MOLECULAR IDENTIFICATION OF DIFFERENTIALLY EXPRESSED ZINC RELATED GENES IN CULTIVATED BREAD WHEAT

By

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ABSTRACT

Zinc (Zn) is an essential micronutrient required for adequate growth of plant species. Zinc is particularly needed for structural and functional integrity of enzymes and biological membranes and directly involved in synthesis of protein. Consequently, Zn deficiency results in severe decreases in growth and yield. Among the crop species, wheat is very sensitive to Zn deficiency. There is very limited information on the molecular mechanisms affecting expression of high Zn deficiency tolerance. Our objective in this study is, therefore, to identify the differentially expressed cDNA fragments in response to varying levels of Zn applications in a tolerant cultivated wheat genotype. For this purpose, we performed a screening experiment by using a number of cultivated bread wheat genotypes which displayed a considerable variation in response to Zn deficiency. Among various modern bread wheat genotypes tested, Bezostaja was selected as the most tolerant genotype. mRNA differential display method has been used to study the expression profile of Bezostaja genotype exposed to different Zn treatments. We observed 20 differentially expressed cDNA bands by using mRNA differential display. Out of 20 cDNA fragments that were isolated, cloned and sequenced, 14 cDNAs displayed similarity with previously identified metal Zn binding proteins and enzymes such as; alcohol dehydrogenase, cystathionine gamma synthase, and cation diffusion facilitator family transporter containing protein.

Keywords: Zn-responsive genes, Zn deficiency, bread wheat, mRNA differential display

ÖZET

Çinko (Zn) bitki türlerinin büyümesi için mutlak gerekli bir mikro besin elementedir. Çinko özellikle biyolojik membranların ve enzimlerin yapısal ve işlevsel bütünlüğü için gerekmektedir. Dolaysıyla, Zn eksikliğinde bitkilerin büyüme ve gelişmesinde şiddetli azalmalar ortaya çıkar. Bitki türleri içinde buğday Zn eksikliğine karşı çok duyarlı bir tür olarak bilinir. Bitkilerin Zn eksikliğine dayanıklılığını belirleyen moleküler mekanizmalar hakkında çok az bilgi bulunmaktadır. Burada sunulan tez çalışmasının amacı seçilmiş bir modern ekmeklik buğday genotipinde değişik dozlardaki Zn uygulamaları sonucu farklı olarak ifade edilen cDNA parçalarını incelemekti. Bu amaç doğrultusunda, belirli sayıda modern ekmeklik buğday genotipi kullanılarak bir tarama çalışması gerçekleştirildi. Bu tarama çalışmasında Zn eksikliğine dayanıklılık açısından genotipsel olarak kayda değer bir varyasyon gözlemlendi. Tüm ekmeklik modern buğday genotipleri arasından, çinko eksikliğine en dayanıklı genotip olarak Bezostaja belirlendi.

Bu çalışmada PCR'a dayalı DNA işaretleyici yöntemlerinden mRNA differential display metodu kullanılarak, farklı çinko doz uygulamaları sonucunda seçilen ekmeklik buğday genotipinin ifade profili incelenmiştir. Bu metod aracılığı ile, 20 farklı ifade edilen cDNA parçası gözlemlenmiştir. İzole edilen, klonlanan ve dizilimleri bulunan bu 20 cDNA parçasından 14'ü daha önceden belirlenmiş, alkol dehidrogenaze, cystathionine gamma synthase, ve cation diffusion facilitator family transporter içeren protein gibi bazı protein dizilimlerine benzerlik göstermiştir.

Anahtar sözcükler: Zn ile ilişkili genler, Zn eksikliği, ekmeklik buğday, mRNA differential display

To my family with all my heart

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
Amp	Ampicillin
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BLAST	Basic local alignment search tool
C	Carbon
CA	Carbonic anhydrase
Cd	Cadmium
cDNA	Complementary Deoxyribonucleic acid
CDF	Cation diffusion facilitator
DEPC	Diethyl pyrocarbonate
EDTA	Ethylene diamine tetra-acetic acid
GSH	Glutathione
Hg	Mercury
IAA	Indole-3-acetic acid
IPTG	Isopropyl β-D-Thiogalactopyranoside
LB	Luria bertani
PC	Phytochelatin
PS	Phytosiderophores
PSI	Photosystem-I
PSII	Photosystem-II
PCR	Polymerase chain reaction
QRT-PCR	Quantitative Real-time Polymerase Chain Reaction
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RuBPC	Ribulose 1,5-biphosphate carboxylase
RT	Reverse transcription
N	Nitrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
O	Oxygen

S	Sulphur
SOD	Superoxide Dismutase
TM	Transmembrane
X-Gal	5-brom-4-chloro-3-indolyl-beta-D-galactopyranoside
ZIP	ZRT-like and IRT-like Proteins
Zn	Zinc

1 INTRODUCTION

Zinc is an essential trace element that is required for the appropriate growth of humans, animals and plants. Small but at the same time critical concentrations of zinc are needed for humans, animals and plants in order to prevent the impairments in number of physiological process and cellular functions (Marschner, 1995). Increasing evidence is available showing that Zn deficiency is a critical micronutrient deficiency problem for human beings and crop production. During the past 20-30 years, widespread cultivation of high yielding cultivars and high input cropping systems with monotonous cropping has induced depletion of Zn in soils and development of Zn deficiency in crop plants (Cakmak, 2002). Compared to the traditional crops, many of the novel crop varieties are much more sensitive to zinc deficiency. The rise of fertilizer utilization, specifically phosphorus fertilization induces zinc deficiency (Alloway, 2004).

There is huge need for increases in food production to meet food demand of the growing world population, especially in the developing countries such as India, China, Pakistan and several African countries. Together with other micronutrient deficiencies Zn deficiency represents an important constraint to crop production (Alloway, 2004). It has been estimated that nearly the half of the cereal-cultivated soils globally have Zn deficiency problem (Graham and Welch, 1996). Zinc deficiency is a global nutritional occurring not only in developing countries but also in majority of the states in the USA, parts of Europe, Australia (Hotz and Brown, 2004). Zinc deficiency is also a critical nutritional problem in soils and crop plants, affecting seriously crop production especially in Central Anatolia (Cakmak et al., 1996; 1999).

Thus, solutions are needed to minimize Zn deficiency related problems in crop production and human health. One important solution to the problem is to develop new genotypes having high capacity to tolerate Zn-deficiency and accumulate high amounts of Zn

in grain. For a successful development of Zn efficient genotypes information is needed on the physiological and molecular mechanisms affecting expression of Zn deficiency tolerance and deposition of Zn in grain. In this study, using a Zn-deficiency tolerant wheat genotype, a qualitative PCR based method has been conducted to gain information on the molecular mechanisms involved in expression of high Zn deficiency tolerance.

2 OVERVIEW

2.1 Form and Function of Zinc in Plants

Firstly Raulin in 1869 observed that a common bread mold; *(Aspegillus niger)* is not able to grow in a Zn deficient medium. That experiment was the first identification of the biological role of Zn. Later on, in both animal and plant tissue, Zn was understood to be involved in various metabolic processes. These findings initiated research on the role of Zn in crop production, and in 1914, Zn deficiency was firstly shown in plants (Maze, 1914). Significance of Zn as being an essential element for plants is demonstrated by Sommer and Lipman (1926). Adverse impacts of Zn deficiency on crop production is now characterized as one of the most common and significant micronutrient deficiencies.

Zinc is mainly taken up as a divalent cation (Zn^{2+}). In long-distance transport in the xylem Zn is either transported as divalent cation (Zn^{2+}) or as chelated with organic acids. Like in xylem Zn probably makes complex with low-molecular-weight organic solutes in the phloem sap (Kochian, 1991) where the Zn concentrations are pretty high. In leaves, the majority of Zn is found in the form of storage metalloproteins, free ions, low molecular weight complexes, and finally found as integrated with cell wall with being insoluble. Ligand formation or by complexation with phosphorous (Olsen, 1972) leads to the inactivation of Zn inside the cell. From 58% to 91% of plant Zn may be soluble displaying variance between different plant species (Brown et al, 1993). The physiologically active portion of Zn is constituted by that water-soluble Zn section that is also considered to be the indicator of total Zn status. The low molecular weight complexes of Zn are the most active and frequently the most predominant and ample forms of Zn (Alloway, 2004).

2.2 Low Molecular Weight Complexes and free Zn

Only a small portion of soluble Zn is found to be available as free Zn ions. Zinc is generally found to be associated with low molecular weight anionic complexes. Soluble zinc is substantially present as an anionic compound and contingently attached to amino acids in plant leaves. For instance, in lettuce, reducing sugars, amino acids and sulphur compose the soluble Zn portion (Walker and Welch, 1987). Moreover, in leaf tissues of tomato, free Zn ion level only forms the 5.8 % of total Zn that is low (Bowen et al, 1962). The cell wall is suggested to be involved in controlling the activity of free Zn. The relative tolerance of various species in response to abundant Zn is found to be associated with affinity of cell wall extracts for free Zn. (Turner, 1970). Diverse of cell wall constituents including cellulose, hemicellulose, and lignin have a high binding affinity to Zn (Torre et al. 1991). In accordance with this, 90% or more of the total Zn in roots is assumed to be adsorbed in the apoplast of cortical and rhizodermal cells (Schmid et al. 1965). There are, however, controversial results in literature on the physiological importance of the binding of Zn to cell wall (Wainright and Woolhouse, 1978).

Low molecular weight Zn complexes may make Zn as physiologically active macromolecules if these complexes are present in considerable amounts. Easy degradation of the low molecular weight Zn complexes provides the physiological effectiveness of Zn. Moreover, Zn that is associated with enzymes is also considered as ‘physiologically active (Olsen, 1972; Cakmak et al., 1997). Although low molecular weight Zn-ligands do not possibly have enough specificity or activity to perform a considerable catalytic function in higher plants (Walker and Welch,1987) these complexes may have catalytic activities like amide hydrolysis by Cu and Zn (Groves and Dias,1979).

Finally, low molecular weight ligands may function in detoxification of Zn. Phytochelatins that are isolated by Grill et al. (1985) are one of the examples of diverse ligands which may behave as a buffer system for absorbing the redundant metal concentrations in the cell. Phytochelatins that are synthesized against the excess levels of heavy metals including Zn, Cd, and Hg are ‘low molecular weight metal-binding peptides’ identified in a broad range of species.

2.3 Zinc Containing Enzymes

Zinc establishes tetrahedral complexes with N-, O- and especially with S-ligands which are involved in metabolic functions of Zn. Via both its catalytic and structural function, Zn greatly influences various enzymatic reactions. Zinc atom is connected to four ligands; one of which is the water molecule with three other amino acids including generally the histidine (His), glutamine (Glu) and asparagine (Asp) in enzymes associated with the catalytic role of Zn. Zinc atoms are attached to four S- groups of cysteine residues with a high stable tertiary structure in enzymes that are associated with structural roles of Zn such as the proteins taking Zinc has been shown to have a significant function in various important enzyme systems (Srivastrava and Gupta,1996) that are;

- Carbonic anhydrase ,
- Several dehydrogenases: alcohol dehydrogenase, glutamic dehydrogenase, L - lactic Dehydrogenase malic dehydrogenase, D - glyceraldehyde - 3 - phosphate dehydrogenase, and D - lactate dehydrogenase,
- Aldolase,
- Carboxypeptidase,
- Alkaline phosphatase,
- Phospholipase
- Superoxide dismutase (converts superoxide radicals to hydrogen peroxide and water),
- RNA polymerase
- Ribulose bi - phosphate carboxylase (significant role in formation of starch)

Alcohol dehydrogenase enzyme is the enzyme that contains two Zn atoms per molecule. One of the Zn atom possesses the catalytic role and the other atom is associated with structural function (Coleman, 1992).The alcohol dehydrogenase enzyme is responsible for reducing the acetaldehyde to ethanol. Under aerobic conditions, the ethanol formation generally occurs in meristematic tissues like root apices in higher plants. The activity of the alcohol dehydrogenase is observed to be reduced in response to Zn deficiency in plants.

In Cu-Zn-Superoxide dismutase (Cu/Zn-SOD) Zn has structural function while Cu is associated with a catalytic role. Consequently, Zn deficiency reduces activity of SOD activity in biological systems and resupply of Zn to Zn-deficient tissues re-activate enzyme (Vaughan et al. 1982). The decline in the activity of SOD in response to Zn deficiency is substantial resulting in the membrane damage by free radicals. The production of O_2^{-1} (superoxide radical) is observed to be enhanced with the decrease in SOD activity. The peroxidation of membrane lipids occur due to the excess level of superoxide radicals and other free radicals produced from O_2^{-1} such as hydroxyl radical, $OH^{\cdot}$ (Cakmak and Marschner, 1988 a,b).

2.4 Physiological Functions of Zinc

2.4.1 Carbohydrate Metabolism

2.4.1.1 Photosynthesis

Photosynthesis and sugar transformations regarding the carbohydrate metabolism are influenced by the Zn status of plants.

Depending on the severity of Zn deficiency and the type of plant species, the net photosynthesis may be diminished by 50% to 70% (Alloway, 2004). . One of the photosynthetic enzymes affected by Zn deficiency is carbonic anhydrases (CA). Its decline under Zn deficiency is one major reason for the Zn deficiency-induced photosynthesis (Marschner, 1995). Compared to the monocotyledons, dicotyledons possess a larger CA molecule incorporating more Zn; six Zn atoms per molecule (Tobin, 1970). In response to Zn deficiency stress, a pronounced reduction of the CA activity is observed (Ohki, 1976). The decline in the CA activity influences also the carbon dioxide assimilation pathway. Carbonic anhydrase is regarded to take place in photosynthesis of C4 plants but the role of the CA in C3 plants which possess the simplest mechanism of photosynthesis is uncertain. Thus, although Zn deficiency is observed to diminish the photosynthesis in all plants, the significance of the CA contribution to that reduction is not same for C3 and C4 plants.

Ribulose 1, 5 – biphosphate carboxylase (RuBPC) is another Zn associated enzyme that takes place in photosynthesis. The enzyme is involved in photosynthesis via catalyzing the initial step of carbon dioxide fixation. The activity of RuBPC is found to decrease in response to Zn deficiency in navy bean (Brown et al, 1993).

The decline in chloroplast content in addition to the abnormal structure of chloroplast is the other factors that lead to the decrease in the rate of photosynthesis. Certainly, a peroxidative damage to chloroplast constituents by Zn-deficiency induced free radicals would be a further reason for decline in photosynthesis (Marschner and Cakmak, 1989; Marschner, 1995).

2.4.1.2 Protein Metabolism

In Zn deficient plants, protein synthesis is severely inhibited resulting in very low levels of total amount of protein. Consequently, a rise in concentration of free amino acids measured by HPLC is increased in the leaves of bean plants in response to Zn deficiency. When the bean leaves are resupplied with Zn, a very distinct decrease is observed in the concentration of free amino acids within 48 hours (Cakmak et al., 1989). The concurrent rise in protein concentration is associated with that decrease in free amino acid levels in response to Zn re-supply, indicating a direct role of Zn in protein biosynthesis (Cakmak et al., 1989). There is also a pronounced decline in RNA concentrations and severe deformations of ribosomes under Zn deficiency that can also impair protein synthesis (Prask and Plocke, 1971; Kitagishi and Obata, 1986). In response to Zn deficiency, the RNA level and the free 80S ribosomes are found to be remarkably declined in the rice seedlings' meristem tissue (Kitagishi et al., 1987).

Zinc is essential for the activity of RNA polymerase (Falchuk et al., 1978; Jendrisak and Burgess, 1975). In higher plants, the activity of RNase is observed to be enhanced in Zn deficient conditions, and the enzyme activity is observed to be reduced when Zn is present (Dwivedi and Takkar, 1974). Accordingly, the reduction in RNA level is one important consequence of zinc deficiency stress in plants. Nevertheless, in rice and pearl millet seedlings, before the rise in RNase level, the decline in RNA level can be observed

(Seethambaram and Das, 1984). Thus, compared to the its effect on RNase activity, Zn deficiency seems to influence more the biosynthesis of RNA. The meristematic tissues where active synthesis of proteins occurs need high concentrations of Zn (Brown et al., 1993). Zinc is also associated with providing stability and function of genetic materials in protein metabolism (Alloway, 2004).

2.4.2 Membrane Integrity

Both in animals and plants Zn is thought to be associated with membranes. Compared to the Zn sufficient conditions, huge amount of ^{32}P leakage is observed from the roots of wheat in Zn deficient conditions (Welch et al., 1982), indicating higher membrane permeability. Moreover enhanced root exudation of K^+, sugars, amino acids and phenolics is observed in Zn deficient plants (Cakmak and Marschner, 1988a). The leakage of compounds from Zn-deficient root is declined in response to the twelve hour resupply of Zn to deficient plants. Thus, Zn has been suggested to have a critical role in maintaining the structural and functional integrity of cell membranes (Welch et al., 1982; Cakmak and Marschner, 1988a).

Via its interaction with phospholipids and membrane protein sulfhydryl groups (Chvapil, 1973), Zn is considered to be necessary for strengthening the biomembranes. The earliest biochemical alteration observed in Zn deficient animal cells is the impairment of membrane integrity (Bettger and O'Dell, 1981). The detoxification and the production of free radicals that destroy the sulfhydryl groups and membrane lipids are catalyzed by Zn and Zn-containing enzymes such as SOD, in addition to its structural role as being a component of biomembranes. In Zn deficient conditions, great level of superoxide radical is detected in plant roots (Cakmak and Marschner, 1988 b,c). The effect of O_2^{-1} in terms of membrane damage is found to be inhibited by the presence of Zn. Besides its function in SOD, Zn has also an inhibitory effects on O_2^{-1} -generating NADPH oxidase (Cakmak and Marschner, 1988bc; Cakmak, 2000). By increasing SOD activity and inhibiting O_2^{-1} -generating NADPH oxidase activity, Zn protects cell membranes from peroxidative attack of free radicals (Cakmak, 2000).

Zn deficiency also results in a decline in the activity of the catalase enzyme that has a function of scavenging the H_2O_2 (Cakmak and Marschner, 1988c). Thus, via O_2^{-1} or O_2^{-1} derived harmful radicals; 'peroxidative damage' of biomembranes is a typical phenomena occurring under Zn deficiency. Accordingly, the leakage of the organic and inorganic substances from root cells is considered to be related to the membrane damage (Welch et al., 1982; Cakmak and Marschner, 1988a).

2.4.3 Auxin Metabolism

Zinc nutritional status of plants greatly affects phytohormone metabolism of plants (Brown et al., 1993). Zinc is known for its well-described role in synthesis of the indole acetic acid hormone (IAA), a natural auxin hormone. Reduction in level of auxine hormone is associated with the characteristic morphological changes in Zn deficient plants such as stunted growth and little leaf. The increased degradation of IAA or the prevention of IAA synthesis may be responsible for the low levels of IAA in plants that are Zn deficient (Marschner, 1995). For biosynthesis of IAA, tryptophan is found to be the most probable precursor, and the evidence available in literature indicates that Zn is needed for biosynthesis of tryptophan (Brown et al., 1993). However, according to Cakmak et al (1989) low levels of IAA in Zn-deficient plants is not a result of inhibited IAA biosynthesis; it is rather a result of IAA degradation by Zn deficiency-induced free radicals.

2.4.4 Reproduction

In peas, beans and other plants, seed production and flowering are observed to be declined in response to Zn deficiency (Brown et al., 1993). Rather than the size of the seed or dry matter production, the number of the inflorescence and yield of seed are enhanced when the Zn deficient subterranean clovers are re-supplied with Zn. (Riceman and Jones, 1959). The damage to the anther and pollen grain physiology and development in addition to the accrued formation of abscissic acid associated with premature loss of leaves and flower buds are considered to be the factors that lead to the decline in seed generation in response to Zn deficiency. The uncommon pollen grains in addition to the development of small anthers are observed in wheat plants in response to Zn deficiency (Sharma et al., 1979).

2.5 Mechanisms of Zinc Uptake by Plants

Zinc is absorbed mainly in the form of Zn^{2+} from the soil by the plant roots. A thermodynamically passive transport of Zn towards a large electrical potential takes place across the plasma membrane (Kochian, 1993). Other than the Poaceae family, in dicotyledons and monocotyledons , the motive force that mediates the Zn transport with the help of the divalent cation channel is that plasma membrane negative electrical potential. In Poaceae, in response to the zinc or iron deficiency, the roots are able to release non-protein amino acids namely; 'phytosiderophores' or 'phytometallophores' (Marschner, 1995). These non-protein amino acids that make complex with Zn mediate the Zn transport to the outer part of the root plasma membrane in Poaceae family (Kochian, 1993). The transport of Zn and the phytosiderophore complex occurs with the help of a Zn- transporter protein. At high pH, Zn is taken up as Zn^{2+} or as $Zn(OH)_2$. Metabolic control and the direct root contact are the factors that determine the Zn uptake considering the low concentrations of Zn in the soil solution. Between the uptake of zinc and other micronutrients, a wide range of interactions occur such as between Zn and Cu or Zn and Fe. All these micronutrients interacting with each other are suggested to be taken up via the same carrier sites, meaning that the each micronutrient prevents uptake of another. Enhanced uptake and accumulation of Fe or Cd in Zn deficient plants seems to be a consequence of the competition for the same transporter protein (Hardt et al., 1998, 2002; Cakmak 2000).

The transportation of Zn takes place in the form of Zn^{2+} or in the form of complex with organic acids. Zn translocation takes place towards the shoot tissues in response to a requirement otherwise, the accumulation of Zn occurs in the root tissues. The partial translocation of Zn occurs towards the developing organs from the old leaves (Marschner, 1995; Alloway, 2004).

2.6 Zinc Deficiency Tolerance Mechanisms in Wheat

There are number of papers showing existence of a large genetic variation for Zn deficiency tolerance between and among the wheat species when grown on zinc deficient calcareous soils in Central Anatolia (Cakmak, 2000). When compared to bread wheat, durum wheat is particularly sensitive to Zn deficiency (Rengel and Graham, 1995; Cakmak et al., 1996). There is also an impressive genetic variation in tolerance to Zn deficiency among the bread wheat genotypes. From the cereal species durum wheat has been found to be the most susceptible cereal species whereas rye was found to be the most resistant cereal species in response to Zn deficiency. The order of increasing tolerance to Zn deficiency is; durum wheat < oat < bread wheat < barley < triticale (a rye and wheat cross) < rye (Cakmak et al., 1997).

It seems that there is not only a single mechanism affecting Zn deficiency tolerance to Zn deficiency. Several physiological mechanisms have been described in literature occurring both during root uptake of Zn and at cellular level in plant tissue (Cakmak et al., 1998). Generally, Zn deficiency tolerant and sensitive genotypes are not different in total concentration of Zn in tissue which may indicate differential utilization of Zn at cellular level (Cakmak et al., 1997). The increased activity of Zn-requiring enzymes such as Cu/Zn superoxide dismutase in addition to the carbonic anhydrase in wheat is found to be associated with Zn efficiency by Hacisalihoglu et al. (2003). Under insufficient Zn supply, Zn deficiency tolerant genotypes may succeed to continue the activity of these two enzymes in addition to other Zn-requiring enzymes. Between the Zn deficiency tolerance and root uptake of Zn with translocation of Zn from the root to shoot, no connection is detected by Hacisalihoglu et al. (2003).

The increased uptake of Zn via roots in addition to the cell level utilization of Zn are the main possible mechanisms that may constitute physiological basis of Zn deficiency tolerance although the efficiency mechanism is not fully understood (Cakmak, 2000). The increased activity of Zn-requiring enzymes such as Cu/Zn superoxide dismutase in addition to the carbonic anhydrase in wheat is found to be associated with Zn efficiency by Hacisalihoglu et al. (2003). Under insufficient Zn supply, Zn deficiency tolerant genotypes may succeed to continue the activity of these two enzymes in addition to other Zn-requiring enzymes.

Between the Zn deficiency tolerance and root uptake of Zn with translocation of Zn from the root to shoot, no connection is detected by Hacisalihoglu et al. (2003).

According to short term uptake experiments, differences in root uptake rate of Zn seem to be also important in differential expression of Zn deficiency tolerance between cereal species (Erenoglu et al., 1999). Differential response of maize, sorghum, rice, oat and wheat to Zn deficiency may be attributable to their roots' differential release of Zn mobilizing compounds; phytosiderophores (phytometallophores). The solubility and mobility of Zn are shown to be increased by release phytosiderophores from their roots in Zn deficient conditions of calcareous soils (Cakmak et al., 1994). It seems that the mechanisms affecting root uptake and seed deposition of Zn are different. Generally, high tolerance to Zn deficiency is not associated with correspondingly high concentrations of Zn in grain. In most cases, high Zn deficiency tolerance is ascribed to better utilization of Zn in tissue, not high Zn concentrations in tissue (Cakmak et al., 1998; 1999).

2.7 Differential Display Technique

At any given time, in any individual cell not all of the genes are expressed. All life processes including the development, differentiation, and homeostasis are determined according to the selective expression of genes. Alterations in gene expressions are the key factors that determine both the flow of normal development in addition to the pathological modifications in organisms including plant systems. Therefore, in different cell types or in response to changed conditions, the search is to find efficient methods for detecting and isolating differentially expressed genes.

Both the types and the quantities of mRNA and proteins reflect the activities of genes. There was need for a technique that possesses various properties such as the representation of the majority of mRNA in a cell, great reproducibility, the act of allowing side-by-side mRNA comparisons from various sources or conditions, being simple and quick, and the convenience for isolation of the genes (Croy and Pardee, 1983).

Differential display or DDRT-PCR name was given (Liang and Pardee, 1992; Liang et al., 1992; Liang et al., 1993) to the method that was developed based on the polymerase chain reaction. Differentially expressed genes associated with the mRNA can be easily detected via that elastic and sensitive method. The method allows the cloning of the selected DNA, after the detection of differentially expressed genes associated with the identification of mRNA species that display alterations in different types of eukaryotic cells or in same cells of different conditions in terms of absence or presence.

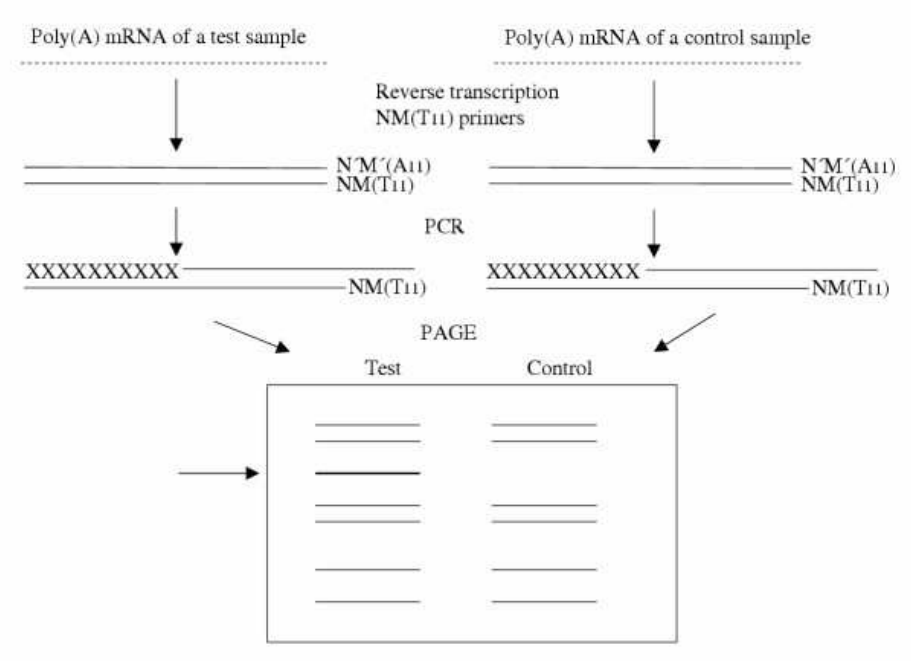

Figure 1: Schematic representation of differential display method

One of the various aims that the differential display technique can be utilized for is to elucidate the sub-groups of mRNAs, short cDNAs or the whole mRNA composition of cells. The sequencing of the selected cDNAs may be performed rapidly. The sequences obtained for each differentially mRNAs may then be compared with the known sequences from databanks. The differential display technique is advantageous since a little quantity of total RNA, little micrograms are sufficient for the utilization of the method. In addition to that, there is no need for waiting until the end of differential display procedure to detect any problem with the procedure because at every stage there is a chance to control the procedure.

In the literature, there are studies utilizing the differential display technique both in plant and other mammalian systems. For example, the effects of Cd on gene expression profile of a liverwort, *Lunularia cruciata* was studied via utilizing mRNA differential display method by Basile et al. (2005). They isolated and identified four genes that are altered associated with differential application of Cd on plants. In addition to that study, another study was also performed in which *Arabidopsis thaliana* plants were grown in the presence of Cd (Suzuki et al. 2001). In order to detect the influence of Cd on *Arabidopsis thaliana* gene

expression profile, they adopted the fluorescent differential display technique; known to be a strong method for visualizing the differential gene expression (Ito *et al*. 1994; Hara *et al*. 2000). Another study that is performed by Carginale et al.(2002) utilized the mRNA differential display method in order to observe the effects of under-lethal doses Cd on gene expression profile of Antarctic fish *Chionodraco hamatus*. They identified seven cDNA fragments that are altered when treated with cadmium. This method was also applied to durum wheat (Cebeci et al., 2006) to identify differential expression of wheat transcriptomes in response varying Cd concentrations. They identified NADH dehydrogenase subunit 1, PsaC gene encoding photosystem 1 genes. In the present study we have used same technique to study gene expression in a Zn deficiency tolerant wheat genotype grown in varying conditions of Zn.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Material

In the screening phase of the experiment, 15 bread wheat cultivars were used. All plant materials were provided by the BD International Agricultural Research Institute located in Konya.

3.1.2 Chemicals

The chemicals and kits used in this study are listed in the Appendix A.1.

3.1.3 Growth Media, Buffers and Solutions

The growth media, buffers, and solutions used in this study were prepared according to the protocols as outlined by Sambrook *et al.*, 2001.

3.1.4 Equipment

Equipments used in this research are listed in Appendix A.2.

3.2 Methods

3.2.1 Plant Growth Conditions and Zinc Treatments

3.2.1.1 Screening Experiment: Soil Culture

Zinc deficient soil (0.1 mg Zn kg^{-1} soil) that is provided from Eskisehir -Central Anatolia was used for the screening experiment. The soil had a ; pH of 8.04, with 14.9 %, $CaCO_3$, 0.69 %, organic matter 0.08 % salt and 60.6 % clay content. Before sowing the plant seeds, for preventing any possible element contamination, pots were washed with diluted HCl and rinsed with water for many times The seeds of plants were sown in plastic pots that had 1700 g soil. In each pot, nearly 15 seeds were sown but after the seedlings growth, they were declined to 10 per a pot. The same basal treatment of 200 mg N kg^{-1} soil in the form of $Ca(NO_3)_2$, 100 mg P kg^{-1} soil in the form of KH_2PO_4, 125 mg K kg^{-1} soil in the form of KH_2PO_4, 20 mg S kg^{-1} soil in the form of $CaSO_4.2H_2O$, 2.5 mg Fe kg^{-1} soil in the form of Fe-EDTA ($C_{10}H_{12}FeN_2NaO_8$) is was applied to the plants. Control plants were treated with Zn but stressed plants were not treated with Zn. Control plants were supplied with 5 mg kg^{-1} soil Zn in the form of $ZnSO_4.7H_2O$. Before sowing the seeds, the soil is mixed entirely with all nutrients. There were 3 replicates for each treatment. For every 5-6 days, the randomization of the pots was done. Plants were watered daily with deionized water. When the leaf symptoms of Zn deficiency became evident, the shoot parts of the 36-day old plants were harvested. In order to determine the element concentration and dry matter production, harvested plant shoots were dried at 70 ºC.

3.2.1.2 Nutrient Solution Experiments

The germination of the seeds was realized in a perlit medium with the addition of saturated $CaSO_4$. Following 6 days of germination, The seedlings have been transferred into 2.5 L plastic pots including the steady aerated nutrient solutions. The nutrient solution contained the following micro and macronutrients: 0.88 mM K_2SO_4, 2 mM $Ca(NO_3)_2$, 0.2 mM KH_2PO_4, 1.0

mM $MgSO_4$, 0.1 mM KCl, 100 µM Fe-EDTA, 1.0 µM H_3BO_3, 1.0 µM $MnSO_4$, 0.2 µM $CuSO_4$, and 0.02 µM $(NH_4)_6Mo_7O_{24}$. Plants were supplied with 5 different Zn applications in form of $ZnSO_4$. These treatments were; -Zn (very severe Zn deficiency), 10^{-8} M Zn (severe Zn deficiency), 10^{-7} M Zn (moderate Zn deficiency), 10^{-6} M Zn (adequate Zn supply), 10^{-4} M Zn (toxic Zn dose).

Plants are then allowed to be grown for 13 days in a growth chamber under controlled conditions (light/dark regime 16/8 h, temperature 24/22ºC, relative humidity 60/70%, and photon flux density of 600-700 µmol m^{-2} s^{-1}).

The root and shoot sections of plants were harvested separately when the leaf symptoms of Zn deficiency appeared on wheat plants. Harvested plants were immediately frozen in liquid nitrogen and kept at -80ºC until molecular analysis. To minimize nutrient contamination of root surfaces, roots were washed with deionized water before storage and then dried on a sterile filter paper.

3.2.2 Total RNA Isolation

With nearly 1.7 ml Biozol (Biogen), 350 mg leaf tissue was ground without liquid nitrogen. With liquid nitrogen, nearly 500 mg root tissue was grounded with 1.7 ml Biozol (Biogen). Into an eppendorf tube, around 1 ml of the sample ground in biozol was placed. While operating the other samples, the sample was allowed to be stored on ice. The samples were incubated at room temperature for 10 minutes after processing all the samples. 0.4 ml chloroform was added to the samples and the tubes were shaken and incubated at room temperature for 5 minutes. Then, the centrifugation of samples was done at 12,000 rpm for 15 minutes at 4ºC. To a fresh eppendorf tube, the upper layer incorporating RNA was transferred. For precipitating the RNA, 0.5 ml isopropanol was added to the samples after chloroform extraction. At room temperature, for 10 min, samples were incubated. After room temperature incubation, the samples were spun at 12,000 rpm for 10 min at 4ºC. Then with 1 ml 75% ethanol the RNA pellets were washed. Via vortexing, samples were mixed and then spun at 7,500 rpm for 5 min at 4ºC. At room temperature for 10 minutes, the RNA pellets were allowed to be dried. Finally, depending on the size of the pellet, 30-60 µl diethyl

pyrocarbonate (DEPC)-treated water (H_2O) or formamide was added to tubes containing the RNA pellets and for dissolving the pellets the samples were let to be incubated in the 55ºC heating block for an hour .

The concentration measurements of RNA samples at 260 nm wavelength were performed via using the NanoDrop spectrophotometer. For the subsequent molecular studies, the samples were kept either at -20 ºC for short-term or at -80 ºC for long-term storage.

3.2.3 Dnase- I Treatment

In order to get rid of any possible chromosomal DNA contamination, isolated RNAs were treated with Dnase I (Fermentas). 10 units of Dnase I enzyme was used for 50 µg of total RNA. The reactions that contained RNA samples, Dnase I, 1X Reaction Buffer including $MgCl_2$, were incubated at 37 ºC for 30 minutes.

Then, ethanol precipitation of RNA samples were performed via mixing RNA with 0.1 volumes of 3 M NaOAc at pH 5.2 and 2 volumes of cold 100 % ethanol. Then the reactions were allowed for incubation at -80 ºC for 1 hour or overnight. The samples were then centrifuged at 4 ºC and the supernatant was eliminated. With using 0.5 ml 70 % cold ethanol, the pellets were washed. For about 10 minutes, the pellets were let to be air-dried. The concentration measurements of RNA samples at 260 nm wavelength were performed via using the NanoDrop spectrophotometer. For the subsequent molecular studies, the samples were kept either at -20 ºC for short-term or at -80 ºC for long-term storage.

3.2.4 cDNA Synthesis

In order to check the quality of RNA in terms of the presence of degradation, 2% agarose gel was utilized for visualization. Then via using the Omniscript reverse transcription kit (Qiagen), the first strand of cDNA was synthesized. In order to use in reverse transcription reaction, OligodT primers were purchased from Invitrogen (0.5 µg /µl). In a 20 µl total volume, the reverse transcription reaction was performed in the presence of 1X Buffer RT,

0.5 mM dNTP mix, 0.5 µg oligo$(dT)_{12-18}$ primer, 10 u RNaseOUT^{TM} Recombinant Ribonuclease Inhibitor, 2 µg DNase I-treated RNA sample and 4 u Omniscript Reverse Transcriptase. At 37 ºC in water bath for about 120 minutes, the reactions were allowed for incubation, then the cDNA samples were kept at -20 ºC for subsequent molecular studies.

3.2.5 mRNA Differential Display

9 different P and T primers of differential display technique were purchased from Biogen. 20 µl PCR reactions were performed via using the synthesized cDNAs as template with utilizing different combinations of P and T primers. The sequences of "P" and "T" primers are listed in Table 1.

Table 1: Primers used in mRNA differential display

Primer designation	Sequence (5' -3')
P1	ATT AAC CCT CAC TAA ATG CTG GGG A
P2	ATT AAC CCT CAC TAA ATC GGT CAT AG
P3	ATT AAC CCT CAC TAA ATG CTG GTG G
P4	ATT AAC CCT CAC TAA ATG CTG GTA G
P5	ATT AAC CCT CAC TAA AGA TCT GAC TG
P6	ATT AAC CCT CAC TAA ATG CTG GGT G
P7	ATT AAC CCT CAC TAA ATG CTG TAT G
P9	ATT AAC CCT CAC TAA ATG TGG CAG G
T1	CAT TAT GCT GAG TGA TAT CTT TTT TTT TAA
T2	CAT TAT GCT GAG TGA TAT CTT TTT TTT TAC
T3	CAT TAT GCT GAG TGA TAT CTT TTT TTT TAG
T4	CAT TAT GCT GAG TGA TAT CTT TTT TTT TCA
T5	CAT TAT GCT GAG TGA TAT CTT TTT TTT TCC
T6	CAT TAT GCT GAG TGA TAT CTT TTT TTT TCG
T7	CAT TAT GCT GAG TGA TAT CTT TTT TTT TGA
T8	CAT TAT GCT GAG TGA TAT CTT TTT TTT TGC
T9	CAT TAT GCT GAG TGA TAT CTT TTT TTT TGG

Each PCR reaction components were; 0.7 µl (nearly 800 ng) first strand cDNA, 2 µl 10X PCR buffer (without $MgCl_2$), 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.25 µM of P primer , 0.25 µM of T primer, 0.5 unit Taq DNA polymerase (Promega). A DNA thermocycler GeneAmp PCR System 9700 (PE Applied Biosystems) was used in each of 20 µl reaction with conditions written in Table 2.

Table 2: PCR Reaction Conditions

1. Heating Lid	T = 105ºC	
2. Denaturation :	T = 94ºC	0:04:00 min
3.Non-specific annealing:	T = 40ºC	0:05:00 min
4. Extension:	T = 72ºC	0:05:00 min
5. Denaturation:	T = 94ºC	0:01:00 min
6.Non-specific annealing:	T = 40ºC	0:01:00 min
7. Extension:	T = 72ºC	0:05:00 min
8. GOTO 5 Repeat cycle 1 time		
9. Denaturation:	T = 94ºC	0:00:30 s
10. Annealing:	T = 58ºC	0:00:30 s
11. Extension:	T = 72ºC	0:02:00 min
12.GOTO 9 Repeat cycle 29 times		
13. Final elongation	T = 72ºC	0:07:00 min

The PCR products were separated via agarose gel electrophoresis utilizing 2% agarose gel and 0.5 X TBE buffer. For visualization of the cDNA fragments ethidium bromide staining was used.

3.2.6 DNA Gel Extraction from Agarose Gels

From 2% agarose gels, differentially expressed cDNA bands were excised with a clean scalpel. QIAquick gel extraction kit (Qiagen) was utilized for purification of the cDNA bands according to the manufacturer's protocol. Then the samples were eluted in 35-40 μL deionized autoclaved water. Then the absorbance of each cDNA fragments at 260 nm was determined via using a NanoDrop spectrophotometer. The samples were kept at -20 ºC.

3.2.7 Ligation

pGEM®-T Vector System I (Promega) was utilized in ligating the purified cDNA fragment. The 10-μL of reaction contained the 5μL 2X ligation buffer, 1-μL 50 ng vector, cDNA insert, 1 -μL 3 unit T4 ligase enzyme and water. The ligations via TA cloning were performed with both the insert-vector ratios of; 3:1 and 5:1. The manufacturer's protocol was performed for the application of ligation reactions. The ligation reactions were either allowed to be incubated for 1-2 hour at room temperature or overnight at 4 ºC if the maximum number of transformants was required.

3.2.8 Transformation

DH5α strain and TOP10F' of *Escherichia coli* chemically competent cells were used in transformation reactions. 5 μl of ligation reaction was gently put into 50 μl chemically competent cells and the mixture was incubated on ice for 20 minutes. The tubes that contain the sample mixture were incubated at 42 ºC for 50 seconds for heat shocking the cells. The samples were then incubated on ice for 2 minutes. 950 μL SOC medium was added to the samples and they were allowed to be incubated around 1.5 hour at 37 ºC. After the centrifugation of the bacterial cells at 5,000 rpm for about 3 minutes, the excessive amount of supernatant was eliminated. After the suspension of bacterial cells in around the remaining 150 μL SOC medium, bacterial cells were spread on LB plates that include ampicillin, IPTG, and X-Gal. Then at 37 ºC for 16-24 hours, the transformation plates were incubated.

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3.2.9 Colony Selection

By exploiting the blue/white selection property of the pGEM®-T Vector System I positive white clones were chosen. The selected colonies are allowed to be grown in another LB plates for plasmid isolation.

3.2.10 Colony PCR

In colony PCR reaction, the same combinations of primers utilized in amplification of cDNA fragments were exploited in order to verify the existence of the cDNA fragments in the expected sizes.

3.2.11 Plasmid Isolation

3 mL LB broth medium that incorporates 100 μg/mL ampicillin was used to incubate the selected white colonies. With shaking at around 270 rpm for 12-16 hours, the cells were incubated at 37 ºC. At room temperature, the centrifugation of the bacterial cells at 8,500 x *g* for around 3 minutes took place after the overnight incubation period. According to the manufacturer's protocol, plasmid isolations were performed via utilizing the QIQprep® Spin Miniprep Kit (Qiagen). The elution of the plasmid samples were finally performed in 35-40 μL autoclaved deionized water. Via using a NanoDrop spectrophotometer, the absorbance of samples at 260 nm was determined. The samples were kept at -20 ºC until the sequencing reactions.

3.2.12 Sequencing

Considering the differential expression of cDNA bands between plants that are exposed to different doses Zn applications, 20 clones were selected and sent to Mclab sequencing (USA) company for sequencing purpose.

4 RESULTS

4.1 Plant Growth and Zinc Concentrations

4.1.1 Screening,Greenhouse Experiment

Fifteen cultivated bread wheat genotypes were grown in soil culture in order to evaluate their Zn efficiency under Zn deficient conditions in greenhouse. By using the dry matter production of genotypes tested, Zn deficiency tolerance indices were calculated.

4.1.2 Dry Matter Production and Zn Deficiency Tolerance Index

Commonly, middle-aged or young leaves develop first slight chlorosis and then necrotic spots when plants suffer from Zn deficiency stress, indicating that Zn is not mobile in plants; the symptoms first appear in young leaves. The old leaves also become entirely chlorotic and short when the Zn deficiency becomes very severe. The leaves are then collapsed in the middle as the necrosis becomes more severe. Zinc deficiency stress was also associated with significant decreases in shoot growth. The necrotic spots on leaves were also detected in almost all genotypes. Among the genotypes tested, 00 KE, Çetinel-2000 and Dagdas genotypes were less affected from Zn deficiency, while the genotypes BDME-10, Bağcı, and Soyer-02 were particularly sensitive to Zn deficiency regarding the severity of Zn deficiency leaf symptoms.

The ratio of shoot dry weight in Zn deficient plants to the shoot dry weight in control plants with sufficient Zn supply is generally used as a parameter for Zn deficiency tolerance index. Based on this parameter there was a considerable variation among the 15 bread wheat genotype (Table 3). Considering the tolerance indices, Bezostaja, Dagdas and Alpu were evaluated as promising genotypes regarding their tolerance to Zn deficiency in controlled greenhouse conditions. Bezostaja genotype was found to be the most tolerant bread wheat considering its tolerance index. Bağcı, Karahan and Ahmetağa genotypes were found to be most susceptible genotypes based on their calculated tolerance indices. The dry matter production and Zn tolerance index of the plants are given in Table 3.

Table 3: The dry matter production and calculated Zn tolerance index of different bread wheat genotypes grown in greenhouse in a Zn deficient calcareous soil with +Zn and without (-Zn) supply for 35 days.

Dry matter production and Zn efficiency			
Genotypes	-Zn	+Zn	Zn Efficiency
	(g plant^{-1})		(%)
Bezostaya-1	0,54 ± 0,05	0,55 ± 0,03	99
Dağdaş	0,47 ± 0,04	0,49 ± 0,04	97
Alpu 01	0,48 ± 0,02	0,51 ± 0,03	95
Yakar	0,42 ± 0,00	0,46 ± 0,03	91
Çetinel 2000	0,44 ± 0,01	0,49 ± 0,02	91
03 SE 18 SEAÇ	0,49 ± 0,02	0,54 ± 0,03	90
ES-14	0,34 ± 0,02	0,40 ± 0,01	85
Yıldız 98	0,40 ± 0,02	0,48 ± 0,02	83
Ziyabey	0,39 ± 0,01	0,48 ± 0,02	83
00 KE 3 KEAÇ	0,47 ± 0,02	0,57 ± 0,02	82
Kırgız 95	0,46 ± 0,02	0,56 ± 0,03	82
İzmir 85	0,44 ± 0,04	0,57 ± 0,06	76
Soyer 02	0,40 ± 0,02	0,52 ± 0,03	76
BDME-10	0,37 ± 0,04	0,51 ± 0,01	74
Ahmetağa	0,41 ± 0,16	0,56 ± 0,05	73
Karahan	0,55 ± 0,10	0,91 ± 0,06	60
Bağcı	0,41 ± 0,01	0,69 ± 0,08	60
Average	0,44	0,55	82

In response to Zn deficiency, the average decrease in shoot dry matter was found to be 82%. Bezostaja, Dagdas and Alpu had tolerance indices of 99, 97, and 95%, respectively whereas in Bagci, Karahan and BDME-10 the tolerance indices were 60, 60 and 74 %, respectively. Preharvest pictures of extreme genotypes are shown in Figure 4 and 5.

Figure 2: Growth of Zn deficiency-tolerant cultivated bread genotypes on a Zn-deficient soil. Picture has been made before harvesting.

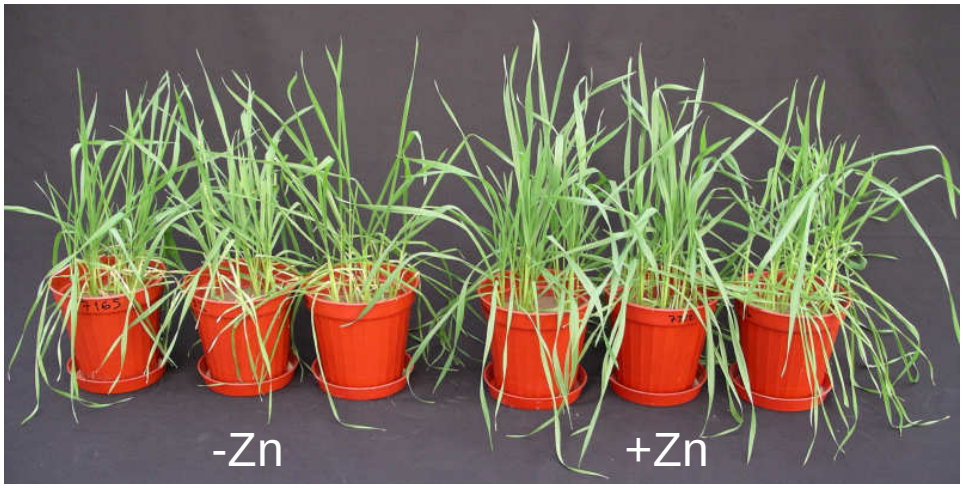


Figure 3: Growth of Zn deficiency-intolerant cultivated bread genotypes on a Zn-deficient soil. Picture has been made before harvesting.

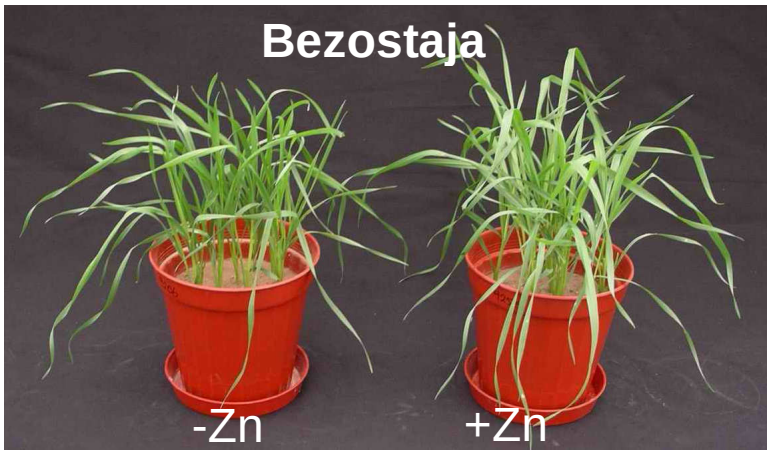


Figure 4: Growth of Zn deficiency-tolerant cultivated bread genotype; Bezostaja on a Zn-deficient soil. Picture has been made before harvesting.

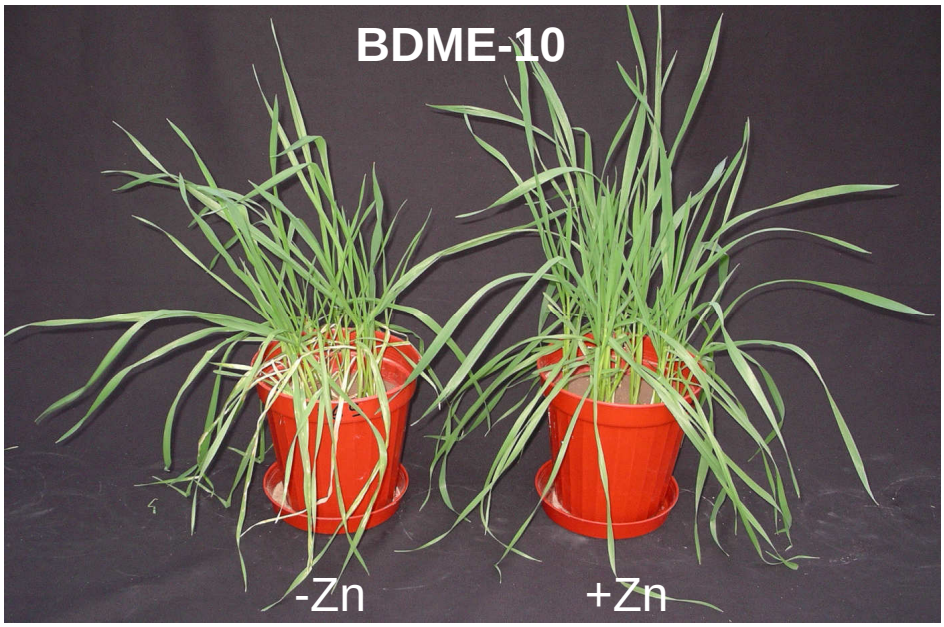


Figure 5: Growth of Zn-inefficient cultivated bread genotype, BDME-10 on a Zn-deficient soil. Picture has been made before harvesting.

Based on the results described above and considering the results from previous field trials (Kalayci et al., 1998), Bezostaja and BDME-10 were selected as a Zn-efficient and Zn-inefficient bread wheat genotypes. However, for the molecular studies, our focus was on Zn-efficient plants to identify gene and gene groups, thus they were grown in nutrient solution in a growth chamber with controlled environmental conditions.

4.1.3 Element Analysis

By using ICP/OES (inductively coupled plasma optical emission spectrometer), micronutrient concentration of plants have been measured ands here only the results of Zn and Fe concentrations were presented. The analysis was only performed for the shoot sections of the wheat plants. The shoot concentrations of Zn and Fe were shown in Table 4 in which the genotypes are ordered according to their decreasing tolerance indices.

Table 4: Shoot Zn and Fe concentrations of cultivated bread wheat genotypes both in Zn deficient and sufficient growth conditions.

	Shoot Zn concentration		Shoot Fe concentration		Zn Efficiency
Genotypes	-Zn	+Zn	-Zn	+Zn	(%)
	(mg kg^{-1} DM)		(mg kg^{-1} DM)		
Bezostaya-1	7,3 ± 0,6	45,2 ± 0,9	86,1 ± 8,8	52,8 ± 0,6	99
Dağdaş	9,0 ± 0,8	41,1 ± 5,0	92,9 ± 3,4	53,1 ± 3,3	97
Alpu 01	6,8 ± 0,3	47,0 ± 8,1	82,2 ± 6,7	50,2 ± 3,5	95
Yakar	7,1 ± 0,0	49,7 ± 2,3	118,4 ± 3,8	56,0 ± 0,8	91
Çetinel 2000	8,0 ± 0,8	47,2 ± 2,8	86,6 ± 4,1	53,8 ± 4,1	91
03 SE 18 SEAÇ	8,1 ± 1,0	43,5 ± 1,0	78,9 ± 14,1	56,8 ± 4,4	90
ES-14	8,0 ± 0,6	51,4 ± 0,5	85,1 ± 0,4	52,8 ± 1,9	85
Yıldız 98	7,1 ± 0,3	46,4 ± 3,9	139,9 ± 34,2	54,4 ± 2,5	83
Ziyabey	8,5 ± 0,3	51,5 ± 5,0	70,9 ± 4,2	57,9 ± 0,8	83
00 KE 3 KEAÇ	8,4 ± 0,6	49,4 ± 0,9	93,3 ± 6,6	56,0 ± 2,2	82
Kırgız 95	6,9 ± 0,3	46,1 ± 1,1	100,6 ± 14,3	51,6 ± 3,5	82
İzmir 85	9,2 ± 0,4	40,5 ± 6,0	84,5 ± 21,4	53,3 ± 1,6	76
Soyer 02	6,6 ± 0,4	46,7 ± 2,6	115,6 ± 16,0	51,8 ± 0,8	76
BDME-10	7,0 ± 0,7	50,4 ± 3,0	124,7 ± 13,7	53,4 ± 1,2	74
Ahmetağa	5,9 ± 0,5	30,7 ± 2,6	56,6 ± 5,6	43,7 ± 4,5	73
Karahan	6,6 ± 0,8	42,3 ± 6,4	78,6 ± 10,1	49,9 ± 2,2	60
Bağcı	6,6 ± 0,8	44,6 ± 1,2	81,2 ± 22,1	54,0 ± 4,4	60
Average	7,5	45,5	92,7	53,0	82

As expected, application of Zn resulted in significant increases in shoot Zn concentration of genotypes. The increases were up to 6-7 folds (Table 4). There was also, to some extend, a genetic variation in shoot Zn concentration between the genotypes under Zn deficiency. Among the genotypes tested Ahmetaga showed the lowest (e.g., 5.9 mg Zn kg^{-1}) and Dagdas had the highest (e.g., 9.0 mg Zn kg^{-1}) Zn concentrations in shoot under Zn deficiency. However, from the Table 4, it seems that there is not always a direct relationship between the Zn concentration and Zn efficiency tolerance of the genotypes. The table clearly illustrated that a genotype with the highest tolerance index may have less Zn concentration

under Zn deficient conditions compared to a genotype with a mean tolerance index. For instance, Bezostaja with the highest tolerance index seemed to have less Zn concentration (7.3 mg kg^{-1}) under Zn deficient conditions compared to 00KE 3 KEAC (8.4 mg kg^{-1}) with a mean tolerance index. Similarly, Bezostaja and BDME-10 which differ in their tolerance to Zn deficiency were more or less similar in shoot Zn concentration under Zn deficiency. According to the Table 4, Fe concentration of wheat genotypes seemed to be increased by more than 1.5 fold in average in Zn deficient plants compared to the plants grown under Zn sufficient conditions.

The Zn and Fe content of plants were calculated for determining the amounts of elements in plants. The shoot Zn and Fe content of plants were shown on Table 5.

Table 5: Shoot Zn and Fe contents of cultivated bread wheat genotypes both in Zn deficient and sufficient growth conditions.

	Shoot Zn content		Shoot Fe content		Zn Efficiency
Genotypes	-Zn	+Zn	-Zn	+Zn	(%)
	(µg $plant^{-1}$)		(µg $plant^{-1}$)		
Bezostaya-1	4,0 ± 0,2	24,9 ± 1,4	46,7 ± 3,0	29,1 ± 1,4	99
Dağdaş	4,3 ± 0,6	20,1 ± 3,1	44,1 ± 5,4	25,8 ± 0,7	97
Alpu 01	3,3 ± 0,1	24,0 ± 5,2	39,6 ± 2,4	25,5 ± 2,9	95
Yakar	3,0 ± 0,0	22,7 ± 0,9	49,4 ± 1,5	25,6 ± 1,7	91
Çetinel 2000	3,5 ± 0,3	23,0 ± 0,6	38,4 ± 2,5	26,3 ± 1,0	91
03 SE 18 SEAÇ	4,0 ± 0,6	23,7 ± 1,8	38,5 ± 6,3	30,9 ± 3,2	90
ES-14	2,7 ± 0,0	20,4 ± 0,7	28,8 ± 1,8	21,0 ± 1,2	85
Yıldız 98	2,9 ± 0,3	22,3 ± 1,1	55,9 ± 12,6	26,2 ± 0,4	83
Ziyabey	3,4 ± 0,2	24,5 ± 2,1	28,0 ± 1,3	27,6 ± 1,3	83
00 KE 3 KEAÇ	4,0 ± 0,2	28,4 ± 1,3	44,0 ± 3,0	32,2 ± 2,4	82
Kırgız 95	3,2 ± 0,2	25,8 ± 1,5	46,0 ± 4,4	28,9 ± 2,4	82
İzmir 85	4,0 ± 0,4	23,0 ± 2,1	37,4 ± 12,2	30,5 ± 2,5	76
Soyer 02	2,6 ± 0,3	24,3 ± 0,9	46,1 ± 9,2	27,0 ± 1,6	76
BDME-10	2,6 ± 0,2	25,5 ± 1,4	46,3 ± 3,0	27,0 ± 0,9	74
Ahmetağa	2,4 ± 1,0	17,1 ± 2,1	22,5 ± 7,0	24,5 ± 4,5	73
Karahan	3,7 ± 1,0	38,3 ± 3,9	43,6 ± 12,9	44,4 ± 1,4	60
Bağcı	2,7 ± 0,4	28,9 ± 0,2	33,6 ± 9,1	35,1 ± 4,0	60
Average	3,3	24,5	40,5	28,7	82

Generally, the genotypes with high Zn deficiency tolerance seemed to possess high or moderate Zn contents under Zn deficient conditions. The genotypes with low tolerance indices appeared to have low levels of Zn content except the Karahan cultivar. Although Karahan cultivar was found to have a low tolerance index, Zn content of the genotype in Zn deficient conditions was higher than the average Zn content. The reason of such higher Zn

content in Karahan could not be understood; it maybe related to an experimental error because of very high standard deviation value (Table 5). However, this genotype had also higher Zn concentrations under Zn adequate conditions. Possibly, this genotype has a higher genetic potential for Zn uptake and this needs for further research.

4.1.4 Nutrient Solution Experiment

In controlled growth chamber conditions, the selected genotypes were grown in hydroponic medium. As described in 3.2.1.2 plants were treated by 5 different Zn treatments as followingŞ -Zn (very severe Zn deficiency), 10^{-8} M Zn (severe Zn deficiency), 10^{-7} M Zn (moderate Zn deficiency) 10^{-6} M Zn (sufficient Zn supply) and 10^{-4} M Zn (toxic dose). After 13 days of growth in nutrient solution Zn deficiency leaf symptoms became evident, and as expected BDME-10 was much more affected from Zn deficiency than Bezostoja (Fig. 4.4). Then, roots and shoots of plants were separately harvested.

There was a close relationship between the intensity of the leaf symptoms of Zn deficiency and concentration of Zn added in nutrient solution until 10^{-6} M Zn. By increasing Zn concentration in nutrient solution plants were grown better and became free from Zn deficiency symptoms. At the highest Zn application (e.g., 10^{-4} M Zn) plants showed some decreases in growth and developed slight chlorosis and some necrosis on leaves and bronzing in roots. As mentioned, in the molecular studies only Bezostaja has been used.

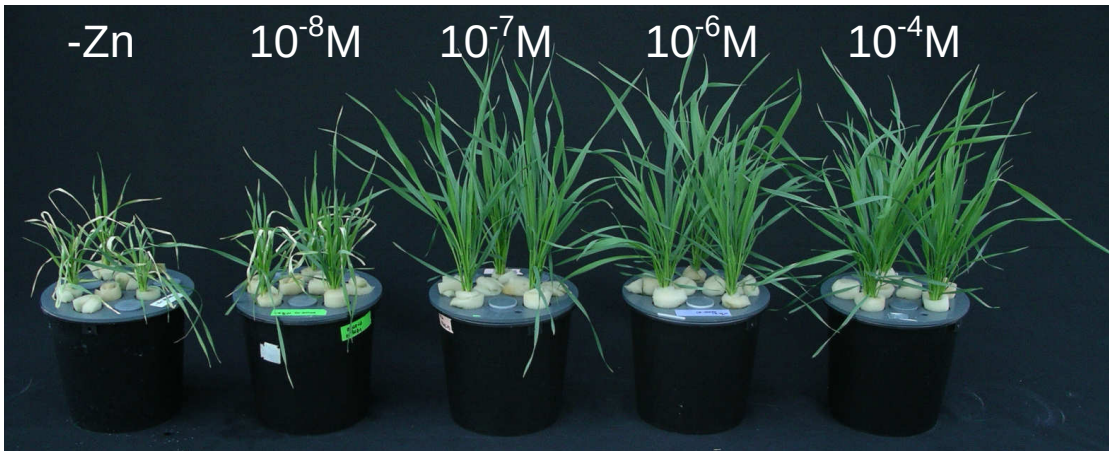


Figure 6: Pre-harvest picture of BDME-10 genotype grown in response to varying Zn treatments.

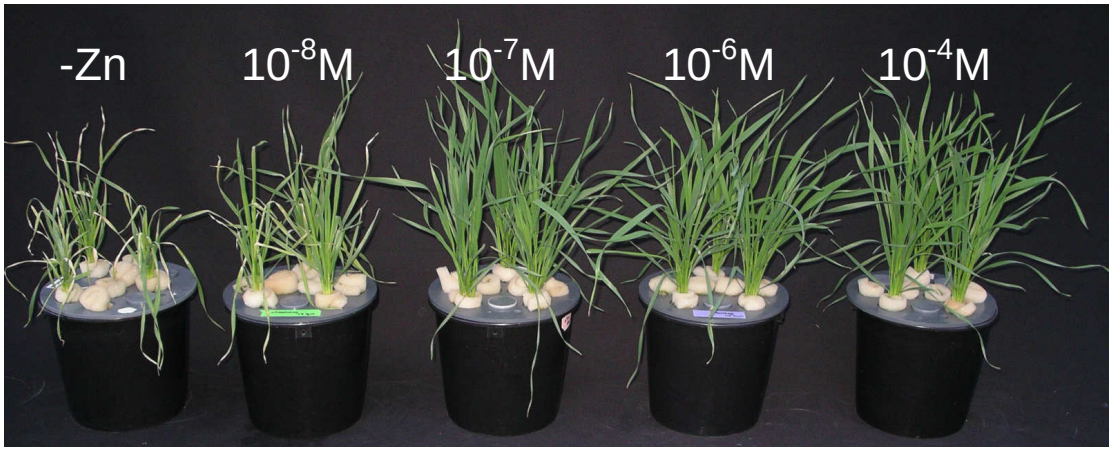


Figure 7: Pre-harvest picture of Bezostaja genotype grown in response to varying Zn treatments.

4.2 Molecular Analysis: mRNA Differential Display

After isolation of total RNAs from the roots of Bezostaja genotype, DNase treatment was performed to the isolated RNAs of the wheat genotype. The DNase treated samples were used as a template for cDNA conversion. The obtained cDNAs were used in mRNA differential display analysis in order to detect the expression profile of a tolerant cultivated bread wheat genotype in response to 4 different doses of Zn applications described above. Different primer combinations were used in PCR reactions for obtaining the differentially expressed cDNA fragments in varying sizes ranged fro 160 to 580 bp.

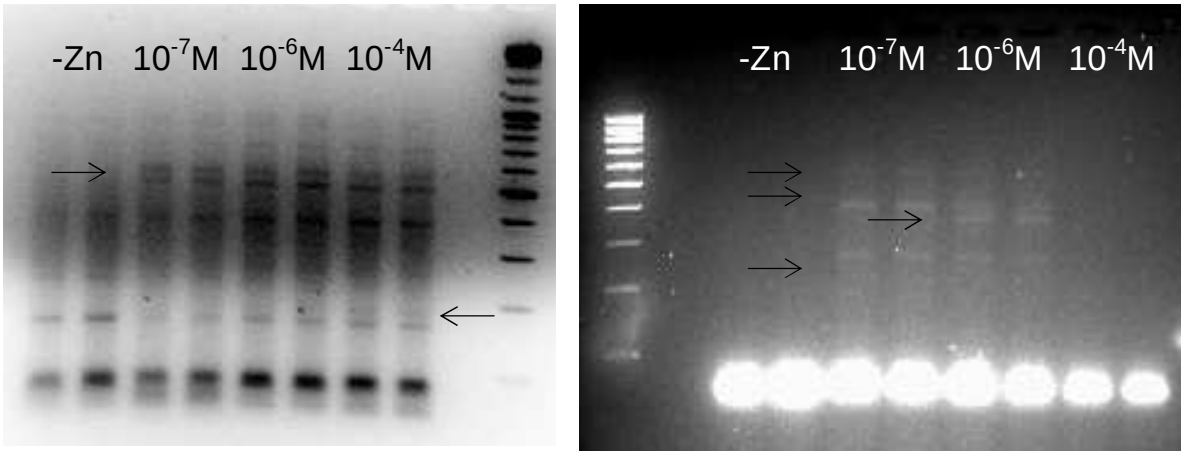


Figure 8: Agarose gel electrophoresis pictures of mRNA differential display PCR products of Bezostaja roots before gel extraction for sequencing. The genotype was exposed to 4 different doses of Zn applications; -Zn (severe Zn deficiency), 10-7 M Zn (slight Zn deficiency), 10-6 M Zn (adequate Zn supply), 10-4 M Zn (toxic Zn dose) (a)PCR products obtained viaT3P3 primers. Both 180 bp and 560 bp fragments displayed with arrows were extracted from the gel. (b)PCR products obtained via T1P9 primers. Both 505, 414, 387 and 263 bp fragments displayed with arrows were extracted from the gel.

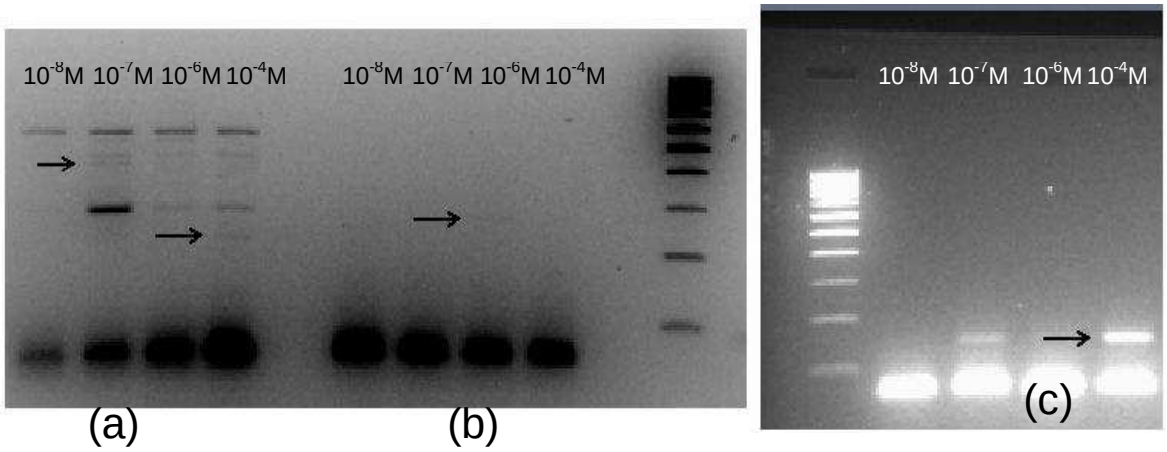


Figure 9: Agarose gel electrophoresis pictures of mRNA differential display PCR products of Bezostaja shoots before gel extraction for sequencing. The genotype was exposed to 4 different doses of Zn applications; 10-8 M Zn (moderate Zn deficiency), 10-7 M Zn (slight Zn deficiency), 10-6 M Zn (adequate Zn supply), 10-4 M Zn (toxic Zn dose). (a)PCR products obtained via T1P9 primers. Both 414 bp and 207 bp fragments displayed with arrows were extracted from the gel. (b) PCR products obtained via T3P2 primers. 253 bp fragment displayed with arrow was extracted from the gel (c)PCR product obtained via T5P2 primers. 147 bp fragment displayed with arrow was extracted from the gel.

The differentially expressed cDNA fragments isolated from both root and shoot parts of the wheat genotype were sequenced and protein sequence similarity search was realized via using NCBI BLASTX algorithm. 457 bp root cDNA fragment obtained by using T1P4 primer combination, was found to be similar to ribosomal protein S5, component of cytosolic 80S ribosome and 40S small subunit of Ostreococcus lucimarinus with 9e-51 e-value and 40S ribosomal protein S5 of Oryza sativa with 8e-50 e-value. The same primer combination also gave 139 bp cDNA fragment that was found to be similar to the protein sequence of Enterococcus faecalis histidine kinase with 7.1 e-value.

A 137 bp cDNA fragment obtained via using T8P6 primer combination displayed similarity to hypothetical protein of Ruminococcus obeum with 0.026 e-value. 560 bp cDNA fragment provided by T3P3 primer combination was found to be similar to alcohol dehydrogenase of Hordeum vulgare with Zinc-binding dehydrogenase region with 2e-43 e-value. The same primer combination also gave 180 bp cDNA fragment that was found to be similar to ribosomal protein L2 of both Arabidopsis thaliana and Triticum aestivum with 4e-24 and 3e-22 e-values respectively.

T1P9 primer combination gave a 505 bp cDNA fragment that was found to be similar to the cysteine synthase of Glycine max and cysteine synthase, O-acetyl-L-serine (thiol)-lyase of Cicer arietinum both with 3e-28 e-value. A 414 bp cDNA fragment obtained via using the same primer combination displayed similarity to cystathionine gamma-synthase of Zea Mays with 1e-66 e-value and Cys/Met metabolism PLP-dependent enzyme family protein of Oryza sativa with 4e-66 e-value. The same primer combination also gave 387 bp cDNA fragment that is similar to the hypothetical protein of Monodelphis domestica with ZIP Zinc transporter region (1.9 e-value) and cation diffusion facilitator family transporter containing protein of Tetrahymena thermophila with Co/Zn/Cd efflux system component (4.2 e-value).

Table 6: BLASTX results obtained from isolated Bezostaja root cDNA fragments via using NCBI BLASTX algorithm.

Primers/ROOT	BlastX Hit	NCBI Accession Number	e-value
T1P4 a)	Ribosomal protein S5, component of cytosolic 80S ribosome and 40S smallsubunit[Ostreococcus lucimarinus CCE9901]	XP_001418582	9e-51
	40S ribosomal protein S5, putative, expressed [Oryza sativa (japonica cultivar-group)].	ABA93723	8e-50
T1P4 b)	histidine kinase, putative [Enterococcus faecalis V583].	NP_815517	7.1
T8P6	hypothetical protein RUMOBE_01293 [Ruminococcus obeum ATCC 29174].	ZP_01963575	0.026
T3P3 a)	alcohol dehydrogenase [Hordeum vulgare subsp. vulgare].with Zinc-binding dehydrogenase region	AAK49116	2e-43
T3P3 b)	60S ribosomal protein L2 [Arabidopsis thaliana].	CAA60445	4e-24
	ribosomal protein L2 [Triticum aestivum].	AAP80668	3e-22
T1P9 a)	cysteine synthase, O-acetyl-L-serine (thiol)-lyase [Cicer arietinum].	CAA06819	3e-28
	cysteine synthase [Glycine max].	AAL66291	3e-28

Table 7: BLASTX results obtained from isolated Bezostaja root cDNA fragments via using NCBI BLASTX algorithm.

Primers/ROOT	BlastX Hit	NCBI Accession Number	e-value
T1P9 b)	cystathionine gamma-synthase [Zea mays].	AAB61347	1e-66
	Cys/Met metabolism PLP-dependent enzyme family protein, expressed [Oryza sativa (japonica cultivar-group)].	ABF96221	4e-66
T1P9 c)	PREDICTED: hypothetical protein [Monodelphis domestica]with ZIP Zinc transporter region and Predicted divalent heavy-metal cations transporter region	XP_001369419	1.9
	cation diffusion facilitator family transporter containing protein [Tetrahymena thermophila SB210] with Co/Zn/Cd efflux system component	XP_001031052	4.2

T5P2 primer combination gave 147 bp shoot cDNA fragment that was found to be similar to the photosystem I assembly protein ycf4 of Hordeum vulgare and ORF 185 of Triticum aestivum with photosystem I assembly protein Ycf4 region both having 4e-16 e-value.

A 264 bp shoot cDNA fragment obtained via T2P4 primer combination was found to be similar to the hypothetical protein of Oryza sativa with 1e-15 e-value.

T6P3 primer combination gave a 323 bp shoot cDNA fragment that displayed similarity to ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit of Triticum aestivum with 4e-38 e-value.

A 414 bp shoot cDNA fragment provided by using T1P9 primer combination was found to be similar to the cystathionine gamma-synthase of Zea mays with 1e-66 e-value, Cys/Met metabolism PLP-dependent enzyme family protein of Oryza sativa with 4e-66 e-value and MTO1 (METHIONINE OVERACCUMULATION 1) of Arabidopsis thaliana with 6e-53 e-value. The same primer combination gave a 207 bp cDNA fragment that showed similarity to ATP synthase CF1 beta subunit of Triticum aestivum and ATP synthase CF1 beta subunit of Hordeum vulgare both with 9e-22 e-value.

A 253 bp cDNA fragment obtained via using T3P2 primer combination, was found to be similar to the Os09g0526200 (Oryza sativa) with 3.3 e-value and MGA protein of Mus musculus containing Myc-like bHLHZip domain; with 5.6 e-value.

T9P6 primer combination gave 401 bp cDNA product that showed similarity to ORF107c of Pinus koraiensis with 1e-16 e-value and hypothetical protein CYtaCp094 of Cycas taitungensis with 7e-14.

Table 8: BLASTX results obtained from isolated Bezostaja shoot cDNA fragments via using NCBI BLASTX algorithm.

Primers/SHOOT	BlastX Hit	NCBI Accession Number	e-value
T5P2	photosystem I assembly protein ycf4 [Hordeum vulgare subsp. vulgare].	YP_874663	4e-16
	ORF 185 [Triticum aestivum] with photosystem I assembly protein Ycf4 region	CAA44030	4e-16
T2P4	hypothetical protein OsJ_030064 [Oryza sativa (japonica cultivar-group)]	EAZ15855	1e-15
T6P3	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit[Triticum aestivum].	AAP92166	4e-38
T1P9 a)	cystathionine gamma-synthase [Zea mays]	AAB61347	1e-66
	Cys/Met metabolism PLP-dependent enzyme family protein, expressed [Oryza sativa (japonica cultivar-group)]	ABF96221	4e-66
	MTO1 (METHIONINE OVERACCUMULATION 1) [Arabidopsis thaliana].	NP_186761	6e-53

Table 9: BLASTX results obtained from isolated Bezostaja shoot cDNA fragments via using NCBI BLASTX algorithm.

Primers/SHOOT	BlastX Hit	NCBI Accession Number	e-value
T9P6 a)	ORF107c [Pinus koraiensis]	YP_001152220	1e-16
	hypothetical protein CYtaCp094 [Cycas taitungensis].	YP_001312259	7e-14
T9P6 b)	ORF107c [Pinus koraiensis]	YP_001152220	1e-04
T1P9 b)	ATP synthase CF1 beta subunit [Triticum aestivum]	NP_114266	9e-22
	ATP synthase CF1 beta subunit [Hordeum vulgare subsp. vulgare].	YP_874660	
T3P2	Os09g0526200 [Oryza sativa (japonica cultivar-group)]	NP_001063724	3.3
	MGA protein [Mus musculus]with contains Myc-like bHLHZip domain;	AAF24761	5.6

Table 10: mRNA differential display results of differentially expressed root cDNA fragments in response to varying levels of Zn applications with fragment sizes obtained by using NCBI BLASTX.

Primers/ROOT	Zn Applications (M)				Size of the Fragment
T1P4 a)	-Zn	10^{-7}	10^{-6}	10^{-4}	457bp
	+	+	+	-	
T1P4 b)	-Zn	10^{-7}	10^{-6}	10^{-4}	139bp
	+	+	+	-	
T8P6	-Zn	10^{-7}	10^{-6}	10^{-4}	137 bp
	+	+	-	-	
T3P3 a)	-Zn	10^{-7}	10^{-6}	10^{-4}	560 bp
	-	+	+	+	
T3P3 b)	-Zn	10^{-7}	10^{-6}	10^{-4}	180 bp
	-	-	-	+	
T1P9 a)	-Zn	10^{-7}	10^{-6}	10^{-4}	505 bp
	-	+	+	-	

Table 11: mRNA differential display results of differentially expressed root cDNA fragments in response to varying levels of Zn applications with fragment sizes obtained by using NCBI BLASTX.

Primers/ROOT	Zn Applications (M)				Size of the fragment
T1P9 b)	-Zn	10^{-7}	10^{-6}	10^{-4}	414bp
	-	+	+	-	
T1P9 c)	-Zn	10^{-7}	10^{-6}	10^{-4}	387bp
	-	-	+	-	

Table 12: mRNA differential display results of differentially expressed shoot cDNA fragments in response to varying levels of Zn applications with fragment sizes obtained by using NCBI BLASTX

Primers/SHOOT	Zn Applications (M)				Size of the fragment
T5P2	10^{-8}	10^{-7}	10^{-6}	10^{-4}	147 bp
	-	+	+	+	
T2P4	10^{-8}	10^{-7}	10^{-6}	10^{-4}	264 bp
	-	-	+	-	
T6P3	10^{-8}	10^{-7}	10^{-6}	10^{-4}	323 bp
	-	+	+	-	
T1P9 a)	10^{-8}	10^{-7}	10^{-6}	10^{-4}	414 bp
	-	+	-	-	
T1P9 b)	10^{-8}	10^{-7}	10^{-6}	10^{-4}	207 bp
	-	-	-	+	
T3P2	10^{-8}	10^{-7}	10^{-6}	10^{-4}	253 bp
	-	-	+	-	

Table 13: mRNA differential display results of differentially expressed shoot cDNA fragments in response to varying levels of Zn applications with fragment sizes obtained by using NCBI BLASTX.

Primers/SHOOT	Zn Applications (M)				Size of the fragment
T9P6 a)	-Zn	10^{-7}	10^{-6}	10^{-4}	401 bp
	-	+	+	+	
T9P6 b)	-Zn	10^{-7}	10^{-6}	10^{-4}	113bp
	-	+	+	+	

5 DISCUSSION

5.1 Physiological Analysis

The objective of this study was to identify the differentially expressed cDNA fragments in response to varying Zn applications in Zn deficiency tolerant Bezostaja cultivar. For this purpose, mRNA differential display technique has been used in addition to sequencing. Selected genotype; Bezostaja was grown in hydroponic culture for obtaining fresh tissue samples needed for the molecular studies. The mRNA differential method is serviceable regarding its quickness compared to other identification techniques. The method allows the detection of differentially expressed cDNA fragments within a single type of cell under varying conditions and also between two or more different cellular populations (Liang and Pardee, 1992; Ito et al., 1994). The method also relies on well settled and commonly utilized molecular techniques. It allows the identification of both up and down regulated cDNA fragments in various cell populations at the same time. Considering that it can elicit rare mRNA fragments, it is a very sensitive method. Moreover, very few amounts of RNA are required for the application of the technique (Lievens et al., 2001).

Decreased shoot growth which is one of the major symptoms observed in Zn deficient plants was observed in bread cultivars when grown under Zn deficient conditions in a greenhouse. Zinc deficiency also caused development of chlorosis and necrotic spots on leaves. Based on the severity of leaf symptoms and decreases in shoot dry matter production, there was a considerable variation among the 15 bread wheat genotypes. Considering the tolerance index values (Table 3), Bezostaja genotype was selected to be the most tolerant bread wheat genotype.

There was also a considerable variation in shoot Zn concentrations among the bread wheat genotypes grown under Zn deficient conditions (Table 4). Table 4 indicated that a genotype with the highest tolerance index may possess less Zn concentration compared to a genotype with a mean tolerance index in Zn deficient conditions. This result may indicate that physiological utilization of Zn in shoot tissue differs between the genotypes. A similar observation was also reported before (Cakmak et al., 1998).

Iron concentration of bread wheat cultivars increased by more than 1.5 fold, in average, in Zn deficient plants compared to the plants grown under Zn sufficient conditions (Tabl0 4.2). This observation seems to be parallel with the literature data which states that Fe uptake is enhanced under Zn deficiency conditions both for the durum and bread wheat plants. According to Rengel and Römheld (2000), regardless of the level of their Zn efficiency, severe Zn deficiency led to the increased Fe uptake of all wheat genotypes studied. Nevertheless, the wheat genotypes that are susceptible to Zn deficiency are observed to possess higher rate of root-to-shoot Fe transport than the Zn efficient wheat genotypes, except the early growth phases under Zn deficiency. Considering Fe concentration difference between the tolerant and susceptible wheat genotypes, there was no remarkable difference in Fe concentrations between the Zn-efficient and Zn-inefficient wheat cultivars except BDME-10 genotype (Table 4). However for BDME-10 genotype, which is a susceptible genotype, an increase in Fe concentration is pronounced. The differential behavior of BDME-10 in Fe concentration value under Zn deficient conditions may be explained by a unique Fe uptake mechanism for BDME genotype.

5.2 Molecular Analysis

14 out of 20 differentially expressed Zn responsive transcripts were identified in Bezostaja genotype with varying doses of Zn application by applying mRNA differential display technique. Except few sequences, the transcripts displayed similarity to previously identified proteins from NCBI protein database. The identified transcripts were found to be associated with Zn. These results indicate that mRNA differential display method was an efficient method in terms of detecting the Zn related genes in bread wheat genotypes.

According to the BLASTX results, a 560 bp cDNA fragment was obtained using T3P3 primer. This differentially expressed fragment corresponds to alcohol dehydrogenase with zinc-binding dehydrogenase region. Alcohol dehydrogenase enzyme is known to contain two Zn atoms per molecule. One of the Zn atoms has the catalytic role and the other atom is associated with structural function (Coleman, 1992). The reduction of acetaldehyde to ethanol is known to be operated by the alcohol dehydrogenase enzyme. Furthermore, the ethanol formation mainly takes place in meristematic tissues like root apices in higher plants under aerobic conditions. In response to Zn deficiency, the activity of the alcohol dehydrogenase is observed to be reduced in plants (Marschner, 1995). In this study, we did not observe the existence of alcohol dehydrogenase cDNA fragment in Zn deficient roots of Bezostaja which seems to be consistent with the literature data. However, real time PCR results should be done in order to detect the quantitative differential expression of this gene between plant roots exposed varying levels of Zn applications.

A cDNA fragment with 387bp length was observed to be differentially expressed among four different Zn applications in roots of Bezostaja genotype using P1T9 primer, That transcript is observed to be expressed in plant roots that are grown in conditions having sufficient amount of Zn (e.g., 10^{-6} M Zn) but not in conditions with toxic Zn supply. The cation diffusion facilitator (CDF) family transporter containing protein with Co/Zn/Cd efflux system component is found to be similar with cDNA fragment identified in this study based on BLASTX analysis. The CDF family transporter family that is found to take place in bacteria, yeast, animals and plants is known to perform the heavy metal transport, specifically Zn, cadmium and Cobalt. (Paulsen and Saier, 1997; Eide, 1998; van der Zaal *et al*., 1999). These proteins are estimated to contain six TM domains with a C-terminal cation binding domain. (Paulsen and Saier, 1997; Mäser *et al*., 2001). A histidine-rich domain between TM-4 and TM-5 responsible for Zn-binding also takes place in eukaryote members. (Huang and Gitschier, 1997; Williams *et al*., 2000). Size variability ranging from 280 to 740 residues was observed with the members of CDF (Paulsen and Saier, 1997). One plant CDF member namely; ZAT that is characterized from Arabidopsis incorporates 398 amino acids (van der Zaal *et al*., 1999). Increased Zn concentrations are found to stimulate the ZAT expression, and throughout the plant, ZAT is known to be expressed constitutively. ZAT is also suggested to take role in Zn homeostasis via providing the sequestration of Zn in vacuole. However, metal homeostasis is not suggested to be the only role for CDF (Haney et al, 2005). According to the functional data, some CDF family proteins may act as cation donators taking role in signal

transduction. (Haney et al., 2005).Our observation of this transporter in the control dose seems to be logical considering that this family of transporters seems to be Zn activated zinc transporters. Considering that, we do not observe the expression of this fragment in toxic dose, one can infer that, rather than taking role in metal homeostasis, this protein may act as zinc donator with having role in signal transduction.

The BlastX result of the 387bp cDNA fragment obtained via T1P9 primer also gave hit for the hypothetical protein containing ZIP Zinc transporter region and predicted divalent heavy-metal cations transporter region. Three ORF regions are found for that cDNA fragment. One of the three ORF regions corresponds to a region called ZIP, Zinc ZIP transporter. ZIP transporter proteins, Zinc and Iron Regulated Proteins incorporate various members in different eukaryotic kingdoms including plants, animals, protists and fungi. Various members of ZIP family transporters which are displayed to take role in metal uptake and transport are characterized (Guerinot, 2000; (Eide et al., 1996; Korshunova et al., 1999; Vert et al., 2001, 2002; Connolly et al., 2002). Having their amino- and carboxyl- terminals located on the outer surface of the plasma membrane, the ZIP proteins are predicted to incorporate eight transmembrane domains (Guerinot, 2000). Because of the variable region between the transmembrane domains of TM-3 and TM-4, these proteins displayed variation in total length. The variable region is predicted to take place on the cytoplasmic region including Histidines where a potential binding may occur. ZIP1, ZIP3, and ZIP4 were suggested to take role in Zn transport considering that, Zn uptake is recovered when mutant yeast (Saccharomyces cerevisiae) are exposed to ZIP1, ZIP3, and ZIP4 from Arabidopsis. ZIP1 and ZIP3 are proposed to take role in transport of Zn from the soil to the plant considering that they are found to be expressed in plant roots in Zn deficient conditions. Whereas, ZIP4 is suggested to provide the transport of Zn intracellularly or between plant tissues considering that ZIP4 is observed to be expressed both in roots and shoots (Grotz et al., 1998; Guerinot, 2000).

We did not observe the expression of this cDNA fragment in toxic doses, but we are not sure about the expression of this transcript in other doses including both the severe Zn deficient, slightly Zn deficient plants. We definitely observed the expression of the transcript in the control dose. Therefore, gene specific primers may be designed and also RACE method may be utilized in order find the full sequence of the cDNA fragment. Then in order to have the accurate information regarding the detection of the transcript expression differences

between the plants exposed to varying levels of Zn applications, real time PCR should be exploited.

414bp cDNA fragment obtained via T1P9 primer is found to have a similarity with cystathionine gamma-synthase (CGS). CGS is the enzyme that catalyzes the committing step in Met and S-adenosyl-L-Met (SAM) synthesis. It catalyzes the irreversible reaction that is the condensation of the sidechain of o-phosphohomoserine (OPH) with the thiol group of Cysteine to form cystathionine. Later cleavage of cystathionine results in the formation of homocysteine, then in order to form Methionine (Met) , the methylation of the homocysteine with 5- 5-methyltetrahydrofolate takes place. Integration into proteins, production of SAM via adenosylation or the formation of *S*-methyl Met (SMM) are the possible metabolic fates of Met after synthesis (Kim et al., 2002) The rate of Met synthesis is suggested to be controlled via CGS based on the evidences. Over accumulation of soluble Met is observed in Arabidopsis mutants which are able provide the over-expression of CGS (Inaba et al., 1994; Chiba et al., 1999). Growth abnormalities due to prevention of Met synthesis are also observed in plants in response to the suppression of CGS expression via antisense-RNA (Gakie´re et al., 2000; Kim and Leustek, 2000).

According to the study that is performed by Chiba et al., (1999) mutant analysis suggested an existence of autogenous mechanism for the regulation of CGS synthesis. Via a posttranslational mechanism, CGS enzyme was observed to be declined by Met or its metabolite in wild type Arabidopsis plant. The posttranslational mechanism was found to function via destabilization of CGS mRNA. However, destabilization via Met was observed to be prevented in Arabidopsis mutants where there is a point mutation in exon 1 of the CGS gene. An enhancement both in the CGS enzyme and soluble Met level was seen in mutant plants. Considering that, observation of the CGS enzyme in control dose in addition to plants grown under slightly Zn deficient conditions may be meaningful since there may be constitutive expression of CGS. Posttranslational mechanisms associated with the regulation of CGS may take place for this genotype under control conditions so that Met biosynthesis may be inhibited.

In a study performed by Kim et al. (2002), a transgenic approach was utilized for observing the role of CGS ampleness on the regulation of free level Met expression in Arabidopsis. They observed the build up Met and SMM only in particular tissues and phases

of development in response to transcriptional up-regulation of CGS. Physiological alterations in addition to remarkable morphological abnormalities similar to the plants that have co-suppressed SAM were observed with the co-suppression of CGS. SAM was suggested to be a regulator of CGS expression considering the relative analysis of CGS and SAM suppressed plants. We did not observe the expression CGS enzyme in plant roots that are exposed to the toxic doses of Zn. Real time analysis application after designing gene specific primers will be more informative regarding the detection of sensitive expression differences between plant roots exposed to varying levels of Zn applications. Generally, in plants that are exposed to Zn deficiency, although the composition does not alter, the quantity of protein is known to be declined. Free amino acid concentration was observed to be enhanced by 6.5 fold in Zn deficient bean leaves compared to the plants grown in adequate levels of Zn. After 48 or 72 hours from Zn supply, a reduction in free amino acid level in addition to the enhancement of protein content was observed (Brown et al., 1993). Considering this literature data, observation of the CGS cDNA may be associated with the alterations in protein metabolism in amino acid level in response to Zn stress. The mechanism behind the link between the amino acid-protein metabolism and Zn deficiency may be accurately understood if the quantitative analysis of that cDNA fragment is performed via utilizing real time PCR method. By real time method, the detailed quantitative expression profile of the CGS cDNA should be done in order to obtain subtle expression differences between plants exposed to different Zn applications.

Photosystem I (PSI) is a large protein complex that is located in the photosynthetic thylakoid membrane. Light-stimulated steps of photosynthesis are facilitated by the PSI together with photosystem II (PSII). Three thylakoid proteins; BtpA Ycf3 and Ycf4 are found to be associated to the stable gathering of PSI. According to the study held by Boudreau et al. (1997), the mutation of ycf3 and ycf4 by the biolistic method resulted in the prevention of the growth of transformants photoautotrophically. The PSI activity of these mutants was also inadequate. In thylakoid membranes of these transformants (ycf3 and ycf4 mutants), PSI complex did not accumulate in a stable manner. Although they are situated on the thylakoid membranes, ycf3 and ycf4 mutants did not stably form the PSI complex. Zinc deficiency reduces the net photosynthesis by 50% to 70% depending on the severity of Zn deficiency stress and the type of plant species (Alloway, 2004). Moreover, the decline in chloroplast content in addition to the abnormal structure of chloroplast associated with Zn deficiency is the other factors that lead to the decrease in the rate of photosynthesis (Alloway, 2004).We

did not observe the ycf4 coding transcript in the Zn deficient shoots of Bezostaja genotype. This finding may be associated with the reduction in chloroplast content in response to Zn deficiency. For more accurate data regarding the quantitative analysis of the ycf4 transcript, quantitative real time PCR should be performed.

In this study, ribulose 1, 5 – biphosphate carboxylase/oxygenase large subunit (323 bp) encoding transcript (having the region Ribulose bisphosphate carboxylase large chain,catalytic domain) was observed in both zinc deficient and control plants, but not in plants exposed to toxic dose of Zn. Ribulose 1, 5 – biphosphate carboxylase (RuBPC) is a Zn associated enzyme that is involved in photosynthetic C metabolism.. The enzyme takes role in photosynthesis via catalyzing the initial step of carbon dioxide fixation. In response to Zn deficiency in navy bean, the activity of RuBPC is found to be reduced (Jung et al. 1972). Here we observed the mRNA transcript encoding the region Ribulose bisphosphate carboxylase large chain under Zn deficient conditions; but, the intensity of the band seemed to be less than the bands observed under slight Zn deficient and control dose treatments. Nevertheless, the sensitive analysis may only be performed with the aid of real time analysis utilizing the gene specific primers against RuBPC.

Via designing gene specific primers, RACE method may be pursued in order to find the full sequence of all the transcripts. More efficient similarity searches with the previously identified proteins may be performed when the full sequences of transcripts are provided. Furthermore, in order to perform biochemical and structural characterization, ORF sections of the full mRNAs may be determined.

6 CONCLUSION

There was a considerable genetic variation in response to Zn deficiency among different cultivated bread wheat genotypes.. The most Zn efficient genotype, *Bezostaja* was selected among those genotypes that display variation regarding tolerance to Zn deficiency. By utilizing mRNA differential display method, 20 transcripts were found to be differentially expressed in response to different Zn applications. Out of 20 cDNA fragments that were isolated, cloned and sequenced, 14 cDNAs displayed similarity with previously identified metal/Zn binding proteins and enzymes.

Some of the transcripts showed similarity to previously known protein sequences. Those protein sequences such as alcohol dehydrogenase., cystathionine gamma-synthase, and cation diffusion facilitator family transporter containing protein were found to be associated with Zn. In view of the fact that differentially expressed transcripts were found to be related to Zn, it may be concluded that mRNA differential display method worked properly.

Further molecular analysis including Real-Time PCR should be realized in order to make accurate evaluation regarding the expression differences of each identified transcripts in response to different Zn applications. Differentially expressed transcript that is found to be similar to ZIP Zinc transporter region may be selected for future molecular studies by using RACE and real-time PCR. The primer combination that is used for obtaining ZIP Zinc transporter region in Zn deficiency tolerant genotype may also be used in Zn inefficient genotype for revealing any possible expression differences between two genotypes both in quantitative and qualitative terms. The molecular mechanisms of Zn deficiency tolerance could be also understood with the help of functional analysis of identified genes.

7 REFERENCES

1. Alloway, B.J. (2004), Zinc in Soils and Crop Nutrition. International Zinc Association: Brussels, Belgium.

2. Basile, A., Nuzzo, R.A.N., Capasso, C., Sorbo, S., Capasso, A., Carginale, V. (2005), Effects of Cd on gene expression in the liverwort *Lunularia Cruciata*. Gene, 356:153-159.

3. Bettger, W.J. and O'Dell, B.L. (1981), A critical physiological role of Zn in the structure and function of biomembrane. Life Sci, 28: 1425-1438.

4. Bowen, H.J.M., Cause, P.A., Thick, J. (1962), The distribution of some inorganic elements in plant tissue extracts. J. Exp. Bot, 13:257-267.

5. Boudreau, E., Takahashi, Y., Lemieux, C., Turmel, M., Rochaix, J.D. (1997), The chloroplast *ycf3* and *ycf4* open reading frames of *Chlamydomonas reinhardtii* are required for the accumulation of the photosystem I complex. The EMBO Journal, 16:6095–6104.

6. Brown, J.C., Jones, W.C. (1975), Phosphorus inefficiency as related to iron inefficiency in sorghum. Agronomy Journal, 67:468 - 472.

7. Brown, P.H., Cakmak, I., Zhang, Q. (1993), Form and function of zinc in plants. In: Robson, AD (ed) Zinc in Soils and Plants, Kluwer Academic Publishers. Dordrecht, The Netherlands, pp. 90 – 106.

8. Cakmak, I. and Marschner, H. (1988a), Increase in membrane permeability and exudation of roots of zinc deficient plants. J. Plant Physiol, 132:356-361.

9. Cakmak, I. and Marschner, H. (1988b), Zinc-dependent changes in in ESR signals, NADPH oxidase and plasma membrane permeability in cotton roots. Physiol. Plant, 73:132-186.

10. Cakmak, I.and Marschner, H. (1988c), Enhanced superoxide radical production in roots of zinc deficient plants. J.Exp.Bot, 39:1449-1460.

11. Cakmak, I., Marschner, H., Bangerth, F. (1989), Effects of zinc nutritional status on growth, protein metabolism and levels of indole-3-acetic acid and other phytohormones in bean. (*Phaseolus vulgaris* L.) J.Exper. Bot., 40: 405-412.

12. Cakmak, I., Gülüt, K., Marschner, H., Graham, R.D. (1994), Effect of zinc and iron deficiency on phytosiderophore release in wheat genotypes differing in zinc efficiency. J. Plant Nutrition , 17:1- 17.

13. Cakmak, I., Yılmaz, A., Ekiz, H., Torun, B., Erenoglu, B., Braun, H.J. (1996), Zinc Deficiency as a critical nutritional problem in wheat production in Central Anatolia. Plant and Soil, 180:165-172.

14. Cakmak, I., Ekiz, H., Yılmaz, A., Torun, B., Köleli, N., Gültekin, 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.

15. Cakmak, I., Oztürk, L., Eker, S., Torun, B., Kalfa, H. I., Yılmaz, A. (1997), Concentration of zinc and activity of copper/zinc-superoxide dismutase in leaves of rye and wheat cultivars differing in sensitivity to zinc deficiency. J. Plant Physiol., 151: 91-95.

16. Cakmak, I., Ekiz, H., Yılmaz, A., Torun, B., Köleli, N., Gültekin, 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.

17. Cakmak, I., Torun, B., Erenoglu, B., Oztürk, L., Marschner, H., Kalaycı, M., Ekiz, H., Yılmaz, A. (1998), Morphological and physiological differences in cereals in response to zinc deficiency. Euphytica, 100: 349-357.

18. Cakmak, I., Kalaycı, M., Ekiz, H., Braun, H. J., Yılmaz, A. (1999), Zinc deficiency as an actual problem in plant and human nutrition in Turkey: A NATO-Science for Stability Project. Field Crops Research, 60: 175-188.

19. Cakmak, I., (2000), http://grain.jouy.inra.fr/ggpages/awn/46/Textfiles/TURKEY.htm.

20. Cakmak, I. (2000), Role of zinc in protecting plant cells from reactive oxygen species. New Phytologist, 146: 185-205.

21. Cakmak, I. (2002), Plant nutrition research: Priorities to meet human needs for food in sustainable ways. Plant Soil, 247: 3-24.

22. Carginale, V., Capasso, C., Scudiero, R., Parisi, E. (2002), Identification of cadmium-sensitive genes in the Antarctic fish *Chionodraco hamatus* by messenger RNA differential display. Gene, 299:117-124.

23. Cebeci, O. (2006), Molecular characterization and expression profiling of cd-responsive genes in triticum durum. Master's thesis, Sabanci University.

24. Chiba, Y., Ishikawa, M., Kijima, F., Tyson, R.H., Kim, J., Yamamoto, A., Nambara, E., Leustek, T., Wallsgrove, R.M., Naito, S. (1999), Evidence for autoregulation of cystathionine gamma-synthase mRNA stability in *Arabidopsis*. Science, 286:1371–1374.

25. Chvapil, M. (1973), New aspects in the biological role of zinc: A stabilizer of macromolecules and biological membranes. Life Sci, 13:1041-1049.

26. Coleman, J.E., (1992), Zinc proteins:enzymes, storage proteins, transcription factors, and replication proteins. Annu. Rev. Biochem, 61: 897-946.

27. Connolly, E.L., Fett, J. P., Guerinot, M.L. (2002), Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. The Plant Cell, 14: 1347–1357.

28. Croy, R.G., Pardee, A.B. (1983), Enhanced synthesis and stabilization of Mr 68,000 protein in transformed BALB/c-3T3 cells: candidate for restriction point control of cell growth. Proc. Natl. Acad. Sci. USA., 80: 4699–4703.

29. Dwivedi, R. S., Takar, P.N. (1974), Ribonuclease activity as index of hidden hunger of zinc in crops. Plant and Soil, 40:170-181.

30. Eide, D., Broderius, M., Fett, J., Guerinot, M. L. (1996), A novel ironregulated metal transporter from plants identified by functional expression in yeast. Proceedings of the National Academy of Sciences, USA, 93: 5624–5628.

31. Eide, D.J. (1998), The molecular biology of metal ion transport in *Saccharomyces cerevisiae*. Annual Review of Nutrition, 18: 441–469.

32. Erenoglu, B., Cakmak, I., Römheld, V., Derici, R., Rengel, Z. (1999), Uptake of zinc by rye, bread wheat and durum wheat cultivars differing in zinc efficiency. Plant and Soil, 209: 245-252.

33. Falchuk, K.H., Hardy, C., Ulpino, L., Vallee, B.L. (1978), RNA metabolism, manganese, and RNA polymerases of zinc sufficient and zinc deficient *Euglena gracilis*. Proc. Nat. Acad. Sci. USA, 75: 4175-4179.

34. Gakie´re, B., Ravanel, S., Droux, M., Douce, R., Job, D. (2000), Mechanisms to account for maintenance of the soluble methionine pool in transgenic Arabidopsis plants expressing antisense cystathionine gamma-synthase cDNA. CR Acad Sci III, 323: 841–851.

35. Graham, R.D., Ascher, J.S., Hynes S.C. (1992), Selecting zinc - efficient cereal genotypes for soils low in zinc status. Plant and Soil, 146: 241 - 250.

36. Grill, E., Winnacker, E., Zenk, M.H. (1985), Phytochelatins:the principle heavy metal complexing peptides of higher plants. Science, 230:674-676.

37. Grotz, N., Fox, T., Connolly, E., Park, W., Guerinot, M.L., 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, USA, 95: 7220–7224.

38. Groves, J.T., Dias, R.M. (1979), Rapid amide hydrolysis mediated by copper and zinc. J.Am. Chem. Soc, 101:1033-1035.

39. Guerinot, M.L. (2000), The ZIP family of metal transporters. Biochimica et Biophysica Acta, 1465:190–198.

40. Hacisalihoglu, G., Hart, J. J., Wang, Y. H., Cakmak, I., Kochian, L. V. (2003), Zinc efficiency is correlated with enhanced expression and activity of zinc-requiring enzymes in wheat. Plant Physiology, 131: 595 – 602.

41. Hall, J.L., Williams, L.E. (2003), Transition metal transporters in plants. Journal of Experimental Botany., 54: 2601-2613.

42. Haney, J.C., Grass, G., Franke, S., Rensing, C. (2005), New developments in the understanding of the cation diffusion facilitator family. Journal of Industrial Microbiology and Biotechnology, 32: 215-226.

43. Hara, K., Yagi, M., Koizumi, N., Kusano, T., Sano, H. (2000), Screening of wound-responsive genes identifies an immediate early expressed gene encoding a highly charged protein in mechanically wounded tobacco plants. Plant Cell Physiology, 41:684–691.

44. Hart, J.J., Welch, R.M., Norvell, W.A., Sullivan, L.A., Kochian, L.V. (1998), Characterization of Cadmium Binding, Uptake, and Translocation in Intact Seedlings of Bread and Durum Wheat Cultivars . Plant Physiol, 116: 1413-1420.

45. Hart, J.J., Welch, R.M., Norvell, W.A. , Kochian, L.V. (2002), Transport interactions between cadmium and zinc in roots of bread and durum wheat seedlings .Physiologia Plantarum, 116: 73–78

46. Holloway, R. E. (1996), Zinc as a Subsoil Nutrient for Cereals. Unpublished PhD, University of Adelaide.

47. Huang, L., Gitschier, J. (1997), A novel gene involved in zinc transport is deficient in the lethal milk mouse. Nature Genetics, 17: 292-297.

48. Inaba, K., Fujiwara, T., Hayashi, H., Chino, M., Komeda, Y., Naito, S. (1994), Isolation of an *Arabidopsis thaliana* mutant, *mto1*, that overaccumulates soluble methionine. Plant Physiol., 104:881–887.

49. Ito,T., Kito, K., Adati, N., Mitsui, Y., Hagiwara, H., Sakaki, Y. (1994), Fluorescent differential display: arbitrarily primed RTPCR fingerprinting on an automated DNA sequencer. FEBS Letters, 351:231–236.

50. Jendrisak, J., Burgess, R.R. (1975), A new method for the large scale purification of wheat germ DNA-dependent RNA Polymerase II. Biochemistry, 14:4639-4645.

51. Jyung, W.H., Camp, M.E., Polson, D.E., Afams, M.W., Witter, S.H. (1972), Differential response of two bean varieties to zinc as revealed by electrophoretic protein pattern. Crop Sci., 12: 26-29.

52. Kanwar, J.S., Youngdal, L.J. (1985), Micronutrient needs of tropical food crops. In: Vlek, P.L.G. (ed) Micronutrients in Tropical Foods: Fertiliser Research;.43 – 67.

53. Kim, J., Leustek, T. (2000), Repression of cystathionine-synthase in *Arabidopsis thaliana* produces partial methionine auxotrophy and developmental abnormalities. Plant Sci., 151: 9–18.

54. Kim, J., Lee, M., Chalam, R., Martin, M. N., Leustek, T., Boerjan, W. (2002), Constitutive Overexpression of Cystathionine _-Synthase in Arabidopsis Leads to Accumulation of Soluble Methionine and *S*-Methylmethionine. Plant Physiology, 128: 95–107.

55. Kitagishi, K., Obata, H. (1986), Effects of Zn deficiency on the nitrogen metabolism of meristematic tissues of rice plants with reference to protein synthesis. Soil Sci. Plant Nutr, 32:397-405.

56. Kitagishi, K., Obata, H., Kondo, T. (1987), Effect of Zn deficiency on 80S ribosome content of meristematic tissues of rice plant. Soil Sci. Plant Nutr, 33: 423-429.

57. Kochian L.V. (1991), Mechanisms of micronutrient uptake and translocation in plants. In: Mortvedt, J.J (ed.) Micronutrients in Agriculture, Soil Science Society of America, Madison, WI, pp 251-270.

58. Kochian, L.V. (1993), Zinc absorption from hydroponic solution by plant roots. In: Robson, A.D (ed.) Zinc in Soils and Plants, Kluwer Academic Publishers. Dordrecht, The Netherlands, pp. 45 - 58.

59. Korshunova, Y.O., Eide, D., Clark, W.G., Guerinot, M. L., Pakrasi, H.B. (1999), The IRT1 protein from Arabidopsis thaliana is a metal transporter with a broad substrate range. Plant Molecular Biology, 40: 37–44.

60. Liang, P., Averboukh, L., Keyomarsi, K., Sager, R., Pardee, A.B. (1992), Differential display and cloning of messenger RNAs from human breast cancer versus mammary epithelial cells. Cancer. Res., 52:6966-6968.

61. Liang, P., Pardee, A.B. (1992), Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science., 257:967-971.

62. Liang, P., Averboukh, L., Pardee, A.B. (1993), Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements. Nucleic Acids Res., 21: 3269-3275.

63. Lievens, S., Goormachtig, S., Holsters, M.A. (2001), Critical evaluation of differential display as a tool to identify genes involved in legume nodulation: looking back and looking forward. Nucleic Acid Res., 29: 3459-3468.

64. Marschner, H. and Cakmak, I. (1989), High light intensity enhances chlorosis and necrosis in leaves of zinc potassium, and magnesium deficient bean (*Phaseolus vulgaris* L.) plant. J. Plant. Physiol., 134: 308-315.

65. Marschner, H. (1995), Mineral Nutrition of Higher Plants, 2'nd edn. Academic, San Diego.

66. Mäser, P., Thomine, S., Schroeder, J.I., Ward, J.M., Hirschi, K., Sze, H., Talke, I.N., Amtmann, A., Maathuis, F.J.M., Sanders, D., Harper, J.F., Tchieu, J., Gribskov, M., Persans, M.W., Salt, D.E., Kim, S.A., Guerinot, M. L.(2001), Phylogenetic relationships within cation transporter families of *Arabidopsis*. Plant Physiology., 126: 1646–1667.

67. Maze, P. (1914) Influences respectives des elements de la solution mineral du mais. Annales de l'Institut Pasteur (Paris), 28: 21-69.

68. Neue, H.U., Quijano, C., Senadhira, D., Setter, T. (1998), Strategies for dealing with micronutrient disorders and salinity in lowland rice systems. Field Crops Research, 56:139 - 155.

69. Ohki, K. (1976), Effect of zinc nutrition on photosynthesis and CA activity in cotton. Physiol. Plant, 38:300-304.

70. Olsen, S. R. (1972), Micronutrient interaction. In: Mortvedt, J.J., Giordano, P. M., Lindsay, W. L (Eds.) Micronutrients in Agriculture, American Society of Agronomy, Madison. pp. 199-227.

71. Paulsen, I. T., Saier, Jr. M.H. (1997), A novel family of ubiquitous heavy metal ion transport proteins. Journal of Membrane Biology., 156: 99–103.

72. Prask, J.A., Plocke, D. J. (1971), A role for zinc in the structural integrity of cytoplasmic ribosomes of Euglena gracilis. Plant Physiol, 48:150-155.

73. Rengel, Z. and Graham R.D. (1995), Importance of seed Zn content for wheat growth on Zn-deficient soil. I. Vegetative growth. Plant Soil, 173: 259–266.

74. Rengel, Z. (1999), Physiological mechanisms underlying differential nutrient efficiency of crop genotypes. In: Rengel, Z. (ed) Mineral nutrition of crops, Fundamental mechanisms and implications. Food Products Press, (Haworth Press) pp. 227 - 265.

75: Riceman, D.C., Jones, G.B. (1959), Distribution of zinc and copper in subterranean clover (*Trifolium subterraneum L.*) grown in culture solution supplied with graduated amount of zinc. Aust. J. Agr. Res, 9:73-122.

76. Sambrook, J., Manniatis, T., Fritsch, E. F. (2001), Molecular cloning: a laboratory manual:Cold Spring Harbor Laboratory Press. 3rd edition.

77. Schmid, W.E., Haag, H.P., Epstein, E. (1965), Absorption of zinc by excised barley roots. Physiol. Plant, 18:860-869.

78. Seethambaram, Y., Das, V.S.R. (1984), RNA and RNase of rice (*Oryza sativa L*) and pearl millet (*Pennisetum americanum L. Leeke*) under zinc deficiency. Plant Physiol. Biochem., 11: 91-94.

79. Sharma, P. N., Chatterjee, C., Sharma, C. P., Nautiya, N., Agarwala, S. C. (1979), Effect of zinc deficiency on development and physiology of wheat polen. J. India Bot. Soc, 58:330-334.

80. Sommer, A.L., and Lipman ,C.B. (1926), Evidence on the indispensable nature of zinc and boron for higher green plant. Plant Physiol., 1:231-249.

81. Srivastava, P.C., Gupta ,U.C. (1996), Trace Elements in Crop Production. Science Publishers, Lebanon, NH. 356pp.

82. Suzuki, N., Kouzumi, N., Sano, H. (2001), Screening of cadmium-responsive genes in *Arabidopsis thaliana*. Plant, Cell, Environ., 24: 1177-1188.

83. Tobin, A. J. (1970), Carbonic anhydrase from parsley leaves. J. Biochem., 245: 2656-2666.

84. Torre, M., Rodriguez, A. R., Saura-Calixto, F. (1991), Effect of diet on fiber and phytic acid on mineral availability. Crit. Rev. Food Sci. Nutr., 1: 1-22.

85. Turner, R. G. (1970), The subcellular distribution of zinc and copper within the roots of metal- tolerant clones of *Agrostis tenuis sibth*. New Phytol., 69,725-731.

86. Wainright, S.J., Woolhouse, H.W. (1978), Inhibition by zinc of cell wall acid phosphatase from roots in zinc-tolerant and non-tolerant clones of Agrostis tenuis sibth. J.Exp.Bot., 29;525-531.

87. Walker, C.D., Welch, R.M. (1987), Low molecular weight complexes of zinc and other trace metals in lettuce leaf. J.Agr.Food Chem., 35:721-727.

88. Welch, R.W., Webb, M. J., Loneragan, J. F. (1982), Zinc in membrane function and its role in phosphorus toxicity. In: Scaife, A. (ed) Plant Nutrition: Proceedings of the Ninth International Plant Nutrition Colloquium, Warwick University, UK, Commonwealth Agricultural Bureaux, Farnham House, Farnham Royal, UK, pp 710-715.

89. Williams, L.E., Pittman, J.K., Hall, J.L. (2000), Emerging mechanisms for heavy metal transport in plants. Biochimica et Biophysica Acta., 1465: 104–126.

90. Van der Zaal, B.J., Neuteboom, L. W., Pinas, J. E., Chardonnens, A. N., Schat, H., Verkleij, J.A.C., Hooykaas, P.J.J. (1999), Over-expression of a novel *Arabidopsis* gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. Plant Physiology., 119: 1047–1055.

91. Vaughan, D., DeKock, P.C., Ord, B.G. (1982), The nature and localization of superoxide dismutase in fronds of *Lemma gibba* L. and the effect of copper and zinc deficiency on its activity. Physiol. Plant., 54, 253-257.

92. Vert, G., Briat, J.F., Curie, C. (2001), *Arabidopsis* IRT2 gene encodes a root periphery iron transporter. The Plant Journal., 26: 181–189.

APPENDIX-A

Supplies

Disposable Labware:

3-part syringes:

Ayset, Turkey (5 mL, 50 mL)

Centrifuge tubes:

Techno Plastic Products AG, Switzerland (91015, 91050)

ClickFit Cap microtubes:

TreffLab, Switzerland (96.8185.9.03, 96.7811.9.03, 96.9329.9.01)

Diamond® Tips:

Gilson, USA (D10, D200, D1000)

PCR-tubes:

TreffLab, Switzerland (96.9852.9.01)

Petri dishes:

ISOLAB Laborgeräte GmbH, Germany (113.02.002)

Polystyrene round-bottom test tubes:

Becton Dickinson Falcon™, USA (352001)

Puradisc™ FP 30 syringe filters:

Whatman® Schleicher & Schuell, UK (10462200)

Tips for pipettes:

TreffLab, Switzerland (96.9515, 96.8700, 96.8276)

Chemical Supplies:

2-Propanol extra pure:

Merck KGaA, Germany (1.00995)

2-Propanol puriss., ≥99.5% (GC):

Riedel-de Haën®, Germany (24137)

6X Loading Dye Solution:

Fermentas, Canada (R0611)

Agar Type A, plant cell culture tested:

Sigma-Aldrich Co., USA (A4550)

Agarose low EEO (Agarose Standard):

AppliChem GmbH, Germany (A2114)

Ampicillin sodium salt *BioChemica*:

AppliChem GmbH, Germany (A0839)

aTaq DNA Polymerase:

Promega, USA (M1245)

Boric acid for molecular biology, ~99%:

Sigma-Aldrich Co., USA (B6768)

Chloroform Biotechnology Grade:

Amresco® Inc., USA (0757)

D-(+)-Glucose monohydrate *BioChemika Ultra*, ≥99.5% (HPLC):

Fluka, Switzerland (49158)

dATP, molecular biology grade:

Fermentas, Canada (R0141)

Deoxyribonuclease I (DNase I), RNase-free:

Fermentas, Canada (EN0521)

Diethyl pyrocarbonate, ≥97% (NMR):

Sigma-Aldrich Co., USA (D5758)

dNTP mix:

Promega, USA (U1515)

Ethanol absolute extra pure:

Merck KGaA, Germany (1.00986)

Ethidium Bromide Solution 1 % in water:

Merck KGaA, Germany (1.11608)

Ethylenediaminetetraacetic acid disodium salt dihydrate for molecular biology, ≥99%:

Sigma-Aldrich Co., USA (E5134)

GeneRuler™ 100bp DNA Ladder Plus:

Fermentas, Canada (SM0321)

IPTG, dioxane-free:

Fermentas, Canada (R0393)

LB Broth:

Sigma-Aldrich Co., USA (L3022)

Luria Agar:

Sigma-Aldrich Co., USA (L3147)

Magnesium chloride hexahydrate puriss., 99-101%, total impurities ≤0.0001% Al:

Riedel-de Haën®, Germany (13152)

MS medium basal salt mixture including vitamins:

Duchefa Biochemie B.V., the Netherlands (M0222)

Oligo$(dT)_{12\text{-}18}$ Primer:

Invitrogen, USA (18418-012)

pGEM®-T Vector System II:

Promega, USA (A3610)

Plant Preservative Mixture™:

Plant Cell Technology, USA

Potassium chloride *BioChemika Ultra*, ≥99.5% (AT):

Fluka, Switzerland (60129)

RNaseOUT™ Recombinant Ribonuclease Inhibitor:

Invitrogen, USA (10777-019)

Sodium chloride EMPROVE®:

Merck KGaA, Germany (1.06400)

Sodium hydroxide pellets pure:

Merck KGaA, Germany (1.06462)

Sucrose Grade I, plant cell culture tested:

Sigma-Aldrich Co., USA (S5390)

Tris Buffer Grade:

AppliChem GmbH, Germany (A1379)

Tris(hydroxymethyl)aminomethane Biotechnology Grade:

Amresco® Inc., USA (0826)

TRIzol® Reagent:

Invitrogen, USA (15596)

Biozol Reagent:

Biogen, Istanbul

Tryptone:

AppliChem GmbH, Germany (A1553)

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside):

Promega, USA (V3941)

Yeast extract *BioChemica*:

AppliChem GmbH, Germany (A1552)

Commercial Kits:

Omniscript RT Kit:

Qiagen Inc., USA (205111)

QIAprep Spin Miniprep Kit:

Qiagen Inc., USA (27106)

QIAquick Gel Extraction Kit:

Qiagen Inc., USA (28706)

QIAquick PCR Purification Kit:

Qiagen Inc., USA (28104)

Appendix B: Equipment

Autoclave Hirayama, Hiclave HV-110, Japan.

Balance Sartorius, BP610, BP221S, BP221D, Germany.

Blot Module Novex, X Cell II Blot Module, USA.

Centrifuge Eppendorf, 5415R, Germany; Hitachi, Sorvall RC5C Plus, USA.

Deep Freeze Bosch, -20±C, Turkey.

ddH2O Millipore, MilliQ Academie, Elix-S, France.

Digital Camera Canon, PowerShot SD 400, USA; Olympus, C-7070, USA.

Electrophoresis BioRad Inc., USA; Novex, X Cell SureLock Electrophoresis Cell, USA.

Element Analysis Varian, Vista-Pro CCD Simultaneous ICP-OES, Australia; LECO, TruSpec CN, USA.

Gel Documentation BioRad, Universal Hood II, USA; BioRad, Quantity One, USA; BioRad, GelDoc XR, USA.

Ice Machine Scotsman Inc., AF20, USA.

Imaging Software GIMP 2.2.12.

Incubator Memmert D06059 Model 300, Germany.

Incubator shaker New Brunswick Scientific Innova 4330, USA.

Laminar Flow Cabinets Heraeus Instruments HS12, Germany.

Lighting Olympus, LG-PS2, USA.

Magnetic Stir VELP Scientifica, ARE Heating Magnetic Stirrer, Italy.

Microliter Pipette Gilson, Pipetman, France.

Microplate Reader BioRad, Model 680 Microplate Reader, USA.

Microscope Olympus, SZ61, USA.

Microwave Oven CEM Corp., Mars Xpress, USA; Bosch, Turkey.

pH Meter WTW, pH540GLP MultiCal, Germany.

Power Supply BioRad, PowerPac 300, USA; Wealtec, Elite 300, USA.

Refrigirator Bosch, +4±C, Turkey.

Sonicator Bioblock Scientific, Vibracell 75043, France; Bandelin, Sonorex, Germany.

Spectrophotometer Schimadzu, UV-3150, Japan; Nanodrop, ND-1000, USA.

Thermomixer Eppendorf, Thermomixer Comfort, Germany.

Vortex VELP Scienti¯ca, 2x3, Ital

APPENDIX C

Gel Picture of DNase and non-DNase Treated RNA Samples

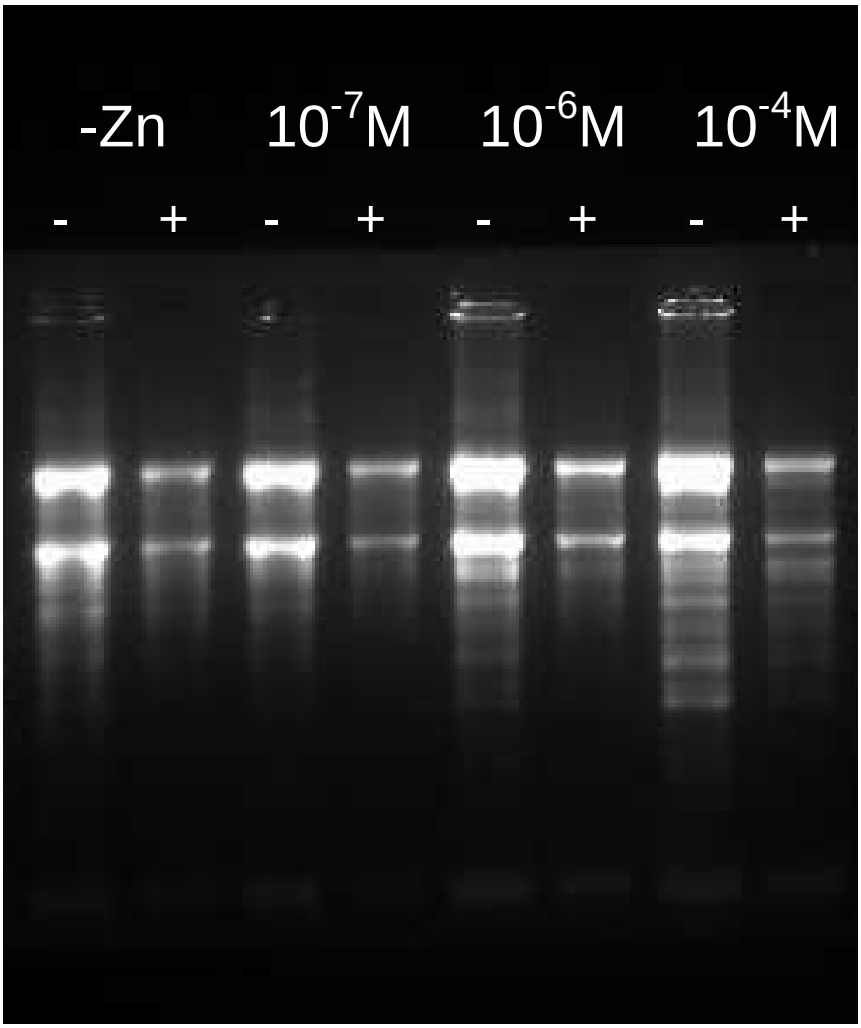


Figure C.1 Gel picture of RNA isolated from the roots of Bezostaja exposed to 4 different Zn concentrations. "+" stands for DNase-treated samples and "-" stands for non-DNase treated samples.

Gel Picture of DNase and non-DNase treated RNA samples

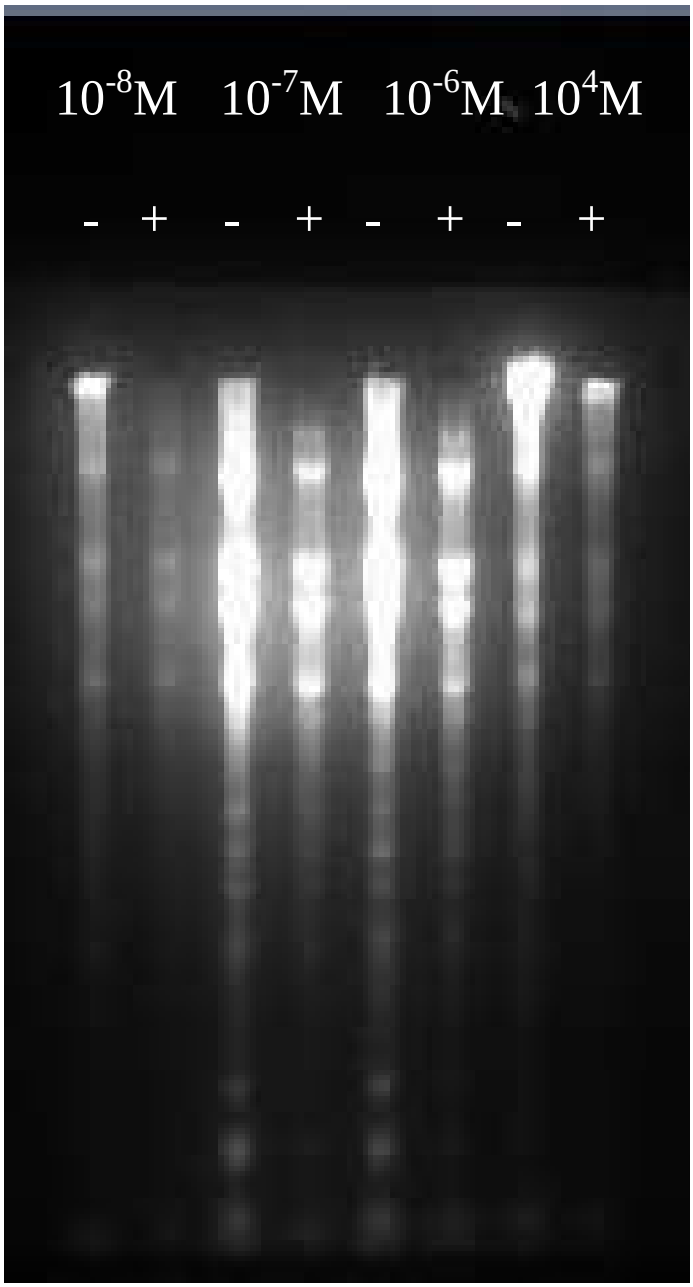


Figure C.2 Gel picture of RNA isolated from the shoots of Bezostaja exposed to 4 different Zn concentrations. "+" stands for DNase-treated samples and "-" stands for non-DNase treated samples.

APPENDIX D

Colony PCR analysis of clones obtained via PCR of Bezostaja shoots

Figure D.1 Colony PCR gel picture obtained via using T1P9 and T3P2 primer combination.The clones which contain the differentially expressed cDNAs have been indicated to be positive clones. The lanes with negative signs were the clones which did not incorporate inserts of interest.

Colony PCR analysis of clones obtained via PCR of Bezostaja roots

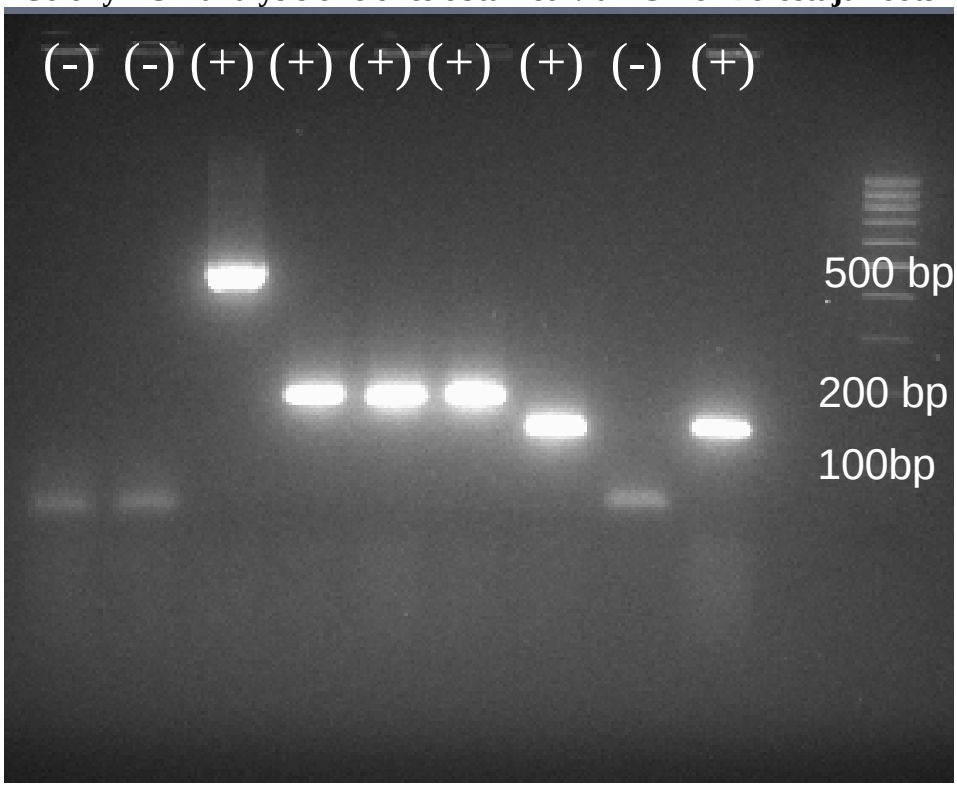
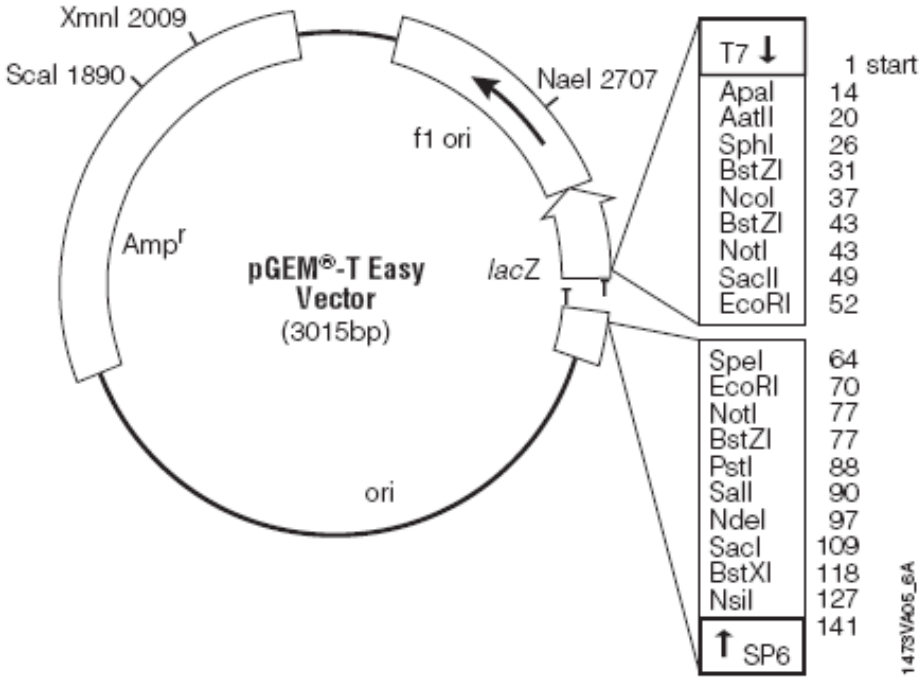


Figure D.2 Colony PCR gel picture obtained via using T1P4 primer combination.The clones which contain the differentially expressed cDNAs have been indicated to be positive clones. The lanes with negative signs were the clones which did not incorporate inserts of interest.

APPENDIX E

pGEM®-T Easy Vector Map and Sequence Reference Points



T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
*lac*Z start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3