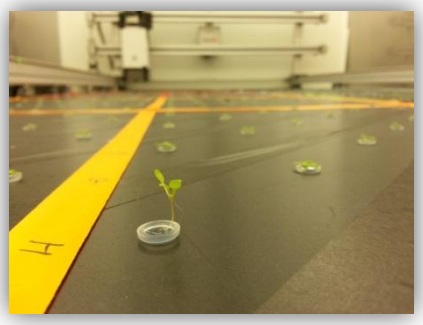
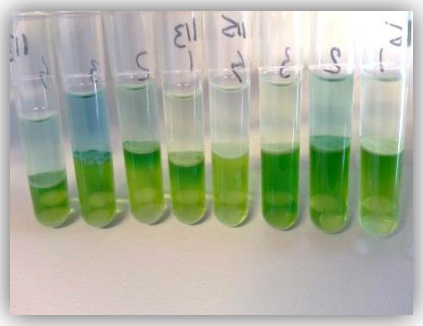
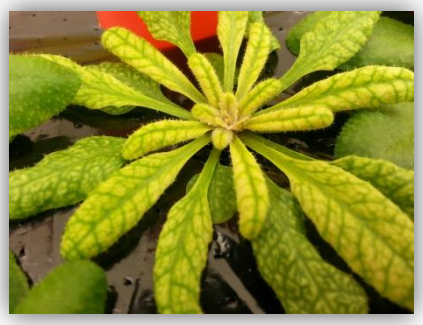
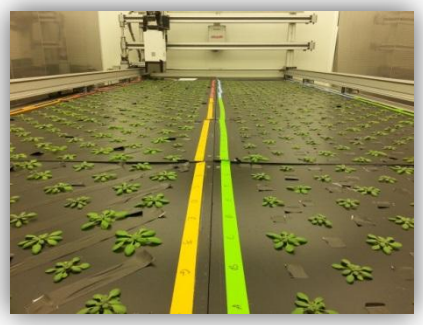
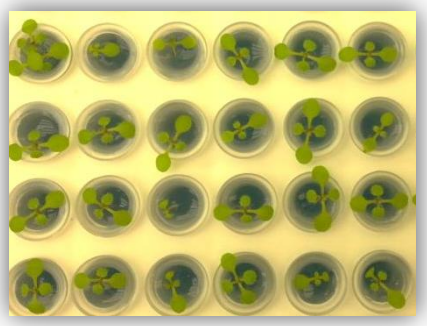
MSc. Thesis (GEN-80436)

Validation of candidate genes involved in Zinc homeostasis in *Arabidopsis thaliana*



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Abstract

Zinc is an essential micronutrient for all organisms. It is involved in a wide spectrum of biological processes and essential for normal functioning of organisms. Large areas of the world suffer from Zinc deficiency, which is reflected in the plants performance, and in human health. One third of the human population is at risk of Zinc deficiency, because of feeding on Zinc deficient crops.

Zinc homeostasis is the network accounting for ideal and balanced concentrations within the organism. Genes which play a critical role in Zinc homeostasis can be pointed out by screening natural populations of *Arabidopsis thaliana* for allelic diversity.

Genes identified by applying a Genome Wide Associating Study were validated by growing their respectively T-DNA insertion knock outs and exposing them to Zinc sufficiency and Zinc deficiency treatments. The physiological effect of Transfer-DNA insertion knock outs was tested based on fresh weight, dry weight, growth rate, photosynthesis efficiency and Ionome profile. Each trait was tested with a Two-way ANOVA, which was expanded with a post hoc two sided Dunnett test to define significant difference in the performance between the wild type background and T-DNA insertion line between the two treatments. Also, performance ratios were calculated for each T-DNA insertion line in Zinc deficiency compared to Zinc sufficiency, normalized on the wild type background performance.

Based on the performance ratio of each individual trait and the overall score I identified the top worst and best performing T-DNA insertion knock out lines out of the 45 candidate genes which were tested in this thesis. The selected candidate genes could reveal more insights in the overall Zinc homeostasis in plants.

Table of Contents

1. Introduction

Zinc (Zn) is an essential micronutrient for all organisms. Zinc is, in its oxidized state 2+, a strong electron acceptor and flexible in geometry. Traits which are ideal to catalyse processes, function in protein-protein interactions, transport proteins and being part of a structural component of proteins (Krämer and Clemens 2006, Broadley, White et al. 2007). Zinc is required as a co-factor in processes like transcription, splicing, translation, tRNA syntheses and is involved in signal pathways (Krämer and Clemens 2006, Broadley, White et al. 2007). Zinc is also involved in the protection against abiotic and biotic stress factors (Marschner 1995). Because Zn is able to neutralize the reactive oxidative species (ROS), which are the result of long exposure to stress conditions (Marschner 1995).

1.1 Zinc Homeostasis in plants

Zinc is an essential micronutrient for plants involved in a wide spectrum of biological processes, therefore a balanced Zn concentration is required. Zinc is acquired by the roots from the soil, is transported by the xylem to the upper parts of the plant and its concentration is controlled by binding to the cytoplasm of the cell. A continuous influx and balanced concentration of Zn in plants is needed to guarantee a balanced Zn concentration in the plant, in which Zn homeostasis accounts for (Sinclair and Kramer 2012).

Zinc homeostasis depends on the genotype, which is reflected in the efficiency of a plant to deal with fluctuations in the external environment to have a stable intern environment (Sinclair and Kramer 2012). Uptake, transport, storage and detoxification of Zn are crucial for Zn homeostasis. Without transporters the plant will be unable to translocate Zn and show symptoms of Zn deficiency in case of shortage, or will result in a toxic state if an excess of Zn cannot be redistributed (Sinclair and Kramer 2012). Several factors influence the efficiency of Zn uptake by the plant, for example root morphology, root-soil interface, mining capacity, number of lateral roots, diameter of the roots, length of the roots, and symbioses with mycorrhiza (Richard, Pineau et al. 2011, Sinclair and Kramer 2012).

Zinc uptake is always symplastic, but Zn translocation is symplastic or apoplastic between the root cells. Efficiency of Zn uptake shows a strong correlation with the activity and number of transmembrane transporters in the root plasma membrane. Specificity and turnover time of the transporters determine the efficiency of Zn uptake (Rengel 2001). Zinc in the roots, will be stored in root vacuoles or transported to the pericycle.

Symplastic uptake and transport to the xylem is mediated by Zinc Regulated Transporters (ZRT) and Iron Regulated Transporters (IRT), which are part of the ZRT-IRT-like protein (ZIP) family. This family consists out of 15 members, ZIP1-12 and IRT1-3, of which ten members are upregulated in Zn deficiency (Grotz, Fox et al. 1998). Two transcript factors bZIP19 and bZIP23 are thought to regulate Zn deficiency response in *Arabidopsis thaliana*. Under Zn deficiency bZIP19 and bZIP23 bind to the Zn deficiency responsive element (ZDRE) and up regulate transcription of ZIP genes, which results in an increased Zn uptake. A double mutant knock out bzip19-23 is therefore hypersensitive to Zn deficiency (Assunção, Herrero et al. 2010, Jain, Sinilal et al. 2013).

Zinc, which is translocated symplastic, will be stored in root vacuoles or transported through the cortex. Apoplastic translocation continues through the endodermis and

Casperian strip to the pericycle (Richard, Pineau et al. 2011, Sinclair and Kramer 2012). When Zn reaches the pericycle, it is translocated by the Heavy Metal ATPases (HMA), Ferric reductase Defective 3 (FRD3), and Plant Cadmium Resistance 2 (PCR2) to the shoots (Pineau, Loubet et al. 2012, Sinclair and Kramer 2012). This process is summarized in figure 1, under normal (left) and Zn deficient (right) conditions. Zinc deficiency upregulates the bZIP19-23 transcription (Sinclair and Kramer 2012, Rogers and Guerinot 2002, Durrett, Gassmann et al. 2007, Pineau, Loubet et al. 2012).

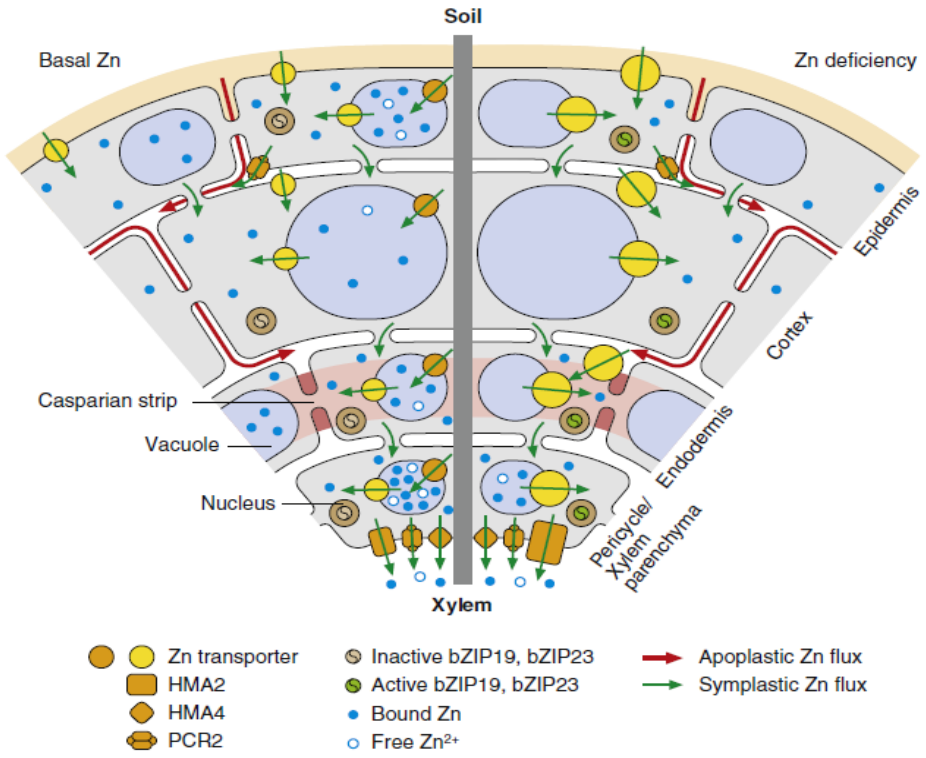

Figure 1: Overview of symplastic (green arrow) and apoplastic (red arrow) translocation of Zn at the root level in Zn sufficieny and Zn deficiency conditions. Zn bound to insoluble complexes is illustrated as a blue dot and soluble Zn is displayed as a blue circle. Transcription of Zn Fe protein (ZIP) family is activated by transcription factors bZIP 19 and bZIP 23 when the plant is exposed to Zn deficiency. Transporters involved in transporting Zn to the pericycle and xylem are Zn transporters, Heavy Metal ATPases 2 (HMA2), Heavy Metal ATPases (HMA4) and Plant Cadmium Resistance 2 (PCR2) (Sinclair and Kramer 2012).

Nicotianamine (NA) serves as a metal chelator. It is ensures Zn cell to cell movement, and transport in the xylem and phloem (Haydon, Kawachi et al. 2012). NA binds to Zn and guarantees a directed transport of Zn molecules. NA is also responsible for the transport of Zn from senescing leaves to the phloem (Waters and Grusak 2008, Stein and Waters 2012). Metal transporter proteins (MTP) are responsible for the efflux of metals chelating compounds, including Zn. They are also known as metal tolerance proteins (Krämer, Talke et al. 2007).

NA, HMA4, and MTP activity together with transcription factors bZIP19-23 are up regulated in plants which are exposed to Zn deficiency. Which are up regulated in hyper accumulators as well (Sinclair and Kramer 2012). Members of NA, HMA, ZIP and MTP genes are a few examples, there are a lot of other genes involved in Zn homeostasis of which I explored more in this thesis.

1.2 Availability of Zinc in nature and problems of Zinc deficiency and excess

1.2.1 Zinc availability in nature

Zinc accounts for 70 µg g^{-1} of the Earth's crust. Zinc increased in concentration with an oxygen enriched atmosphere. Although, concentrations of Zn in nature show large differences (Marschner 1993, Sinclair and Kramer 2012).

Composition of the soil affects the concentration of Zn in the soil. Soils which consist for example out of quartz, have a lower Zn concentration compared to soils which lack quartz. This also applies to sandy, phosphorous and clay soils. Humidity of the soil also affects the concentration of Zn. Zinc is unstable in an aqueous environment and its concentration is higher in dry soils, compared to high humidity environments. Next to the composition and the humidity level of the soil, the pH determines the solubility of Zn. A high pH in the soil correlates with a low solubility of Zn, which hardens Zn uptake from the soil by plants (Marschner 1993, Sinclair and Kramer 2012).

Mankind influences the bio-availability of Zn in the soil as well. Industries, Zn mines and applications of fertilizers in agriculture resulted in heavily polluted Zn soils. In some cases it resulted in toxic circumstances for plants and organisms (Sinclair and Kramer 2012). On the other hand, Zn deficiency is a worldwide problem, there are large areas which show Zn deficiency (Figure 2). It has a huge impact on the environment and the organisms within those areas (Alloway 2004).

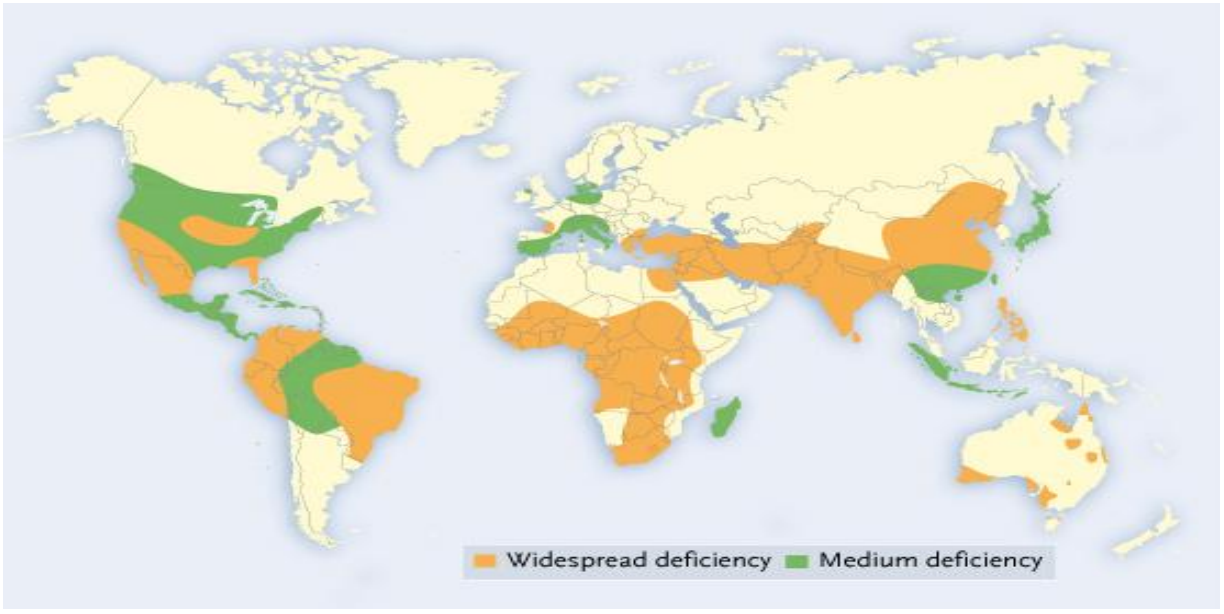

Figure 2: World map of Zn concentration in the soil. Soils which show a widespread Zn deficiency in the soil are marked orange. Area's which show medium Zn deficiency are marked green. Figure retrieved from: http://www.fertilizer.org.

1.2.2 Symptoms of Zinc deficiency and excess in plants

Susceptible plants which are exposed to Zn deficiency (<20 µg Zn per g leaf dry weight) or Zn excess (>100 µg Zn per g leaf dry weight) present an unbalanced Zn homeostasis (Marschner 1995, Sinclair and Kramer 2012). This results in Zn deficiency symptoms or toxic state, and trouble the functioning of the plant.

First response of Zn deficiency in a susceptible plant, is reduced Zn transport from the roots to the shoots. This also applies to transport from old to new plant tissue. Therefore young leaves will show the first symptoms of Zn deficiency (Hacisalihoglu and Kochian 2003). Symptoms of Zn deficiency are: chlorosis of young leaves, smaller leaf size, stunted growth, thin stems, wilting, curling, lower biomass production, and necrosis in plants (Hacisalihoglu and Kochian 2003).

Stress tolerance against other biotic and abiotic stresses is reduced under Zn deficiency. Zn is required for the production of Super oxide dismutase (SOD). SOD neutralizes radicals, which are the result of ROS. ROS will accumulate during the exposure of stress and will damage membranes, membrane proteins, chlorophyll and enzymes (Hacisalihoglu and Kochian 2003, Sinclair and Kramer 2012). If ROS are not neutralized, it will result in a hyper sensitive response and lower the plant performance.

Zinc excess will result in toxic conditions for plants. Stunted growth and chlorosis are observed. Uptake of Phosphor (P), Manganese (Mn) and Magnesium (Mg) are also reduced and result in a poor overall plant performance (Broadley, White et al. 2007).

Plants which are susceptible to Zn deficient or excess environments will show effects on their fitness (Cakmak 2008). Although there are tolerant plants, which are able to survive under Zn deficiency or excess conditions. Plant natural populations contain therefore a large variation in genotypes for Zn deficiency and Zn excess tolerance (Alonso-Blanco, Aarts et al. 2009). Insights in their genetic variation will teach us more about Zn homeostasis.

1.2.3 Correlation Zinc deficiency in soils and mankind

The World Health Organisation (WHO) states: Zn deficiency is a problem for one third of the world's population. Recommended daily intake is 15 mg of Zn (Allowances 1989). Whole grain wheat, which is consumed in large portions, contains 20 to 35 mg of Zn per kg (Rengel, Batten et al. 1999). Which will result in large amounts of consumption to meet the daily uptake of Zn. Increased concentration of Zn in crops are therefore important to overcome Zn deficiency.

Symptoms of Zn deficiency in mankind are: reduced growth rate of children, reduced taste, anorexia, impaired brain development, lower immune response, skin disorders and diarrhoea (Sinclair and Kramer 2012, C. Dye 2013).

Plants which are susceptible to Zn deficiency will have a low concentration of Zn, if they are located in an area which is low in Zn concentration (Cakmak 2008). They cannot take up Zn, since it is simply not there in sufficient amounts. Therefore, we see a correlation between soils which show Zn deficiency and the degree of risk to be exposed to Zn deficiency in humans (Figure 3) (Alloway 2004). People in South and Central America, Africa, and large parts of Asia are at a high potential risk of Zn deficiency. Those areas overlap with the world map of Zn deficiency in soils (Figure 2) (Alloway 2004). While the United States of America do no not have a significant overlap, due to a varied diet.

Plants serve as a main food source in third world countries (Alloway 2004). Plants contain phytatic acids and polyphenols, which make it difficult to take up Zn from the diet. Phosphate groups of phytatic acids react with Zn and result in insoluble compounds (Rengel, Batten et al. 1999). Phytase, a phosphytase which functions as an enzyme, is required to detach Zn from those insoluble compounds. Although, this enzyme is almost

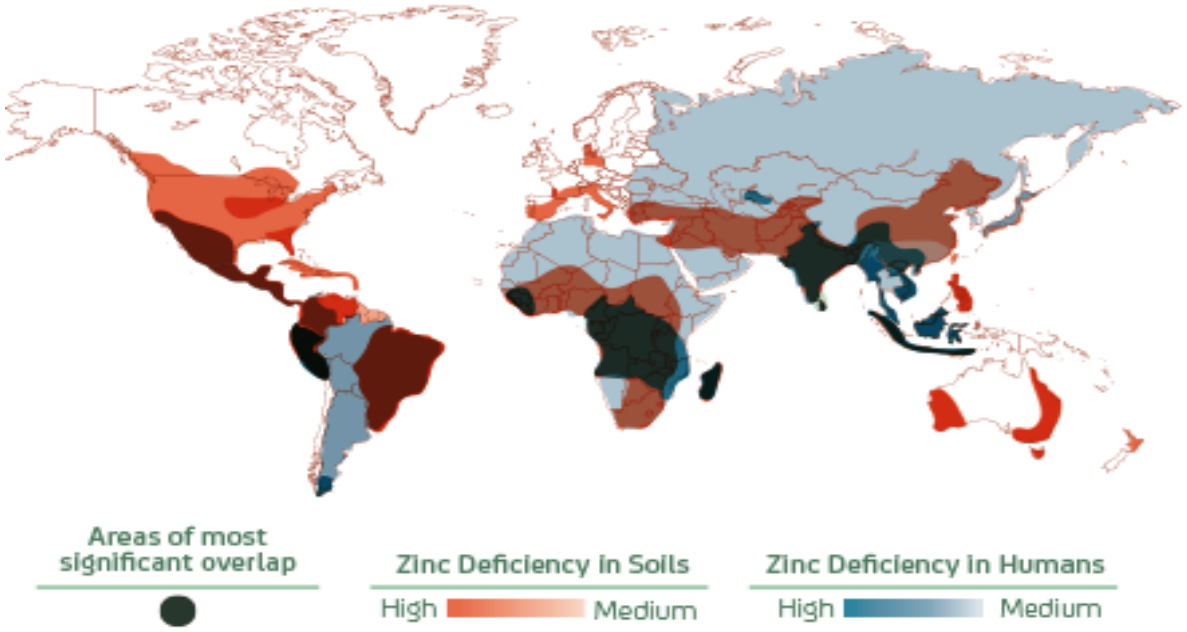

Figure 3: Integrated world map of Zn deficiency in soils (Blue) and the risk in human populations (Red) to be exposed to Zn deficiency. Significant overlap is marked black. Image retrieved from: http://rootsforgrowth.com/soilhealth/.

absent in humans. Polyphenols bind to enzymes and other proteins as well, lowering their efficiency. This results in a lower uptake of Zn by humans, if they are able to eat sufficient amounts of food (Rengel, Batten et al. 1999, Lönnerdal 2000, Cakmak 2008, C. Dye 2013). Efficient uptake of available Zn deserves therefore special attention as well.

There is a clear correlation between Zn deficiency in soils and mankind, which consume the crops on those soils (Figure 3). Insight in Zn homeostasis of plants which are able to survive under Zn deficiency circumstances, could reveal genotypes and pathways which are helpful to reduce Zn deficiency in mankind.

1.3. Need to study Zinc homeostasis in plants

Consumption of large amounts of staple foods, low variation in the diet or nothing to consume, contribute to Zn deficiency in mankind. Solutions for this problem need to be found.

In plant pathways which are activated at an excess of Zn could correlate with pathways involved in Zn deficiency tolerance (Sinclair and Kramer 2012). Plants which are Zn deficient tolerant have developed a more efficient translocation of Zn from the roots towards the shoots. This also applies to storage in edible parts of the plant (Wu, Lu et al. 2010, Sinclair and Kramer 2012). This agronomic trait can contribute in the sustainable and cost-effective method to alleviate Zn deficiency in mankind (Cakmak 2008).

1.3.1 How to study Zinc homeostasis in plants

Understanding Zn homeostasis and the genes involved in Zn Homeostasis are essential to increase Zn availability in plants. Genetic variation coupled to the phenotypic performance of plants gives insights into Zn homeostasis.

A. thaliana is a suitable plant for research due to its short generation time, small size, complete genome sequence and the availability of T-DNA insertion knock outs for almost

every gene. In addition, large amounts of seeds are obtained through selfing and will result in homozygote lines in natural populations, which makes it suitable to study natural variation (Mitchell-Olds and Schmitt 2006, Alonso-Blanco, Aarts et al. 2009).

Figure 4: *A. thaliana* grown in hydroponic medium without Zn. The phenotypic shoot difference underlined the genetic variation present within this accession, and therefore the performance of the plant (Campos 2015).

Study of natural variation will help us to understand and use this genetic variation to select genes correlated with Zn deficiency tolerance. Atala Lombelo Campos, 2015 indicated that natural variation for Zn deficiency tolerance is present within the HapMap population of *A. thaliana*. The HapMap is a collection of 360 natural accessions of which most are sequenced. The specific haplotypes present within certain accessions could correlate with a specific trait, for example tolerance in Zn deficiency. This variation is reflected at the physiological level, as is illustrated in figure 4 for the shoots in a hydroponic medium without Zn.

Genome wide association study (GWAS) is a reverse genetics method, to correlate a phenotypic trait to Single Nuclear Polymorphism (SNP) markers (Korte and Farlow 2013). GWAS is a similar technique to Quantitative Trait Loci (QTL) mapping. Although QTL analysis is performed over large intervals and only able to correlate allelic diversity of a genetically related population. QTL analysis therefore requires a cross between two known parents and looks for recombination in certain regions of the genome. GWAS is able to point out associated SNP's in the whole genome and across different populations. The mapping resolution is therefore higher in GWAS, compared to QTL studies (Korte and Farlow 2013). Nevertheless, GWAS is not able to point out epistatic gene interactions and false positives are likely to be present (Korte and Farlow 2013). Therefore, an additional selection tool which can be integrated into GWAS analysis is gene expression data to avoid false positives.

Natural variation for Zn homeostasis is present within *A. thaliana* (Campos 2015). This contributes to pin point candidate genes which are essential for a balanced Zn homeostasis under Zn deficiency and excess. GWAS is a powerful tool to define those candidate genes for natural variation experiments. Integrating QTL mapping into GWAS is the best selection method and will complement each other (Korte and Farlow 2013).

1.3.2 Strategies to study genes function: T-DNA insertion knock out mutants

GWAS analysis is able to point out genes which could contribute to Zn homeostasis. Silencing or over expression of the gene will reveal more insight about the function and involvement of genes in metabolic pathways, which is reflected in the phenotype of the plant under specific conditions (Wang 2008).

Genes can be over expressed by fusing their coding sequence with the Cauliflower Mosaic 35S promoter. Or a gene can be knocked out by disrupting its sequence with a Transfer-DNA insertion (T-DNA) (Krysan, Young et al. 1999).

T-DNA insertion mutants are a valuable tool for reverse genetics to determine the direct correlation of the gene with the corresponding phenotype. The location of T-DNA insertion is important for the inactivation of the candidate gene. If the insertion is in the middle of the gene it will disrupts its function and result in a knock out. If the T-DNA is inserted at the promoter, it may result in a reduced expression of the gene (Krysan, Young et al. 1999, Wang 2008).

Due to the use of T-DNA insertion mutants, biomass production of the plant will change. Based on this difference in biomass production under Zn deficiency and sufficiency the function of the gene can be correlated to Zn homeostasis. This method is also known as Zn efficiency (ZnE) and is the most used method to define Zn deficiency effects (Marschner 1995, Rengel and Graham 1996, Sinclair and Kramer 2012, Campos 2015).

Due to the T-DNA insertion the Ionome of the plant will change as well if the gene's function is related to an element. The Ionome comprises the element composition within a plant. Which is based on the change of the element concentration in response to physiological stimuli, the developmental stage, and genotype. Therefore, the functional stage of the plant can be examined, for example in relation to Zn homeostasis under Zn deficiency conditions (Salt, Baxter et al. 2008).

Therefore, I hypothesised GWAS is able to determine candidate genes, which are involved in Zn deficiency tolerance, within the HapMap population. Which allowed me to correlate the phenotypes of T-DNA insertion knock outs in relation to the candidate genes in Zn homeostasis, due to exposure of Zn deficiency and sufficiency treatments.

2. Material and methods

2.1 Candidate genes and knockout mutants

Previously, a GWAS analysis was done with the HapMap population of *A. thaliana*. In the HapMap 360 natural accession were tested under normal and deficient Zn conditions. The response of these accessions to the two Zn conditions applied was measured in terms of their Ionome at shoot and root level. The GWAS was conducted combining the Ionome data and a data set of 250000 SNPs in the genome of *A. thaliana*. The most highly associated SNPs were determined to select a list of 46 candidate genes (appendix A). Selection criteria were: logarithm of the odds (LOD), linkage disequilibrium (LD), occurrence with several elements, gene ontology and expression profile. If possible at least two T-DNA lines for each candidate gene were selected to double validate the gene function. No suitable lines were available for five candidate genes. Resulting in 41 candidate genes with 70 T-DNA insertion lines. In addition from a gene expression study six more genes were selected with eleven T-DNA insertion lines, resulting in a total of 81 T-DNA insertion lines.

2.2 Growing knock outs & homozygote selection

Seeds were stratified for three days at 4°C to break the dormancy, whereupon the seeds were transferred to a growing chamber to germinate. The conditions were sixteen hours of light (24°C) with eight hours of night (24°C). Geminated seeds were transferred to Rockwool in the climate cells. In the climate cells the conditions were twelve hours of light (20°C) with twelve hours of night (15°C). The plants were automatically irrigated with hydroponic nutrient solution by flooding the table three times a week. The plants were transported after the genotyping to the greenhouse, before flowering.

T-DNA lines were genotyped to select homozygote plants. Initially, four plants per line were screened, on which CTAB DNA isolations were performed (Appendix B). The quality and concentration of the samples were checked by agarose gels and Nanodrop™ 2000c of Thermo Scientific®. DNA was diluted to a concentration of ~100 ng/µL. The PCR was performed as detailed by Promega for the GoTaq® G2 DNA Polymerase protocol. The

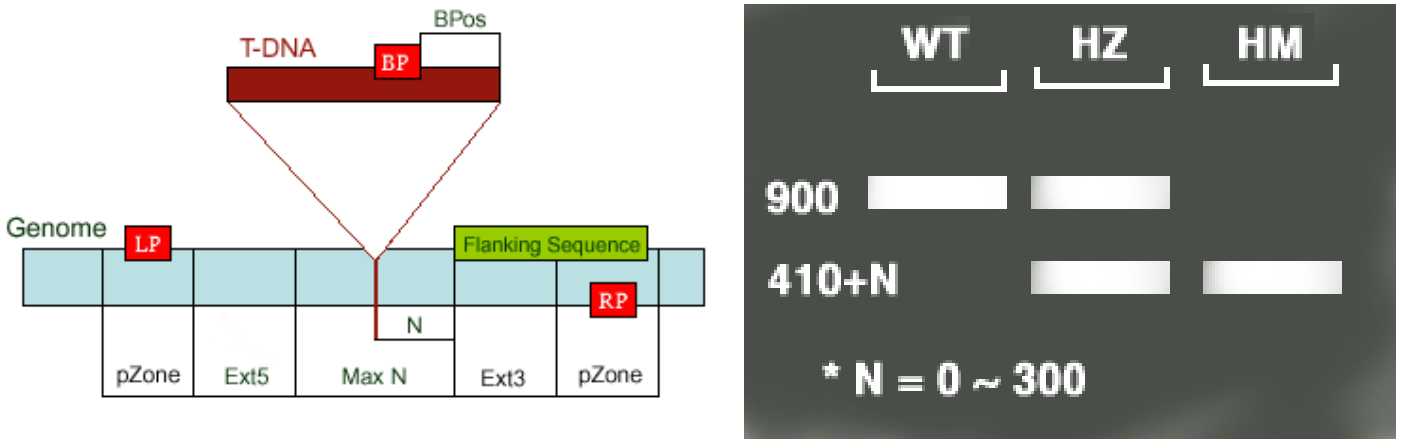

Figure 5: If a T-DNA insertion was successful an amplification product from the BP (left border primer of T-DNA insertion) to RP (right border primer) occured. This resulted in a shorter amplification product compared to the original transcription product of the gene, which was transcribed from the LP (left border primer) to the RP (right border primer). N=difference of insertion site BPos=distance BP to insertion site. In heterozygote individuals both amplifications products were present and resulted in a specific band pattern in the electrophoresis gel. Based on the amplification products, it was possible to discriminate the homozygous wild type (≈900-1300 bp), true knock out (≈400-900 bp) and heterozygote (which contain both amplification products) offspring. Image retrieved from: http://signal.salk.edu/.

primers were designed in the SALK website: http://signal.salk.edu/ or manually with Primer3 v. 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm) (Appendix C). The PCR amplification product was loaded in an agarose gel to distinguish homozygote's wild types from heterozygote's and homozygous T-DNA insertion knock outs, due to the usage of three primers (Figure 5). Seeds of homozygous T-DNA insertion lines were harvested for the validation step and heterozygous plants were grown again to give rise to ¼ homozygous T-DNA insertion offspring.

2.3 Substrate selection for Zinc deficiency experiments

To know if Polygrow or Rockwool were suitable substrates to achieve Zn deficiency. Seeds were grown as stated in subject 2.2. Seeds of *A. thaliana* Col-N60000, bzip19-23 (transcript factors 19 and 23 knock out), nas (Nicotianamine synthase knock out) and At5G52720 (Copper transporter knock out) were grown on Rockwool or Polygrow. The plants were exposed to a Zn deficiency of 0µM ZnSO4; sufficiency of 4µM ZnSO4 and excess of 30µM ZnSO4. The tables were flooded three times a week with Hyponex solution with the corresponding Zn treatment.

Photosynthesis efficiency of Col-N60000 was defined by measuring the chlorophyll florescence with a fluorescence Photon Systems Instruments™ SN CCD 371 camera. Plants were placed in the dark to adapt and gradually pulses were applied. Based on the level of fluorescence the camera calculated the photosynthesis efficiency with the formula: $(F_m-F_0)/Fm=Fv/Fm$ (Maxwell and Johnson 2000). The output was analysed with the software program Fluorcam 7©.

2.4 Hydroponic physiological experiment in a phenotype platform

Beforehand, Polygrow and Rockwool were both tested as possible substrate, but neither of them was suitable for the experiment. Therefore, homozygote T-DNA insertion lines and their wild type controls (Col-N60000;Col-2 and Col-3) were grown under hydroponic conditions. Controls for Zn deficiency were nas and bzip19-23, as hyper sensitive Zn deficiency mutants. bg0011 was used for ß-glucorinidase (GUS) staining. In total 55 genotypes were included of which 49 T-DNA insertion lines for 45 candidate genes.

The seeds were vapour sterilized (Appendix D). A total of twenty seeds were sown per genotype, per treatment. The seeds were sown on agar tubes, which contained a mixture of 0.5x Hoagland nutrient solution and 0.55g/L of Daishin agar. The tubes were placed in a container with 0.5x Hoagland nutrient solution (Appendix E). The containers were closed and put to a three days stratification treatment at 4°C to break the dormancy. After which they were transferred to the hydroponics room. In which the conditions were twelve hours of light (20°C) with twelve hours of night (15°C). The containers were opened after one week from

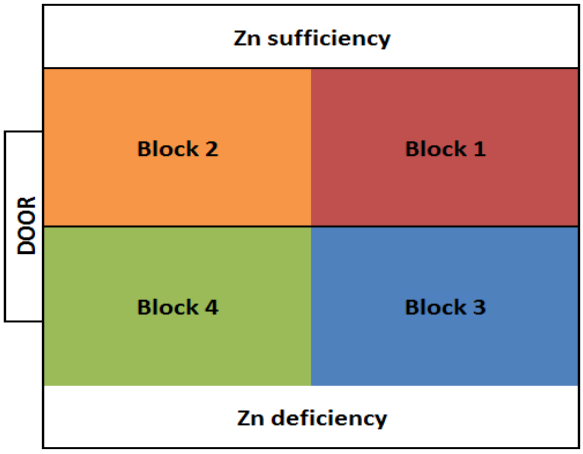

Figure 6: Setup of phenotype platform climate cell. Zinc sufficiency was applied to block 1 and 2. Zinc deficiency was applied to block 3 and 4. Per block 3 replicates were present of a genotype, which were completely randomized within each block.

germination. Ten days after germination the plants were transferred to the phenotype platform climate cell.

The design for the phenotype platform experiment was a complete randomized block design. In which the plants were randomized per block. In total twelve replicates per genotype were used of which six replicates were placed per treatment. Three replicates were placed per block (Figure 6).

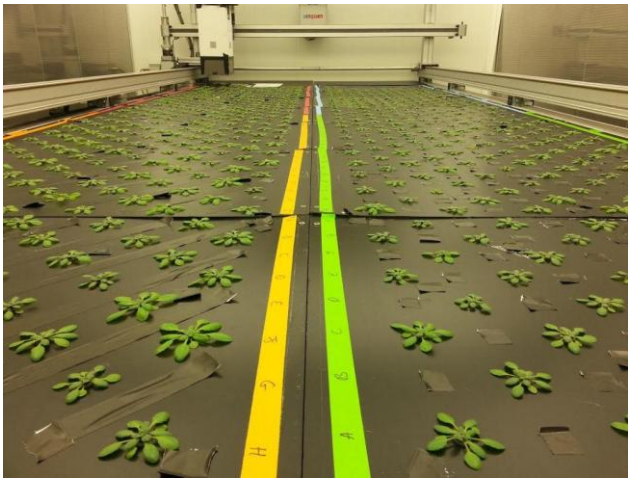

Figure 7: Phenotype platform climate cell. A high throughput camera was used to phenotype the plants in a climate controlled chamber. The left side of the table was Zn sufficient (4μM $ZnSO_4$) and the right side Zn deficient (0μM $ZnSO_4$).

The phenotype platform climate cell ensured a stable environment cycle of twelve hours of light (20 °C) with twelve hours of night (15°C). The high throughput camera of this climate cell measured six times a day photosynthesis efficiency (PAM) and rosette area (NIR)(figure 7). Every plant was photographed daily at the same time for several measuring points. (van Rooijen, Aarts et al. 2015).

Plants were harvested 35 days after germination. Shoots were cut, pictured, and weighted to obtain shoot fresh weight (SFW), put into a glassine bag and dried in the oven for four days at 60 °C to obtain the shoot dry weight (SDW). Harvested roots were also dried in the oven to obtain the root dry weight (RDW). SDW and RDW were defined to measure the biomass on a scale. The whole plant was taken out of the bag in this procedure, as well for the root out of the tube.

2.5 Hydroponic physiological experiment in individual trays

Seeds were pre-germinated as mentioned in section 2.4. The only difference was the 0.5x Hoagland solution in the containers of germination contained normal levels of Zn (2μM ZnSO4). After eight days from germination the plants were exposed to their corresponding treatment, Zn deficiency (0μM ZnSO4) and Zn sufficiency (2μM ZnSO4). This was, because the experiment was initially meant for the Zn excess experiment. I used 12 trays of 9 litre 0.5x Hoagland nutrient solution each. Of which half of the trays were exposed to Zn deficiency (0μM $ZnSO_4$) and the other half to Zn sufficiency (2μM $ZnSO_4$). Each tray contained 70 plants which were completely randomized. The Hoagland solution in those trays was refreshed every week in order to prevent shortage of nutrients and to ensure a stable pH.

Plant material of three plants per treatment were harvested to analyse the Ionome profile. Two middle leaves were harvested per plant, after SFW was defined. The roots were first washed with 1mM Na-EDTA (pH 8) and then washed in Milli-q (MQ) water. After this step the material was put into Eppendorf tubes and dried for 4 days at 60 °C in the oven. After SDW and RDW were defined, the material was sent to the University of Aberdeen to analyze the elemental content by Inductively coupled plasma mass spectrometry (ICP-MS).

2.6 Data analysis

After checking the data was normally distributed and had equal variances. Independent T-tests and Two-Way-ANOVA's were performed to check for significant difference between the lines, the treatment and the blocks. The statistics were executed with the software package IBM SPSS Statistics 23. On SFW, SDW, RDW, NIR, PAM and Ionome the two-way ANOVA was performed with an additional Post-Hoc two sided Dunnett test. The two sided Dunnett test was used to check for significant difference between the T-DNA line and the Col-N60000; Col-2 and Col-3 control as reference group.

Significant differences enabled me to couple the genotype of a certain phenotype by linkage analysis, and therefore validated the candidate gene's function in the Zn homeostasis pathway of *A. thaliana*.

An overview of the workflow of the project is illustrated in Appendix F.

3. Results

3.1 Optimal substrate for Zinc deficiency experiments

As a pilot experiment to achieve Zn deficiency and Zn excess the substrates Rockwool and Polygrow were tested. SFW, SDW and PAM were measured (Figure 8). None of the Zn treatments was significant different for SFW and SDW from the Zn sufficiency treatment (Table 1). The plants performed the best on Rockwool in Zn excess and sufficiency treatment. Nevertheless, there was no significant difference between the two substrates within the treatment for SFW, SDW and PAM (Table 2). Although, Polygrow showed less variance for SFW and SDW compared to Rockwool.

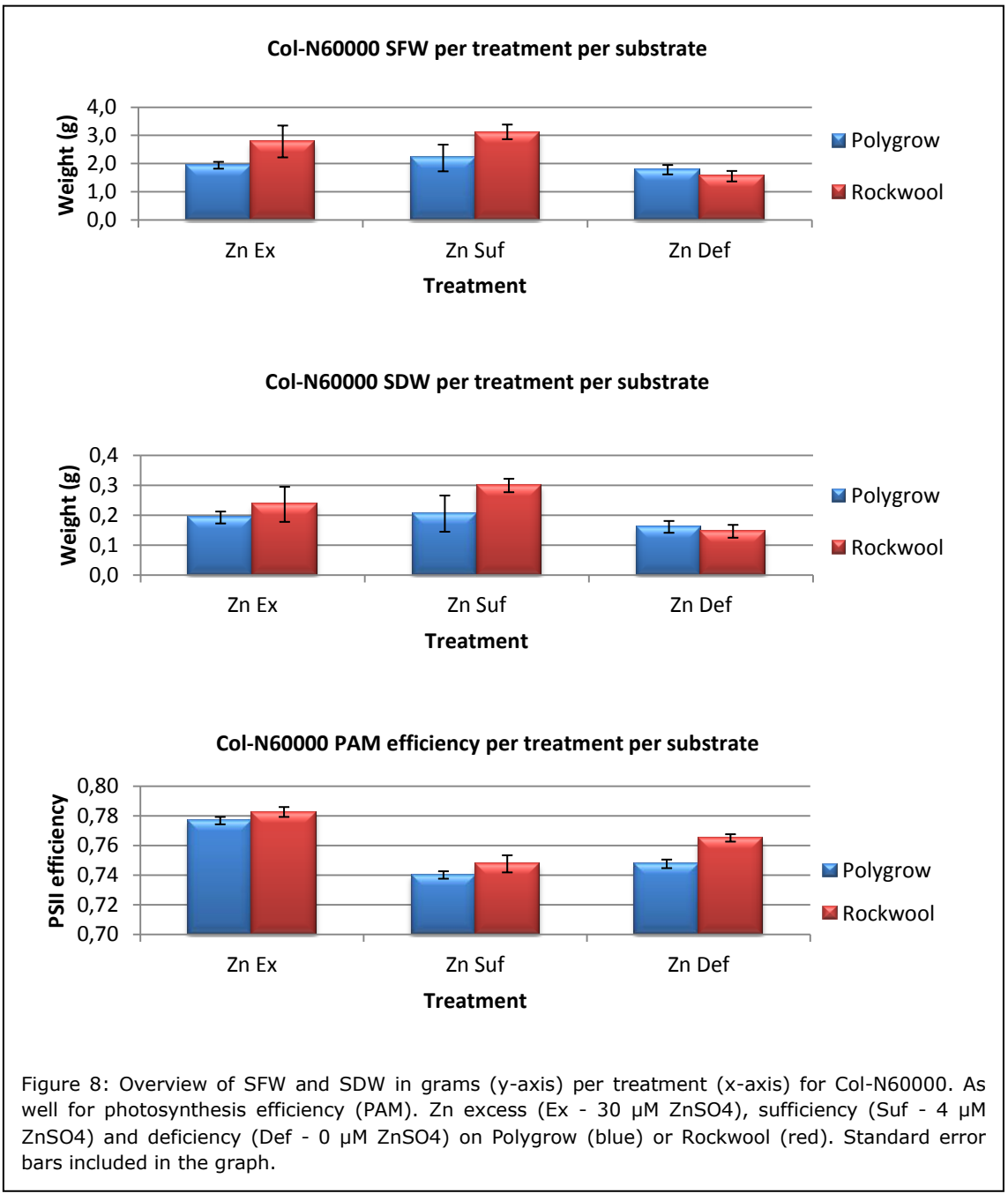

Figure 8: Overview of SFW and SDW in grams (y-axis) per treatment (x-axis) for Col-N60000. As well for photosynthesis efficiency (PAM). Zn excess (Ex - 30 µM ZnSO4), sufficiency (Suf - 4 µM ZnSO4) and deficiency (Def - 0 µM ZnSO4) on Polygrow (blue) or Rockwool (red). Standard error bars included in the graph.

Table 1: Tukey HSD to check for treatment difference

SFW and SDW compared per substrate between the treatment Zn sufficiency with Zn deficiency and Zn sufficiency with Zn excess. Mean difference, Std Error difference and two sided p-value were calculated between the treatments. No significant difference between the treatments was observed.

Trait	Treatment	Compared to treatment	Substrate	Mean Difference	Std. Error Difference	p-value
SFW	Zn sufficiency	Zn deficiency	Polygrow	0,4156	0.384	0.554
		Zn excess		0,2581	0.480	0.856
SDW	Zn sufficiency	Zn deficiency	Polygrow	0,044	0,048	0.642
		Zn excess		0,024	0.059	0.913
SFW	Zn sufficiency	Zn deficiency	Rockwool	1,2477	0,709	0.211
		Zn excess		0,625	0.622	0.583
SDW	Zn sufficiency	Zn deficiency	Rockwool	0,121	0,068	0.209
		Zn excess		0,0835	0.060	0.366

Table 2: Independent T test to check substrate effect within treatment

SFW, SDW and PAM are compared between the two substrates within the treatment. Mean difference, Std Error difference and two sided p-value were calculated between the substrates. No significant difference between the substrates was observed.

Trait	Treatment	Mean Difference	Std. Error Difference	p-value
SFW	Zn deficiency	0.23004	0.249991	0.384
	Zn sufficiency	-0.60203	0.715105	0.422
	Zn excess	-0.36034	1.271.676	0.785
SDW	Zn deficiency	0.01474	0.029206	0.627
	Zn sufficiency	0.0618	-0.06178	0.071
	Zn excess	-0.023	0.123	0.914
PAM	Zn deficiency	0.018	-0.0333	0.057
	Zn sufficiency	0.005	-0.0075	0.243
	Zn excess	0.003	-0.0175	0.374

Polygrow or Rockwool showed no significant difference between the treatments (Table 1) and within the treatments (Table 2). Therefore, neither of them was suitable for achieving Zn deficiency or excess conditions. This was due to the large variance between the phenotypes within the same treatment. Therefore, I decided to grow the T-DNA insertion knock outs on hydroponics media in the phenotype platform climate cell.

3.2 Selection of homozygote T-DNA lines

In total I had 81 T-DNA knock out lines. Out of those 81 T-DNA insertion knock outs, 70 lines needed to be confirmed homozygous T-DNA insertion (T) lines (Figure 9).

According to Nottingham Arabidopsis Stock Center (NASC) 65 out of the 70 T-DNA lines were homozygous T-DNA insertion lines, the other lines were segregating. The screening resulted in 43 (66.2%) T-DNA insertion lines contained at least one homozygous T-DNA insertion individual. Out of these 43 lines, 22 lines were complete homozygous T-DNA insertion knock out lines (33.8%). Sixteen lines (24.6%) were complete wild type (Wt) and discarded from the experiment. An overview of the 65 lines which were homozygous T-DNA insertions according to NASC and in which classes the four individuals were actually scored are illustrated in figure 10.

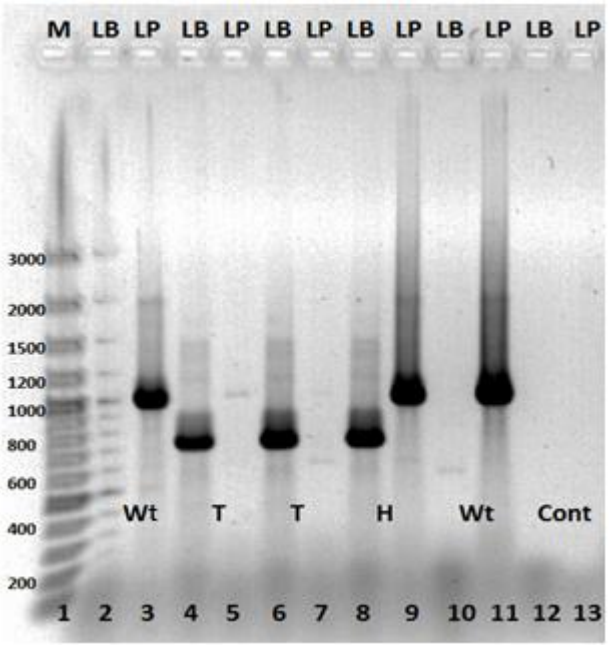

Figure 9: Example of a segregating line. The lanes were separated for each LB-RP and LP-RP amplification product. Lane 1 contained a marker (Generuler™ 100 bp Plus ladder) to distinguish the length of the amplification product in base pairs. Next pair of lanes (2 to 9) each contained DNA from a single plant per LB+LP reaction. Lane 10 and 11 contained DNA from Col-N60000. Lane 12 and 13 contained H_2O to check for contamination in the process. Based on the band pattern the lines were scored as: Wt = wild type; H = heterozygote; T = homozygous T-DNA insertion knock out.

Genotype				Nr of lines	Percentage (%)	Lines
T	T	T	T	22	33.8	37,38,41,67,68,69,73,74,82,83,87,89,91,96,98,112,117,120,122,126,129,132
T	T	T	H	5	7.7	84,119,123,141,144
T	T	H	H	2	3.1	90,99
T	H	H	H	1	1.5	64
H	H	H	H	1	1.5	66
H	H	H	Wt	1	1.5	110
H	H	Wt	Wt	0	0.0	
H	Wt	Wt	Wt	0	0.0	
Wt	Wt	Wt	Wt	16	24.6	33,40,65,76,86,88,92,97,100,115,124,125,130,133,137,140
Wt	Wt	Wt	T	0	0.0	
W	Wt	T	T	4	6.2	42,85,111,113
Wt	T	T	T	0	0.0	
Wt	-	-	-	1	1.5	121
Wt	Wt	-	-	2	3.1	26,80
Wt	Wt	H	T	1	1.5	138
Wt	H	H	-	1	1.5	104
Wt	H	H	T	2	3.1	39,107
Wt	H	T	T	2	3.1	45,108
T	T	T	-	2	3.1	135,143
T	-	-	-	2	3.1	28,48
-	-	-	-	0	0.0	
Total				65	100	

Figure 10: Scored genotype per line for T-DNA insertion efficiency. Per line it is indicated which genotype profile was found and in which percentage corresponding to the lines being complete T-DNA insertion homozygous according to NASC. Each plants was scored wild type (Wt), heterozygote (H) or homozygote T-DNA insertion knock out (T). Classification (-) corresponds with the fact no more plants were available or no amplification product was visualized on the agarose gel.

Complete heterozygous (H) T-DNA insertion lines were highly unlikely. This chance is 0.5^4=0.0625 within a line. In this case more plants were genotyped and in most cases scored as H. Therefore, it was likely the primers were unspecific and bound to homologous regions. New primers were designed for those lines and genotyped again. This resulted in six additional T-DNA insertion homozygous lines, which were originally scored as a complete heterozygote line.

Genotype profile of line 42,85,111 and 113 were highly suspicious. In an expected ratio of 1:2:1 the middle class, heterozygote, was completely lacking. Screening of the other plants within the line resulted in heterozygous genotypes. Therefore homozygous T-DNA insertion individuals in those lines could be selected.

Out of the five segregating lines corresponding to NASC, four lines contained at least one homozygous T-DNA insertion plant. All the plants which were genotyped as homozygous T-DNA insertion knock outs were used in the validation step of the candidate genes.

3.3 Validation of candidate genes in phenotype platform experiment

3.3.1 Treatment and block effect

After 31 days from germination the plants showed Zn deficiency symptoms, which were chlorotic lesions and stunted growth. The symptoms were observed at a later stage, compared to the occasional 28 days after germination. Plant material was harvested 35 after germination. To find a possible explanation for this delay in symptoms appearance, I measured the concentration of the Zn deficiency treatment. The concentration was 0.2µM Zn in the Zn deficiency treatment (appendix G).

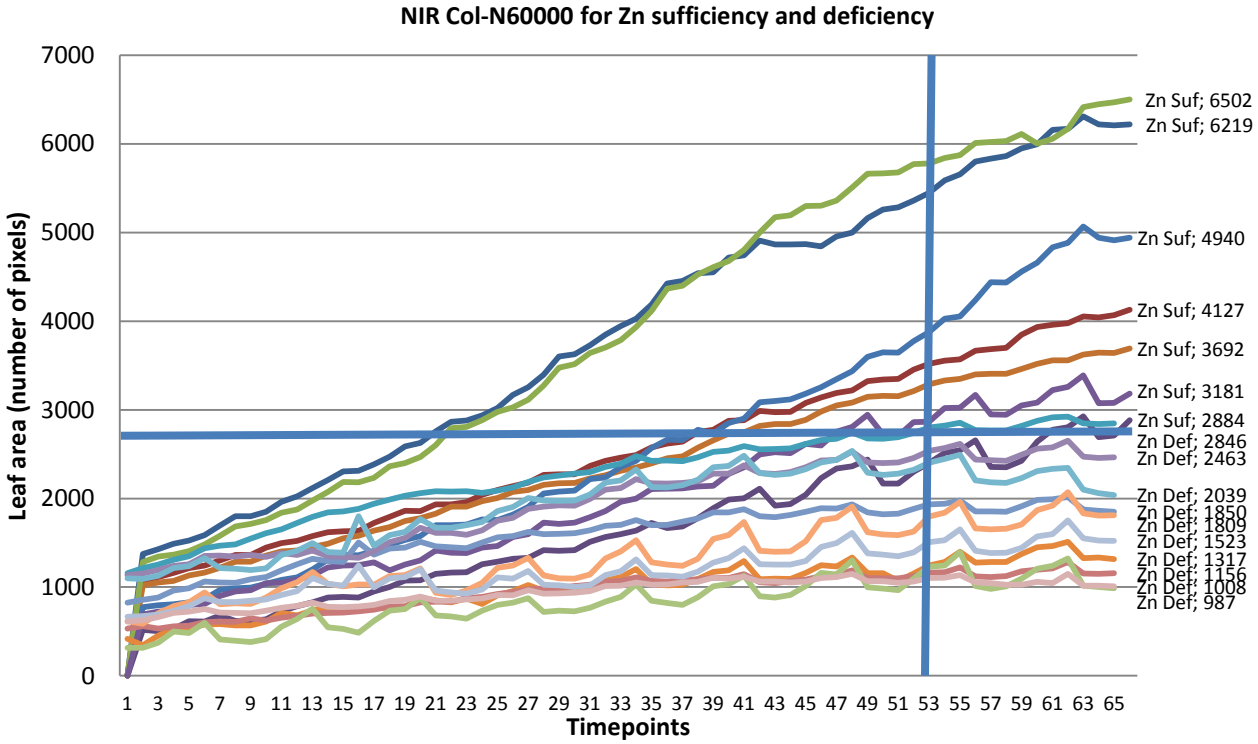

Figure 11: Illustration of set time point to check for difference between the treatments in Col-N60000.In total 65 time points have been measured. Time point 53 was selected (vertical blue bar) to distinguish two groups for the treatments Zn Sufficiency (Zn Suf) and Zn deficiency (Zn Def) (horizontal blue bar). This is 33 days from germination. Individuals of Col-N60000 were corrected for outliers.

To analyse the data for NIR and PAM one certain time point had to be taken. This reference point was 33 days after germination. Two days before harvest and two days after the first chlorotic lesions and stunted growth were visible. Two groups can be distinguished for the two treatments on day 33 for time point 53 (Figure 11).

Independent T-tests were performed to check if the treatments worked. Treatments for the traits were all significantly different, except RDW (p=0.172) (Table 3).

Table 3: Independent T test to check treatment effect

Independent T-test performed per trait to compare Zn sufficiency with Zn deficiency treatment. Mean difference, standard error difference and two sided p-value were calculated between the treatments per trait. All traits showed a significant difference for the treatments, except RDW.

Trait	Mean Difference	Std. Error Difference	p-value
SFW	-0.70558	0.0470919	0.000
SDW	-0.07017	0.0041355	0.000
RDW	-0.00137	0.0010039	0.172
NIR	-1193.89	69.9173	0.000
PAM	-0.037	0.003	0.000

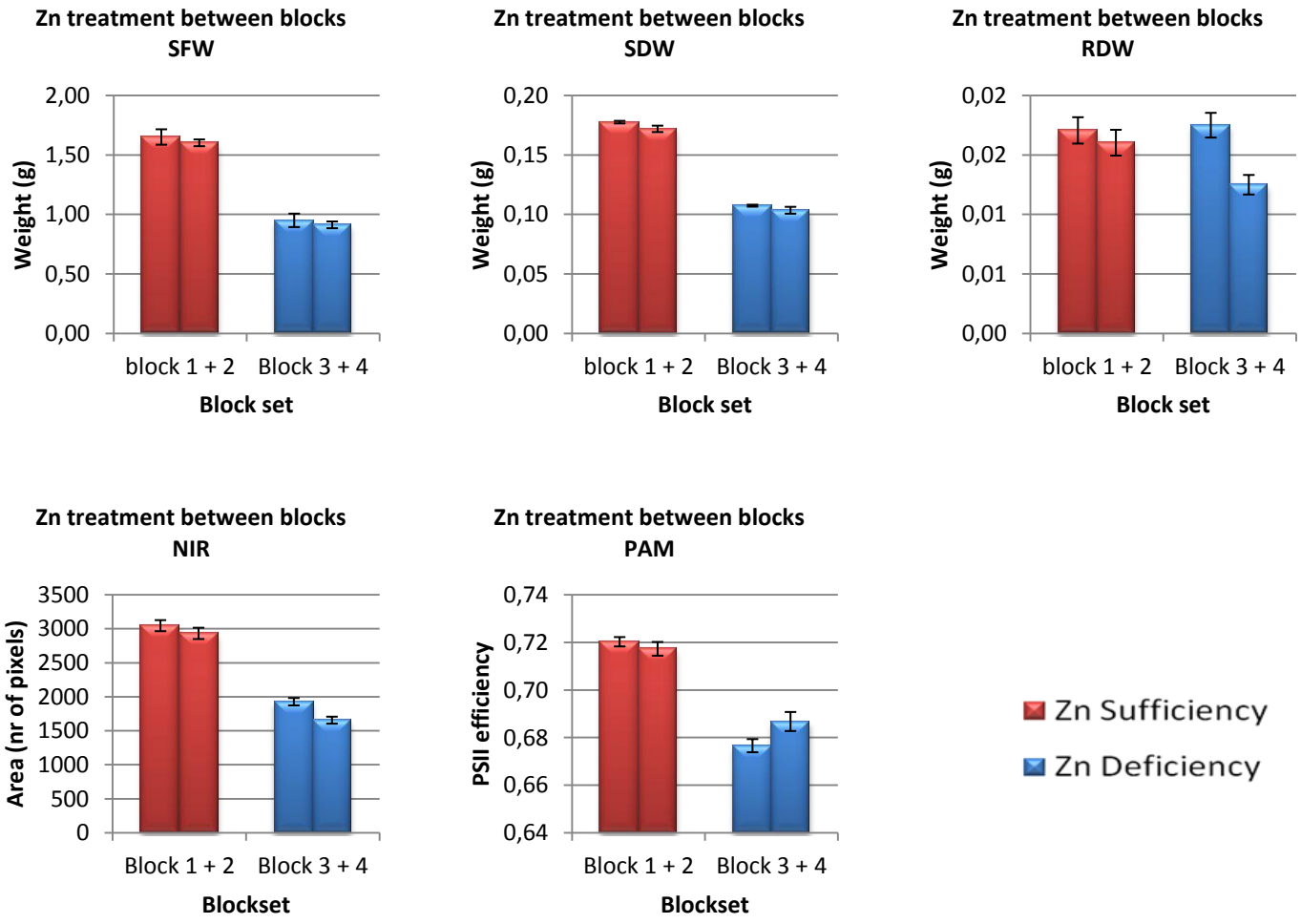

Figure 12: Treatment effect on SFW, SDW, RDW, NIR and PAM per block set (y-axis). One block set contains a block of Zn sufficiency (block 1 and 2) and Zn deficiency (block 3 and 4) treatment (x-axis). Block 1 and 3 were located at the back and block 2 and 4 at the entrance of the phenotype platform climate cell. Standard errors bars included in graph.

I also checked for significant difference between the blocks within each treatment. Because plants in the back of the phenotype platform climate cell performed better compared to the plants at the entrance (Figure 12). Within Zn sufficiency no significant difference between the blocks was observed. Although, there was a significant difference between the Zn deficiency blocks in the RDW, NIR and PAM (Table 4).

Table 4: Independent T test to check difference within treatment between blocks

Difference between the blocks within the Zn sufficiency and Zn deficiency treatment per trait was tested with independent T-tests. Mean difference, standard error difference and two sided p-value were calculated between the treatments per trait. Significant differences between the blocks per treatment are highlighted in yellow, and only observed in the Zn deficiency treatment.

Treatment	Trait	Mean Difference	Std. Error Difference	p-value
Zn sufficiency	SFW	0.072023	0.08499	0.397
	SDW	-0.00403	0.007254	0.579
	RDW	0.001719	0.001444	0.235
	NIR	1.137.602	1.162.061	0.328
	PAM	0.003	0.003	0.397
Zn deficiency	SFW	0.037629	0.040269	0.351
	SDW	-0.00387	0.003947	0.328
	RDW	0.005094	0.001368	0.000
	NIR	2.715.476	749.135	0.000
	PAM	-0.01	0.005	0.036

Because there was a significant difference between the blocks in the same treatment, it was not allowed to combine the results of the blocks. Normally you can correct for this to take the block as a co-factor in a Two Way ANOVA, although in my case I did not have enough degree of freedom to execute the test. A solution for this was a nested Two Way ANOVA to prevent re-parameterisation. Although, the variance between the plants in my experiment remained. Therefore I defined the overall mean of the blocks at the entrance and in the back of the phenotype platform to define the factor to correct for the data (Table 5).

Table 5: Factor to correct for effect phenotype platform climate cell

Mean per block was calculated. Block 1 and 2 were exposed to Zn sufficiency. Block 3 and 4 were exposed to Zn deficiency. The factor to correct between the blocks within the treatment was calculated by dividing the mean of the block in the back of the room by the mean of the block at the entrance of the corresponding treatment. Based on this factor the blocks at the entrance were corrected for, to reduce the variance within the treatment.

Trait	Block 1	Block 2	Factor Zn Suf	Block 3	Block 4	Factor Zn Def
SFW	1.651	1.602	1.030	0.951	0.913	1.041
SDW	0.172	0.178	0.968	0.104	0.107	0.964
RDW	0.017	0.016	1.064	0.018	0.013	1.400
NIR	2466.299	2691.053	0.916	2377.220	2063.647	1.152
PAM	0.698	0.705	0.990	0.709	0.690	1.027

If the block effect was not corrected, no statistics could be performed at all due to the large variance in the data and the low degree of freedom for line*genotype*block interaction. RDW was not a reliable factor in this experiment, because a lot of roots were mixed, which was observed in the harvesting process. Therefore it was not analyzed in detail in the rest of the experiment. Because of the mixed roots the plant material of the phenotype platform was not selected for the Ionome analysis.

3.3.2 T-DNA insertion line performance

T-DNA insertion lines were individually checked with background Col-N60000; Col-2 and Col-3 by the post hoc two sided Dunnett test. This test pointed out a significant result for the traits SFW,SDW and PAM per T-DNA insertion line (Table 6). Although, only the plants which performed better compared to the wild type in the Zn deficiency treatment were significant. T-DNA insertion lines 23,28,29,31,32, 90 and 120 performed all better in the Zn deficiency treatment compared to the wild type.

Table 6: Two sided Dunnett test to check for significant difference in performance T-DNA insertion line referred to performance control

Significant lines illustrated for two sided Dunnett test in which the homozygous T-DNA insertion knock out line was compared to the performance of the wild type in Zn deficiency compared to Zn sufficiency per trait. Mean difference, standard error difference and two sided p-value were calculated. All lines performed better in Zn deficiency treatment compared to the control. Except line 128. Which performed poorer compared to the control in Zn deficiency treatment.

Trait	Line	Compared to:	Mean Difference	Std. Error	p-value
SFW	23	Col-N60000	-,735604*	0.203	0.012
	28	Col-N60000	-,646006*	0.203	0.048
	29	Col-N60000	-,830855*	0.199	0.001
	31	Col-N60000	-,722229*	0.203	0.015
	32	Col-N60000	-,719698*	0.209	0.021
	90	Col-3	-,790752*	0.216	0.002
	120	Col-N60000	-,716108*	0.199	0.012
SDW	23	Col-N60000	-,061977*	0.017	0.010
	28	Col-N60000	-,060475*	0.017	0.014
	29	Col-N60000	-,074149*	0.017	0.000
	31	Col-N60000	-,062301*	0.017	0.010
	32	Col-N60000	-,065742*	0.017	0.007
	90	Col-3	-,057924*	0.020	0.014
	120	Col-N60000	-,059764*	0.017	0.012
PAM	128	Col-N60000	-,041850*	0.012	0.024

Although, significant difference was observed for the two sided Dunnett test in line 128 for photosynthesis efficiency (p=0.024). Clearly two groups were visualized within line 128 for the two treatment, in contrast to the control. Line 128 performed worse in the Zn deficiency treatment compared to the Zn sufficiency treatment. It flowered earlier as well, compared to the other lines in the experiment. Since the gene AT2G25930, which was knocked out, is required for circadian clock and flowering time (TAIR 2015).

In addition, performance ratios were calculated based on the Zn deficiency performance compared to Zn sufficiency performance. This ratio was referred to the performance of the wild type. The ratio's were calculated to set the trait of the Zn sufficiency line at 1 and check how the line performed in a ratio corresponding to the Zn deficiency treatment. Formula: ratio = (1/Zn sufficiency trait)* Zn deficiency trait. This performance factor was calibrated on the performance factor of the wild type background. Ratio >1 if the T-DNA line performed better in Zn deficiency compared to the Col background, and worse if ratio < 1. Ratio for SFW, SDW, PAM and NIR were calculated in this way and illustrated in figure 13.

Overview of all the calculated ratio's are illustrated in appendix H.

The best performing T-DNA lines per trait based on those ratio's and their function are illustrated in table 7.

Table 7: Overview top five best and worst performing T-DNA insertion lines in phenotype platform experiment

Overview of the five best and worst performed T-DNA insertion lines compared to the control performance in Zn deficiency treatment, based on the calculated ratio's. The name of the gene, the traits on which the selection was based and the function of the gene as far it has been studied are included (NCBI 2015, TAIR 2015).

	Phenotype platform climate cell			
Classification	Line	Gene	Based on	Function
Worst performing	34	AT5G22740	SFW,SDW	Cellulose synthase function in mucilage seed structure
	46	AT3G52850	SFW,SDW	Epidermal growth receptor like protein
	98	AT5G52760	SFW,SDW,NIR	Copper transporter
	126	AT4G39700	SFW,SDW	Heavy metal transport/detoxification superfamily protein
	68	AT4G26970	SFW	Catalyse the conversion of citrate
Best Performing	23	AT3G13772	SFW,SDW,NIR	Transmembrane nine (TMN9) protein
	32	AT4G20230	SFW,SDW,NIR	Lyase activity, magnesium ion binding
	31	AT2G05830	SFW,SDW,NIR	Translation initiation factor activity
	29	AT1G04220	SFW,SDW,NIR	Biosynthesis of VLCFA (very long chain fatty acids)
	28	AT3G07600	SFW,SDW,NIR	Heavy metal transport/detoxification super family protein

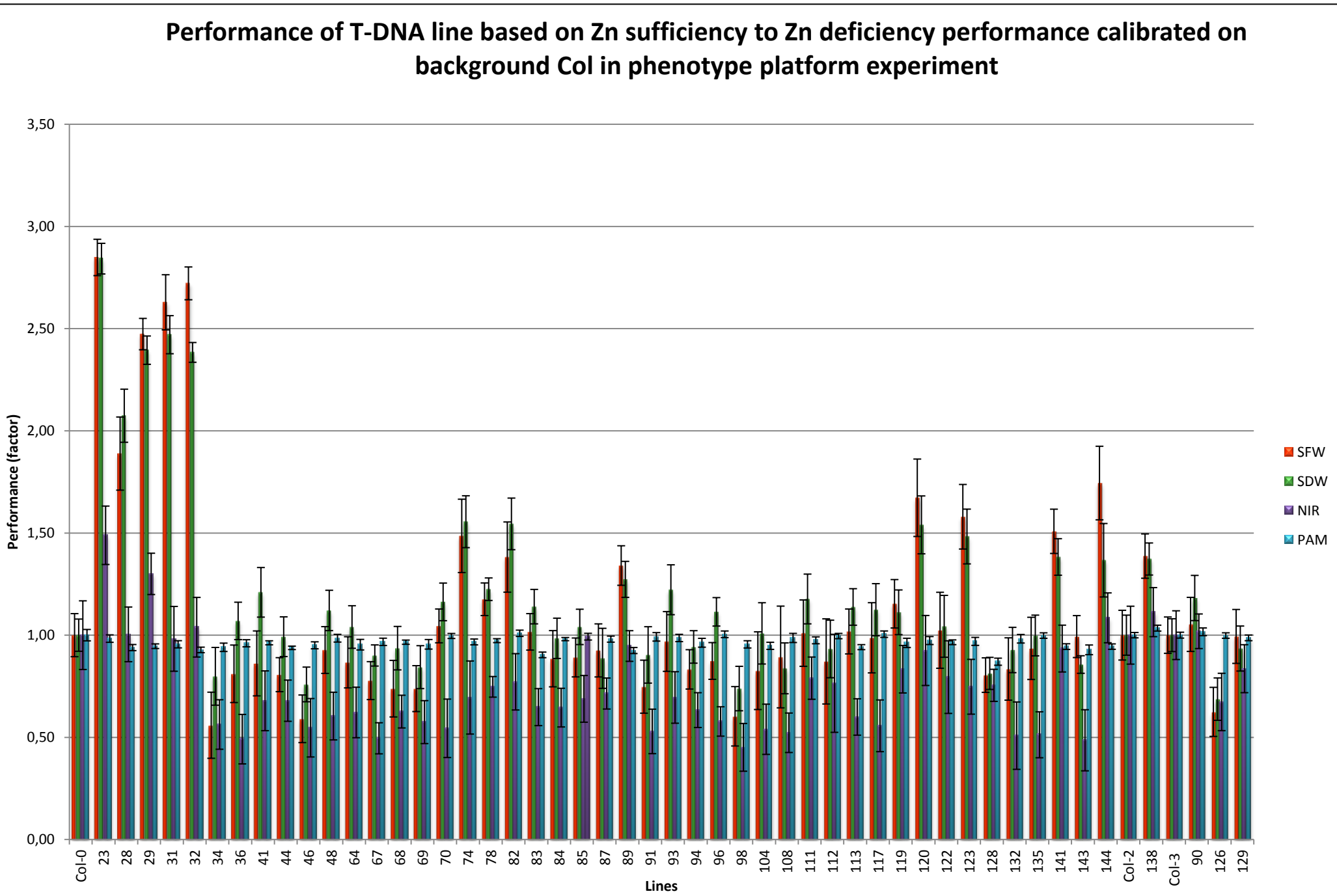

Figure 13: Overview of performance factor of T-DNA line in Zn deficiency treatment based on performance in Zn sufficiency treatment. Calibrated on the performance of the corresponding background Col-N60000;2;3. Every trait which scored above 1, the corresponding T-DNA insertion line performed better in Zn deficiency compared to the control. If the ratio was below 1, the T-DNA insertion line performed worse in the Zn deficiency treatment compared to the control. Traits illustrated are SFW, SDW, NIR and PAM. Error bars are included. Col-0 = Col N60000

3.4 Validation of candidate genes in hydroponics room

3.4.1 Treatment effect

Originally six Zn sufficiency and six Zn deficiency trays were in the hydroponics room experiment. Although after 26 days from germination half of the Zn deficiency trays showed Zn deficiency symptoms. Therefore, half of the material was harvested after 28 days from germination and the other trays were discarded, because there was too much variation between the other trays.

Effect of the treatment was checked with independent T-tests and all were significant (Figure 14) (Table 8).

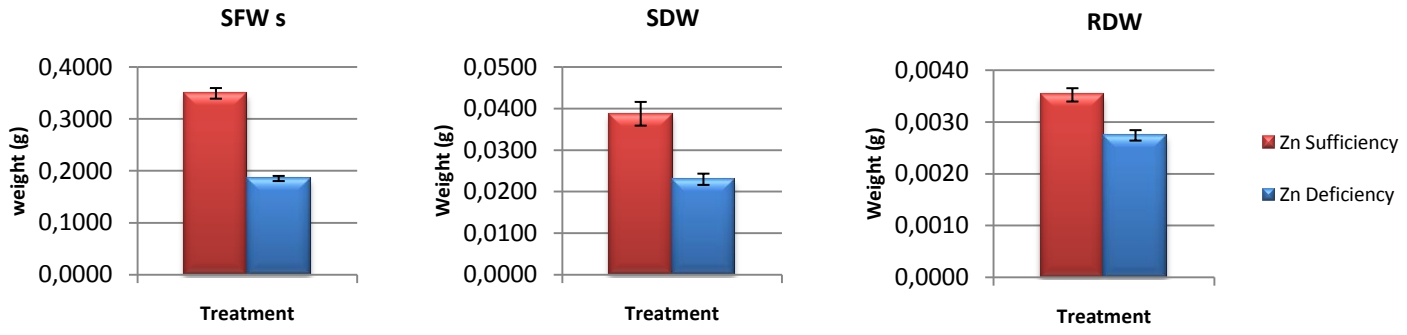

Figure 14: SFW, RDW and RDW (y-axis) displayed for all the lines exposed to Zn sufficiency (red) and Zn deficiency (blue) treatment (x-axis). Standard errors included in graphs.

Table 8: Independent T test between treatments

Independent T-test performed per trait to compare Zn sufficiency with Zn deficiency treatment. Mean difference, standard error difference and two sided p-value were calculated between the treatments per trait. Significant differences were observed for all traits, which means treatments worked.

Trait	Mean Difference	Std. Error Difference	p-value
SFW	0.165	0.011	0.000
SDW	0.0138365	0.001097	0.000
RDW	0.000803	0.000162	0.000

The treatment for Zn deficiency and Zn sufficiency therefore worked in the hydroponics room experiment for the selected trays.

3.4.2 T-DNA insertion line performance

Significant difference in performance compared to the wild type background was underlined by the post-hoc two sided Dunnett test (Table 9). Line 34, 90 and 93 performed all better compared to the wild type. Performance factors as in the phenotype platform climate cell were also calculated for the hydroponics room experiment (Figure 15).

Due to the big variance in the phenotype platform experiment, the physiological essay was repeated in the hydroponics room. Twelve trays were used, although there was variance observed between the trays as well within the same treatment, which forced me to discard half of the trays. Because of this large variance and low number of replicates,

in contrary with the setup of the experiment, it was hard to do statistics and pointed out almost no significant results.

Table 9: two sided Dunnett test per line

Significant lines illustrated for two sided Dunnett test in which the T-DNA insertion knock out line was compared to the performance of the wild type in Zn deficiency compared to Zn sufficiency per trait. Mean difference, standard error difference and p-value were calculated. All significant lines performed better compared to the control.

Trait	Line	Compared to:	Mean Difference	Std. Error	p-value
SFW	93	Col-N60000	,18*	.054	.024
RDW	34	Col-N60000	,077540*	.0219633	.014
	90	Col-3	,00262*	.000810	.014

Overview of all the calculated ratio's are illustrated in Appendix H.

The top five worst and best performing T-DNA insertion lines and their corresponding functions are illustrated in table 10.

Table 10: Overview top five best and worst performing T-DNA insertion lines in hydroponics room experiment

Overview of the five best and worst performing T-DNA insertion lines compared to the control performance in Zn deficiency treatment, based on the calculated ratio's. The name of the gene, the traits on which the selection was based and the function of the gene as far it has been studied are included (NCBI 2015, TAIR 2015).

	Hydroponics room experiment			
Classification	Line	Gene	Based on	Function
Worst performing	129	AT5G59250	SFW,SDW,RDW	Transmembrane transport
	85	AT2G34830	SFW,SDW	Member of WRKY Transcription Factor
	98	AT5G52760	SFW,RDW	Copper transport
	87	AT5G47450	SFW,SDW,RDW	Tonoplast intrinsic protein
	138	AT1G49660	SFW,SDW,RDW	Encodes a protein with carboxylesterase
Best Performing	122	AT1G80770	SFW,SDW,RDW	Iron transport
	46	AT3G52850	SFW,SDW	Epidermal growth receptor like protein
	32	AT4G20230	SFW	Lyase activity, magnesium ion binding
	144	AT2G40460	SFW,RDW	Major facilitator super family protein
	132	AT5G48250	SFW,SDW	Transcription factor activity, zinc ion binding

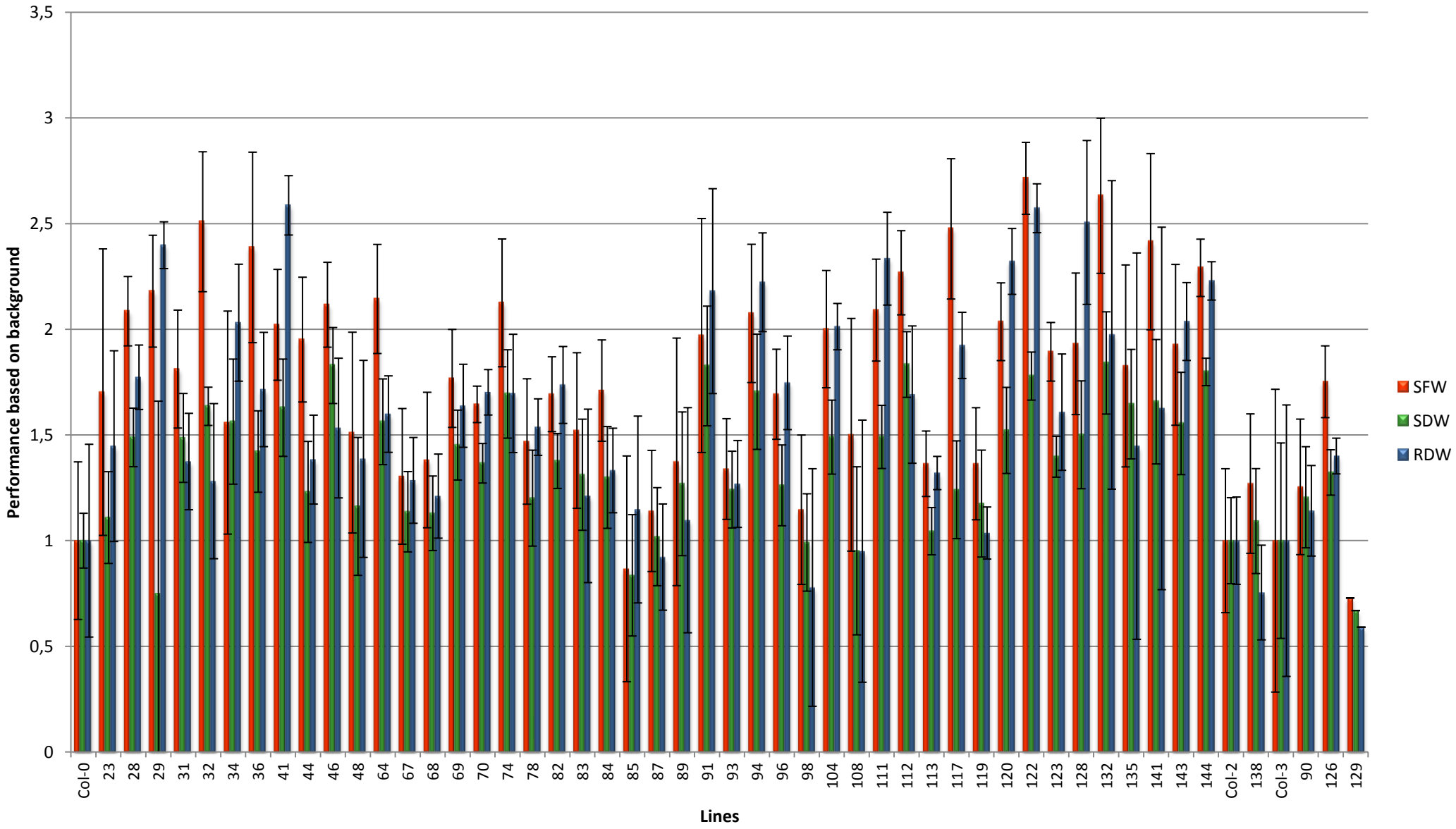

Figure 15: Hydroponics room experiment. Overview of performance factor of T-DNA insertion line in Zn deficiency treatment based on performance in Zn sufficiency treatment. Calibrated on the performance of the corresponding background Col-N60000; Col-2;Col-3. Every traits which scored above 1, the corresponding T-DNA insertion line performed better in Zn deficiency compared to the control. If the ratio was below 1, the T-DNA insertion line performed worse in the Zn deficiency treatment compared to the control. Traits illustrated are SFW, SDW and RDW. Error bars are included. Col-0 = Col N60000

3.4.3 Ionome

The Ionome analyses gave me the concentration of twenty elements. Based on correlations between Zn and the other elements on the broad heritability of Campos 2015, I analysed the concentration of P, Mn, Fe, Cu, Zn, Mo and Cd. Independent T-test were performed for roots and shoots between the two treatments. For shoots there was a significant difference for the treatment for all the analysed elements (Table 11). In roots there was only no significant difference for P (Table 12). P was therefore discarded in further analysis.

Table 11: Independent T-test Shoots

Independent T-test were performed to check for significant difference in the element concentrations in the shoots between Zn sufficiency and Zn deficiency treatment. Mean difference, standard error Difference and two sided p-value are illustrated. All elements were significant for the treatment.

Element	Mean Difference	Std. Error Difference	p-value
P	1752,2497	193,0463	,000
Mn	40,7349	3,6733	,000
Fe	33,9193	4,0713	,000
Cu	-1,6068	0,1507	,000
Zn	-83,6755	4,2378	,000
Mo	9,3746	0,5931	,000
Cd	0,0511	0,0137	,000

Table 12: Independent T-test Roots

Independent T-test were performed to check for significant difference in the element concentrations in the roots between the treatments. All elements, except from P, were significant for the treatment. Mean difference, standard error Difference and two sided p-value are illustrated.

Element	Mean Difference	Std. Error Difference	p-value
P	-1347,2049	2634,6025	,609
Mn	7,0464	3,4406	,041
Fe	-910,2065	51,8150	,000
Cu	-3,6680	0,6516	,000
Zn	-403,9555	17,1559	,000
Mo	46,0387	11,4852	,000
Cd	0,1694	0,0601	,005

Two Way ANOVA was performed between the treatments per line for shoots and roots. Two sided Dunnett test pointed out significant difference for lines 48, 70, 94 and 135 in shoots (Table 13). The concentrations of the elements were higher in the Zn deficiency treatment compared to the control in the shoots.

Table 13: Two sided Dunnett test for Shoots Ionome

Two sided Dunnett test to point out significant difference in element concentration in shoots for Zn sufficiency and Zn deficiency treatments calibrated on control.

Element	Line	Background	Mean Difference	Std. Error	p-value
Fe	48	Col-N60000	77,42962*	17.72870	.001
Cu	70	Col-N60000	2,33593*	.71154	.037
Zn	94	Col-N60000	90,61073*	20.69509	.001
Cd	135	Col-N60000	,33782*	.072214	.000

Lines 132, 135, 132, 135, 141 and 135 were significant in the two sided Dunnett test for roots (Table 14). They all contained a higher concentration of the element compared to the control in the roots.

Table 14: Two sided Dunnett test for Roots Ionome

Two sided Dunnett test to point out significant difference in element concentration in roots for Zn sufficiency and Zn deficiency treatments calibrated on control.

Element	Line	Background	Mean Difference	Std. Error	p-value
Mn	132	Col-N60000	75,16584*	14.818487	.000
	135	Col-N60000	56,74383*	14.81848	.006
Cu	132	Col-N60000	12,60866	3.05274	.002
	135	Col-N60000	9,76224*	3.05274	.047
	141	Col-N60000	9,83542*	3.05274	.044
Mo	132	Col-N60000	191,64603*	55.07590	.020
	135	Col-N60000	178,33725*	55.07590	.042
Cd	135	Col-N60000	,93858*	.28791	.039

Hypersensitive Zn deficiency mutant nas was included. It showed trouble with transport of Zn from the root to the shoot. High concentrations of Zn in the roots and low concentrations of Zn in the shoots were observed. Simply, because it was unable to transport the available Zn. Under normal conditions plants contain 30-100 Zn ppm (Marschner 1995, Sinclair and Kramer 2012). The average concentration for Zn in nas for Zn sufficiency was 42.4 Zn ppm in shoots and 4081.1 Zn ppm in the roots. While in Zn deficiency it was 30.37 Zn ppm in shoots and 40.6 Zn ppm in the roots. Performance was calculated based on the Zn deficiency state corresponding to the Zn concentration in Zn sufficiency: (1/(Zn suf)*Zn deficiency) and calibrated to the background of Col-N60000; Col-2 and Col-3 (Zn sufficiency 8.75 ppm Zn; Zn deficiency 10.9 ppm Zn) as was stated before in the phenotype platform experiment. The ratio for shoot (8.17449) and root (0.09122) were calculated (Figure 16). If the ratio of the shoot was then divided by the root. It was possible to estimate how well plants transported Zn from the roots to the shoots (Figure 17). For nas this factor was: 8.17449/0.09122=89,611. High ratio's illustrated a plant which was unable to transport Zn to the shoots. Low ratio's illustrated the plant was unable to process the transported Zn in the shoots. Overview of all the calculated ratios are illustrated in appendix I. Top five worst and best lines are shown in table 15.

Table 15: Overview top five worst genes transported Zn from roots to shoot and correlated metals. Plus top five worst performed genes to execute the processes Zn is involved in the shoots

Compared to the control performance in Zn deficiency treatment, based on the calculated ratio's. The name of the gene, the traits on which the selection was based and the function of the gene as far it has been studied are included (NCBI 2015, TAIR 2015).

	Ionome identification experiment			
Classification	Line	Gene	Based on	Function
Worst performing genes in transporting Zn from roots to shoots	94	AT5G52730	Zn	Copper transport protein family
	82	AT1G67040	Cu, Fe, Mn, Cd, Mo	Unknown
	128	AT2G25930	Cu, Fe, Mn	Component of the core circadian clock
	122	AT1G80770	Zn, Cu, Fe, Mn, Cd, Mo	Iron transport
	48	AT5G46210	Cu, Fe, Mn	Repression of photo morphogenesis
Worst performing genes to use transported Zn in shoots	108	AT5G67180	Cu ,Fe, Mn,	Pathogenesis-related transcriptional factor
	138	AT1G49660	Zn, Cu, Fe	Encodes a protein with carboxylesterase
	90	AT4G14630	Cu, Fe,	Germin-like protein
	132	AT5G48250	Cu, Fe, Mn	Transcription factor activity, zinc ion binding
	98	AT5G52760	Zn, Cu, Fe, Mn	Copper transport

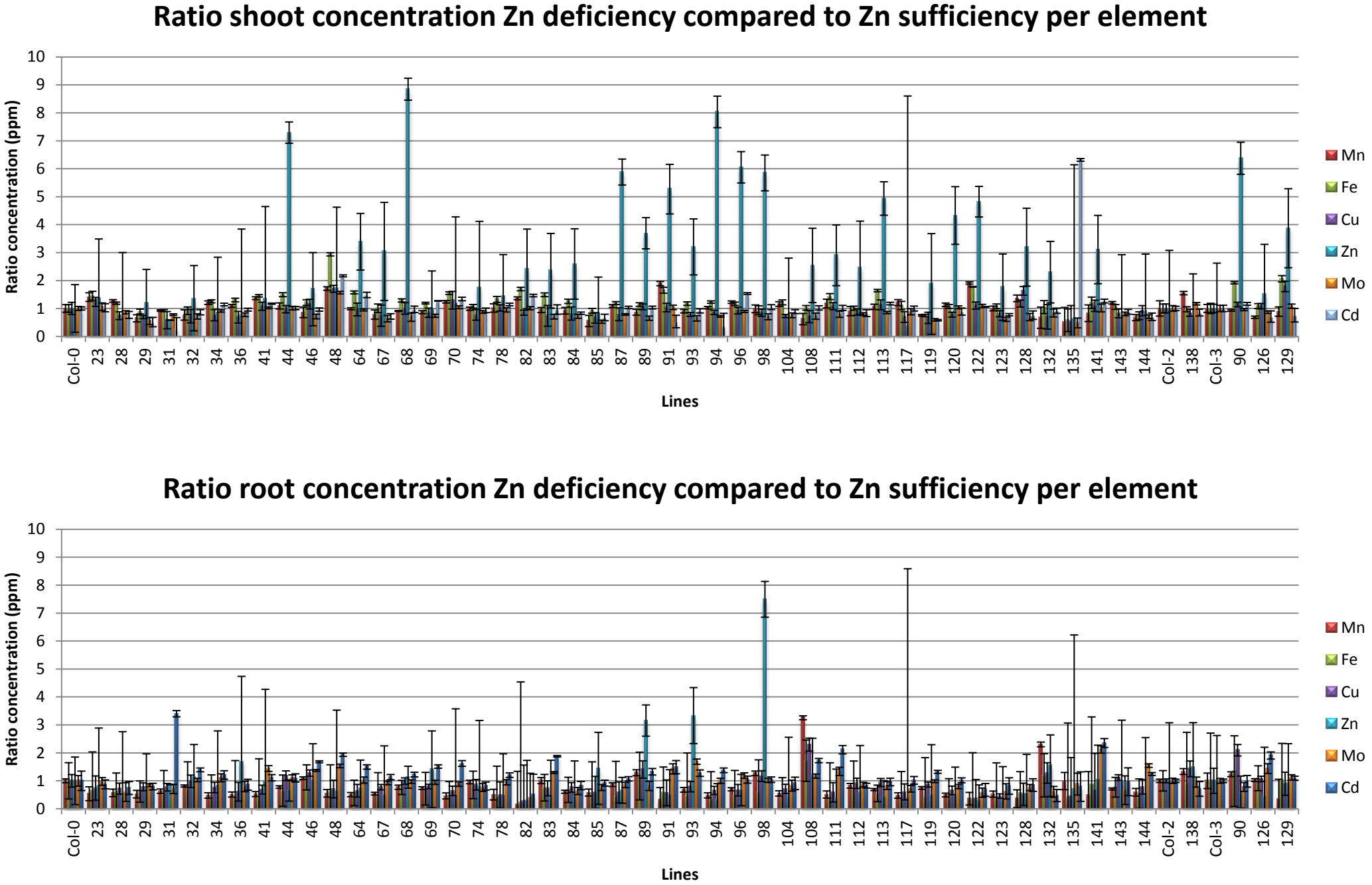

Figure 16: Ratio root and shoot concentration for Zn deficiency compared to Zn sufficiency per element. Ratios were calculated based on the percentage concentration of Zn deficiency to Zn sufficiency. Based on those ratio's all lines were calibrated on the control background Col-N60000; Col-2 and Col-3. Elements P (Phosphor) Mn (Manganese) Fe (Iron) Cu (Copper) Zn (Zinc), Mo (Molybdenum) and Cd (Cadmium) are illustrated. Standard error bars are included. Col-0 = Col N60000

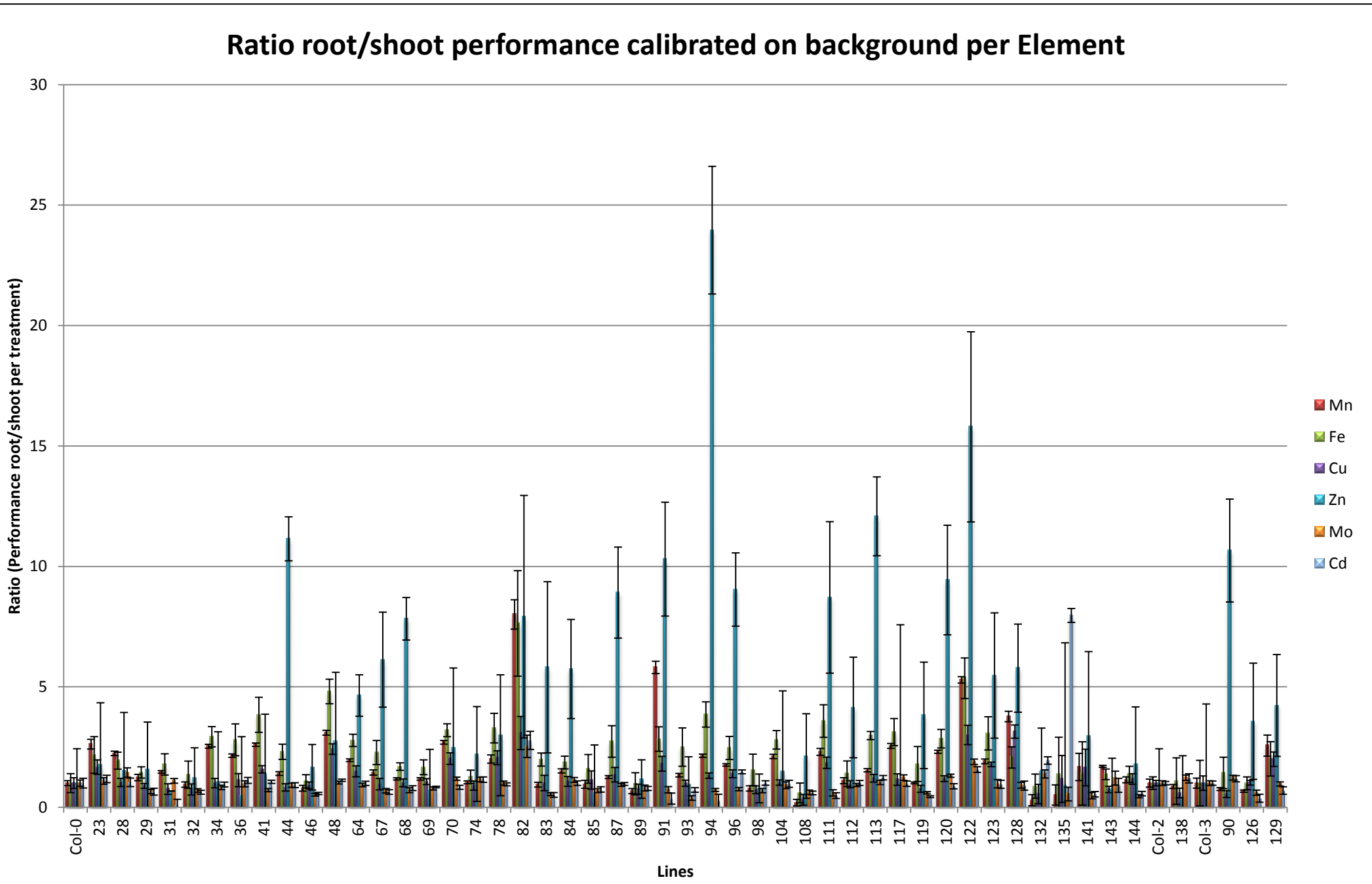

Figure 17: Ionome overview of ratio shoot/roots performance factor of T-DNA line in Zn deficiency treatment based on concentration in Zn sufficiency treatment. Calibrated on the performance of the corresponding background Col-N60000;Col-2 and Col-3. Every trait which scored above 1 performed worse in the transport from the root to the shoot. Below 1 the plants was good at transporting the element to the shoot, but was unable to process it. Elements P (Phosphor) Mn (Manganese) Fe (Iron) Cu (Copper) Zn (Zinc), Mo (Molybdenum) and Cd (Cadmium) are illustrated. Standard error bars are included. Col-0 = Col N60000

3.5 Overview

The best traits to score the plants in the phenotype platform experiment were SFW and SDW, because in RDW mixing of the roots was observed during the harvest. In NIR analysis plants overlapped partly and PAM only gave one significant result for line 128. For the hydroponics room experiment SFW, SDW and SRW all were used, because no interference was observed within the traits. As well for the Ionome identification and correlation in accumulation of the elements linked to Zn Homeostasis. Although, the number of replicates was low in the hydroponics room experiment.

For every gene it was calculates how well or how bad the T-DNA insertion line performed in Zn deficiency compared to the Zn sufficiency treatment referred to the wild type. The top five worst and best performed lines are outlined in table 16. As well for the capability to transport the Zn to the shoots and process the Zn in the shoots.

Table 16: Overview top five worst and best performing lines coupled to candidate gene

Overview of all the three experiments and the selected genes based on the performance ratio's. The Gene name, function and in which experiment it was in the top five are illustrated (NCBI 2015, TAIR 2015).

	Phenotype platform climate cell		Hydroponics room			Ionome	
Classifica-tion	**Gene**	**Function**	**Gene**	**Function**	**Classifica-tion**	**Gene**	**Function**
Worst perform-ing	AT5G22740	Cellulose synthase	AT5G59250	Transmembrane transport	Worst performing genes in transporting Zn from roots to shoots	AT5G52730	Copper transport protein family
	AT3G52850	Epidermal growth receptor like protein	AT2G34830	Member of WRKY Transcription Factor		AT1G67040	Unknown
	AT5G52760	Copper transporter	AT5G52760	Copper transport		AT2G25930	Component of the core circadian clock
	AT4G39700	Heavy metal transport/detoxification super family protein	AT5G47450	Tonoplast intrinsic protein		AT1G80770	Iron transport
	AT4G26970	Catalyse the conversion of citrate	AT1G49660	Encodes a protein with carboxylesterase		AT5G46210	Repression of photo morphogenesis
Best Perform-ing	AT3G13772	Transmembrane nine (TMN9) protein	AT1G80770	Iron transport	Worst performing genes to use transported Zn in shoots	AT5G67180	Pathogenesis-related transcriptional factor
	AT4G20230	Lyase activity, magnesium ion binding	AT3G52850	Epidermal growth receptor like protein		AT1G49660	Encodes a protein with carboxylesterase
	AT2G05830	Translation initiation factor activity	AT4G20230	Lyase activity, magnesium ion binding		AT4G14630	Germin-like protein
	AT1G04220	Biosynthesis of VLCFA (very long chain fatty acids)	AT2G40460	Major facilitator super family protein		AT5G48250	Transcription factor activity, zinc ion binding
	AT3G07600	Heavy metal transport/detoxification super family protein	AT5G48250	Transcription factor activity, zinc ion binding		AT5G52760	Copper transport

There was a lot of difference between the top five best and worst performing genes between the experiments. Although, AT5G52760 was among the top five worst performing lines in the phenotype platform experiment, hydroponics room experiment and among the accumulation of Zn in the shoots. It is known as a Cu transporter, but seems to be important for Zn Homeostasis as well. This can also be due to the strong correlation between the elements Zn and Cu (Wintz and Vulpe 2002). AT5G52730, which is close to AT5G52760, is involved in the Cu transport protein family (TAIR 2015). It showed a huge accumulation of Zn in the roots in the hydroponics room experiment.

AT1G49660 was among the worst performed lines in the hydroponics room experiment and accumulated Zn in the shoots. AT4G20230 was among the top five best performing lines in the phenotype platform experiment, and hydroponics room experiment. AT4G20230 is therefore interesting to investigate in more detail as a good performing gene. Next to At3g13772 which was the best performing line in the phenotype platform experiment.

Although, in this experiment false positives have to be taken into account. AT1G80770 is for example the best performing line in the hydroponics room experiment, while it accumulated large proportions of Zn in the roots. As well for AT3G52850 which was among the worst performing lines in the phenotype platform experiment, and in the hydroponics room among the best performing lines.

The proteins coded by the genes AT5G17450, AT1G69600 and AT4G39700 showed an interaction (BAR Arabidopsis Interactions Viewer: http://bar.utoronto.ca). AT5G17450 is part of the Heavy metal transport/detoxification super family protein and is involved in binding, transport and detoxification (TAIR 2015). AT1G69600 encodes a member of the Zn finger homeodomein transcriptional factor family (ZFHD1). AT1G69600 encodes proteins for the HMA domain in Heavy metal-associated isoprenylated plant protein (HIPP) 26 which could lead to binding with Cu and Zn, and regulate the transcription of the Zn finger domain (TAIR 2015, Barth, Vogt et al. 2009). AT4G39700 is part of Heavy metal transport/detoxification super family protein and involved in Cu homeostasis (TAIR 2015). It ensures a controlled distribution of Cu within the plant by chelators and accounts therefore in Cu Homeostasis and Zn Homeostasis, due to the strong correlation between Cu and Zn (Wintz and Vulpe 2002).

AT5G17450 and AT1G69600, in contrary to AT4G39700, did not seem to be affected that much by the Zn deficiency treatment in the phenotype platform and hydroponics room experiments. AT4G39700 was among the top five worst performing lines in the phenotype platform experiment, performed average in the hydroponics room experiment and was four times worse in transporting Zn from the roots to the shoots corresponding to the Ionome identification.

Nevertheless, some genes return in more than one experiment in the top five worst performing or best performing lines. AT5G52760, AT1G49660, AT4G20230 are three genes which could be studied in more detail, because those genes might play a critical role in Zn homeostasis.

4. Discussion

The provided material from NASC was not 100% reliable, because out of 65 T-DNA insertion lines claimed to be homozygote, 43 T-DNA insertion lines were confirmed. For that reason not all the genes which were selected from the GWAS analysis were validated in this experiment.

In this case it can be useful to use natural accessions which have the corresponding SNP in our candidate gene. Based on information of the database 1001 genomes it is possible to use the natural accessions to validate our candidate genes (Campos 2015).

New in this experiment was the usage of hydroponics in the phenotype platform climate cell on a large scale. Normally Rockwool is used in phenotyping experiments, although it showed a large variance between the plants and the treatments were not significant different. As well, it is known Rockwool contains too much Zn over a tenfold difference between the blocks (Grodan®; personal communication Tânia Lobato Paulo Serra). A solution could be the neutral Polygrow, although the roots of *A. thaliana* are facing problems with penetrating Polygrow during germination (personal communication Tânia Lobato Paulo Serra). However, none of these substrate achieved a stable Zn deficiency or excess. Therefore I applied hydroponic conditions in my experiments.

The phenotype platform experiment showed a large variance in plant performance. The plants at the entrance of the climate cell performed worse than at the back. The difference in performance did not follow a gradient. Otherwise, a factor could be applied. It can be due the door-effect, different concentrations of elements within the table, the steepness of the table, the up and down pumping of the Hyponex, the light etc. If new experiments for Zn deficiency are executed in the phenotype platform climate cell the number of replicates should be increased. The Hyponex could also be replaced with 0.5x Hoagland solution prepared with 2-(*N*-morpholino)ethanesulfonic acid (MES) to buffer the solution. No Zn is present in the media, MES does not interacts with ions and MES prevents pH's shifts as a buffer agent, which otherwise leads to refreshing of the media (Bugbee and Salisbury 1985). The 0.2 μM $ZnSO_4$ contamination was observed in the fresh Hyponex, as well in the 1 week old Hyponex. Therefore, it was likely the phenotype platform was almost free of Zn before the Hyponex was added.

As well, the steepness of the table should be considered and at the backside of the table a second drain should be installed to have a circulation of the media. Instead of supply and drainage at the same side of the table. For the hydroponics room new trays should be ordered and maybe it is good to work with another material which is unable to absorb Zn. As well the number of replicates should be increased to lower the variance between the plants within the same T-DNA insertion line. Due to the fact, both experiments showed large variance within the genotypes.

In this thesis the biomass production under Zn deficiency and Zn sufficiency was compared to each other in terms of the whole biomass plant production, also known as ZnE. In order to find genes which contribute to the overall Zn Homeostasis (Marschner 1995, Rengel and Graham 1996, Sinclair and Kramer 2012, Campos 2015). As an additional step the calculated ratio's were referred to the Col-N60000;Col-2 and Col-3 biomass. In order to correct for phenotype effect by the T-DNA insertion and not by the treatment effect. In most papers you can find the ZnE ratio calculations, although not directly referred to the performance ratios of the control. This is more often based on ratio's between maximum and minimum values within the same genotype (Buescher,

Achberger et al. 2010). Or sub-grouping of several mutants within a group (Yu, Danku et al. 2012). As well Pearson correlation tests are performed to define the ZnE (Conn, Berninger et al. 2012, Campos 2015). Plus, most calculations are performed with a couple of genotypes, instead of screening large populations (Rengel, Batten et al. 1999). Or are performed in the stage of functional analysis, instead of validation of candidate genes. This hardens to compare my approach with existing ratio calculations. Therefore, the performance ratios which I defined may be used as a guideline, in the phenotypic platform experiment, hydroponic room experiment and Ionome experiment. Especially, the Ionome translocation calculations have to be taken with caution. Translocation is an important factor in Zn Homeostasis, but even more important is to keep the processes going which depend on the requirement of Zn at a certain moment in the plant life (Sinclair and Kramer 2012). Based on the calculated performance ratio's it was possible to point out some possible genes which might play a role in Zn homeostasis.

Based on the ratio performance results of the phenotype platform experiment candidate gene At3g13772 showed to be the least affected by the Zn deficiency treatment. It was the best performing line in SFW, SDW and NIR. This line had an insertion within the gene At3g13772, which encodes Transmembrane nine (TMN9) protein. TMN9 is involved in the cell adhesion and phagocytosis (Hegelund, Jahn et al. 2010). Cu and Zn homeostasis are altered when this gene is over expressed (TAIR 2015). If knocked out, Mn compensates for the low Cu and Zn concentration (Hegelund, Jahn et al. 2010). Increased Mn uptake was observed within the Ionome identification experiment. Mn concentrations were higher in the roots compared to other elements in the plant. Nevertheless, TMN9 has a lot of homologous present within the plant kingdom (Hegelund, Jahn et al. 2010). This hardens to define the true effect of the mutated gene.

Candidate gene At5g22740 performed the worst in SFW in the phenotype platform experiment. At5g22740 is involved in cellulose synthase. If this gene is knocked out it showed no significant difference in hemi cellulosic polysaccharides in stems under normal conditions (Goubet, Barton et al. 2009, Yu, Shi et al. 2014). Low concentrations of Zn in the plant reduced growth and cellulose composition. Cellulose is required for transport along membranes and therefore influence Zn transport within the plant (Weber, Deinlein et al. 2013). Therefore it might play an indirect role in Zn homeostasis.

If AT5G52730 was knocked out, it showed large accumulation of Zn in the roots and performed likewise nas, which showed big problems in the transport of Zn from the roots to the shoots. T-DNA insertion was within gene AT5G52730, which is involved in the Cu transport family, also known as HIPP (Abreu-Neto, Turchetto-Zolet et al. 2013). HIPPS are involved in element Homeostasis and detoxification. Zn and Cu are strongly correlated, which explained this gene pops up in the Ionome identification (Conn, Berninger et al. 2012). Therefore HIPPS and also this gene can contribute in a better understanding of Cu and Zn Homeostasis in *A. thaliana*.

The genotype with the T-DNA insertion in front of AT5G59250 was the worst performing plant in the Zn deficiency treatment of the hydroponics room experiment corresponding to the SFW, SDW and RDW ratio. Expression analysis has to be performed to check if the gene is a true knock out. AT5G59250 is involved in a Major facilitator superfamily protein like AT5G52730. Although, this gene is involved in glucose transport (Büttner 2007). Glucose transport is regulated by Hexose transporters (HXT) which depend on glucose and Zn concentrations. Zn binds to the promoters of HXT genes and reduce the glucose transportability, which affect the Zn homeostasis (Lalonde, Boles et al. 1999). In *A.*

thaliana AT5G59250 also affects germination time and flowering time (Aluri and Büttner 2007). This could be due to the transportability of Zn and glucose in the plant.

For the interaction between the proteins encoded by AT4G39700, AT5G17450 and AT1G69600 the role of AT5G17450 and AT1G69600 have to be examined in more detail. As well the expression of AT4G39700 and AT1G69600. If they are true knock outs, it is possible to make a triple hypersensitive mutant to Zn deficiency, like nas, by crossing the lines with each other in two generations. It is therefore worthwhile to investigate AT4G39700. Although, it has to be considered knocking out AT5G17450 and AT1G69600 did not resulted in a poorer performance compared to the wild type in the Zn deficiency treatment.

The genes AT5G52760, AT1G49660 and AT4G20230 returned in at least two top five worst or best performing genes per experiment, and deserve special attention in follow up experiments.

AT1G49660 was among the top five worst performing lines in the hydroponics experiment and in processing the accumulated Zn, Fe and Cu in the shoots. AT1G49660 encodes a protein which is associated with *A. thaliana* and contains multiple carboxyesterases which function in herbicide bio activation (Gershater, Cummins et al. 2007). No interaction with metals have been reported. Therefore no link with Zn homeostasis can be laid yet. Gene expression have to be tested before further investigation, since the T-DNA insertion was in front of the gene.

AT4G20230 was among the top five best performing lines in the phenotype platform and hydroponic room experiment. This gene is part of the Protein prenyltransferases superfamily protein and catalyzes thioether bond formation (Hartman, Hicks et al. 2005). Zinc is essential to form a complex prior to the catalytic process of farnesyl diphosphate. Mg is required to optimize the catalytic process and reveals the correlation with Zn (Hartman, Hicks et al. 2005).

The T-DNA insertion within gene AT5G52760 resulted in poor phenotype performance along the three experiments. In the phenotype platform this was based on the SFW, SDW and NIR performance. SFW and RDW in the hydroponics room experiment and the accumulation in shoots of the elements Zn, Cu, Fe, Mn in the Ionome identification. AT5G52760 is involved in Cu transport and ion binding. It is a member of the Heavy metal transport/detoxification super family protein (TAIR 2015). Cu and Zn are strongly correlated and it is therefore likely the Cu is linked with the Zn Homeostasis (Wintz and Vulpe 2002). A knock out in the region of the HIPP's from AT5G52720 to AT5G52760 could be interesting as well. Since this region is correlated with the Cu transport and therefore Cu Homeostasis, which is strongly correlated with Zn homeostasis. This experiment underlines therefore the importance and correlation between Cu and Zn.

Due to the large variance and difference in the top five worst and best performing lines it was not possible to define the accuracy of the GWAS. If there was large overlapping in the top five worst and best performing lines and all showed direct interactions with Zn, indication of the quality of GWAS analysis could be given. Although, it's accuracy will be truly tested in the functional analysis with the proposed genes.

5. Conclusions

Zn deficiency can be reached under hydroponics growth conditions. Although, it resulted in large variance between the plants in the three experiments. This hardened the execution of statistics. The defined ratio's indicated a possible relationship of the candidate gene in Zn Homeostasis. Which enabled me to highlight the candidate genes, which might be involved in Zn Homeostasis.

Overlapping genes in the top five worst and best performing genes among the experiments were: AT5G52760, AT1G49660 and AT4G20230. Those genes could be studied in more detail. They seem to play a role in Zn homeostasis, since they are highlighted in at least two out of the three experiments.

Functional analysis can point out what the true functions of the selected genes are. The Cu transporter, AT5G52760, deserves special attention in this step. It is known as a Cu transporter, but does have an effect in Zn Homeostasis as well. Functional analysis could confirm this role in Zn homeostasis as well.

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8. Appendix

Appendix A

Appendix table 1: List of candidate genes pointed out by GWAS and corresponding function

Gene selected	Function
AT3G05150	Major facilitator superfamily protein; FUNCTIONS IN: carbohydrate transmembrane transporter activity, sugar:hydrogen symporter activity; INVOLVED IN: transport, transmembrane transport; LOCATED IN: nucleus, membrane; EXPRESSED IN: 12 plant structures; EXPRESSED DURING: L mature pollen stage, M germinated pollen stage, 4 anthesis, petal differentiation and expansion stage; CONTAINS InterPro DOMAIN/s: Sugar transporter, conserved site (InterPro:IPR005829), Major facilitator superfamily (InterPro:IPR020846), General substrate transporter (InterPro:IPR005828), Sugar/inositol transporter (InterPro:IPR003663), Major facilitator superfamily, general substrate transporter (InterPro:IPR016196); BEST Arabidopsis thaliana protein match is: Major facilitator superfamily protein (TAIR:AT5G18840.1); Has 29751 Blast hits to 29100 proteins in 2018 species: Archae - 566; Bacteria - 13294; Metazoa - 5300; Fungi - 6650; Plants - 2634; Viruses - 0; Other Eukaryotes - 1307 (source: NCBI BLink).
AT4G26850	Encodes a novel protein involved in ascorbate biosynthesis, which was shown to catalyze the transfer of GMP from GDP-galactose to a variety of hexose-1-phosphate acceptors. Recessive mutation has a reduced amount of vitamin C, lower level of non-photochemical quenching, and reduced rate of conversion of violaxanthin to zeaxanthin in high light.
AT4G26970	Encodes an aconitase that can catalyze the conversion of citrate to isocitrate through a cis-aconitate intermediate, indicating that it may participate in the TCA cycle and other primary metabolic pathways. The protein is believed to accumulate in the mitochondria and the cytosol. It affects CSD2 (At2g28190 - a superoxide dismutase) transcript levels and may play a role in the response to oxidative stress. One member of the family (ACO1 - At35830) was shown to specifically bind to the 5' UTR of CSD2 in vitro.
AT4G26990	unknown protein; BEST A. thaliana protein match is: unknown protein (TAIR:AT5G54920.1); Has 30201 Blast hits to 17322 proteins in 780 species: Archae - 12; Bacteria - 1396; Metazoa - 17338; Fungi - 3422; Plants - 5037; Viruses - 0; Other Eukaryotes - 2996 (source: NCBI BLink).
AT5G17420	Encodes a xylem-specific cellulose synthase that is phosphorylated on one or more serine residues (on either S185 or one of S180 or S181).
AT5G17450	Heavy metal transport/detoxification superfamily protein ; FUNCTIONS IN: metal ion binding; INVOLVED IN: metal ion transport; EXPRESSED IN: 14 plant structures; EXPRESSED DURING: 7 growth stages; CONTAINS InterPro DOMAIN/s: Heavy metal transport/detoxification protein (InterPro:IPR006121); BEST A. thaliana protein match is: Heavy metal transport/detoxification superfamily protein (TAIR:AT1G71050.1); Has 1807 Blast hits to 1807 proteins in 277 species: Archae - 0; Bacteria - 0; Metazoa - 736; Fungi - 347; Plants - 385; Viruses - 0; Other Eukaryotes - 339 (source: NCBI BLink).
AT5G17460	unknown protein; FUNCTIONS IN: molecular_function unknown; INVOLVED IN: response to salt stress.
AT5G34850	Encodes a dual-targeted purple acid phosphatase isozyme AtPAP26. Involved in phosphate metabolism.
AT1G67030	Encodes a Zn finger protein containing only a single Zn finger.
AT1G67040	unknown protein; FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; LOCATED IN: cellular_component unknown; EXPRESSED IN: 20 plant structures; EXPRESSED DURING: 11 growth stages; BEST A. thaliana protein match is: unknown protein (TAIR:AT5G26910.3); Has 89 Blast hits to 84 proteins in 15 species: Archae - 0; Bacteria - 0; Metazoa - 5; Fungi - 2; Plants - 82; Viruses - 0; Other Eukaryotes - 0 (source: NCBI BLink).
AT2G34830	member of WRKY Transcription Factor; Group II-e
AT5G47450	Tonoplast intrinsic protein, transports ammonium (NH3) and methylammonium across the tonoplast membrane, gene expression shows diurnal regulation and is upregulated by ammonium (NH3).
AT1G24120	encodes a DnaJ-like protein similar to ARG1 and ARL2 that are both involved in root and hypocotyl gravitropism response. However, null mutation in this gene does not result in defects in gravitropism. Gene is expressed in all tissues examined.

Gene selected	Function
AT4G14630	germin-like protein with N-terminal signal sequence that may target it to the vacuole, plasma membrane and/or outside the cell.
AT4G34950	Major facilitator superfamily protein; CONTAINS InterPro DOMAIN/s: Nodulin-like (InterPro:IPR010658), Major facilitator superfamily, general substrate transporter (InterPro:IPR016196); BEST A. thaliana protein match is: Major facilitator superfamily protein (TAIR:AT2G16660.1); Has 3379 Blast hits to 3250 proteins in 882 species: Archae - 26; Bacteria - 1593; Metazoa - 4; Fungi - 302; Plants - 604; Viruses - 0; Other Eukaryotes - 850 (source: NCBI BLink).
AT5G52720	Copper transport protein family; BEST A. thaliana protein match is: Copper transport protein family (TAIR:AT5G52670.1); Has 113 Blast hits to 106 proteins in 12 species: Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 113; Viruses - 0; Other Eukaryotes - 0 (source: NCBI BLink).
AT5G52730	Copper transport protein family; BEST A. thaliana protein match is: Copper transport protein family (TAIR:AT5G52720.1); Has 1807 Blast hits to 1807 proteins in 277 species: Archae - 0; Bacteria - 0; Metazoa - 736; Fungi - 347; Plants - 385; Viruses - 0; Other Eukaryotes - 339 (source: NCBI BLink).
AT5G52740	Copper transport protein family; FUNCTIONS IN: metal ion binding; INVOLVED IN: metal ion transport; LOCATED IN: cellular_component unknown; EXPRESSED IN: 11 plant structures; EXPRESSED DURING: 8 growth stages; CONTAINS InterPro DOMAIN/s: Heavy metal transport/detoxification protein (InterPro:IPR006121); BEST A. thaliana protein match is: Copper transport protein family (TAIR:AT5G52760.1); Has 1807 Blast hits to 1807 proteins in 277 species: Archae - 0; Bacteria - 0; Metazoa - 736; Fungi - 347; Plants - 385; Viruses - 0; Other Eukaryotes - 339 (source: NCBI BLink).
AT5G52750	Heavy metal transport/detoxification superfamily protein ; FUNCTIONS IN: metal ion binding; INVOLVED IN: metal ion transport; LOCATED IN: cellular_component unknown; EXPRESSED IN: 20 plant structures; EXPRESSED DURING: 12 growth stages; CONTAINS InterPro DOMAIN/s: Heavy metal transport/detoxification protein (InterPro:IPR006121); BEST A. thaliana protein match is: Copper transport protein family (TAIR:AT5G52760.1); Has 1807 Blast hits to 1807 proteins in 277 species: Archae - 0; Bacteria - 0; Metazoa - 736; Fungi - 347; Plants - 385; Viruses - 0; Other Eukaryotes - 339 (source: NCBI BLink).
AT5G38000	Zn-binding dehydrogenase family protein; FUNCTIONS IN: oxidoreductase activity, binding, Zn ion binding, catalytic activity; INVOLVED IN: response to oxidative stress; CONTAINS InterPro DOMAIN/s: GroES-like (InterPro:IPR011032), NAD(P)-binding domain (InterPro:IPR016040), Alcohol dehydrogenase, C-terminal (InterPro:IPR013149), Alcohol dehydrogenase superfamily, Zn-containing (InterPro:IPR002085); BEST A. thaliana protein match is: Zn-binding dehydrogenase family protein (TAIR:AT5G37940.1); Has 1807 Blast hits to 1807 proteins in 277 species: Archae - 0; Bacteria - 0; Metazoa - 736; Fungi - 347; Plants - 385; Viruses - 0; Other Eukaryotes - 339 (source: NCBI BLink).
AT4G35080	high-affinity nickel-transport family protein; FUNCTIONS IN: nickel ion transmembrane transporter activity, metal ion binding; INVOLVED IN: nickel ion transport, metal ion transport, transmembrane transport; LOCATED IN: integral to membrane, chloroplast; CONTAINS InterPro DOMAIN/s: Nickel/cobalt transporter, high-affinity (InterPro:IPR011541); BEST A. thaliana protein match is: high-affinity nickel-transport family protein (TAIR:AT2G16800.1).
AT3G60100	citrate synthase 5 (CSY5); FUNCTIONS IN: citrate (SI)-synthase activity, transferase activity, transferring acyl groups, acyl groups converted into alkyl on transfer; INVOLVED IN: cellular carbohydrate metabolic process, tricarboxylic acid cycle; LOCATED IN: mitochondrion; EXPRESSED IN: 8 plant structures; EXPRESSED DURING: M germinated pollen stage, 4 anthesis, 4 leaf senescence stage, petal differentiation and expansion stage; CONTAINS InterPro DOMAIN/s: Citrate synthase-like, large alpha subdomain (InterPro:IPR016142), Citrate synthase, eukaryotic (InterPro:IPR010109), Citrate synthase-like (InterPro:IPR002020), Citrate synthase active site (InterPro:IPR019810), Citrate synthase-like, core (InterPro:IPR016141); BEST A. thaliana protein match is: Citrate synthase family protein (TAIR:AT2G44350.1); Has 10430 Blast hits to 10424 proteins in 2755 species: Archae - 158; Bacteria - 6931; Metazoa - 258; Fungi - 314; Plants - 178; Viruses - 0; Other Eukaryotes - 2591 (source: NCBI BLink).
AT5G66890	Leucine-rich repeat (LRR) family protein; BEST A. thaliana protein match is: Disease resistance protein (CC-NBS-LRR class) family (TAIR:AT5G66900.1); Has 1807 Blast hits to 1807 proteins in 277 species: Archae - 0; Bacteria - 0; Metazoa - 736; Fungi - 347; Plants - 385; Viruses - 0; Other Eukaryotes - 339 (source: NCBI BLink).
AT5G67140	F-box/RNI-like superfamily protein; CONTAINS InterPro DOMAIN/s: F-box domain, cyclin-like (InterPro:IPR001810), F-box domain, Skp2-like (InterPro:IPR022364); BEST A. thaliana protein match is: F-box/RNI-like superfamily protein (TAIR:AT1G21410.1); Has 1807 Blast hits to 1807 proteins in 277 species: Archae - 0; Bacteria - 0; Metazoa - 736; Fungi - 347; Plants - 385; Viruses - 0; Other Eukaryotes - 339 (source: NCBI BLink).
AT5G67180	target of early activation tagged (EAT) 3 (TOE3); CONTAINS InterPro DOMAIN/s: DNA-binding, integrase-type (InterPro:IPR016177), Pathogenesis-related transcriptional factor/ERF, DNA-binding (InterPro:IPR001471); BEST A. thaliana protein match is: Integrase-type DNA-binding superfamily protein (TAIR:AT4G36920.2); Has 1807 Blast hits to 1807 proteins in 277 species: Archae - 0; Bacteria - 0; Metazoa - 736; Fungi - 347; Plants - 385; Viruses - 0; Other Eukaryotes - 339 (source: NCBI BLink).

Gene selected	Function
AT1G69550	disease resistance protein (TIR-NBS-LRR class); FUNCTIONS IN: transmembrane receptor activity, nucleoside-triphosphatase activity, nucleotide binding, ATP binding; INVOLVED IN: signal transduction, defense response, apoptosis, innate immune response; LOCATED IN: intrinsic to membrane, endomembrane system; EXPRESSED IN: 21 plant structures; EXPRESSED DURING: 13 growth stages; CONTAINS InterPro DOMAIN/s: ATPase, AAA+ type, core (InterPro:IPR003593), NB-ARC (InterPro:IPR002182), Leucine-rich repeat (InterPro:IPR001611), Disease resistance protein (InterPro:IPR000767), Toll-Interleukin receptor (InterPro:IPR000157); BEST A. thaliana protein match is: Disease resistance protein (TIR-NBS-LRR class) (TAIR:AT5G11250.1); Has 8455 Blast hits to 8197 proteins in 261 species: Archae - 0; Bacteria - 113; Metazoa - 3; Fungi - 4; Plants - 8303; Viruses - 0; Other Eukaryotes - 32 (source: NCBI BLink).
AT1G69600	Encodes ZFHD1, a member of the Zn finger homeodomain transcriptional factor family. Binds to the 62 bp promoter region of ERD1 (early responsive to dehydration stress 1). Expression of ZFHD1 is induced by drought, high salinity and abscisic acid.
AT5G15290	Uncharacterised protein family (UPF0497); CONTAINS InterPro DOMAIN/s: Uncharacterised protein family UPF0497, trans-membrane plant (InterPro:IPR006702), Uncharacterised protein family UPF0497, trans-membrane plant subgroup (InterPro:IPR006459); BEST A. thaliana protein match is: Uncharacterised protein family (UPF0497) (TAIR:AT1G14160.1); Has 635 Blast hits to 635 proteins in 21 species: Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 635; Viruses - 0; Other Eukaryotes - 0 (source: NCBI BLink).
AT1G09750	Eukaryotic aspartyl protease family protein; FUNCTIONS IN: aspartic-type endopeptidase activity; INVOLVED IN: proteolysis; LOCATED IN: apoplast, cell wall, plant-type cell wall; EXPRESSED IN: 22 plant structures; EXPRESSED DURING: 14 growth stages; CONTAINS InterPro DOMAIN/s: Peptidase aspartic (InterPro:IPR021109), Peptidase aspartic, catalytic (InterPro:IPR009007), Peptidase A1 (InterPro:IPR001461); BEST A. thaliana protein match is: Eukaryotic aspartyl protease family protein (TAIR:AT3G54400.1); Has 2762 Blast hits to 2753 proteins in 256 species: Archae - 0; Bacteria - 2; Metazoa - 415; Fungi - 350; Plants - 1861; Viruses - 0; Other Eukaryotes - 134 (source: NCBI BLink).
AT1G11670	MATE efflux family protein; FUNCTIONS IN: antiporter activity, drug transmembrane transporter activity, transporter activity; INVOLVED IN: drug transmembrane transport, N-terminal protein myristoylation, transmembrane transport; LOCATED IN: membrane; EXPRESSED IN: 22 plant structures; EXPRESSED DURING: 13 growth stages; CONTAINS InterPro DOMAIN/s: Multi antimicrobial extrusion protein MatE (InterPro:IPR002528); BEST A. thaliana protein match is: MATE efflux family protein (TAIR:AT1G61890.1); Has 11525 Blast hits to 11473 proteins in 2135 species: Archae - 258; Bacteria - 8318; Metazoa - 144; Fungi - 328; Plants - 1428; Viruses - 0; Other Eukaryotes - 1049 (source: NCBI BLink).
AT1G12845	unknown protein; FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; LOCATED IN: endomembrane system; EXPRESSED IN: 22 plant structures; EXPRESSED DURING: 13 growth stages; Has 8 Blast hits to 8 proteins in 4 species: Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 8; Viruses - 0; Other Eukaryotes - 0 (source: NCBI BLink).
AT1G12950	root hair specific 2 (RSH2); FUNCTIONS IN: antiporter activity, drug transmembrane transporter activity, transporter activity; INVOLVED IN: drug transmembrane transport, transmembrane transport; LOCATED IN: membrane; EXPRESSED IN: sperm cell, root hair, root; CONTAINS InterPro DOMAIN/s: Multi antimicrobial extrusion protein MatE (InterPro:IPR002528); BEST A. thaliana protein match is: MATE efflux family protein (TAIR:AT3G26590.1); Has 9730 Blast hits to 9684 proteins in 1960 species: Archae - 180; Bacteria - 6829; Metazoa - 143; Fungi - 326; Plants - 1351; Viruses - 0; Other Eukaryotes - 901 (source: NCBI BLink).
AT1G80770	pigment defective 318 (PDE318); FUNCTIONS IN: ferrous iron transmembrane transporter activity, GTP binding; INVOLVED IN: ferrous iron transport; LOCATED IN: integral to membrane; EXPRESSED IN: 21 plant structures; EXPRESSED DURING: 13 growth stages; CONTAINS InterPro DOMAIN/s: GTP1/OBG (InterPro:IPR006073), Nucleolar GTP-binding 1 (InterPro:IPR010674), Ferrous iron transport protein B, N-terminal (InterPro:IPR011619); BEST A. thaliana protein match is: Nucleolar GTP-binding protein (TAIR:AT1G10300.1); Has 11067 Blast hits to 11052 proteins in 2859 species: Archae - 320; Bacteria - 7505; Metazoa - 379; Fungi - 348; Plants - 242; Viruses - 0; Other Eukaryotes - 2273 (source: NCBI BLink).
AT1G80780	Polynucleotidyl transferase, ribonuclease H-like superfamily protein; FUNCTIONS IN: ribonuclease activity, nucleic acid binding; INVOLVED IN: RNA modification; LOCATED IN: nucleus; EXPRESSED IN: 24 plant structures; EXPRESSED DURING: 15 growth stages; CONTAINS InterPro DOMAIN/s: Ribonuclease CAF1 (InterPro:IPR006941), Polynucleotidyl transferase, ribonuclease H fold (InterPro:IPR012337); BEST A. thaliana protein match is: Polynucleotidyl transferase, ribonuclease H-like superfamily protein (TAIR:AT2G32070.1).
AT1G80820	Encodes an cinnamoyl CoA reductase isoform. Involved in lignin biosynthesis.

Gene selected	Function
AT4G39700	Heavy metal transport/detoxification superfamily protein ; FUNCTIONS IN: metal ion binding; INVOLVED IN: metal ion transport; LOCATED IN: cellular_component unknown; CONTAINS InterPro DOMAIN/s: Heavy metal transport/detoxification protein (InterPro:IPR006121); BEST A. thaliana protein match is: Heavy metal transport/detoxification superfamily protein (TAIR:AT1G22990.1); Has 1218 Blast hits to 1176 proteins in 83 species: Archae - 0; Bacteria - 23; Metazoa - 6; Fungi - 46; Plants - 1141; Viruses - 0; Other Eukaryotes - 2 (source: NCBI BLink).
AT2G25930	Encodes a nuclear protein that is expressed rhythmically and interacts with phytochrome B to control plant development and flowering through a signal transduction pathway. Required component of the core circadian clock regardless of light conditions.
AT5G59250	Major facilitator superfamily protein; FUNCTIONS IN: carbohydrate transmembrane transporter activity, sugar:hydrogen symporter activity; INVOLVED IN: transport, transmembrane transport; LOCATED IN: chloroplast, membrane, chloroplast envelope; EXPRESSED IN: 21 plant structures; EXPRESSED DURING: 13 growth stages; CONTAINS InterPro DOMAIN/s: Sugar transporter, conserved site (InterPro:IPR005829), Major facilitator superfamily (InterPro:IPR020846), General substrate transporter (InterPro:IPR005828), Sugar/inositol transporter (InterPro:IPR003663), Major facilitator superfamily, general substrate transporter (InterPro:IPR016196); BEST A. thaliana protein match is: Major facilitator superfamily protein (TAIR:AT5G17010.3); Has 35333 Blast hits to 34131 proteins in 2444 species: Archae - 798; Bacteria - 22429; Metazoa - 974; Fungi - 991; Plants - 531; Viruses - 0; Other Eukaryotes - 9610 (source: NCBI BLink).
AT5G48250	B-box type Zn finger protein with CCT domain; FUNCTIONS IN: sequence-specific DNA binding transcription factor activity, Zn ion binding; LOCATED IN: plasma membrane; EXPRESSED IN: 23 plant structures; EXPRESSED DURING: 13 growth stages; CONTAINS InterPro DOMAIN/s: CCT domain (InterPro:IPR010402), Zn finger, B-box (InterPro:IPR000315); BEST A. thaliana protein match is: CONSTANS-like 9 (TAIR:AT3G07650.4); Has 1807 Blast hits to 1807 proteins in 277 species: Archae - 0; Bacteria - 0; Metazoa - 736; Fungi - 347; Plants - 385; Viruses - 0; Other Eukaryotes - 339 (source: NCBI BLink).
AT5G48290	Heavy metal transport/detoxification superfamily protein ; FUNCTIONS IN: metal ion binding; INVOLVED IN: metal ion transport; LOCATED IN: cellular_component unknown; EXPRESSED IN: 6 plant structures; EXPRESSED DURING: 4 anthesis, petal differentiation and expansion stage; CONTAINS InterPro DOMAIN/s: Heavy metal transport/detoxification protein (InterPro:IPR006121); BEST A. thaliana protein match is: Heavy metal transport/detoxification superfamily protein (TAIR:AT3G07600.1); Has 30201 Blast hits to 17322 proteins in 780 species: Archae - 12; Bacteria - 1396; Metazoa - 17338; Fungi - 3422; Plants - 5037; Viruses - 0; Other Eukaryotes - 2996 (source: NCBI BLink).
AT1G49350	pfkB-like carbohydrate kinase family protein; FUNCTIONS IN: kinase activity, ribokinase activity; INVOLVED IN: D-ribose metabolic process; LOCATED IN: cellular_component unknown; EXPRESSED IN: 22 plant structures; EXPRESSED DURING: 13 growth stages; CONTAINS InterPro DOMAIN/s: Carbohydrate/purine kinase (InterPro:IPR011611), Ribokinase (InterPro:IPR002139), Carbohydrate/puine kinase, PfkB, conserved site (InterPro:IPR002173); Has 2896 Blast hits to 2893 proteins in 1061 species: Archae - 1; Bacteria - 2475; Metazoa - 103; Fungi - 59; Plants - 64; Viruses - 0; Other Eukaryotes - 194 (source: NCBI BLink).
AT1G49420	Heavy metal transport/detoxification superfamily protein ; FUNCTIONS IN: metal ion binding; INVOLVED IN: metal ion transport; LOCATED IN: cellular_component unknown; CONTAINS InterPro DOMAIN/s: Heavy metal transport/detoxification protein (InterPro:IPR006121); BEST A. thaliana protein match is: Heavy metal transport/detoxification superfamily protein (TAIR:AT4G16380.1); Has 30201 Blast hits to 17322 proteins in 780 species: Archae - 12; Bacteria - 1396; Metazoa - 17338; Fungi - 3422; Plants - 5037; Viruses - 0; Other Eukaryotes - 2996 (source: NCBI BLink).
AT1G49660	Encodes a protein with carboxylesterase whose activity was tested using pNA.
AT2G40300	Encodes FERRITIN 4, AtFER4. Ferritins are a class of 24-mer multi-meric proteins found in all kingdoms of life. Function as the main iron store in mammals. Evidence suggests that Arabidopsis ferritins are essential to protect cells against oxidative damage, but they do not constitute the major iron pool. Localize to mitochondria. Knock out mutants are not sensitive to abiotic stress.
AT2G40435	BEST A. thaliana protein match is: transcription regulators (TAIR:AT3G56220.1); Has 289 Blast hits to 289 proteins in 30 species: Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 289; Viruses - 0; Other Eukaryotes - 0 (source: NCBI BLink).
AT2G40460	Major facilitator superfamily protein; FUNCTIONS IN: transporter activity; INVOLVED IN: oligopeptide transport, response to nematode; LOCATED IN: membrane; EXPRESSED IN: 22 plant structures; EXPRESSED DURING: 13 growth stages; CONTAINS InterPro DOMAIN/s: Oligopeptide transporter (InterPro:IPR000109), Major facilitator superfamily, general substrate transporter (InterPro:IPR016196); BEST A. thaliana protein match is: peptide transporter 3 (TAIR:AT5G46050.1); Has 7555 Blast hits to 7238 proteins in 1401 species: Archae - 0; Bacteria - 3710; Metazoa - 634; Fungi - 431; Plants - 2228; Viruses - 0; Other Eukaryotes - 552 (source: NCBI BLink).

Appendix B

Protocol 96 Well format DNA extraction with CTAB (adapted from Maloof lab)

1. Prepare 1 L of 2x CTAB and heat to increase solubility (Can release toxic fumes)
   - 20g CTAB (cetyl trimethyl or hexyl trimethyl ammonium bromide)
   - 81.82g NaCl
   - 100mL 1M Tris, pH 8
   - 40 mL 0.5 M EDTA
2. Add a single ball bearing to each 1.2 mL tube containing tissue. (This can be done using a sealing mat with 96 indentations and sprinkling the ball bearings over the top). Put samples into liquid nitrogen.
3. Disrupt tissue by shaking with paint shaker on a frequency of 30 times per second for 2 minutes. Repeat if clumps of tissue remain.
4. Centrifuge briefly to bring down tissue dust.
5. Add 300 µL CTAB and vortex and centrifuge briefly. Place sealing mat loosely on top of plate with paper and heat at 65 °C in water bath for 30 minutes.
6. Let cool to room temperature
7. Add 300 µL chloroform (done in fume hood). Use a TIP BOX for lid chloroform; it melts the boats! Seal tight with the sealing mat and vortex vigorously for 10-20 seconds. Or use the mix step on the apricot.
8. Centrifuge at 3500 rpm for 15 minutes.
9. During centrifugation, prepare a 96 well deep plate by adding 200µL of (-20°C) 2-propanol to each well.
10. Transfer 200 µL of the chloroform-extracted supernatant to the new plates. Be very carefully not to transfer any of the goop from the interface, or any of the organic layer. Mix carefully by inverting the tubes gently.
11. Cover the tubes with the sealing mat and centrifuge at 3500 rpm for 15 minutes.
12. Pour off the liquid into the sink; the pellet of DNA should stay behind.
13. Wash with 200 µL of 70% ethanol (-20°C), re-cover the tubes and centrifuge for 10 minutes at 3500 rpm.
14. Repeat step 12 and 13.
15. Dump of the liquid again and blot the plates dry on paper towels. Air dry for 1 hour in fume hood (or until no liquid can be seen inside and the tubes don't smell like ethanol).
16. Resuspend the pellets in 100 µL of Milli-Q and let sit for 3 hours at 4°C. The DNA can then be transferred to skirted 96 well PCR plates for storage.

Appendix C

Appendix table 2: List of T-DNA insertion lines were grown, candidate genes and primers required for homozygous knock out validation.

Line ID	AGI code	LP	RP
L.26	AT5G48290	TCTTTGGTGGTTCAACTTTGG	AATTTCAGTCCAGCTGAGCAG
L.28	AT3G07600	TTCTGGCTTTTTGTCACCATC	TGCGAAAGTACGTATTGCAAG
L.33	AT4G20230	ATCTTTTGTTGTCGCCAAATG	ATTTGCATTATCGCCGTAATC
L.37	AT5G22750	ACCATGACAAAAGCATTGGAC	TTGCTGGGTTTAACTTCCATG
L.38	AT1G78290	TCCAAAGAGCTTTTCGCTGT	GGCAACGAATATCCGAGAGA
L.39		ATTTTCCAAAGAGCTTTTCGC	GGTGATAGGTTTCCGAGCTTC
L.40		ATTTTCCAAAGAGCTTTTCGC	GGTGATAGGTTTCCGAGCTTC
L.41	AT3G13750	TTTCATTTAAGCACAAACGGC	TTGGGTCCAGTTTCATTGAAC
L.42		TTTCATTTAAGCACAAACGGC	TTGGGTCCAGTTTCATTGAAC
L.45	AT3G44990	GTTGTCACTCTTTGGCTCGAC	CATTCTGGTGTTTGGGTATGG
L.48	AT5G46210	ATTGGCTAATCCATGACATCG	AATCCAGAGCATCAATTGCAC
L.64	AT3G05150	ACGCTTAACCAGGTGATGATG	GCCTAGTGCTGTGAGAACGAC
L.65		GGTTTTGACAGGCTCATGGT	AAAGCTGAAGGCCCAAAAGT
L.66	AT4G26850	CTGGTGAAGATGCCCAAGTT	CTCCATGGGTTCTGTTGCTT
L.67		GTGTTCTTGACTGCTTGCCTC	CCAAGAAGCTTCAAATGCAAC
L.68	AT4G26970	GCATCTTCTGACCTTGCAAAG	TCTTCTTCCCTCTCCAGGATC
L.69		TTCTGTGACTCACATGATCGC	TGAAAATGGCAGAAGGAGATG
L.70	AT4G26990	TATCCGAATTTGCAGTGAAGC	ATGTCCTTGATCTGGACAAGC
		CAGTTGCAGCACTTAGGGATC	GCATTTGTCTTTTGCTTGAGG
L.73	AT5G17420	AGAGAAGCTTAAGGAAACCGC	GAACAACACAAGAGCAGAGGG
L.74	AT5G17450	CGGTCGGTTTCAACATTCAAG	GAATCTTGAATGCATGTCACG
L.76	AT5G17460	TCTCAAAACGATGGTGTTTCC	TTCCACGAACACCTGGTCTAC
L.78	AT5G34850	CTTCATGATTTCCTGCAGACC	TTAAGACCCAATCCATTTCCC
L.80	AT1G67030	TCCACCATGAAAGTACCCAAC	TCACGCCAACACTTTAAACTTG
		TCCTTGAAAAGAAAAACTCGG	GCCAACAAACACGAAAGAGAG
L.82	AT1G67040	TCTGTTTCTATAACCCGGCAG	CTCGCAAATCACTTCTTCCTG
L.84	AT2G34830	CTGAACAACCTTTTGAGCTGC	TTCGTCCTTTTTCTGCTGTTC
L.85		TGATTAGGTTACTCGCCGTTC	TTACGCTCAATTGGATCAAGG
L.86	AT5G47450	TCCAGATTTGGGACAAGTCAC	ATCAAATAAGAGGCGGTTTGG
L.87		TTAGAGCGAGAATTCGCAAAC	TGACCACCAGAAATGTTAGCC
L.88	AT1G24120	TCTTTTTCTTGCGTAAAAGCG	CTAAGCCCCCTTTTATGCAAG
L.89		TCTTTTTCTTGCGTAAAAGCG	CTAAGCCCCCTTTTATGCAAG
L.90	AT4G14630	CCAAAATCTTTCAATGAAACTTCAC	GCGAAAACTATCCTTGGATCC
L.91	AT4G34950	GACCGAAGAGTTCTGATGCTG	TCATCGGAATCAAAACAGGAG
L.92		ATATCCCACGGCCATAATGA	AATCGCATTCGCTTCCATAC
L.94	AT5G52730	CATGAAGCTCGAAAAGATTGC	TTTGTGGTTCCTCAACCATTC
L.96	AT5G52740	TGGATTTGTCAGACGGAAGAC	CAGTTGACATAGCTTTTTGTTTGG

Line ID	AGI code	LP	RP
L.97	AT5G52750	TGATTTCGATTTCAGTTTGG	AATTTCAGCCGGTTTAGCTG
L.98	AT5G52760	CTCCACGTCACCCTAGACTTG	TTCTGGAGTAGTTTCTCGAGCC
L.99	AT5G38000	CCAAACCCAGAGATTGGcta	CGTCATGCGTTTAAACATGG
L.100	AT4G35080	TGATGACAATTCCCAATCCA	TGGGTTTTAATGGGGTTTGA
L.103	AT3G60100	TGGAAGTTAAACCCAGCAATG	TCACCTGAGTTTCGAATGGTC
		TACCGTCGTTGAAGTTTCTGG	GCCTACAATGGGAGGACTAGG
		CTTTGCCTTCAACAGCTCATC	ATTTTGTCAACCTTGCCACTG
L.104	AT5G66890	CAGTCTCAGCGTTTCAAGGTC	CGAATTCGATTCAAAGCTTTG
L.107	AT5G67140	ATTGCTAGCTCTCCTCGGTTC	GAAAGCAAGATGAACGAGACG
L.108	AT5G67180	TTAACGACTCACCCGATCATC	TGTTGAGGAATTGACCCAAAC
L.110	AT1G69550	ATTCCTTCCCACAGTTTCTCG	TGCTTATTGTTCTTGATGGGG
L.111		AACCTGGGAAACGTCAATTTC	GATGAGGGAAGCTCCACTAGG
L.112	AT1G69600	AAATGCAGGGGTGATACAGTG	TTGGATCTTGATCTGACGGTC
L.113	AT5G15290	TGCATGGAAAGTTGGAAACAC	TGCATGGAAAGTTGGAAACAC
L.117	AT1G11670	CAGAAGCTGACTTCAGGATGG	CAGGGTCTTTAAGAAGTCCGG
L.115		GTTTGGGAAGTATGCAACTCG	TTCGGCTGTTAGTGTTTTCATG
L.119	AT1G12950	TGGCGTGAGTTCTCTAACCAC	AAAGCAAGATCATGGTCATGG
L.120		GCAACTTCTGACAAGCGAGTAG	AAGCATGAACATTTTGGGATG
L.121	AT1G80770	AAAATGGGATCGGTATGAAGC	GAGCTTGGTTCCTCATCAATG
L.122		CACACTTTCTGGTCTCCAAGC	TTGTTGGAAAACCAATTCCAG
L.123	AT1G80780	CTCGGTGAAAACAGAACAAGG	TGCAATACTTGTCAGTGCCAC
L.124		TAGGTGGCCTGTAATCACCAG	TTTATTTTCCGACCGTGTACG
L.125	AT1G80820	TTCGCTACCAAAAGACATATAAATC	GGTTCCTCTAACGGTGTAGCC
L.126	AT4G39700	TGGTGGGGGTAGTTGTATGT	CTCAGCCGTACATAGCTCAGG
L.128	AT2G25930	GTTTCTTGTGATTGCCCTGAG	GAAATATGCCCTACCACAGTCC
		GAAATATGCCCTACCACAGTCC	CCAGCATCTTGATTCTTCAGC
L.129	AT5G59250	TTAGCTGGTTGTCTTCCAAGC	ATACGCACGGGAGTATCACAG
L.130		TGGTCTTGAACAGTCACCTGC	CAAAGCTTTCAAATTAGGCCC
L.132	AT5G48250	TCCAGAAAATTCATCAATGGC	TCACAGTCAAGTCATGATGCC
L.133	AT5G48290	TCTTTGGTGGTTCAACTTTGG	AATTTCAGTCCAGCTGAGCAG
L.135	AT1G49350	CTTGGGAGCTTAGTTTGGTCC	ACCGGTTTGGTGAACATACAG
L.137	AT1G49420	TCAGTTTGTTCTCGGTTCGAC	TGGATGGTGGAGAAGAAAAAC
		TGATAATCGTGGGTCCTTTTG	GACTCATACATCGGTCGCTTC
L.138	AT1G49660	GCAATCTTCTCCCAAATTTCC	CACCTCATTCTCAACTCCAGC
L.140	AT2G40300	AATTCCAGCAACGACAAAAAG	ACCACGTTTGTTCTGCAATTC
L.141		ACCACGTTTGTTCTGCAATTC	TGAAGCTGCAATTTCAATTTTC
L.143	AT2G40435	TCCAGTTTGGCTTTATAAACCC	CCGACTCTTTTAGCTTGCATG
L.144	AT2G40460	TGATAATCGTGGGTCCTTTTG	GACTCATACATCGGTCGCTTC

Appendix D

Protocol Vapor-phase sterilization

1. Obtain a vessel for seed sterilization, typically a dessicator jar. Place in fume hood.
2. Place seed that is to be sterilized into appropriate resalable containers (for example 1.5 mL Eppendorf tubes). Do not use gloves or metal spatula to prevent static charge of the tubes. This could lead to seed contamination between the tubes.
3. Place two 100 mL beakers containing 50 ml bleach each into the desiccator jar.
4. Place open containers of seed into a rack or stand inside desiccator jar.
5. Immediately prior to sealing the jar, carefully add 1.5 ml of HCL to the beakers with bleach. When a small desiccator is used, it's easier to put the rack or stand with the seed on the beaker after the concentrated HCL is added. Wear gloves when the HCL is added and when the rack is placed in the jar. Para film is used to seal the jar.
6. Allow sterilization by chlorine fumes to proceed for 3 hours, which is often sufficient for reasonable clean seed.
7. Turn on flow cabinet 30 min before finishing the sterilization process.
8. Open the lid of the desiccator jar a little bit and let the chlorine gas escape. Close the lid after 15 minutes and open it again in the sterile lamina flow cabinet. After opening in the lamina flow cabinet take the rack or stand out of the jar and immediately close the jar again to be sure the amount of gas that escapes is as small as possible.
9. Leave the seed overnight in the flow cabinet and close the Eppendorf tubes to store the seeds for use.

This protocol of Andrew Bent (http://plantpath.wisc.edu/ ~afb/vaster.html) is modified by Peter Goossens.

Appendix E

Protocol Hoagland nutrient solution

1. Prepare stock solution of macronutrient, micronutrients (in one bottle) and Fe(Na)EDTA.

Macronutrients	Stock (g/L)	Stock (M)	Element
KNO_3	101.10	1	N/K
$Ca(NO_3)_2.4H_2O$	236.15	1	Ca
$NH_4H_2PO_4$	115.03	1	P
$MgSO_4.7H_2O$	246.47	1	Mg/S

Micronutrients (1X)	Stock (g/L)	Stock (mM)	Element
KCL	0.075	1	Cl
H_3B_3	1.546	25	B
$MnSO_4.4H_2O$	0.446	2	Mn
$CuSO_4.5H_2O$	0.025	0.1	Cu
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.1241	0.1	Mo

Other	Stock (g/L)	Stock (mM)	Element
Fe(Na)EDTA	7.342	20	Fe/Na
$ZnSO_4.7H_2O$	0.575	2	Zn

Cover Fe(Na)EDTA stock in aluminum foil or store in a dark room.

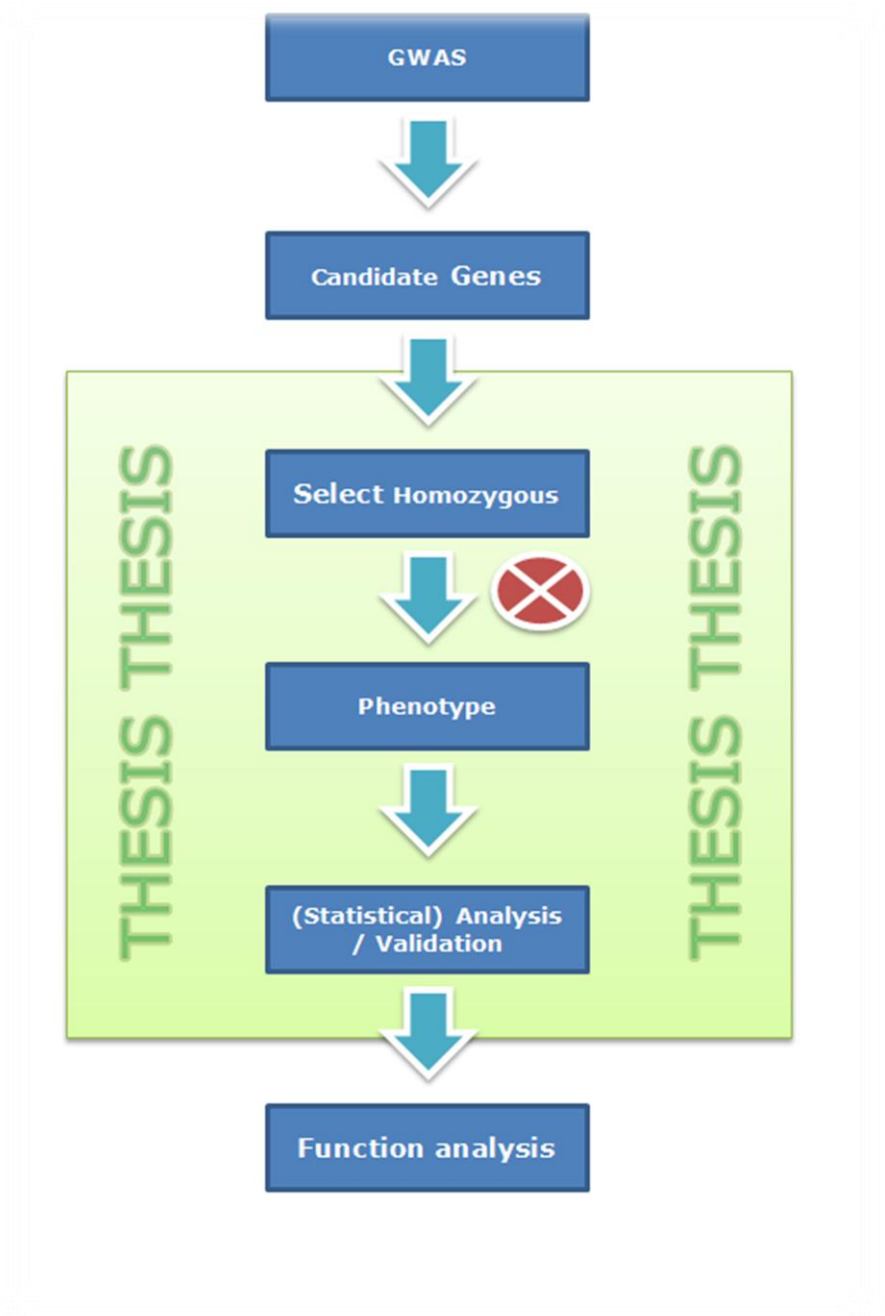
2. Fill the barrels half with Mili-Q (Zn deficiency) or demi-water (Zn sufficiency) and buffer solution with 2mM MES (0,39 g/L).
3. Add the following mL of stock solution per liter of nutrient solutions.

Stocks:	0.5x Hoagland solution	1x Hoagland solution
KNO_3	3	6
$Ca(NO_3)_2.4H_2O$	2	4
$NH_4H_2PO_4$	1	2
$MgSO_4.7H_2O$	0.5	1
Fe(Na)EDTA	1	2
1x Micronutrients	1	2

4. Add 20 µL of 2mM $ZnSO_4.7H_2O$ stock per liter for Zn sufficiency treatment.
5. Shake the barrels for at least 30 seconds to mix all the nutrients.
6. Adjust volume with Mili-Q or demi-water to the desired volume.
7. Mix the barrels again for 30 seconds.
8. Ready for use after right temperature or store in dark cold room.

Appendix F

Overview workflow



Appendix figure 1: Workflow in which every step of the project is summarized. The project started at the growing of knock-outs and ended after the validation ste⊗ = selfing of homozygous T-DNA insertion knock-outs.

Appendix G

Appendix table 3: Sample Hoagland solution of phenotype platform experiment Zn deficiency treatment

Molar concentration	Elements	Molar concentration
Cation mmol/L	NH_4	1.8
	K	8.1
	Na	0.2
	Ca	2
	Mg	1.7
Anions mmol/L	NO_3	4.9
	Cl	0.2
	S	5.8
	HCO_3	<0.1
	P	1.35
Micro elements µmol/L	Fe	25
	Mn	11
	Zn	0.2
	B	36
	Cu	0.4
	Mo	0.8
mmol/L	Si	<0.01

Hyponex solution pH:5.9

Appendix H

Appendix table 4: Overview ratios calculated per trait in Zn deficiency referred to Zn sufficiency calibrated on wild type

	Phenotype platform climate cell										Hydroponics room				
Line	SFW	Line	SDW	Line	RDW	Line	NIR	Line	PAM	Line	SFW	Line	SDW	Line	RDW
34	0,56	126	0,69	104	0,22	98	0,52	128	0,88	129	0,73	129	0,67	129	0,59
46	0,59	98	0,74	135	0,27	143	0,52	83	0,91	85	0,87	85	0,84	138	0,75
98	0,6	46	0,76	132	0,28	36	0,56	143	0,93	Col-N60000	1	108	0,88	98	0,78
126	0,62	34	0,8	113	0,28	67	0,57	89	0,93	Col-2	1	98	0,99	87	0,92
69	0,74	128	0,81	98	0,29	132	0,58	32	0,93	Col-3	1	Col-N60000	1	108	0,95
68	0,74	69	0,84	68	0,33	135	0,59	44	0,94	87	1,14	Col-2	1	Col-N60000	1
91	0,75	108	0,84	96	0,33	108	0,6	34	0,95	98	1,15	Col-3	1	Col-2	1
67	0,78	143	0,86	84	0,33	91	0,61	104	0,95	90	1,25	87	1,02	Col-3	1
128	0,8	87	0,89	85	0,36	104	0,62	113	0,95	138	1,27	113	1,04	119	1,04
44	0,81	91	0,9	48	0,37	46	0,63	141	0,95	67	1,3	138	1,09	89	1,1
36	0,81	67	0,9	94	0,38	70	0,63	144	0,95	93	1,34	23	1,11	90	1,14
132	0,83	132	0,93	87	0,38	117	0,64	28	0,95	113	1,36	68	1,13	85	1,15
94	0,83	112	0,93	143	0,38	34	0,65	29	0,95	119	1,36	67	1,14	68	1,21
104	0,83	129	0,93	111	0,38	69	0,66	46	0,96	89	1,37	48	1,16	83	1,21
41	0,86	68	0,94	69	0,41	96	0,66	98	0,96	68	1,38	119	1,18	93	1,27
112	0,87	94	0,94	91	0,46	126	0,67	69	0,96	78	1,47	78	1,2	32	1,28
64	0,87	84	0,98	41	0,46	48	0,69	64	0,96	108	1,5	44	1,23	67	1,29
96	0,87	44	0,99	112	0,46	113	0,69	31	0,96	48	1,51	90	1,24	113	1,32

Line	SFW	Line	SDW	Line	RDW	Line	NIR	Line	PAM	Line	SFW	Line	SDW	Line	RDW
84	0,88	135	1	122	0,47	64	0,71	68	0,97	83	1,52	93	1,24	84	1,33
108	0,89	Col-N60000	1	46	0,49	68	0,72	67	0,97	34	1,56	117	1,24	31	1,37
85	0,89	Col-2	1	70	0,49	94	0,73	36	0,97	70	1,65	96	1,26	44	1,38
87	0,93	Col-3	1	64	0,5	84	0,74	94	0,97	96	1,69	89	1,27	48	1,39
48	0,93	104	1,01	36	0,51	83	0,74	41	0,97	82	1,69	84	1,3	126	1,4
135	0,94	64	1,04	67	0,53	44	0,78	122	0,97	23	1,7	83	1,31	23	1,45
93	0,97	85	1,04	108	0,53	41	0,78	119	0,97	84	1,71	126	1,32	135	1,45
143	0,99	122	1,04	126	0,54	85	0,79	74	0,97	126	1,75	128	1,36	46	1,53
129	0,99	36	1,07	128	0,63	93	0,8	111	0,98	69	1,77	70	1,37	78	1,54
117	0,99	96	1,11	93	0,65	74	0,8	78	0,98	31	1,81	82	1,38	64	1,6
Col-N60000	1	119	1,11	44	0,66	87	0,82	123	0,98	135	1,83	120	1,38	123	1,61
Col-2	1	48	1,12	34	0,67	129	0,84	120	0,98	123	1,89	123	1,4	141	1,63
Col-3	1	117	1,12	82	0,69	78	0,86	132	0,99	128	1,93	36	1,42	69	1,64
111	1,01	113	1,14	78	0,7	123	0,86	84	0,99	143	1,93	69	1,45	112	1,69
122	1,02	83	1,14	117	0,72	128	0,87	108	0,99	44	1,95	31	1,49	70	1,7
113	1,02	70	1,16	141	0,83	112	0,88	87	0,99	91	1,97	104	1,49	74	1,7
83	1,02	111	1,18	123	0,83	82	0,89	48	0,99	104	2	28	1,49	36	1,72
70	1,05	90	1,18	119	0,85	111	0,91	93	0,99	41	2,02	111	1,49	82	1,74
90	1,05	41	1,21	83	0,94	122	0,91	129	0,99	120	2,04	143	1,55	96	1,75
119	1,15	93	1,22	138	0,97	119	0,96	23	0,99	94	2,08	34	1,56	28	1,77
78	1,18	78	1,22	Col-N60000	1	Col-N60000	1	126	1	28	2,09	64	1,56	117	1,92
89	1,34	89	1,27	Col-2	1	Col-2	1	91	1	111	2,09	41	1,63	132	1,97
82	1,38	138	1,37	Col-3	1	Col-3	1	112	1	74	2,13	32	1,64	104	2,01
138	1,39	144	1,37	74	1	90	1,02	85	1	64	2,14	135	1,65	34	2,03

Line	SFW	Line	SDW	Line	RDW	Line	NIR	Line	PAM	Line	SFW	Line	SDW	Line	RDW
74	1,49	141	1,38	120	1,06	120	1,06	135	1	29	2,18	141	1,66	143	2,04
141	1,51	123	1,48	129	1,07	141	1,07	Col-N60000	1	112	2,27	74	1,69	91	2,18
123	1,58	82	1,54	144	1,08	89	1,09	Col-2	1	132	2,28	94	1,7	94	2,22
120	1,67	120	1,54	89	1,13	138	1,11	Col-3	1	144	2,29	29	1,7	144	2,23
144	1,74	74	1,56	29	1,16	31	1,13	70	1	36	2,39	122	1,78	120	2,32
28	1,89	28	2,07	23	1,32	28	1,15	96	1,01	141	2,41	144	1,8	111	2,33
29	2,47	32	2,38	28	1,33	32	1,19	117	1,01	117	2,48	91	1,83	29	2,4
31	2,63	29	2,39	90	1,35	144	1,25	90	1,02	32	2,51	112	1,83	128	2,51
32	2,72	31	2,47	31	2,41	29	1,49	82	1,02	46	2,53	46	1,83	122	2,57
23	2,85	23	2,84	32	1.18	23	1,71	138	1,03	122	2,71	132	1,84	41	2,59

Appendix I

Appendix table 5: Element concentration shoot/root transportability

	Ionome												
Line	Zn shoot/root ratio	Line	Cu shoot/root ratio	Line	Fe shoot/root ratio	Line	Mn shoot/root ratio	Line	P shoot/root ratio	Line	Cd shoot/root ratio	Line	Mo shoot/root ratio
94	23,96	128	3,14	128	3,14	82	8	108	2,53	135	7.96	82	2.53
122	15,79	82	3,08	82	3,08	91	5,8	90	2,28	82	2.77	122	1.89
113	12,08	122	3	122	3	122	5,29	89	2,18	132	1.94	28	1.44
44	11,14	48	2,41	48	2,41	128	3,77	98	2,13	122	1.56	132	1.38
90	10,66	78	2,05	78	2,05	48	3,09	132	2,12	96	1.47	120	1.31
91	10,3	70	2,02	70	2,02	70	2,7	46	1,77	113	1.23	90	1.23
120	9,43	129	1,99	129	1,99	23	2,62	138	1,42	138	1.20	117	1.23
96	9,04	111	1,84	111	1,84	41	2,6	32	1,39	90	1.18	138	1.22
87	8,91	91	1,8	91	1,8	129	2,56	93	1,34	23	1.17	143	1.21
111	8,71	123	1,78	123	1,78	117	2,54	67	1,31	74	1.15	70	1.18
82	7,91	23	1,67	23	1,67	34	2,53	135	1,29	48	1.12	74	1.15
68	7,82	141	1,65	141	1,65	120	2,3	69	1,28	36	1.11	84	1.13
67	6,13	41	1,57	41	1,57	111	2,3	44	1,27	41	1.05	31	1.10
83	5,81	64	1,49	64	1,49	28	2,24	143	1,27	28	1.03	23	1.09
128	5,77	96	1,39	96	1,39	94	2,14	68	1,23	112	1.01	113	1.03
84	5,74	87	1,35	87	1,35	36	2,14	83	1,23	98	1.01	48	1.02
123	5,47	94	1,31	94	1,31	104	2,11	144	1,22	84	1.00	78	1.00
64	4,64	113	1,2	113	1,2	78	2	74	1,17	Col-N60000	1.00	Col-N60000	1.00
129	4,22	120	1,18	120	1,18	64	1,95	119	1,15	Col-2	1.00	Col-2	1.00

Line	Zn shoot/root ratio	Line	Cu shoot/root ratio	Line	Fe shoot/root ratio	Line	Mn shoot/root ratio	Line	P shoot/root ratio	Line	Cd shoot/root ratio	Line	Mo shoot/root ratio
112	4,14	144	1,17	144	1,17	123	1,9	31	1,15	Col-3	1.00	Col-3	1.00
119	3,81	135	1,17	135	1,17	96	1,76	126	1,14	64	0.99	123	0.98
126	3,57	117	1,15	117	1,15	143	1,7	84	1,13	117	0.98	129	0.97
78	2,99	85	1,13	85	1,13	141	1,67	85	1,09	87	0.97	36	0.97
141	2,95	36	1,12	36	1,12	113	1,53	36	1,07	104	0.97	87	0.93
48	2,72	84	1,07	84	1,07	84	1,51	87	1,05	123	0.95	64	0.92
70	2,47	69	1,06	69	1,06	31	1,47	96	1,03	78	0.95	112	0.92
74	2,21	28	1,03	28	1,03	67	1,44	Col-N60000	1	34	0.94	44	0.91
108	2,13	104	1,02	104	1,02	44	1,4	Col-2	1	44	0.92	128	0.91
144	1,79	68	1,01	68	1,01	93	1,33	Col-3	1	143	0.91	104	0.91
23	1,75	83	1,01	83	1,01	87	1,25	34	0,98	128	0.90	34	0.82
46	1,66	126	1,01	126	1,01	29	1,23	104	0,96	120	0.87	89	0.80
29	1,57	34	1	34	1	68	1,18	29	0,95	69	0.84	69	0.78
28	1,52	Col-N60000	1	Col-N60000	1	69	1,17	112	0,94	70	0.83	96	0.75
104	1,5	Col-2	1	Col-2	1	138	1,16	41	0,92	68	0.79	91	0.73
132	1,49	Col-3	1	Col-3	1	144	1,14	94	0,9	89	0.78	94	0.72
41	1,38	93	1	93	1	112	1,1	113	0,85	85	0.74	68	0.71
32	1,21	67	0,96	67	0,96	74	1,03	28	0,85	93	0.72	41	0.71
89	1,17	112	0,95	112	0,95	119	1,01	120	0,81	129	0.69	32	0.69
34	1,06	46	0,91	46	0,91	Col-N60000	1	129	0,8	29	0.65	98	0.69
117	1,04	74	0,9	74	0,9	Col-2	1	123	0,76	67	0.63	85	0.69
Col-N60000	1	29	0,88	29	0,88	Col-3	1	117	0,76	32	0.62	67	0.68

Line	Zn shoot/root ratio	Line	Cu shoot/root ratio	Line	Fe shoot/root ratio	Line	Mn shoot/root ratio	Line	P shoot/root ratio	Line	Cd shoot/root ratio	Line	Mo shoot/root ratio
Col-2	1	44	0,82	44	0,82	85	0,95	111	0,74	108	0.59	29	0.65
Col-3	1	119	0,76	119	0,76	83	0,94	70	0,7	46	0.57	108	0.62
93	0,96	89	0,75	89	0,75	32	0,94	141	0,64	144	0.56	119	0.60
135	0,89	31	0,75	31	0,75	46	0,79	91	0,63	141	0.54	126	0.60
98	0,78	32	0,74	32	0,74	98	0,78	64	0,63	83	0.51	111	0.59
143	0,74	143	0,74	143	0,74	90	0,75	23	0,59	111	0.48	83	0.54
69	0,69	98	0,72	98	0,72	126	0,67	78	0,58	119	0.44	135	0.54
31	0,67	138	0,59	138	0,59	89	0,67	128	0,51	126	0.37	46	0.52
85	0,58	132	0,55	132	0,55	135	0,51	48	0,48	91	0.36	141	0.48
36	0,47	90	0,54	90	0,54	132	0,29	122	0,38	94	0.27	144	0.46
138	0,43	108	0,31	108	0,31	108	0,19	82	0,26	31	0.09	93	0.38