Response of *bZIP10*-Overexpressing *Brachypodium distachyon* to Zinc

by
Tinh An Nguyen

A PROJECT

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Biology
(Honors Scholar)

Presented May 20, 2015
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Tinh An Nguyen for the degree of Honors Baccalaureate of Science in Biology presented on May 20, 2015. Title: Response of *bZIP10*-Overexpressing B*rachypodium distachyon* to Zinc.

Abstract approved:

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Kari van Zee

The micronutrient zinc (Zn) plays a key role in the animal and plant kingdoms. Zn-deficient soils impact plant production and can cause yield reductions of 40% or more. Zn is also important for both human and plant development as a cofactor for over 200 enzymes. Zn-deficiency symptoms in plants include interveinal chlorosis, abnormally shaped leaves, stunting and rosetting. *Brachypodium distachyon* has emerged as an experimental model for temperate cereal crops and forage grasses. BZIP10 is a protein with basic domain binding DNA and leucine zipper dimerization motif found to enhance oxidative stress resistance and may be involved in Zn homeostasis. My objective was to assess the effect of *bZIP10*-overexpressing *Brachypodium distachyon* to varying Zn concentrations. Wildtype (WT) and 3 transgenic type (TR) seedlings were grown in optimized hydroponics system for 3 weeks prior to initiation of experimental Zn conditions (no Zn, normal Zn, high 15x Zn). Samples were collected for gene expression analysis at 12hours, 24hours and 7 days after treatment. Preliminary results suggest no observable phenotypic differences between the different growing conditions and plant types. Preliminary gene expression analysis of WT and two TR plants show higher expression levels of *BdbZIP10, BdIRT-1* and *BdZIP4* in TR than WT.

Key Words: Brachypodium distachyon, zinc, hydroponics, transgenics, bZIP10

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

_____

Tinh An Nguyen, Author

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Introduction

The micronutrient zinc (Zn) plays a key role as a structural constituent or regulatory co-factor for enzymes in plants and animal pathways concerned with carbohydrate metabolism, protein metabolism, auxin metabolism, pollen formation, maintenance of integrity of membranes and infection resistance. Inside cells, Zn is not oxidized nor reduced so its behavior is mainly that of a divalent cation with a strong tendency to form stable tetrahedral complexes, since its monovalent form is highly unstable (Reichman, 2002; Grotz et al., 1998). Also, Zn does not tend to be significantly reduced under low redox conditions within organic matter (Reichmann, 2002).

Zinc Deficiency in Soil

Millions of acres of cropland are Zn deficient and this is one of the most widespread limiting factors in crop production (Alloway, 2009; Hacisalihoglu and Kochian, 2003). Factors affecting Zn availability include low total Zn content, high pH, high calcite and organic matter, and high concentrations of Na, Ca, Mg, bicarbonate and phosphate in soil (Alloway, 2009). In a survey conducted by Sillanpaa in 1982, researchers found at least 10% of soil samples were potentially Zn-deficient in 10 of the 29 countries surveyed (Alloway, 2009). The forms of Zn available to plants in soil are free ions (Zn^{2+} and $ZnOH^{+}$), soluble organic complexes and labile Zn (Alloway, 2009). Zn solubility in soil has also been found to be inversely linked to soil pH (Alloway, 2009). Soils with low Zn content are likely to give rise to two types of Zn deficiencies: ‘primary deficiency’ where the deficiency arises from soil with low Zn content as found primarily in sandy soils, while ‘secondary deficiencies’ are due to unavailability of Zn in soils with higher total

contents (Alloway, 2009). Other soil types that commonly exhibit Zn deficiency include high pH calcerous soils and high phosphorous-containing fertilized soils (Hacisalihoglu and Kochian, 2003). Methods for overcoming Zn deficiency include Zn fertilization and biofortification (Alloway, 2009). Applications of zinc fertilizers can be either banded during pre-planting or while planting. During the season, after Zn deficiency has been determined, plants may be sprayed with zinc sulfate solution, but this is mainly a salvage method. Inorganic sources of Zn include zinc sulfate and zinc oxide, while the more effective organic sources include zinc chelates (Westfall and Bauder, 1992). But these methods may not always be feasible due to agronomic and economic factors (Hacisalihoglu and Kochian, 2003).

Since loss of yield of 40% or more has been found in Zn deficient soils, two options that may help reduce the loss of yield would be to either decrease the amount of land that is Zn deficient, or to create crop varieties that are more stress resistant and higher yielding (Glover-Cutter et al., 2014). With the increasing world population, solving problems related to crop production to feed this growing population will be necessary. Furthermore, this will increase the economic impact of Zn deficient soils in developing countries by necessitating increased imports of grains to make up for the yield loss from Zn deficient soils.

Zinc Deficiency in Humans

In humans, Zn acts as a cofactor for over 200 enzymes and is required for many processes like normal development and function of the immune system, neuro-sensory functions, reproductive health and brain function and intestinal iron (Fe) absorption (reviewed in Impa and Johnson-Beebout, 2012). Consequences of Zn deficiency include stunted growth, poor immune function and increased rate of premature birth in Zn deficient mothers (reviewed in Impa and Johnson-Beebout, 2012). Zinc is considered a ‘Type 2’ nutrient for humans and higher animals meaning

its concentration in blood does not decrease in proportion to the degree of deficiency thus causing slow physical growth and reduced excretion to conserve Zn (reviewed in Alloway, 2009). Hotz and Brown 2004 (reviewed in Alloway, 2009) estimates 33% of the world population have Zn deficient diets but this may range between 4-73% in different countries.

Zinc Deficiency in Plants

Extreme Zn deficiency in developing plants may show visible symptoms of stress that can include interveinal chlorosis, bronzing of chlorotic leaves, abnormally shaped leaves, stunting and rosetting (Reichmann, 2002). Plants may also have greatly reduced yield without visible symptoms in cases of moderate deficiency. Possible explanations for variations in Zn uptake between plants include differences in volume and length of roots, presence of proteoid roots, root-induced changes in rhizosphere pH, and efficiency of utilization of Zn once it is absorbed into the plant, recycling of elements within tissues of growing plants and tolerance of inhibiting factors (Alloway, 2009). Even though there are several key sites where Zn uptake might be involved (root processes increasing the bioavailability of soil Zn uptake, enhanced root uptake and translocation of Zn from root to shoot, enhanced biochemical utilization of Zn in cells), there seem to be no correlations between Zn efficiency and root uptake of Zn, Zn translocation to the shoot, or shoot Zn accumulation (Hacisalihoglu and Kochia, 2003). Zn has not been found to compete for sites that form complexes with organic matter (Reichmann, 2002). In Colorado, Zn deficiencies were associated with soil low in organic matter; soils with alkaline pH (pH greater than 7.0) and areas where topsoil has been removed by irrigation, erosion or channel construction. Under high yielding cropping conditions, responses to applied Zn were greater, and Zn deficiency was more severe during years with cold and wet springs than years with warm and dry springs (Westfall and Bauder, 1992). Zinc deficiency has been shown to depress leaf photosynthetic capacity due to reduction in chlorophyll levels and the destruction of chloroplast ultrastructure

(Tavallali et al., 2009). Zinc may interfere with production of reactive oxygen species (ROS) by membrane-bound NADPH oxidase (Tavallali et al., 2009) which are toxic to plants at high levels (reviewed in Tavallali et al., 2009).

‘Stuart’ Pecan (*Carya illinoensis*)

Despite much research in methods of prevention and correction of Zn deficiency for proper growth of pecan trees, Zn deficiency remains a recurring problem. In 1991, Hu and Sparks determined the physiological impacts of Zn deficiency in leaves by looking at leaf chlorophyll content, stomatal conductance and photosynthesis. The severity of Zn deficiency was inversely correlated to leaf Zn concentration. Leaf chlorosis decreased and chlorophyll concentrations increased with increasing leaf Zn concentrations. Photosynthesis was regulated by Zn while Zn was within a deficiency range. As leaf Zn increased, responses to intercellular CO_2 were the reverse of net photosynthesis. Their results showed stomatal response capacity to carbon fixation may be lost partially under Zn deficiency but was not the mechanism that restricts photosynthetic capacities (Hu and Sparks, 1991).

Pistachio (*Pistacia vera* L.)

Carbonic anhydrase activity was enhanced in pistachio when grown in soil with added zinc. Salt (NaCl) stress leads to decrease in Zn concentration within pistachio seedlings (Tavallali et al., 2009). When Zn was added to soil, increased Zn concentrations were found in shoot tissue, and decreased sodium (Na) concentrations in leaf and shoot tissue (Tavallali et al., 2009) which was similar to results seen in tomato plants (Alpaslan et al., 1999; reviewed in Tavallali et al., 2009).

Wheat (*Triticum aestivum*)

Zn-deficient wheat showed decreased carbonic anhydrase activity and decreases in net photosynthetic CO_2 assimilation (Rengel, 1995; reviewed in Tavallali et al., 2009). When grown under Zn or Fe deficiency, wheat roots have been shown to exude phytosiderophores at levels proportional to the extent of deficiency stress (reviewed in Rengel and Romheld, 2000). Within the first 20 days of growth, greater amounts of phytosiderophores were exuded under Fe deficiency than Zn deficiency. Wheat grown under Zn or Fe deficiency had decreased shoot and root growth as well as decreased Zn and Fe concentrations in shoot and root (Rengel et al., 1998; Rengel and Romheld, 2000). While Zn deficient plants were not able to reach the high level of phytosiderophore exudation as in Fe deficient plants, they were more capable of sustaining phytosiderophore exudation for longer periods (Rengel and Romheld, 2000). This greater tolerance to Zn deficiency was a result of a lower transport of Fe from roots to shoots, thus causing an Fe deficiency in the shoots which sent a signal to the roots to increase exudation of phytosiderophores (reviewed in Rengel and Romheld, 2000). There is some evidence that Zn deficient plants have less disease resistance and that low grain Zn levels may result in Zn deficiency in the next generation (reviewed in Impa and Johnson-Beebout, 2012).

Rice (*Oryza sativa*)

Rice is susceptible to soil Zn deficiency and is also a staple food for human populations who are Zn deficient. Rice differs from other staple crops, as it is produced in soils submerged under water for most of its growing season (reviewed in Impa and Johnson-Beebout, 2012) with a small proportion of rice grown in rain-fed soil instead of continuously submerged soil. Flooded soil has lower zinc availability for plant uptake compared to non-flooded soil (reviewed in Impa and Johnson-Beebout, 2012). Soils with low total zinc typically also have low plant-available zinc.

"Plant available" forms of zinc include zinc associated with soluble inorganic compounds and zinc weakly absorbed to surfaces of organic or inorganic soil particles. Suzuki et al., 2008 (reviewed in Impa and Johnson-Beebout, 2012) have shown rice root membranes take up free Zn^{2+} (aq) and Zn complexed with organic compounds from nutrient solutions. Flooded soils undergo many chemical changes including variations in pH, forms of nitrogen, plant-available phosphorus, end products of organic matter decomposition, production and release of greenhouse gases, and plant available forms of micronutrients like Zn, Fe, Mn, etc… Other important changes that affect zinc chemistry are redox potentials and pH. Oxygen depletion can occur due to oxygen consumption by respiration of roots, soil fauna and microorganisms. When oxygen is depleted, anaerobic microorganisms further reduce soil redox potentials by using atoms instead of oxygen as electron acceptors (Impa and Johnson-Beebout, 2012). Increases in pH after flooding tends to make zinc less available but may be offset by other redox-induced changes that may temporarily increase zinc availability (reviewed in Impa and Johnson-Beebout, 2012). The roots are the primary mode of nutrient uptake from soil so plants tend to change their root size and morphology for more efficient nutrient uptake. In lowland rice genotypes, Zn deficiency tolerance has been associated with enhanced root growth both in length and number of roots during Zn deficiency (reviewed in Impa and Johnson-Beebout, 2012). Finer roots tend to be more branched than coarse roots thus increasing the surface area of the more active roots. This root structure has been shown to be a tolerance trait to low available zinc and high bicarbonate in rice (reviewed in Impa and Johnson-Beebout, 2012).

Arabidopsis

In the model organism *Arabidopsis*, two homologous transcription factors have been shown to play a role in regulating the adaptation to Zn deficiency: *bZIP19* and *bZIP23*. BZIP is a transcription factor that contains a highly conserved basic domain binding DNA and a leucine

zipper dimerization motif (Assuncao et al., 2010; Glover-Cutter et al., 2014). In the absence of zinc, *bZIP19* and *bZIP23* were found to have expression levels two times higher than when presented with normal Zn supply (Assuncao et al., 2010). In 2007, Haydon and Cobbett reported that the Zinc-Induced Facilitator 1 (*AtZIF1*) gene in *Arabidopsis thaliana* belongs to a family of transporters and is involved in Zn homeostasis. *AtZIF1* is expressed in the tonoplast and may be involved in transport of zinc, a Zn-ligand, or the ligand alone into the vacoule. During Zn excess and/or Fe deficiency, *AtZIF1* is up regulated in roots and leaves (reviewed in Ricachenevsky et al., 2011).

Hydroponics

The term "hydroponics" was first coined by William Gericke in 1929 but has been in use since the late 17th century (Conn et al., 2013) and refers to the cultivation of plants in solution rather than soil. Different hydroponic methods have been tried, but the most common and efficient way is placing plants within a rockwool plug on the top of a container lid. The tub is filled with media and aerated constantly by bubbling air through airstones maintaining constant oxygenation levels. New media is switched out about once a week. The advantages of using hydroponics include easy manipulation of nutrient profiles of medium in which the plants are grown compared to soil, uniformity and reproducibility of plant development given the easily controlled growth conditions, and accessibility to both shoot and root material (Conn et al., 2013; Robinson et al., 2006). Disadvantages include morphological differences in roots between soil and hydroponics as seen in a potential decrease or lack of root hairs (Conn et al., 2013). There are differing opinions on whether hydroponics aggregate algal contamination and other diseases (Robinson et al., 2006; Conn et al., 2013). When Conn et al. 2013 grew *Arabidopsis* using the hydroponics system, they could manipulate the leaves of the plants relatively cleaner compared to soil grown leaves, and avoided mechanical damage since they did not have to wipe down the leaves before measurement

of gas exchange. Furthermore, measurements for leaf gas exchange were not significantly different between the hydroponic and soil grown plants. Robinson et al. 2006's hydroponics system had consistent quality within and between experiments and a nearly 100% seedling survival rate after first germinating seeds on agar plates. By first germinating seedlings on agar plates, it can be ensured that seedlings are of equal quality and development at time of transfer into rockwool. Rockwool provides a preformed path for root development, thus potentially aiding in further uniform growth.

Brachypodium as a Model Organism

As a member of the grass species, the model organism *Brachypodium distachyon* (Bd) has emerged a valuable experimental model for temperate cereal crops and forage grasses (Glover-Cutter et al., 2014; Draper et al., 2001; Yordem et al, 2011). When compared to *Arabidopsis*, *Brachypodium distachyon* also has a small genome (300 Mbp), short generation time (8-12 weeks) and simple growing conditions (Glover-Cutter et al., 2014, Draper et al., 2001). Furthermore, *Brachypodium distachyon* is self-fertile, is of relatively small size (about 20 cm in height at maturity), lacks seed-head shatter, and its immature embryos exhibit high capacity for plant regeneration via somatic embryogenesis (Draper et al., 2001). Within *Brachypodium distachyon*, the ZIP transcription factor *bZIP10* was found to be highly homologous to *AtbZIP19* and *AtbZIP23* of *Arabidopsis*. Overexpression of *bZIP10* results in enhanced oxidative stress resistance and increased viability (Glover-Cutter et al., 2014).

The goal of my project is to examine the response of *bZIP10*-overexpressing *Brachypodium distachyon* to varying zinc concentrations. As overexpression of *AtbZIP19* and *AtbZIP23* play a role in Zn deficiency regulation, I expect to see that overexpression of *bZIP10* will enhance *Brachypodium distachyon's* tolerance to Zn deficiency. Specifically, I will compare expression of

several genes involved in zinc homeostasis in wildtype and *bZIP10*-expressing *Brachypodium distachyon* in low, normal and high concentrations of zinc.

Materials and Methods

Agrobacterium-mediated Transformation of *Brachypodium*

While I did not produce the transgenic plants that were used in this experiment, I did have the opportunity to go through the transformation procedures with a different bZIP construct being studied in the lab. *Brachypodium* was transformed following Vogel's notes found on the following United States Department of Agriculture (USDA) website: (**brachypodium**.pw.usda.gov/files/Brachy**Transform**Protocol). *Brachypodium* were grown in the greenhouse until the seeds were just beginning to fill. The best embryos are ~0.3-0.7 mm in length. The lemma was removed from the seeds, and the seeds were surface sterilized by soaking in a 10% bleach solution containing 0.1% triton X-100 for 4 minutes, decanting the solution, and rinsing three times with sterile distilled water. The embryos were removed from the seeds (under a dissecting microscope) and placed on Petri plates containing Callus Induction Media (CIM; 4.43g MS salts, 30g sucrose, 1mL of 0.6mg/mL $CuSO_4$, pH adjusted to 5.8, and 2g of phytagel/L). After autoclaving, 2.5mg of 2,4-D (dissolved in dimethyl sulfoxide (DMSO)) was added to the cooled media prior to pouring the plates. The plated embryos were incubated at 28°C in the dark to induce embryogenic calli. The yellowish embryogenic calli was transferred to new CIM plates after ~3 weeks. The embryogenic culture was transferred 2 weeks later to fresh media, approximately 1 week prior to transforming the embryogenic calli. *Agrobacterium* containing a transformation vector with a constitutive promoter and the *bZIP10* gene was grown up 2 days prior to the transformation date. Transformation was performed as described in the Brachypodium transformation protocol (**brachypodium**.pw.usda.gov/files/Brachy**Transform**Protocol). The embryos were co-cultivated with the *Agrobacterium* for 3 days in the dark at 22°C. The calli were then transferred to CIM

plates containing 150mg/L Timentin (to kill the *Agrobacteria*) and 40 mg/L hygromycin to select for transformants and placed in the dark at 22°C to allow transformed cells to grow. Calli were subcultured on selection media every two weeks for ~4-5 weeks to get chunks of healthy looking calli. Healthy callus pieces were move onto regeneration media (Murashige and Skoog (MS) media with maltose, 2g/L phytagel, 40mg/L hygromycin and 0.2 mg kinetin/L) and the plates were moved into a light chamber (16 hr light at 28°C) for production of shoots, which appear in 2-4 weeks. Once shoots were present, they were moved to MS media with sucrose to facilitate rooting. Plantlets with roots were transplanted into soil and placed in the greenhouse, being careful to acclimate them by keeping them under a plastic dome for about a week. Seeds were collected from the transformants and tested for their ability to germinate in the presence of hygromycin. Tests for homozygosity could have also been done by germination on hygromycin. Ideally, it would have been good to have homozygous lines for these studies. However, due to time constraints, Transgenic plant (TR) 1 was a homozygous line, but Transgenic plant 2 was not. This could lead to variable expression levels of the transgene in these experiments.

Hydroponics System

Seedling Preparation

The palea and lemma were removed from seeds prior to surface-sterilization. Seeds were placed in 50% household bleach and 0.01% Triton for 30 min, followed by three washes in sterile distilled H_2O. Excess water was removed, and the seeds were incubated overnight at room temperature. The following day, seeds were soaked in 70% ethanol for 3 minutes, followed by one wash in sterile distilled H_2O. Seeds were sterilized for an additional 20 min in 30% household bleach containing 0.01% Triton, followed by three washes with sterile distilled H_2O. Ten to fifteen seeds were transferred to each petri dish containing either ½-strength MS or ½-

strength MS with hygromycin (to select for transformants) and timentin (to minimize contamination). The plates were wrapped with Nescofilm sealing film and then placed in a dark cold room for 1 week to ensure synchronized germination, prior to placing them in an incubator maintained at 25°C with 10 hours of light. As plants develop, they were moved into the greenhouse or growth chamber, as described above.

Hydroponic System Set-up

Holes (24) were drilled at least 5 centimeters apart on top of a removable lid of a 5L plastic storage container to hold a ¾-½ PVC reducer bushing (Fig 1) for setting plants into the hydroponics system. The bottom of the container was painted completely black to prevent light penetration. Two additional holes were drilled in the top of the lid to accomodate tygon tubing, with one end connected to two aeration stones in the tub and the other end connected to an air pump. Rockwool plugs were created by cutting each piece of rockwool square into 7/8 x 3/4 in cylinders to fit inside of the reducer bushings. Three to four week old seedlings were planted into wet rockwool plugs by cutting the rockwool lengthwise and placing the seedling root within the middle part of the cut with the shoot protruding from top. Rockwool was kept in a bed of water during transfer to the hydroponics system to ensure seedling roots did not dry out. Each of the hydroponics containers had equal numbers of transgenic and wildtype plants.

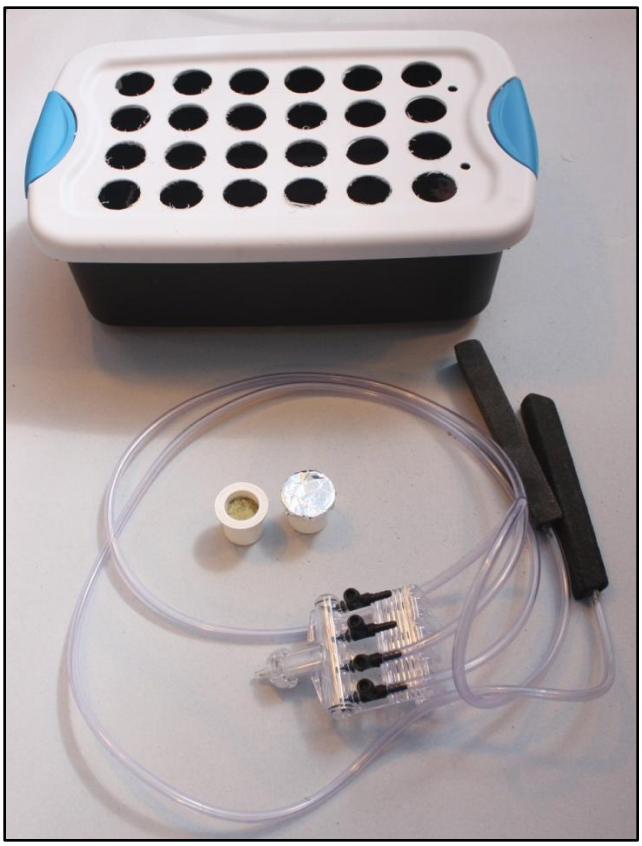

Figure 1. Hydroponics components

Plant Growth

Plants were grown using a modified Hoagland solution with a modified Fe stock following Philip Stoddard's lab at Florida International University's protocol (www2.fiu.edu/~efish/lab_business/Protocols_and_recipes/Fish_and_pool_care/Hoagland%20solution.doc.): 5mM $NH_4H_2PO_4$; 30mM KNO_3; 20mM $Ca(NO_3)_2$; 10mM $MgSO_4$; 0.25mM KFeEDTA solution stock/L (stock made of 26.1g EDTA, 19g KOH, 24.9g $FeSO_4 \cdot 7H_2O$, pH 7.1); 0.77mM $ZnSO_4 \cdot 7H_2O$; micronutrient stock containing 46µM H_3BO_3, 9.1µM $MnCl_2$; 0.32µM $CuSO_4$, 0.83µM H_2MoO_4•H2O. The solution was buffered with 2ml of 1M 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.0, per 5L of solution. For first two weeks of growth in hydroponics ¼-strength Hoagland's solution was used to prevent burning the roots. The plants

were switched to ½-strength solution the following week and levels of solution were replenished by adding freshly made solution every other day, and the solution was totally replaced every 7 days.

Plant Collection

To expose plants to differing levels of zinc, three containers (with different zinc concentrations) each holding 24 plants were arranged as indicated in Table 1. Plants were grown in ¼-strength Hoagland solution for the first two weeks then in ½-strength Hoagland solution during the 3rd week prior to exposure to different Zn concentrations, using the same modified Hoagland solution. Each treatment was represented by two plants of each type (Control, Transgenic 1, 2, and 3) at each zinc concentration. The experiment was repeated twice. At time 0 hours, solutions differing only in the amount of Zn used (zero Zn (Z-), ½-strength Zn (Z) and 15X Zn (Z+++)) were prepared and kept at greenhouse temperature for 8-12 hours before being added to the appropriate tub to bring solutions to greenhouse temperature to avoid shocking the plants. Two plant of each type (WT, TR1 and TR2) were collected at times 12 hours, 24 hours and 7 days by removing the plant from rockwool, gently drying the roots with paper towels, separating the roots from the shoots by cutting the plant at the crown, placing each sample in an aluminum packet, freezing the samples in liquid nitrogen, and then storing at -80°C until use for RNA extraction. Plants were randomly placed into the tubs, as shown in Table 1 with abbreviations shown in Table 2, and labeled as indicated in Table 3-4 for roots and shoots, respectively.

Orientation in Tub						
Position	1	2	3	4	5	6
Plant Type	TR3-7d	**TR2-7d**	TR3-12	**C1-7d**	TR1-7d	C1-7d
Position	7	8	9	10	11	12
Plant Type	TR2-12	**C1-12**	TR2-7d	TR1-24	C1-12	C1-24
Position	13	14	15	16	17	18
Plant Type	**TR1-24**	**TR1-7d**	**TR1-12**	**TR2-24**	TR2-24	TR3-7d
Position	19	20	21	22	23	24
Plant Type	**TR2-12**	TR3-24	**C1-24**	TR3-12	TR1-12	TR3-24

Table 1. Orientation of plants in hydroponics tubs

Legend	
Bolded	Chosen for RNA extraction and testing
C1	WT plants
TR1	*ZIP10* Plant 1
TR2	*ZIP10 GFP* Plant 1
TR3	*ZIP10 GFP* Plant 3
-12	Collected at time 12 hrs
-24	Collected at time 24 hrs
-7d	Collected at time 7 days
Z-	No Zn
Z	Normal Zn
Z+++	High 15x Zn

Table 2. Legend for abbreviations used

	Orientation	Type	Collection Time	Zinc Conc			Orientation	Type	Collection Time	Zinc Conc
1	1	TR3	7d	Z-		**37**	**13**	**TR1**	**24h**	**Z**
2	1	TR3	7d	Z		**38**	**13**	**TR1**	**24h**	**Z+++**
3	**2**	**TR2**	**7d**	**Z-**		**39**	**14**	**TR1**	**7d**	**Z-**
4	**2**	**TR2**	**7d**	**Z**		**40**	**14**	**TR1**	**7d**	**Z**
5	**2**	**TR2**	**7d**	**Z+++**		**41**	**14**	**TR1**	**7d**	**Z+++**
6	3	TR3	12h	Z-		**42**	**15**	**TR1**	**12h**	**Z-**
7	3	TR3	12h	Z		**43**	**15**	**TR1**	**12h**	**Z**
8	3	TR3	12h	Z+++		**44**	**15**	**TR1**	**12h**	**Z+++**
9	**4**	**C1**	**7d**	**Z-**		**45**	**16**	**TR2**	**24h**	**Z-**
10	**4**	**C1**	**7d**	**Z**		**46**	**16**	**TR2**	**24h**	**Z**
11	**4**	**C1**	**7d**	**Z+++**		**47**	**16**	**TR2**	**24h**	**Z+++**
12	5	TR1	7d	Z-		48	17	TR2	24h	Z-
13	5	TR1	7d	Z		49	17	TR2	24h	Z
14	5	TR1	7d	Z+++		50	17	TR2	24h	Z+++
15	6	C1	7d	Z-		51	18	TR3	7d	Z-
16	6	C1	7d	Z		52	18	TR3	7d	Z
17	6	C1	7d	Z+++		53	18	TR3	7d	Z+++
18	7	TR2	12h	Z-		**54**	**19**	**TR2**	**12h**	**Z-**
19	7	TR2	12h	Z		**55**	**19**	**TR2**	**12h**	**Z**
20	7	TR2	12h	Z+++		**56**	**19**	**TR2**	**12h**	**Z+++**
21	**8**	**C1**	**12h**	**Z-**		57	20	TR3	24h	Z-
22	**8**	**C1**	**12h**	**Z**		58	20	TR3	24h	Z
23	**8**	**C1**	**12h**	**Z+++**		59	20	TR3	24h	Z+++
24	9	TR2	7d	Z-		**60**	**21**	**C1**	**24h**	**Z-**
25	9	TR2	7d	Z		**61**	**21**	**C1**	**24h**	**Z**
26	9	TR2	7d	Z+++		**62**	**21**	**C1**	**24h**	**Z+++**
27	10	TR1	24h	Z-		63	22	TR3	12h	Z-
28	10	TR1	24h	Z		64	22	TR3	12h	Z
29	10	TR1	24h	Z+++		65	22	TR3	12h	Z+++
30	11	C1	12h	Z-		66	23	TR1	12h	Z-
31	11	C1	12h	Z		67	23	TR1	12h	Z
32	11	C1	12h	Z+++		68	23	TR1	12h	Z+++
33	12	C1	24h	Z-		69	24	TR3	24h	Z-
34	12	C1	24h	Z		70	24	TR3	24h	Z
35	12	C1	24h	Z+++		71	24	TR3	24h	Z+++
36	**13**	**TR1**	**24h**	**Z-**						

Table 3. Plant roots collected for RNA isolation organized by orientation in tub, plant type, and Zn growth condition (numbered 1-71)

	Orientation	Type	Collection Time	Zinc Conc		Orientation	Type	Collection Time	Zinc Conc
72	1	TR3	7d	Z-	**108**	**13**	**TR1**	**24h**	**Z-**
73	1	TR3	7d	Z	**109**	**13**	**TR1**	**24h**	**Z**
74	1	TR3	7d	Z+++	**110**	**13**	**TR1**	**24h**	**Z+++**
75	**2**	**TR2**	**7d**	**Z-**	**111**	**14**	**TR1**	**7d**	**Z-**
76	**2**	**TR2**	**7d**	**Z**	**112**	**14**	**TR1**	**7d**	**Z**
77	**2**	**TR2**	**7d**	**Z+++**	**113**	**14**	**TR1**	**7d**	**Z+++**
78	3	TR3	12h	Z-	**114**	**15**	**TR1**	**12h**	**Z-**
79	3	TR3	12h	Z	**115**	**15**	**TR1**	**12h**	**Z**
80	3	TR3	12h	Z+++	**116**	**15**	**TR1**	**12h**	**Z+++**
81	**4**	**C1**	**7d**	**Z-**	**117**	**16**	**TR2**	**24h**	**Z-**
82	**4**	**C1**	**7d**	**Z**	**118**	**16**	**TR2**	**24h**	**Z**
83	**4**	**C1**	**7d**	**Z+++**	**119**	**16**	**TR2**	**24h**	**Z+++**
84	5	TR1	7d	Z-	120	17	TR2	24h	Z-
85	5	TR1	7d	Z	121	17	TR2	24h	Z
86	5	TR1	7d	Z+++	122	17	TR2	24h	Z+++
87	6	C1	7d	Z-	123	18	TR3	7d	Z-
88	6	C1	7d	Z	**124**	**18**	**TR3**	**7d**	**Z**
89	6	C1	7d	Z+++	**125**	**18**	**TR3**	**7d**	**Z+++**
90	7	TR2	12h	Z-	**126**	**19**	**TR2**	**12h**	**Z-**
91	7	TR2	12h	Z	127	19	TR2	12h	Z
92	7	TR2	12h	Z+++	128	19	TR2	12h	Z+++
93	**8**	**C1**	**12h**	**Z-**	129	20	TR3	24h	Z-
94	**8**	**C1**	**12h**	**Z**	130	20	TR3	24h	Z
95	**8**	**C1**	**12h**	**Z+++**	131	20	TR3	24h	Z+++
96	9	TR2	7d	Z-	**132**	**21**	**C1**	**24h**	**Z-**
97	9	TR2	7d	Z	**133**	**21**	**C1**	**24h**	**Z**
98	9	TR2	7d	Z+++	**134**	**21**	**C1**	**24h**	**Z+++**
99	10	TR1	24h	Z-	135	22	TR3	12h	Z-
100	10	TR1	24h	Z	136	22	TR3	12h	Z
101	10	TR1	24h	Z+++	137	22	TR3	12h	Z+++
102	11	C1	12h	Z-	138	23	TR1	12h	Z-
103	11	C1	12h	Z	139	23	TR1	12h	Z
104	11	C1	12h	Z+++	140	23	TR1	12h	Z+++
105	12	C1	24h	Z-	141	24	TR3	24h	Z-
106	12	C1	24h	Z	142	24	TR3	24h	Z
107	12	C1	24h	Z+++	143	24	TR3	24h	Z+++

Table 4. Plant shoots collected for RNA isolation organized by orientation in tub, plant type, and Zn growth conditions (numbered 72-143)

RNA Isolation

Each packet of frozen plant material was ground in liquid nitrogen in the presence of 0.1g of polyvinylpyrrolidone (PVP) and immediately resuspended in TRIzol reagent (1mL Trizol per 0.1g of plant material). RNA was extracted from samples following a modified protocol of

Ambion using Life Technology's TRIzol Reagent. Samples were centrifuged at 10,000 x g for 10 minutes at 6°C and the supernatant was transferred to a new tube and incubated at room temperature for 5 minutes. Chloroform (0.2mL/mL of Trizol used) was added to the sample. The samples were shaken vigorously by hand for 15 seconds, incubated at room temperature for 5-10 minutes, and then centrifuged at 10,000 x g for 15 minutes at 6°C. The upper aqueous layer was removed to new tube, and 0.5mL of 100% isopropanol was added per 1mL of TRIzol reagent used during homogenization. Samples were incubated in a -20°C freezer overnight before centrifuging at 12,000 x g for 10 minutes at 4°C. Liquid was discarded, being careful not to lose the pellet. The RNA pellet was washed using 1mL of 75% ethanol per 1mL of TRIzol reagent used during initial homogenization. Samples were vortexed briefly and allowed to sit on ice for ~10 minutes before centrifuging at 7,500 x g for 5 minutes at 6°C. The wash was discarded and RNA pellet was allowed to air dry. The RNA pellet was resuspended in 30-50µL RNase-free water. The pellet was incubated in a 55-60°C water bath for 15 minutes to help solubilize the RNA before quantification using the Nanodrop.

The purity of the RNA extracted from roots had a very low 260/230 ratio indicating possible phenol contamination left over from RNA extraction. To further purify the root RNA, the RNA sample was resuspended in TRIzol reagent and purified using the Zymo Research Direct-zol MiniPrep kit. One volume of 100% ethanol was added to the sample and mixed by vortexing. The mixture was loaded into a Zymo-Spin IIC Column in a collection tube and centrifuged at 12,000 x g for 30 seconds. The column was then transferred to a new collection tube, washed with 400µl of RNA Wash Buffer, and centrifuged at 12,000 x g for 30 seconds. The wash-through was discarded and then 80µl of DNase I Reaction Mix (5µl of DNase I and 75µl of DNA Digestion Buffer) was added to each column. The samples were incubated at 20-30°C for 15 minutes to facilitate the DNase digestion process. The column was then washed with 400µl of Direct-zol RNA PreWash, centrifuged at 12,000 x g for 30 seconds and wash-through was again discarded.

This step was repeated one more time. Then, the column was washed with 700µl of RNA Wash Buffer and centrifuged at 12,000 x g for 2 minutes. The column was transferred to new tube and RNA was eluted with 50µl of nuclease-free water by centrifuging at 12,000 x g for 30 seconds. The amount of RNA was then quantified using the Nanodrop.

The RNA from the shoots also had low 260/230 ratio indicating possible phenol contamination left over from RNA extraction and was further purified by treating with Bio-Rad's Aurum Total RNA Mini Kit. To each sample, 700µl of lysis solution was added and the sample was disrupted by pipetting up and down multiple times. The sample was then centrifuged at 12,000 x g for 3 minutes and the supernatent was transferred to a new microcentrifuge tube. Ethanol (700µl of 70% ethanol) was added to each sample and mixed thoroughly by pipetting up and down. Homogenized lysate was pipetted onto the RNA binding column in a 2ml capless wash tube, centrifuged at 12,000 x g for 1 minute, and then the wash-through was discarded. Low stringency wash solution (700µl) was added to the column, and the samples were centrifuged at 12,000 x g for 30 seconds and the wash-through was again discarded. Samples were treated with 80µl of diluted DNase I mix (5µl of reconstituted DNase I and 75µl of DNase dilution solution) by addition to each column, and columns were incubated at room temperature for 15 minutes. Each column then had 700µl of high stringency wash solution added before centrifuging at 12,000 x g for 30 seconds. The wash-through was discarded before 700µl low stringency wash solution was added to the column. Column was then centrifuged at 12,000 x g for 30 seconds and the wash-through was discarded before centrifuging again at 12,000 x g for an additional 2 minutes to remove residual wash solution. The RNA binding column was then transferred to a new tube and 80µl of elution buffer was added to the membrane stack and columns were incubated for 1 minute to allow saturation of the membrane. Column was then centrifuged at 12,000 x g for 2 minutes to elute RNA from column. The RNA was then quantified using the Nanodrop.

cDNA Synthesis

SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Life Technologies) was used to synthesize cDNA following the manufacturer's instructions.The reaction mixes consisted of 1.5µg of RNA, 15µl of 2 x reaction mix (includes oligo$[dT]_{20}$, random hexamers, $MgCl_2$ and dNTPs) and 3µl of RT Enzyme mix (includes Superscript® III RT and RNaseOUT™). *Escherichia coli (E. coli)* RNase H provided with the kit was used to remove the RNA template prior to running qPCR reactions. The cDNA samples were stored at -20°C until used for expression analysis.

Primer Design

Batch Primer3 was used to design primers for quantitative reverse transcription polymerase chain reaction (RT qPCR) (You et al., 2008). Primers (Table 5) were designed to amplify a product of ~150bp, with a GC content between 40 and 60%, and a melting temperature T_m of ~62°C.

Primer	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
BdUBC18	TCTTGTCCATGCTGTCTAGCTC	TTCCGTTGCGGCAGTTC
BdbZIP10	ACAGCTACTTCGACGAGAT	CAGGTGTGGGTGTGGGA
BdZIF1	TTGGCTTTGTTGGTCCTGGT	ATTGTGGGGCAATCTCCTGA
BdIRT-1	CGACCACGAGAGCCCTGAT	TGCCCATCTCCAGAACCTGAAC
BdZIP6	CTTGGGCATGTCACAGGACG	CAAAACCAGCCTGCGCGAT
BdZIP1	CATCAAAGAGCAACCGCAAA	AGCCAGAAGGAAGAACACCAT
BdZIP4	CGGTTGCATTGTTCAGGCAAAA	GATCGCGATACCGAGTGCGA

Primer	Gene Family Name
BdUBC18	Ubiquitin-Conjugating Enzyme
BdbZIP10	Basic domain binding DNA and leucine zipper dimerization motif transcription factor
BdZIF1	Zinc-Induced Facilitator
BdIRT-1	Iron-Regulated Transporter
BdZIP6	Zinc-regulated transporter, Iron-regulated transporter-like Protein (*Zea mays* homolog)
BdZIP1	Zinc-regulated transporter, Iron-regulated transporter-like Protein (*Arabidopsis* homolog)
BdZIP4	Zinc-regulated transporter, Iron-regulated transporter-like Protein (*Zea mays* homolog)

Table 5. List of primers used for gene expression analysis with full gene names

Expression Analysis

Samples were diluted 1:40 and 4µl of diluted cDNA was used per reaction.The Bio-Rad iTaq™ Universal SYBR® Green Supermix was used to set up the samples for qPCR analysis on the Bio-Rad® CFX96 Real-Time system. The program was as follows: 95°C for 2 min; 40 cycles of 95°C for 10 sec, 60°C for 30 sec (camera); followed by a melt curve analysis of 65-95°C, increments 0.5°C with 5 sec/step (camera). *BdUBC18* was used as a reference gene. Expression levels for each gene (*BdUBC18, bZIP10, BdZIF1, BdIRT-1, BdZIP6, BdZIP1, BdZIP4*) and for each of the selected samples were analyzed on each of three individual qPCR runs for both root and shoot samples. Samples were analyzed based on the $\Delta\Delta C_T$ calculations. All samples were normalized to *BdUBC18*. Samples were then compared by different zinc levels (within each type of sample, WT, TR1, and TR2); so the no zinc and high zinc samples (Z- and Z+++) were compared to the

optimal zinc level samples (Z). In addition, gene expression levels for TR1 and TR2 samples were compared to WT levels of gene expression at all zinc concentrations.

Results

Please note that all results reported in this thesis are only preliminary results.

Hydroponics System

Plant Growth

WT plants grown in hydroponics system had huge root masses and began to set seed about 7 weeks after transfer into the hydroponics system (Figure 2) and continued to produce fully mature seeds (Figure 3).

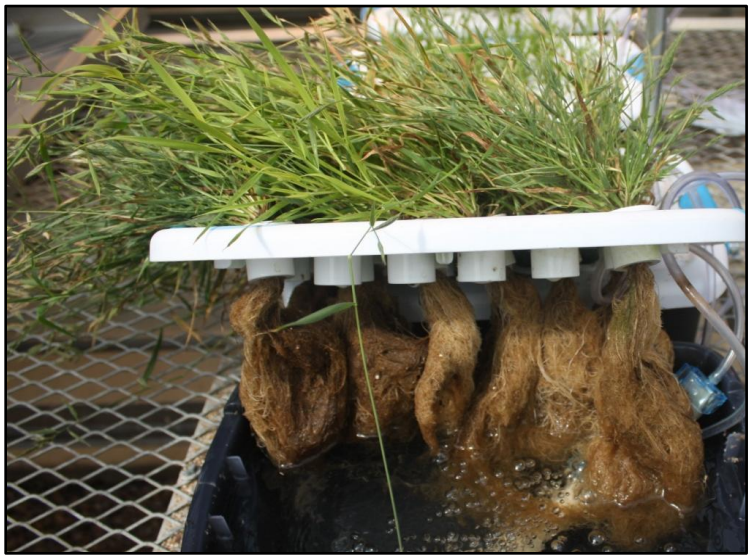

Figure 2. WT plants grown for 10 weeks in the hydroponics system

Figure 3. Mature WT *Brachypodium* about 12 weeks after transfer into the hydroponics system

Implementation of experimental conditions did not seem to cause noticeable phenotypic differences on the plants in any plant type or growth conditions. Figure 4 shows a picture comparison of each hydroponics system containing all plant types (WT, TR1, TR2 and TR3). Of the few plants that did exhibit symptoms such as chlorosis or whitening of tips or leaf edges, symptoms were not consistent between plants of the same type nor the same Zn conditions. Otherwise, shoot growth and root development was consistent across all wildtype and transgenic plants between Zn conditions and within the same Zn condition.

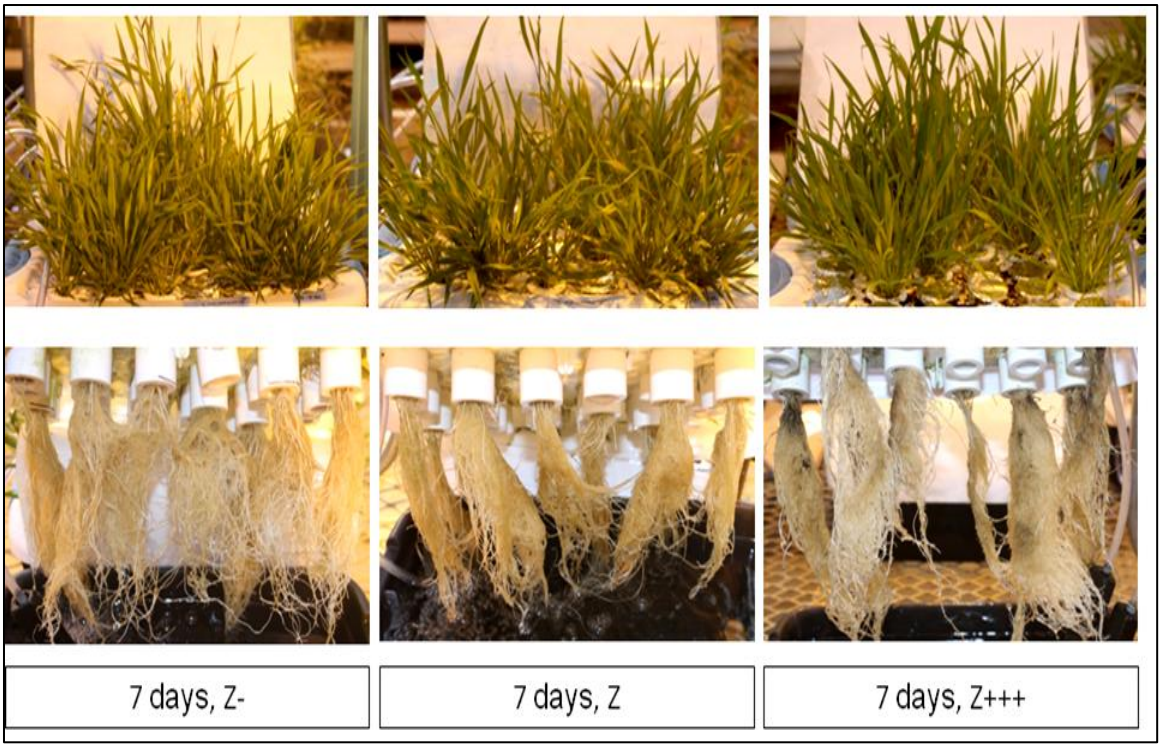

Figure 4. Comparison of roots and shoots at time 7 days after initiation of experimental Zn conditions

Gene Expression

Gene expression was analyzed on the Bio-Rad® CFX96 Real-Time system. Tables 6 and 7 show the qPCR threshold cycle (C_t) values obtained when running the subset of samples chosen for RNA isolation and cDNA synthesis with selected primers (Table 5). The average C_t values for the three independent runs are also shown in the table. The average C_t value was used to calculate the relative expression levels for each gene when comparing different treatments. The lower C_t values of *BdbZIP10* seen in transgenic roots and shoots show the transgenic plants do express the *BdbZIP10* gene that was transformed into the plant at much higher levels than the control (wild type) plants, as would be expected.

		BdUBC18		*BdbZIP10*		*BdZIF1*		*BdIRT-1*		*BdZIP6*		*BdZIPL1*		*BdZIP4*	
	Run	Cq	Avg	Cq	Avg	Cq	Avg	Cq	Avg	Cq	Avg	Cq	Avg	Cq	Avg
C1 Z-	1	24.70	24.58	27.33	27.27	25.88	25.81	26.06	25.92	25.55	25.57	27.01	26.95	27.13	27.08
	2	24.65		27.35		25.94		26.17		25.64		27.19		27.11	
	3	24.38		27.12		25.61		25.87		25.51		26.67		26.99	
C1 Z	1	24.26	24.13	25.22	25.10	25.23	25.22	24.79	25.08	25.40	25.45	26.43	26.36	28.93	29.03
	2	24.32		25.23		25.39		24.99		25.71		26.43		29.12	
	3	23.81		24.84		25.04		24.53		25.22		26.21		29.05	
C1 Z+++	1	24.25	24.28	26.12	26.45	25.26	25.35	24.84	25.15	25.24	25.40	26.36	26.38	31.55	31.54
	2	24.64		26.90		25.54		25.39		25.62		26.48		31.72	
	3	23.94		26.33		25.26		24.96		25.33		26.29		31.35	
TR1 Z-	1	24.17	23.99	21.64	21.31	25.06	24.90	23.26	24.41	24.25	24.57	25.78	26.05	25.52	25.71
	2	24.11		21.20		24.84		23.65		24.78		26.25		25.98	
	3	23.67		21.09		24.79		23.42		24.68		26.13		25.64	
TR1 Z	1	24.08	24.11	20.77	20.67	25.02	24.96	24.60	24.86	24.97	25.00	26.97	26.84	27.77	27.67
	2	24.29		20.64		25.01		24.50		25.04		26.77		27.68	
	3	23.95		20.62		24.84		24.51		25.01		26.78		27.56	
TR1 Z+++	1	23.77	23.79	21.70	21.56	24.75	24.69	24.43	24.30	25.07	25.00	26.35	26.36	29.52	29.51
	2	24.01		21.56		24.76		24.31		24.90		26.45		29.63	
	3	23.58		21.42		24.57		24.16		25.03		26.28		29.37	
TR2 Z-	1	24.08	24.14	20.82	20.82	25.08	25.09	24.45	24.34	25.33	25.30	26.45	26.37	25.23	25.31
	2	24.15		20.89		25.06		24.17		25.28		26.38		25.22	
	3	24.21		20.76		25.13		24.39		25.31		26.30		25.49	
TR2 Z	1	25.17	24.48	22.11	21.77	25.46	25.25	24.36	24.25	25.09	25.17	26.32	26.49	26.78	27.00
	2	24.30		21.70		25.18		24.20		25.19		26.51		27.10	
	3	23.96		21.49		25.12		24.19		25.22		26.63		27.11	
TR2 Z+++	1	29.68	29.73	27.77	27.78	30.44	30.52	30.83	30.67	30.24	30.46	32.25	31.90	34.24	34.67
	2	29.77		27.79		30.60		30.52		30.69		31.56		35.10	

Table 6. Summary of root Ct values for three independent qPCR runs

		BdUBC18		*BdbZIP10*		*BdZIF1*		*BdIRT-1*		*BdZIP6*		*BdZIPL1*		*BdZIP4*	
	Run	Cq	Avg	Cq	Avg	Cq	Avg	Cq	Avg	Cq	Avg	Cq	Avg	Cq	Avg
C1 Z-	1	25.80	25.31	26.16	26.07	25.49	25.50	32.94	32.80	26.60	26.38	27.86	27.64	26.69	26.64
	2	24.96		26.11		25.49		32.88		26.31		27.67		26.69	
	3	25.18		25.94		25.51		32.58		26.23		27.38		26.55	
C1 Z	1	25.15	24.95	26.18	26.36	25.16	25.19	33.49	33.44	26.05	26.02	27.46	27.29	27.98	27.88
	2	24.71		26.55		25.13		33.37		26.05		27.27		27.83	
	3	24.98		26.35		25.29		33.47		25.96		27.14		27.83	
C1 Z+++	1	25.17	25.47	26.66	26.74	25.70	25.74	33.56	33.81	26.51	26.63	28.11	27.99	29.42	29.54
	2	25.59		26.73		25.75		33.85		26.81		28.16		29.79	
	3	25.66		26.82		25.79		34.03		26.57		27.70		29.41	
TR1 Z-	1	25.22	25.35	21.71	21.76	25.69	25.57	33.06	33.04	26.26	26.33	27.96	27.97	24.62	24.78
	2	25.40		21.73		25.47		33.18		26.32		28.07		24.93	
	3	25.43		21.85		25.53		32.86		26.41		27.88		24.78	
TR1 Z	1	25.90	25.76	20.39	20.58	25.76	25.89	32.25	32.43	26.77	26.76		27.85	27.25	27.26
	2	25.53		20.62		25.83		32.87		26.72		28.03		27.20	
	3	25.85		20.73		26.08		32.17		26.79		27.67		27.31	
TR1 Z+++	1	25.23	25.21	21.14	21.32	25.20	25.27	32.39	32.15	26.35	26.40	27.59	27.55	28.33	28.40
	2	25.01		21.24		25.18		32.01		26.34		27.59		28.29	
	3	25.39		21.58		25.42		32.04		26.49		27.45		28.59	
TR2 Z-	1	25.23	25.26	20.27	20.37	25.52	25.55	32.62	32.74	26.46	26.42	27.80	27.74	24.33	24.37
	2	25.24		20.26		25.60		32.47		26.43		27.67		24.35	
	3	25.32		20.60		25.53		33.14		26.38				24.45	
TR2 Z	1	25.28	25.22	21.49	21.46	25.33	25.32	32.47	32.09	26.39	26.35	27.90	27.71	26.67	26.55
	2	25.00		21.22		25.22		31.59		26.26		27.69		26.26	
	3	25.40		21.68		25.40		32.22		26.38		27.55		26.73	
TR2 Z+++	1	25.40	25.32	21.27	21.35	25.59	25.53	32.59	32.81	26.51	26.47	27.89	27.86	27.86	27.89
	2	24.93		21.07		25.37		32.69		26.26		27.75		27.63	
	3	25.64		21.71		25.64		33.16		26.65		27.94		28.17	

Table 7. Summary of shoot Ct values for three independent qPCR runs

The $\Delta\Delta C_T$ calculations (Livak and Schmittgen, 2001) were used to compare expression levels within each plant type (Control, TR1 and TR2) at low and high zinc concentrations (Z- and Z+++) compared to the normal zinc concentration (Z). Expression levels for each gene were also compared between each transgenic plant and the control plant at the same zinc concentration. The results for the roots are presented in Figures 5-7. Comparisons between gene expression levels in roots of control plant grown under different zinc concentrations are shown in Figure 5. The Bd homolog of the *Zea mays* ZIP4 gene was expressed 5-fold higher under low zinc conditions

compared to the normal zinc conditions and showed a decrease in expression under high levels of zinc (Figure 5). This was also seen in the transgenic plant roots, but to a lesser degree (Figures 6-7). Within the WT roots (Figure 5), *BdbZIP10* and *BdIRT-1* showed slightly lower expression levels within Z- compared to Z. TR1 roots from plants grown in Z- showed higher expression of genes *BdIRT-1*, *BdZIP1* and *BdZIP4* compared to TR1 roots grown in Z (Figure 6). TR1 plants grown under high zinc conditions expressed *BdZIP1* at higher levels (3 fold) in the roots compared to plants grown on normal zinc levels (Figure 6). Similar to TR1, TR2 also showed elevated levels of the *BdZIP4* homolog in the roots of plants grown under zinc deficient conditions compared to normal conditions, and also showed a decreased level of *BdZIP4* (in roots) under zinc excess conditions (Figure 7).

In the WT shoots, plants grown in Z- showed elevated expression of the *BdZIP4* (3 fold) and a 2 fold increase in *BdIRT-1* compared to plants grown in normal zinc levels, and a slight increase in *BdbZIP10*, while control plants grown in Z+++ showed a slightly lower expression of the *BdZIP4* compared to those grown at Z (Figure 8). Interestingly the *BdZIP4* gene was expressed at higher level in shoots of both transgenic plants grown under zinc deficient conditions, and was expressed at lower levels under zinc excess when compared to shoots from plants grown at normal zinc levels (Figures 9-10). This is similar to the expression pattern present the transgenic roots.

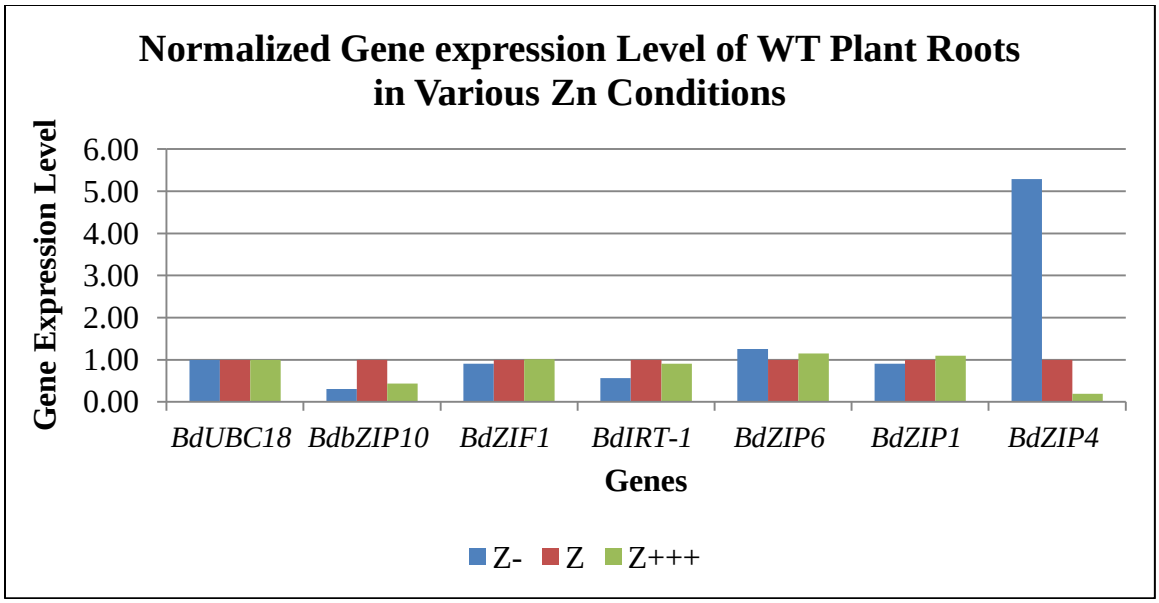

Figure 5. Graph comparing gene expression (normalized to *BdUBC18)* in roots of select WT plants grown in various Zn conditions

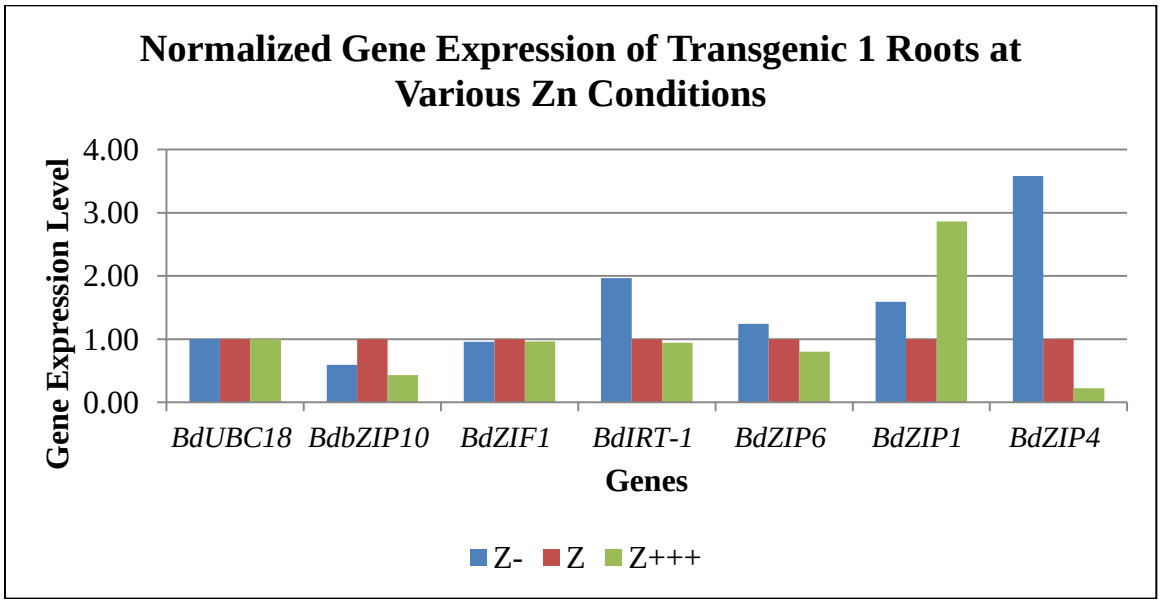

Figure 6. Graph comparing gene expression (normalized to *BdUBC18*) in roots of select TR1 plants grown in various Zn conditions

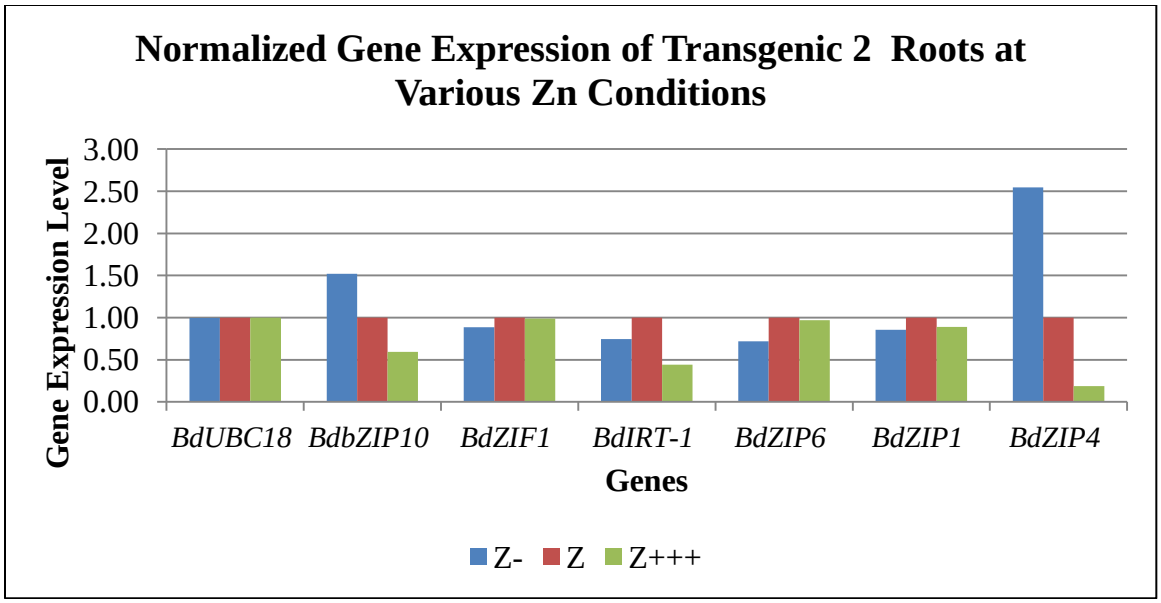

Figure 7. Graph comparing gene expression (normalized to *BdUBC18*) in roots from select TR2 plants grown in various Zn conditions

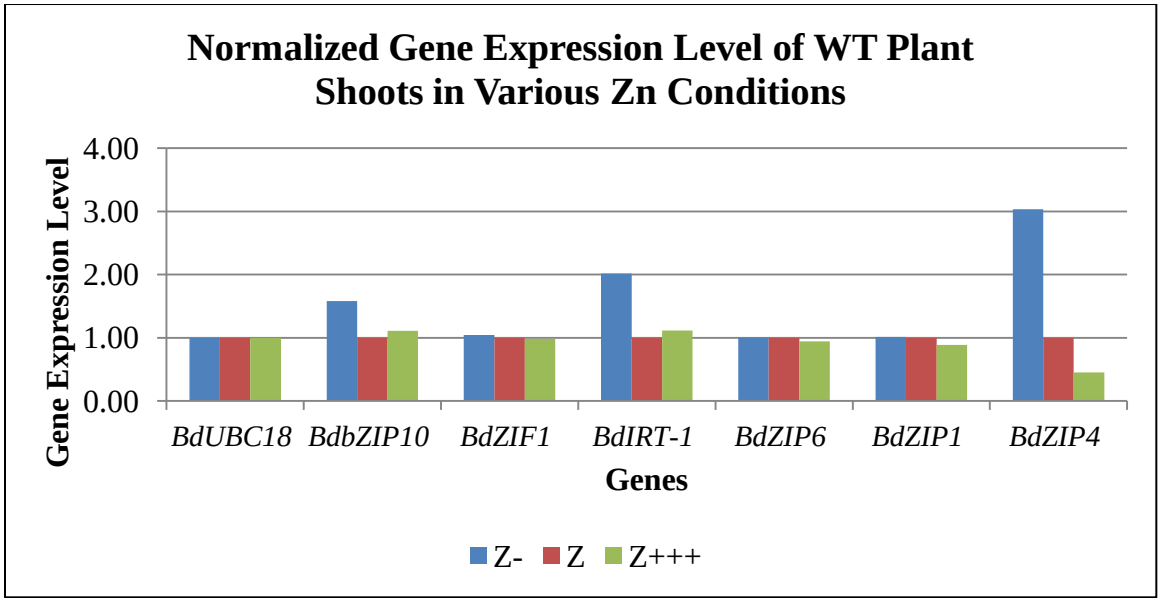

Figure 8. Graph comparing gene expression (normalized to *BdUBC18*) in shoots from select WT plants grown in various Zn conditions

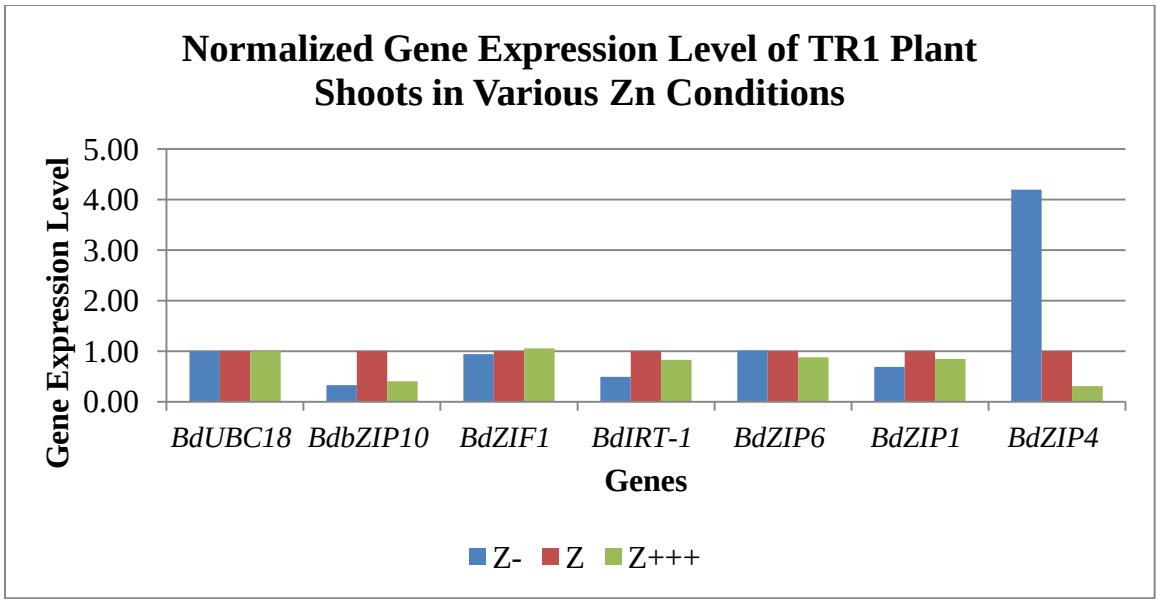

Figure 9. Graph comparing gene expression (normalized to *BdUBC18*) in shoots from select TR1 plants grown in various Zn conditions

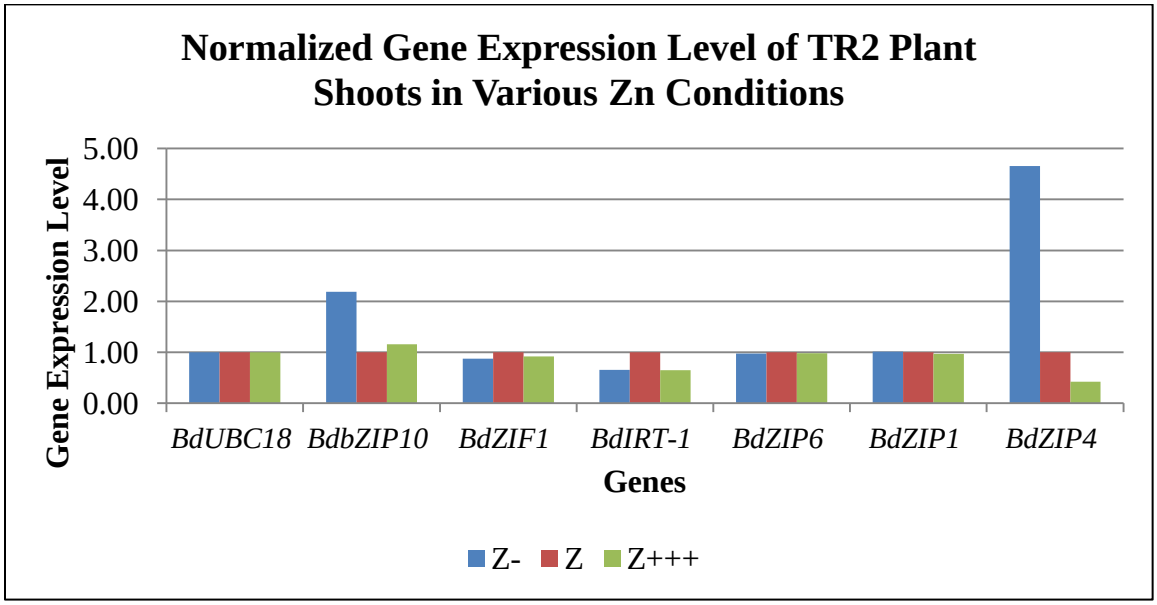

Figure 10. Graph comparing gene expression (normalized to *BdUBC18*) in shoots from select TR2 plants grown in various Zn conditions

Figures 11-14 show the fold difference of gene expression of transgenic plants compared to WT plants across the various Zn conditions. Figures 12 and 14 show fold difference of *BdbZIP10*

between the transgenic and the WT plants were separated from the rest of the genes so the more subtle differences in the other genes could be seen, since the *BdbZIP10* expression levels are much higher than the others as expected. Interestingly, the *BdbZIP10* levels of gene expression are quite variable between the roots and the shoots. Most strikingly is the almost 5-fold difference in expression between TR1 roots (approximately 20-fold, Figure 12) and shoots (about 100-fold, Figure 14) under normal zinc conditions when comparing these to WT levels. Under Z- conditions, the *BdbZIP10* gene was expressed at higher levels in the roots compared to the shoots of both transgenics. The transgene roots (Figure 11) and shoots (Figure 13) show different fold differences of gene expression relative to WT, with the only similarity being *BdZIP4* showing the highest fold difference in both. TR2 roots grown in normal and Z+++ conditions having a 5-fold difference, TR1 roots grown in Z+++ having a 3-fold difference, TR1 roots grown in Z having a 2.5-fold difference, TR2 roots grown in Z- having a 2.5-fold difference and TR1 roots grown in Z- having a 1.7-fold difference. When looking at *BdIRT-1* in the roots, TR1 roots grown in Z- conditions showed a 4-fold difference from the WT while TR2 grown in Z- showed a 2.3-fold difference compared to WT (Figure 11). As for *BdbZIP10*, TR2 and TR1 roots grown in Z- conditions showed the greatest fold differences at 64-fold and 41-fold respectively (Figure 12), followed by TR1 roots in Z and in Z+++ at 21-fold, and finally with TR2 in Z+++ and TR2 in Z showing the least fold difference of this batch at 17.4-fold and 12.8-fold respectively (Figure 12).

In the shoots, the greatest variability in fold differences of transgenic shoots are *BdZIP4* and *BdIRT-1* (Figure 13), while the other genes do not show much fold difference between transgenic and WT plants. The shoots grown in Z- conditions showed the greatest fold difference for *BdZIP4* at 4.7-fold and 3.7-fold for TR2 and TR1 respectively (Figure 13). The other TR2 shoots showed a 3-fold and 2.8-fold difference for Z and Z+++ conditions respectively with TR1 in Z and TR1 in Z+++ showing the lowest fold difference of this batch at 2.7-fold and 1.8-fold. The transgenic plants grown under Z and Z+++ conditions showed the greatest fold difference for *BdIRT-1*

compared to WT plants grown under the same conditions: 3.5-fold for TR1 in Z, 3-fold for TR2 in Z, 2.6-fold for TR1 in Z+++ and 1.8-fold for TR2 in Z+++ (Figure 13). As for *BdbZIP10*, TR1 in Z showed the greatest fold difference from WT at 96-fold, 50-fold for TR2 in Z-, 38-fold for TR2 in Z+++, 36-fold for TR2 in Z, 36-fold for TR1 in Z+++ and 20-fold for TR1 in Z- (Figure 14).

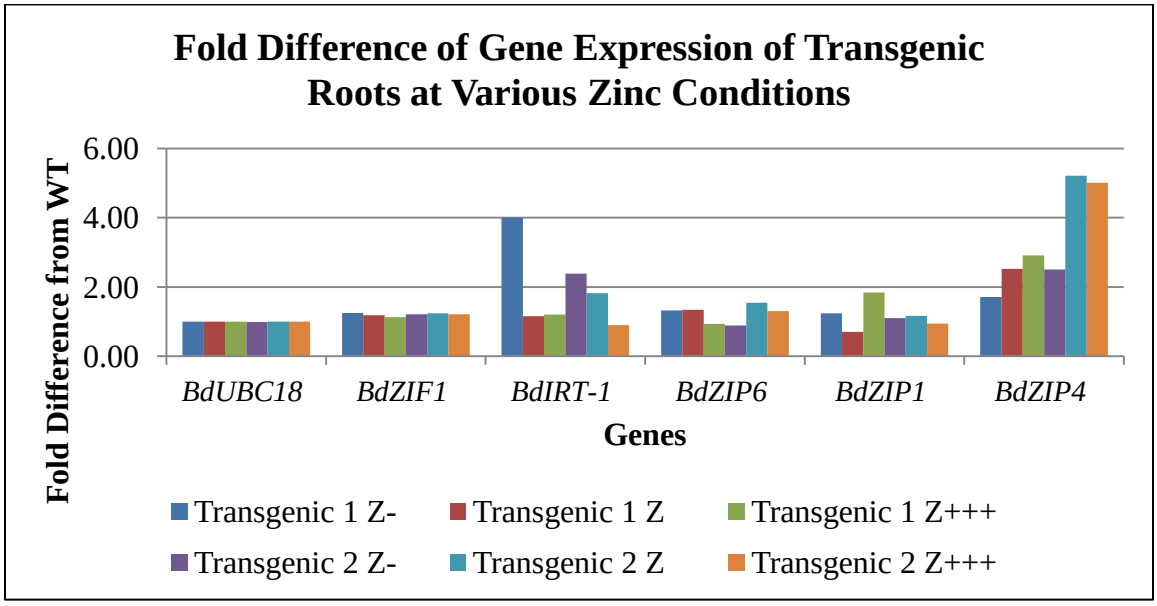

Figure 11. Graph comparing fold difference of gene expression of transgenic plant roots from wildtype plant roots grown in various Zn conditions

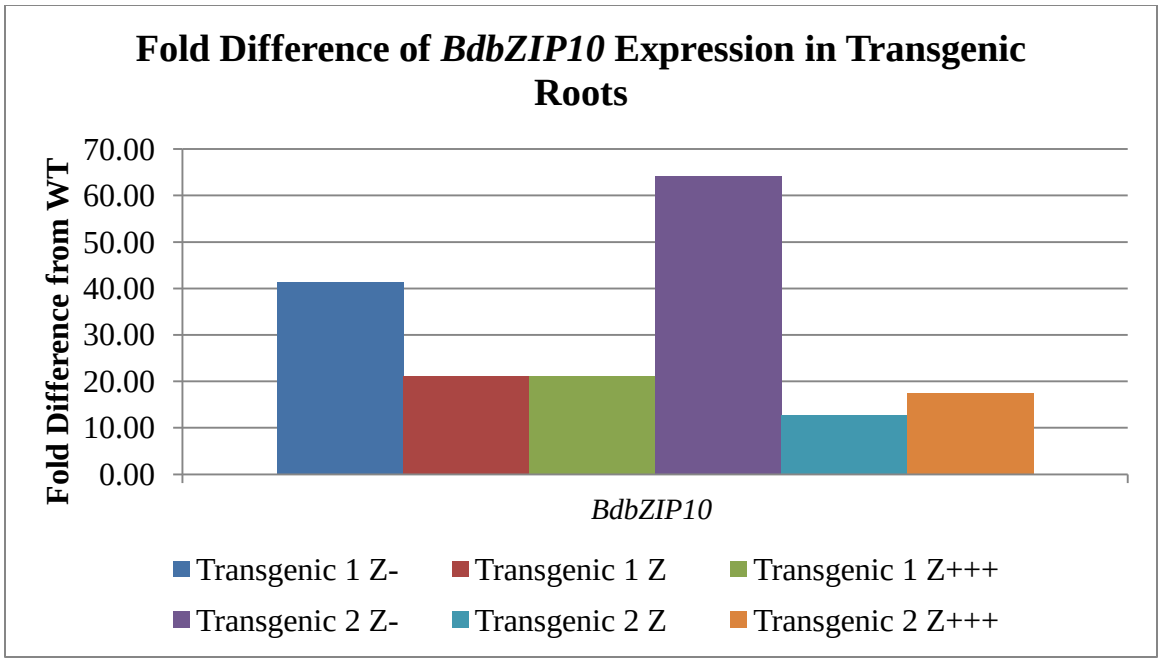

Figure 12. Graph comparing expression of *BdbZIP10* in transgenic roots to wildtype roots grown at various Zn conditions

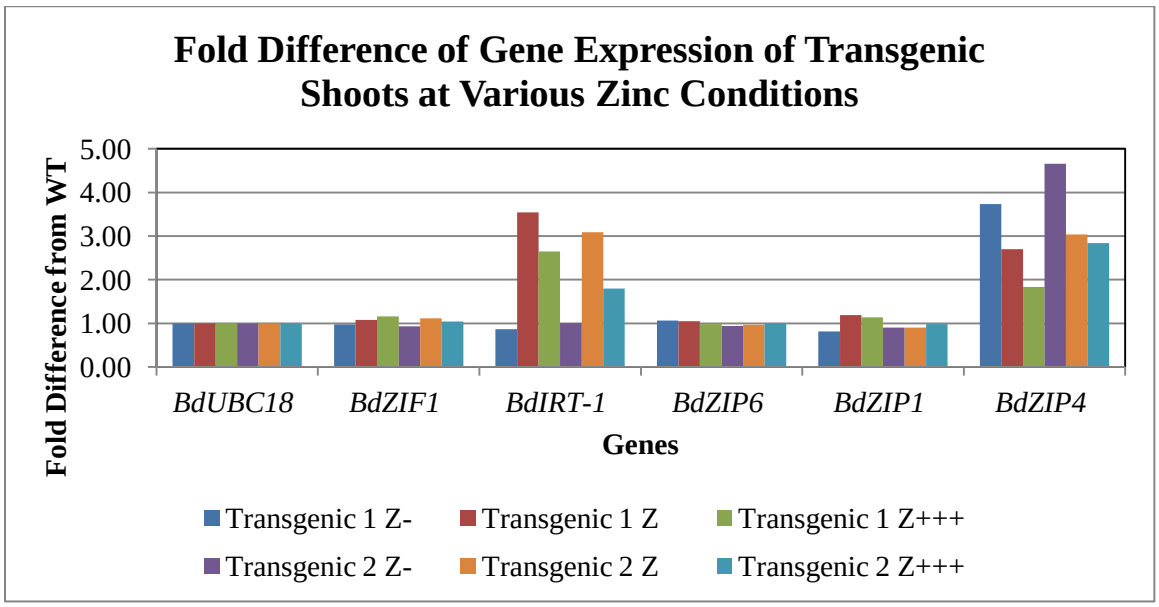

Figure 13. Graph comparing fold difference of gene expression of transgenic plant shoots from wildtype plant shoots grown in various Zn conditions

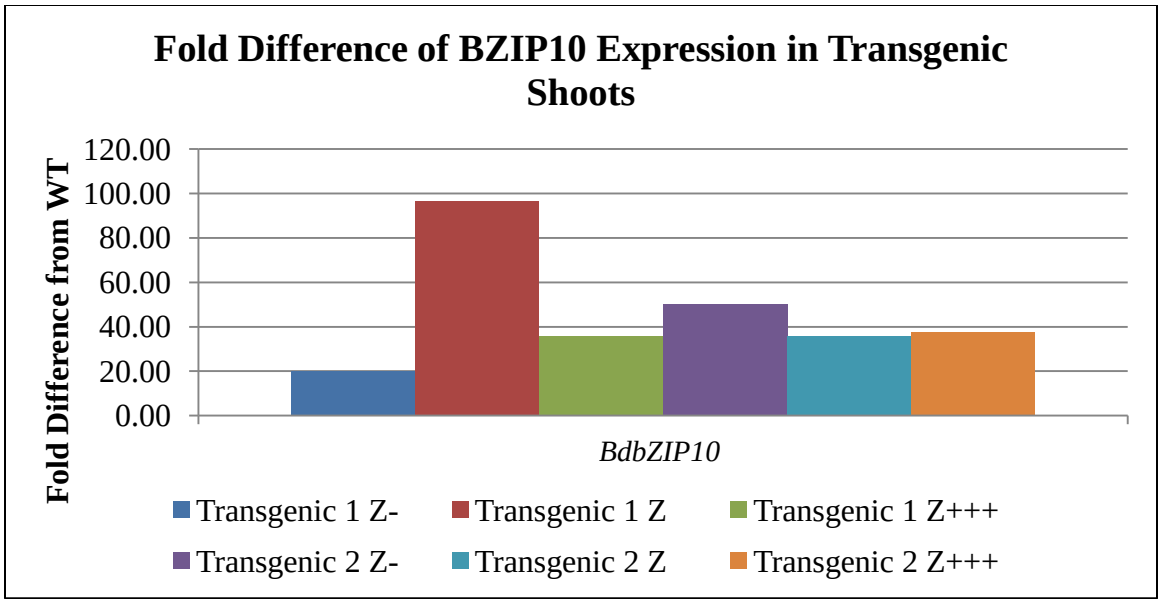

Figure 14. Graph comparing fold difference of gene expression of transgenic shoots to wildtype shoots grown in various Zn conditions

Discussion

Initial experiments were performed to set up a hydroponics system and identify the best nutrient solution for growing *Brachypodium* hydroponically. Good aeration of the hydroponic solution is essential for good plant growth. Initially, fish pumps and round aeration stones were used but were later switched to an inline air supply and 6-inch long aeration stones (2 per container), where I could increase the aeration rate and provide more even aeration over the entire area of the container. Moss can be a problem in hydroponics, so all containers were sprayed black on the outside to prevent moss from growing. Three hydroponics nutrient solutions were tested for their ability to grow healthy WT *Brachypodium* plants including a modified Hewitt 1966 solution as published in Barhoumi et al., 2010; a modified Hoagland solution as published by Yordem et al. in 2011; and the third solution which is based on Hoagland and Arnon, 1950's complete nutrient solution with a modification in the iron stock following the protocol of Philip Stoddard's lab at FIU found at the following website: (www2.fiu.edu/~efish/lab_business/Protocols_and_recipes/Fish_and_pool_care/Hoagland%20solution.doc.). Based on early experiments, *Brachypodium* grew best and were able to produce lots of healthy seed under our greenhouse conditions in the Stoddard's lab Hoaglands solution with the modified iron stock. Changes in pH are common during hydroponic growth and can affect the uptake of micronutrients. To control the pH of the hydroponic solution, the solution was adjusted to pH 6.0 and then MES-KOH (buffered at pH 6.0) was added for a final concentration of 0.4mM MES-KOH. The pH of the nutrient solution remained constant during the week. This hydroponics system was used for all zinc-related experiments.

There were no observable phenotypic differences between wildtype and transgenic plants grown under different zinc conditions within the first seven days after initiation of experimental

conditions. However, there were changes in gene expression. Further research is needed to determine if extended growth under Zn- or Z+++ conditions would cause observable phenotypic differences or changes in growth rates. Timelines for these studies were based on a previous paper that looked at zinc and iron regulated transporters in maize (Li et al., 2013). Previous nitrogen and salinity studies on *Brachypodium distachyon* where observable effects on growth were observed were conducted for longer periods of time (Barhoumi et al., 2010). Other plants like *Arabidopsis*, rice and wheat that reported noticeable phenotypic differences were also conducted for longer than 7 days (Impa et al., 2013; Rengel and Romheld, 2000; Conn et al., 2013; Assuncao et al., 2010; Yamaji et al., 2013). However, looking at gene expression, one would expect to see genotypic changes much earlier than the phenotypic changes.

The preliminary results showed similarities to published research in *Arabidopsis* where they found higher expression levels of *AtbZIP23* and *AtbZIP19* (homologs of *BdbZIP10*) in roots when plants were grown in Zn deficient conditions compared to plants grown on normal or high levels of zinc. They also reported higher expression of *AtZIP4* in seedlings of *Arabidopsis* grown in Zn deficient conditions (Grotz et al., 1998) and I found higher levels of *BdZIP4* in shoots and roots of WT grown in Zn deficient conditions. These preliminary results are also supported by results found in maize (Li et al., 2013) where expression of *ZmZIP4* was shown to be decreased during early time points of Z- conditions, but as they sampled over 24, and 48 hours, *ZmZIP4* was gradually increasing and at 96 hours, the expression of *ZmZIP4* was actually elevated in shoots grown in Z- conditions compared to control conditions. In their studies, *ZmIRT-1* was strongly induced in the shoots from plants grown in the presence of excess zinc, increasing gradually over time, but expression levels in the roots were elevated early on, but not to the extent seen in the shoots. In my studies, there was only a two fold increase in *BdIRT-1* expression in shoots growing under Z- conditions.

It is interesting to look at the expression of the two genes that are most affected by Z- and Z+++ conditions in the transgenic plants compared to the WT plants. Both of these genes are increased above the relative levels found in the WT plants in both transgenic plants, indicating that their expression may be enhanced by the overexpression of the *bZIP10* gene. In the future, I hope to have data to look at other time points and other genes in the zinc pathway to try to develop a more complete picture of what is happening in the WT and transgenic plants. It will also be interesting to test earlier time points to determine the expression patterns leading up to what was observed at the 7 day time point.

In conclusion, my preliminary results indicate that the developed hydroponics system is working to grow viable plants that produce seed. It will be interesting to see how the other members of the gene families examined here, and other gene families like the heavy metal ATPase (HMA) and zinc-induced facilitator-like (ZIFL) genes will respond to different levels of zinc. Another aspect to be examined includes seeing if TR plants show differential expression compared to WT plants under various Zn concentrations at different collection times as was seen in other plants.

References

Alloway, J. (2009). Soil factors associated with zinc deficiency in crops and humans. *Environmental Geochemistry and Health*, 31, 537-548.

Assuncao, A.G., Herrero, E., Lin, Y., Huettel, B., Talukdar, S., Smaczniak, C., Immink, R.G.H., van Eldik, M., Fiers, M., Schat, H., Aarts, M.G.M. (2010). *Arabidopsis thaliana* transcription factors bZIP19 and bZIP23 regulate the adaptation to zinc deficiency. *Proceedings of the National Academy of Science of the United States of America*, 107 (22), 10296-10301.

Cakmak, I., Ekiz, H., Yilmaz, A., Torum, B., Koleli, N., Gultekin, I., Alkan, A., Eker, S. (1997). Differential response of rye, triticale, bread and durum wheats to zinc deficiency in calcareous soils. *Plant and Soil*, 188, 1-10.

Conn, S.J., Hocking, B., Dayod, M., Xu, B., Athman, A., Henderson, S., Aukett, L., Conn, V., Shearer, M.K., Fuentes, S., Tyeerman, S.D., Gilliham, M. (2013). Protocol: optimising hydroponic growth systems for nutritional and physiological analysis of *Arabidopsis thaliana* and other plants. *Plant Methods*, 9, 4-14.

Draper, J., Mur, L.A.J., Jenkins, G., Ghosh-Biswas, G.C., Bablak, P., Hasterok, R., Routledge, A.P.M (2001). *Brachypodium distachyon*. A New Model System for Functional Genomics in Grasses. *Plant Physiology*, 127, 1539-1555.

Giri, J., Vij, S., Dansana, P., Tyagi, A. (2011). Rice A20/AN1 zinc-finger containing stress-associated proteins (SAP1/11) and a receptor-like cytoplasmic kinase (OsRLCK253) interact via A20 zinc-finger and confer abiotic stress tolerance in transgenic *Arabidopsis* plants. *New Phytologist*, 191, 721-732.

Glover-Cutter, K.M., Alderman, S., Dombrowski, J.E., Martin, R.C. (2014). Enhanced oxidative stress resistance through activation of a zinc deficiency transcription factor in *Brachypodium distachyon*. *Plant Physiology*, 166 (3), 1492-1505.

Grotz, N., Fox, T., Connolly, E., Park, W., Guerinot, M., Eide, D. (1998). Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency. *Proceedings of the National Academy of Sciences*, 95, 7220-7224.

Hacisalihoglu, G., Kochian, L.V. (2003). How do some plants tolerate low levels of soil zinc? Mechanisms of zinc efficiency in crop plants. *New Phytologist*, 159, 341-350.

Hong, S., Seo, P.J., Yang, M., Xiang, F., Park, C. (2008). Exploring valid reference genes for gene expression studies in *Brachypodium distachyon* by real-time PCR. *BMC Plant Biology*, 8, 112-122.

Hu, H., Sparks, D. (1991). Zinc Deficiency Inhibits Chlorophyll Synthesis and Gas Exchange in 'Stuart' Pecan. *HortScience*, 26 (3), 267-268.

Impa, S.M., Johnson-Beebout, S.E. (2012). Mitigating zinc deficiency and achieving high grain Zn in rice through integration of soil chemistry and plant physiology research. *Plant Soil*, 361, 3-41.

Li S., Zhou X., Huang Y., Zhu L, Zhang S., Zhao, Y., Guo, J., Chen, J., Chen, R. (2013). Identification and characterization of the zinc-regulated transporters, iron-regulated transporter-like protein (ZIP) gene family in maize. *BMC Plant Biology*, 13: 114.

Livak, K.J., Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* 25 (4): 402-408.

Martinka, M., Vaculik, M., Lux, A., (2014). Plant Cell Responses to Cadmium and Zinc. *Applied Plant Cell Biology* Plant Cell Monographs, 22: 209-246.

Olsen, L.I., Palmgren., M.G. (2014). Many rivers to cross: the journey of zinc from soil to seed. *Fronteirs in Plant Science*, 5, 1-6.

Reichman, S.M (2002). The Responses of Plants to Metal Toxicity: A review focusing on Copper, Manganese and Zinc. *Australian Minerals & Energy Environment Foundation*. 1-54.

Rengel, Z., Romheld, V. (2000). Root exudation and Fe uptake and transport in wheat genotypes differing in tolerance to Zn deficiency. *Plant and Soil*, 222, 25-34.

Ricachenevsky, F.K., Sperotto, R.A., Sperb, E.R., Lopes, K.L., Fett, J.P. (2011). ZINC-INDUCED FACILITATOR-LIKE family in plants: lineage-specific expansion in monocotyledons and conserved genomic and expression features among rice (*Oryza sativa)* paralogs. *Plant Biology*, 11: 20-41.

Robinson, M.M., Smid, M.P.L., Wolyn, D.J. (2006). High-quality and homogenous *Arabidopsis thaliana* plants from a simple and inexpensive method of hydroponic cultivation. *Canadian Journal of Botany*, 84, 1009-1012.

Sasaki, A., Yamaji, N., Ma, J.F. (2014). Overexpression of *OsHMA3* enhances Cd tolerance and expression of Zn transporter genes in rice. *Journal of Experimental Botany*, 65 (20), 6013-6021.

Sinclair, S.A., Kramer, U. (2012). The zinc homeostasis network of land plants. *Biochimica et Biophysica Acta*, 1823, 1553-1567.

Tavallali, V., Rahemi, M., Maftoun, M., Panahi, B., Karimi, S., Ramezanian, A., Vaezpour, M. (2009). Zinc influence and salt stress on photosynthesis, water relations, and carbonic anhydrase activity in pistachio. *Scientia Horticulturae*, 123, 272-279.

Yamaji, N., Xia, J., Mitani-Ueno, N, Yokosho, K., Ma, J.F (2013). Preferential Delivery of Zinc to Developing Tissues in Rice Is Mediated by P-Type Heavy Metal ATPase OsHMA2. *Plant Physiology*, 162, 927-939.

Yordem, B.K., Conte, S.S., Ma, J.F., Yokosho, K., Vasques, K.A., Gopalsamy, S.N., Walker, E.L. (2011). *Brachypodium distachyon* as a new model system for understanding iron homeostasis in grasses: phylogenetic and expression analysis of Yellow Stripe-Like (YSL) transporters. *Annals of Botany*, 108, 821-833.

You, F.M., Huo, N., Yong, Q.G., Luo, M., Ma, Y., Hane, D., Lazo, G.R., Dvorak, J., Anderson, O.D. (2008). BatchPrimer3: a high throughput web application for PCR and sequencing primer design. *BMC Bioinformatics*, 9, 253.