ARIADNE RIBEIRO HENRIQUES

IMPROVING TOLERANCE TO ZINC DEFICIENCY AND SALT STRESS IN PLANTS BY MODIFICATION OF F-CLASS bZIP GENES

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Supervisors

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Dedicated to my beloved fiancé,
Lauber Zorzolli de Farias

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GENERAL ABSTRACT

Growth and development are dynamic and complex processes frequently subjected to environmental stresses. Stress is an external factor that exerts a disadvantageous influence on the plant and induces a series of molecular and physiological changes in order to normalize the life processes. Zinc is an essential micronutrient responsible for the maintenance of vital processes in all living organisms, however, zinc deficiency is a serious global problem that results in lost productivity and nutritional quality of food, which has a direct and indirect negative effect on human health and well-being. Currently, the techniques that aim to modulate gene expression by controlling initial transcription rates have helped to unravel the complex network of homeostasis of the plants subjected to stress. The research described in this thesis was designed with the main purpose of increasing tolerance to zinc deficiency and enhance zinc accumulation in plants by modifying the expression of the bZIP gene. The results of this study are very promising for agriculture because they may contribute to increased productivity and plant robustness in areas that are suffering from low zinc bioavailability.

Keywords: Zinc deficiency. bZIP genes. abiotic stress.

RESUMO

O crescimento e desenvolvimento das plantas é um processo complexo e dinâmico freqüentemente sujeito a estresses ambientais. O estresse é um fator externo que exerce uma influência desvantajosa sobre a planta e induz uma serie de mudanças moleculares e fisiológicas com a finalidade de normalizar os processos vitais. Zinco é um micronutriente essencial responsável pela manutenção de processos vitais em todos os organismos vivo apesar disso, a deficiência deste elemento é um sério problema global que acarreta perdas na produtividade e na qualidade nutricional dos alimentos. Atualmente, as técnicas que visam modular a expressão gênica através do controle de taxa iniciais de transcrição têm auxiliado a desvendar a complexa rede da homeostase das plantas submetidas ao estresse. Desta maneira, esta tese foi desenvolvida com o objetivo principal de aumentar a tolerância e acumulo de zinco nas plantas através da modificação da expressão da classe F dos genes bZIP. Os resultados obtidos neste trabalho são bastante promissores e podem contribuir para aumentar a produtividade e a robustez da plantas em áreas que sofrem com a baixa disponibilidade de zinco.

Palavras-chave: Deficiência de zinco. genes bZIP. estresse abiótico

SUMMARY

Chapter 1

General Introduction

INTRODUCTION

Plants are autotrophic organisms that possess the ability to use solar energy to synthesize vital components from carbon dioxide, water and nutrients. The minerals, macronutrients and micronutrients, are essential to plant growth and development. Their absence impedes the plant from completing its life cycle (RAMESH; CHOIMES; SCHACHTMAN, 2004) due to their essential role in physiological and metabolic processes of the plant.

Zinc is a vital micronutrient for the functionality of more than 300 enzymes, protein synthesis, transcriptional and post-transcriptional processes, as well as the structural and functional integrity of cell membranes (CLEMENS, 2006; MORTEL et al., 2006). Moreover, zinc is involved in detoxification of reactive oxygen species (ROS). Most of the environmental stress factors, such as heat, drought, salinity and freezing, alter plant ion homeostasis and cause cell damage by producing ROS. Zinc is part of the structural component of Cu-Zn superoxide dismutase (SOD), a key enzyme in combating oxidative stress and protecting membranes. It accomplishes the dismutation of superoxide radiation (O_2^-) in oxygen and hydrogen peroxide, which is then detoxified by catalase. The reactive oxygen species (ROS) are also determinants of the cell damage under stress such as drought, heat and salt. So, Cu-Zn superoxide dismutase plays a key role in the plants defense systems against ROS (ARAVIND and PRASAD, 2005; CAKMAK, 2000).

In addition to its essentiality in plants, zinc is essential to human nutrition and its deficiency causes a nutritional problem worldwide (CAKMAK, 2011). It is estimated that one-third of the world's population (about 2 billion people) suffers from (mild) zinc deficiency (CAKMAK; PFEIFFER; MCCLAFFERTY, 2010; WELCH; GRAHAN, 2004). An important tool in combating the shortage of this nutrient is biofortification, which aims to increase the nutritional value of crops through convectional breeding or genetic engineering. Several research programs have reported positive results from the application of Zn-containing fertilizers that represents a quick solution to the zinc deficiency problem (CAKMAK; PFEIFFER; MCCLAFFERTY, 2010). However, in the long term, plant breeding and genetic engineering will be needed to develop new high-Zn content genotypes to be a promising alternative approach for increasing the zinc content in food and crops (CAKMAK; PFEIFFER; MCCLAFFERTY, 2010; GOMES-GALERA et al., 2010).

Interaction zinc-plant

Zinc is a transition metal essential for terrestrial life since it has a number of crucial functions in plant cell. However, although essential for growth and development of plants, this micronutrient is necessary at low concentrations. Concentrations between 30 and 100 µg zinc g^{-1} DW support adequate plant growth and zinc toxicities are observed in concentrations above 300 µg zinc g^{-1} DW for species not adapted to high zinc exposure (MARSCHNER, 1995; MORTEL et al., 2006). Therefore, plants need to keep tight control over zinc homeostasis.

A complex network of homeostatic mechanism is involved in plants to control metal uptake, accumulation, trafficking and detoxification of metals. The ability to take up Zn of higher plants depends on both the concentration in soil and bioavailability, which is modulated by various soil physical and chemical factors. Zinc solubility in soil decreases due to high levels calcium carbonate, metal oxides, pH and low levels of organic matter and soil moisture (CAKMAK, 2011; ROBSON, 1994).

Thus, when available in the soil solution, zinc is absorbed and transported predominantly in the divalent (Zn^{+2}) form from roots to shoots through the xylem, being easily re-translocated by phloem (CLEMENS, 2001). This transport of ions and molecules from epidermal and cortical cell to xylem can occur through the symplastic or apoplastic route.

When, traversing the apoplastic route, zinc and other minerals (essential and not essential), are dragged with the water through cell walls, intercellular spaces and tissues located outside the membrane to the endoderm, where casparian bands force the substances to into the endoderm cells by crossing the plasma membrane. The symplastic route consists of a continuous system of cytoplasmatic cells interconnected by the plasmodesmata. The ions enter the exoderm cell's cytosol and are transported from cell to cell into the xylem (BROADLEY et al., 2007; TAIZ; ZEIGER, 2004).

However, regardless the chosen path, the solutes which reach the xylem parenchyma cells are transferred to cells of conducting elements of xylem in a tightly controlled process mediated by membrane transport. Via the xylem sap, the metal is released in the apoplast of the leaves and subsequently distributed intracellularly (CLEMENS; PALMGREN; KRAMER, 2002).

The zinc distribution, transport and accumulation are affected by the zinc supply level in plant. Either in low or in adequate zinc supply, roots, vegetative shoots and reproductive tissues have higher zinc concentration in growing tissue than in mature tissue. When in toxic zinc levels, tolerant plants accumulate zinc in the roots cortex and in leaves, specifically in cell wall or vacuoles (ROBSON, 1994).

A quick reduction in both growth and yield occurs at low zinc concentrations in the shoot, because zinc plays an important role in plants cells (CAKMAK, 2011).

Zinc plays a fundamental role in enzymes. It can act as a functional, structural or regulatory cofactor of large number of enzymes. Some enzymes, such as alcohol dehydrogenase, Cu-Zn superoxide dismutase and carbonic anhydrase have zinc in their structure. Other enzymes, such as aldolases, enolase, isomerases, peptidases, transfosforilases and RNA and DNA polymerases, require zinc for enzymatic activation (BUCHANAN; GRUISSEN; JONES, 2000; GUERINOT; EIDE, 1999).

The structural and functional integrity of cell membranes is also influenced by this metal, which acts in stabilization of biomembranes by interaction with phospholipids and sulphydril groups of membrane proteins. Zinc deficiency in plants causes biochemical changes in membranes modifying the permeability and architecture of biological membranes. Nevertheless, the protection against the peroxidation of membrane lipids and proteins has been shown to be the major role of zinc in membranes (CAKMAK, 2011; MARSCHNER, 1995).

Common symptoms of plants growing on low zinc supplements are as follows: delayed and reduced growth, small and malformed leaves, short internodes and yellowing effects, all largely attributed to the disturbance of auxin metabolism. However, the mechanism of zinc deficiency effects on indole-3-acetic acid (IAA) metabolism is yet not clear. It is believed that zinc is involved in both IAA biosynthesis and in protection of IAA from oxidative degradation by ROS (CAKMAK, 2011; ROBSON, 1994).

Finally, as a result of its vital importance to plants, zinc deficiency affects the plant as a whole, causing serious problems in carbohydrates metabolism, principally by the severe decline in photosynthesis and sugar transformation, and the synthesis of proteins, attributed to a sharp reduction in RNA and deformation and reduction of ribosomes (MARENCO; LOPES, 2007). However, the great damage of low zinc

supply on protein metabolism is on stability and function of genetic material, because besides affecting the structure of both RNA and DNA, zinc shortages affects zinc finger proteins that are required for the expression and regulation of genes (MARSCHNER, 1995).

Role of zinc in human health

Zinc deficiency in soils is a serious global problem that affects many agricultural soils. Currently, it is estimated that about half of the cultivated soil in the world contains low amount of soluble zinc (Figure 1). This problem is aggravated mainly in arid and semi-arid regions due to low organic matter and soil moisture as well as high levels of pH and $CaCO_3$ (CAKMAK, 2008).

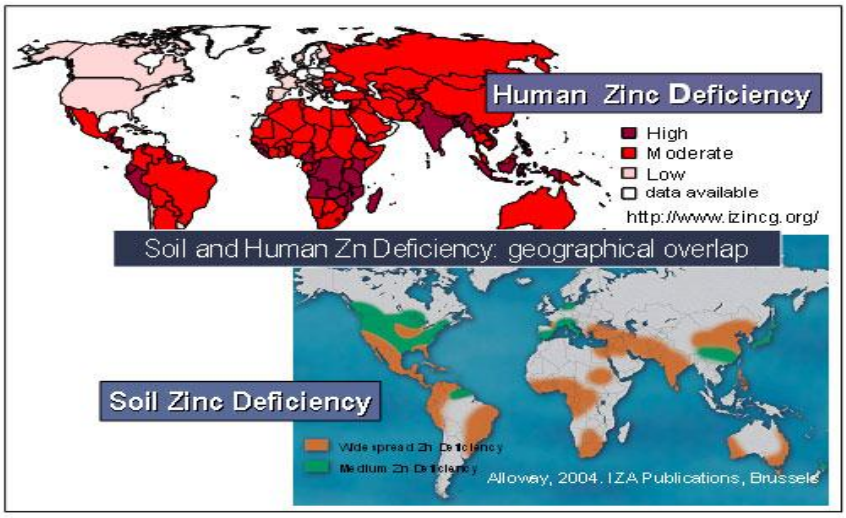

Figure 1: Overlap between geographical regions with soil zinc deficiency (CAKMAK, 2011).

The low availability of this metal in the soil limits zinc uptake by plants resulting in significant decreases in both productivity and nutritional quality of food. The regions with zinc-deficient soils are strongly correlated with regions with high incidence of human Zn deficiency. The cultivation of food crop on Zn-deficient soil is a critical problem, especially when the crop in question is a source of the population's basic diet. Cereals-based foods are the most important source of calories of developing countries. The cultivation of such cereals in Zn-deficient soils, like in Turkey, leads to the production of cereal grains which are very poor in zinc, with approximately 50% reduction in zinc content (CAKMAK, 2011). Then, the insufficiency of the dietary

intake is the major cause of zinc deficiency in humans and affects billions of people worldwide (ERENOGLU et al., 2010).

According to the World Health Organization, zinc deficiency in humans can results in several undesirable consequences including diminished learning ability, impaired immune response, dysfunction of reproductive system and reduced growth rates on infants (WORLD HEALTH ORGANIZATION - WHO, 2003). Therefore, there is a need to find new approaches and technologies that seek to either solve or mitigate this problem that affects one third of the world's human population.

Biofortification of crops

Currently, several programs with promising strategies are engaged in trying to remedy the zinc and other micronutrients deficiency problem in humans. Traditional strategies intended for providing vitamins and minerals supplements and fortification of foods through post-harvest processes have reached favorable results in combating malnutrition. However, the fortification of foods as well the supply of commercial supplement require a good infrastructure of distribution, have a high cost-effectiveness, and demand a continuous funding over time, when these strategies are government-subsidized (GOMES-GALERA et al., 2010).

As an alternative for nutritional enrichment of foods post-harvest, arose the biofortification proposal with a new paradigm for agriculture and as a tool for improving human health. The biofortification aims at the improvement of plants promoting not only an increase in the nutritional value of foods but also agronomic benefits, because it is technically focused in the source of problem, making the growing of a plant more productive and nutritious rather than having added nutrients to the food when they are being processed (NUTTI et al., 2007). In order this method to succeed, the new lines of micronutrient-rich staple food should take into consideration some aspects such as: crop productivity (must be either maintained or increased), the micronutrient enrichment level (should have significant impact on human health), the stability the micronutrient enrichment trait in the environment and the bioavailability of micronutrients in humans (WELCH; GRAHAM, 2002).

Many biofortified crops have already been produced successfully. The β-carotene-enriched rice (YE et al., 2000), maize with high protein content (RASCON-

CRUZ et al., 2004) and lycopene-enriched tomato (FRASER et al., 2002) are some examples of crops developed to have high nutritional value.

The utilization of plant genetic resources to achieve cultivars with enhanced nutritive content can be done either through either biotechnology or conventional breeding, and the both appear to provide cost-effective and sustainable solution for improving the food crop quality (WELCH; GRAHAM, 2002). However, even though the transfer nutrients-rich traits by conventional breeding cheaper and less controversial than genetically engineering crops, it is a long-term method that presents problems when some desirable nutritionals trait are not present in plant genome (ZHU et al., 2007). So, the exploitation of genes involved to capture of essential nutrients associated with the modern biotechnological tools seems to be the most promising way to solve to the growing problem of global malnutrition.

Genes involved in homeostasis of zinc in plants

Accumulation of zinc or other metals is due to uptake capacity and intracellular binding sites. The metal accumulation rates are affected by concentration and affinities of chelating molecules and by the presence and selectivity of transport activities (CLEMENS; PALMGREN; KRAMER, 2002). Metal transporters are involved in metal uptake or metal efflux within plant and metal chelators contribute to metal detoxification by buffering cytosolic metal concentration, therefore both have a major role in metal homeostasis. Based on this, there has been several research focused on the possibility of indentify and manipulate candidates genes for proteins zinc transporter and chelators biosynthesizing enzymes.

The NRAMP (natural resistance associated macrophage protein family) is a family of proteins whose function is to take up and transport metal. This family is present in several species of plants such as tomato, rice, maize and Arabidopsis. In Arabidopsis, research showed that transporters encoding by NRAMP have limited metal specificity. The AtNRAMP3, upon iron starvation, controls the accumulation of zinc and manganese (MORTEL et al., 2006).

Another family of transporters involved in zinc efflux is the P_{1B}-ATPase. Arabidopsis has eight genes encoding P_{1B}-ATPase that differ in their structure, function and regulation (EREN; ARGÜELLO, 2004). Among these, *AtHMA2* and *AtHMA4* are related to zinc transport. The *AtHMA2* gene encodes a Zn^{+2}-ATPase

in transmembrane and is activated by cadmium and other divalent metals. The *AtHMA4* gene is possibly involved in zinc hyperaccumulation, specifically in loading of zinc into the xylem (MORTEL et al., 2006; WATERS; SANKARAN, 2011).

MTP1 is a gene belonging to the cation diffusion facilitator (CDF) protein family and seems to be involved in the sequestration of zinc because when *MTP1* is overexpressing in Arabidopsis, an increased resistance to zinc and high zinc content in roots takes place (KOBAE et al., 2004). In addition, the *MTP2* is also involved in zinc homeostasis. Under zinc deficiency *MTP2* gene has increased its expression. It suggests a specific function in counteracting the effect of zinc deficiency (MORTEL et al., 2006).

Members of the ZIP genes family transport zinc into the cytosol are thought to play an important role in zinc uptake in plants (PALMER; GUERINOT, 2009; SONG et al., 2010). ZIPs have eight transmembrane domains and a histidine-rich variable loop between transmembrane domains III and IV that appears to be fully conserved among all family members (COLANGEL; GUERINOT, 2004). In Arabidopsis there are fifteen ZIP genes members *ZIP1-12*, *IRT1*, *IRT2* and *IRT3* and at least ten different members this family (*ZIP1, 2, 3, 4, 5, 9, 10, 11, 12* and *IRT3*) play a role in zinc uptake in roots. They can be involved in transport of cation across the plasma membrane (MORTEL et al., 2006). Other research has shown that approximately half of ZIP genes are induced in response to zinc deficiency (ASSUNÇÃO et al., 2010) demonstrating, through analysis of gene expression, that steady-state mRNA levels for some of zinc transporters are metal responsive under certain condition.

Metal chelators are also important for metal homeostasis. Nicotianamine (NA) is a metal chelating compound that acts binds zinc and other metals. NA is thought to be involved in long distance transport, perhaps to play a role in the entry of metals into the phloem or xylem. Nicotianamine synthase is an enzyme that catalyzes the synthesis of nicotianamine. This enzyme has in Arabidopsis four members, *AtNAS1-AtNAS4*, but only *AtNAS2* and *AtNAS4* are highly expressed in roots under zinc deficiency (MORTEL, 2006).

Significant advancement about candidate genes for zinc transports and molecules chelating has been reported, however, little is known about the regulators of zinc homeostasis network in plants (ASSUNÇÃO et al., 2010). Recently two genes of the basic region leucine zipper motif (*bZIP*) family of transcription factors, *bZIP23* and

bZIP19, that regulate the adaptation to low zinc supply have been identified. *bZIP23* and *bZIP19* the show up conserved in high plants and they represent the first transcription factor of the plant zinc homeostasis network, that control the zinc deficiency response in Arabidopsis (ASSUNÇÃO et al., 2010).

Therefore, in terms of understanding of genes involved and uptake and translocation of zinc in plant, a lot has been achieved, but the information on where in the plant each transporters function and how each one is controlled in response to nutrient availability it remains unclear. Perhaps, the driven expression of set genes by promoters can be the best strategy, since activation of multiple genes is necessary to micronutrients movement through the plant and these may need to be targeted simultaneously to increase the accumulation of zinc and zinc deficiency tolerance in plants.

The availability of *bZIP23* and *bZIP19* transcription factors that recognize palindromic motifs, called Zinc Deficiency Response Elements, found in tandem in several zinc homeostasis genes offer interesting options to try and modify the plant zinc deficiency response, either by making plants less sensitive to zinc deficiency or by conferring a constitutive zinc deficiency response, which can induce plants to over accumulate metals. So, the aim of this thesis was to modify expression of *bZIP19*, *bZIP23* and *bZIP24* for improved zinc deficiency tolerance and zinc accumulation. In the chapter 2 the expression of *bZIP19* and the *bZIP23* gene under the transcriptional control of the strong constitutive CaMV 35S promoter is compared to wild type expression in different of Zn supply conditions. Another overexpression evaluation is described in the chapter 3, in which plants contain the full Arabidopsis *bZIP19* cDNA fused downstream of the promoter of the zinc deficiency responsive Arabidopsis *ZIP4* gene have compared their expression with wild type. In the chapter 4 a similar approach was taken to compare the overexpression of bZIP19 function mediated by the *ZIP4* promoter with wild type in Coffee transformants. The relation of *bZIP24*, another gene from this F-class of bZIP proteins, with salt stress tolerance is described in the chapter 5.

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Chapter 2

The overexpressing *bZIP23* or *bZIP19* in Arabidopsis to confer tolerance to zinc deficiency

ABSTRACT

Zinc deficiency among crops is a widely occurring phenomenon causing decreased crop yields and crop quality. Recently identified transcription factors, *bZIP19* and *bZIP23*, are essential for switching on the zinc deficiency response of *Arabidopsis thaliana.* Thus, this study was focused on modifying the expression of *bZIP19* and *bZIP23* for providing improvement of Zn deficiency tolerance in plants. Arabidopsis plants, homozygous for high expressing p35S::bZIP19 or p35S::bZIP23 constructs, were generated and grown under deficient, sufficient and excessive supplies of zinc. Transgenic lines showed less sensitivity to zinc deficiency and higher zinc use efficiency, although gene expression analysis indicated a slight disturbance in gene regulation. These findings demonstrated that modified expressions of (one of) these *bZIP* genes can be an efficient tool in improving plant Zn tolerance and to contribute in elucidation of the complex network of zinc homeostasis regulation.

Keywords: Zinc deficiency. bZIP genes. stress.

INTRODUCTION

Zinc deficiency among crop is a widely occurring phenomenon resulting in severe losses in yields and nutrition quality (CAKMAK; PFEIFFER; MCCLAFFERTY, 2010; WELCH; GRAHAN, 2004). Zinc is a micronutrient essential to human nutrition and insufficient dietary intake of zinc is the major cause of zinc deficiency in humans, affecting billions of people worldwide (ERENOGLU et al., 2010). Cereals-based foods are the most important source of calories, especially in developing country, but their cultivation in Zn-deficient soils leads to production of cereal grains very poor in zinc content and decrease in crop productivity (CAKMAK, 2011). The low bioavailability of zinc in soil hinders its absorption by plants. Then, when the supply of this nutrient to plants is inadequate, one or more of its many essential physiological functions of zinc are unable to operate normally and the cellular homeostasis of plant is adversely affected (ALLOWAY, 2004). When facing shortage in zinc supply the carbohydrates metabolism, proteins synthesis, indoli-3-acetic acid (IAA) metabolism and biomembranes stabilization are deeply affected (CAKMAK, 2011; ROBSON, 1994).

In order to minimize the damage and to attempt to maintain the correct zinc concentration in cellular compartments, plants increase the expression of several genes encoding zinc transporters and metal chelator biosynthesis enzymes (MORTEL et al., 2006). Member of ZIP genes family transport zinc into the cytosol and are thought to play an important role in zinc uptake in plants since many members of the ZIP family are upregulated in roots under zinc-deficiency (PALMER; GUERINOT, 2009; SONG et al., 2010; WINTZ et al., 2003). Gene expression analysis suggests that about half of ZIP genes are induced in response to zinc deficiency (ASSUNÇÃO et al., 2010), but it remains unclear which member plays the dominant role in zinc uptake in roots.

Recently two transcription factors, bZIP23 and bZIP19, that are involved in the regulation of zinc deficiency response in *Arabidopsis thaliana* were identified. These transcription factors recognize 8-10 bp palindromic motifs called Zinc Deficiency Response Elements, found in tandem in several zinc homeostasis genes, which constitutes the primary response to zinc deficiency (ASSUNÇÃO et al., 2010). So, this finding may offer interesting options to try and modify the plant zinc deficiency

response, either by increasing zinc deficiency tolerance or by increasing zinc accumulation in plants.

In this report we analyzed to response of overexpression either bZIP23 or bZIP19 constructs in plant grown under different zinc supply conditions. We examined the biomass production, the zinc concentration in tissues and percentage of Zn Use Efficiency (ZUE) in transgenic lines to evaluated the sensibility of this plant to face zinc deficiency stress and the capacity of utilization and translocation of zinc present in the medium. We also compared the change of gene expression of the target genes to identify the genes involved in zinc homeostasis regulation. Therefore, this work was carried out with the objective of investigating the effects of expression modification of transcription factors *bZIP19* and *bZIP23*, which can offer an attractive advantage for crop in areas suffering from low zinc bioavailability.

MATERIALS AND METHODS

Generation of p35S::bZIP19 and p35S::bZIP23 constructs.

To generate overexpressor constructs for transformation of the *Arabidopsis* wild-type plants, full-length cDNAs of *AtbZIP19* and *AtbZIP23* (clones GSLTSIL54ZH09 and GSLTFB35ZE06, respectively) were obtained from the CNRGV (Centre National de Ressources Génomiques Végétales, France) in pCMV SPORT6 cloning vector, containing Gateway recombination sites. The cDNAs were cloned into the entry vector pDONR207 (Invitrogen) by *in vitro* site-directed recombination, for further recombination into the overexpressor vector pGD625 (FOLTER et al; 2005). The constructs pCaMV35S::bZIP19 (OX19) and pCaMV35S::bZIP23 (OX23) were verified by digestion analysis and sequencing and transformed by electroporation into *Agrobacterium tumefaciens* strain AGL0. Subsequently, *Arabidopsis* wild-type plants were transformed by floral dipping (CLOUGH; BENT, 1998). Independent transformed lines were selected for a single insertion locus by antibiotic resistance and 3:1 segregation ratio of T2 seedlings. The overexpression of *bZIP19* or *bZIP23* was confirmed by RT-PCR. Homozygous lines were selected for further analysis.

Plant growth condition

Arabidopsis thaliana ecotype Columbia (col-0) plants homozygous for high expressing p35S::bZIP19 and p35S::bZIP23 constructs were generated and three independent transgenic lines containing either OX19 (genotype 14, 15, 19) or OX 23 (genotype 16, 17, 18) were selected. Seeds of these lines along with wild-type seeds were kept for 3 days at 4°C in the dark in order to promote uniform germination and after these seeds were sown on 0.55% agar filled tubes and plants grown in two replicates, each of nine plants, on hydroponic medium containing a modified half-strength Hoagland's nutrient solution (SCHAT; VOOIJS; KUIPER, 1996) prepared either with low zinc supply (0.05 µM $ZnSO_4$), sufficient zinc supply (2 µM $ZnSO_4$) or excess zinc supply (25 µM $ZnSO_4$). The pH buffer MES at 2 mM concentration was included in preparation of the all nutrient solution and the pH was adjusted to 5.5. During the first two weeks, the nutrient solution was refreshed once a week and thereafter twice a week. Germination and plant growth were performed in a climate chamber with 12h/d at 20/15 ºC day/night temperature, 250 µmol photons $m^{-2}s^{-1}$ and 75% relative humidity.

Determination of zinc concentration

Two pools of three plants for each treatment and replicate were harvested after six weeks growing on hydroponic systems. The root systems were desorbed with ice-cold 5 mM $PbNO_3$ for 30 minutes and then roots and shoots were dried overnight at 65°C. All samples were digested with a mixture of HNO_3 (65%) and HCl (37%) for 7 hours at 140°C and analyzed for zinc concentration using flame atomic absorption spectrometry (Perkin Elmer 1100B).

Real-time quantitative RT-PCR

To analyze the expression levels in leaves and roots of six-week-old plants grown under distinct zinc conditions, the total RNA was extracted with the RNAeasy plant RNA Kit (Qiagen) and treated with DNAse (Fermentas) to eliminate any genomic DNA, following the manufacturer's instructions. One µg of total RNA was used to synthesize first-strand cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad). For the PCR reaction 5 µL of a 100x dilution of the cDNAs, 12,5 µL iQ™

SYBR® Green Supermix (Bio-Rad) and 5 pmol of forward and reverse primers (Invitrogen) were used in a total volume of 25 µL. The reactions were carried out in an iCycler Thermal (Bio-Rad).

The following standard thermal profile was used: 3 min at 95.0°C, followed by 40 cycles of 15 sec at 95.0 °C and 1 min at 60.0 °C. All gene expression analyses were performed with 4 biological replicates and 3 technical replicates per biological repeat for each genotype x treatment. The samples first were normalized to an internal control gene, 18S rRNA, and then the relative target gene expression was determined by performing a comparative ΔΔCt (LIVAK; SCHMITTGEN, 2001). The following primers were used for the validation of the expression analysis (forward then reverse): HMA2 (5'AGAATGGCGTCGA AGAAGATGACC 3', 5'ACGATGACGGTTCT TGACGG 3'); ZIP4 (5' GATCTT CGTCGATGTTCTTTGG 3', 5'TGAGAGGTATG GCTACACCAGCAGC 3'); bZIP23 (5'TAATCAGCTGTTGAAGAGGT 3', 5'TCA TGTATGAGTAAGGCACG 3'); bZIP19 (5'TTCTCCCGGATGA GAGCG ATGA 3', 5' GCTGATTCACCGCC CTAAGCCT 3'); NAS4 (5' CTGTGGTGAGGCTGAAGG TTACTTA 3', 5'GACCA GAGCCAATGAAAGCTAC 3'); 18S (5' TGACGGAGAA TTAGGTTCG 3', 5'- CC TCCAATGGATCCTCGTTA 3').

Calculations and Statistical analysis

The percentage of Zn Use Efficiency (ZUE) was calculated by dividing the root and shoot dry biomass yields either in low zinc or excess zinc by those in optimal zinc and multiplying by 100. The statistical analysis of zinc concentration and dry weight data was performed by one-way ANOVA and significant differences between means were determined using a Tukey test at the 5% level of significance ($P \leq 0.05$).

RESULTS

Experimental design

To test whether overexpressing either bZIP23 or bZIP19 confers an increased tolerance to zinc deficiency or else some adaptive advantage on low zinc bioavailability compared to wild-type Arabidopsis plant, the plants were germinated and grown in hydroponic system under low zinc supply (0.05 µM $ZnSO_4$), sufficient

zinc supply (2 µM $ZnSO_4$) or excess zinc supply (25 µM $ZnSO_4$). We used a hydroponic system to minimize variation in the bioavailability of zinc and other nutrients. Plants were grown on hydroponic solution for six weeks and after this time the material was harvested and analyzed. The evaluation of the dry matter and zinc content in plants allowed us to determine the fitness of plants in response to stress, and the capacity of utilization and translocation of zinc present in the solution. Through of the analysis of gene expression we investigated the involvement of *bZIP23*, *bZIP19* and other genes in the adaptation of the plants at different conditions of zinc.

Overexpressing *bZIP23* or *bZIP19* plants are less sensitive to zinc deficiency.

When comparing plants grown at either sufficient or excess zinc supply, there was no obvious difference in visible phenotype (Fig.1). Although, the plants growing under excess zinc showed a drastic reduction in the initial phase of growth and a delay in the flowering. At Zn deficient medium, the plants exhibited poor growth and strong chlorosis, mainly after four-week-old (Fig.1), but the lines 19 (OX 19), 16 and 17 (OX23) were generally larger and greener (Fig. 2) when compared with wild-type plants. Under these conditions, all transgenic lines, when examined for biomass production, were found to produce more biomass than wild type plants (Fig. 3A) although, this was only found to be significant for line # 16 (Fig 3A). The same lines that looked better under low zinc, showed a significant reduction of biomass at excess zinc (Fig 3C). Plants grown at normal Zn supply were also examined for biomass production. They were generally found to have less or equal biomass when compared to wild type plants (Fig 3B), although the observed differences were not statistically significant.

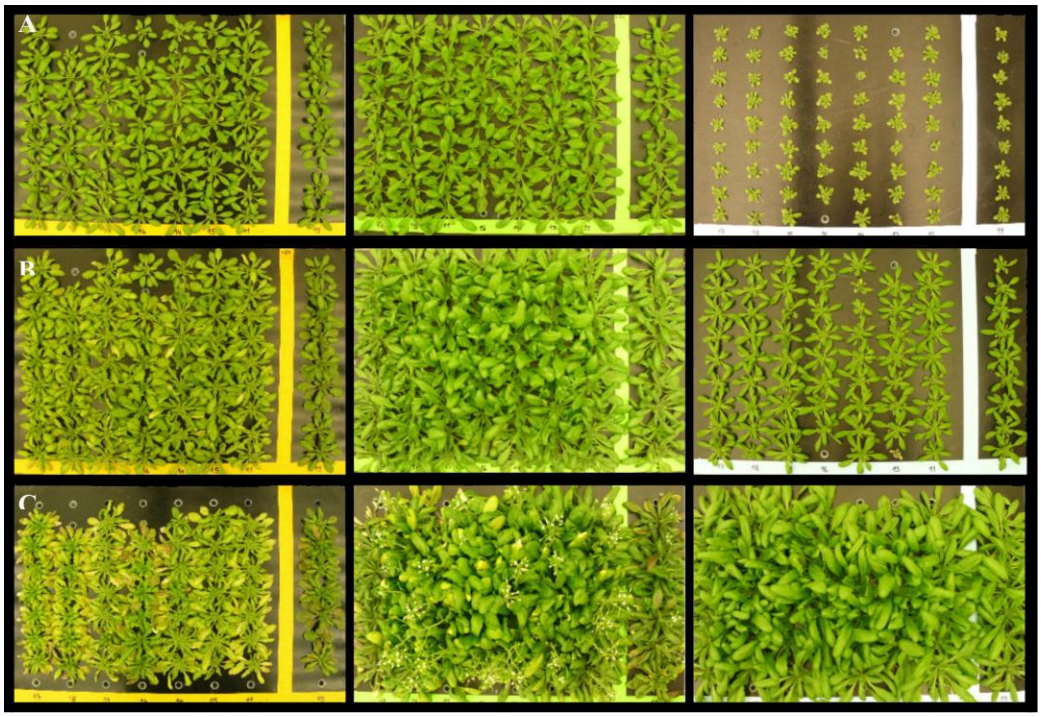


Figure 1: Visible phenotypes of OX 19 (# 19, 14, 15), OX 23 (# 16, 17, 18) and untransformed Arabidopsis Col plants (WT), grown for three weeks (A) four weeks (B) and six weeks (C) on hydroponics medium with excess Zn supply (white tape), sufficient Zn supply (green tape) or low zinc supply (orange tape). Each tray is showing respectively lines 17; 18; 11; 16; 14; 15; 11; 19; from left to right.

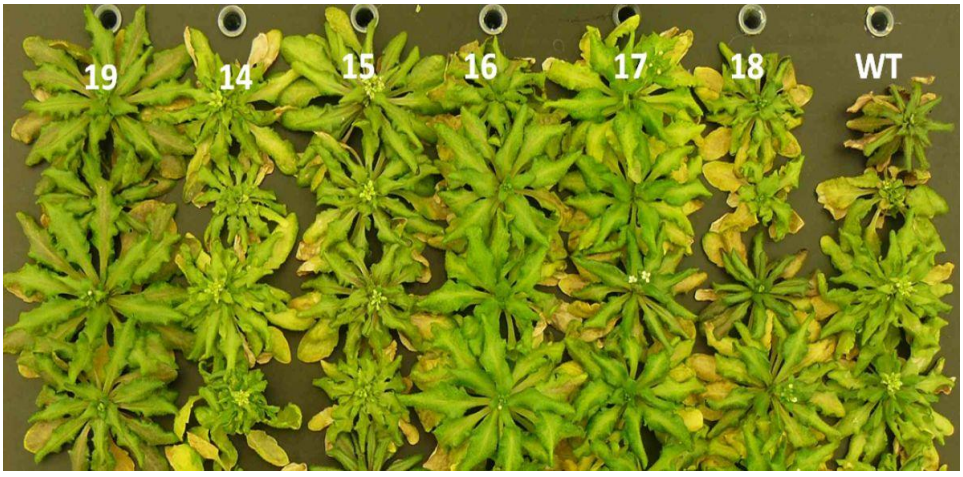


Figure 2: Visible phenotypes of OX 19 (# 19, 14, 15), OX 23 (# 16, 17, 18) and untransformed Arabidopsis Col plants (WT), grown for 6 weeks on hydroponic medium to which 0.05 µM $ZnSO_4$ has been added, creating strong zinc deficiency symptoms. Lines 19, 16 and 17 appear to have larger rosettes and darker green leaves, showing less sensitivity to zinc deficiency, when compared to WT.

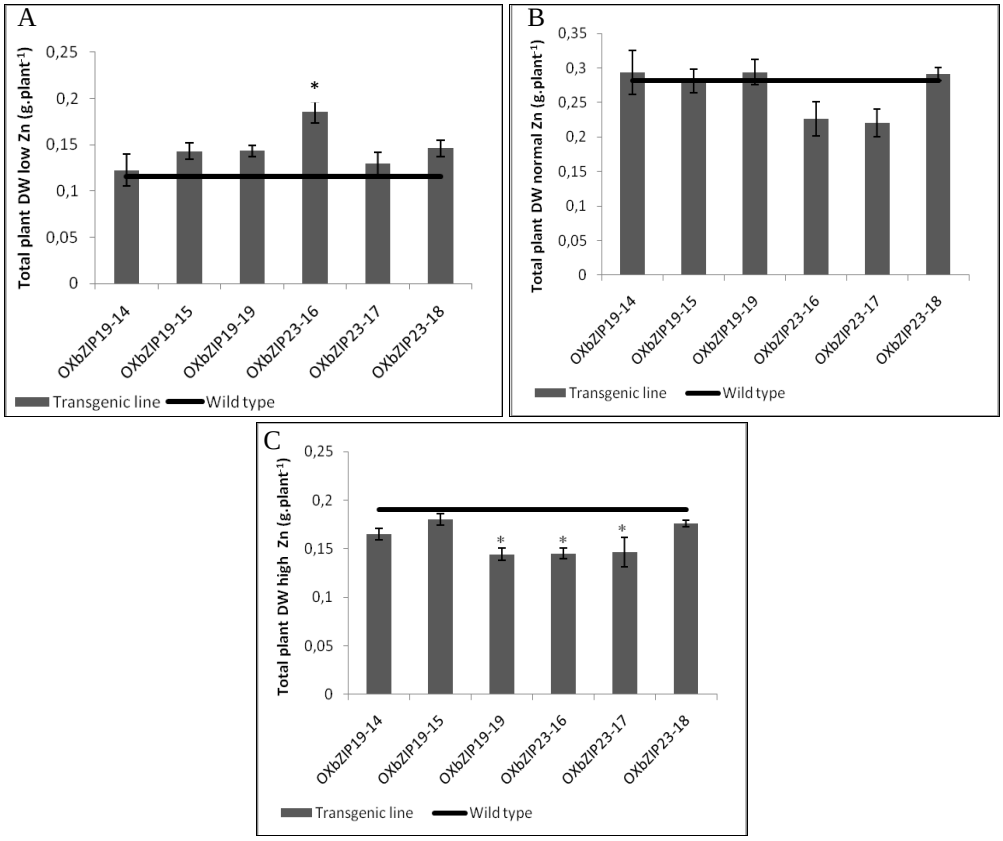


Figure 3: Total plant dry weight comparisons of OX 19 (# 19, 14, 15), OX 23 (# 16, 17, 18) and untransformed Arabidopsis Col plants (WT), grown for 6 weeks on hydroponics medium containing low Zn (0.05 µM $ZnSO_4$)(A); or on hydroponic medium containing normal Zn (2 µM $ZnSO_4$) (B) or excess zinc (25 µM $ZnSO_4$) (C). *Asterisks indicate values that are significantly different (P<0.05) from WT.

Zinc content in *Arabidopsis thaliana* growth on different Zn supply

In order to investigate the capacity of utilization and translocation of zinc contained in the solution, we determined the zinc concentration in roots and shoots of overexpressing *bZIP23* or *bZIP19* plants and wild type plants grown at deficient, sufficient and excess zinc. The Zn concentrations of plants grown at normal zinc or excess zinc, in both root and shoot, were in general not different from wild type (data not shown). However, at zinc deficiency, the shoot zinc concentration in all lines was lower than in wild type plants (Fig. 4), with especially lines OXbZIP23-18 and OXbZIP19-19 showing only 60% of the Zn concentration of wild type plants.

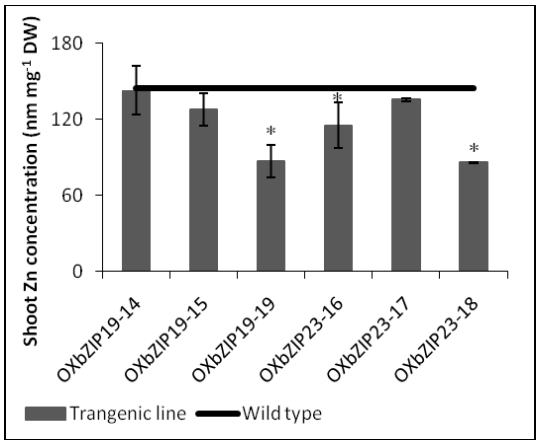


Figure 4: Zinc concentrations of OX 19 (# 19, 14, 15), OX 23 (# 16, 17, 18) and untransformed Arabidopsis Col plants (WT), grown for 6 weeks on hydroponics medium containing low Zn (0.05 μM $ZnSO_4$). *Asterisks indicate values that are significantly different (P<0.05) from WT.

Zinc Efficiency

To identify the ability of both transgenic plants and untransformed plants to grow and yield better under Zn deficiency and excess Zn, I analyzed the percentage of Zn Use Efficiency that is expressed as the ratio of dry matter produced under Zn deficiency or excess zinc to dry matter produced at Zn sufficient.

In zinc deficiency, transgenic lines were more zinc efficient than wild type plants (Fig. 5A). On average, Zn efficiency ratios were 47% for OX *bZIP19*, 64% for OX *bZIP23* and 41% for wild type plants. However, the advantage in zinc use efficiency of transformed plants was lost under excess zinc. In this condition, all lines, except the line 16, showed less Zn efficient than wild type plants (Fig. 5B).

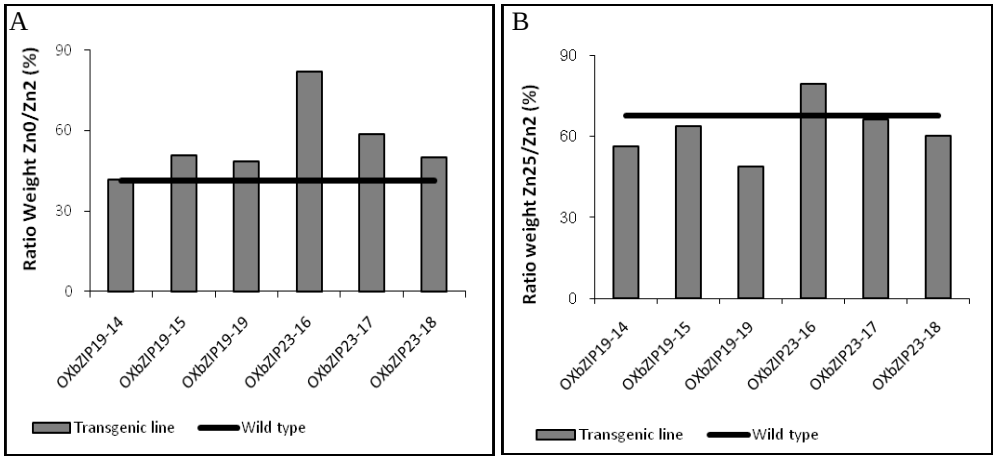


Figure 5: Zn Use Efficiency, ratio weight low zinc/sufficient Zn (A) and excessive Zn/ sufficient Zn (B), based on the total dry biomass of OX 19 (# 19, 14, 15), OX 23 (# 16, 17, 18) and untransformed Arabidopsis Col plants (WT), grown for 6 weeks on hydroponics medium.

Relative transcript levels of overexpressing bZIP23 and bZIP19 plants

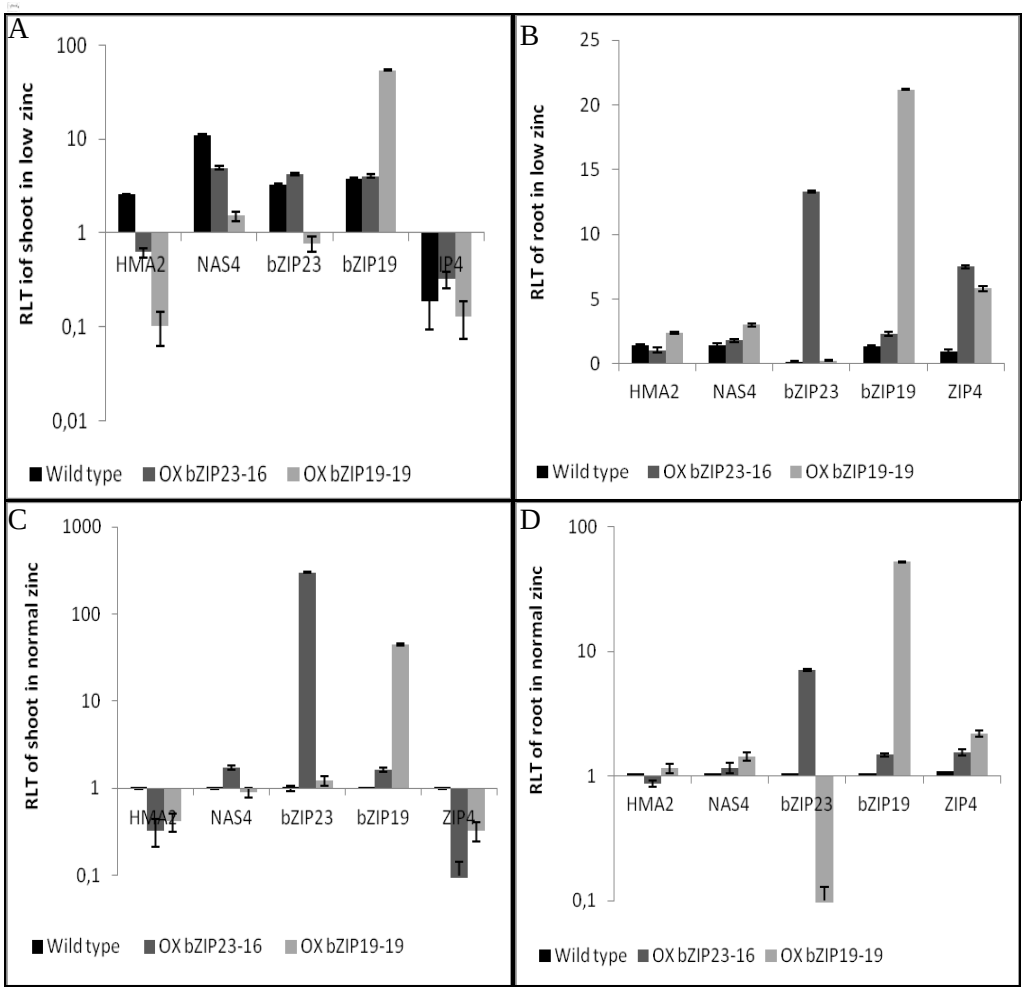
To study the expression of the target genes in both root and leaf tissues, wild type plants (WT) and transgenic lines overexpressing *bZIP19* or *bZIP23* were grown on hydroponics medium with 25 μM $ZnSO_4$ (excess Zn), 2 μM $ZnSO_4$ (optimal Zn) or 0.05μM $ZnSO_4$ (low Zn) for 6 weeks. After this time the relative transcript level of the target genes was determined.

In general, under all conditions of zinc supply, the expression of both bZIP19 and bZIP23 genes in roots and shoots was strongly induced in the respective transgenic overexpression lines (Fig. 6). Wild type plants only showed high expression of bZIP19 and bZIP23 genes in shoot, under low zinc supply, where the expressions were about 3 fold more than expression of wild type in normal conditions (Fig 6A).

The ZIP4 gene was upregulated in root under low zinc condition (Fig. 6B). This gene was much higher expressed in transgenic line, about 8 fold (OX19) and 6 fold (OX23) higher expression, than when compared with untransformed plants. The transgenic roots grown on sufficient Zn conditions showed similar expression pattern, but the increase in the expression was very slight, around 1.5 times more than in wild type plants. In shoot of transgenic and wild type plants under low, sufficient and excessive zinc supply conditions, ZIP4 gene was downregulated (Fig 6 C, E).

Also under zinc deficiency, HMA2 gene was downregulated of 25 and 4 fold, respectively, in OX19 and OX23 shoots of plants compared to wild type plants (Fig 6A). The same way, in sufficient and excess zinc supply the HMA2 gene was downregulated in transgenic lines, but the difference of expression levels when compared to wild type plants was not so significant (Fig. 6 C, D, E).

The expression of NAS4 gene in overexpressing bZIP19 or bZIP23 plants remained practically unchanged under sufficient zinc supply, in both shoots and roots, and in roots under low zinc supply when compared with wild type plants. However, there was a strong increase in expression of NAS4 (2 at 7 fold) in wild-type plants for transgenic plants at low zinc supply (Fig. 6A).



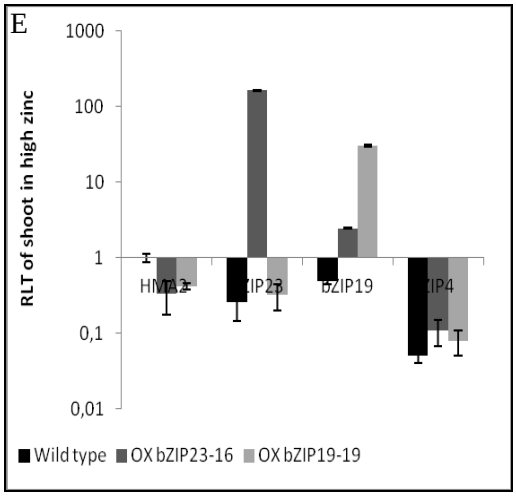


Figure 6: Relative transcript levels (RTL) of ZIP4, bZIP23, bZIP19, NAS4, HMA2, in 6-week-old of the wild-type plants (WT) and of transgenic lines overexpressing bZIP19 or bZIP23 grown on hydroponic medium containing excess Zn (25 μM $ZnSO_4$), sufficient Zn (2 μM $ZnSO_4$) or low Zn (0.05 μM $ZnSO_4$). Expression of genes in each individual sample reflects the fold-change increase or decrease when compared to the average expression level of wild-type plants under normal Zn supply.

DISCUSSION

In this study we investigated the effects of expression modification of transcription factors *bZIP19* and *bZIP23.* These transcription factors are directly involved in plant zinc homeostasis network and play an important role in switching on the zinc deficiency response of Arabidopsis thaliana. We postulate that overexpressing of *bZIP19* or *bZIP23* genes can confer to increase the accumulation of zinc and zinc deficiency tolerance in plants.

Zinc mineral deficiencies generally affect plant growth as well as the physiological and metabolic process of plants (CAKMAK, 2011; HARRIS et al., 2007; MARWAHA, 2007; RAMESH; CHOIMES; SCHACHTMAN, 2004). However, our results showed that overexpressing of *bZIP19* or *bZIP23* plants are less sensitive and more tolerant to zinc deficiency. Transgenic lines grown under zinc limiting conditions decreased the symptoms of zinc deficiency, such as very small leaf, internodes short, necrotic spots as well as chlorosis, mainly in lines 19, 16 and 17 (Fig 2). Moreover, these plants had continuing growth and obtained higher production of biomass after 6 weeks (Fig. 3A). It suggests a best performance of transgenic lines to face zinc-

deficient environments. Similar result was obtained in rice plants overexpressing OsIRO2, responsible for regulation of the genes involved in Fe homeostasis in rice, grew better and had improved the tolerance to low Fe availability in calcareous soil (OGO et al., 2011).

The mineral analysis displayed that the increase of biomass are not correlated with zinc accumulation in plants, since that biomass production total was inversely proportional to zinc accumulation in plants grown on low zinc supply. The wild type plants obtained higher shoot zinc concentration than all transgenic lines plants (Fig. 4). The lines 18 and 19 showed only 60% of the Zn concentration of wild type. Nevertheless, the zinc use efficiency were more high in overexpressing *bZIP23* or *bZIP19* plants than in untransformed plants, indicating that not only enhanced Zn uptake capacity but also enhanced internal Zn utilization capacity of plants play important role in the maintenance of cellular homeostasis at zinc scarcity conditions. Different wheat ploidys were also tested to zinc efficiency and the hexaploid wheat ‘Bezostaja', which is well adapted to Zn-deficient soils, was found contain the lowest Zn concentration, although had the highest Zn efficiency of all the genotypes (CAKMAK et al., 1999). Regel (2001) reports that micronutrient-efficient genotypes have a greater yield in towards to inefficient genotypes even when fertilized with smaller amounts and that Zn-efficient genotypes have more efficient utilization of Zn in tissue contributing to overall Zn efficiency. Thus, these researches corroborate that high zinc efficiency is not always a reflection of the high concentrations or zinc content in the plant.

By the contrast, overexpressing *bZIP19* or *bZIP23* plants grown at sufficient or excess zinc showed equal or less biomass production (Fig. 3 B,C) and zinc efficiency (Fig. 5B) when compared with wild type plants. The Zn concentration, were in general not different from WT (data not shown). This proves that transformed plants grow well in different zinc supply conditions and that this plants not exhibiting difference in visible phenotype (Fig 1). Additionally, our results reinforce the findings of Assunção et al. (2010) which report that transcription factors *bZIP19* and *bZIP23* are essential for ability to respond to zinc deficiency.

To investigate the changes of gene expression caused by the overexpressing of *bZIP23* or *bZIP19* in plants under different conditions of zinc supply, some genes already known to be involved in zinc homeostasis such as ZIP4, HMA2, NAS4, bZIP19 and bZIP23 had their expression quantified in roots and shoot (Fig. 6). We expected to find difference in gene expression between the transgenic lines and wild type, especially those which were grown on low zinc and showed a difference in response to biomass production and zinc use efficiency. Our results showed that all the target genes were induced in transgenic roots by zinc deficiency. The ZIP4 were strongly expressed in roots of overexpressing *bZIP23* or *bZIP19* plants upon shortage in zinc supply. Many previous reports indicated high expression of ZIP4 under zinc deficiency (GROTZ et al., 1998; GUERIOT; EIDE, 1999; ISHIMARU et al., 2005; MORTEL et al., 2006), because this gene plays a role in zinc uptake in roots and is involved in transport of cations across the plasma membrane in the roots (MORTEL et al., 2006). The high expression this gene in both *bZIP23* and *bZIP19*, around seven-folds when compared to wild type has lead us to speculate that it might be strongly related with increase zinc deficiency tolerance in plants. Also on low zinc supply, the transcript levels of NAS4 increased in overexpressing plants. Nicotianamine is responsible by chelates and transports micronutrient metal ions in plants (KLATTE et al., 2009), then a high expression of NAS4 suggests that ZIP4 and NAS4 are in the same regulatory network as genes involved in root zinc uptake and activated on zinc low zinc supply. The mRNA level of HMA2, which is involved in transport of zinc into the vasculature (MILLS et al., 2005), had increased slightly in both, transgenic and wild type roots under zinc deficiency. According with Mortel et al. (2006) the increase of expression of HMA2 can be a response to a higher zinc demand from the shoot. However, the expression of both bZIP19 and bZIP23 was equally high at deficiency, sufficient and excess zinc, unlike was reported by Assunção et al. (2010) which found that these transcription factors are more expressed under low zinc supply and regulate the adaptation of plants to zinc deficiency.

In conclusion, the CaMV-mediated overexpressing of either of the two bZIP transcription factors, controlling the essential Arabidopsis zinc deficiency response has effect towards improving zinc deficiency tolerance. Although, transgenic lines are not able to acquire more zinc, due to depletion in the medium, some OX19 and OX23 lines are able to produce more biomass with the same amount of zinc, indicating they are more Zn deficiency tolerant and more Zn efficiency. However, we reasoned that general overexpression of the *bZIP* genes might disturb the precise regulation required for proper action of the downstream genes, and such deregulation would not lead to the required effect. Therefore, for a best performance, a controlled enhanced expression of these transcription factors under control of the zinc deficiency responsive ZIP4 promoter can be most effective in enhancing Arabidopsis zinc deficiency tolerance.

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Chapter 3

Expression of *bZIP19* under control of the Zn deficiency responsive *ZIP4* promoter can lead to enhanced the zinc deficiency tolerance in Arabidopsis

ABSTRACT

Zinc is an essential micronutrient responsible for the maintenance of vital processes in all living organism. On encountering a shortage in zinc supply, plants undergo a range of changes in molecular and physiological levels in order to adapt to stress. Transcription factors from *Arabidopsis thaliana*, bZIP19 and bZIP23, are essential for their ability to respond to zinc deficiency and the Zinc Deficiency Responsive Element, a *cis*-element found in the promoter of genes such as *ZIP4*, to which these transcription factors bind, is also directly involved in Zn deficiency response. I investigated the expression of the *bZIP19* gene in control of the Arabidopsis zinc deficiency responsive *ZIP4* promoter. Transgenic Arabidopsis lines expressing pZIP4::bZIP19 were grown under deficient and sufficient zinc supplies. The experiments provided evidence that the pZIP4::bZIP19 construction is very effective in enhancing Arabidopsis zinc deficiency tolerance and that the response to zinc deficiency is switched on at a very early stage of zinc deficiency. Furthermore, our results suggest that bZIP19 may also play an important role in plant development and proposes that adequate zinc nutrition could alleviate effects of heat stress. These finding are promising and may offer an attractive advantage for crops in areas suffering from low zinc bioavailability as well as contributing to enhanced yields and plant robustness.

Keywords: Zinc deficiency. bZIP genes. ZIP4.

INTRODUCTION

Plants are capable of absorbing a wide variety of mineral ions which is of extreme importance to both nutrition and human health. Nevertheless, mineral concentrations in many plant foods are low due to insufficient availability of minerals such as zinc and iron in agricultural soils (CAKMAK, 2008). Zinc (Zn) is an essential micronutrient for all living organisms since it has a number of crucial functions in cells. In plants, this mineral is vital for the functionality of more than 300 enzymes, protein synthesis, transcriptional and post-transcriptional processes, structural and functional integrity of cell membrane as well as for detoxification of superoxide (KRÄMER; CLEMENS, 2006; SONG et al., 2010). Thus, a low availability of this mineral in the soil, a problem often found in arid and semi-arid regions, limits the zinc uptake by plants resulting in significant decreases in growth, yield and in zinc content of the plants, facts which are totally undesirable given the current increase in demand for food with high nutritional quality and high productivity (WELCH; GRAHAM, 2002).

On encountering a shortage in zinc supply, plants undergo a range of adaptive changes to try to maintain zinc at an acceptable level in all cellular compartments, and thus avoid strong alterations in the dynamic and complex process of development (GRUSAK, 2002). As natural response mechanism to overcome low Zn bioavailability, plants increase the expression of several genes involved in Zn homeostasis, including genes encoding Zn transporters and chelating enzymes (MORTEL et al., 2006). In recent years, various genes have been identified which encode membrane transporters and chelating molecules that have a major role in metal homeostasis. The expression differential analysis has revealed that a number of genes are induced in plants grown under low Zn supply conditions (GROTZ et al., 1998; MORTEL et al., 2006; WINTZ et al., 2003). These are thought to carry zinc into the cytosol (PENCE et al., 2000) and appear to be important for acquisition of Zn from the soil. Many members of the ZIP family, such as *ZIP1, ZIP3, ZIP4, ZIP5, ZIP6, ZIP9, ZIP10, ZIP12* and *IRT3,* are upregulated in roots under zinc-deficiency (MORTEL et al., 2006; PALMER and GUERINOT, 2009; SONG et al., 2010; WINTZ et al., 2003). The *HMA2* and *HMA4* genes, both belonging to the P_{1B}-ATPase family, are involved in zinc transport out of the cell. The Zn^{+2}-ATPases they encode locate to the plasma membrane and are implicated in translocation of zinc from the root to the shoot

(SONG et al., 2010). Another family of transporters involved in zinc efflux is the members of ZIP genes.

Recently two bZIP transcription factors, bZIP19 and bZIP23 were identified. These transcription factors were found, in yeast screening, associated to promoter regions of the zinc deficiency induced *ZIP4* and belong to bZIP group F. They stimulate the expression of set of the target genes, called Zinc Deficiency Response Elements, which constitutes the primary response to zinc deficiency (ASSUNÇÃO et al., 2010). The *bZIP19* and *bZIP23* genes share 69% of amino acid sequence identity and act redundantly, although bZIP19 is only partially redundant (ASSUNÇÃO et al., 2010). Therefore, as these transcription factors are essential for switching on the zinc deficiency response of *Arabidopsis thaliana* we hypothesize that the expression of the *bZIP19* gene under control of the Arabidopsis zinc deficiency responsive *ZIP4* promoter may be an interesting option to try make plants less sensitive to zinc deficiency and/or to confer a constitutive zinc deficiency response, contributing to enhance yield and the robustness of crops in areas suffering from low zinc bioavailability.

MATERIALS AND METHODS

Generation of pZIP4::bZIP19 construct

To generate ZIP4::bZIP19 construct for transformation of the Arabidopsis wild-type plants, the AtZIP4 promoter was cloned as a BamHI-NcoI fragment from pBG0011. The AtbZIP19 cDNA was cloned as an NcoI-SpeI fragment from a cloned PCR fragment of the cDNA, cloned into the vector pGEM-T-Easy (Promega), after amplification with 5'CCTCTTTTGGCCTCCATCGGAA'3 and 3´CTGATTCA CCCCCCTAACCGT´5 primers, generating an NcoI site at the ATG start codon and an SpeI site at pos. 1051 of the cDNA. Both fragments were cloned into pBG0060, cut with BamHI and XbaI, thus removing the AtZIP4 promoter and the eGFP gene. The constructs pZIP4: bZIP19 were verified by digestion analysis and sequencing and transformed by electroporation into *Agrobacterium tumefaciens* strain AGL0. Subsequently, Arabidopsis wild-type plants were transformed by floral dipping (CLOUGH; BENT, 1998). Independent transformed lines were selected for a single insertion locus by antibiotic resistance and 3:1 segregation ratio of T2 seedlings. The

expression of bZIP19 was confirmed by RT-PCR. Homozygous lines were selected for further analysis.

Plant growth condition

Arabidopsis thaliana accession Columbia (Col-0) plants homozygous for the pZIP4::bZIP19 constructs introduced by *Agrobacterium tumefaciens* transformation were generated and three independent transgenic lines each represented by two lines, 7(3) and 7(4); 10(3) and 10(9); 14(9) and 14(10) were selected and used in all experiments. For the hydroponically grown plants, seeds of transgenic lines along with wild-type seeds were treated as described in the chapter 2, then seeds were sown and grown for 4 weeks in hydroponic medium containing a modified half-strength Hoagland's nutrient solution (SCHAT; VOOIJS; KUIPER, 1996). The Hoagland's solution was prepared either with low zinc supply (0.05 µM $ZnSO_4$) or sufficient zinc supply (2 µM $ZnSO_4$). During the first two weeks, the nutrient solution was refreshed once a week and thereafter twice a week. Germination and plant growth were performed in a climate chamber for 12h/d at 20/15 °C day/night temperature, 250 µmol photons $m^{-2}s^{-1}$ and 75% relative humidity. For the plate-agar assay, seeds were sterilized through vapour-phase seed sterilization and sown on Petri dishes containing half-strength MS (Murashige and Skoog) medium, 1% sucrose and Daishin agar. The MS medium was prepared either with optimal zinc (15 µM $ZnSO_4$) or deficient zinc supply (0 µM $ZnSO_4$). After 3 days of stratification treatment at 4°C, seeds were grown in a climate chamber (16h/d at 22/20°C day/night temperature; 120 µmol photons $m^{-2}s^{-1}$; 50% relative humidity), for four weeks. After this time, biomass production was determined and the WinRHIZO *Pro* 2007a system was used to evaluate morphological attributes of root systems and leaf parameters. The plant material was placed in an acrylic container (20 X 30 cm) then scanned using a 400 dpi resolution. For each zinc supply treatment five plants per genotype were analyzed. For the soil grown plants, seeds were germinated and grown in separate pots in a temperature-controlled greenhouse with 22 °C at 14/10 hours light/dark.

Heat stress assay

Germination and plant growth were performed in a climate chamber on modified half-strength Hoagland's nutrient solution at low zinc supply (0 µM $ZnSO_4$) or sufficient zinc supply (2 µM $ZnSO_4$) as described above. When the plants were five-weeks old, one part of the experiment was harvested and dry weight determined. The remaining plants were kept on the hydroponic system and transferred to the high temperature growth room where the temperature was gradually increased from 20°C to 30°C at a rate of 2°C h^{-1}. The temperature was maintained at 30/25°C day/night for 18 days. During the heat stress treatment, the hydroponic solution either with low zinc or sufficient zinc was filled daily to ensure that plants were not under water stress.

Determination of metal concentrations

Three blocks of three plants for each treatment and replicate were harvested after four weeks on hydroponic medium. Roots and shoots were dried overnight at 65°C and all samples were digested with a mixture of HNO_3 (65%) and HCl (37%) for 7 hours at 140°C. Then they were analyzed for zinc, iron, manganese and copper using flame atomic absorption spectrometry (Perkin Elmer 1100B).

Real-time quantitative RT-PCR

To analyze the different expression levels in leaves and roots at four weeks, grown under low or sufficient zinc conditions, qRT-PCR was performed as described in Chapter 2. The following standard thermal profile was used: 3 min at 95.0°C, followed by 40 cycles of 15 sec at 95.0 °C and 1 min at 60.0 °C. All gene expression analyses were performed with four biological replicates and three technical replicates per biological replicate for each genotype x treatment combination. The samples were first normalized to a selected internal control gene, 18S rRNA, and then the relative target gene expression was determined by performing a comparative ΔΔCt. The following primers were used for the validation of the expression analysis (forward then reverse): HMA2 (5'AGAATGGCGTCGAAGAAGATGACC3', 5'ACGATGACG GTTCTTGACGG3'); ZIP4 (5'GATCTTCGTCGATGTTCTTTGG 3', 5'TGAG AGGTAT GGC TACACCAGCAGC3'); bZIP23 (5'TAATCAGCTGTTGAAGAGGT 3', 5'TCATGTATGAGTA AGGCACG 3'); bZIP19 (5'TTCTCCCGGAT GAGAGC

GATGA 3', 5'GCTGATTCACCGCCCTAAGCCT 3'); NAS4 (5' CTGTGGTGAG GCTG AAG GTTACTTA 3', 5'GACCAGAGCCAATGAAAGCTAC 3'); 18S (5' TGAC GGAGAATTAGGTTCG 3', 5'- CC TCCAATGGATCCTCGTTA 3').

Statistical analysis

The statistical analysis of mineral concentration and dry weight data were performed by one-way ANOVA and significant differences between means were determined using a Tukey test at the 5% level of significance ($P \leq 0.05$).

Experimental design

This study was designed to analyze the response of ZIP4::bZIP19 plants for tolerance to zinc deficiency and zinc accumulation compared to wild-type Arabidopsis plants. The hydroponic system was used to minimize variation in the bioavailability of zinc and other nutrients. In this experiment, seeds were germinated and grown in a hydroponic system under low zinc supply (0.05 µM $ZnSO_4$) or optimal zinc supply (2 µM $ZnSO_4$) for 4 weeks. The determination of biomass production and mineral concentrations in plants allowed us to evaluate both the health of plants faced with stress and the capacity of utilization and translocation of zinc and other mineral contained in the solution. The expression of direct and indirect zinc homeostasis target genes of bZIP19 was determined aiming to unravel genes involved in zinc deficiency response. The development of ZIP4::bZIP19 plants compared to wild type plants grown in soil and on agar plates was also investigated. Finally, the relation between zinc nutrition and thermotolerance was examined by applying a combined stress.

RESULTS

ZIP4::bZIP19 plants are more zinc deficiency tolerant

After three weeks of growth differences in visible phenotype between transgenic lines and wild-type plants became apparent. At that stage, the wild type plants grown on low zinc supply showed growth retardation, exhibiting smaller rosettes than the pZIP4::bZIP19 transformed plants (Fig.1). Nevertheless, none of the plants was showing visible zinc deficiency symptoms.

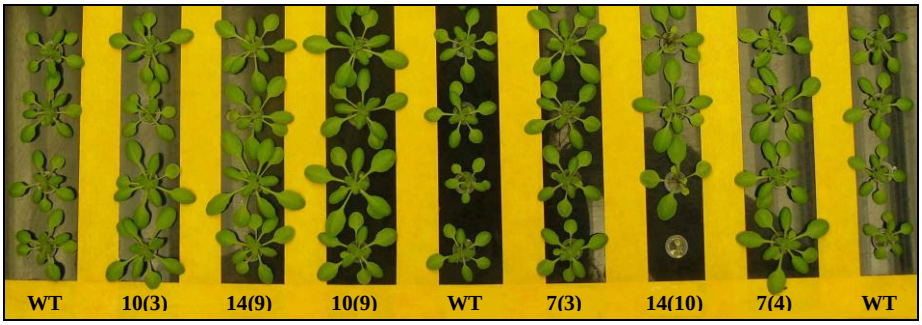

Figure 1: Visible phenotypes of pZIP4::bZIP19 (three independent transformants 7, 10, 14) and untransformed Arabidopsis Col plants (WT), grown for 3 weeks in hydroponics medium to which 0.05 μM $ZnSO_4$ has been added.

The effect of growth retardation was also reflected in the dry weight of Arabidopsis plants after four weeks (Fig 2). Under zinc deficient medium, all independent transformants expressing pZIP4::bZIP19 were showing almost double the dry weight when compared to wild type plants (Fig 2A). Surprisingly, at sufficient zinc supply, the biomass production of the transgenic lines was also significantly higher, about 30%, than of untransformed plants (Fig. 2B). After four weeks growing under zinc shortage, we observed the first zinc deficiency symptoms, such as chlorosis in older leaves. In general, these were more obvious in wild type plants which also showed smaller rosette diameters than transgenic lines plants (Fig.3).

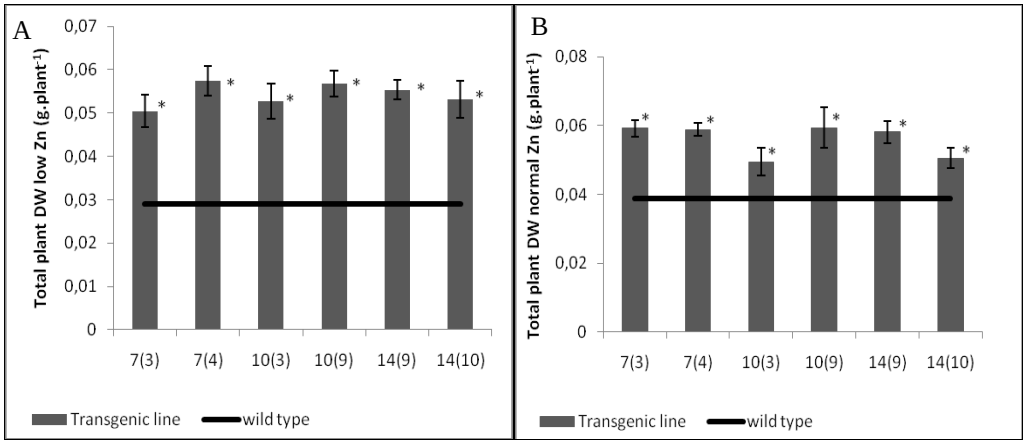

Figure 2: Total plant dry weight of two lines from three independent transformants (7, 10, 14) expressing proZIP4::bZIP19 grown for four weeks in hydroponic medium either with low (0.05 µM)(A) or normal (2 µM)(B) $ZnSO_4$ supply, compared to Col-0 wild-type plants (WT).

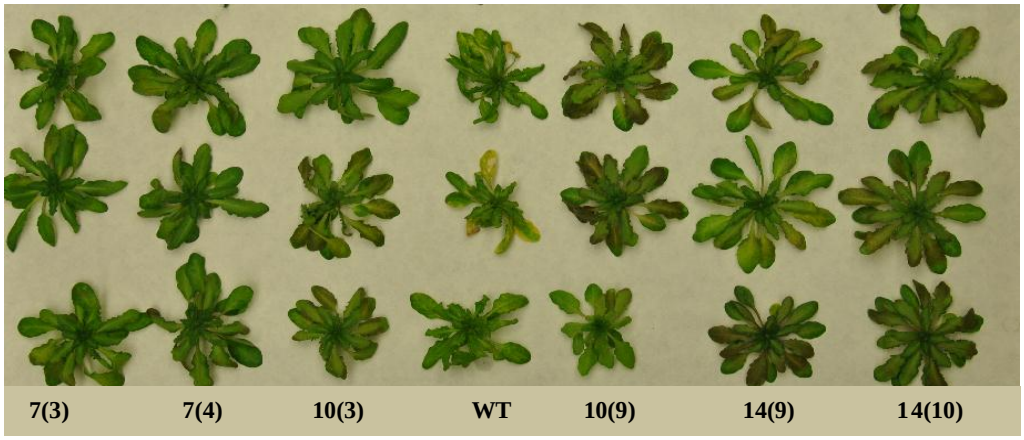

Figure 3: Visible phenotypes of pZIP4::bZIP19 (three independent transformants 7, 10, 14) and untransformed Arabidopsis Col plants (WT), grown for 4 weeks in hydroponics medium to which 0.05 µM $ZnSO_4$ has been added.

Mineral concentration is affected by zinc nutrition

The concentration of zinc was determined in tissues of both pZIP4::bZIP19 transformed plants and wild type plants in order to investigate the capacity of utilization and translocation of zinc. For both growth conditions the zinc concentration in shoots of transgenic lines was higher than that of wild-type plants (Fig 4), although the difference was only statistically significant for few lines. At sufficient Zn supply, the best lines accumulated ~20% more Zn when compared to untransformed plants (Fig. 4B).

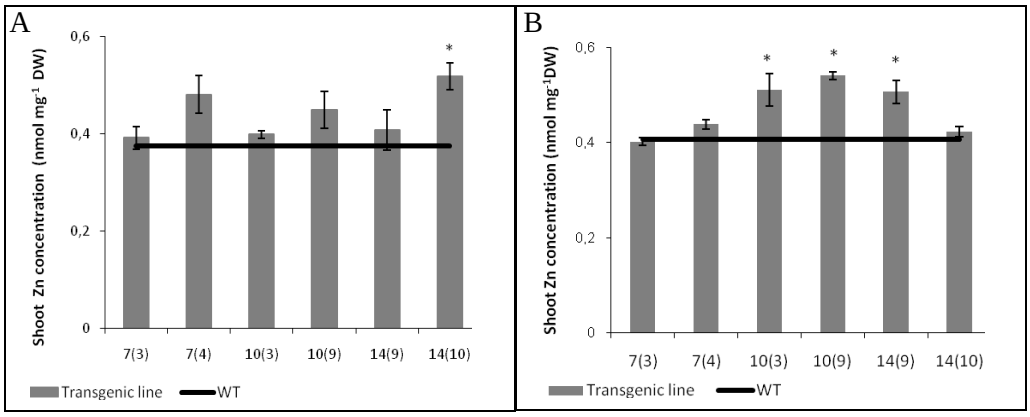

Figure 4: Zinc concentrations of pZIP4::bZIP19 (three independent transformants 7, 10, 14) and untransformed Arabidopsis Col plants (WT), grown for 4 weeks in hydroponics medium containing low Zn (0.05µM $ZnSO_4$)(A) or sufficient Zn (2µM $ZnSO_4$)(B). *Asterisks indicate values that are significantly different (P<0.05) from WT.

Since it is expected that different zinc supply conditions affect the concentrations of other metals, we also measured the concentrations of iron, manganese and copper in roots and shoots of plants grown either in low or sufficient zinc supply (Fig. 5). The iron concentration was about 10 fold higher in roots than in shoots in both zinc supply conditions (Fig. 5). In general, the concentration of iron was similar similar when comparing transgenics with wild type plants. Only for shoots of transgenic plants, grown under low zinc supply, the iron concentration in one line decreased significantly (8-32%) when compared to wild type plants (Fig. 5A).

For manganese the situation is opposite to iron, because Arabidopsis plants accumulated more manganese in shoots than in roots. There was no effect of zinc supply and differences between transgenics and wild type were not statistically significant (Fig 5B and D).

The copper concentration was higher in roots than in the shoots , however the concentration has a tendency to decrease, mainly in roots, with a decreasing zinc supply (Fig 5). The accumulation of copper in shoots was higher in pZIP4::bZIP19 transformed plants than in wild type plants in both low and sufficient Zn supply, but the difference is significant only at sufficient Zn supply. In this situation, the transgenic lines accumulated on average ~ 0.3 nmol/mg DW of copper (Fig. 5B) which is 46% more Cu than in wild type plants.

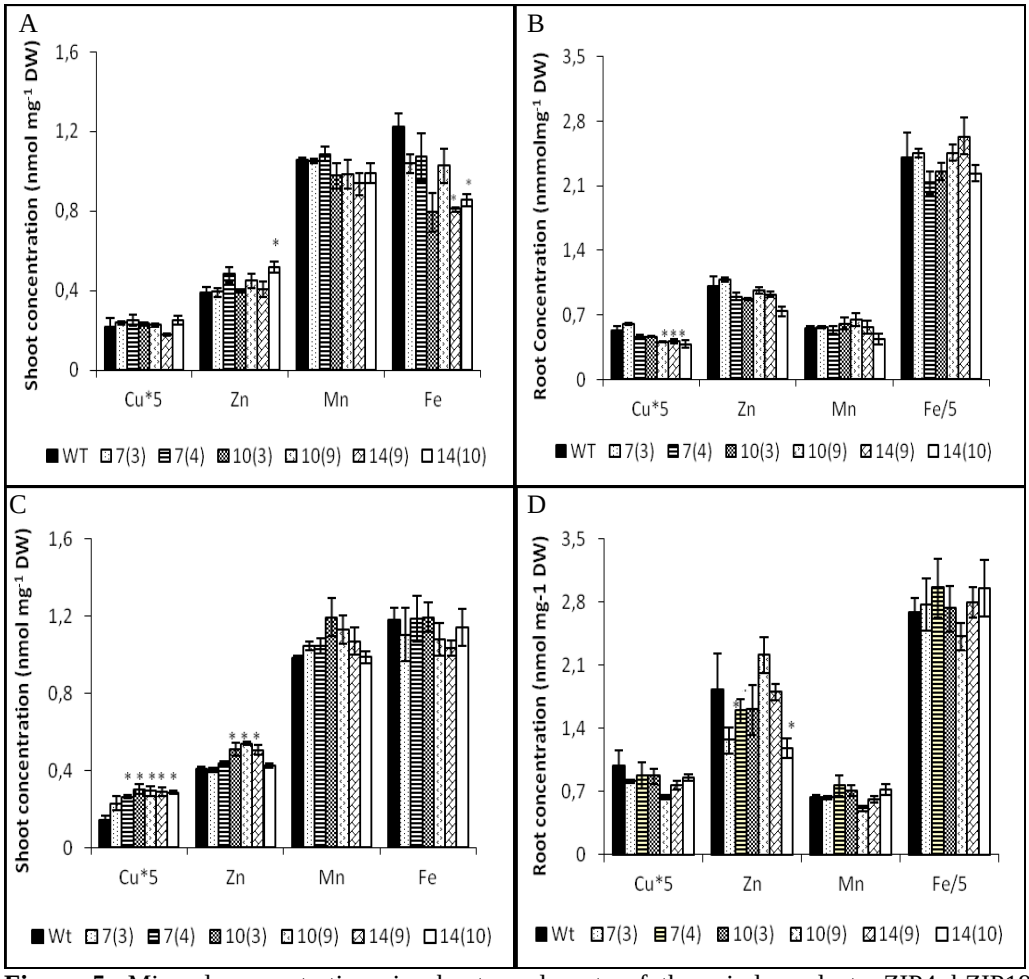

Figure 5: Mineral concentrations in shoots and roots of three independent pZIP4::bZIP19 transformants (7, 10, 14) and untransformed Arabidopsis Col plants (WT), grown for 4 weeks in hydroponics medium containing low Zn (0.05µM $ZnSO_4$)(A, B) or sufficient Zn (2µM $ZnSO_4$)(C, D). *Asterisks indicate values that are significantly different from WT (P<0.05). Fe concentrations in roots are divided by 5 (Fe/5) and Cu concentrations in roots and shoots are multiply by 5 (Cu*5), in order to fit the scale.

Quantitative RT-PCR analysis

After 4 weeks in hydroponics medium containing low zinc supply (0.05 µM $ZnSO_4$) or sufficient zinc supply (2 µM $ZnSO_4$), the expression of zinc homeostasis target genes of bZIP19 was determined in roots and shoots of *pZIP4::bZIP19* transformed plants and wild type plants (Fig. 6).

The expression of *bZIP19* in tissue, shoot and root, was higher in transformed plants than in wild type plants grown on sufficient and deficient zinc supply. Transformed shoots showed higher increase than transgenic roots. At sufficient zinc supply transformed shoots were strongly induced, mainly in line 10(9) which showed about 45 times higher expression than wild type shoots (Fig. 6A) and in plants grown under low zinc supply the expression of *bZIP19* was upregulated at least 5 times more than wild plant (Fig. 6C).

At optimal zinc supply, the *NAS4*, *bZIP23*, *ZIP4* genes maintained similar expression levels in transgenic roots when compared to wild type root (Fig. 6B).The *HMA2* expression in roots was 2 to 5 times higher in lines 7(3) and 14(9) than in wild type root (Fig. 6B). Under the same conditions, the expression levels of *bZIP23*, *ZIP4* and *HMA2* in transgenic shoots were comparable to untransformed shoots (Fig. 6A). The *NAS4* gene showed a little increase of expression in transgenic shoots (Fig. 6A).

At low zinc supply, in general transformed leaves and roots showed no differences in expression levels of both *bZIP23* and *NAS4* genes when compared to wild type plants (Fig 6C and D). Line 7(3), in both tissues, expression of *HMA2* is about twice higher than in other transgenic lines and untransformed plants. The *pZIP4::bZIP19* transformed plants showed a high expression of *ZIP4* genes in tissues of plants grown on zinc deficient medium. In transgenic roots, *ZIP4* was upregulated about 2 to 4 fold when compared to the expression of wild type root (Fig. 6D). A similar expression pattern can be seen for shoots of transformed plants that showed high expression levels of *ZIP4* from 5 to ~ 30 times more than untransformed shoots (Fig. 5C).

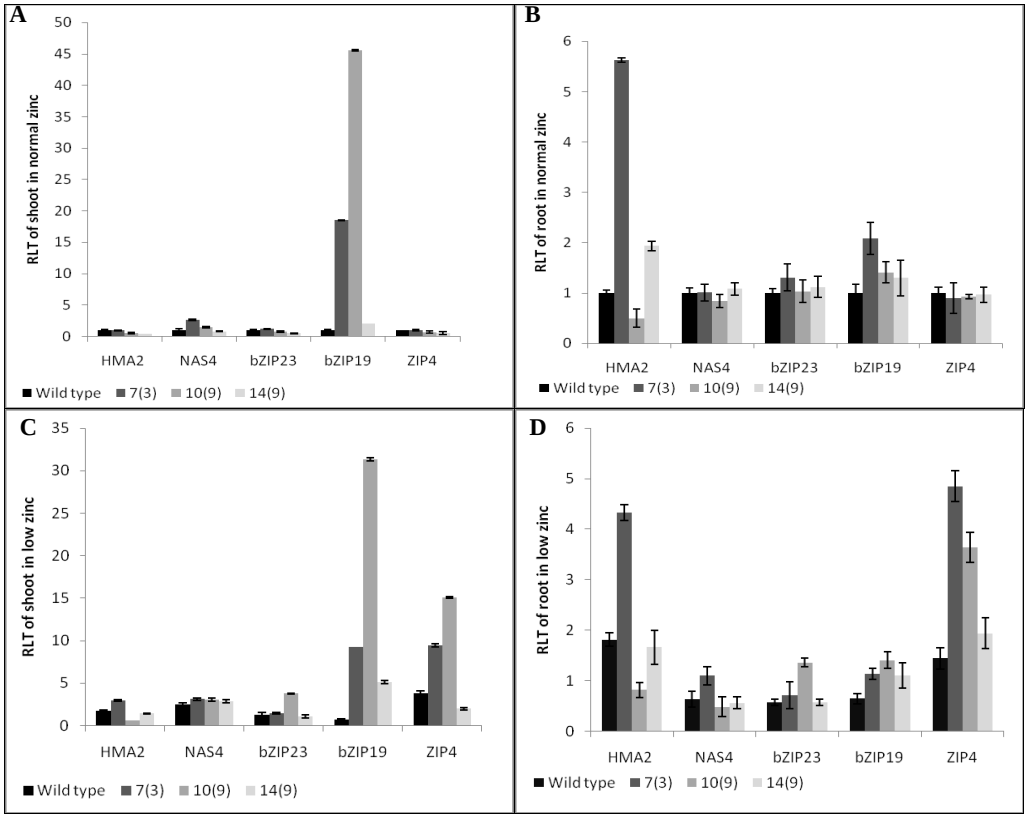

Figure 6: Relative transcript levels (RTL) of *ZIP4*, *bZIP23*, *bZIP19*, *NAS4*, *HMA2*, in 4-week-old wild-type plants (WT) and plants of three independent transformants 7(3), 10(9), 14(9) expressing *proZIP4::bZIP19* grown in hydroponic medium to which 2µM $ZnSO_4$ has been added (normal zinc; A, B) or containing 0.05µM $ZnSO_4$ (low zinc; C, D). Expression of genes in each individual sample reflects the fold-change increase or decrease relative to the average expression level of wild-type plants under normal Zn supply.

Agar plate assay

After four weeks of growth on agar plate to which no zinc had been added (0 µM $ZnSO_4$), Arabidopsis plants were showing clear zinc deficiency symptoms (Fig.7A). Under this conditions, pZIP4::bZIP19 transformed plants in general exhibited phenotype with greener rosettes than wild type plants (Fig. 7A). On the other hand, plants grown on agar containing sufficient zinc supply kept uniform growth and did not show zinc deficiency symptoms (Fig. 7B). When examined for biomass production, the two lines from three independent transformants (7, 10, 14) expressing proZIP4::bZIP19 were found to have higher dry weight than wild type plants. This was statistically significant for lines 7(3) and 14(9) that showed about 38% more biomass (Fig 8A).

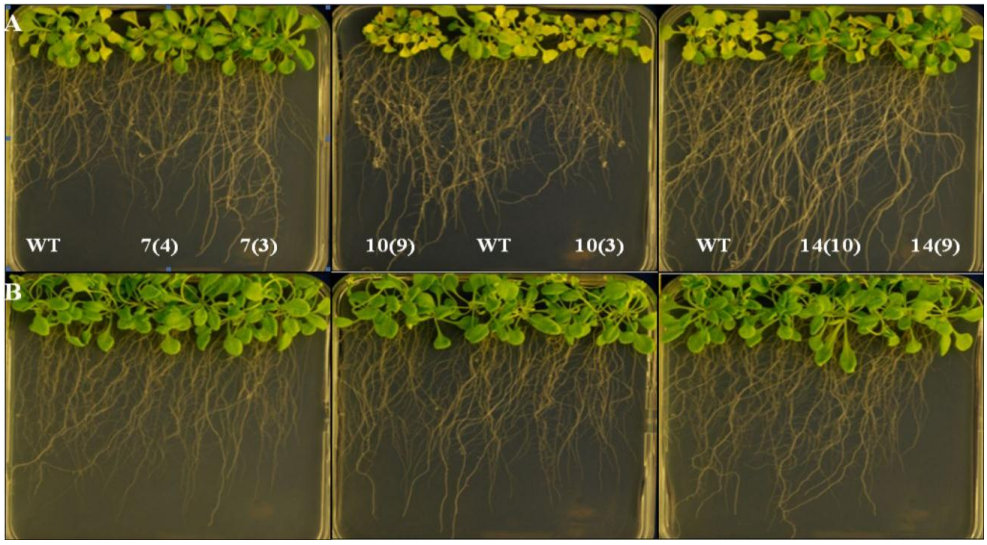

Figure 7: Visible phenotypes of pZIP4::bZIP19 (three independent transformants 7, 10, 14) and untransformed Arabidopsis Col plants (WT), grown for four weeks on agar plate containing 0 µM $ZnSO_4$ (low zinc) (A) or 15 µM $ZnSO_4$ (sufficient zinc) (B).

Through WinRHIZO *Pro* 2007a software it was possible to analyze projected leaf area and to quantify the extension of zinc deficiency symptoms in foliar area, as well as evaluate morphological attributes of root systems. With sufficient zinc supply, no differences between transgenic lines and wild type plants were observed in the projected leaf area (Fig. 8B), while transgenic plants grown on agar plate without zinc added showed a projected area equal to or higher than WT, especially for lines 7(3) and 14(9). In these lines, the projected area under zinc shortage was even higher than with optimal zinc conditions (Fig. 8B).

Quantification of zinc deficiency symptoms was performed by distinguishing the color of the leaf blade. The area covered by green or dark green was classified as healthy and the yellow or light green area was classified as poor. Transgenic lines 7(3), 7(4), 14(9) and 14(10) showed less zinc deficiency symptoms (≥ 33%), the other two transformed lines, 10(3) and 10(9), and the wild type plants exhibited an area larger than 40% with zinc deficiency symptoms (Fig. 9).

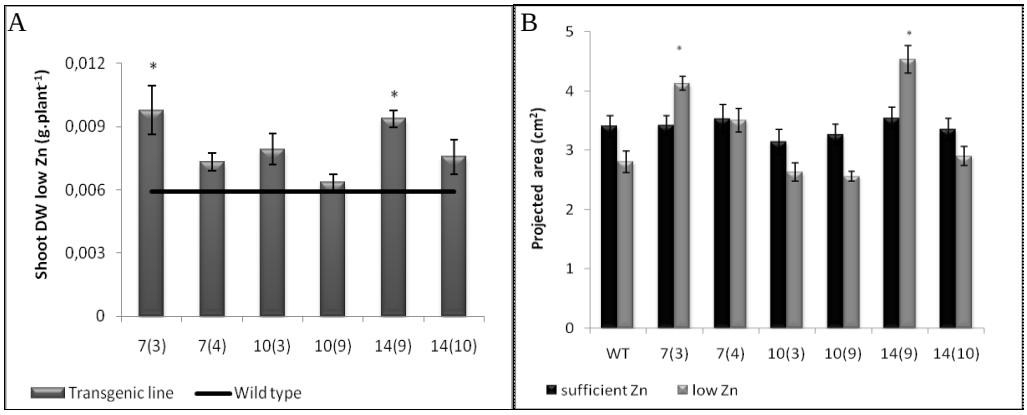

Figure 8: Shoot dry weight and projected leaf area of two lines from three independent transformants (7, 10, 14) expressing proZIP4::bZIP19 and untransformed Arabidopsis Col plants (WT). For shoot dry weight, plants were grown for four weeks on an agar plate to which no zinc (0μM $ZnSO_4$) had been added(A).The projected area was analyzed by WinRhizo in four week-old plants grown on agar plates containing 0μM $ZnSO_4$ (low zinc) or 15μM $ZnSO_4$ (sufficient zinc) (B). *Asterisks indicate values that are significantly different (P<0.05) from WT.

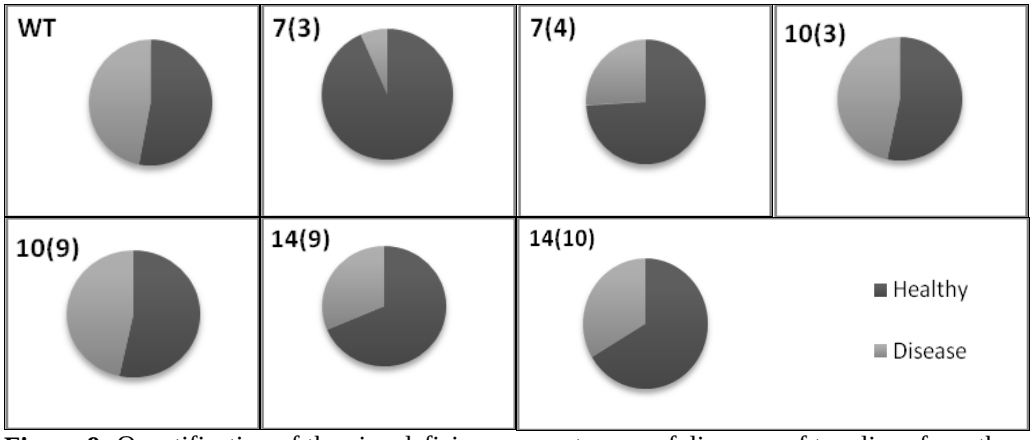

Figure 9: Quantification of the zinc deficiency symptoms on foliar area of two lines from three independent transformants (7, 10, 14) expressing proZIP4::bZIP19 and of untransformed Arabidopsis Col plants (WT), grown for four weeks on agar plates to which no zinc (0μM $ZnSO_4$) had been added. The area covered by green or dark green was classified as healthy area and yellow or light green areas were classified as poor.

The morphological analysis of root systems revealed that in wild type lines root length, decreased with around 10 cm when grown under Zn-limiting conditions in comparison to plants grown under sufficient zinc conditions. However, the opposite was observed for most transgenic lines, except for line 10(9). The same was observed with surface area, where in general the surface area was higher in pZIP4::bZIP19 transformed plants. Nevertheless, as can be observed in table 1, both diameter and root volume did not present drastic changes when compared to untransformed plants.

Table 1. Total length, surface area, diameter and mean volume root of three independent transformants (7, 10, 14) expressing proZIP4::bZIP19 and of untransformed Arabidopsis Col plants (WT), grown for four weeks on agar plate containing 0 μM $ZnSO_4$ (low zinc) or 15 μM $ZnSO_4$ (sufficient zinc)

Genotypes		**Zinc Levels**	
		Normal	**Low**
		Total length ($cm.plant^{-1}$)	
Wild type	WT	45.4	34.2
ZIP4::bZIP19	7(3)	49.5	54.5*
ZIP4::bZIP19	7(4)	36.7	55.6*
ZIP4::bZIP19	10(3)	39.4	48.2
ZIP4::bZIP19	10(9)	54.4	39.6
ZIP4::bZIP19	14(9)	57.2	66.0*
ZIP4::bZIP19	14(10)	36.5	64.3*
		Surface area ($cm^2.plant^{-1}$)	
Wild type	WT	9.0	8.3
ZIP4::bZIP19	7(3)	10.1	9.5
ZIP4::bZIP19	7(4)	7.8	10.5*
ZIP4::bZIP19	10(3)	9.1	10.2*
ZIP4::bZIP19	10(9)	10.3	7.6
ZIP4::bZIP19	14(9)	10.1	10.1*
ZIP4::bZIP19	14(10)	7.2	11.5*
		Diameter ($mm.plant^{-1}$)	
Wild type	WT	0.4	0.5
ZIP4::bZIP19	7(3)	0.5	0.5
ZIP4::bZIP19	7(4)	0.4	0.5
ZIP4::bZIP19	10(3)	0.5	0.5
ZIP4::bZIP19	10(9)	0.5	0.4
ZIP4::bZIP19	14(9)	0.5	0.6
ZIP4::bZIP19	14(10)	0.5	0.5
		Root Volume ($cm^3.plant^{-1}$)	
Wild type	WT	0.1	0.1
ZIP4::bZIP19	7(3)	0.1	0.1
ZIP4::bZIP19	7(4)	0.1	0.1
ZIP4::bZIP19	10(3)	0.1	0.1
ZIP4::bZIP19	10(9)	0.1	0.1
ZIP4::bZIP19	14(9)	0.1	0.2
ZIP4::bZIP19	14(10)	0.1	0.2

*Asterisks indicate values that are significantly different ($P<0.05$) from Wild type

The pZIP4::bZIP19 transformed plants grown in soil

To determine the phenotype of pZIP4::bZIP19 transformed plants in soil, these plants were grown along with wild type plants for five weeks in a greenhouse in individual pots containing soil. Transgenic lines grew healthy and the phenologic development stage was achieved at a similar time as the wild type plants, except for line 7(4) that showed growth retardation (Fig.10). The dry weight of transformed plants was higher than for untransformed plants, mainly for lines 7(3), 10(9) and 14(10) that obtained about 15% more dry weight (Fig. 11). These results confirm the better performance of transgenic lines even under normal zinc supply conditions.

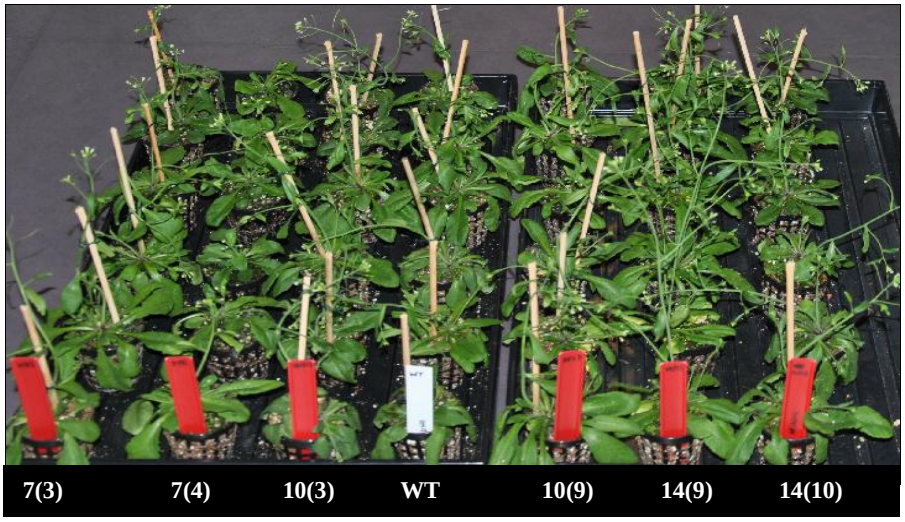

Figure 10: Visible phenotypes of pZIP4::bZIP19 (three independent transformants 7, 10, 14) and untransformed Arabidopsis Col plants (WT), grown in a greenhouse in individual pots containing soil.

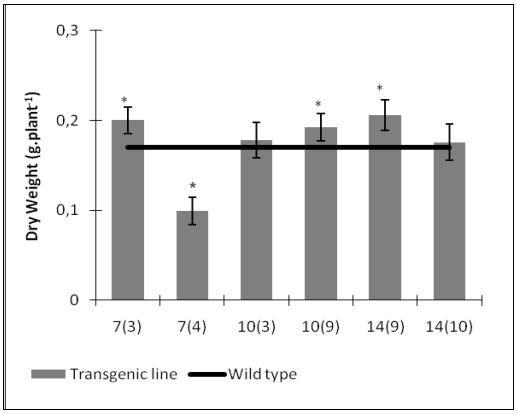

Figure 11: Dry weight of two lines from three independent transformants (7, 10, 14) expressing proZIP4::bZIP19 and untransformed Arabidopsis Col plants (WT) grown in a greenhouse in individual pots containing soil. *Asterisks indicate values that are significantly different (P<0.05) from WT.

Effects of heat stress are alleviated in pZIP4::bZIP19 transformed plants with adequate zinc nutrition

Before the heat stress, Arabidopsis seeds were sown in agar tubes containing 2 μM $ZnSO_4$, to guarantee good seedling establishment, then grown for 5 weeks in hydroponics systems to which no (0 μM $ZnSO_4$) had been added or containing optimal zinc supply (2 μM $ZnSO_4$). After this time, one part of the experiment was harvested and dry weight determined. Although visible phenotypes of the five-week old plants grown on Zn shortage were not showing zinc deficiency symptoms (not shown), these plants obtained smaller rosettes and lower dry weight when compared to plants grown on optimal zinc supply, suggesting they are zinc deficient.

On Zn-limiting conditions, pZIP4::bZIP19 transformed plants showed to have about 30% higher biomass production than untransformed plants (Fig 12A).

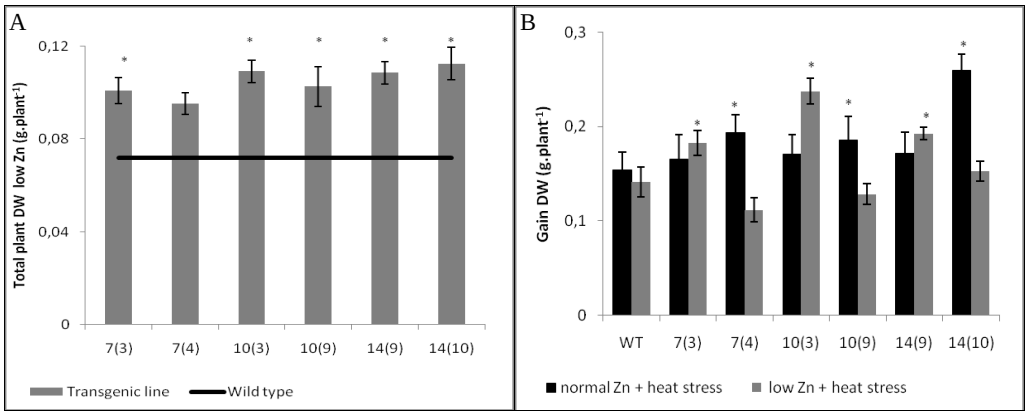

Figure 12: Dry weight, before heat stress, of two lines from three independent transformants (7, 10, 14) expressing proZIP4::bZIP19 and untransformed Arabidopsis Col plants (WT) (A). Dry weight gain of plants grown for 5 weeks in hydroponics medium containing low Zn (0 μM $ZnSO_4$) or sufficient Zn (2 μM $ZnSO_4$) and then submitted to 18 days of heat stress (B). *Asterisks indicate values that are significantly different (P<0.05) from WT.

The ability to continue plant growth when subjected to either heat stress + normal zinc supply or heat stress + low zinc supply was also evaluated. Transgenic lines under single stress (normal zinc + heat stress) exhibited larger rosettes (Fig. 13A) and higher dry weight gain than wild type plants (Fig. 12B), showing to be more thermotolerance. Under a combined stress (low zinc + heat stress) transgenic lines obtained equal or higher dry weight compared to WT, with the exception of line 7(4) that showed less dry weight (Fig. 12B). Besides having a lower dry weight gain, wild type plants and the transgenic line 7 (4), also showed delay in flowering (Fig. 13A).

Unfavorable environmental conditions, such as high temperature, affect the flowering time of plants. To ensure plant survival and reproductive success, when exposed to stress conditions they show early flowering induced by changes in expression pattern of flowering gene, usually associated to epigenetic changes. Epigenetic processes help to stabilize the systems facing adverse conditions therefore many researchers reported that there is a strong correlation between stress tolerance and epigenetic modifications (BOYKO; KOVALCHUK, 2008; CHINNUSAMY; ZHU, 2009; YAISH et al., 2011).

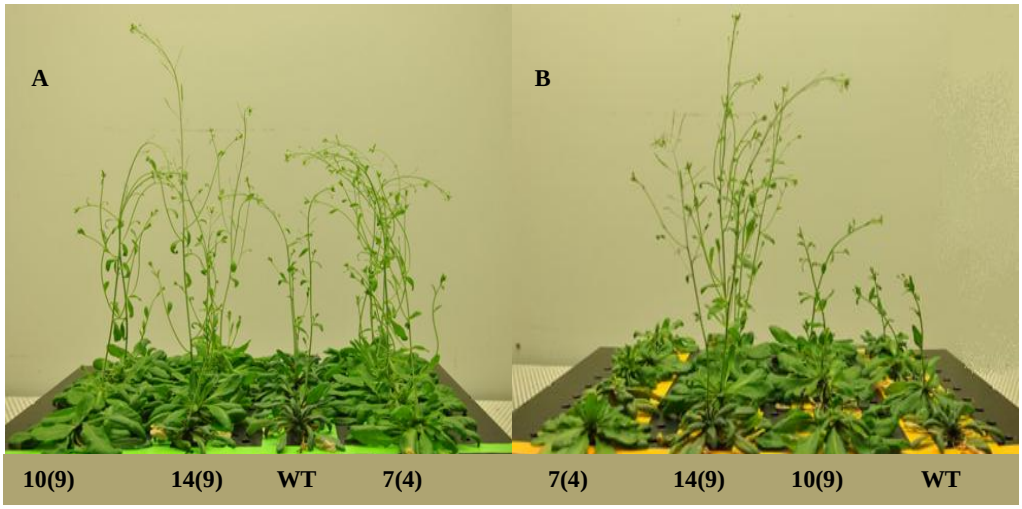

Figure 13: Visible phenotypes of pZIP4::bZIP19 (three independent transformants 7, 10, 14) and untransformed Arabidopsis Col plants (WT), grown for 5 weeks in hydroponics medium and after submitted to 18 days of heat stress. Hydroponics medium with 2 μM $ZnSO_4$ + heat stress (A); 0 μM $ZnSO_4$ + heat stress (B).

DISCUSSION

In this study we investigated whether the additional expression of *bZIP19* mediated by the Arabidopsis zinc deficiency responsive *ZIP4* promoter can improve zinc deficiency tolerance and zinc accumulation in plants. The results have provided evidence that pZIP4::bZIP19 transformed Arabidopsis plants have increased zinc deficiency tolerance and that the overexpression of *bZIP19* confers both superior yield and robustness to Arabidopsis plants even under adequate zinc nutrition.

After 3 weeks under zinc-limiting condition it was already possible to find phenotypic differences among transgenic plants and wild type plants. Although at this stage plants did not yet show visible zinc deficiency symptoms, pZIP4::bZIP19 transformed Arabidopsis plants were exhibiting larger rosettes than wild type plants (Fig. 1) suggesting that the response to zinc deficiency was switched on at a very early stage of zinc deficiency. Moreover, when plants were at 4 weeks the biomass production was determined revealing that all independent transformants expressing pZIP4::bZIP19 grown on low zinc supply showed almost double the dry weight of wild type plants (Fig 2A). The increased Zn uptake capacity as seen in the transgenics indicated that overexpression of *bZIP19* is very effective in enhancing Arabidopsis zinc deficiency tolerance.

Transcription factors of the basic leucine zipper (bZIP) family, such as bZIP19, regulate many processes in all eukaryotes including pathogen defence, development, nitrogen/carbon balance control as well as environmental signaling (ASSUNÇÃO et al., 2010; CICERI et al., 1999; CORRÊA et al., 2008; WELTMEIER et al., 2006), although no specific function is assigned to many bZIP members (JAKOBY et al., 2002). Assunção et al. (2010) reported that bZIP23 and bZIP19 regulate the adaptation to zinc deficiency, but our results showed that also under optimal zinc supply pZIP4::bZIP19 transformed Arabidopsis plants showed higher performance in both biomass production and Zn uptake capacity when compared to wild type plants (Fig.2B, 4B). This suggest that the transcription factor *bZIP19* besides being involved in the regulation of the zinc deficiency response may have one additional function, perhaps related to the development process or that the overexpression of *bZIP19* in cell without specific environmental stimuli, zinc deficiency, may have influenced positively or negatively in expression of broad range of unrelated genes, providing advantages for transgenic plants.

Plant development is a dynamic and complex process frequently subjected to environmental stresses (JAIN et al., 2009). On encountering Zn-limiting conditions, plants undergo an array of adaptive changes in order to try to maintain intracellular zinc at an acceptable level. Transgenic lines in both zinc conditions (low/sufficient zinc supply) showed a higher zinc concentration in leaves although the increases were not statistically significant for all lines. Copper plays an important role in plant metabolism, it participates in photosynthetic electron transport, mitochondrial respiration as well as in oxidative stress response (YRUELA, 2005), as the increase of Cu could have helped in the growth and development of transgenic plants on different zinc nutrition conditions. The zinc and iron uptake are closely linked as zinc has an inhibitory effect on iron absorption (AMBLER; BROWN; GAUCH, 1970; THOMINE et al., 2003). The iron levels decreased in transgenic shoots grown on low zinc supply when compared to wild type plants, perhaps because transgenic plants have activated a specific transporter to improve the zinc uptake, reducing the chances of iron complexing with a system capable of reacting with iron or zinc. In general, the manganese concentration was unchanged, as similarly reported by Mortel et al. (2006) that only found difference in the amount of manganese in Arabidopsis plants grown under an excess zinc supply.

The gene expression analysis revealed that overexpression of *bZIP19* was predominant not only in tissue in which the zinc deficiency is normally active, but also in transgenic lines grown under optimal zinc conditions (Fig. 6). The higher levels of *bZIP19*, mainly in shoots, when compared to wild type plants suggest that overexpression of *bZIP19* in cells confer better performance to Arabidopsis plants. The *ZIP4* gene was strongly expressed in roots of all independent transformants expressing pZIP4::bZIP19 grown with low zinc supply (Fig.6 C, D), the same as reported by Assunção et al. (2010) and Mortel et al. (2006). Under optimal zinc condition, the *ZIP4* expression in roots and shoots was not increased, despite of high levels expression of *bZIP19* in transgenic shoots. This suggests post-transcriptional regulation of *ZIP4* gene.

HMA2 is involved in the transport of zinc into the vascular tissues (HUSSAIN et al., 2004; MILLS et al., 2005). This gene is a Zn^{2+} dependent ATPase and also can be activated by Cu^{2+} and other divalent heavy metals. The mRNA level of *HMA2* increased in roots of the 7(3) and 14(9) transgenic lines in both low and sufficient zinc supply (Fig. 6B, D) unlike that described by Mortel et al. (2006) that found higher expression of *HMA2* gene in Arabidopsis plants grown under zinc deficiency.

The hydroponic systems have long been recognized in the production of more uniform and larger plants than those grown in soil or agar plate (BARKER et al., 2006). However, the culture of seedling on agar plates allows an easy observation of the root systems. The pZIP4::bZIP19 transformed plants on agar plates, in which no zinc had been added, in general exhibited similar performance to plants grown in hydroponic systems with greener rosettes and higher dry weight than wild type plants (Fig. 8A). However, the biomass production was only statistically significant from WT for lines 7(3) and 14(9), perhaps an explanation for this is the difference in nutrient diffusion rate on the plate. According to Barker et al. (2006), there are various agar inhibitory chemical compounds that cause changes in the medium and can alter the nutrient diffusion rate hampering the seedling growth. Lines 10(3) and 10(9) did not develop well on agar plates and showed, for all analyzed characteristics, performance most similar to wild type plants.

The morphological attributes analysis of root systems revealed that independent transformants expressing pZIP4::bZIP19 grown on Zn-limiting conditions showed longer total length and higher surface area, except genotype 10 (table 1). The root length and surface area are closely related, and play an important role in controlling water and nutrient uptake. Then, it is suggested that these two attributes of roots system are the responsible for the better performance of transgenic lines. The same results were found Genc, Huang and Langridge (2007), which report that barley seedlings grown under zinc deficient conditions developed longer roots and greater surface area than barley seedlings grown under adequate zinc condition, moreover the author highlighted that a effective surface area is the most crucial trait of roots in terms of nutrient uptake, mainly for diffusion-limiting nutrients in soils with low availability. Gao et al. (2005) described that root surface area explained 32% of the variations observed in zinc uptake and that zinc uptake is an important determinant of zinc efficiency.

The pZIP4::bZIP19 transformed plants grown in soil under normal zinc supply grew healthy and showed higher dry weight than untransformed plants (Fig. 10, 11), emphasizing the hypotheses that the transcription factor *bZIP19* also has a function related to the development process or influence in the expression of unrelated genes that contributing to increase plant robustness.

After confirming the superiority of the independent transformants expressing pZIP4::bZIP19 on different substrates, the relation between zinc nutrition and thermotolerance was examined by applying a combined stress (heat stress + low zinc supply) or single stress (heat stress + normal zinc supply). Zinc deficiency occurs frequently in arid and semi-arid regions (CAKMAK, 2008), places that regularly experience high temperature stress. Zinc plays an important role in plants helping to protect them from the damages of heat stress. This metal is responsible for maintaining membrane integrity and activity of superoxide dismutase enzyme, this way is expected that plants with more zinc deficiency tolerance show better performance under heat stress. Before the heat stress, transgenic lines showed significantly higher biomass production than wild type plants evidencing zinc deficiency tolerance (Fig. 12A) and confirming previously reported results. After heat stress, transgenic lines in both single and combined stress showed less sensitivity to heat and a higher dry weight gain

revealing them to be more thermotolerant. Peck and McDonald (2010) also confirmed the importance of adequate zinc nutrition to alleviate the effect of heat stress. Their experiments showed that plants poorly supplied with zinc can show greater sensitivity to high temperature and obtain more harmful effects on kernel growth and chloroplast function.

In conclusion, the experiment provided evidence that the increased control of the expression of *bZIP19* by placing this gene under control of zinc deficiency responsive *ZIP4* promoter is very effective in enhancing Arabidopsis zinc deficiency tolerance and that the response to zinc deficiency is switched on at an early stage of zinc deficiency. The results suggest that the transcription factor *bZIP19*, besides regulating the response to zinc deficiency, may play an important role in plant development by improving the productivity even under adequate zinc nutrition. Furthermore, this work suggests that overexpression of *bZIP19* provides more thermotolerance to Arabidopsis plants. Therefore, these results are promising for agriculture because they indicate that the expression of pZIP4::bZIP19 in plants may offer an attractive advantage for crops in areas suffering from low zinc bioavailability as well as contributing to enhance yields and plant robustness.

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Chapter 4

Expression of *bZIP19* under control of the Zn deficiency responsive *ZIP4* promoter in *Coffea arabica*

ABSTRACT

Coffee is one of the most valuable agricultural export commodities. The expansion of coffee crops to less fertile soils has led to an increase of the zinc deficiency problem, which compromises coffee growth and production. In chapter 3 we showed that the increase of expression of *bZIP19* mediated by the promoter of the zinc deficiency responsive *ZIP4* gene is a promising strategy in enhancing Arabidopsis zinc deficiency tolerance. The *bZIP19* transcription factor is conserved in the plant kingdom, so the pZIP4::bZIP19 construction appears promising to test in coffee. Coffee seeds transformed with either pZIP4::bZIP19 or pDsRed-Root (control), by *A. rhizogenes* were grown in normal conditions for five months, after this time the plants were transferred to a solution containing a deficient or sufficient zinc supply. The results of this pilot project reinforce our previous findings. The pZIP4::bZIP19 transformed Coffee plants showed better adaptation to zinc deficiency, suggesting that this strategy may promote the development of zinc deficiency tolerant crops.

Keywords: zinc deficiency. bZIP genes. coffee.

INTRODUCTION

Coffee is one of the most important primary products in world trade (LASHERMES; ANDRADE; ETIENNE, 2008). This commodity is grown in around 80 countries, with Brazil being the largest coffee producer followed by Colombia and Vietnam. For tropical and subtropical countries it represents the key export product and cash crop, which in general causes a positive impact on the social and physical environment (INTERNATIONAL COFFEE ORGANIZATION - ICO, 2011) as this activity, from production to final consumption, provides employment for hundreds of millions of people (DAMATTA; RAMALHO, 2006). Among the species, *Coffea arabica* is responsible for 70% of the world coffee production due to the quality and superiority of the beverage derived from its beans (COSTE, 1992), however *C. Arabica* plants generally seem less vigorous and more susceptive to environmental stress (ICO, 2011).

The expansion of coffee crops to less fertile soils and the need to maximize production is leading to the use of larger amounts of corrective fertilizers that raise the pH of the soils producing mineral imbalance such as zinc deficiency, a micronutrient which is highly important in the coffee culture. These facts cause a widespread occurrence of zinc deficiency in most coffee crops (MARTINEZ et al., 2003; ROSOLEM; SACRAMENTO, 2001), which affects plant performance in terms of growth, development, and yield. Zinc is a vital micronutrient for the functionality of more than 300 enzymes, protein synthesis, transcriptional and post-transcriptional processes, structural and functional integrity of cell membranes, as well as detoxification of superoxide (CLEMENS, 2006; MORTEL et al., 2006). So, in order to prevent zinc deficiency, Zn supplementation by soil application or foliage spraying has been recommended. Nevertheless, application of fertilizers to soil is compromised due to some physical and chemical characteristics of soil that reduces the availability of Zn to plants. The correction of Zn deficiency by foliage spraying requires a several applications, because Zn displays very low mobility or even immobility when applied to mature leaves (POLTRONIERI; MARTINEZ; CECON, 2011). This leads to a rise in production costs.

The advance of coffee biotechnological techniques and the significant progress in understanding the regulators of zinc homeostasis in plants have offered new possibilities for the development of improved cultivars in terms of zinc deficiency tolerance. Recently, an important step forward towards unraveling the regulation of zinc homeostasis networks was made with the identification of two bZIP transcription factors, *bZIP19* and *bZIP23* which are essential for switching on the zinc deficiency response of *Arabidopsis thaliana*. Both genes are widely conserved in the plant kingdom, suggesting conservation of their function (ASSUNÇÃO et al., 2010). Previous experiments focused on the expression of the *bZIP19* gene under control of the Arabidopsis zinc deficiency responsive *ZIP4* promoter. This provided evidence that the increased control of expression of *bZIP19* is very effective in enhancing Arabidopsis zinc deficiency tolerance and may offer attractive advantages for crops in areas suffering from low zinc bioavailability as well as contributing to enhanced yields and plant robustness. Therefore, this work was carried out with the objective to investigate if the overexpression of *bZIP19* mediated by the *ZIP4* promoter contributes to enhanced biomass yield and robustness of coffee crops under zinc shortage.

MATERIALS AND METHODS

Seed germination

Coffea arabica L. (Catuaí Vermelho IAC 144**)** seeds without parchment were surface-sterilized by immersing the seeds in 5% HClO (w/v) bleach solution for 15 minutes then rinsed three times in sterile water and placed individually in Petri-dishes containing half-strength MS (Murashige and Skoog) medium and phytagel. Coffee seeds were maintained for about 4 weeks in the dark at 28°C for full hypocotyls expansion.

Coffee transformation

As soon as the hypocotyls became fully expanded the root transformation procedure was carried out. Coffee seeds were cut above the hypocotyls-root boundary with a scalpel that was previously dipped in an *Agrobacterium rhizogenes* culture. This was collected from LB-agar plates with freshly grown *A. rhizogenes* cultures containing either the pZIP4::bZIP19 construct or the pDsRed-Root empty vector.

Transformed seeds were grown in a climate chamber (16h/d at 22/20°C day/night temperature; 120 µmol photons $m^{-2}s^{-1}$; 50% relative humidity), for four weeks. After this time, the seedlings were transferred to pots containing half-strength MS (Murashige and Skoog) medium supplemented with tricarcilin (200 $mg.L^{-1}$) and continued growing for 10 days in the climate chamber. For the selection of transformed roots, seedlings were moved to new pots containing ½ MS medium added with tricarcilin (200 $mg.L^{-1}$) and hygromycin (25 $mg.L^{-1}$) for pZIP4::bZIP19 transformed roots or kanamycin pDsRed transformed roots and kept for 10 more days. Transformed roots were checked with markers by Stereo-macroscopy (pDsRed) or by PCR analysis (pZIP4::bZIP19).

PCR analysis

To confirm if the new formed roots were co-transformed with pZIP4::bZIP19 construct, DNA was extracted following a CTAB method as described by Doyle & Doyle (1991) and the pellet was re-suspended in 20 µL sterile water. For PCR reaction we used 4 µL of DNA, 0.5 µL of 5 mM dNTPs, 2.5 µL of 10x Taq buffer, 0.25 µL Taq DNA polymerase, 1.25 µL of 10 pmol forward and reverse primers (Invitrogen) in a total volume of 25 µL. The experiment was carried out in an iCycler Thermal machine (Bio-Rad). The mixture was then subjected to 94.0°C for 5 min, followed by 29 cycles of 30 sec at 94.0 °C, 30 sec at 54.0 °C and 20 sec at 72.0 °C. After the last cycle was completed, an additional 7 min at 72 °C elongation step was performed. The following primers were used, 5'TTCTCCCGGATGAGAGCGATGA3' and 3'GCTGATTCAC CGCCCTAAGCCT5'. The size of the amplification was 150 pb. In order to confirm that the amplification results were not due to contamination of bacterial DNA we performed a second PCR, with the same thermal profile described above, which used primers for *Coffea* alpha tubulin (292 pb), 5'ATTGGTCAGGCCGGTATCCAGG3' and 3'AGATCGACGAACACGGCACG5'.

Zinc deficiency stress

The plants with roots co-transformed either with pZIP4::bZIP19 or pDsRed-Root (empty vector) constructs were transferred to pots containing a modified hydroponic solution (SCHAT; VOOIJS; KUIPER, 1996) and placed in a climate chamber for 12h/d at 20/15 day/night temperature, 250 µmol photons $m^{-2}s^{-1}$ and 75% relative humidity. The hydroponic medium initially contained quarter-strength Hoagland's nutrient solution, which was gradually increased until 10 days when it reached half-strength Hoagland's nutrient solution (LAVIOLA; MARTINEZ; MAURI, 2007). The hydroponic solution was maintained this way until coffee plants completed five months. After this time, a new hydroponic solution was prepared either with low zinc supply (0.05 µM $ZnSO_4$) or optimal zinc supply (2 µM $ZnSO_4$) to test the zinc deficiency tolerance of the pZIP4::bZIP19 transformed coffee plants. The pH buffer MES was included in preparation of the all nutrient solutions and the pH was adjusted to 5.5. The plants were kept under this condition for about 30 days, of which during the first two weeks the nutrient solution was refreshed once a week and thereafter twice a week.

RESULTS AND DISCUSSION

After 30 days of being grown under zinc shortage both overexpressing bZIP19 plants and DSRed transformed plants (control) were not exhibiting any visible phenotype zinc deficiency symptoms (Fig. 1). However, the control plants were generally smaller than over expressing bZIP19 plants (Fig. 1). Zabini et al. (2007) found zinc deficiency symptoms in coffee seedlings only after 10 months in hydroponic medium without zinc supply. Pedrosa (2008) also reports the appearance of deficiency symptoms in coffee seedlings under zinc shortage in a similar time, 9 months. Thus most likely the absence of visual zinc deficiency symptoms was due to the short duration of the experiment.

Figure 1: Visible phenotypes of DsRed (control)(A) and pZIP4::bZIP19(B) plants, grown for 30 days on hydroponics medium to which 0.05 µM $ZnSO_4$ has been added.

When examined for biomass production, coffee plants grown on optimal zinc supply showed no significant difference in fresh weight (Fig.2), but difference was found for plants growing on zinc shortage. Under these conditions, pZIP4::bZIP19 transformed coffee plants showed significantly 27% more fresh weight than control plants and this accumulation was higher even than under optimal Zn, although no significant.

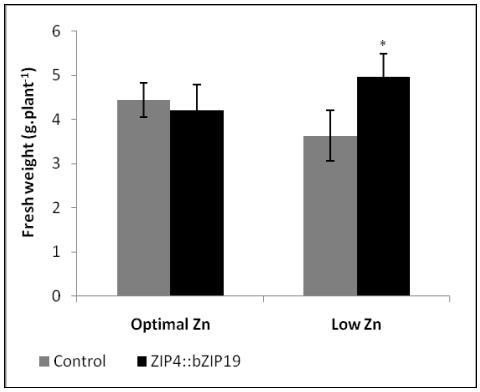

Figure 2: Fresh weight of pZIP4::bZIP19 and DsRed (control) plants, grown for 30 days on hydroponics medium either in low zinc supply (0.05 µM $ZnSO_4$) or optimal zinc supply (2 µM $ZnSO_4$)**.** *Asterisks indicate values that are significantly different (P<0.05) from control.

Zinc is a vital micronutrient required in physiological and metabolic processes of coffee plants, although zinc deficiency is a problem frequently found in coffee regions (MELO et al., 1999; POLTRONIERI; MARTINEZ; CECON, 2011; REIS; MARTINEZ, 2002; SANTOS et al., 2005). Plants facing zinc deficiency stress divert the destined growth energy to cope with the stress, causing reduction in biomass production (LARCHER, 2003). However, over expression of bZIP19 coffee plants did not show decrease in fresh weight indicating that transcription factor bZIP19 is involved in regulating zinc deficiency response and can confer more zinc deficiency tolerance to coffee plants.

So, even though this experiment was a pilot and small-scale project and consequently small-scale, the results reinforce our previous experiments performed with Arabidopsis which also showed that pZIP4::bZIP19 transformed plants to have a higher zinc deficiency tolerance. Therefore, based on these results and the fact that the transcription factor *bZIP19* is conserved in the plant kingdom (ASSUNÇÃO et al., 2010) strongly suggested that strategies of expression *bZIP19* gene under control of zinc deficiency responsive *ZIP4* promoter may promote the development of a zinc deficiency tolerant crop enabling plants growing without significant yield penalties in areas suffering from low zinc bioavailability, but better knowledge on physiological and molecular behavior of pZIP4::bZIP19 transformed plants in a wide range of crops is crucial to elucidate how these genes regulate the zinc deficiency response.

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Chapter 5

Role of transcription factor *bZIP24* in salt adaptation

ABSTRACT

Salt stress negatively impacts agricultural yield throughout the world. Expression of the *bZIP24* transcription factor is strongly down-regulated in halotolerant species unlike that of *A. thaliana* in response to salt stress. So, modification of the transcriptional control by regulation of bZIP24 expression is a promising approach to improve salt tolerance. The screening of transgenic *A. thaliana* lines with activation or inhibition of bZIP24 function was performed in agar plates with increasing addition of NaCl. KObZIP24 plants were less sensitive to salt stress, showing higher biomass production and an improvement of morphological attributes of roots, adaptive strategies that corroborate with the hypothesis that repression of bZIP24 regulates may improve salt tolerance. However, overexpression bZIP24 plants also showed superior performance when compared with wild type plants, indicating that the T-DNA insertion may not have led to a complete knockout and that it may be necessary to knockout more than one transcription factor to obtain the desired phenotype. there is need for further study to understand how the modification of bZIP24 expression may contribute to the regulation of salt stress acclimation responses.

Keywords: Salt stress. bZIP genes. Arabidopsis.

INTRODUTION

Growth and development of all organisms dependents on the adequate regulation of gene expression. Transcription factors play one of the most important functions of modulating gene expression by controlling of transcription initiation rates (MESHI; IWABUCHI, 1995; WARREN, 2002). Transcription factors of the basic leucine zipper (bZIP) family, characterized by containing a basic region mediating sequence-specific DNA-binding and a leucine zipper required for dimerization, are found in many DNA binding eukaryotic proteins and regulate many processes in the plant such as pathogen defence, development, nitrogen/carbon balance control, flower development , as well as environmental signaling (ASSUNÇÃO et al., 2010; CICERI et al., 1999; CORRÊA et al., 2008; WELTMEIER et al., 2006).

75 *bZIP*-type genes have been identified in the *A. thaliana* genome that were classified into groups based on structural similarities in their DNA binding domains (CORREA et al., 2008; JAKOBY et al., 2002). Little is known about the F-group of bZIPs, to which belong *bZIP19*, *bZIP23* and *bZIP24*. Experimental evidence suggests that both *bZIP19* and *bZIP23* are involved in the regulation of the response to zinc deficiency (Assunção et al, 2010) while the *bZIP24* gene is involved in salt response (FUJII et al., 2000).

Salt stress negatively impacts agricultural yield throughout the world (YOKOI; BRESSAN; HASEGAWA, 2002) since salinity affects the growth and development of plants. The accumulation of salt ions in plants causes osmotic stress, ion cytotoxicity and nutritional imbalances that together lead to osmotic stress (ZHU, 2001). In response to salinity, plants adjust their osmotic pressure through the stimulated biosynthesis of osmoprotectants, such as proline, glycinebetaine and soluble sugars, that help to control cell water status as well as the accumulation of antioxidant metabolites (HUSSAIN et al., 2008). Plant adaptation to salt is a multi-genic trait involving the regulation of specific molecular and physiologic process that seek to maintain intracellular ion homeostasis by capturing and accumulating salt in the cellular vacuoles and by regulating influx/ efflux of Na^+ and Cl^- ions at the plasma membrane (HASEGAWA et al., 2000; SERRANO; RODRIGUEZ-NAVARRO, 2001). Based on this, most approaches aim to introduce salt tolerance in salt-sensitive

plants by modifying biochemical pathways to stimulate the production of osmolytes, to restrict cellular Na^+ uptake, and/ or increase vacuolar Na^+ sequestration. However, current strategies that seek to modify the expression of transcription factors in order to regulate salt tolerance responses have shown to be a more promising approach than the modification of a single metabolic feature (YANG et al., 2009).

Recent findings indicate that the bZIP24 transcription factor is a key regulatory element controlling salt tolerance in plants (CORRÊA et al., 2008; POPOVA et al., 2008). It is hypothesized that *bZIP24* functions as a negative transcriptional regulator of salt stress acclimation response, since in halotolerant species, such *Lobularia maritima*, this gene is down-regulated upon salt exposure, inversely to what occurs with salt-sensitive plants (POPOVA; GOLLDACK, 2007). Yang et al (2009) reported that RNAi-mediated repression of AtbZIP24 improve salt tolerance in *Arabidopsis thaliana* by reducing Na^+ accumulation and improving development of plants. Therefore, this work was carried out with the objective to verify if modification of the *bZIP24* expression in Arabidopsis, a salt-sensitive plant, may contribute to the regulation of salt stress acclimation responses.

MATERIALS AND METHODS

Plant material and plant growth condition

Arabidopsis thaliana ecotype Columbia (Col-0) plants homozygous for overexpression p35S::bZIP24 constructs and T-DNA knockout plants were generated and three independent transgenic lines each containing either OX bZIP24 (1.8; 7.3; 8.7), or KO bZIP24 (1; 2; 3) were selected. Seeds were sterilized through vapour-phase seed sterilization and sown on Petri dishes containing half-strength MS medium (Murashige and Skoog) supplemented with different concentrations of NaCl (0; 50 mM; 75 mM and 100 mM). After 3 days of stratification treatment at 4°C in the dark, seeds were grown in a climate chamber (16 h/d at 22/20 °C day/night temperature; 120 µmol photons m^{-2} s^{1}; 50% relative humidity), for four weeks.

WinRhizo analysis

After four weeks, both fresh and dry weight were determined and the morphological attributes of root systems were evaluated by the WinRHIZO *Pro* 2007a system. The plant material was placed in an acrylic container (20 X 30 cm) then scanned using a 400 (dpi) resolution. For each salt treatment five seedlings per genotype were analyzed for total root length (cm), root surface area (cm^2), mean root diameter (mm) and root volume (cm^3).

Statistical analysis

The statistical analyses of data were performed by one-way ANOVA and significant differences between means were determined using a Duncan test at a 5% level of significance ($P \leq 0.05$).

RESULTS AND DISCUSSION

To identify salt tolerance in overexpression p35S::bZIP24 and knockout Arabidopsis plants was performed screening using high levels of NaCl. The visible phenotypes showed that both root and shoot of transgenic lines grew slightly better than wild type plants in all NaCl treatments (Fig. 1). Moreover, overexpression p35S::bZIP24 and knockout plants showed a significantly higher fresh weight suggesting that wild type plants presented more difficulty in water absorption and in maintenance of water potential under salt stress conditions (Fig. 2).

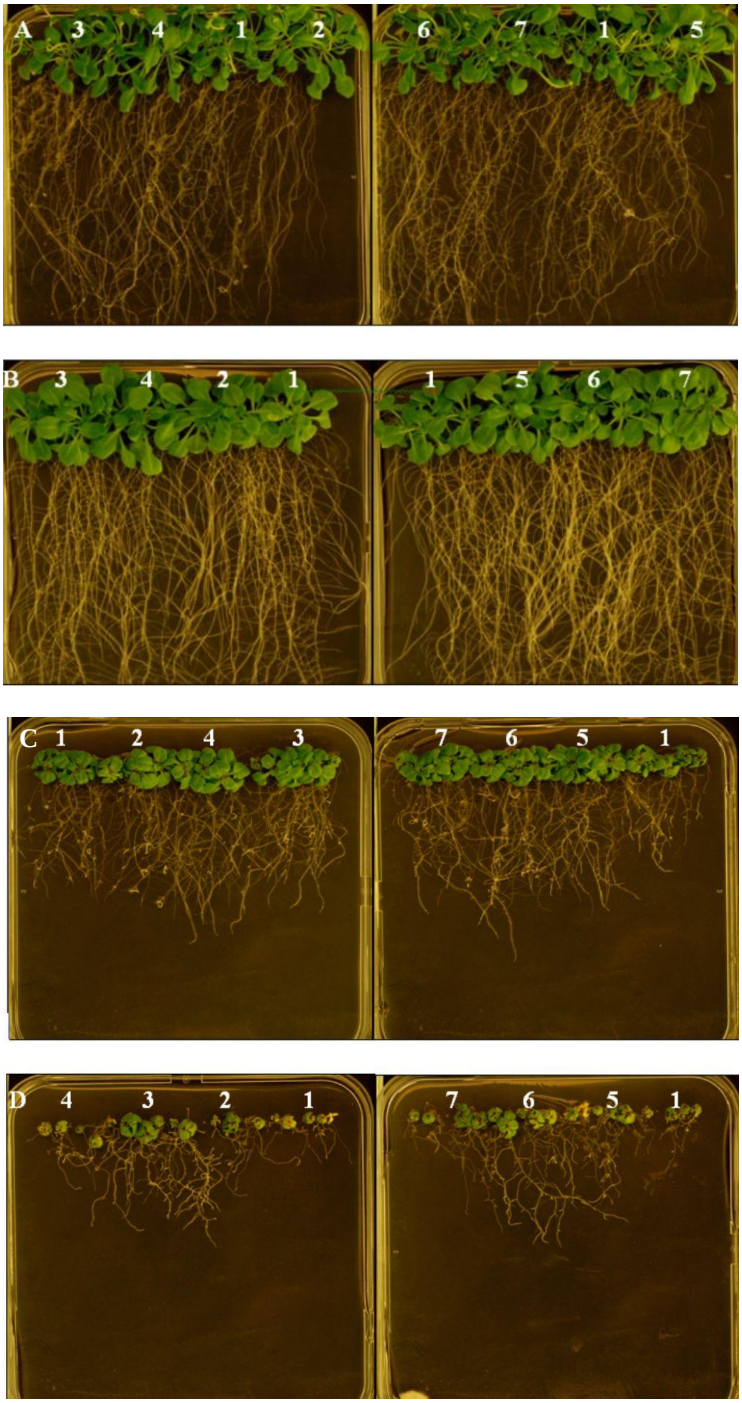

Figure 1: : Visible phenotypes of wild type (1), KO bZIP24 (2; 3; 4) and OX bZIP24 (5; 6; 7) plants, grown for 4 weeks on agar plates containing half-strength MS medium supplemented with 0 mM NaCl (A); 50 mM NaCl (B); 75 mM NaCl (C) and 100 mM NaCl (D).

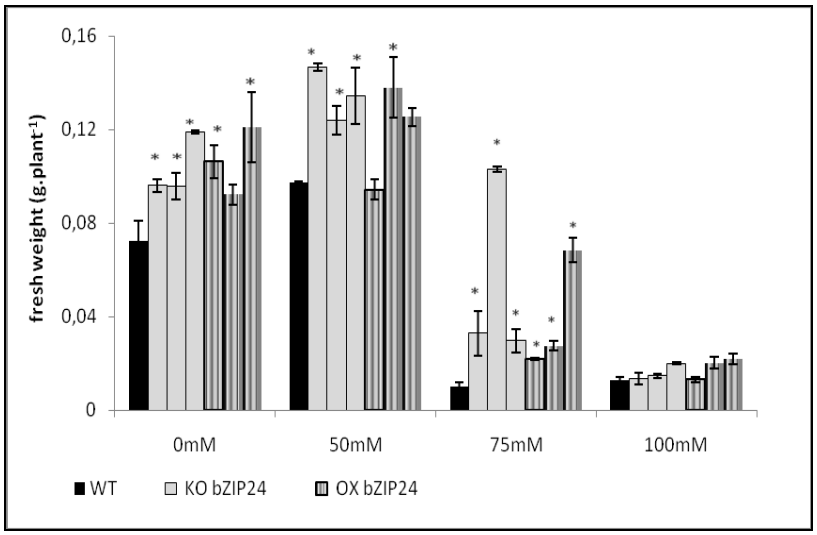

Figure 2: Fresh weight of wild type, KO bZIP24 (2; 3; 4) and OX bZIP24 (5; 6; 7) plants, grown for 4 weeks on agar plates containing half-strength MS medium supplemented with different concentration of NaCl (0 mM, 50 mM; 75 mM, and 100 mM).*Asterisks indicate values that are significantly different (P<0.05) from control.

The dry weight of knockout plants grown on agar plates supplemented with 50 mM and 75 mM NaCl was significantly higher when compared to wild type and OXbZIP24 plants (Fig. 3). Under 50mM NaCl the plants with suppression of bZIP24 showed, respectively, 35% and 25% more dry weight than untransformed and OXbZIP24 plants and this difference increased when the salt concentration in the medium was raised to 75 mM NaCl. Under this condition, in general, dry weight was reduced by about 30% in OXbZIP24 plants and 60% in wild type plants when compared with knockout lines. Thus, the higher biomass production indicates that KObZIP24 plants are less sensitive to salt stress and are able to continue growing under moderate salt concentration environments. The results obtained corroborate with findings of Popova and Golldack (2007) that report that *bZIP24* functions as a negative transcriptional regulator of salt stress acclimation response. The down-regulation of this gene may provide a faster adjustment cell, reducing cell injuries. So, once the cellular homeostasis is reestablished, stress is decreased, and the energy diverted to cope with the stress is again used for plant growth (MOUD; MAGHSOUDI, 2008).

Popova et al. (2008) also showed that both growth and development was improved in transgenic *AtbZIP24*-RNAi lines, but emphasized that improving salt stress tolerance in this seedling is dependent on the NaCl concentration applied. Many factors determine how the plants respond to salt stress. The severity (NaCl concentration) and development stage of plants are characteristics of crucial importance (KHAJEH-HOSSEINI; POWELL; BINGHAM, 2003). Janmohammadi, Dezfuli and Sharifzadeh (2008) related that salt tolerance usually increases with plant ontogeny and the germination and early seedling growth stages are very vulnerable to salt stress. This possibly explains the fact that suppression of bZIP24, apparently, did not improve salt acclimation in plants grown under 100 mM NaCl. At a concentration of 100mM NaCl, the growth of wild type plants, OXbZIP24 and KObZIP24 was drastically affected, despite transgenic plants showing a slightly better performance (Fig. 3). Therefore, as the Arabidopsis seeds were sown directly on agar plates containing a high concentration of salt, the salt effects were probably potentiated, causing a collapse of cellular integrity before the activation of defenses mechanisms.

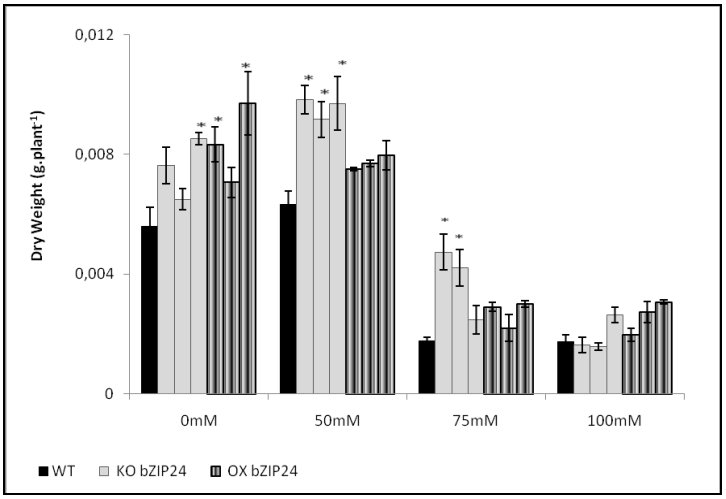

Figure 3: Dry weight of wild type, KO bZIP24 (2; 3; 4) and OX bZIP24 (5; 6; 7) plants, grown for 4 weeks on agar plates containing half-strength MS medium supplemented with different concentrations of NaCl (0 mM, 50 mM; 75 mM, and 100 mM).*Asterisks indicate values that are significantly different (P<0.05) from control.

Roots are the primary place of salinity perception, therefore, the evaluation of morphological attributes of root systems are an accurate indicator of seedling growth and saline environment tolerance. The total root length was severely reduced under high NaCl concentrations, because salt present in growth medium may hamper the absorption of calcium, causing reduction in emergence and growth of roots (CRAMER, 2004; ZAHRAN; SPRENT, 1986). However, OXbZIP24 and KObZIP24 plants showed significantly less reduction in total root length when compared with wild type plant grown on 75 and 100 mM NaCl concentrations. When analyzed surface area and root volume in general OXbZIP24 and KObZIP24 also presented superior performance. Nevertheless, as can be observed in table 1, no drastic changes in diameter were seen when compared to plants grown on agar plates with and without salt added. Many studies have shown that there is a correlation between root growth rates and salt tolerance (BARTELS; SUNKAR, 2005; LORENZO et al., 2007; MUNNS et al., 2000). This way, it is assumed that the improvement of morphological attributes of roots was an adaptation strategy developed by KObZIP24 plants when continued root growth under a saline environmental may offer additional surfaces for sequestration of toxic ions, leading to a decrease in salt concentration, and enabling exploration of new soil areas with lower salt concentrations (LORENZO et al., 2007).

Table 1. Total length, surface area, diameter and mean volume root of wild type, KO bZIP24 (2; 3; 4) and OX bZIP24 (5; 6; 7) plants, grown for 4 weeks on agar plates containing half-strength MS medium supplemented with different concentration of NaCl (0 mM, 50 mM; 75 mM, and 100 mM).*Asterisks indicate values that are significantly different (P<0.05) from control.

Genotypes	NaCl Concentration			
	0mM	**50mM**	**75mM**	**100mM**
		Total length (cm.plant^{-1})		
Wild type (1)	41,5	51,4	2,08	3,35
KO bZIP24 (2)	48,5	55,8	6,76*	4,48*
KO bZIP24 (3)	52,5*	47,3	14,3*	4,30*
KO bZIP24 (4)	61.3*	49,9	6,66*	4,57*
OX bZIP24 (5)	50,2*	40,3	5,22*	4,74*
OX bZIP24 (6)	41,0	49,0	6,17*	5,14*
OX bZIP24 (7)	40,5	45,4	11,0*	5,21*
		Surface area (cm^2.plant^{-1})		
Wild type (1)	9,18	9, 2	1,56	1,96
KO bZIP24 (2)	10,26	9,2	3,18*	2,14
KO bZIP24 (3)	9,21	9,9	4,11*	2,36
KO bZIP24 (4)	10,34	9,2	4.08*	2,12
OX bZIP24 (5)	9,30	8,1	2,67	2,01
OX bZIP24 (6)	7,01	9,1	3,04	2,25
OX bZIP24 (7)	6,71	9,1	2.59	2,38
		Diameter (mm.plant^{-1})		
Wild type (1)	0,27	0,33	0,31	0,29
KO bZIP24 (2)	0,29	0,32	0,26	0,30
KO bZIP24 (3)	0,37	0,33	0,26	0,28
KO bZIP24 (4)	0,31	0,29	0,25	0,27
OX bZIP24 (5)	0,30	0,32	0,30	0,26
OX bZIP24 (6)	0,38	0,39	0,30	0,26
OX bZIP24 (7)	0,34	0,40	0,28	0,32
		Root Volume (cm^3.plant^{-1})		
Wild type (1)	0,05	0,115	0,004	0,005
KObZIP24 (2)	0,066	0,128	0,008*	0,006*
KObZIP24 (3)	0,072*	0,14	0,015*	0,004
KO bZIP24 (4)	0,116*	0,102	0,005	0,005
OX bZIP24 (5)	0,125*	0,176	0,007	0,004
OX bZIP24 (6)	0,804	0,203*	0,007	0,006*
OX bZIP24 (7)	0,07	0,196	0,018*	0,008*

*Asterisks indicate values that are significantly different (P<0.05) from Wild type

Plant salt tolerance is a complex trait involving many genes. Currently, the advances in plant genome mapping and the new molecular techniques have offered new strategies for understanding salt tolerance in plants. In this pilot study, we showed that the transcription factor bZIP24 is an important regulator of salt stress response in plants and the modification of transcriptional control by suppression of bZIP24 can be a useful strategy for improving salt tolerance. The results reported in this study corroborate with the hypothesis that repression of bZIP24 regulates the maintenance of osmotic and ionic balance and allows continued growth and development in response to increased salinity. However, it was expected that only plants with knockout bZIP24 functions presented better growth and development under high salt concentration, but overexpression bZIP24 plants also showed superior performance when compared with wild type plants, indicating that the T-DNA insertion may not have led to a complete knockout and that it may be necessary to knockout more than one transcription factor to obtain the desired phenotype.

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GENERAL CONCLUSION

Understanding plant response to environmental stressors is an important topic for agriculture which has the challenge of conciliating the ever increasing demand for food and dealing with soil fertility loss, accelerated by unfavorable environmental conditions. The elucidation of the complex network of homeostatic mechanisms involved in stress adaptation will facilitate the development of crops in less favorable environments without significant yield penalties. In this work, we showed that the modulating gene expression through control of F-class bZIP transcription factor genes is a promising approach that may contribute to increased yields and plant robustness, mainly in areas suffering from low zinc bioavailability.