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GENEESKUNDE

master in de biomedische wetenschappen: milieu en gezondheid

2010
2011

Masterproef

Proteomic study of Arabidopsis thaliana with silenced RCC1 gene

Promotor :
Prof. dr. Ann CUYPERS

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

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Contents

List of abbreviations

35S CaMV: Cauliflower mosaic virus 35S promoter

21nt: an oligonucleotide composed of 21nucleotides

AGO1: argonaute-1

cDNA: reverse-transcribed DNA

DNA: deoxyribonucleic acid

dsRNA: Double stranded-RNA

GEF: Guanine exchange factor

IR-PTGS: inverted-repeat post-transcriptional gene silencing

LC: *T. caerulescens* accession La Calamine

LE: *T. caerulescens* accession Lellingen

Ran: RAs-related Nuclear protein

RanBP: Ran-binding protein

RanGAP: Ran GTPase activating protein

RCC1: Regulator of chromatin condensation 1

RISC: RNA induced silencing complex

RLD: RCC1-like domain

RNA: ribonucleic acid

RNAi: RNA interference

siRNA: Small interfering-RNA

T-DNA: Transfer-DNA

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Joris Comhair

Abstract (EN)

For many years great efforts have been made in reducing anthropologic impact on the environment. There are many types of pollution with organic and inorganic components. In this research the relevance is the inorganic pollution with heavy metals. Phytoremediation, the use of plants to extract the pollutant from the soil, could be a solution to this problem. The study of naturally occurring plants with the capacity to extract, accumulate and tolerate heavy metals can give us more insights in making phytoremediation work. Understanding these mechanisms is essential for future applications. In recent studies of V. H. Hassinen at the Department of Biosciences, University of Eastern Finland (UEF) found numerous genes which could be important in metal accumulation and tolerance mechanisms of the *Thlaspi caerulescens*. The gene of interest was the Regulator of chromatin condensation (*RCC*) 1. About the function of this gene in plants not much is known. In this research our aim was to increase our knowledge about the *RCC1* gene. We found that *A. thaliana* containing a silenced *RCC1* gene showed abnormalities in root development with loss of gravitrophism. In order to understand these reactions a proteomic investigation was started using the same RNAi lines which showed the altered phenotype. Three genotypes were compared using 2DE-gel image analysis. The Wild-type *A. thaliana* ecotype Columbia (Col-0) was compared with RNAi4.5 and RNAi4.3 *RCC1* silenced lines. In the gels, 805 spots were matched and statistical analysed. From these spots 13 where significantly ($P<0,05$) different. Seven of these spots were significantly higher in the Wild-type group were as 5 were significantly ($P<0,05$) higher in RNAi lines. We identified 5 spots that were significantly higher in the Wild-type group using mass-spectrometry analysis. The results indicated that at least 2 proteins identified had a link to root development. The protein actin was especially an important finding because of its involvement in root growth and gravitrophism. A protein associated with actin, WAV2, had also functions in root development. We now have an idea about how RCC1 causes these altered phenotypes. Interesting future investigations could use overexpressors plants to investigate how they react to heavy metal exposure and other abiotic stresses.

Abstract (NL)

De impact van de mensheid op het milieu is al sinds enkele decennia een groot probleem. Niet enkel het milieu, maar ook de gezondheid van de mens leidt hier onder. Om deze reden wordt veel onderzoek geweid aan het oplossen van dit probleem. Dit onderzoek heeft relevantie met de problematiek rond zware metaal pollutie. Phytoremediatie is het uitvoeren van een bodemsanering door gebruik te maken van planten, dit zou een milieuvriendelijke en economische oplossing kunnen zijn voor de zware metaal problematiek. Onderzoek naar natuurlijk voorkomende metaal tolerante en accumulerende plant species kunnen ons hierbij helpen. In studies uitgevoerd door V. H. Hassinen aan het Deparatment of Biosciences, University of Eastern Finland op *Thlaspi caerulescence* heeft men verschillende genen ontdekt, welke belangrijk zouden kunnen zijn in metaal accumulatie en tolerantie. Eén van deze genen is het Regulator of Chromatin Condensation (*RCC*) 1 gen. Dit onderzoek is ertoe gericht de functie van dit relatief onbekend gen uit te diepen door gebruik te maken van het model organisme *Arabidopsis thaliana*. Voorafgaand aan dit onderzoek werd een *A. thaliana* genotype ontwikkeld waarvan het *RCC1* gen ge silenced werd door middel van de RNA interference techniek. Bij deze planten werd een veranderd fenotype waargenomen. De planten vertoonden verstoorde wortel groei in de vorm van gekrulde wortels en een verlies van gravitrofisme. Om de onderliggende verklaring voor dit fenotype te ontrafelen werd een proteoom studie uitgevoerd op Wild-type *A. thaliana* ecotype Columbia planten en twee *A. thaliana* plant lijnen met een ge-silenced *RCC1* gen (RNAi4.3 en RNAi4.5). Gebruikmakend van 2DE-gel image analyses werd gezocht naar veranderingen in proteoom profiel. Bij deze analyse werden 805 proteïne spots vergeleken tussen de 3 verschillende genotype (Wild-type, RNAi4.3 en RNAi4.5). Van deze 805 spots bleken er 13 significant ($P<0,05$) te verschillen tussen de genotypen. Voor 7 van deze spots was er een significante($P<0,05$) stijging in spot intensiteit gemeten in de Wild-type groep. Voor 5 van deze spots was een significante ($P<0,05$) stijging gedetecteerd in ten minste één van de RNAi groepen. Vanuit deze gels werden 5 proteïne spots geïdentificeerd door middel van in- gel trypsine digestion. Alle 5 spots hadden een significante hogere intensiteit in de Wild-type groep. Vervolgens werd gebruik gemaakt van ESI-TOF-MS/MS om de identificatie te voltooien. Uit deze resultaten werden 2 proteïnen gelinkt aan de ontwikkeling van wortels. Het actine, dat deficiënt bleek te zijn in de RNAi lijnen, draagt bij aan de waargenomen fenotypische veranderingen, alsook het geassocieerde WAV2 proteïne. Toekomstige onderzoeken zullen gebruik maken van overexpressor planten van *RCC1*. Op deze planten zou een eventueel effect kunnen waargenomen worden van verhoogde wortel groei en ontwikkeling, dit zou ook het geval kunnen zijn bij blootstelling aan zware metalen, waardoor de plant een hogere tolerantie zou kunnen bekomen.

1. Introduction

1.1 Plants and heavy metals

Every known element is potentially toxic to humans and plants. The toxicity of an element or substance is determined by its concentration and availability for the surrounding organisms. This is a well known fact within the toxicological research field. Like humans plants must be able to take up a number of essential elements (e.g. N, P, K) for various biochemical processes. Some elements are only necessary in small amounts and are called the micro-nutrients (e.g. Cu^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}). Both these groups can be potentially toxic for humans and plants. Metals, particularly heavy metals such as lead, mercury, cadmium and arsenic constitute a significant potential threat to human health and environmental well being. Heavy metals are spread throughout the world in air, soil and aquatic systems. This is often a direct or indirect consequence of metal mining, processing and melting activities. The environmental persistence of metals in addition with their intensive use by modern society has, over the years, created an increased concentration of metals in the biosphere [1].

However, the toxicity and the mobility of heavy metals in soils depend not only on the total concentration, but also on their specific chemical form, the metal properties, environmental factors and soil properties like pH, organic matter content and type, redox conditions and root exudates acting as chelates [2]. Understanding these parameters is important for reducing the bioavailability of these metals.

Current methods used for restoration of metal contaminated soils are aiming to reduce the bioavailability and mobility of the metals. These techniques can be a solution for the problem when the affected area is too large and economically unfavourable for physical removal of the contaminated soil.

Although these techniques can be effective, they do not remove the heavy metal pollution from the soil. In the recent years a newer and environmentally-friendly technique is being developed which is called phytoremediation. In this technique plants are used to extract heavy metals from the soil. Extensive research has been done in this field but there are still obstacles to be cleared out. Naturally occurring hyperaccumulator and metal tolerant plants are often used to investigate basic mechanisms of metal uptake and tolerance. Because

hyperaccumulators are mostly low-biomass and slow-growing plants, current research is focused mainly on designing transgenic plants that can overcome these deficiencies. The complexity of plant–metal interactions and influences of the environment are making this research difficult and multidisciplinary [3]. For this reason more efforts have to be undertaken in this field of environmental research.

1.2 Plant responses and adaptation

Since plants have been evolving for millions of years they have been able to adapt their physiology towards their environmental conditions. This is the same for plants growing in soils polluted with heavy metals. Because of the relevance within this paper only the heavy metal associated phenotypes will be discussed.

1.2.1 Plant phenotypes

There are three main types of heavy metal associated phenotypes. The relationship between the metal content in the soil and the content found in the plant is the main way of classifying to which phenotype a plant species belongs to.

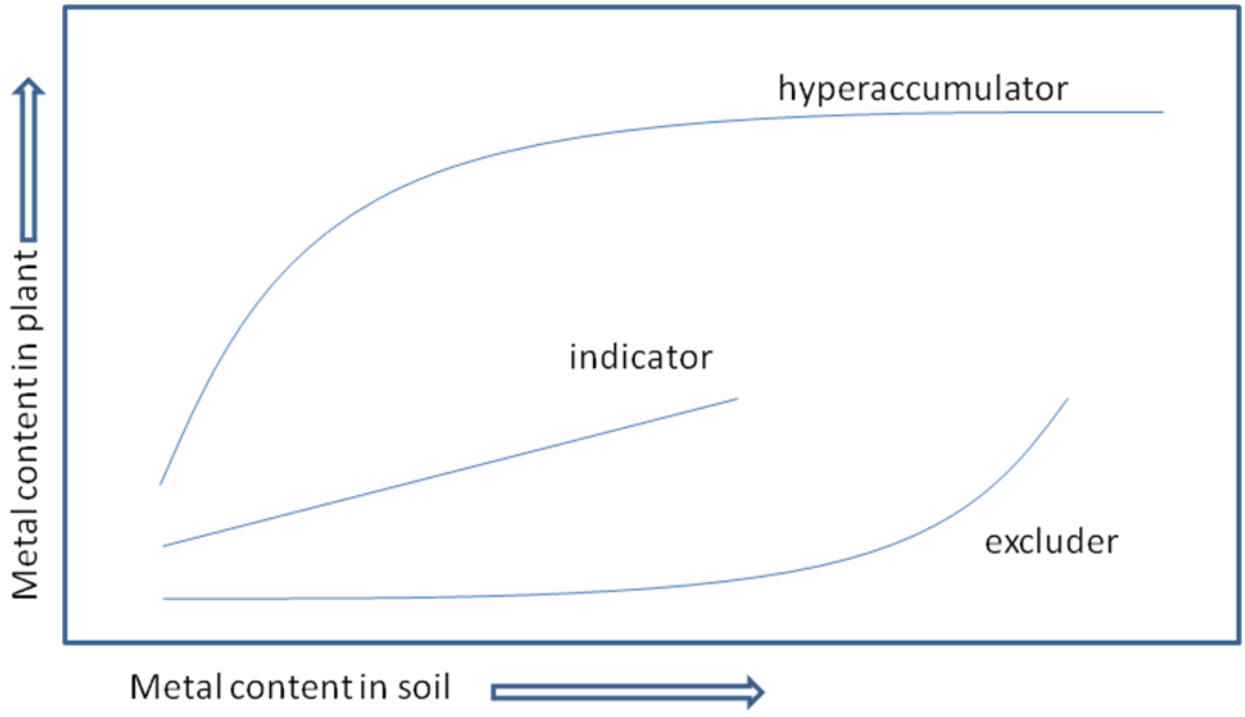

Figure 1: Different phenotypes of plants in regard with metals (metallophytes). One selection criteria is the relationship between metal content in the soil and the concentration found in the plant material. Plants can be categorised in two large groups: metal excluders and metal non-excluders (indicator and hyperaccumulator) [4,5].

The first publication concerning the classification of the different metallophytes was done by Baker (1981) [5]. However, these criteria and the metal specificity are currently redefined. Recently, conditions for above-mentioned classification of plant strategies were improved by two further characteristics: bioaccumulation factor (BF or BAF) and translocation factor (TF). Both factors have to be considered for hyperaccumulator category. The term BF, defined as the ratio of metal concentrations in plant dry mass (µg g^{-1} d.m.) to those in soils (µg g^{-1} soil), has been used to determine the effectiveness of plants in removing metals from soils [4]. *Thlaspi caerulescens* and *Arabidopsis halleri* are two hyperaccumulator and heavy metal tolerant plant species. They are well known experimental tools to study these characteristics. Their occurrence in nature is not restricted to metalliferous soils and they are rather widely spread throughout Europe [6]. Extensive screening of these two plant species (*T. caerulescens* and *A. halleri*) has been done in search of extreme ecotypes. The importance of this research is to investigate the genome of these plants in order to unravel the molecular basis behind the hyperaccumulation and heavy metal tolerance. This knowledge could eventually be exploited in environmental technologies, for example: phytostabilization, phytoremediation and phytomining [5,6].

1.2.2 Plant genotypes

Genomic screening has led to a wide variety of possible genes contributing to metal tolerance and hyperaccumulation. Genes that react to metal exposure can be an important lead in understanding molecular mechanisms of metal tolerance or hyperaccumulation. These genes have been found to be active in various pathways of plant metabolism and physiology. This led to an increasing investigation of how certain plant species can: mobilise and extract metals from the soil, sequestrate metal complex formation and deposition in vacuoles for detoxification within the roots, competence of metal(loid) translocation to shoots via symplast or xylem (apoplast), distribution to aboveground organs and tissues, sequestration within tissue cells and an eventually expulsion of accumulated metals to less metabolically-active cells [7,8].

Subsequently, using microarray technologies, large scale genomic and transcriptomic analysis of the *T. caerulescens* and *A. halleri* transcriptome have shown a broad amount of genes that appear to be responsive to (heavy) metal exposure. Several studies have compared the transcriptome of different *T. caerulescens* accessions using commercially available gene chips

[9]. Phenotypic screening makes it possible to select accessions with different characteristics in regard with metal tolerance and accumulation. The analysis of this data is often a difficult procedure due the vast amount of information generated. Some of the discovered genes are yet unknown in function and demand additional research. Reverse genetics is a useful tool in solving these questions.

1.3 Biotechnology

Since 2000 the international efforts for sequencing the genome of *A. thaliana* ecotype Columbia (Col-0) was concluded (The Arabidopsis Genome Initiative (AGI), 2000). 26.828 potential genes where predicted, of these genes 25.540 where annotated as protein coding. Only 70% of these annotated genes could be given a biological or biochemical function based on homology searches. Even less than 10% of these individual genes could be given a definitive individual function [10]. In plant sciences great efforts are being undertaken in defining these individual genes. Biotechnological tools are often of great importance in this research area.

One technique that has proven its effectiveness in several studies concerning molecular biology is T-DNA insertion. T-DNA insertion can be used to for multiple purposes like tagging parts in the genome for latter isolation (enhancer, gene, intron-exon and promoter trapping) [11]. In this research the reverse genetic technique of T-DNA knock-out was used. The targeted insertion of bacterial (*Agrobacterium tumefaciensis*) plasmid DNA into the genome of *A. thaliana* results in a gene with reduced functionality. The complete silencing of a gene by this insertional mutagenesis technique is often difficult to accomplish. Nevertheless observations of phenotypic change due to this technique have been helpful in understanding the function of numerous genes [11,12].

In contrast to this technique, plants with the capacity to overexpress a certain gene are also part of the molecular tool package. There are more ways to achieve this and in this case ectopic expression was used. In ectopic expression a gene from a foreign organism is used to be placed under a hyperactive promoter. In this case the CaMV 35S promoter was used. The *RCC1* gene of *T. caerulescens* was placed under the influence of this hyperactive promoter and finally the construct was incorporated in the genome of *A. thaliana.*

1.3.1 RNA interference

RNAi is a relatively new technique that was discovered in the early 90's when plant scientists were looking for ways to change flower color. Since its discovery, RNAi has been a valuable technique to use within reverse genetic approaches [13].

The mechanism of RNAi is based in the formation of dsRNA with mRNA within the cell. This dsRNA is broken down by specific proteins domestic to every cell [13,14,15]. This is however a simplified description. The breakdown of mRNA is always the end result (translation inhibition has also been observed) but the means of how this is achieved can be different [14]. For relevance to this paper the inverted-repeat post-transcriptional gene silencing (IR-PTGS) will be briefly explained. In these transgenic *A. thaliana* strains construct which are transformed into the plant contain a part of the target gene first in sense orientation, followed by an (artificial) intron and the same partial target in antisense orientation. This leads to a hairpin-like structure, where the sense and antisense strands bind together. The presence of the intron causes it to form a loop and hence the hairpin-like structure [14,16]. This complex is broken down by endogenous protein complexes called dicer-like enzymes (DCL) into 21nt dsRNA pieces or siRNA. One siRNA-strand incorporates into AGO1-loaded RISC to guide endonucleolytic cleavage of homologous RNA, leading to its degradation [14,17]. The exact mechanisms are not completely understood. This investigation makes use of this last technique.

1.4 Previous research findings

This paper is an extension to previously conducted research at the Department of Biosciences, University of Eastern Finland (UEF).

The first findings leading to this research was a paper written by V. H. Hassinen. In this paper, Zn-responsive genes of two *T. caerulescens* accessions where discovered [18]. The two accessions were: La Calamine (LC) originates from soil contaminated with calamine ore waste (Zn, Cd, Pb) near La Calamine (Belgium) and Lellingen (LE) from non-metalliferous soil at Lellingen (Luxembourg) [18]. LC has a higher Zn-tolerant capacity whereas LE has a higher Zn-accumulation capacity. Numerous cDNA fragments (16) where confirmed to be differentially expressed [18]. Of these 16, one *RCC1* is of interest in this paper.

1.4.1 *RCC1* gene

In the previously mentioned paper and the doctoral dissertation of Viivi H. Hassinen the Regulator of chromatin condensation (*RCC*) 1 gene was found differentially expressed in roots. The different accession where compared with each other. Besides comparing the accession, they were also exposed to 500µM Zn. This led to the compression of four different groups: LE C(ontrol), LE Zn, LC C and LC Zn (control 0,2µM Zn, Zn 500 µM Zn) [19]. In the LC accession the *RCC1* gene was found to be down-regulated by Zn exposure. Not much is known about this gene in *A. thaliana* (sequence of *RCC1* is given in supplementary data 1).

More is known about the human homolog of this protein/gene. The RCC1 protein is part of a highly conserved protein superfamily named the RCC1 superfamily. Every protein with a RCC1-like domain or RLD is part of this group [20]. There are 18 proteins identified to have RLD's and they are divided in 5 subgroups. The RCC1 protein we are interested in is part of the RCC1 subgroup and the RLD spans the complete protein and related gene [20]. In humans and other mammals RCC1 functions as a guanine exchange factor or GEF [20,21].

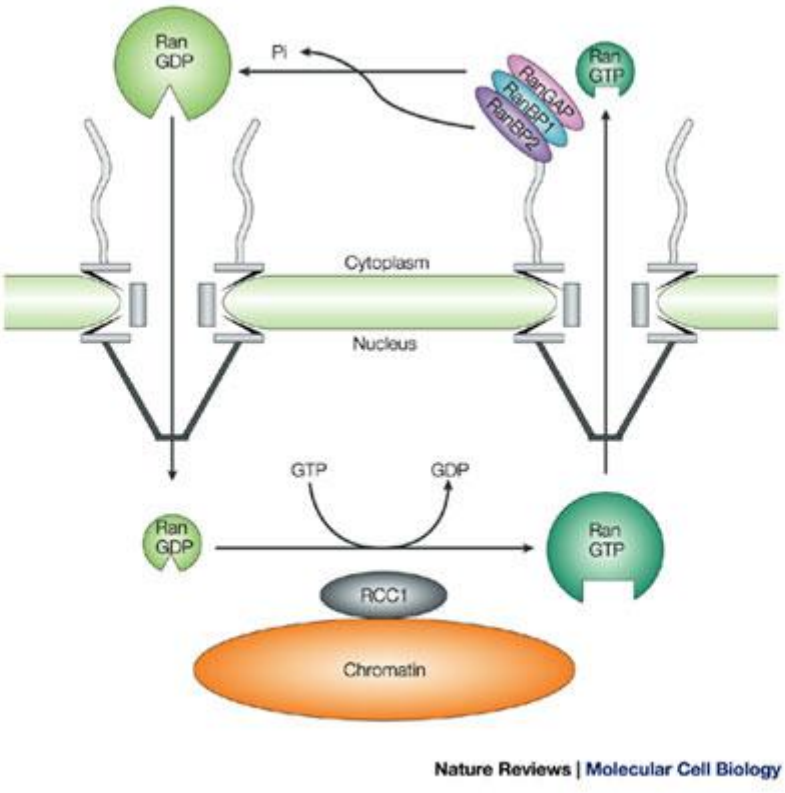

Figure 2: Schematic representation of RCC1 function. A Ran-gradient in the cell is maintained by the action of RCC1 in the nucleus and RanGAP in the cytoplasm. RCC1 bound to the chromatin promotes the dissociation of Ran-GDP and allows it to bind GTP, in consequence a high level of

RanGTP is present. In the cytoplasm RanGAP together with RanGTP-binding proteins (1 and 2) induces GTP hydrolysis at the filaments of the nuclear pore complex, inconsequence to this a low level of RanGTP is present in the cytoplasm an a gradient is created [22].

As mentioned before not much is known about RCC1 in plants but more is known about the RAs-related Nuclear (Ran)- protein. Ran has been shown to be involved in transport of macromolecules. Movement of molecules larger then 50-60kDa (proteins and RNAs) across the nuclear membrane is known to be an active process. The gradient of RanGTP/GDP is of great importance in this transport of macromolecules [23,24]. RanGTP can also act as an active form which can participate in signal transduction. Its' major effector molecule is RanBP. These effector molecules of RanGTP are found to be diverse which can suggest a wide range of funtions [23]. RCC1 is also the only GEF known at this moment for Ran [24]. A changed activity or functionality of RCC1 can thus indirectly influence many processes in the plant.

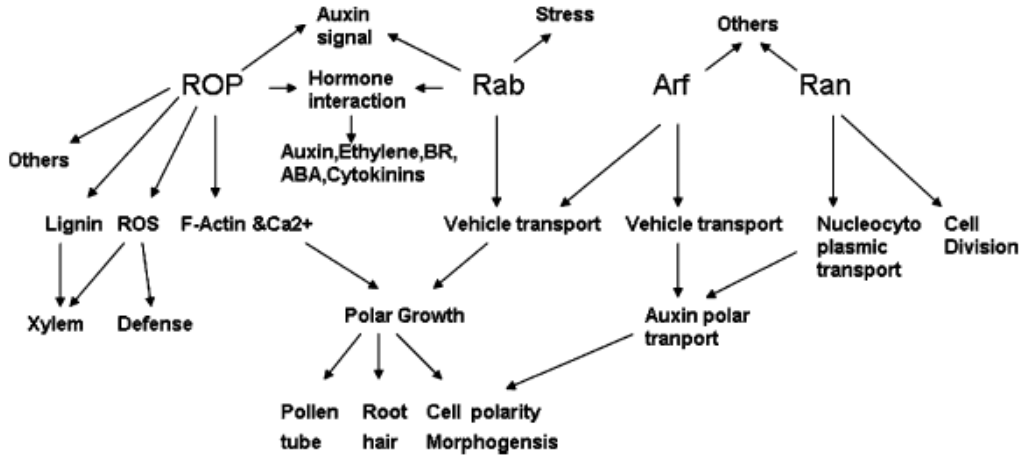

Figure 3: Schematic overview of functions of small GTP-binding proteins in plants. Ran is also part of this group of signalling molecules and under influence of the RCC1 protein. An important effect of Ran is the influence of cell polarity and morphogenesis by auxin transport [23].

In other research a loss of gravitropism and the curling of roots were detected when exogenous ATP was added to the growth medium. This was the result of a decrease in transport of auxin [25]. Additional research has pointed out that when the RanGTP is depleted and a thus the RanGTP/GDP is disturbed, the nuclear import and export of Ran-dependent molecules is inhibited [26].

The characterisation of these pathways is still necessary to understand all the cross-talk between these molecules. However the discovery of a differentially expressed *RCC1* gene in these LC lines can be important for understanding the biochemical pathways controlled by this gene.

1.4.2 Previous experiments

Because of its expression in a Zn-tolerant non-exposed *T. caerulescens*, its' unclear function and involvement in important biochemical pathways, makes *RCC1* an interesting lead towards understanding molecular mechanisms involved in metal tolerance. To study its function, transgenic *A. thaliana* strains were created and different experiments conducted using them. The strains used in previous experiments were; overexpressors, T-DNA knock-out mutants and RNAi knock-down plants. One of the first observations was a phenotypic change of the RNAi plants (supplementary data 2). Another important test was the responsiveness of the *RCC1* gene to various abiotic stress factors. In this experiment *A. thaliana* plants overexpressing the *T. caerulescens RCC1* gene seemed to have a decrease in root length compared to the wild-type strains (supplementary data 3). The effects of plant growth in the absence of *RCC1* expression and in the presence of heavy metals could also be of interest. To investigate this, knock-out and knock-down plants were used (supplementary data 4). No conclusive results were obtained from these experiments. However, it is known that *RCC1* is a differentially expressed gene in *T. caerulescens* and that when silenced in *A. thaliana* phenotypic changes occur in root development.

1.5 Research methods and aim

There is very little data about biochemical interactions mediated, directly or indirectly, by RCC1. One of the ways to investigate this is to use proteomic techniques. This makes it possible to search for proteins that are differentially brought to expression in different transgenic *A. thaliana* strains.

Recent whole root proteome analysis identified approximately 5159 proteins in 10-day-old roots, and 4466 in 23-day-old roots [27].

We can silence the endogenous *RCC1* gene using RNAi in *A. thaliana.* By comparing the root proteome of these stains with the roots of wild-type *A. thaliana* ecotype Columbia proteins can discover which are influenced by *RCC1*. The comparison is done by using a technique

called two-dimensional gel electrophoresis (2-DE). In this technique proteins are separated according to their molecular size and iso-electric point [28]. The gels are analysed digitally with software designed for these purposes. The software will allow me to search for spots that can be present or absent in the different genotypes. Only roots were investigated because of the discovery in root samples and the phenotypic changes observed.

Changes in protein expression can be expected because of a clearly altered phenotype in the root development. The biochemical pathways in which RCC1 is involved are also influenced by its absence and can thus reflect in the protein profile of the transgenic strains. Identification of these proteins could give an explanation for the observed phenotypic change and increases our knowledge of the *RCC1* gene.

2. Materials and methods

2.1 Plants cultures

2.1.1 Plant material

There were three different genotypes used in this research. The control genotype was an *A. thaliana* ecotype Columbia. The other two genotypes were separate transgenic plant lines with a silenced *RCC1* gene. The plasmid pB7GWIWG2(I) (supplementary data 5) was obtained from VIB, Gent [29]. Construction of *RCC1* RNAi plasmid and transformation of the plants was performed by MSc Pauliina Halimaa at the Department of Biosciences, UEF. Two different RNAi transformant lines were used: RNAi4.5 and RNAi4.3. These were selected based on the outcome of previously obtained results of qRT-PCR experiments and the observation of phenotypic changes (supplementary data 2,6).

2.1.2 Transformation of *A. thaliana* and vector construction

Plasmids containing *RCC1* DNA for endogenous gene suppression were constructed using the Gateway® cloning system. This technology makes use of bacteriophage lambda site-specific recombination within *E. coli* and the switch between the lytic and lysogenic pathways [30,31].

Lambda recombination is catalyzed by a mixture of enzymes that bind to specific sequences (*att* sites), bring together the target sites, cleave them, and covalently attach the DNA. Recombination occurs following two pairs of strand exchanges and ligation of the DNAs in a novel form.

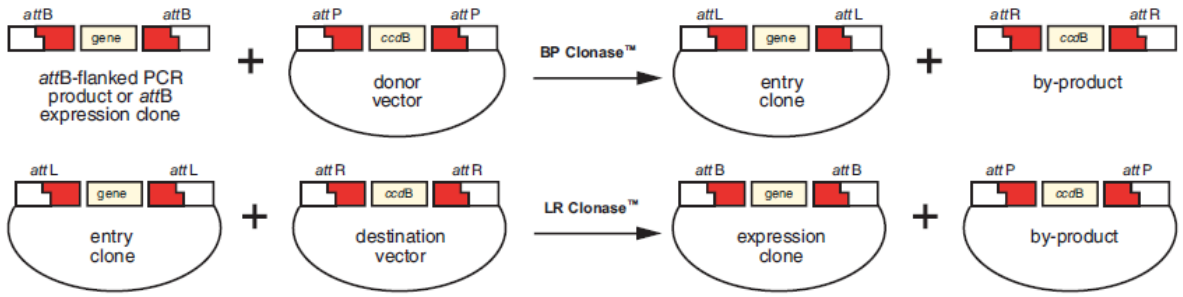

Figure 4: A scheme presenting the reactions in the Gateway® cloning system between the different type of vectors. The first **BP Reation** facilitates recombination of an attB substrate (attB-PCR product gene of intrest) with an attP substrate (donor vector) to create an attL-containing entry clone (see diagram below). This reaction is catalyzed by BP Clonase™ enzyme mix. The second step is the **LR Reaction** which facilitates recombination of an attL substrate (entry clone) with an attR

substrate (destination vector) to create an attB-containing expression clone. This reaction is catalyzed by LR Clonase™ enzymemix [32,33]

DNA fragments flanked by recombination sites (att) can be transferred into vectors that contain compatible recombination sites (attB × attP or attL × attR) in a reaction mediated by the Gateway™ BP Clonase™ or LR Clonase™ enzyme Mix (Invitrogen Co). The entry clone, which can be considered as a general donor plasmids, is made by recombining the DNA fragment of interest with the flanking attB sites into the attP site mediated by the Gateway™ BP Clonase™ II enzyme Mix. Subsequently, the fragment in the entry clone can be transferred to any destination vector that contains the attR sites by mixing both plasmids and by using the Gateway™ LR Clonase™ II enzyme Mix [30,31]. For the vectors used in the RNAi 4.5 and 4.3 lines an *RCC1* fragment was amplified from *Arabidopsis thaliana* ecotype Col-0 using CATMA primers *att*B1 GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC GTA ATC GAC ATG CAA ACG TGCT and *att*B2 GGGG AC CAC TTT GTA CAA GAA AGC TGG GTC TAT TCC CCT TTT TGT ACA AGC TTC C, *att*B1 and *att*B2 adapters underlined. CATMA primers were used to ensure gene-specific amplification. The PCR fragment was subsequently cloned into pDONR201 vector according to Gateway BP Gateway™ BP Clonase™ II enzyme Mix (Invitrogen Co) and transferred to *E. coli. E. coli* containing the entry clone were selected on plates containing 50 µg/ml kanamycin. Recombination between the entry clone and Gateway destination vector pB7GWIWG2 (I) was performed according to manufacturer instructions (Gateway™ LR Clonase™ II Enzyme Mix,Invitrogen). The destination vector was transferred in *E. coli* and transformants were selected on agar plates containing 50 µg/ml spectinomycin. The destination vector construct was transferred to *Agrobacterium tumefaciens* and transformation of *A. thaliana* was carried out as described in Logemann et al. (2006).

The transformation of the plants was started by using healthy *A. thaliana* plants for floral dip. The optimal plant is one with many immature flower clusters. The *Agrobacterium tumefaciens* (LB4404) with the gene of interest on a binary vector was selected using spectinomycin. *Argobacterium* was selected on agar plates. Transformed *Agrobacterium* was transferred from plates to transformation media prior to floral dip.

Before the dipping takes place Silwet L-77 detergent is added to the dipping solution. Plants are then afterwards grown under high-humidity and low sunlight conditions. Harvest of dry seeds is accomplished by diminishing the watering of the plants before maturation of the

seeds. Selection of transformed seeds can be done by using antibiotic resistance selection or herbicide treatment. In this case, the transformants were selected using a non-selective total herbicide to which only the desired plants were resistant to. This is also called, Basta (glufosinate ammonium) selection.

2.1.3 Seed sterilisation

The seeds were sterilized in a 8.5% $Ca(ClO)_2$ solution containing 0.1% Tween-20 detergent. After the components were resolved the solution was filtered through Whatman 1M filter paper. The seeds were mixed in the clear solution for 30-40 min and centrifuged gently afterwards. The supernatants was removed from the eppendorf tubes under a laminar hood and 70% ethanol was added for an incubation of 1min. Three washing steps followed with sterile H_2O. The seeds were dried in a laminar hood.

2.1.4 Hydroponics

The hydroponic system used for this research is based on a paper about a novel low maintenance, high efficient and synchronous system of growing *A. thaliana* [34].

As a preparation to this technique 1ml pipet tips were filled with 0.6% agar (Duchefa biochemie, 9002) mixture in sterile H_2O. One seed was used per pipet tip. For every hydroponic experiment one pipet tip box (96)/genotype was used. Vernalisation of the seeds was accomplished by placing the pipet tip boxes in 4°C for 2 days.

Germination of the seeds was done by placing the pipet boxes in an in vitro room for one week. The climate was controlled at a temperature of 21°C and light intensity of 36 $\mu E\ m^{-2}S^{-1}$. The light cycle had a duration of 19h. After germination the hydroponics were started. Six 10L tanks were used on which 14 plants of every genotype were placed. The best germinated plants were selected for this. The media, based on Hoagland protocol (supplementary data 7), was refreshed twice a week. The pH of the media was brought to 5.5 before use. Because of regulations for working with transgenic organisms the media was deactivated by adding Virkon®S (Dupont, Pharmaxim) in a 1% ratio for 24h. The climate conditions in the growth chambers were aligned with real-time day and night cycles. The light period was 16h, at the start of this period the temperature increased from 20°C to 25°C. The humidity decreased during day cycle from 80% relative humidity to 60% relative humidity. The period from seed

to harvest was one month. The harvest was done so that roots and shoots were separately stored at -80°C and with efforts to minimize proteomic changes or overturn.

2.2 Proteomics

2.2.1 Sampling method for proteome analysis

The main objective of the sampling was the homogeneity between the plants of different genotypes and within the same genotype. Overall evaluation of the condition and the health of the plants was done by certain parameters like: number and weight of leaves, colour of the leaves and size and weight of the roots. Two cultures were used, in the first culture no pooled samples were used, while for the second culture samples were pooled. In the pooling of the samples three roots per genotype per tank were selected based on highest root weight and overall health of the plant. The pooling took place in the first step of the protein extraction i.e. grinding of the roots. The position of the growth thanks was changed during the growth experiment to prevent positional effects.

2.2.2 Protein extraction

The roots of selected samples were homogenised by liquid nitrogen deep freezing and grinding them using pestle and mortar. The powder was collected and suspended in a Tris-EDTA-thiourea buffer (supplementary data 8). A 10x volume of second buffer was added to this mixture, a 10% trichloacetic acid (TCA) 0.07% β-mercaptoethanol in acetone (-20°C) solution. Overnight precipitation of the samples was made at -20°C. The precipitated samples were washed by centrifugation at 4000 rpm for 10 min at 4°C (Eppendorf centrifuge 5810r). The washing solution used was a cold 0.07% β-mercaptoethanol in acetone. The washed pellets were dried in a speed vac for 20 to 30 minutes. Resuspension of the pellet was done using a second Tris-EDTA buffer (0.1M Tris-HCl pH 8.0 – 50mM EDTA) added in a 5X amount of the first buffer. A Tris-buffered phenol was added in 0.5x amount of the second buffer Tris-EDTA buffer. The mixture was slightly mixed for 5 minutes. Centrifugation of the samples took place at 4000 rpm for 10 min at 4°C (Eppendorf centrifuge 5810r). If the phenol phase was not clear additional centrifugation could be performed. The phenol phase was collected and the precipitation of the proteins was accomplished by adding cold 0.1M ammonium acetate in methanol in a 5X amount of the phenolic phase. The mixture was put on -20°C for overnight precipitation. After a centrifugation at 4000 rpm for 10 min at 4°C (Eppendorf centrifuge 5810r) the supernatants was removed. The pellet was washed three

times with a 1% β-mercapthoethanol in acetone. The pellet was dried to the air and resolubilised in resolubilisation buffer (supplementary data 8). Resolubilised protein samples were stored at -70°C.

2.2.3 Protein quantification

Total protein quantification was performed using the Bio-Rad Protein Assay dye reagent. A calibration curve was used with the resolubilisation as a correction for the milli-Q blank. Bovine serum albumin was used as a standard. All standards were created in triplicate like the samples. Absorption of standards and samples was done using a Pharmacia Biotech Ultraspec 2000 cuvet reader at a λ=595 nm.

2.2.4 First dimension

The data obtained from the protein quantification was used to create samples of equal protein concentration which are ready to be loaded for the first dimension. The first dimension is started with a rehydration of the IPG Immobiline DryStrip linear pH 4-7 of 24cm (GE Healthcare). In this step protein samples and rehydration buffer (supplementary data 8) were loaded in one mixture on the Immobiline DryStrip reswelling tray. The protein samples were diluted using rehydration buffer to get a final volume of 450µl. Once the dilution is made and the rehydration buffer is added the samples are loaded in the rehydration tray. The strips are placed over the sample with the gel side facing down and no air bubbles in between. Each strip is covered with mineral oil and was left to rehydrate over night on room temperature. After this step the proteins are present in the gel of the strips.

The IPG strips were rinsed with milli-Q after rehydration and subjected for isoelectric focusing. The focusing was done using a IPGphor IEF system (GE Healthcare). Strips in the ceramic manifold (Amersham, Biosciences) were covered with mineral oil. Paper wicks damped with 100µl of milli-Q were applied on both ends of the strip.

The total running program spans 16:35h with parameters:

- 50µA/ strip
- 300V step-n-hold 3:00h
- 1000V gradient 6:00h
- 8000V gradient 3:00h
- 8000V 36700 Vhr

After isoelectric focussing strips were rinsed with milli-Q and stored at -70°C.

2.2.5 Second dimension

For the equilibration step a standard buffer was used: 50mM Tris-HCl pH 8.8, 6M urea, 30% glycerol, 2% SDS and a few grains of bromophenol blue. Equilibration was done in two steps: in the first step DTT (Sigma) was added to the standard equilibration buffer so that it was present in a 1% (w/v) amount, the second step was done by using the same buffer but DTT was replaced by 2.5% (w/v) of iodoacetamide (Sigma). During the equilibration steps the strips were gently shaked for 10min.

SDS-PAGE was performed with 1.5mm 12% acrylamide gels. The gel solution was prepared using a readymade 30% acrylamide/bis solution (Bio-Rad), 375mM Tris-HCl pH 8.8, 0.1% SDS, 0.1% APS (Bio-Rad) and 0.014% TEMED (Bio-Rad). Casting of the gels was done by using the Hoefer DALT system (Amersham, Biosciences). In the casting process a displacing solution (50% (v/v) glycerol, 375mM Tris-HCl pH 8 in H_2O) was used and poured in the system after the gel solution. During two hours polymerisation took place after which the gels were stored until use at 4°C in storage buffer (375mM Tris-HCl pH 8, 0.1% SDS in H_2O).

Once the gels had polymerised the equilibrated IPG Immobiline DryStrip's were loaded on the gels. A molecular weight marker (PageRuler™ Unstained protein ladder, Fermentas) was added to one gel/2nd dimension run. The gels were sealed with a 0.5% agarose in running buffer solution coloured with a few grains of bromophenol blue. The running itself was done using a the DALT vertical slab system electrophoresis (Amersham, Biosciences). A standard SDS-running buffer with 25 mM Tris, 192 mM glycine and 0.1% SDS cooled to 10°C was used. The current selected in the first 20min of the run was set at 14µA/gel, after which the current was increased to 21µA/gel. The program was continues and total running length was 18h.

2.2.6 Staining and imaging

After the second dimension the gels were fixated by shaking the gels in a 10% methanol 7% acetic acid solution for 30 min. The staining solution used was a SYPRO Ruby protein stains (Biorad). After an overnight staining the gels were washed with a 10% methanol 7% acetic acid solution for 1h before they were put on milli-Q water. Scanning of the gels was done by using a FUIJFILM-3000 FLA scanner with an excitation of λ= 473 nm and an emission of λ= 580 nm.

2.3 Image analysis and statistics

The software used to analyse the gel scan images was PDQuest version 7.1.1 (Bio-Rad). This software made it possible to get the intensities of the gel spots from the images. The intensities were normalised by dividing the spot intensities of each spot by the total intensity of all valid spots on the gels.

All statistical analysis were performed with using the SPSS software version 14.0 (SPSS Inc. Chicago IL, USA). The analysis of root weight was done using a oneway-ANOVA with a Bonferroni correction as post-hoc parameter.

2.4 Mass spectrometric analyses

The gels were silver-stained according O'Connel and Stults (1997) except in the first step the gels were incubated 2x 30 min in 30 % ethanol and 0.5 % acetic acid were used. For the mass-spectrometric analysis the spots were excised from the stained gels and cut in small pieces. Gel particles were washed three times with 25 mM ammonium bicarbonate pH 8 / 50% acetonitrile and dried in a vacuum centrifuge. Digestion was performed with equal volumes of 0.05 µg µl Sequencing Grade Modified Trypsin (Promega, Madison, USA) and 50 mM ammonium bicarbonate pH 8. Gel particles were immersed with 25 mM ammonium bicarbonate pH 8 and the samples were digested overnight at 37 ˚C. Peptides were recovered with 50% ACN/5% TFA. After two extractions the gel particles were rehydrated with water, extracted twice and lyophilized [35].

For the mass spectrometric analyses peptides were resolubilized with 0.1 % formic acid, 2 % acetonitrile and separated using the Ultimate/Famos capillary liquid chromatography (LC) system (LC Packings, Amsterdam). Before loading to precolumn, with the flow rate of 30 µl/min, the samples were filtered on-line through PEEK encapsulated titanium filter (0.5 µm

pore; VICI/ Valco, Houston, TX, USA). The precolumn was automatically switched in-line with C18 Mass Spec (75 μm x 150 mm, Grace Vydac, Hesperia, CA, USA). The peptides were eluted using 100 % of eluent A (0.1% formic acid - 5% acetonitrile) for 3 min and followed with a linear gradient starting with 100 % of eluent A to 45% of eluent B (0.1% formic acid - 95% acetonitrile) till 25 min, to 85% of B till 35min followed by hold up to 45 min. Eluent A was increased to 100 % during 1 min and held-on till 80 min. Flow rate was 1μl/min. The liquid chromatograph was connected to mass spectrometer by a nano-ESI ion source (MDS Sciex, South San Francisco, CA, USA) using distally coated 10 μm PicoTip emitters (New Objective, MS Wil GmbH, Switzerland). The time-of-flight (TOF) mass spectra were recorded on a QSTAR XL hybrid quadrupole TOF instrument (Applied Biosystems, Foster City, CA). The instrument was calibrated using tryptic peptides from ACTH clip (Sigma Aldrich). The MS/MS data were analyzed (Yates *et al.* 1995) with the MASCOTSearch v1.6b13 script

3. Results and discussion

In the first proteomic experiments protein samples from single plants (no pooled samples) were used for the 2-DE gel electrophoresis. For every genotype three biological repeats were done with a protein loading of 80µg per gel. The images were not subjected to analysis with the PDQuest software program.

The second set of proteomic analysis was done using pooled samples. From each tank three root samples were pooled. The weights of the roots harvested from this hydroponic cultivation were also statistically compared. For every genotype five biological repeats were tested in proteomic analysis. For image and statistical analysis four gels of every genotype was used based on the quality of the gel image. In total 12 gels were analysed. All the root protein spots present in the gel are separated with a p*I* within pH range of 4 to 7. The total number of spots were detected was between 800 and 2000 per gel. Of these spots, 805 spots were included into statistical analysis because of their clear distinction in the image and software detection.

Protein identification was done on samples that were accessible for analysis. Proteins that could not be identified had lower then background concentration of proteins and therefore could not be used in further discussion.

3.1 Root weight

RCC1 is assumed to be involved in root development. For this reason the weight measured from all the roots harvested were also statistically compared.

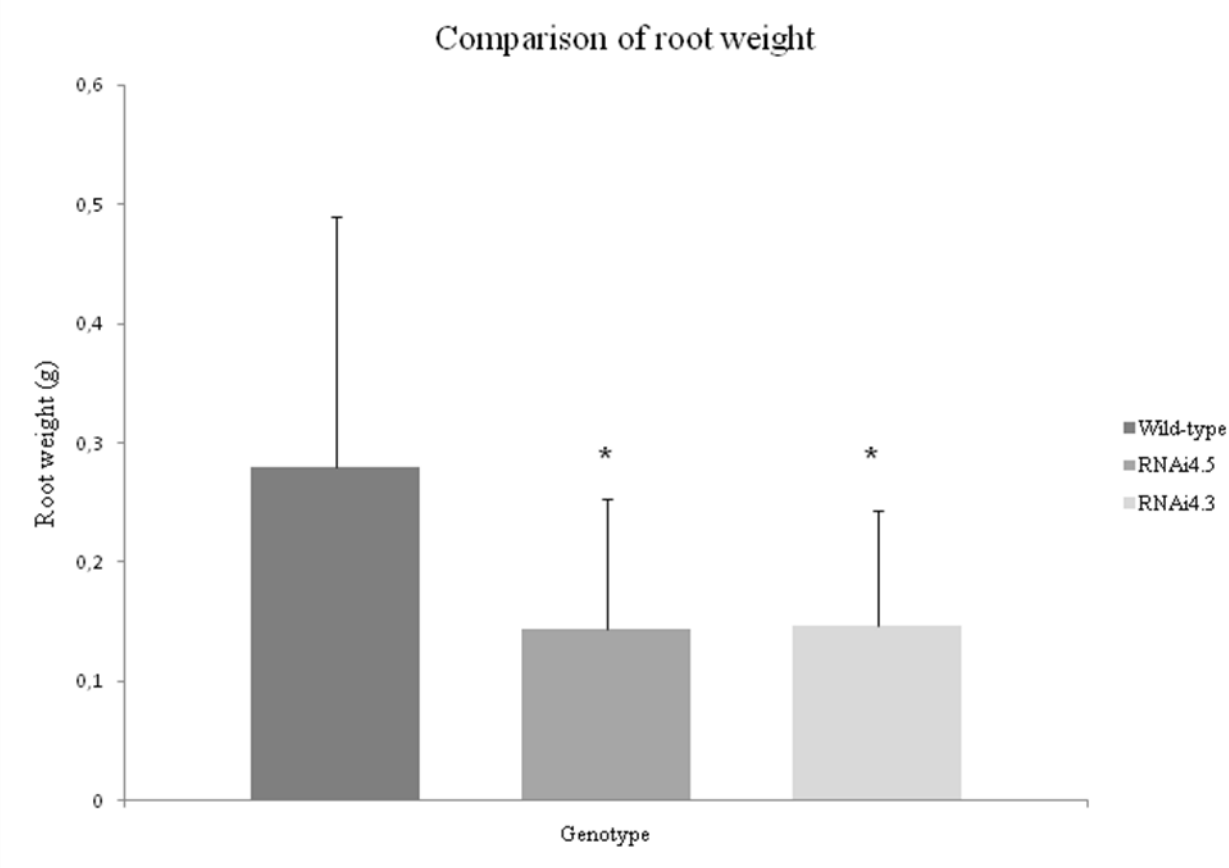

Figure 5: Comparison of root weight between the genotypes used in the proteomic experiments. Significant difference in root weight was detected between the Wild-type (Col-0) roots (n=23) and the RNAi4.5 (n=29) and 4.3 (n=31) plants. Significance was measured at α=0.05 using an ANOVA analysis with Bonferroni correction as post-hoc test.

A significant difference in root weight is detected between the Wild-type (Col-0) and RNAi lines. Between the different RNAi genotypes (4.3 and 4.5) no difference is detected in root weight. A high standard deviation was however detected for the Wild-type (Col-0) plants.

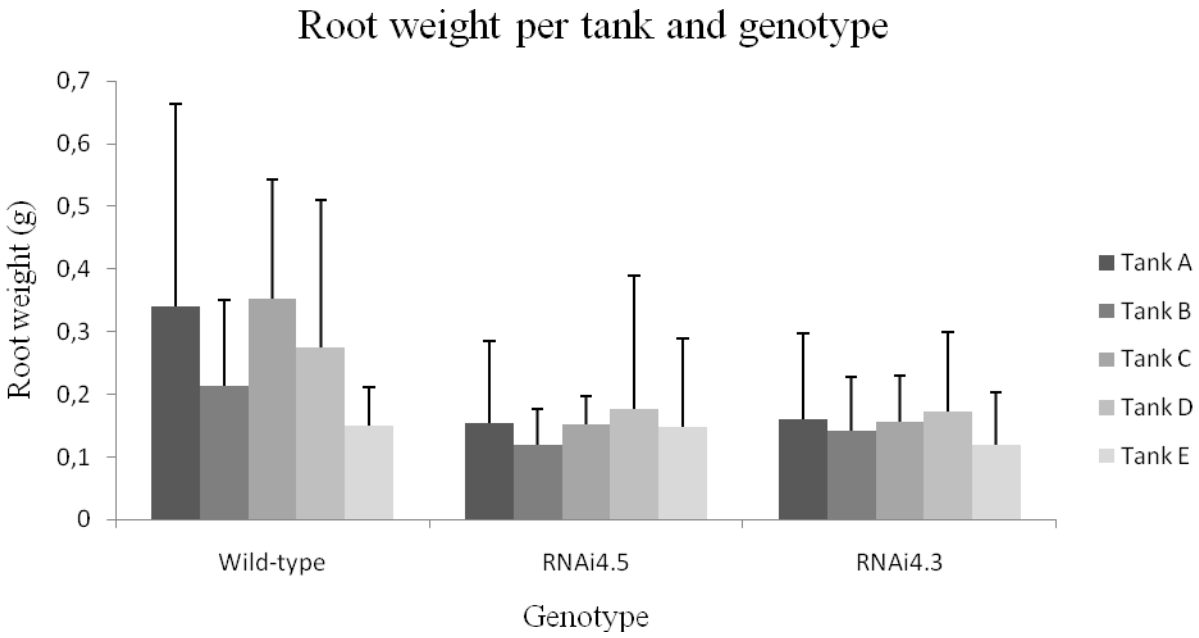

Figure 6: Comparison of the root weight between different growth tanks used and within each genotype. No significant difference detected (α=0.05) between the plants from different tanks within the same genotype using an ANOVA analysis with Bonferroni correction as post-hoc test. The number of samples differs for each tank and genotype. The sample size for RNAi4.5 was respectively for tank A, B, C, D and E: n=9, n=9, n=4, n=3 and n=4. For RNAi4.3 the sample size was respectively for tank A, B, C, D and E: n=7, n=8, n=6, n=3 and n=7. Error bars constructed using standard deviation.

Like in figure 5 an overall higher root weight is visible in the Wild-type group. Variation between the different tanks in the Wild-type group is more pronounced than in the other groups. The sample size for the Wild-type group was respectively for tank A, B, C, D and E: n=5, n=5, n=6, n=4 and n=3. The variation in the RNAi lines is less pronounced then in the Wild-type group. It is however detectable that the overall root weight is lower than the Wild-type group and relatively equal between the RNAi groups.

The RNAi 4.3 and 4.5 lines also showed the altered phenotypes with loss of gravitrophism and curly roots. This is in accordance with the previous research and observations. This could indicate that the auxin pathways are affected. Like mentioned in the introduction, plants that show decreased auxin sensitivity have similar altered phenotypes as these RNAi lines [36]. In recent published data of plants over-expressing RanGAP, a group of molecules that keep the cytoplasmatic Ran in the GDP state, showed an increase root length and auxin hypersensitivity [37]. In other words these molecules have opposite effects than the RCC1 protein. The involvement of these molecules in nuclear transport was known before but the proteomic change in the plants harbouring curly roots and loss of gravitrophism has never

been investigated. When these plants used in this investigation with the silenced *RCC1* gene harbour the same phenotypic alterations as plants with decreased auxin sensitivity and opposite as plants with increased auxin sensitivity a strong assumption could be made that transport of auxin from nucleus to cytoplasm is disturbed by the silencing of *RCC1*. The loss of gravitrophism was investigated before by proteolysis of the PIN2, an *Arabidopsis* auxin-efflux facilitator of the AUX1 family, with phenotypic changes similar as observed in this research setup [38]. Although in our case the gravitrophism loss is rather the consequence of diminished auxin transport. Further proteomic analysis could elucidate this question.

3.2 Proteomic experiments

3.2.1 pH range evaluation

Before starting the main proteomic experiments a small pre-experiments was performed to check for the p*I* range of the spots for isoelectric focusing. A broad pH range was used to get an idea of the optimal pH for a maximal separation.

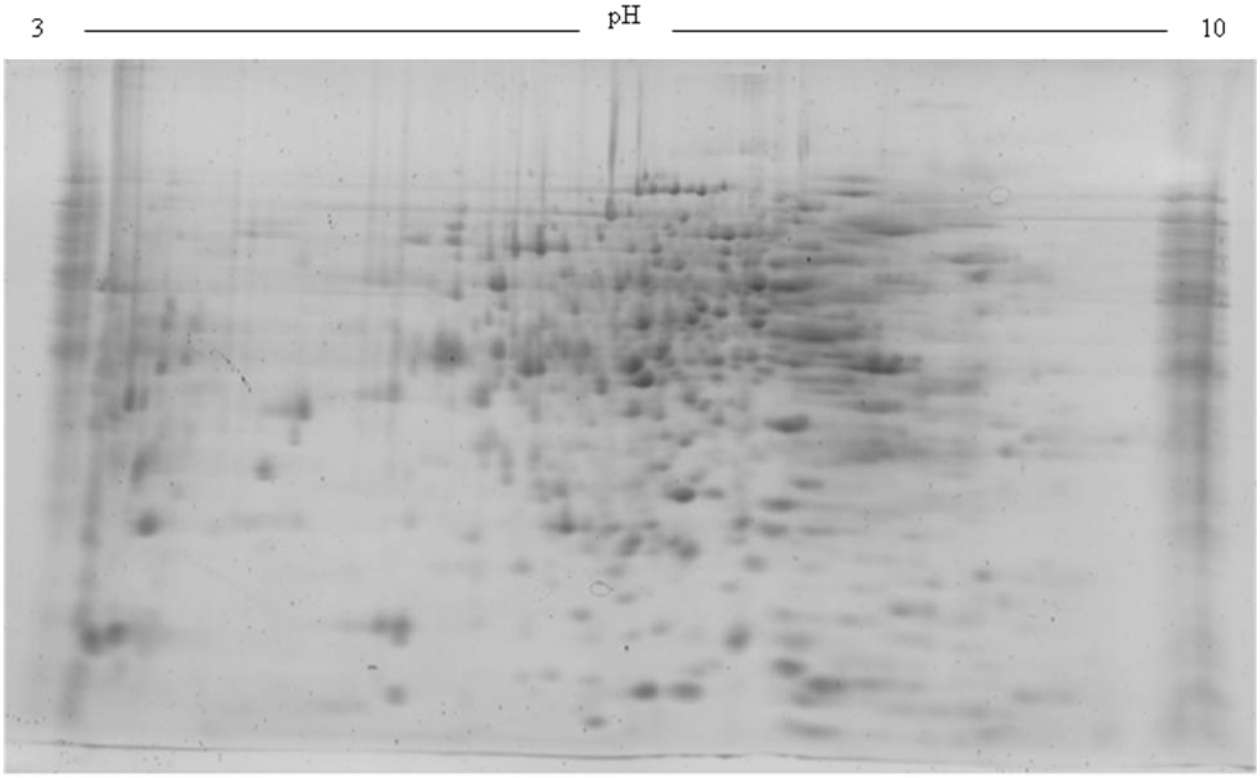

Figure 7: This small gel image was created using a 7cm strip. The linear pH range used was 3-10. No image or statistical analysis was performed on this image. This image is one of the two gels tested. The biological sample used was harvested from roots of two Wild-type plants.

The small gel image shows a cluster in the central region of the pH range. The outside margins of the gel contained a less dens amount of spots. This was an indication to use a pH range of 4-7 in the main experiments in order to get a good separation in the focusing steps to get most of the information.

3.2.2 2DE-gel image analysis

All the gels used in the image and statistical analysis are derived from pooled samples of three roots of different from three different plants within the same hydroponic tank. For every sample there were 5 biological repeats. From the 5 biological repeats four of them were selected based on the quality of the gel image.

The normalised intensity data extracted from the PDQuest image analysis was subjected to statistical analysis. Spots shown to be significantly different were selected and tested with the oneway-ANOVA and Bonferroni correction as post-hoc test ($P<0.05$) to locate the statistical difference. As mentioned before the complete matchset included 805 spots. In the statistical analysis 13 spots seemed to differ significantly ($P<0.05$) from each other.

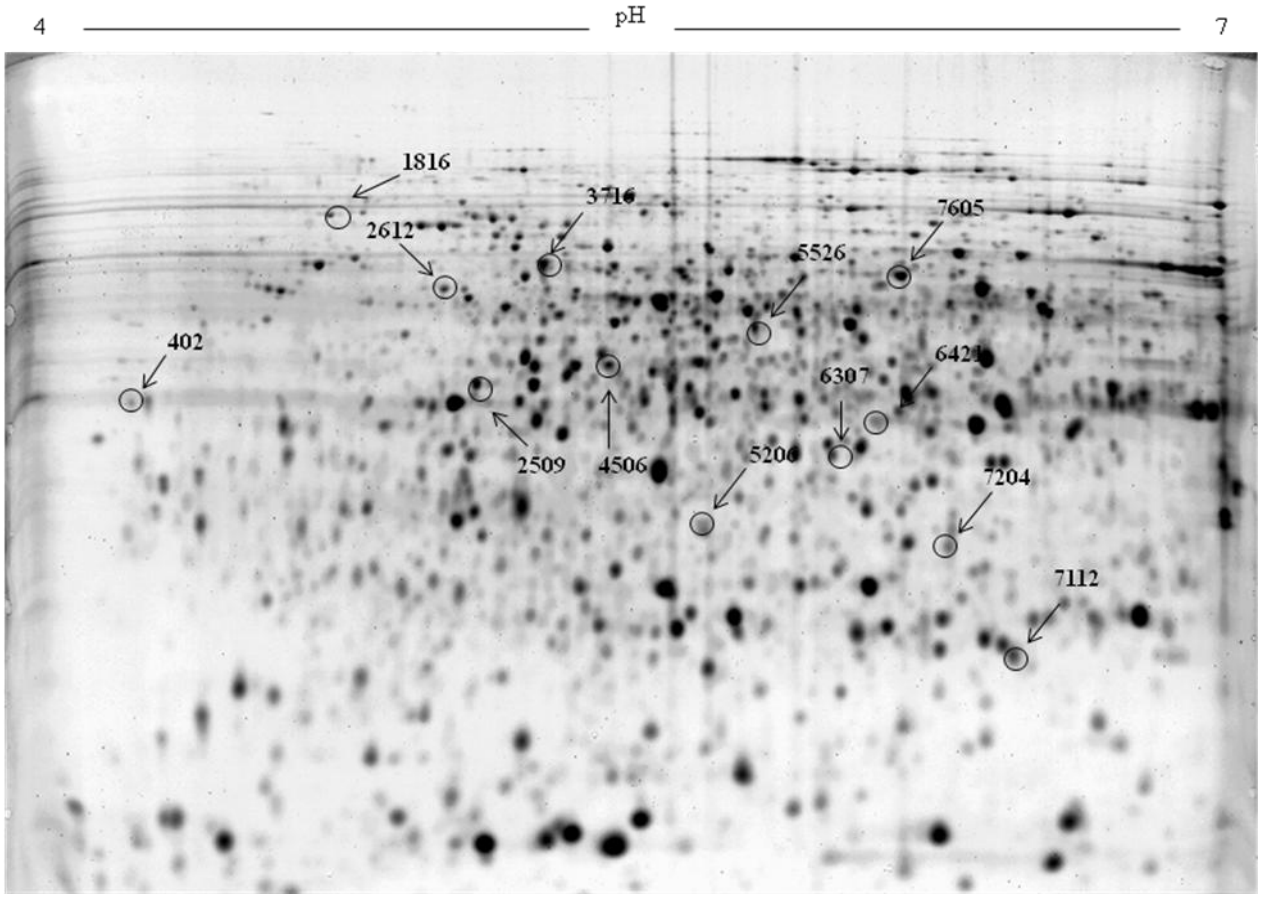

Figure 8: Gel scan image from Wild-type samples (WTB) with arrows indicating significantly different spots. The spots (13) were found to be significantly different between the genotypes using a oneway-ANOVA test (n=5) with $P<0.05$ and Bonferroni correction. The numbers indicate the identification given by the PDQuest software.

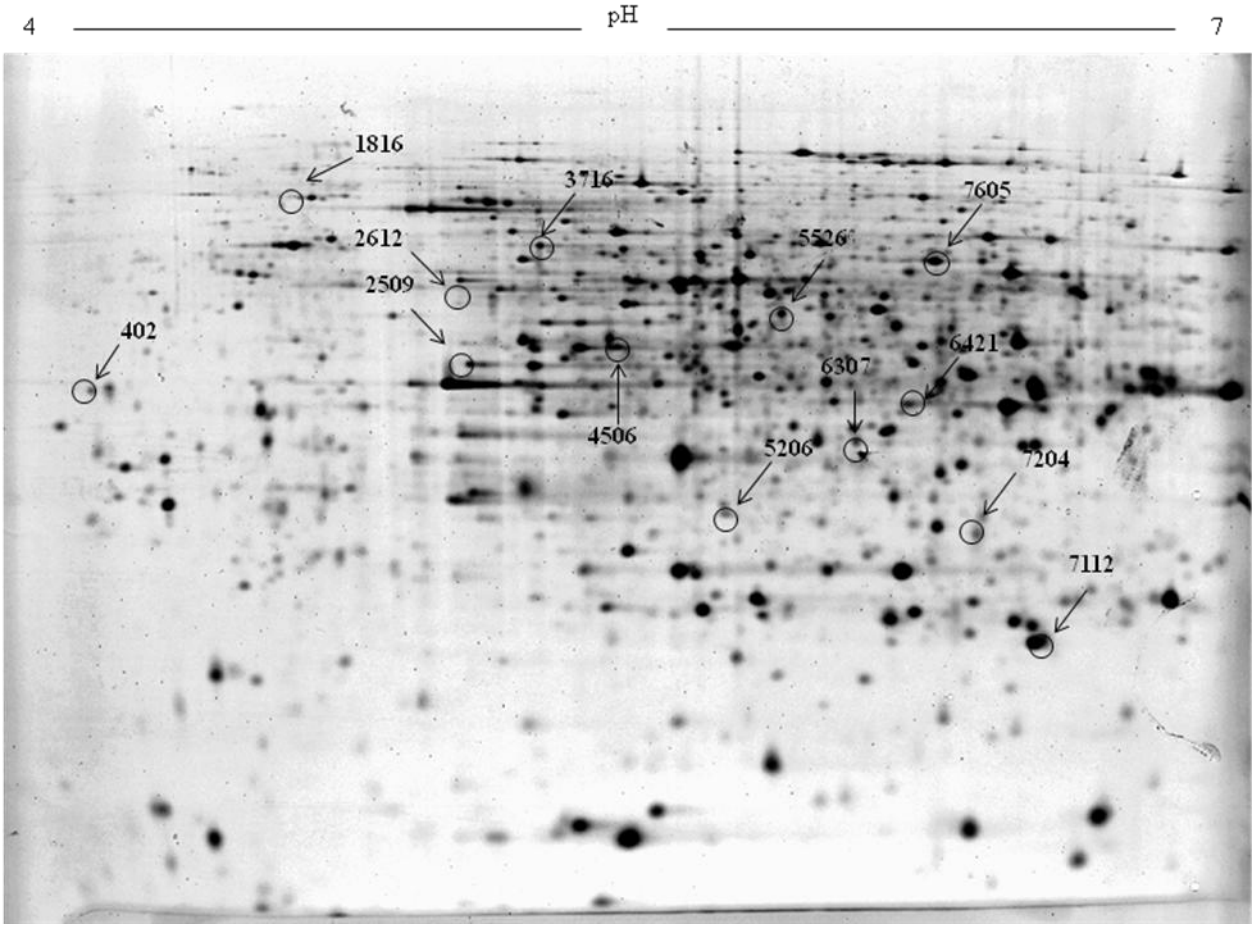

Figure 9: Gel scan image from RNAi 4.5 (4.5B) with arrows indicating significantly different spots. The spots (13) were found to be significantly different between the genotypes using a oneway-ANOVA test (n=5) with $P<0.05$ and Bonferroni correction. The numbers indicate the identification given by the PDQuest software.

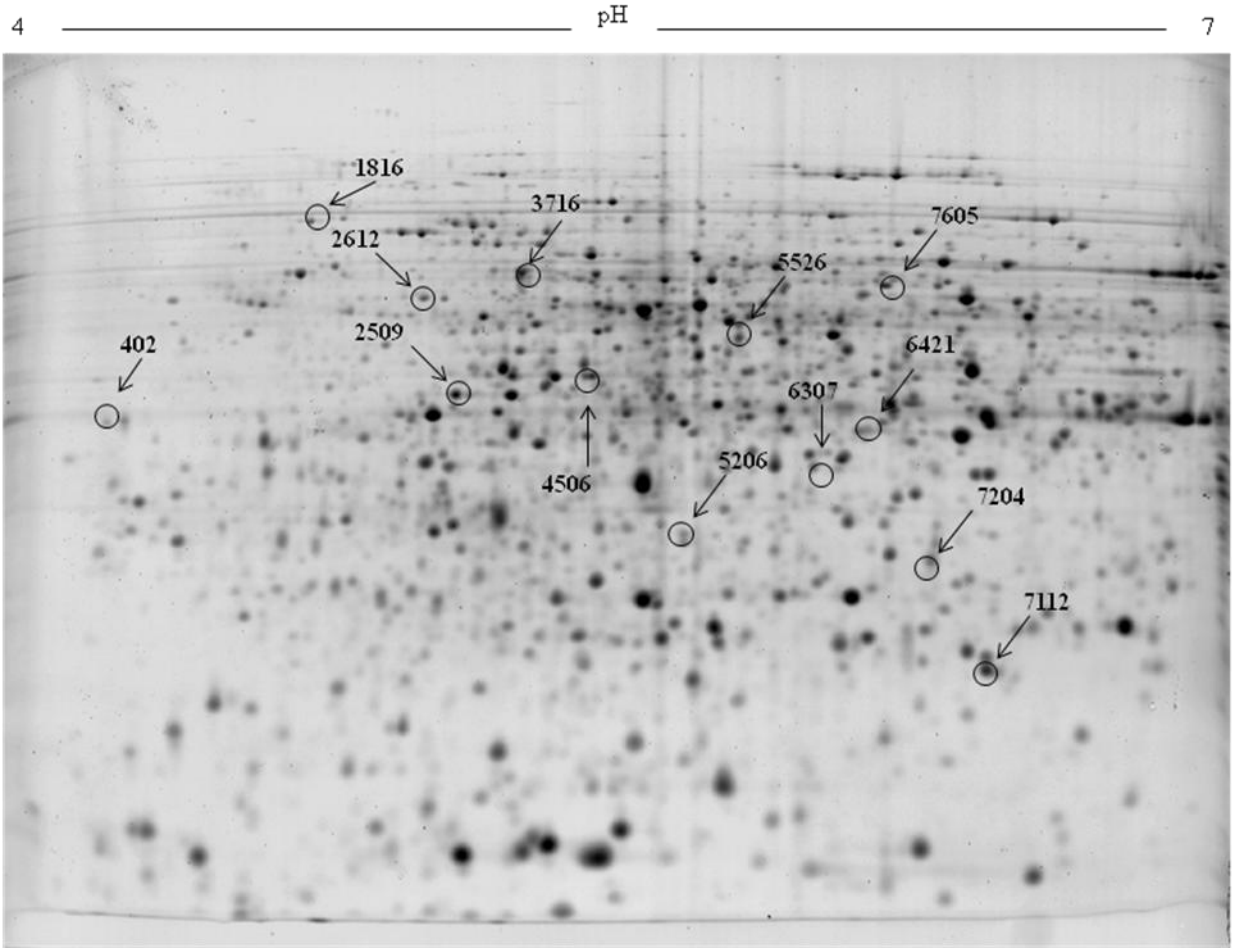

Figure 10: Gel scan image from RNAi 4.3 (4.3A) with arrows indicating significantly different spots. The spots (13) were found to be significantly different between the genotypes using a oneway-ANOVA test (n=5) with $P<0.05$ and Bonferroni correction. The numbers indicate the identification given by the PDQuest software.

All three images (Figures 8, 9, 10) presented are one of the four gels analysed statistically. The spots found to be statistically significant were also confirmed with the image analysis software PDQuest in order to avoid errors in the matchset which could bias statistical outcome.

Some gels showed streaking and possible double spots. This could be caused abnormal iso-electrical focusing. The reason for this could be impurities in the protein sample. For this reason a visual check of each protein spot analysed was preformed.

3.2.3 Spot intensity measurements

In this section we compare the normalised intensity values from the different lines. The 13 spots represent the same spots as indicated in the figures above. The visualisation of the spots is also represented with the ones found in the gel images from the above section.

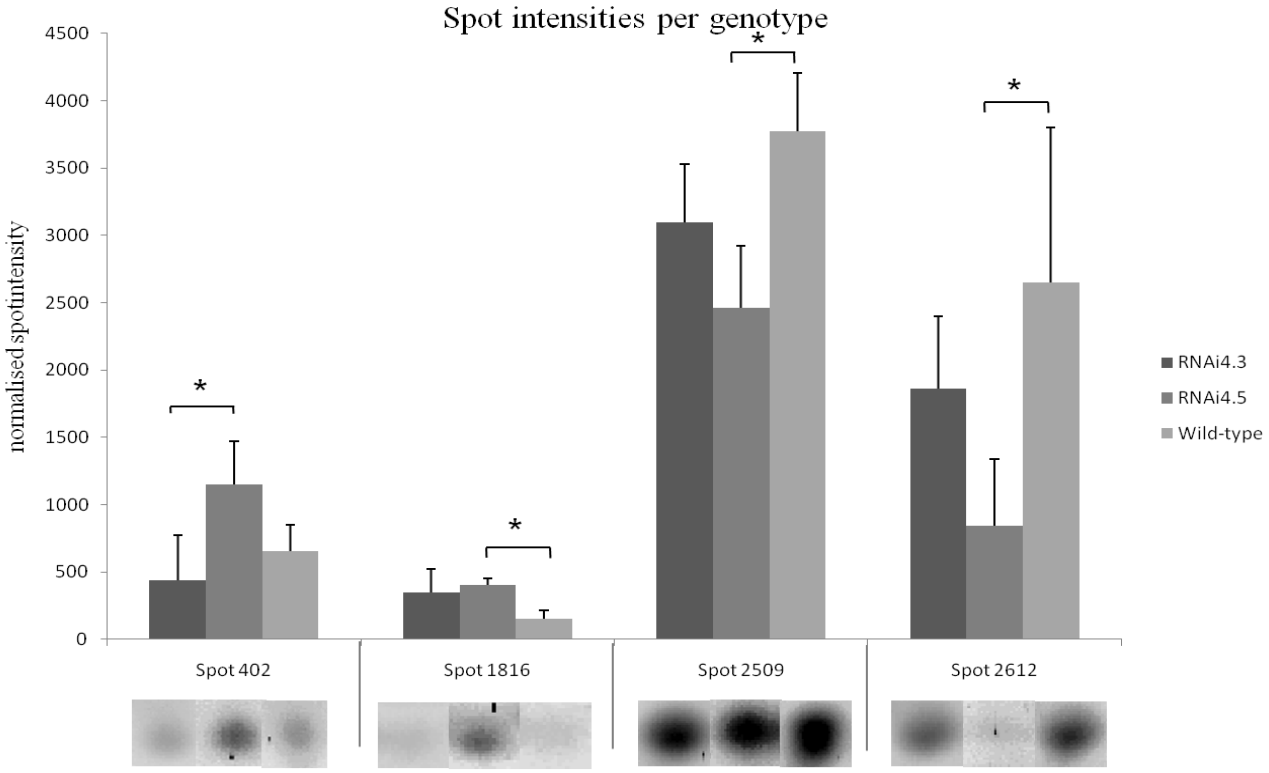

Figure 11: This figure displays the normalised intensity values per genotype and compares them statistically. The spot images corresponds with the gel image of the correlated spot. The intensities are normalised averages (n=4) derived from PDQuest. A oneway-ANOVA test with Bonferroni correction was used to detect significant ($P<0.05$) differences. Error bars are constructed using standard deviation values.

Spot number 402 shows a significant lower intensity in the RNAi4.5 and RNAi4.3 lines. The intensity of this spot seems to be relatively low for all the genotypes analysed. For spot number 1816 the significant difference was observed between the RNAi4.5 and Wild-type lines. Intensity is significantly lower in the Wild-type lines compared with the RNAi4.5. For the spots 2509 and 2612 a significantly higher spot intensity is detected in the Wild-type line compared with the RNAi4.5 lines. The RNAi4.3 line follows this trend but no significance was detected.

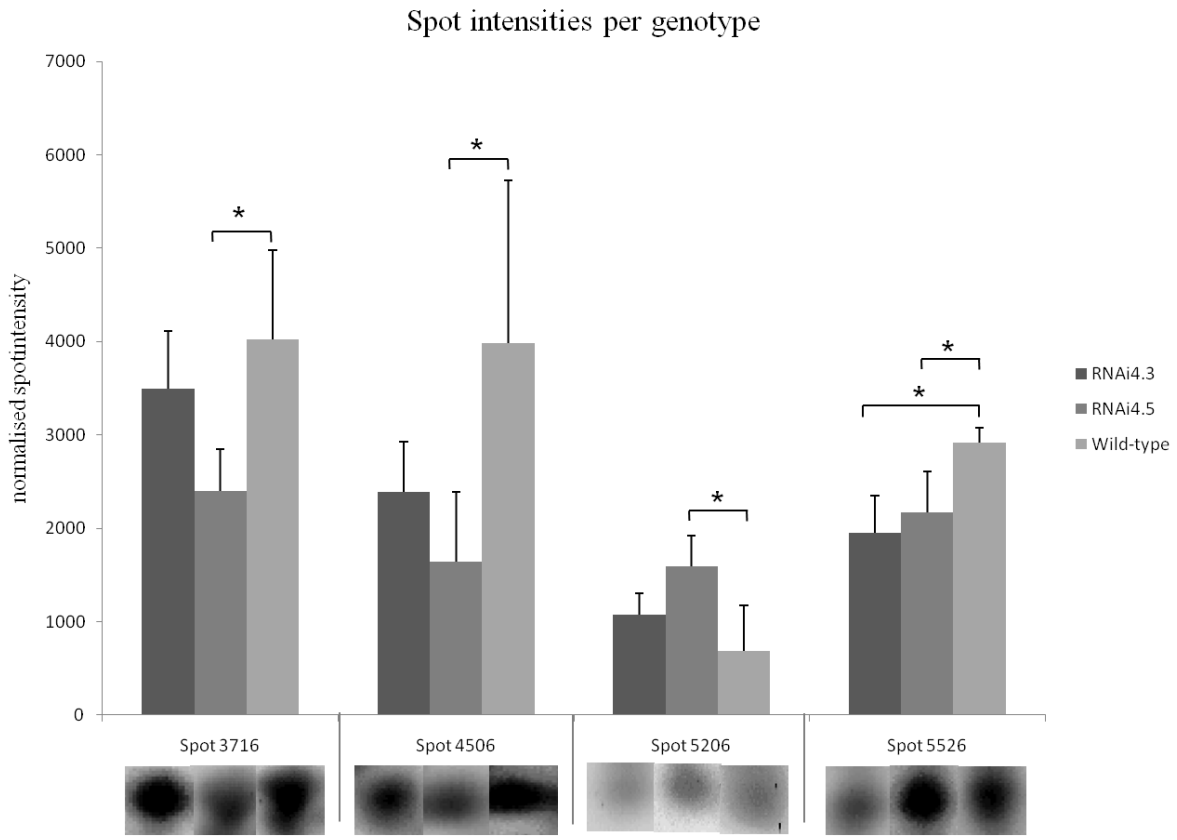

Figure 12: This figure displays the normalised intensity values per genotype and compares them statistically. The spot images corresponds with the gel image of the correlated spot. The intensities are normalised averages (n=4) derived from PDQuest. A oneway-ANOVA test with Bonferroni correction was used to detect significant ($P<0.05$) differences. Error bars are constructed using standard deviation values.

A significantly higher intensity in the Wild-type lines was detected for every spot in the figure above except for spot 5206 were lower intensity was detected. For spot 5526 the significant difference was also detected between the Wild-type and RNAi4.3 lines. In the other spots the RNAi4.3 lines reacts in the same way as RNAi4.5, but the difference to Wild-type is not statistically of significance.

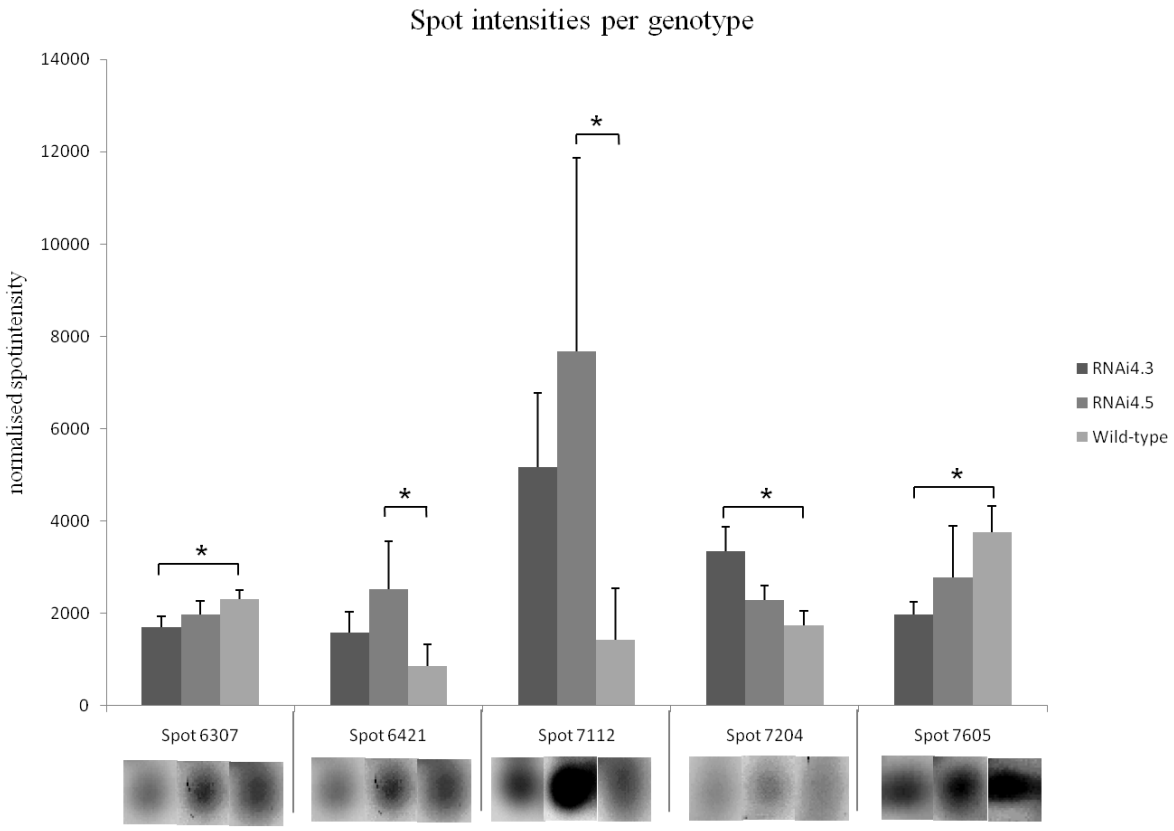

Figure 13: This figure displays the normalised intensity values per genotype and compares them statistically. The spot images corresponds with the gel image of the correlated spot. The intensities are normalised averages (n=4) derived from PDQuest. A oneway-ANOVA test with Bonferroni correction was used to detect significant ($P<0.05$) differences. Error bars are constructed using standard deviation values.

For spots 6307, 7204 and 7605 had a significant difference between the Wild-type and RNAi4.3 lines. Spots 6307 and 7605 showed a higher intensity in the Wild-type line which was statistically significant compared with the RNAi4.3 lines. Spot 7204 showed a higher intensity in the RNAi lines compared with the Wild-type line, this was only significant for the RNAi4.3 line. In spots 6421 and 7112 the RNAi lines showed a higher spot intensity compared with the Wild-type line. This increase was only significant for the RNAi4.5 line.

The difference in intensities between the two RNAi lines could be due to the fact that they were constructed separately prepared vectors. The integration in the genome could be different which could have an influence in the spots.

There were no spots detected that were unique for one genotype (RNAi vs. Wild-type) which could be a result of the fact that the silencing is not total. In the search for proteins involved one candidate pathway could be involve in mitotic processes. In studies concerning these

pathways it was proven that the RanGTP/GDP ratio's controlled by RCC1/RanGAP were important in the correct formation of the mitotic spindle. This findings could also be of relevance in altered root development [39].

3.2.4 Protein identification

Of the 13 spots 5 were identified. The other 8 spots could not be identified because of the technical reasons. All of the identified spots were found to be increased in the Wild-type lines compared to the RNAi lines.

Table 1: Identified proteins. The spot identification number corresponding with above given figures. Protein identification was performed using a MASCOT Online search (http://www.matrixscience.com/). Gi refers to the identificitation number in NCBI protein database.

Spot identification number	Difference (P<0,05)	Protein identification
Spot 2612	Wild-type > RNAi4.5	Nucleotide-binding subunit of vacuolar ATPase (gi:166627) Nearly identical to vacuolar ATP synthase subunit B (V-atpase B subunit)(V-atpase 57 KD subunit) and is a member of ATP synthase alpha/beta family that contains an ATP synthase beta chain domain (gi:9558599) V-type proton ATPase subunit B1 (gi:15222929) V-type proton ATPase subunit B3 (gi:240254125)
Spot 3716	Wild-type > RNAi4.5	Chaperonin hsp60 (gi:16221) HSP60 (HEAT SHOCK PROTEIN 60); ATP binding (gi:240254125)
Spot 4506	Wild-type > RNAi4.5	Actin 8 (gi:1669389) ACT8 (ACTIN 8); copper ion binding / structural constituent of cytoskeleton (gi:15222075) AT3G18780 (gi:222424327) ACT2 (ACTIN 2); structural constituent of cytoskeleton (gi:15230191)
Spot 5526	Wild-type > RNAi4.5, RNAi4.3	6-phosphogluconate dehydrogenase family protein (gi:15238151)
Spot 7605	Wild-type > RNAi4.3	Mitochondrial processing peptidase beta subunit, putative (gi:30678485) Mitochondrial processing peptidase, putative (gi:110740617) Mitochondrial processing peptidase beta subunit, putative (gi:15232845)

Five proteins were found to be higher present in the Wild-type plants. The peptides detected from the spot 2612 were found from four different proteins. The different proteins however showed high similarities towards function in the cell. The protein found higher present in the Wild-type plant is a subunit B of the V-type proton ATPase pump. The indication here was that it comprises a pump spanning the vacuolar membrane. The main function of this pump is the synthesis of ATP but it can also be reversible. ATP is synthesized when the ionic

electrochemical potential is greater than the free energy of ATP hydrolysis. In contrast, when the free energy of ATP hydrolysis is greater than the ionic electrochemical potential, the hydrolysis of ATP drives the uphill transport of ions. The subunit B is situated in the V_1 part of the polypeptide. This means it is situated in the cytoplasm. Also it has been shown that this subunit B has nuclear binding capacities. Interestingly more results indicate that subunit B also has an affinity to actin filaments [40,41]. This binding is probably a way of cellular transport. Spot 3716 that was detected to higher in the Wild-type lines is identified as a HSP (heat shock protein) 60. Commonly this protein is known to react as a stress signalling molecule and interacting with misfolded proteins but current insights reveal a wider function. The HSP60 or chaperonin hsp 60 in eukaryotes, often referred to as CCT (chaperonin containing t-complex polypeptide-1), is a heterooligomeric protein composed of 8 to 9 different subunit species of approximately 60 kDa. The chaperonin hsp 60 has abilities to bind ATP in order to enclose substrates [42,43]. Recently more substrates have been identified for this chaperonin hsp 60 protein like: actin, tubulin, and β-propeller proteins such as transducin-β [42]. The relation with actin is according to recent studies that CCT is of importance in formatting the cytoskeleton by repressing the depolymerisation of actin [43,44]. Spot 4506 was identified as actin protein or more specifically actin (ACT) 2 or actin (ACT) 8. One annotated proteins was found which is probably actin related. ACT2 and ACT8 differ only in one amino acid. This protein was found to be present in higher amounts in the Wild-type group compared with the RNAi4.5 line. ACT2 function has been suggested by previous biochemical studies that showed that interference with actin stops root hair growth. Actin accomplishes these processes by giving directional vesicle transport of cell wall materials towards the root hairs [45]. Actin filaments also play a critical role in vacuolar trafficking of proteins, this was especially detected with the ACT2 [46]. One other interesting finding concerning ACT2 is that it is found to be associated with wavy growth (WAV) 2. The ATTEDII database shows that transcriptomic analysis indicate a similar expression of the ACT2, ACT8 and WAV 2 (not detectable in the present 2DE-analysis, because the p*I* used is higher than 7). This last protein negatively regulates root bending when roots alter their growth direction. It is possible that WAV2 plays a role in regulation of differential growth of roots, in addition to the regulation of root tip rotation during root bending [47]. There are indications that the WAV2 plays a role in actin filament organisation but so far no conclusive evidence has been found to support this.

The only spot found to be significantly higher in both RNAi (4.3 and 4.5) lines was spot 5526. This was identified as 6-phosphogluconate dehydrogenase family protein, known to have a role in the pentose phosphate pathway. It forms ribulose 5-phosphate from 6-phosphogluconate. Other finding of this enzyme is that it is involved in the response to cadmium [48]. 6-phosphogluconate dehydrogenase family protein has been linked with numerous genes based on references and bio-informatics (ATTEDII). One of these is root ferredoxin:$NADP^+$ oxidoreductase (FNR) 2. This protein is relatively unknown but there is evidence that this it provides the reducing power for converting NADPH into a form available for ferredoxin (Fd)-dependent enzymes, assimilations of nitrogen and sulphur and other Fd-linked reductive metabolism reactions such as lipid desaturation [49]. All of these processes can be linked back again with root growth and development. The last protein identified was mitochondrial processing peptidase beta subunit (MPPbeta) This was identified from spot number 7605 and was significantly higher in the Wild-type lines compared with the RNAi4.3 line. Its most known function is the cleavage of the presequence of proteins in the matrix of mitochondria [50]. Recent published data indicates that it is part of a larger mitochondrial complex called pentatricopeptide repeat (PPR) domain protein which provides a signalling link between mitochondrial electron transport and regulation of stress and hormonal responses in *A. thaliana* [51].

4. Conclusion and synthesis

This research was started based on the findings of previous investigations. The most important discovery of these investigations was the possible involvement of the *RCC1* gene in metal accumulation and tolerance. The model organism *A. thaliana* was used to investigate this gene of which only a few articles are published concerning its function in plants. In transgenic *A. thaliana* plants where the *RCC1* gene was silenced abnormal root development was observed. This was an indication that the gene is involved in important pathways of plant development. These facts made the RCC1 protein an interesting start for further research.

In the first results (figure 5 and 6) we can clearly see the impaired root development because the lower weight and phenotypic changes. We could expect this since previous research had similar results. More interesting now is that for the first time a proteomic study has been conducted on *A. thaliana* with a silenced *RCC1* gene. The first observations made it highly likely to expect change in proteome profile.

The image analysis gave 13 significant spots and after mass-spectrometry analysis we identified 5 of them. One of the most interesting is the ACT2 discovery which is clearly shown to be involved in root development and protein transport. We know that RCC1 is involved in the transport of auxin from nucleus to cytosol and silencing RCC1 should impair this transport. The Actin-auxin relation is not always clear but it has been well studied. It is assumed that actin is necessary for polar transport of auxin [52]. In addition to this other studies indicate the involvement of actin in polar transport of plant-specific pinformed (PIN) proteins. These proteins are auxin efflux carriers that are essential in directional gravitrophic growth by establishing an auxin gradient [52,53]. According to our findings we can assume that in the *A. thaliana* RNAi lines the auxin transport was impaired because of the silencing, but we know now that the actin system is deficient. This based on the decreased protein expression and phenotypic changes that are characteristic for loss of the actin-auxin system. The other identified proteins can add to the phenotypic change or they can be the result of impaired transport by actin. Besides this, the disturbed formation of mitotic spindle, in which actin is essential, can also contribute to impaired root development. Actin is involved in vacuolar trafficking, the protein V-type proton ATPase subunit B was found to be increased in the Wild-type line. This could be a consequence of impaired trafficking mechanisms directed

by actin. Another protein associated with actin was the WAV2. A direct biological link has not yet been found but they are known to be highly co-expressed which could indicate similar transcriptional control. This protein is also probably a regulator of root morphogenesis and dysfunctional roots cannot react sufficiently to changing stimuli (gravity). Another potentially important protein is 6-phosphogluconate dehydrogenase family protein. It performs essential metabolic reactions and is closely linked with co-expressed FNR 2. 6-phosphogluconate dehydrogenase family protein could however play a role in metal tolerance based on its involvement in cadmium response. If this is to be disturbed by decreased levels inside the roots, the development of the root could also be impaired. The reason why chaperonin hsp 60 and mitochondrial processing peptidase beta subunit were found significantly lower in the RNAi lines is less clear. Actin is a substrate for chaperonin hsp60, and they both were down-regulated in RNAi lines. It is also possible that the lower amount of chaperonin hsp60 played a role in the malformation of the actin network and thus contributing to the phenotype observed. For mitochondrial processing peptidase beta subunit we know that it can take part in hormone signaling, although the exact mechanism is not fully understood, one could implement that a reduced signaling towards mitochondria would not be beneficial for plant (root) development.

Proteins identified in this thesis are very likely to be, at least partially, related to the altered phenotype in RNAi lines. More indications of the RCC1 link with auxin have also been established. We have shown clearly that the *RCC1* gene is of great importance in the normal development of roots in *A. thaliana*.

More studies are needed to further characterize the RCC1 gene and its' function. There are more questions to be addressed. One interesting would be, how would the protein profile of overexpressor plants react to heavy metals or other pollution? If a silenced *RCC1* gene impairs root development, does an increased expression of this gene enforce root development? Could this be the case also on exposure to heavy metals and maybe be one of the reasons why *Thlaspi caerulescens* has a metal tolerant phenotype?

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Supplementary data 1

Genomic *RCC1* sequence in *Arabidopsis thaliana*

ATGGATGCTACGAGTGGAACTCCGGCTTTACAGTACCATAACTTACCGGAGCAACCGGTTTC
GACTACTTCTCCTCCACTGTCTCCATTTCAGAGGCAAAAACGCCATTGCTTTGGAGACTCAA
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AGATTGGATCCTCGTGACCTCGCAAATCTTGAGGTTCCATGTCGACAAAATGCTTTACTGAA
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TAGCTGCTCTCGACATGTGTAACAAAAGAGTGATCTTCAAGCCGATGGACGAAGAAGAACGT
CAAGAAATGAAACGCAGGTGCGGAGGTTCGTGGAAACTGGTCCTTAGGTTTTTGCTGGCTGG
TGAAGCGTGTTGTCGAAGAGAGAAATCTCAAGCCGTCGCTGGCCCCGGTCACAGCATCGCCG
TGACATCCAAAGGCCAGGTTTTTACATTCGGTTACAATAACTCCGGTCAGCTAGGACACGGC
CACACCGAAGAAGAAGCTCGGATCTTGCCCGTCAGGTATCAATAACCGTTTTAACACACCTA
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TACGCTTGCGGGAAAGACTCGTTCGGTGAAGCTGAATACGGAGGGCCAGGGACTAAACCGGT
TACAACTCCTCAGTATTGACTCTTTGAAGAACACTTTCCGTGTTAG**CTAGTGACTTCTTTGA**
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ACACTCTTTATTTTCGGTTAAGACCGGCTCTGACGCAATAGCGTGGTTTATTTTAGGT**CGGT**
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GCTTGGCACGCCGCGGTGGTTGGTCAGGACGGGAGAGTGTGCACTTGGGGTTGGGGAAGGTA
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TCTTTGAATCTTATGTTTTGGTTTTGGTTTGTAGG**GTAATCGGCAAGCGAATGTGCTGAGTC**
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TCGATATATTGGAACGCTCATACGTTTGCGCTCGCTGAATCGGGGAAAGCTGTTGCGTTTGG
TGCGGGTGATAAGGGTCAGCTTGGAGCAGAGCTTGGTCGTAACCAAGCAGAAAGGTGTGTAC
CGGAGAAAGTGGATATTGATCTCAGCTAACCATCTTTCTAGAAGATCT

RCC1 cDNA-sequence

ATGGATGCTACGAGTGGAACTCCGGCTTTACAGTACCATAACTTACCGGAGCAACCGGTT
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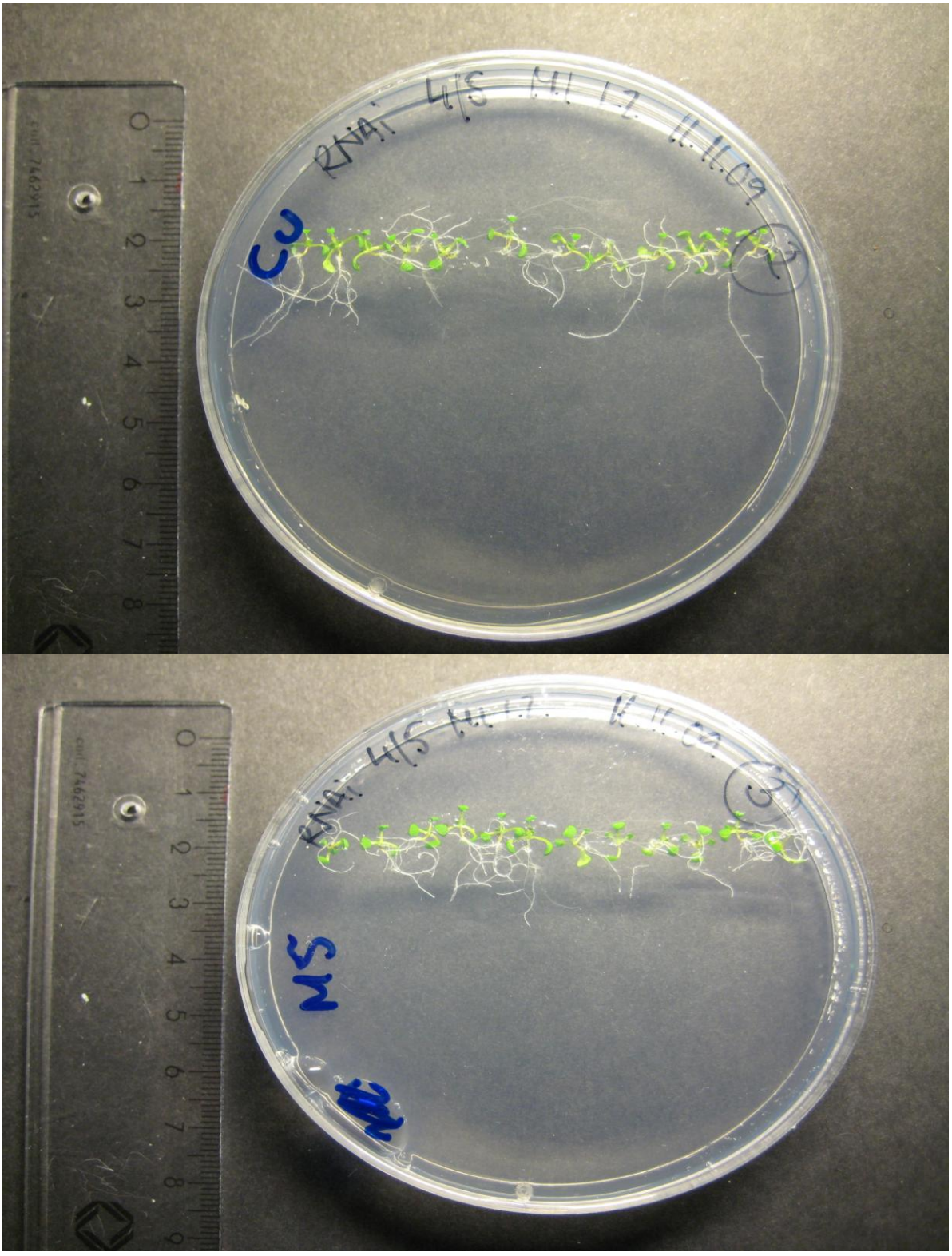
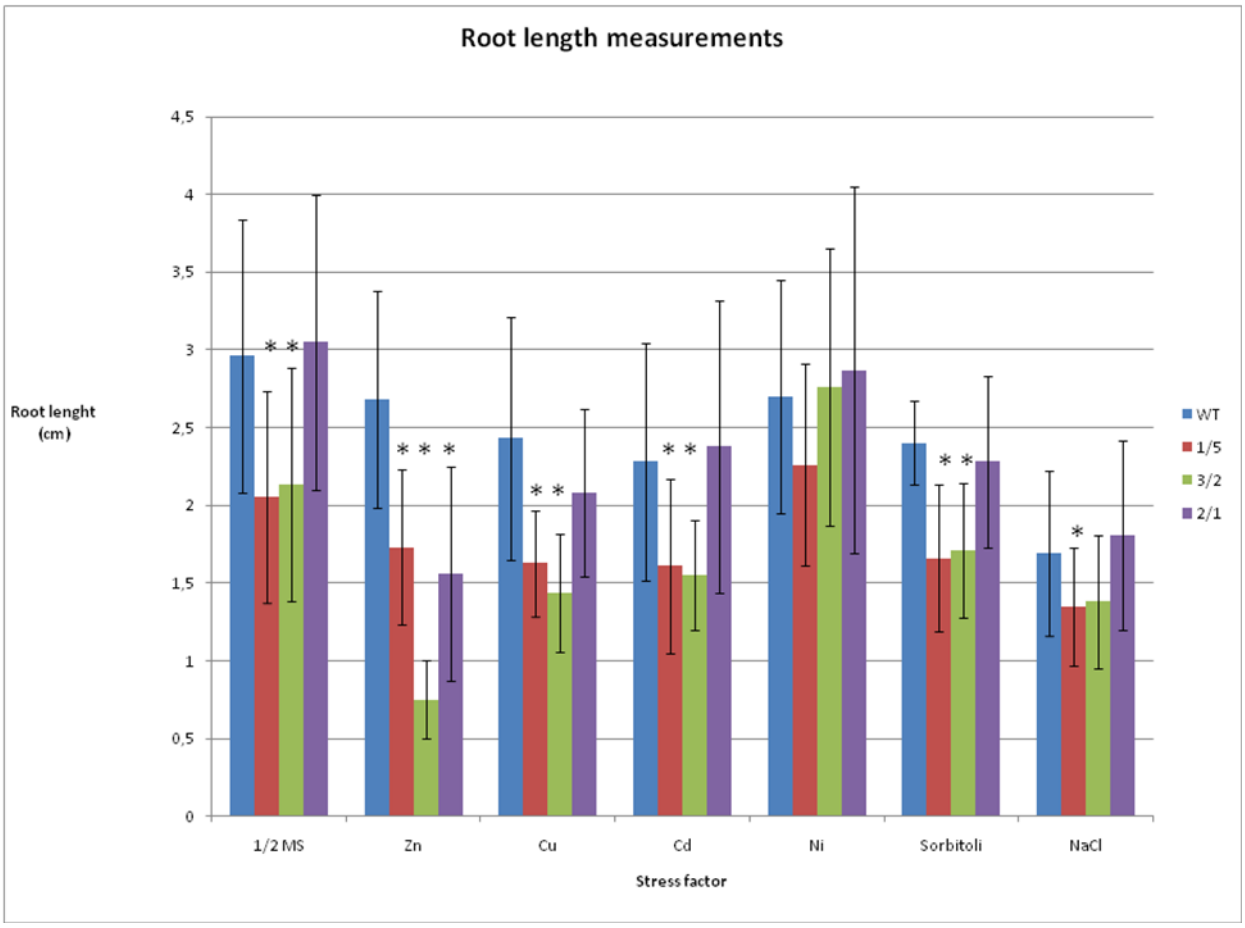
Supplementary data 2



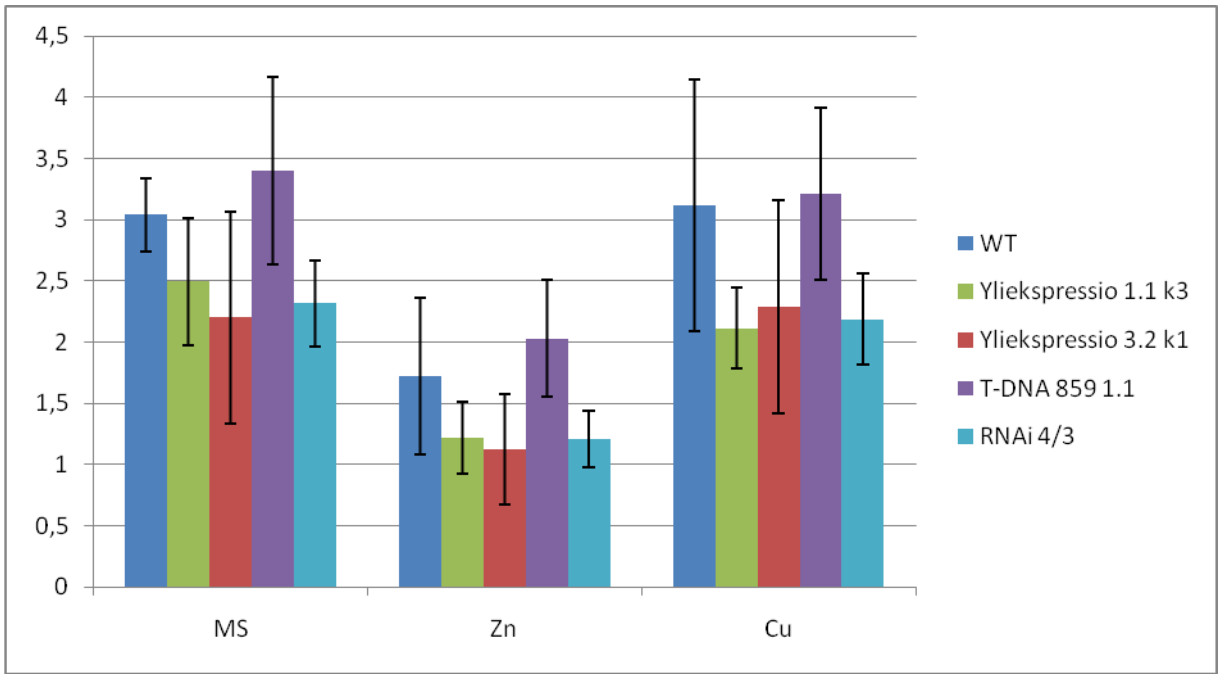
Supplementary figure 1: Phenotypic comparison. These 4.5 RNAi plants have abnormal root development. In the top figure there is exposure to Cu.

Supplementary data 3



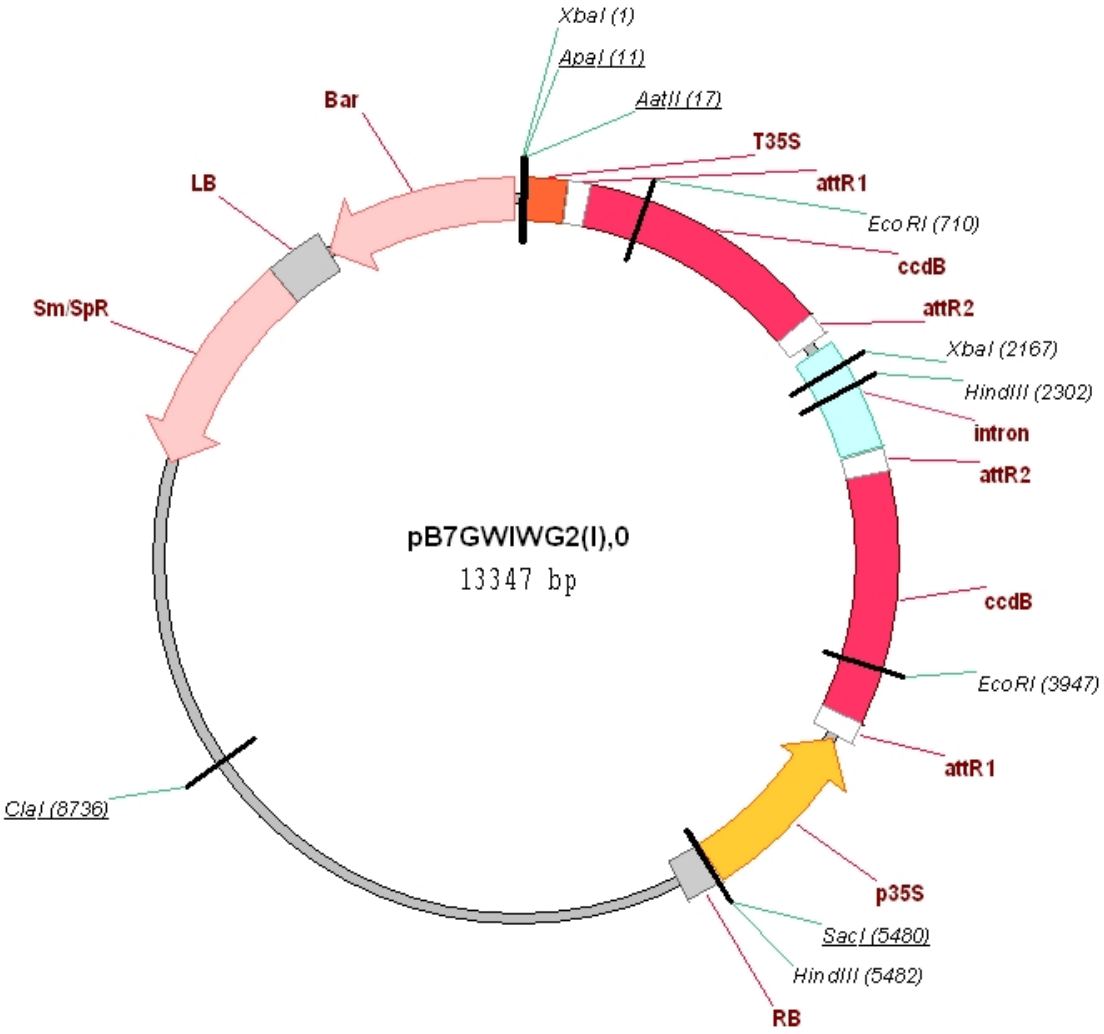
Supplementary figure 2: Measurement of root length under various different stress conditions. In the first (1/2 MS) plants where grown in media with a lower amount of nutrients. The next four stress conditions are exposure to heavy-metals. The last two conditions are osmotic stress. There are four genotypes used in this experiment. Except for the wild-type (WT) all of these plants are overexpressors. The 1/5 and 3/2 are homozygote overespressors whereas the 2/1 is a heterozygote. The * indicate a significant difference in root length (α=0.05).

Supplementary data 4



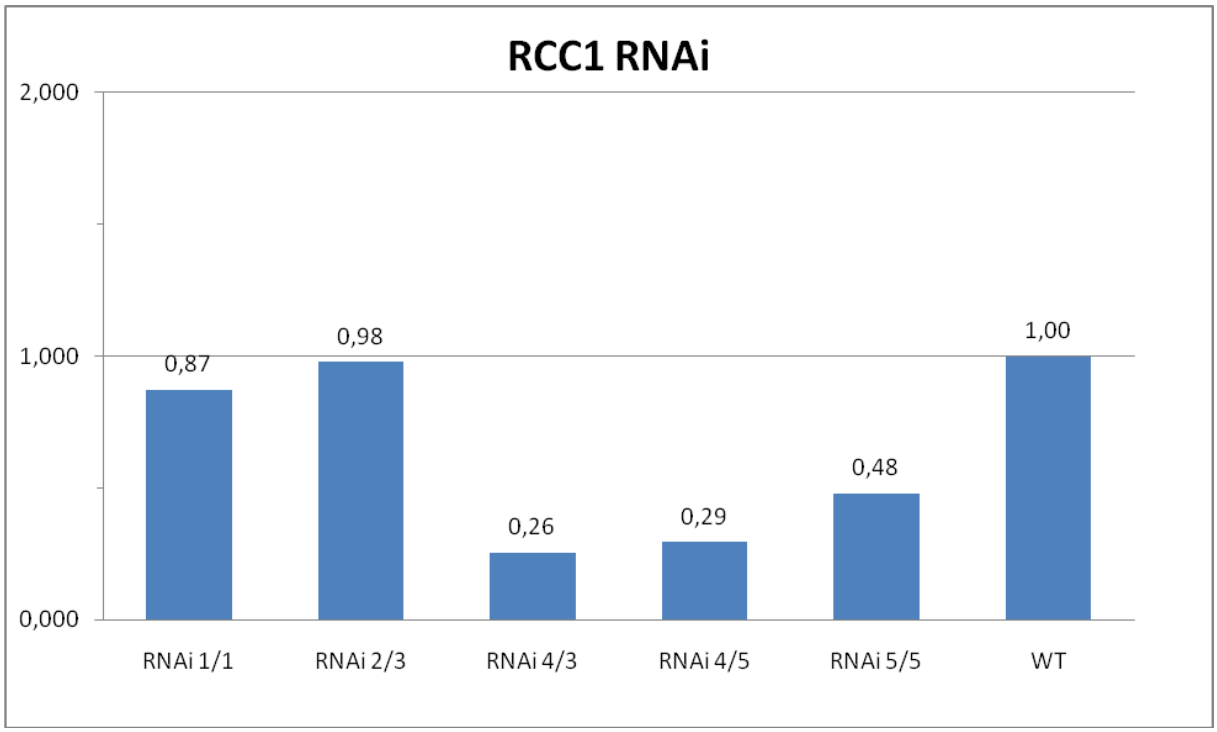
Supplementary figure 3: comparison of different knock-out, knock-down and overexpressors in regard to root length and heavy-metal exposure. The 1.1 k3 and 3.2 k1 genotypes are both overexpressors of the RCC1 gene. The T-DNA strain is the knock-out genotype whereas the RNAi 4/3 is the knock-down strain. Besides the control MS condition two other conditions were used with heavy-metal exposure. No significant differences were detected.

Supplementary data 5



Supplementary figure 4: Plasmid used for transformation of the transgenic RNAi lines. The red part in this figure is the part where the *RCC1* gene was brought into in sense and antisense direction. The blue part in the middle, the intron is essential for obtaining the hairpin-like structure of the RNA. *P35S* is a strong constitutive promoter (*T35S* is the terminator) that ensures sufficient transcription. The LB and RB are respectively left border and right border, everything between these is incorporated in the plant genome. *attR* sites are important in bringing the *RCC1*gene into this construct. *ccdB, Bar* and *Sm/SpR* are antibiotic resistance genes which are necessary in selecting the right clones.

Supplementary data 6



Supplementary figure 5: qRT-PCR experiments performed in different RNAi lines with normalisation towards the wild-type line. These results show that silencing of the target gene *RCC1* is best achieved in the lines RNAi 4.5 and 4.3. This is also in accordance with the phenotypic changes observed in these two lines.

Supplementary data 7

Growth media

Supplementary table 2: Composition of the media used for hydroponics. The collard section indicates the micronutrients which were made as one stock solution. Fe(III)NaEDTA was added fresh.

Substance	Stock solution	Media concentration	Obtained from
KNO_3	1.5M (500x)	3mM	Fluka, 60419
$Ca(NO_2)_3$	2M (1000x)	2mM	Riedel-de Haën, 31218
$NH_4H_2PO_4$	1M (1000x)	1mM	Fluka, 09708
$MgSO_4$	0.5M(1000x)	0.5mM	Fluka, 00627
KCl	0.1mM(100 000x)	1µM	Merck, 4936
H_3BO_3	0.25M(10 000x)	25µM	Oy FF-Chemicals Ab, 80340
$MnSO_4$	0.2M(100 000x)	2µM	Merck, 5963
$ZnSO_4$	0.2M(100 000x)	2µM	Riedel-de Haën, 31665
$CuSO_4$	0.01M(100 000x)	0.1µM	Merck, 6508
$(NH_4)_6Mo_7O_{24}$	0.01M(100 000x)	0.1µM	Merck, 6414
Fe(III)NaEDTA	/	20µM	Sigma, 6543
MES	1M(500x)	2mM	Duchefa, 4432

Supplementary data 8

Protein extraction

Supplementary table 2: Components and concentration used to make the buffer for resolving the root powder.

Component	Concentration	Obtained from
Tris-HCl pH 8	50mM	MP-Biomedicals, 819620
EDTA	25mM	Sigma, 6453
Thiourea	500mM	ICN-Biomedicals, 152588
β-mercaptoethanol	0.5% v/v	Fluka, 6940

Protein resolubilisation

Supplementary table 3: Components and concentration used to make the resolubilisation buffer. The extracted proteins were resolved using this buffer

Component	Concentration	Obtained from
Urea	7M	Invitrogen, 11668027
Thiourea	2M	Sigma-Aldrich, 62-56-6
CHAPS	4% (w/v)	Bio-Rad, 161-0460
Bio-lyte	2% (w/v)	Bio-Rad, 163-1193
DTT	65mM	Sigma-Aldrich, 3483-12-3

Protein equilibration buffer

Supplementary table 4: Components used to prepare the equilibration buffer. The IPG buffer is added as a ready to use mixture. Additional to these components a few grains of bromophenol bleu is added.

Component	Concentration	Obtained from
Urea	7M	Invitrogen, 11668027
CHAPS	2% (w/v)	Bio-Rad, 161-0460
IPG buffer (pH 4-7)	0.5% (w/v)	GE Healthcare, 17-6002-46

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Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:
Proteomic study of Arabidopsis thaliana with silenced RCC1 gene

Richting: **master in de biomedische wetenschappen-milieu en gezondheid**
Jaar: **2011**

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Comhair, Joris

Datum: **14/06/2011**