Zn effect.

Conclusion / outlook The ability to overwrite the inhibition effect exerted by excess zinc confirms that genes and gene products are involved, also the successful production and confirmation of *diz* mutants by means of EMS mutagenesis confirms this statement. Confirmation of the *diz* mutants implies that the causal mutations can be transferred throughout generations. Backcrossing of these confirmed *diz* mutants could enlighten the nature of the causal mutation by displaying its segregation pattern, which reveals a single or multi locus origin. The F2 *diz* generation formed in this backcrossing can be pooled and used for sequencing by means of next generation sequencing and SHOREmap technologies.

ABSTRACT (NEDERLANDS)

Acthtergrond De abiotische stress die een omgeving kan veroorzaken weegt zwaar op de groei, overleving en voortplanting van planten en verminderd het aanbod van bruikbare grond dat ook lijdt onder de competitie tussen gewassen voor voeding- en bio-energie. Het adaptatievermogen van het wortel systeem is gekend voor zijn aandeel in het verhogen van de overleving door het exploiteren van nutriënt rijke zones en het vermijden van gecontamineerde zones in de bodem. Het verrijken van de kennis over deze gerichte wortelgroei kan tot een oplossing leiden door het minimaliseren of maximaliseren van de opname van contaminanten door het manipuleren van de blootstelling. Een uitgesproken voorbeeld van het vermijden van een contaminant wordt gegeven door blootstelling aan een overmaat aan zink, wat primaire wortelgroei en laterale wortel initiatie inhibeert.

Objectief Het doel van dit onderzoek ligt in het initiëren van het onderzoek naar de onderliggende mechanismen die het inhibitie effect van overmatige zink blootstelling en bepalen of dit effect zijn oorzaak vind in overmatige toxiciteit en cel dood of als gen producten een rol spelen. Een genetische screen voor onderliggende genen zal worden opgezet.

Methodologie Blootstelling aan overmatig zink veroorzaakt een bepaald phenotype, wat het geschikt maakt voor gebruik in een forward genetics screen om onderliggende genen en gen producten te achteralen. Een populatie van *Arabidopsis thaliana* zaden wordt onderworpen aan het mutagen EMS, en de mutaties betrokken in het zink inhibitie effect worden eruit gehaald door middel van een screen. Om te bepalen of genen en hun producten inderdaad een rol spelen in dit effect wordt er een auxine complementatie experiment uitgevoerd, welk exogeen auxine toevoegt in een poging het inhibitie effect van zink te overschrijven.

Resultaten Het auxine complementatie experiment indiceert dat het inhibitorisch effect uitgeoefend door zink omzeild kan worden door phytohormonen, wat wijst op een oorzaak die eerder ligt bij genen en hun producten dan bij excessieve toxiciteit en celdood. De *diz* mutanten die een gewenst afwijkend phenotype vertonen in de forward genetics screen en hun bevestiging in de volgende generatie onderstuenen verder het idee van de betrekking van genen in het inhibitorisch effect van Zn

Conclusie/Vooruitzichten De mogelijkheid om het inhibitie effect uitgeoefend door overmatig zink te overschrijven bevestigd dat genen en gen producten betrokken zijn, ook het succesvol vinden en bevestigen van *diz* mutanten door EMS mutagenese wijst in deze richting. Bevestiging van de *diz* mutanten impliceert dat de causale mutaties overgedragen kunnen worden doorheen generaties. Een terugkruisting van deze bevestigede *diz* mutanten zal het segregatie patroon weergeven en onthullen of het gaat om mutatie met een oorsprong in een enkele of meerder loci. De gevormde F2 *diz* generatie in deze terugkruising kan worden gepoold om zo sequentionering mogelijk te maken door het gebruik van next generation sequencing en de SHOREmap technologie

1. INTRODUCTION

1.1. Plant roots may provide a solution for agricultural problems

Plant growth, survival and reproduction are greatly affected by biotic and abiotic environmental stresses. Suitable arable land is becoming sparser due to erosion of hill-slides, landslides and the occurrence of soil degradation due to abiotic stresses such as climate change, nutrient and water shortages or anthropogenic industrial pollution. A sustainable solution is needed to meet the demands of the growing world population in terms of food, feed and biofuels.[4; 5] A solution can be found in optimizing plant growth for remediation or safe biomass production[27], opening up contaminated lands for non-food biomass production, e.g. for bio-fuels. Mainly focussing on increased shoot biomass and seed yield, the relevance of the root system for biomass production has often been overlooked in the past. Nevertheless the root system is an important point of interaction between the soil environment and the organism, and thus plays an important role in sustaining the plant by supplying nutrients and water. Immaculately displaying their developmental plasticity, roots have already shown their adaptability under certain nutritional circumstances. Coping with phosphorus (phosphate) deficiency, an increase in root surface was achieved by changes in branching patterns, elongation rate, and root hair density, enlarging the root surface-coil contact area[11; 28]. Another example of the nutrient-induced plasticity of root development can be found in the stimulated colonization of nitrate rich patches by localised lateral root proliferation[19]. Emphasized by these examples, it can be said that the root system is an important feature in the research towards plant growth optimization [4].

Apart from assuring the uptake of essential elements, the root system unavoidably takes up excess of essential elements and toxic non-essential elements as well. With its developmental plasticity, the exposure of the root system to these excess concentrations of contaminants will unavoidably have an effect on the root system, changing the root system architecture (RSA) to minimalize exposure. As such, stress-induced morphogenic responses (SIMR's) in the presence of excess elements such as metals have been postulated to redirect plant growth to achieve a diminished stress exposure[17]. Besides the avoidance of contaminated areas, colonization by the roots system of less contaminated areas may be considered prerequisite to ensure sufficient growth[21]. Thus RSA manipulation may be a solution to either minimize (safe biomass production) or maximize (phytoremediation) uptake when faced with contaminated soils. Prior to this, an understanding of the effects the contaminants exert on the root system must be established.

1.2. Root system development

Root cell types and their development, organization and patterning are typically described using terminology that encompasses the radial and longitudinal structure of an individual root, of which a schematic overview is given in figure 1. Regarding the longitudinal classification of the root tissues, above the root cap, consisting of columella- and lateral root cap cells, the apical meristem can be found holding stem cells, which provide new cells in the growing root by active and rapid mitosis[14]. The transition zone between the apical meristem and the elongation zone is referred to as the basal meristem. Following the basal meristem, an elongation zone and differentiation zone can be marked, respectively achieving the elongation and differentiation of the newly formed root cells. The elongation of newly formed cells is the driving force of root growth, as expanding the newly formed root cells forces the root tip along with the apical meristem forward, protruding through the surrounding substrate.

1.2.1.Lateral root formation

Post-embryonic formation of the plant root system relies on *de novo* formation of lateral roots. Development of a lateral root requires primed pericycle cells and tightly regulated asymmetric cell divisions to generate cell diversity and tissue patterns. Priming of the pericycle cells occurs close to the root tip in the basal meristem under the influence of accumulation of the phytohormone auxin (figure 1). After priming of the pericycle cells only some acquire the founder cell (FC) status closely preceding lateral root initiation (LRI). In the differentiation zone, a pair of FC undergoes asymmetrical divisions initiating lateral root primordia (LRP) formation. The stages the lateral root development are presented in figure 2 alongside with a representation of the auxin distribution during these developmental stages. After formation, the LRP has to emerge through several intervening parental root tissues comprising single layers of endodermal, cortical and epidermal cells in *Arabidopsis*. Recently, genes encoding several *Arabidopsis* cell wall remodelling (CWR) enzymes have been reported to be expressed in root cells next to new LRP, presumably causing cell separation in overlaying root tissues and facilitating LRP emergence. Triggering by auxin-dependent degradation of repressor proteins (e.g. IAA3/SHY2) has been reported to activate endodermal CWR genes aiding lateral root emergence[10; 16]

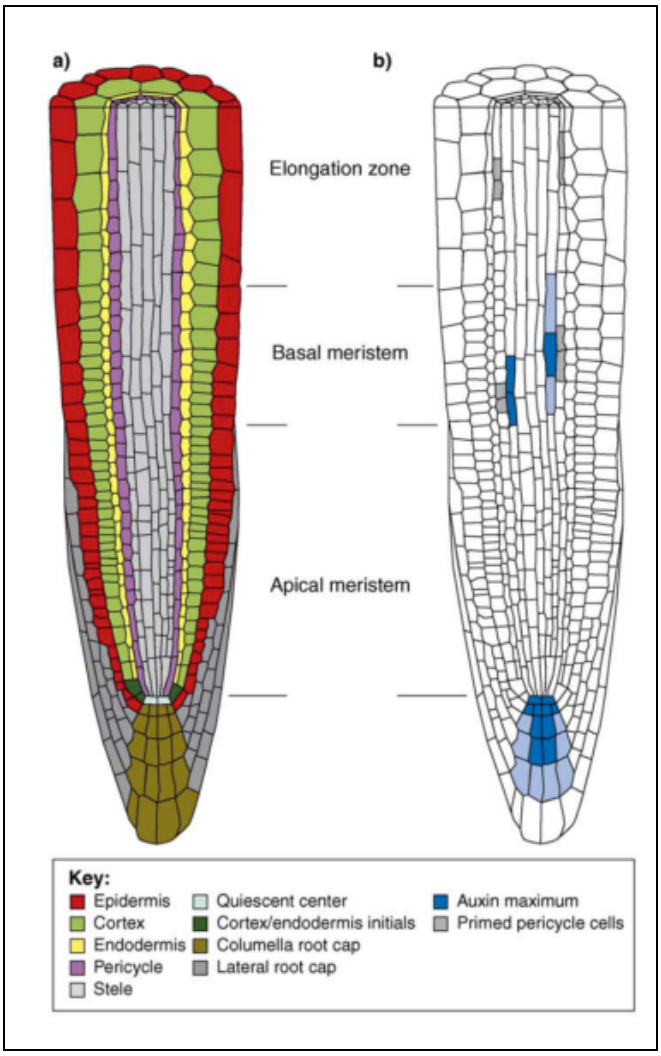

FIGURE 1: Longitudinal section of a root
a) Schematic overview of different root tissues
b) Auxin signalling maximum as reported by the DR5::GUSmarker line (De Smet et al. 2007).
Adapted from Peret et al. (2009)

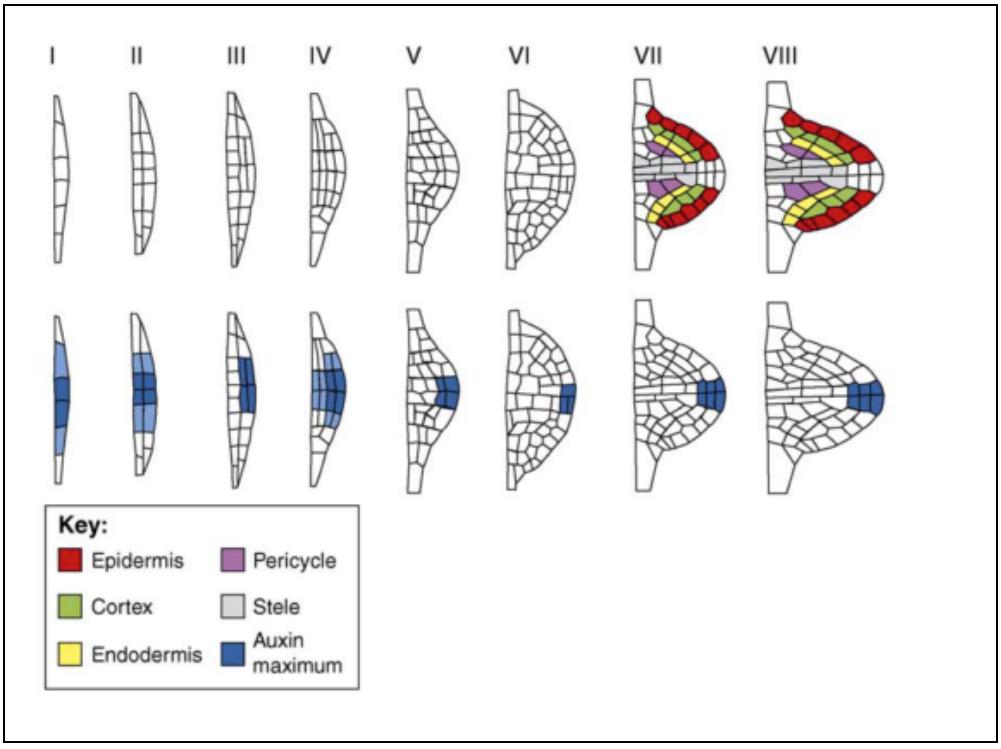

FIGURE 2: Morphological changes during lateral root development *Adapted from Peret et al (2009).*

1.2.2.Phytohormones in root development

The study of developmental plasticity of roots strongly suggest a pivotal role for auxin in root development, influencing both primary and lateral roots as well as root hair (RH) formation. The biosynthesis and transport as wel as auxin signalling dictates the development of roots in plants. Other phytohormones, such as cytokinin (CK), brassinosteroids (BS), ethylene (ET), absisic acid (ABA), gibberellic acid (GA), jasmonic acid (JA), polyamines (PA) and strigolactones (SL) integrate their signals into the important auxin processes triggering cascade events leading to root development[22]. The complexity of this network is displayed in figure 3. Repressing both transport and signalling, the antagonistic interaction of auxin with cytokinin ensures the maintenance of root meristem size and root growth. CK directly activates transcription of the *short hypocotyl2* (SHY2) gene, which in turn, negatively regulates pin-formed (*PIN)* genes, hindering auxin transport and distribution, affecting root growth. Also intervening at molecular level is the synergistic interaction between auxin and ethylene, noted to regulate root gravitropism, root growth, LR development and RH differentiation and elongation. Auxin induces a key enzyme in ethylene biosynthesis by upregulation of the *1-aminocyclopropane-1-carboxylate synthase* (ACS) genes[13; 15]. Ethylene is also known to influence auxin levels by interfering in gene activity regulating the tryptophan (Trp) biosynthesis, a key element in the Trp-dependent auxin biosynthesis pathways[12; 22]. Whereas tightly balanced hormone levels are important for regulation of RSA under normal conditions, environmental factors can also affect the hormonal balance in the root systems of *Arabidopsis* leading to an alteration of the RSA. Lequeux et al (2010) reported the reorganization of the RSA due to a local signal induced by excess Cu^{2+}, coinciding with an increase in cytokinin and auxin pools in the root tips. Apart from non-essential elements, essential elements such as phosphorus can also disturb the hormonal balance. P_i deficient circumstances led to decreased cytokinin levels and an ethylene-jasmonic acid causing meristem exhaustion and remodelling of the RSA [24]. Demonstrating various ways of altering the RSA under stress conditions, the complex phytohormonal network can not be overlooked when researching the effect of excess zinc on the root system. The role that phytohormones play during this specific stress may be elucidated by means of an open screen, in which genes involved in RSA adaptations may be identified that are related to phytohormone synthesis, signalling and responses under excess Zn conditions.

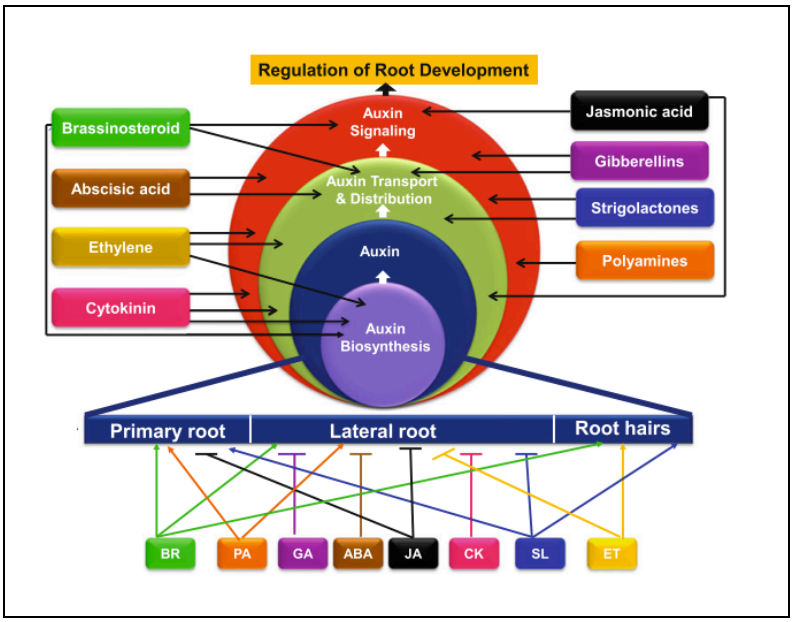

FIGURE 3: Cross-regulatory interactions of different phytohormones with auxin biosynthesis, transport, distribution and signalling. *Adapted from Sani et al (2013)*

1.3. Root development in the presence of contaminants

Potters et al. (2009) postulated that different abiotic stresses trigger similar morphological outcomes. The underlying determinants of these morphological outcomes would be related to altered gradients of plant hormones (e.g. ethylene, auxin), reactive oxygen species (ROS) and antioxidants that result in altered cell division, elongation and/or differentiation. Indeed, for a number of stress conditions, similar changes in auxin gradients could be linked to similar changes in root morphology[18]. Being a key hormone and part of intrinsic root development pathways, as described above, it is not surprising that changes in root architecture are likely to be connected with changes in auxin gradients or sensitivity. However, the question remains how these altered gradients or altered sensitivity to auxin come in place. Furthermore, it can be argued that the postulation of "different stresses, similar responses" (Potters 2009)[17] is inconclusive as it is based on the observation of increased root densities only after exposure of juvenile plants without emerged lateral roots.

However also lateral root elongation is an important parameter defining root architecture under metal stress. Indeed, Remans et al. (2012) documented stress-specific effects on lateral roots induced by the excess metals cadmium (Cd), copper (Cu) and zinc (Zn). At the same level of primary root growth inhibition, changes in the lateral root development were found to be metal-specific. Figure 4 depicts the findings for each metal, comparing them to a control plant. Lateral root density was increased by Cd and Cu but decreased by exposure to excess zinc. Exposure to Cu affected the elongation of lateral roots less than exposure to Cd. The most remarkable finding was the negative effect of excess Zn application on *A. thaliana* roots, which inhibited both the density and elongation

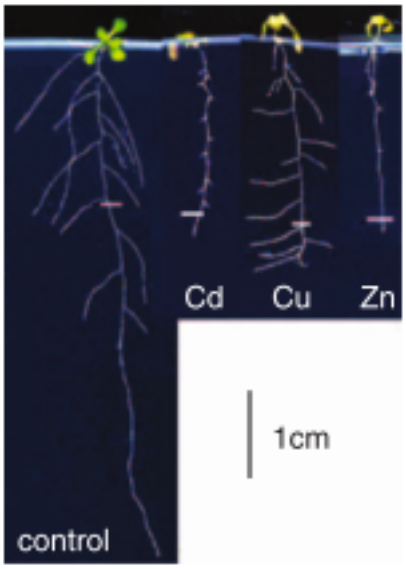

FIGURE 4: Seven-day old *A. thaliana* plants, with a primary root length up to the white mark were exposed for another 7 days to 5 µM $CdSO_4$, 10 µM $CuSO_4$ or 75 µM $ZnSO_4$

of lateral roots, strongly contrasting with the postulation that abiotic stresses cause increased lateral root density[17]. In the research towards revealing the underlying molecular mechanisms of these metal-specific phenotypes, an opportunity is to use the strongly negative effect of Zn on lateral root growth in a genetic screen for deviating phenotypes. This could reveal new molecular insights in the mechanisms of developmental plasticity in the presence of excess Zn.

1.4. The essential element zinc

The element zinc has been used increasingly by life throughout evolution. Since the oxygenation of the Earth's atmosphere, the availability of Zn increased, giving life the opportunity to incorporate it into its structures. The earlier formed zinc sulphides reacted with the atmospheric oxygen (0_2) and ozone (O_3) exposing life to the formed zinc sulphates and zinc oxides[26]. Most frequently occurring is the oxidized Zn(II), which is found throughout the biology. The oxidized state of Zn(II) excludes Zn from direct participation in electron transport chains or other electron transfer reactions, unlike other ions. The non-redox active character of Zn in biological systems ensures a comparably safe usage in zinc-finger transcription factors operating in the proximity of sensitive macromolecules such as DNA. Zinc is also incorporated into some metalloenzymes such as carbonic anhydrase and alcohol dehydrogenase and is the only metal represented in the six enzyme classes (oxidoreductases, transferases, hydrolases, lysases, isomerases and ligases)[1; 26].

1.4.1.Excess zinc generates oxidative stress

Although Zn is an essential element necessary for normal functioning of plants biosynthesis and growth, exposure to excess Zn causes stress. As the first encountering surface, the roots try to cope with excess zinc levels by root to shoot translocation, complexation by chelation (e.g. glutathione and phytochelatins), and compartmentalization in vacuoles[1; 9]. When these primary mechanisms become insufficient, excess zinc generates oxidative stress by indirect mechanisms. Although Zn itself is not redox active in plants, antioxidative enzyme capacities (catalase, peroxidase, superoxide dismutase, enzymes of the ascorate glutathione cycle), changed oxidation states or altered levels of the major antioxidants (ascorbate, glutathione) and induced lipid peroxidation have been shown as evidence for Zn-induced oxidative stress alterations by several studies[3; 20]. In the roots an overall oxidation of metabolites occurs together with inhibition of the ascorbate-glutathione cycle, which is an important metabolic mechanism to detoxify reactive oxygen species (ROS)[3; 23]. Potters et al. 2007 pointed out a direct link between ROS, antioxidants, phytohormones at one side and cell cycle progression on the other, stating that changes in any of these parameters can control cell cycle activity in a location- specific manner. This sort of control over cell cycle activity can, for example, simultaneously cause a cessation of meristematic activities in the primary root apex, while inducing the initiation of new lateral primordia. Taking note of this, the generation of oxidative stress and ROS must be considered when the effects of excess zinc on the root system are studied. Thus, an open genetic screen may identify the involvement of redox-status related molecular parameters in Zn induced RSA effects.

1.5. Identification of molecular mechanisms of Zn induced effects on root growth using a forward genetics screen and next generation sequencing

Given metal specific morphogenic responses, as well as metal specific changes oxidative stress related parameters, metal specific parameters underlying zinc-induced stress responses may be identified. The inhibitory effect of excess zinc on the primary root elongation and lateral root emergence of *Arabidopsis thaliana* provides an opportunity that can be used to discover the underlying mechanism(s) of the Zn specific stress response by means of a forward genetics screen. Investigation of the mechanisms behind this response will provide knowledge achieving further understanding of the developmental plasticity of the root system. Forward genetics works by screening a mutant population for phenotypes deviating from the wild type under selective conditions. Respecting the reproducibility and desired facility of a visual screen, the absence (wild type phenotype) or presence (desired deviating phenotype) of lateral root emergence under excess

zinc can be used as a screening phenotype. Screening for a phenotype will circumvent the lack of knowledge concerning the underlying mechanisms of the stress effects.

Mutant populations can be formed by several kinds of mutagenesis. Insertional mutagenesis, also called T-DNA mutagenesis, uses a tumour-inducing (Ti) plasmid to insert a small sequence into the genome inducing mutations in genes[2]. Physical mutagenesis uses the exposure to radiation or ionized beams to induce random changes in the DNA sequence, often deletions. The chemical mutagenesis is perhaps the most available form of mutagenesis [7], exposing germ cells to a mutagen, such as ethyl methanosulfate (EMS) for several hours, induces random point mutations in the genomic sequence.

Following Hartwig et al. (2012) an isogenic bulked segregant analysis of ethyl methanosulfate (EMS)-induced mutants with desired phenotype, combined with deep sequencing could provide a fast workflow to reveal the causal mutation in a forward genetic screen of *A. thaliana*. A schematic illustration of the fast isogenic mapping approach is depicted in figure 5. Historically, the mapping of causal mutations needed backcrossing with a different *A. thaliana* ecotype to introduce polymorphisms for the creation of a mapping population. On the other hand, the isogenic method uses the EMS induced polymorphisms in an isogenic segregating population after backcrossing to the same ecotype. With the isogenic approach, the interference of another ecotype, which may have different root growth effects, can be circumvented, facilitating the process of selecting deviating phenotypes in the F2 population. After selection of mutants with the desired phenotype in the M2 EMS population, these plants are backcrossed with their parental-line and selfed forming an F2 population[6]. This population is subjected to the same phenotypical screening, phenotypes are selected and bulked forming a pool of bulked segregants. The phenotypical selection of mutants in the segregating population ensures that the causal mutation occurs at the highest frequency, whereas non-causal mutations are diluted with the wild-type variant. Deep sequencing can than be performed on the combined DNA pools of the bulked segregants, yielding multiple readings for every single nucleotide position to provide high genome coverage and increased sequence accuracy. Separately aligning the bulked segratant population sequence data to the reference genome will reveal the positions that are in the segregating population only covered by the mutations and thus not represented by wild type cross-over in the F2 population. Thus these are the areas that are selected for in the F2 screen. Schneeberger et al. (2009) provides the software, SHOREmap, to identify the location of the causal mutation, providing information necessary to link the mutation to exact chromosomal location on the reference genome[25].

1.6. Objectives and hypothesis

Hypothesising that certain gene products are involved in the molecular mechanism underlying the inhibition of lateral root appearance by excess zinc, two interdependent research questions are formulated: (1) ARE CERTAIN GENE PRODUCTS RESPONSIBLE FOR THIS EFFECT OR IS IT MERELY CAUSED BY EXCESSIVE TOXICITY AND CELL DEATH and (2) WHICH ARE THE GENES INVOLVED. In a first research objective, it will be tried to metabolically interfere with the inhibition by excess zinc. Successfully overwriting the inhibition by applying the root-growth inducing phytohormone auxin may serve as indication of the involvement of certain genes in the inhibitory pathway, rather than just excessive toxicity leading to cell death. The second objective states the identification and conformation of EMS mutants with deviating phenotypes. Utilizing the powerful and unbiased forward genetics approach, EMS mutated *Arabidopsis thaliana* seeds will be phenotypically screened for desired lateral root growing-phenotypes under zinc stress.

2. MATERIALS AND METHODS

2.1. Preparation of vertical agar plates

For determining the screening conditions, preliminary tests were executed using three growth media, (1) 10 times diluted Gamborg's B5 medium, (2) 50 times diluted B5-medium and (3) 4 times diluted Murashige and Skoog (MS) medium. Each media was supplemented with 10g/L plant tissue culture agar, for germination plates media were supplemented with 5g/L D-sucrose. The nutritional value of each of these media can be found in the supplemental table 1 and 2. Adjustment of pH value to pH 5.7-5.8 was achieved by addition of KOH or HCl before autoclavation at 121°C for 10 minutes and overnight storage at 65°C in an oven. Square petri dishes of 12x12 (Greiner bio-one, Wemmel, Belgium) were filled with 40 mL of 65°C medium in a laminar air flow, and left open until the medium had solidified.

Media for Zn treatment plates were supplemented under a laminar air flow, either by adding filter sterilised (0.2 µm) $ZnSO_4$ to the autoclave bottle and , mixing using a magnetic stirrer, or by pippeting into the petri dishes before adding media in the required volumes. For treatment plates, 1 cm of agar was removed at the top to form an air gap for the shoots. For vertical split root experiments, agar plates containing a top section without excess Zn and a bottom section with excess Zn, were prepared by removing 2.5 cm of agar from the top of the Zn containing solidified agar plates, then filling the gap with control medium. After the solidifying of the control patch, a 2 mm gap was made between the two agar conditions to avoid diffusion. When not used directly, the agar plates were stored in the original plastic bags at room temperature for maximum one week.

2.2. Plant material

Arabidopsis thaliana Col-0 seeds were obtained from Lehle seeds (Round Rock, Texas, USA) and transferred to a 90 mm filter paper (Whatman No. 542, hardened ashless) that was folded in a cone shape and sealed by a paper clip. The seeds were sterilised by means of 100 ml of 0.1% (v/v) NaOCl (Sigma-Aldrich, St. Louis, USA) with 0.1% (v/v) of Tween 80 (Fluka, Steinheim, Germany) for 1 minute. The seeds were rinsed four times with a small volume of sterilized distilled water (dH_20) and then soaked four times for five minutes with large volumes (>200 ml) of sterile distilled water. Sterile forceps were used to open the filters under a laminar flow in a round petri dish, they were left under the laminar air flow to dry completely. The dried seeds were sown or stored in a tightly closed round petri dish.

2.3. Growth conditions

Arabidopsis thaliana seeds were sown per twenty on germination plates in a straight line 1 cm below the top. The plates were sealed with parafilm and two 2 cm air gaps were incised on the lower left and right side to allow for air exchange. Sown plates were incubated in the dark at 4°C to rehydrate the seeds, ensuring a uniform germination. The plates were then placed vertically in a climate chamber (21°C) with cycles of twelve hours dark and twelve hours light (delivered by fluorescent lamps). Treatment plates were alternated and empty petri plates surrounded treatment or germination plates to diminish the border effect. After seven days of incubation a homogenous set of seedlings (±2 cm primary root length) were transferred to the treatment plates, placing the shoots in the 1cm air gap, each plate containing five or ten seedlings, depending on the experiment. After transfer the plates were sealed with parafilm, two air exchange gaps of 2cm were provided at the lower left- and right side. The plates were incubated as described above.

2.4. Imaging

After transfer to treatment plates, the growth of the seedlings was monitored by marking the root tips and for the *diz* screens also the lateral roots each day, at the end of the experiment the plates were scanned with an Epson Perfection V330 photo scan at 300 dpi to allow root imaging, result documentation and root analysis of the primary and lateral root growth in IAA complementation experiments.

2.5. Determination of screening conditions

The above-mentioned media were supplemented with a concentration range of $ZnS0_4$, plates were supplemented with $K2SO_4$ to complement all plates to the same sulphate concentration. Germination plates were prepared for the three different three different media as mentioned above and sterile *Arabidopsis thaliana* seeds were sown. After seven days the seedlings were transferred to treatment plates with same medium supplemented with KSO_4 or $ZnSO_4$ (Table 1). After seven days of treatment, the petri plates were scanned and the root growth was visually analysed.

Table 1: Screening conditions experiment 1

Screening condition determination 1: µM Zn added				
B 5/50	0	15	25	35
B 5/10	0	50	75	100
¼ MS	0	150	200	250

Table 2: Screening conditions experiment 2

Screening condition determination 2: µM Zn added								
B 5/50	0	15	25	30	40	50	75	100
¼ MS	0	100	150	300	350	400	500	600

2.6. IAA complementation experiment

The media selected for the screening of *diz* mutants was also used for a metabolic complementation experiment using IAA (3-indole acetic acid; SIGMA catalog nr.I2886). Adding a range of IAA concentrations to the Zn concentration will determine if the inhibition of lateral root growth can be overcome by administering this root growth promoting hormone. The IAA concentration range is presented in *table 3*. After seven days of exposure, the petri plates were scanned, the jpeg files were used foor root growth analysis focussing on lateral root number and lateral root length.

Table 3: Zn and IAA concentrations used for complementation experiment

Background medium ¼ MS							
Zn	**(µM)**	0	500				
IAA	**(nM)**	0	0.1	1	10	50	100	1000

2.7. Preparation of an EMS mutagenized population

Arabidopsis thaliana Col-0 seeds (lehle seeds, Round Rock, Texas, USA) were exposed to a range of four ethyl methanse sulphonate (EMS – SIGMA catalog nr. M0880) concentrations. In 50 ml polypropylene tubes, each time 6000 *A. thaliana* seeds were exposed to a solution with 1.0%, 0.75%; 0.50% or 0.25 % (v/v) EMS in dH2O for 16h in the dark at room temperature, without shaking but by placing the 50 ml tubes flat to ensure spreading of the seeds and contact with the solution. After exposure, the seeds were rinsed four times with distilled water and soaked ten times for one minute. The distilled water was poured out of the falcon tube as much as possible, the remaining fluid was removed by means of a pipette with extreme care not to pipette seeds along with the fluid. Liquid waste and glassware was treated with sodium thiosulphate as it destroys EMS, plastic disposables contaminated with EMS were treated as carcinogenic waste.

The mutated M1 seeds were sown onto purified aquarium sand in five trays per EMS concentration, at 1200 seeds per tray. M1 plants were grown in the greenhouse and watered regularly with ½ Hoagland nutrient solution. Siliques were left to dry on the plant for an additional four weeks after stopping the watering with nutrient solution. Per container, two siliques of 5 plants were harvested and placed into a paper cone filter, for the determination of the mutation frequency. The rest of the plant material was harvested in a paper bag and left to dry further for another 3 weeks at 28°C. The M2 seeds were then collected in five pools per EMS concentration, each pool corresponding to one tray of 1200 M1 seeds. Seeds were harvested from the 0.25 to 0.75% pools, and not from the 1% EMS population as it had a high lethality and high level of sterility.

2.8. Sterilisation of M2 seeds

After applying the same sterilisation protocol as in the establishment of the screening conditons (see above), there was still a contamination during germination on agar plates, hence a stronger sterilisation protocol was used. The mutagenised seeds in cone shape folded filters were sterilized by submersing them in a 70% (v/v) ethanol for 2 minutes followed by 5 minutes in a 10%(?) 1%? NaOCl (Sigma-Aldrich, St. Louis USA) with 0.1% (v/v) Tween 80 (Fluka, Steinheim, Germany) solution. Seeds were further washed and dried as described above.

2.9. Determination of mutant frequencies

Plants in the M1 population with white sectors and albino's occurring in the seeds from the harvested siliques and throughout the *diz* screens were scored. The mutation frequency was determined during the screens using following formula's.

Albino segregation in M1 population:
Mf= (# M1 segregating for Albinism)/(#M1's screened * GECN)
With GECN for *A.thaliana* being 2.

Lethality in *diz* screens:
Mf= (m') / (n*f)
= (non germinated seeds)/(sterile seeds*0,25)
With f for *A.thaliana* being 0,25 given a normal segregation of recessive mutations

2.10. Mutant *diz* screen

The *Arabidopsis thaliana* seeds exposed to 0.5% of EMS were selected to screen for *diz* mutants. Sowing and germination conditions were the same as mentioned above, and a 500 µM zinc in ¼ MS medium was chosen to use during the phenotypical screen. After germination and growth on germination plates (no excess Zn) all of the grown seedlings were transferred to treatment plates. Lateral roots were marked each day, using different colours to identify the day of emergence. Seedlings that initiated and grew lateral roots after day 4 of treatment were considered *diz* mutants. On day 7 of treatment the markings were evaluated and *diz* mutants were selected. Selected M2 mutants were carefully transferred to purified sand and watered with ½ Hoagland nutrient solution, to allow further growth and form M3 seeds. The first days after this transfer the seedlings were covered with a petri plate lid to allow slow adaption to humidity difference between closed agar plates and open air culture on sand.

2.11. Confirmation of *diz* mutants

For confirmation of the identified *diz* mutants the same growth conditions as the screen for *diz* mutants were used. A total of eight *diz* mutants formed siliques and seeds and fourty M3 seeds per potential *diz* mutant were sown alongside Col-0 seeds and phenotypes were compared.

2.12 Statistical analysis

All the statistical analysis were preformed using R 2.15.0. Using the Shapiro test all the data was found not normal distributed, a non parametrical anova was preformed using Kruskal Wallis and 'Pairwise wilcoxon and rank sum'

3. RESULTS AND DISCUSSION

3.1. Screening conditions

To determine the screening conditions used for the *diz* mutant screen, wild type *Arabidopsis thaliana* seedlings were characterised phenotypically under control and excess zinc conditions. The introduction already pointed out that a clear, easy recognizable and reproducible phenotype is needed for a forward genetic screen. Non-mutagenized control plants that undergo the screening conditions must then also display a clear reproducible effect of excess zinc from which the desired mutants can deviate. When plants with a 2 cm long primary root are transferred from control media to excess Zn media, two effects resulting from excess zinc contamination can be considered: (1) a severe inhibition of primary root growth and lateral root emergence in the existing primary root axis, exerted by high concentrations of zinc or (2) the inhibition of lateral root development in the *de novo* synthesized primary root zone under moderate concentrations of excess Zn.

The course of the media selection and the used zinc concentrations are given in figure 5 depicted below. Three growth media were tested: 4x diluted Murashige and Skoog (1/4 MS), 50x diluted Gamborg's B5 (B5/50), and 10x diluted Gamborg's B5 (B5/10). At first evaluating the lower zone of the treated plants was considered, handing an opportunity to study the primary root and lateral root initiation synthesised *de novo* in treatment conditions. Of the two B5 media, B5/10 displayed a stronger primary root inhibition (PRI) effect throughout the zinc range tested which left little to no lower zone to evaluate. The second experiment subdued the two remaining media to a wider range of zinc concentrations so an increasing gradient of the Zn inhibition effect was made visible. Interpreting the results of this experiment, it was decided that a phenotype with severe primary root inhibition would serve better in a forward genetics screen shifting the focus towards the lateral roots initiated and grown in the upper zone of the seedlings.

Finally, the ¼ Ms media with a 500µM Zn concentration was selected for the mutant screen. The 500µM Zn concentration gives a full inhibition of primary root growth, directing the focus of the screen to lateral root formation in the pre-existing primary root tissue that had developed under control conditions.

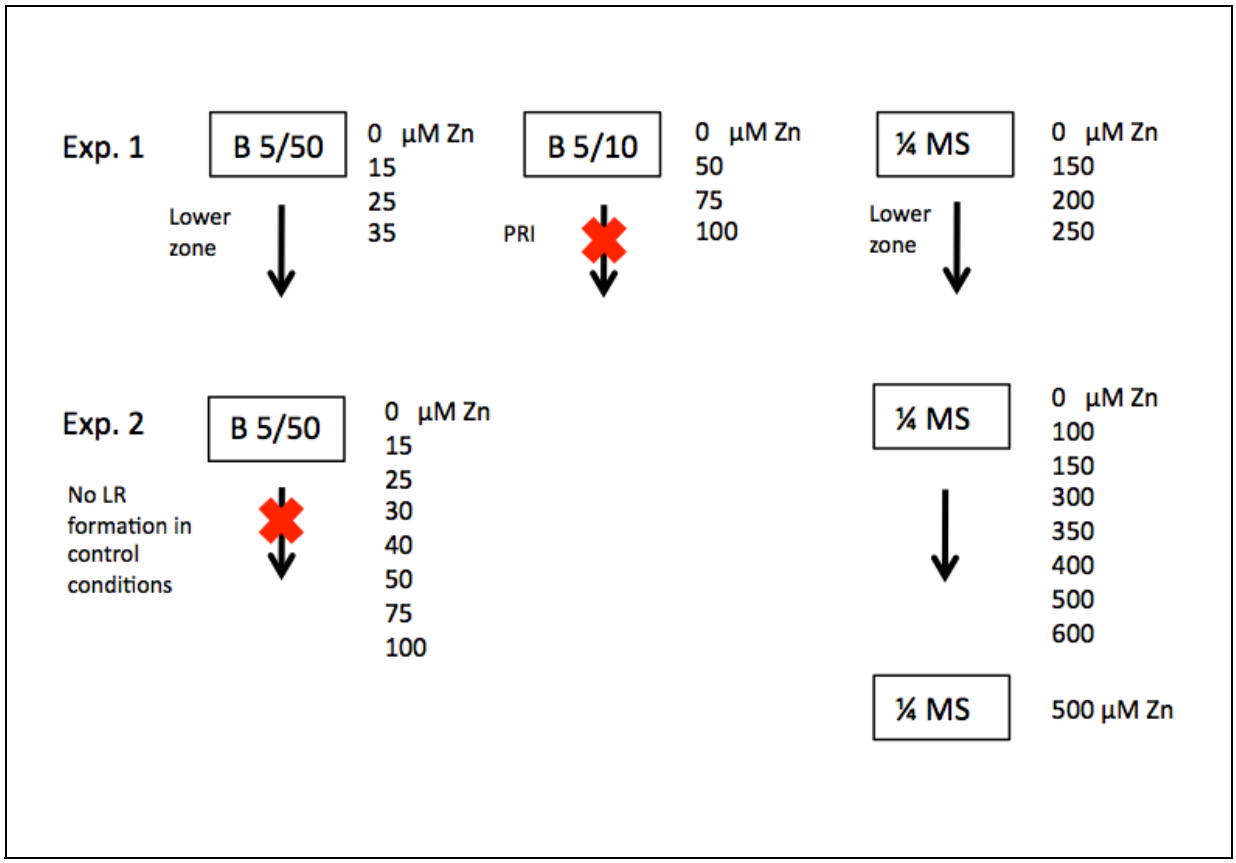

FIGURE 5: Course of media selection.
Selection criteria involve primary root inhibition (PRI) and lower zone (LZ) evaluation.

3.2. IAA complementation experiment

To enlighten how the inhibition that excess zinc exerts on the lateral root growth occurs, the root growth-promoting hormone auxin (IAA) was added to treatment plates with or without excess zinc. Whether or not the auxin effect is visible in the seedlings inhibited by excess zinc sheds light on the mechanisms the metal does or does not use. After a seven-day treatment of the *Arabidopsis thaliana* seedlings to certain zinc (0 or 500µM) and auxin (0; 0,1; 1; 5; 10; 50; 100 or 1000 nM) concentration, end-point lateral root number and mean lateral root length were analysed. The data (Figure 6) show the control conditions and the auxin effect on control plants, the inhibitory effect of excess Zn with and without added IAA. Whereas exposure to 500 µM Zn strongly inhibited LR number and length, addition of exogenous IAA restored lateral root growth. A peak in lateral root number and mean lateral root length is observed around 10 nM IAA indicating a maximum effect in this concentration range. These result hint towards a possible metabolic rewrite of the excess zinc inhibition effect by auxin with a maximum at addition of 10 nM IAA.

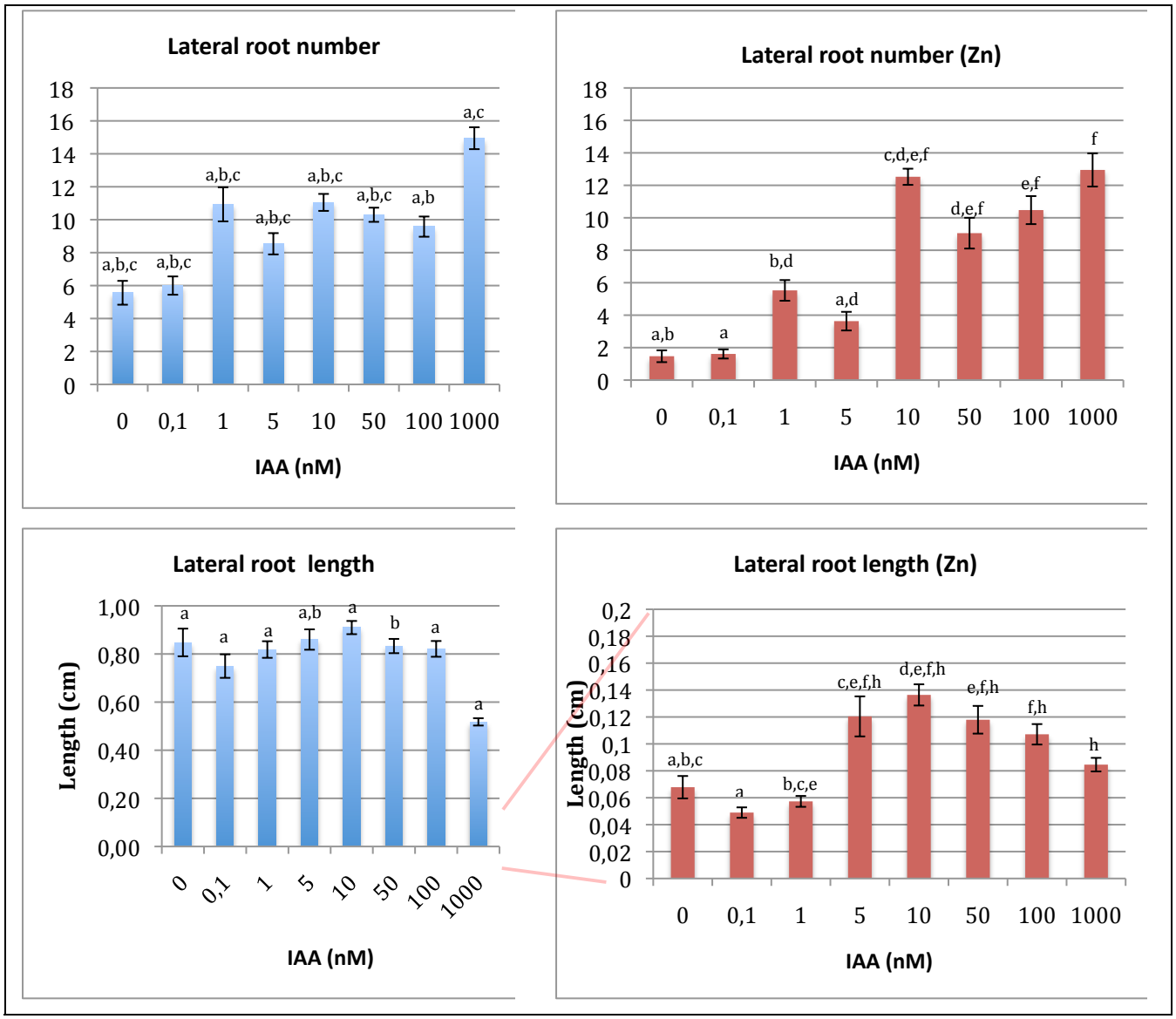

FIGURE 6: Lateral root number and mean length of Arabidopsis thaliana plants grown for 7d under control conditions, then transferred to growth medium supplemented with indicated concentrations of IAA, without (blue graphs) or with (red graphs) 500 µM ZnSO4. A one way Anova analysis was preformed to analyse this data.

3.3. EMS mutagenesis

3.3.1. M1 populations and growth evaluation

Four pools of 6000 A.thaliana seeds were mutagenized using 0.25%, 0.50%, 0.75% and 1.0% EMS, respectively, then sown into 20 trays at 1200 seeds per tray. After growing for three weeks on the purified aquarium sand, a first consequence of the mutagenesis became visible as the growth of the mutated *A. thaliana* seedlings diminished both in quantity and size with increasing EMS concentrations. Photographs taken at the end of the third week in the greenhouse are displayed below in figure 7 and depict this finding, showing three out of the five trays for each EMS concentration.

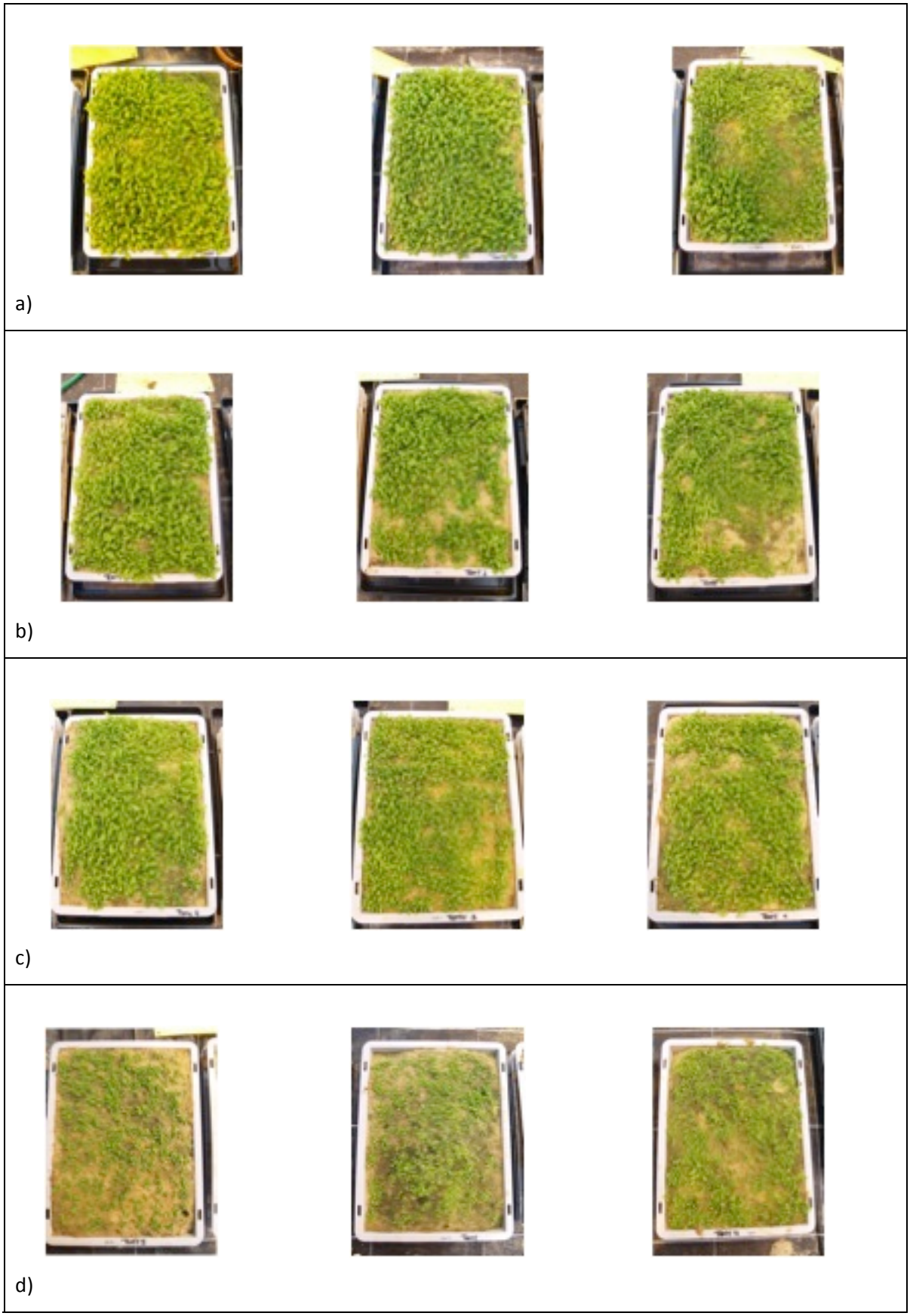

FIGURE 7: photographs of trays with mutated seeds. a) 0.25 % EMS b) 0.5% EMS c) 0.75 % EMS d) 1% EMS

3.3.2.Mutation frequency determination

Another indication of mutagenesis is the occurrence of white sectors in the leaves of the mutated *A. thaliana* seedlings. These white sectors are caused by mutation in genes involved in chlorophyll production in one of the genetically effective cells. Examples of such white sectors were photographed and are shown in figure 8. After counting and documenting the white sectors, an increase in their occurrence with increased EMS concentration was observed (table 4).

FIGURE 8: Example of the with sectors occurring in the EMS treated *Arabidopsis thaliana* populations.

Table 4 White sector occurrence in EMS mutated *A. thaliana* populations

EMS concentration	Occurrence of white sectors per concentration (6000 estimated plants)	Occurrence of white sectors (percentage)
0,25%	42	0,7
0,5 %	68	1,1
0,75	89	1,5

To assess the occurrence of mutations, mutation frequencies were calculated as described in materials & methods for the EMS treated M1 generation in which albinism was scored, and for the M2 plants (in the *diz* screens for deviating root phenotypes under excess Zn) in which the embryo lethality was estimated (table 5 and 6). These results form an indication of a succeeded EMS mutagenesis as the values calculated are representative in comparison with those found in other studies confirming the used method and concentrations in the EMS mutagenesis.[8; 7]

Table 5 mutation frequency observed in siliques of M1 plants

Seed batch	# M_1 plants	Total # M_1 siliques	# M_1 plants segregating M2 seeds for albinism (considering GECN)
Col-0 +0,25 % EMS	25	50	2/25 (4%)
Col-0 + 0,50% EMS	25	50	2/25 (4%)
Col-0 + 0,75% EMS	25	50	3/25 (6%)

Table 5 Lethality in M2 seeds, observed during *diz* screens

Screen	EMS conc. (%)	not germinated	not sterile	total # seeds	Lethaility
***diz* S1**	0,5	170	238	960	0,06
***diz* S2**	0,25	175	89	960	0,05
***diz* S3**	0,5	275	190	960	0,09
***diz* S4**	0,5	908	595	3840	0,07

3.4. Mutant selection

The first *diz* screen was performed on a pool of 0,5% EMS treated seeds. We started with the middle concentration to leave room for adjustment in the following screens if none or perhaps only sterile *diz* mutants are found. The primary root axis of the seedlings had developed during 7d under control conditions, and the seedlings were scored for lateral root development in the next 7d on 500 µM ZnSO4. The first screen yielded 26 potential *diz* mutants that had a more than usual lateral root growth compared to the bulk of other mutants. The second screen was performed using the 0,25% EMS treated pool and yielded only three potential *diz* mutants. Therefore, the remaining two screens that could be executed in the remaining time again utilised 0,5% EMS treated seed pools yielding another 9 and 15 potential *diz* mutants respectively. The figure below depicts one such selected *diz* mutant and the deviating phenotype it displays.

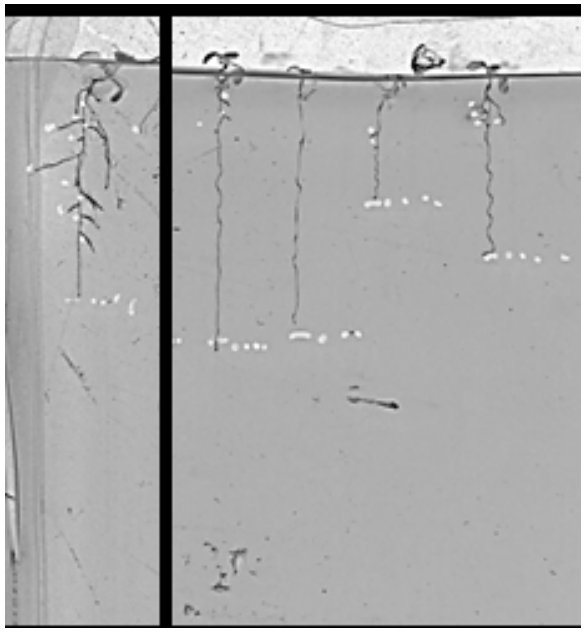

FIGURE 9: *diz* mutant screen
On the left a selected *diz* muntant from the screen, on the right other seedlings in the same screen, not displaying the desired phenotype.

3.5. Confirmation of diz mutants

From the total 32 *diz* mutants selected from the four *diz* screens, five were unable to cope with the rescue from the agar plate and transfer to growth on sand and died. Another 15 plants only formed a rosette but lacked stele and flower formation. Of the remaining 12 mutants, only 9 produced siliques and seeds. These seeds were subjected to a confirmation experiment to find out if the causal mutation was passed on to the next (M3) generation, which should be expected as A. thaliana is self-fertilizing, and the selected M2 plants were either homozygous mutant for a recessive mutation or, less likely but possibly, heterozygous for a dominant mutation. Also, formation of more seeds from the same genotype allowed a more reliable confirmation, compared to a decision based on one seedling during the screen that is more prone to identification of false positives. Only one *diz* mutant has been confirmed so far, displaying the *diz* phenotype where other mutants again display the wild type phenotype. The figure below depicts the seedlings from the confirmed *diz* mutant and a non-confirmed mutant, displaying the wild type phenotype.

CONCLUSION

Though this research hasn't provided hard evidence or pinpointed genes responsible for the effect on *Arabidopsis* roots caused by exposure excess zinc, the very first steps have been undertaken and the first hints towards the involvement of genes in this effect were found. The successful complementation using the phyothormone auxin presented a mechanism capable of overwriting the inhibitory effect that zinc excerts on lateral root growth and thus confirming the hypothesis that genes are involved in the effect. Confirmation should be achieved by repeating the experiments. Pending the discovery of genes, the underlying mechanisms can be further explored by means of a metal-content determination of wild type *A.thaliana* seedlings subjected to an auxin complementation experiment. Exploring the possibility of the induced lateral root number and growth and their uptake of a range of zinc levels provided by the media could further enlighten the processes used by excess zinc.

Forward genetic screening needs an easy, robust and reproducible visible screening system. The establishment of the screening conditions has been fulfilled and is the basis of the identification of potentially many genes involved in the Zn effect. The existence of responsible genes has been confirmed by the mere excistence of *diz* mutants and possibility to create such *diz* mutants by EMS mutagenesis, which causes point mutations that could also occur randomly throughout life of a plant, although in a highly accelerated and saturated manner. Using high EMS concentrations, a high mutation rate is achieved which increase the possibility of finding the desired mutation, facilitating the screen. Through the improvements in next generation sequencing technology, no extensive backcrossing for purification of the causal mutation is necessary neutralizing another downside of the usage of high EMS concentrations.

The confirmed *diz* mutants can provide the genome necessary for locating the causal mutation and revealing the underlying genes. The localisation of these genes can be accomplished by usage of the next generation sequencing technique combined with the SHOREmap technology at hand today. Providing a cost effective, time effcicient technique further research will benefit if this method is considered. A next step in the discovery of causal genes would be a backcross with the parental phenotype Col-0 to reveal segregation pattern in F2, which reveals if the confirmed *diz* mutant makes us of a single or multi locus mutation. The F2 *diz* mutants will then be selected and pooled, after which the pooled genomes will be sequenced.

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SUPPLEMENTAL DATA

Supplemental table 1: Nutritional value of B5/50 media

Macronutrients	MW	B 5/50 mg/L	Final concentration B5/50 mM
$(NH_4)_2SO_4$ Sigma-Aldrich, St louis,USA	132.14	2.68	0,02
$MgSO_4$ Sigma-Aldrich, St louis,USA	120.37	2.44	0.02
$CaCl_2$ Sigma-Aldrich, St louis,USA	110,98	2,26	0,02
$NaH_2PO_4H_2O$ Sigma-Aldrich, St louis,USA	156.01	3.39	0.02
KNO_3 (Merck, Darmstadt, Germany)	101.10	50	0.49
Micronutrients			
H_3BO_3 (Merck, Darmstadt, Germany)	61.83	0.06	0.970
$MnSO_4.H_2O$ (Merck, Darmstadt, Germany)	169.02	0.2	1.183
$CuSO_4.H_2O$ (Merck, Darmstadt, Germany)	249.69	0.025	0.100
$ZnSO_4.H_2O$ (Merck, Darmstadt, Germany)	287.56	0.04	0.139
$Na_2MoO_4.2H_2O$ (Merck, Darmstadt, Germany)	241.95	0.005	0.021
$CoSO_4.H_2O$ Sigma-Aldrich, St louis,USA	155	0.00032	0.0021
KI (Merck, Darmstadt, Germany)	166	0.015	0.090
Fe			
$FeNO_3.9H_2O$ (Merck, Darmstadt, Germany)	404	0.808	0.002

0,5g/L MES buffer was added to the solution before pH adjustment

For the preparation of B5/10 media, the B 5/50 was 5 times diluted.

Supplemental table 2 Nutritional content MS medium

Component	¼ Murashige and skoog basal salt mixture (MS) M5524 (mg/L)
Ammonium Nitrate	412,5
Boric Acid	1,55
Calcium Chloride	83,05
Cobalt Chloride.6H_2O	0,00625
Cupric Sulfate.5H_2O	0,00625
Na_2-EDTA	9,315
Ferrous Sulfate	6,95
Magnesium Sulfate	45,175
Manganese Sulfate	45,175
Manganese Sulfate.H_2O	4,225
Molybdic Acid (Sodium)	0,0625
Potassium Iodid	0,2075
Potassium Nitrate	475
Potassium Phosphate	42,5
Zinc Sulfate.7H_2O	2,15

supplemental table 3: Nutritional content ½ diluted Hoagland, used to grow the rescued *diz* mutants

Hoagland stock solutions						1x Hoagland	**Our diluted Hoagland**
					conc	final	**final**
Macro elements	**10x**		For 2L	*Mr*	mM	mM	**µM**
KNO_3	10,2	g/l	20,4 g	101,11	100,88	10,1	**505**
$Ca(NO_3)_2.4H_2O$	7,08		14,16 g	236,15	29,98	3,0	**150**
$NH_4H_2PO_4$	2,3		4,6 g	115,03	19,99	2,0	**100**
$MgSO_4.7H_2O$	4,9		9,8 g	246,48	19,88	2,0	**100**
	6/10000ste				conc	final	
Fe	**Toevoegen**		For 250 mL	*Mr*	mM	µM	
$FeSO_4.7H_2O$	7,6	g/l	1,9 g	278,02	27,34	16,4	**1,64**
Na_2-EDTA	5,0		1,25 g	372,24	13,43	8,1	**0,81**
					conc	final	
Micro elementen	**1000x**			*Mr*	mM	µM	
H_3BO_3	2,86	g/l		61,83	46,26	46,3	**4,63**
$MnCl_2.4H_2O$	1,81			197,91	9,15	9,1	**0,91**
$CuSO_4.5H_2O$	0,08			249,68	0,32	0,3	**0,03**
$H_2MoO_4.H_2O$	0,09			161,95	0,56	0,6	**0,06**
$ZnSO_4.7H_2O$	0,22		ZnCl2	136,28	1,61	1,6	**0,16**

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A forward genetics screen towards identifying mechanisms and genes involved in lateral root inhibition by excess zinc

Richting: **master in de biomedische wetenschappen-milieu en gezondheid**
Jaar: **2013**

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