IDENTIFICATION AND CHARACTERIZATION OF A cDNA ENCODING ZIP TRANSPORTER PROTEIN IN *Triticum dicoccoides*

by

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ABSTRACT

Wild emmer wheats exhibit a potential genetic resource for wheat improvement and increased food production. In this study, different accessions of wild emmer wheat, *Triticum dicoccoides*, were studied for their Zn deficiency tolerance which is particularly prevalent in wheat leading to significant reduction in yield and nutritional quality of grains. *T. dicoccoides*, selected from a screening study, were grown under Zn deficient conditions. Three *T. durum* wheats, Balcali 85, Ç-1252 and Meram, were also included in this study as standard.

Increasing data are being accumulated to identify proteins that are involved in Zn deficiency. Hence, ZIP family of metal transporter proteins are extensively being studied. In this study, identification of cDNA encoding ZIP transporter protein in wild emmer wheat was studied. The result revealed that cDNA does not have high level of polymorphism compared to cultivated wheats. The expression of identified ZIP transcript was measured using root and shoot of plants subjected to Zn deficiency. Quantitative Real-time PCR results revealed that ZIP transcript levels are elevated with decreasing Zn supply in all accessions. ZIP transcript accumulation was lower in root of MM5/4 accession, which is the most tolerant accession to Zn deficiency, than that of 19-36 accession, which is one of the most susceptible accessions. This is most likely because the susceptible genotype senses the Zn deficiency stress earlier than the tolerant does so that the response of 19-36 root cells are much quicker than the response of MM 5/4 root cells. The low level expression and the absence of ZIP transcript at 10^{-4} M Zn concentration suggested that this ZIP metal transporter found in these accessions was Zn-specific.

YABANİ BUĞDAYDA, *Triticum dicoccoides*, ZIP TAŞIYICI PROTEİNİ SENTEZLEYEN cDNA DİZİSİNİN TANIMLANMASI VE KARAKTERİZASYONU

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ÖZET

Yabani emmer buğdayı, ekilen buğday türlerinin verimini yükseltmek ve kalitesini artırmak açısından potansiyel genetik zenginlik içermektedir. Çinko (Zn) eksikliği, buğdayda hem verimin hem de tane besin kalitesinin düşmesine yol açmaktadır. Bu çalışmada, altı farklı yabani buğday genotipi, *Triticum dicoccoides*, Zn stresine maruz bırakıldı ve yapılan sera çalışmaları Zn eksikliğine karşı verilen cevaplarda genotipler arası çeşitlilik bulunduğunu ortaya koydu. Yabani buğday genotiplerinin yanı sıra üç tane *T. durum* buğdayı (Balcalı 85, Ç-1252 , Meram) da referans bitki olmaları açısından bu çalışma içine alındı.

Zn eksikliği stresinde, dayanıklılığa katkıda bulunan mekanizmalar henüz tam olarak bilinmemektedir. Fakat gün geçtikçe Zn eksikliğinde rol alan daha çok protein belirlenmektedir. Bu çalışmada, yabani buğdaylardaki ZIP proteininin tamamlanmış DNA (cDNA) dizisi belirlenmiş ve bu dizilerle kültüre alınmış buğday genotiplerinin dizileri arasında önemli bir fark bulunmadığı gözlemlenmiştir. Zn noksanlığına maruz bırakılan bitkilerin kök ve yeşil aksamlarından elde edilen örneklerindeki ZIP ekpresyon seviyeleri QRT-PCR yöntemiyle ölçülmüştür. Elde edilen sonuçlara göre, tüm genotiplerde besi yerindeki Zn miktarı azaldıkça ZIP cDNA ekpresyon seviyesi artmıştır. Zn stresine en dayanıklı olan genotip MM 5/4'ün kök hücrelerinde, Zn stresine en duyarlı genotiplerden biri olan 19-36'nın kök hücrelerine nazaran daha az ZIP ekspresyonu saptanmıştır. Bunun nedeni büyük olasılıkla, duyarlı genotipin, 19-36, kök hücrelerinin dayanıklı genotipin, MM 5/4, kök hücrelerine nazaran stresi daha çabuk hissedip daha çabuk ZIP seviyesini artırmaya çalışmasından kaynaklanmaktadır. Toksik Zn uygulamasında (10^{-4} M Zn) ZIP ekpresyon seviyesinin kontrol bitkisine kıyasla artmaması, bu çalışmada kullanılan yabani buğdaylardan dizisi çıkarılan ZIP proteininin Zn'ye özel olabileceği olasılığını kuvvetlendirmektedir.

To my family with all my heart...

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LIST OF ABBREVIATIONS

Amp	Ampicillin
CA II	Carbonic anhydrase II
cDNA	Complementary Deoxyribonucleic acid
CDF	Cation diffusion facilitator
DEPC	Diethyl pyrocarbonate
EDTA	Ethylene diamine tetra-acetic acid
EST	Expressed sequence tag
IAA	Indole-3-acetic acid
IRT	Iron-Regulated Transporter
PC	Phytochelatin
PS	Phytosiderophores
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
MTP1	Metal Transport Protein 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NRAMP	Natural resistance-associated macrophage protein
SOD	Superoxide Dismutase
TM	Transmembrane
X-Gal	5-brom-4-chloro-3-indolyl-beta-D-galactopyranoside
ZAT1	Zinc Transporter of *Arabidopsis thaliana*
ZIP	ZRT-like and IRT-like Proteins
Zn	Zinc
ZRE	Zn-responsive element
ZRT	Zinc-Regulated Transporter

1) INTRODUCTION

1.1 Zinc in Plant Nutrition

1.1.1 Physiological Aspects of Zinc in Plants

Zinc, as a biological requirement, was first observed by Raulin in 1869. He realized that the common bread mould (*Aspergillus niger*) was unable to grow in the absence of Zn. However, Zn deficiency under field conditions was not identified until 1932. Since 1932, Zn has been found to be an essential micronutrient in crop production and its deficiency has been shown to be more prevalent over the world when compared to other micronutrient deficiencies. (Brown et al. 1993) Marschner asserted that Zn plays both a functional and a structural role in various enzymatic reactions via its strong tendency to form tetrahedral complexes with N-, O- and S-ligands (Alloway, 2004).

The predominant forms of Zn in plants are found to be as: low molecular weight complexes, storage metalloproteins, free ions, and insoluble forms associated with the cell walls. Complexation with organic ligands or phosphorus causes Zn to become inactivated within the cells. The water-soluble form (low molecular weight complexes and free ions) of Zn ranges from 58 % to 91 % among various species. Since the water soluble fraction of Zn is considered to be the most physiologically active, it is often referred as a better indicator of plant Zn status than total Zn contents (Alloway, 2004). The most abundant soluble form of Zn which is found in the low molecular weight complexes is said to be the most active form of the metal since they can be degraded and might be involved in homeostatic mechanisms where they may bind to excess free Zn ions, acting like a buffer system. In this context, phytochelatins are found in a wide range of species and synthesized in response to excess cadmium, zinc and mercury exposure (Brown et al. 1993).

1.1.1.1 Zinc in Proteins

Zinc has in functional, structural and regulatory roles in more than 300 enzymes (McCall et al., 2000). It has been identified to be found in more than 70 metallo-enzymes which are classified in six different classes: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. (Barak and Helmke, 1993) Zn is generally tightly bound to the apoenzyme which can only be removed with severe chemical treatments (Brown et al., 1993). Zn binding sites of proteins are often consisted of the sulfur of cystein, the nitrogen of histidine or the oxygen of aspartate and glutamate, or a combination (McCall et al., 2000). Zn is fully coordinated with four protein ligands in enzymes where it plays a structural and regulatory role. Conversely, catalytic Zn is bound with three ligands and a water molecule. It is considered that the presence of a water molecule, offering an open coordination site is essential for the catalytic function of Zn (Brown et al., 1993). Figure 1.1 illustrates the zinc-binding site of carbonic anhydrase II (CA II) where three histidines are bound to Zn (McCall et al., 2000).

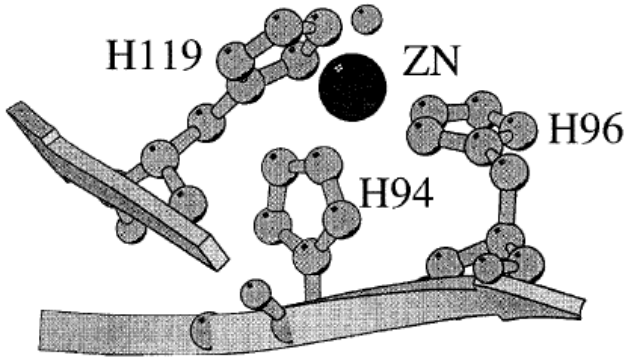

Fig. 1.1 The Zn-binding site of CA II. The first two ligands, H_{94} and H_{96}, are on the same strand of the β-sheet whereas the third ligand, H_{119}, is on the neighboring strand of the β-sheet.

1.1.1.2 Physiological Functions of Zinc

1.1.1.2.1 Carbohydrate Metabolism

Zn is involved in carbohydrate metabolism through its effects on photosynthesis and sugar transformations.

1.1.1.2.1.a Photosynthesis

Zn causes a reduction in net photosynthesis by 50 %-70 % depending on the plant species and the severity of the deficiency. One of the enzymes in photosynthesis, whose activity is declined by Zn deficiency, is carbonic anhydrase. The presence of carbonic anhydrase activity in C_3 plants is uncertain whereas it is generally considered to be involved in C_4 plants (Alloway, 2004).

C_3 plants, such as wheat, rice and soybean, exhibit the most basic photosynthetic mechanism which fixes CO_2 once using the Calvin-Benson cycle only. It was asserted that there is no direct relationship between carbonic anhydrase activity and photosynthetic CO_2 assimilation or growth of C_3 plants with different Zn applications (Graham et al., 1992). Carbonic anhydrase affects Zn content through its effect on photosynthesis and dry matter production. Its activity is absent with extreme Zn deficiency (Alloway, 2004).

In contrast to the C_3 plants, C_4 plants, such as maize, sugarcane and sorghum, fix CO_2 twice and possess a mechanism to increase the CO_2 levels in their leaves. They utilize a four carbon pathway and the Calvin-Benson cycle consecutively. High carbonic anhydrase activity is required in mesophyll chloroplasts to provide substrates for photosynthesis in C_4 plants. Therefore, Zn deficiency may cause more pronounced effect on the rate of photosynthesis in C_4 plants than that in C_3 plants (Marschner, 1995).

Another Zn containing-enzyme that plays a role in photosynthesis is ribulose 1,5-biphosphate carboxylase (RuBPC) which has been identified to catalyze the initial step of CO_2 fixation in photosynthesis (Brown et al., 1993). Additionally, the decrease in chlorophyll content and the abnormal structure of chloroplasts can also contribute to the reduction in photosynthesis rate in Zn deficiency plants (Alloway, 2004).

1.1.1.2.1.b Sucrose and Starch Formation

It has been shown that enzymes involved in sucrose metabolism are severely affected by Zn deficiency. It was found that the reduction in the activity of sucrose synthetase caused a decline in the level of sucrose in sugar beet and maize. Depression of starch content, activity of the enzyme starch synthetase, and the number of starch grains in Zn deficient plants suggests that Zn may probably play a crucial role in the metabolism of starch (Alloway, 2004).

On the other hand, another study suggested that Zn deficiency has been led to the increased concentration of sucrose and starch in the leaves of cabbage whereas the concentrations were decreased in the roots of bean carbohydrate. These studies suggest that Zn deficiency may impair the translocation of sucrose from the source leaves to the roots. It has also been shown that phloem loading of sucrose can be restored with Zn application. Although the whole mechanism involved in the impaired sucrose transport has not been totally elucidated, it could be due to the presence of Zn in the integrity of the biomembranes (Alloway, 2004).

1.1.1.2.2 Protein Metabolism

There is a high correlation between protein content and Zn deficiency caused a reduction in protein content while the composition remains almost the same (Brown et al., 1993). Study on beans revealed that even a 6.5 fold difference in the concentration of free amino acids between Zn supplied and Zn deficient plants can be recovered after

the application of Zn for 48 or 72 hours. Zn deficiency is considered to be involved in protein synthesis via the reduction in RNA and the reduction and deformation of ribosomes. In the meristem of rice seedlings, it was shown that the level of RNA and the number of free ribosomes were significantly decreased under Zn deficiency (Alloway, 2004).

Ribonuclease activity has been found to be increased by Zn deficiency in higher plants. This is due to the necessity of Zn for the enzyme RNA polymerase which protects the ribosomal RNA from attack by the enzyme ribonuclease. Consequently, the preliminary effect of Zn deficiency is reflected in the sharp decline in the level of RNA. However, the reduction in RNA is not directly associated with the increase in the ribonuclease activity because it was shown that the reduction in RNA can occur before the increase in ribonuclease activity. Due to the importance of Zn in protein metabolism, it was suggested that the higher concentrations of Zn is crucial especially for the meristematic tissues where cell division with nucleic acid and protein synthesis is regularly taking place. It is important to note that Zn has a fundamental role in the stability and function of the genetic material (Alloway, 2004).

1.1.1.2.3 Membrane Integrity

Zn is considered to play a critical role both in the structure and function of biomembranes in plants, as well as in animals. It has been observed that efflux of K^+, amino acids, sugars and phenolics are increased in Zn-deficient plant roots. However, the leakage has been found to decrease after Zn application for at least 12 hours (Alloway, 2004). Another study utilized root exudates, as an indicator of root plasma membrane integrity and showed that there is a greater leakage of ^{32}P isotope out of roots of Zn-deficient wheat than from Zn-sufficient roots (Welch et al., 1982). Thus, it has been suggested that Zn may contribute to the integrity of cellular membranes through the structural orientation of macromolecules and the maintenance of ion transport systems.

Other than structural importance of Zn, it has also been considered to have a role in controlling the generation and detoxification of free oxygen radicals ($O_2^{\cdot-}$) which damage membrane lipids and sulphydryl groups of membrane proteins. The major role of Zn in membrane integrity is considered to be involved in the protection of membrane lipids and proteins from peroxidation caused by the free oxygen radical attacks. Structural and functional impairments in root cell membranes associated with the enhanced activity of $O_2^{\cdot-}$ -generating NADPH oxidase by different stress factors, including Zn deficiency, have been shown in different studies (Cakmak and Marschner, 1987, 1988, 1998). Cakmak and Marschner showed that Zn-deficient cotton, bean and tomato root cells have reduced level of superoxide dismutase (SOD) activity and an increased NADH- dependent free oxygen radical production (Cakmak and Marschner, 1987, 1988b).

It has been found that Zn-deficient plants showed a toxic accumulation of phosphorus (P) in the oldest leaves. However, Zn-treated plants had higher accumulation of P without displaying toxicity symptoms. This is due to the fact that Zn deficient plants “leak” more P than the Zn-treated ones since Zn is involved in the cell membrane integrity. This observation led to suggest that Zn-deficient roots, due to impaired membrane integrity, could allow non-selective entry of boron and phosphorus into the roots which could then be transported and accumulated to the transpiring older leaves (Alloway, 2004).

This hypothesis was further reinforced by Marschner (Marschner et al., 1987) who figured out that the roots of Zn-deficient cotton plants excreted 3.3 times more amino acids and 2.6 times more carbohydrates than Zn-sufficient control plants. Cakmak and Marschner also reported that potassium leakage was significantly greater from the roots of Zn-deficient cotton, wheat and tomato plants. The loss of potassium is particularly significant since it is a constituent of the cell sap and its leakage indicates that the integrity of the cell membrane is impaired. However, it has been found that this leakage could be mitigated by supplying Zn for 12 hours (Cakmak and Marschner, 1998). Leakage of nitrate and amino acids from Zn-deficient cotton plants and the leakage of sugars and phenols from Zn-deficient apple trees were observed to be much pronounced than that of their controls (Cakmak and Marschner, 1988a).

The major role of Zn in membrane integrity is through its protection of membrane proteins and lipids from the destructive effects of free radicals and Zn, together with copper (Cu), is involved in SOD activity which scavenge free radicals (Cakmak and Marschner, 1998). Wheat subjected to Zn deficiency showed a decline in the activities of superoxide dismutase, peroxidase, ascorbate peroxidase, glutathione reductase and particularly that of cyanide-sensitive superoxide dismutase. However, supplementation of Zn within 24 h significantly increased activities of cyanide-sensitive and total superoxide dismutase and ascorbate peroxidase, and concentration of H_2O_2, and decreased malondialdehyde significantly (Sharma et al., 2004).

1.1.1.2.4 Auxin Metabolism

Reduced growth and stunted leaf are the most distinguished features under Zn deficiency which are probably associated with the disturbances in the auxin metabolism. Zn is required for the synthesis of auxin (a growth promoting phytohormone, particularly indole-3-acetic acid (IAA)). It is still uncertain that either inhibited synthesis or enhanced degradation of IAA causes low levels of IAA. There is some evidence that Zn is involved in the synthesis of tryptophan which is the most likely precursor for the biosynthesis of IAA. It has been observed that Zn fertilization of rice plants growing on a calcareous soil, where Zn availability is limited for plant roots, increased tryptophan content in rice grains (Brown et al., 1993). Cakmak et al. found that the level of IAA in the shoot tips and young leaves of Zn deficient bean decreased about 50% to that of Zn-sufficient plants. It was observed that IAA levels increased by re-supplying Zn to the deficient plants for up to 96 h. Tryptophan level was found to be parallel to that of most of the other amino acids under Zn deficiency. Therefore, it was claimed that the decrease in IAA level in Zn-deficient plants is not brought about by impaired synthesis of tryptophan instead Zn in protein synthesis was impaired. It was also argued that it is unlikely that the conversion of tryptophan to IAA is specifically inhibited in Zn deficient plants (Cakmak et al., 1989).

1.1.1.2.5 Reproduction

Zn-deficient plants show reduction in seed production and flowering. There are two suggestions for reduced seed production in Zn-deficient plants:

a) increased formation of abscissic acid which causes premature loss of leaves and flower buds,
b) disruption of the development and physiology of anthers and pollen grains. It was reported that Zn-deficient wheat developed small anthers and abnormal pollen grains (Brown et al., 1993).

1.1.2 Uptake and Translocation of Zinc by Plants

In recent years, an increasing number of genes that encode membrane proteins involved in metal transport are being identified. Figure 1.2 illustrates a model of the potential regulation points in the regulation of metal homeostasis in crop plants (Grusak et al., 1999).

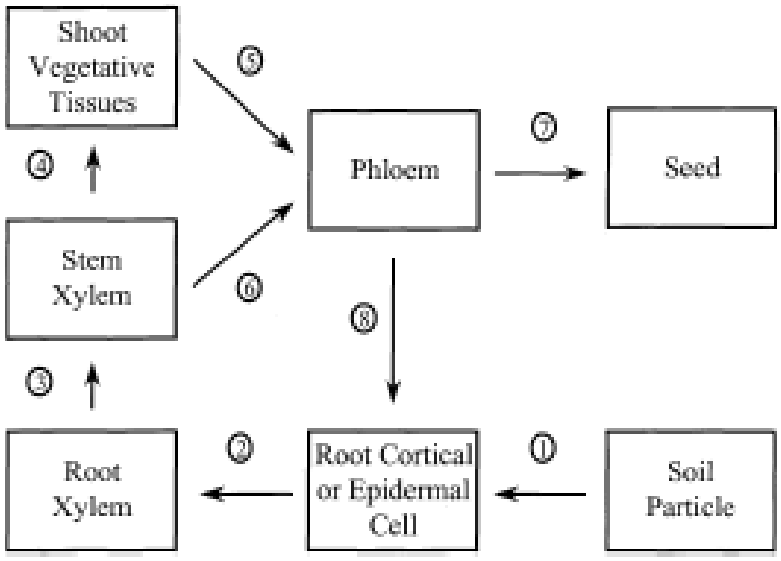

Figure 1.2 Potential control points in the regulation of metal homeostasis in crop plants. This scheme is generalized to indicate analogous steps for all micronutrients. Regulatory processes which would influence the movement of metals from one compartment to the next include: (1) root acquisition/uptake phenomena; (2)

intracellular transport, including the involvement of xylem parenchyma; (3) cell-wall-cationic binding sites within the xylem pathway; (4) transpiration rates of vegetative tissues; (5) capacity for phloem loading of metal ions, including the necessity of chelators for some metals; (6) xylem-to-phloem exchange; (7) phloem transport capacity of photoassimilates from a given source region; (8) communication of shoot micronutrient status via phloem-mobile signal molecules (Grusak et al., 1999).

Essential trace metal elements are sometimes limited in soil so that plants have difficulties to utilize these elements for their proper growth. Therefore, they have developed two main strategies to overcome this problem. Strategy I refers to plants that rely on reductive mechanisms to mobilize elements. Release of protons and reductase systems in order to acidify the soil to enhance mineral uptake can be considered in Strategy I. Dicotyledons and nongrass monocotyledons can utilize Strategy I. However, grasses can be given as an instance for Strategy II plants since they secrete phytosiderophores (PS) to chelate metals for improved mineral uptake (Grusak et al., 1999; Williams and Hall, 2003).

Zn is considered to be taken up by plant roots primarily in the form of Zn^{2+} from the soil solution which is mediated by a protein with a strong affinity for Zn. Unlike Fe, Zn does not require to be reduced before transport. Once taken up, Zn is neither oxidized nor reduced, therefore its function is based on its behavior as a divalent cation to form tetrahedral complexes with other molecules (Guerinot, 2000). Kochian suggested that transport of Zn across the plasma membrane was towards a large negative electrical potential so that the process is thermodynamically passive (Kochian, 1993). The presence of divalent cation channel in dicotyledons and monocotyledons other than the Poacae serves as a driving force for Zn. Kochian proposed that non-protein amino acids called “phytosiderophores” or “phytometallophores” form a complex with Zn and facilitate its transport from soil to the outer phase of the root-cell plasma membrane in the Poacae. PSs are released under Fe and Zn deficiency and their complex with the metal is then transported to the cell with the aid of a transport protein. Several reports demonstrated an increase in PS extrusion under Zn deficient conditions (Cakmak et al., 1996a and 1996b; Zhang et al., 1989). Cakmak et al. showed that increased release of PS resulted in increased Zn efficiency (Cakmak et al., 1994). It was

further claimed that growth conditions affect the reproducibility of PS extrusion (Pedler et al., 2000).

When Zn availability in soils is limited, uptake is restricted to direct root contact and is metabolically controlled. There are various mechanisms that take place in the uptake of several micronutrients. For instance, Zn and Cu mutually inhibit each other which suggest that both of their absorption may utilize the same mechanism (Neue et al., 1998). Zn generally accumulates in roots and it is translocated to the shoots when needed. It is partially translocated from source organs, such as old leaves, to developing organs. It was reported that alkaline earth cations inhibited Zn^{2+} absorption by plants, non competitively, in the following order: $Mg^{2+} > Ba^{2+} > Sr^{2+} = Ca^{2+}$ (Alloway, 2004).

Several studies were conducted in order to elucidate the mechanisms of translocation of Zn within plants. Uptake and translocation of foliar-applied ^{65}Zn in bread and durum wheats were studied. All of the studied cultivars differing in Zn efficiency, irrespective of leaf age and Zn status of plants, showed similar Zn uptake rates with application of $^{65}ZnSO_4$ to leaf strips in a short-term experiment. It was further observed that immersing the tip of the oldest leaves to $^{65}ZnSO_4$ solution resulted in no difference in Zn uptake among and within both wheat species. However, it was found that Zn-deficient plants translocated more ^{65}Zn from the treated leaf to the roots and remainder parts of shoots. Thus, it was reported that variation in Zn efficiency in between two wheat species was not associated with translocation or distribution of foliar-applied ^{65}Zn within plants, instead, compartmentalization of Zn at the cellular level might determine variation in Zn efficiency in wheat (Erenoglu et al., 2002). Additionally, Haslett et al. found that not only foliar-applied $^{65}ZnSO_4$ but also chelated Zn, such as ZnEDTA, provided sufficient Zn for proper plant growth in which phloem transport of Zn from leaves to roots was observed. Shoot Zn concentrations of 7-week-old plants which are supplied with foliar-applied $^{65}ZnSO_4$ and chelated Zn forms were two-fold greater to the plants which are supplied with Zn in the root environment or foliar-applied ZnO (Haslett et al., 2001). Plants grown in EDTA-containing nutrient solution showed a reduced rate of Zn transport from roots to shoots when compared to plants grown in EDTA-free nutrient solution (Rengel, 2002).

Bacteria, fungi, plants and animals have members of Cation Diffusion Facilitator (CDF) family which are responsible for transporting metals from the cytoplasm, either by efflux to the extracellular environment or compartmentalization in the intracellular organelles. Metal Transport Protein 1 (MTP1), which is also known as Zinc Transporter of *Arabidopsis thaliana* (ZAT1), is the only characterized CDF family protein found in *Arabidopsis thaliana*. MTP1 in *Arabidopsis thaliana* appears to be functioning in Zn sequestration rather than an efflux mechanism since MTP1 overexpressing plants showed higher Zn contents in roots than wild-type plants. This suggestion was also reinforced by the localization of MTP1 on the vacuolar membrane (tonoplast). However, it was shown that MTP1 functioned in efflux of Zn when heterologously expressed in *Xenopus oocytes* and bacterium *Ralstonia metallidurans* (Guerinot and Grotz, 2006).

1.1.3 Relative Sensitivity and Tolerance Mechanisms of Wheat to Zinc Deficiency

Zn deficiency is thought to have an important role in most of the crop plants, however, the sensitivity to Zn deficiency is dependent on the species. Both inter- and intra-species may differ in response to Zn deficiency stress. There are some cases where the difference in Zn deficiency response is higher among intra-species than inter-species. In the case of wheat, durum wheat (*Triticum durum*) is considered to be more sensitive to Zn deficiency than bread wheat (*Triticum aestivum*). Nevertheless, the response in both types of wheat show differences among different varieties (Cakmak et al., 1999; Alloway, 2004).

Although wheat has a relatively lower sensitivity to Zn deficiency among other plants, wheat crops in many parts of the world are still badly affected by Zn deficiency. For instance Zn-deficient calcareous soils of Central Anatolia of Turkey cause a significant reduction in wheat yield (Cakmak et al., 1996c). However, a wide range of variation in tolerance exists within wheat varieties (Cakmak et al., 1999). It was reported that the most Zn-efficient cultivars were from crosses with local landraces

(Cakmak, 2000). Anatolian bread wheat landraces are very tolerant to Zn deficiency. It was observed that rye was more tolerant than wheat. The order of decreasing tolerance regarding to several cereals was determined as follows: rye > triticale (hybrid of wheat and rye) > barley > bread wheat > oat > durum wheat (Cakmak et al., 1997). Considering the variation in Zn deficiency response between different species of wheat, it was suggested that AA and DD genomes would possibly possess the genes that confer Zn-efficiency trait since the most primitive hexaploid wheats and primitive and modern diploid wheats had a higher tolerance to deficiency than primitive and modern hexaploid wheats (Cakmak, 2000).

It was stated that plants respond to a micronutrient stress via a genetically controlled adaptation system which includes the ability to absorb nutrients from the soil, to secrete root exudate for increasing element mobilization in the soil so that the absorption via the roots are enhanced, and the ability to retranslocate absorbed nutrients within the plant (Brown and Jones, 1875; Kanwar and Youngdal, 1985). Graham et al. indicated that the varieties of crops which are defined as being more tolerant of Zn deficiency (i.e. Zn-efficient) is not directly associated with efficiency for other minerals (Graham et al., 1992). This suggests that genetically controlled Zn efficiency mechanism is independent.

It was found that Zn efficiency traits for nutrient-poor sandy and nutrient-rich clayey soils were genetically different. For instance, Zn efficient genotypes absorb more Zn from deficient soils, produce more dry matter and grain yield. However, they do not necessarily incorporate the highest Zn concentration in leaves or grain. Therefore, Zn efficiency is not directly associated with high Zn grain content which has a significant contribution in seedling vigor and cereal-based human diets (Alloway, 2004). According to Rengel, four different possible mechanisms of Zn efficiency are as follows (Rengel, 1999):

a) a greater proportion of longer, fine roots
b) chemical and biological manipulation of rhizosphere, e.g. release of Zn chelating phytosiderophores
c) increased uptake rate resulting in a net increase in Zn accumulation,

d) more efficient utilization mechanisms, e.g. compartmentalization of Zn within cells, tissues, organs

Although Rengel defined these mechanisms as principle factors of getting tolerant to Zn deficiency, there are studies with contradictory results. For instance, there was no correlation between Zn efficiency and root uptake in an experiment conducted by Hacisalihoglu et al. (2003). It was claimed that root surface area is not directly related to Zn tolerance because some Zn-inefficient wheat cultivars had higher root surface area than the ones which are Zn-efficient (Alloway, 2004).

1.2 ZIP (ZRT-like and IRT-like Proteins) Family of Metal Transporters

Essential minerals, such as some metal ions should be transported from the soil and then distributed throughout the plant to provide proper plant growth. Plants developed several high-affinity transporter proteins in order to utilize trace elements that are present at low amounts in the soil solution. Three major groups (ZIP, IRI and Nramp proteins) of trace metal transporter systems have been identified (Reid and Hayes, 2003). Over 100 members of ZIP family of proteins, which contribute for the metal homeostasis, are found in all phylogenetic levels –animals, plants, protists, bacteria and fungi (Eide, 2006; Guerinot and Grotz, 2006). Mammalian members of this protein were designated as "SCL39".

1.2.1 Overview of the ZIP family

The ZIP family takes its name from the yeast ZRT1 (Zinc-Regulated Transporter) and *Arabidopsis* IRT1 (Iron-Regulated Transporter). IRT1 was observed to be expressed in the roots of Fe-deficient *Arabidopsis* plants while ZRT1 and ZRT2, respectively, were found to be high-affinity and low-affinity Zn transporter in yeast (Eide et al., 1996; Guerinot, 2000). Although IRT1 was regarded to be induced under Fe-deficiency, it was suggested that it may also allow for transport of Zn^{+2}. IRT1-

mediated accumulation of other divalent cations is reasonable since, to some extent, they are known to replace Fe in some cellular processes under low Fe conditions (Vert et al, 2002; Reid and Hayes, 2003).

Over 25 ZIP family members have been identified and they were classified into two subfamilies due to their amino acid similarities. Subfamily I consists of 15 genes from plants (11 from *Arabidopsis*, two from tomato, one from pea, one from rice), 2 genes from yeast (*ZRT1* and *ZRT2*), and a gene from the protozoan *Tyrpanosoma brucei*. Subfamily II is comprised of 8 genes from the nematode *Caenorhabditis elegans*, one gene from *Drosophila* and two genes from humans (Guerinot, 2000). So far, 16 ZIP transporters were identified in *Arabidopsis* (Guerinot and Grotz, 2006).

Most ZIP family proteins are predicted to have eight transmembrane domains (TM) and similar membrane topologies with their N- and C- termini located on the extracellular face of the membrane. As illustrated in Fig. 1.3 many members have relatively long variable region between TM 3 and TM 4. This region is predicted to be histidine rich and reside in the cytoplasm (Guerinot, 2000). Although the function of this region has not been elucidated yet, his-rich structure is predicted to be involved in metal binding thereby functioning in Zn transport or its regulation. However, mutations in these residues of the ZRT1 protein in yeast did not disrupt the protein function but altered its subcellular localization (Gitan et al., 2003). Eide et al. figured out that Zn transport by the yeast ZRT1 requires energy whereas the human Zip2 (SLC39A2) transporter is energy independent (Zhao and Eide, 1996; Gaither and Eide, 2000). In humans, this Zn uptake may be driven by the gradient f HCO_3^- across the membrane of cells (Gaither and Eide, 2000).

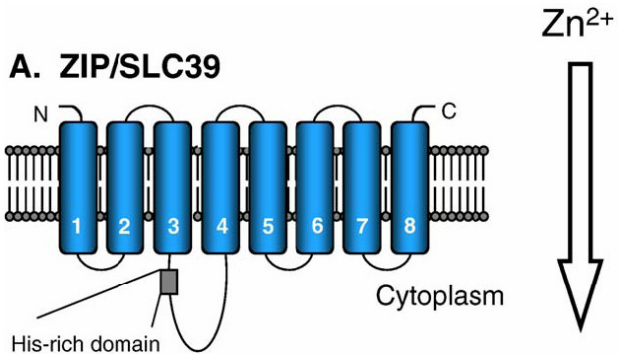

Figure 1.3 The predicted structure of ZIP/SLC39 family

of metal ion transporters. Numbers are referred to transmembrane domains (Eide, 2006).

It was found that the variation in the size of different ZIP proteins is due to the variation in length of this variable region. The difference in the length of this variable region leads to different ZIP proteins with amino acid numbers ranging from 309 to 476. Conversely, the most conserved region of the ZIP family transporters is found in TM 4 which is predicted to form an amphipathic helix with a fully conserved histidine residue (Guerinot, 2000). Heterologous expression of *At*IRT1 in yeast revealed that the transport function is disrupted when conserved histidines or certain adjacent residues are mutated. For instance, substitution of an alanine residue to a glutamic acid residue at position 103, lying in the loop region between TM II and TM III, eliminated IRT1 ability to transport Zn but not Fe, Cd and Mn (Rogers et al., 2000).

1.2.2 ZIPs in the Zinc transport

1.2.2.1 Yeast

Zn transporters ZRT1 and ZRT2 were first found in yeast on the basis of their similarity to IRT1 (Zhao and Eide, 1996a, 1996b). It was observed that they are 44 % identical and 67 % similar to each other and approximately 30-35 % identical and 54-65 % similar to IRT1. Kinetic studies in yeast revealed that these two proteins serve as different uptake systems. *ZRT1* and *ZRT2* genes encode a high-affinity and a low-affinity transporter proteins, respectively. It was found that ZRT1 protein is glycosylated and localized to plasma membrane of the cell (Guerinot, 2000). ZRT1 expression levels are upregulated by Zn deficiency at the transcriptional level through the Zap1 Zn-responsive activator protein. Although upregulation of ZRT2 by Zap1 was also detected in mild Zn-deficient conditions, it was observed that ZRT2 expression was suppressed under more severe Zn-deficient conditions. The differentiation in regulation system is carried out by three different binding sites (ZRE –as Zn-responsive element) of Zap1 within the promoter. Two binding sites are found upstream of TATA

box which stimulate the activation of gene expression whereas third binding site is located downstream of TATA box near the start site of transcription. *ZRT2* gene expression is repressed upon Zap1 binding to this third binding region (Eide, 2006). The ZRE consensus sequence is found to be as follows: 5'- ACCYYNAAGGT -3' (Guerinot, 2000).

Post-translational regulation is also found in yeast system in order to maintain metal homeostasis. It was observed that when cells are exposed to high extracellular levels of Zn, ZRT1 uptake activity is rapidly lost in order to prevent damage from Zn overaccumulation. The decrease in ZRT1 uptake is due to endocytosis of the ZRT1 protein and its subsequent degradation in the vacuole. Several experiments suggested that ubiquitination of the ZRT1 protein is essential prior to endocytosis (Guerinot, 2000).

1.2.2.2 Arabidopsis

Arabidopsis ZIP transporters were mainly identified by yeast complementation assays. Like IRT1, ZIP1, ZIP2 and ZIP3 genes of Arabidopsis were isolated via functional expression cloning in a *zrt1zrt2* mutant yeast strain. It was shown that the expression of these Arabidopsis ZIP genes could rescue mutant yeast strain in a Zn-limited environment. The experiment in the yeast revealed that ZIP1, ZIP2 and ZIP3 have different time-, temperature-, and concentration-dependent Zn uptake activities. Moreover, it was found that these ZIP genes do not contribute Fe transport and defined as the first Zn transporter genes to be cloned from plant species (Guerinot, 2000).

Transcripts of *ZIP1*, *ZIP3* and *ZIP4* were shown to be accumulated in response to Zn-deficiency in plants. It was found that *ZIP1* and *ZIP3* are root specific while *ZIP4* mRNA accumulated in both the shoots and the roots of Zn-deficient plants. When ZIP4 expressed in *ctr1* (copper transporter) mutant yeast, Cu uptake was detected, suggesting that *ZIP4* gene may transport Cu, as well (Guerinot and Grotz, 2006).

1.2.2.3 *Medicago trancatula*

A recent study on *Medicago trancatula* identified six new metal ion transporters showing a high similarity to ZIP family. Sequence analysis revealed that they possess eight transmembrane domains, including a highly conserved ZIP signature motif. Several of them also had a histidine-rich region between TM3 and TM4. Functional complementation studies with metal-uptake defective yeast contributed for understanding metal-specificity of these transporters. It was found that *zrt1zrt2* mutant yeast (unable to grow in Zn-deficient conditions) cells expressing MtZIP1, MtZIP5 and MtZIP6 proteins restored yeast growth on Zn-limited medium, indicating the ability of these proteins to transport Zn. MtZIP4 and MtZIP 7 proteins restored yeast (defective in Mn transporter *smf*) growth on Mn-limited medium, indicating that MtZIP4 and MtZIP7 transport Mn. Finally, *fet3fet4* mutants (unable to grow without Fe) expressing MtZIP3, MtZIP5 and MtZIP6 proteins was detected to restore yeast growth on Fe-limited medium, indicating the ability of these proteins to transport Fe. In compatible with yeast complementation studies, semi-quantitative Real-time PCR results showed that MtZIP1 transcripts, which is the most similar protein to AtZIP1, were only detected in roots and leaves of Zn-deficient plants (Lopez-Millan et al., 2004).

1.2.2.4 Rice

Several ZIP transporter proteins have been characterized in rice using yeast complementation assays. For instance, *OsZIP4* expression was recorded to increase in yeast defective in plasma membrane Zn uptake under Zn-limited conditions. When *OsZIP4* fused to GFP was transiently expressed in onion epidermal cells, fluorescence was detected at the plasma membrane. According to this finding, *OsZIP4* presumably functions to transport Zn from rhizosphere into the cytoplasm. However, it may also be involved in the distribution of Zn throughout the plant since *in situ* hybridization experiments showed that *OsZIP4* transcripts accumulate in the phloem cells of the stem as well as in the vascular bundles of the roots and leaves (Guerinot and Grotz, 2006).

Real-time PCR experiments recorded that *OsZIP4* transcripts were more abundant than those of *OsZIP1* or *OsZIP3* in Zn-deficient roots and shoots (Nishizawa et al., 2005).

OsZIP1 and *OsZIP2*, which are more similar to Arabidopsis ZIP2, are more highly expressed under Zn-deficiency. *OsZIP1* is accumulated in Zn-deficient shoots and roots while *OsZIP2* is accumulated preferentially in Zn-deficient roots and was observed to a lesser extent in shoots. *OsZIP3* mRNA levels are found in both the roots and shoots of Zn-deficiency plants (Guerinot, 2006). *OsZIP3* transporter, which differ from *OsZIP1* and *OsZIP2*, was detected as more selective for Zn than for other divalent cations. The cDNAs of *OsZIP1* and *OsZIP3* partially compensated ZHY3 yeast mutant defect in growth on low-Zn medium (Ramesh et al., 2003).

1.2.2.5 Zn-hyperaccumulating Plants

Plants capable of sequestering metals in their shoots at exceptionally high concentrations that would be toxic to their non-hyperaccumulator counterparts are referred as hyperaccumulators. Around 16 Zn-hyperaccumulating (containing 10000 μg Zn g^{-1} in shoot dry matter) plants were detected among 400 hyperaccumulating plants (Guerinot, 2000). Relatively little knowledge exists about the genetic basis of hyperaccumulation. With the development of phytoremediation technology, mechanisms that contribute to sequestering high levels of metals are being studied more extensively. Currently, a plant is said to be a hyperaccumulator if it is capable of accumulating trace metals at tissue concentrations approximately 100 times greater than those of "normal" plant species (Baker and Brooks, 1989).

Although the majority of hyperaccumulators were defined to accumulate only single metal, some plant species are capable of accumulating more than one metal such as *Thlaspi caerulescens*. At different fields, Thilaspi was recorded to accumulate high levels of Zn, Ni, Cd and Pb. Certain species of *Thlaspi caerulescens* were recorded to tolerate up to 40000 μg Zn g^{-1} in shoot dry matter whereas normal Zn concentration for most plants is between 20 to 100 μg g^{-1} tissue (Guerinot, 2000). Many of the known hyperaccumulators are both small and slow growing and often they are rare species of

limited population size (Pollard et al., 2002). Hyperaccumulation appears generally to be a species-level phenomenon, though there is within-species variation in degree and specificity of accumulation (Macnair, 2003).

It was found that although Michaelis constant (K_m) values of *T. caerulescens* and its non-hyperaccumulating related species, *T. arvense* were not significantly different, the maximum initial velocity (V_{max}) for Zn^{+2} influx in *T. caerulescens* root cells was 4.5-fold greater than that in *T. arvense*. This finding suggests that Zn absorption into the roots is involved in Zn hyperaccumulation. Moreover, the fact that 10-fold more Zn was translocated to the shoots of *T. caerulescens* after 96 hours implies that other transport mechanisms are also stimulated. These findings suggest that both transport across the plasma membrane and tonoplast of leaf cells are critical for Zn-hyperaccumulation (Lasat et al., 1996). More recent study revealed that metal hyperaccumulation feature of *T. caerulescens* also caused this species to be sensitive to Zn deficiency when compared to its non-hyperaccumulator plants (Ozturk et al., 2003).

ZNT1 gene, which is a ZIP gene homolog was identified in hyperaccumulator *T. caerulescens* (Pence et al., 2000). This gene is predicted to function as a Zn transporter since it survives *zrt1zrt2* mutant yeast strain. Northern blot analysis revealed that *ZNT1* transcript is abundant in the roots and shoots of *T. caerulescens* irrespective of Zn status. Unlike *ZNT1*, *ZIP4* expression of Arabidopsis was dependent on the Zn status. Therefore, the presence of *ZNT1* zinc transporter gene expression at all times regardless of Zn status may contribute for the hyperaccumulation process found in *T. caerulescens* (Guerinot, 2000).

1.3 Wild Wheat *Triticum dicoccoides*

A long-term research program, which includes study on wild cereals, wild barley (*Hordeum spontaneum*) and wild emmer wheat (*Triticum dicoccoides*) has been extensively conducted at the Institute of Evolution, University of Haifa, Israel. *Triticum dicoccoides* is a major model organism, which has been studied in the University of Haifa since 1979, is the progenitor of wheat and most of the background information

regarding to this species was contributed by the studies of this group (Nevo et al., 2002).

Crop domestication of humankind contributed to the evolution in an artificial way. Throughout the processes of plant domestication, yield was often the major criterion and a considerable progress has been achieved in wheat. Utilizing the morphological and genetic analyses, the wild progenitors and domesticated plants are being compared which reveals the genetic changes brought by the evolution. It was found that not only the hybridization, which contributed to the tetraploidy of *T. dicoccoides*, but also the natural selection played the crucial role in wheat evolution primarily through the mechanisms of diversifying and balancing selection regimes. Elucidation of the genomes of tetraploid and hexaploid (12,000 Mb and 17,000 Mb, respectively) would contribute to understanding of genome structure, function and evolution. In this context, wild emmer, *T. dicoccoides*, is a plant of fundamental importance. The comparison of domesticated wheat with its wild progenitors would eventually lead to optimizing the utilization of the rich genetic resources for wheat improvement, increased food production, challenging spreading starvation in the developing countries (Nevo et al., 2002).

Wheat crops are the universal cereals both for the Old World agriculture and modern times. It is the most widely cultivated food crop and is the staple food in more than 40 countries and for over 35 % of the global population. The earliest utilization of wild, brittle tetraploid wheat *T. dicoccoides*, dated as 19,000 years old, was found to be in Israel (Nevo et al., 2002).

1.3.1 Cytogenetic and Taxonomic Background of Wheats

Cultivated wheats, barleys, ryes, oats and a number of important grasses come from the same tribe Triticeae which is the most economically important group of the family Gramineae. Polyploidy and the exchange of genetic material occurred by the hybridization among genera. The genus *Triticum* (i.e. the wheat genus) includes a series of diploid, tetraploid and hexaploid forms which are formed by polyploidy having

arisen by amphiploidy between *Triticum* species and diploid species of the genus *Aegilops* (Nevo et al., 2002).

Although wild diploid species diverge from each other, which is evident in the morphologically well-defined seed-dispersal units of the species and their specific ecological requirements, they are presumably monophyletic in origin. It was shown that diploid species contains a distinct genome where the related chromosomes of the different genomes have little affinity to each other so that they do not pair regularly in interspecific hybrids, resulting in complete sterility and isolation of the diploid species from each other (Nevo et al., 2002).

On the other hand, the chromosomes of the polyploid species pair in a diploid-like fashion which shows a classic example of evolution through amphiploidy. Hybridization between different levels of ploidy led to the formation of allopolyploid nature of *Triticum* polyploids (Nevo et al., 2002).

Considering the diploid level, there are two main species of einkorn wheat, *Triticum monococcum L*. and *T. urartu* Thum. and their hybrids are sterile. *T. monococcum* includes cultivated ssp. *T. monococcum monococcum* (*T. monococcum* L.) and wild ssp. *T. monococcum aegilopoides* (Link) Thell. However, *T. urartu* presumably exists only in its wild form (Nevo et al., 2002).

At the tetraploid level there are two species, *T. turgidum* L., which includes wild ssp. *T. turgidum dicoccoides* (Korn.) Thell. (i.e. *T. dicoccoides*) and other several cultivated subspecies, and *T. timopheevi*, which includes wild ssp *T. timopheevi araraticum* (Jakubz.) and cultivated ssp. *timopheevi = T. turgidum* ssp. *timopheevi* (Zhuk.) (Nevo et al., 2002).

At the hexaploid level there are also two species, *T. aestivum* L., which has several subspecies and *T. zhukovskyi* Menab. et Ericz. Table 1.3.1 illustrates the classification of cultivated wheat and closely related wild species (Nevo et al., 2002).

Table 1.3.1 Classification of cultivated wheat and closely related wild species. (Feldman *et. al,*
1995)

Species	Genomes	Wild	Cultivated	
		Hulled	Hulled	Free-threshing
Diploid (2n = 14)				
Aegilops speltoides	S(G)	All		
Ae. bicornis	S^b	All		
Ae. longissima	S^l	All		
Ae. searsii	S^a	All		
Ae. squarrosa	D	All		
T. urartu	A	All		
T. monococcum	A	Var. *boeoticum* (wild einkorn)	Var. *monococcum* (cultivated einkorn)	Var. *Sinskajae* (cultivated einkorn)
Tetraploid (2n = 28)				
T. timopheevi	AG	Var. *araraticum*	Var. *timopheevi*	Var. *Militinae*
T. turgidum	AB	Var. *dicoccoides* (wild emmer)	Var. *dicoccum* (cultivated emmer)	Var. *Durum* Var. *Turgidum* Var. *Polonicum* Var. *Carthlicum* Var. *Turanicum*
Hexaploid (2n = 42)				
T. aestivum	ABD		Var. *spelta* Var. *macha* Var. *vavilovii*	Var. *Aestivum* Var. *Compactum* Var. *sphaerococcum*

According to cytogenetic studies, polyploids constitute two evolutionary lineages where *Triticum turgidum* (genomes AABB) and *Triticum aestivum* (genomes AABBDD) comprise one lineage, while *Triticum timopheevi* (genomes AAGG) and *Triticum zhukovskyi* (genomes AAAAGG) comprise the other evolutionary lineage. *Triticum aestivum* is evolved from the hybridization of *T. turgidum* with *T. tauschii* (Coss.) Schmalh (= *Ae. squarosa*) which contributed the D genome. *Triticum zhukovskyi* is evolved from the hybridization of *T. timopheevi* with an einkorn wheat, which contributed its second A genome (Nevo et al., 2002).

1.3.2 Origin of Wild Emmer, *Triticum dicoccoides*

Based on the genetic and morphological studies, it was concluded that the cultivated tetraploid *turgidum* wheats (both hulled *dicoccum* forms and free threshing *durum* varieties) are closely related to the wild wheat *T. dicoccoides* (wild emmer wheat). A and B genomes of *T. dicoccoides* and *T. aestivum* is of same origin and make fertile hybrids. *T. dicoccoides* represents the origin of all cultivated bread wheats, *T. aestivum* (Nevo et al., 2002).

Wild emmer wheat (i.e. *T. dicoccoides*) is an annual, pre-dominantly self-pollinated, tetraploid wheat with large and brittle ears and big elongated grains, as observed in cultivated emmer and durum wheat. It is the only wild ancestor in the genus *Triticum* that is cross-compatible and fully inter-fertile with cultivated *T. turgidum* wheats since the chromosomes of both species can pair in meiosis. This compatibility between *T. dicoccoides* and *T. turgidum* species suggested that they possess homologous chromosomes of the AABB genomic constitution. Due to its cross-compatibility with *T. aestivum* and *T. turgidum* wheats, *T. dicoccoides* is said to play the central role in wheat evolution. Figure 1.4 illustrates the wild emmer wheat, *T. dicoccoides* (Nevo et al., 2002).

Figure 1.4 Wild emmer wheat, *Triticum dicoccoides*
Ref: http://www.osel.cz/index.php?clanek=2380&akce=show2

1.3.3 Classification of Wild Emmer, *Triticum dicoccoides*

Unfortunately, there is no consensus in the nomenclature and classification of wheat. Miller (1992) embraces the traditional view of *T. dicoccoides* as a valid biological species. It is known that the speciation can occur with very little genomic and morphological changes. There are several distinct morphological differences between *T. dicoccoides* and other cultivars. For instance, *T. dicoccoides* has brittle ears which shatter upon maturity into individual spikelets. Each spikelet then disseminate the seeds by inserting them into the ground. This spikelet morphology found in wild-type wheats reflects the adoption for seed dissemination to ensure survival in nature. Manipulation by humankind with reaping, threshing and sowing broke down this adaptation and resulted in the selection of non-brittle types. Miller (1992) stated that more than one major gene were involved in this shift from brittle spike in *T. dicoccoides* to a non-brittle spike in *T. dicoccum* (Nevo et al., 2002).

Van Zeist (1976) stated that kernel morphology differs between wild and cultivated wheats. The grain is wider, thicker and rounder in cross-section in cultivated emmer (*T. dicoccum*) than in wild emmer (*T. dicoccoides*). This trait of the grain facilitates differentiation between the wild and cultivated types. Important unique chromosomal translocations and genetic polymorphisms are also utilized in characterization of *T. dicoccoides* (Nevo et al., 2002).

1.3.4 Ecology of Wild Emmer, *Triticum dicoccoides*

Triticum dicoccoides is found in region called “Fertile Crescent” which embraces Israel, Jordan, Syria, south-east Turkey, northern Iraq and western Iran. Figure 1.5 depicts the distribution of wild tetraploid wheat species throughout the Fertile Crescent. It was first discovered in 1906 in eastern Galilee and on the slopes of mountain Hermon of Israel by Aaronsohn, who then recognized its potential importance for wheat improvement. *T. dicoccoides* is genetically highly polymorphic allozymically in its

center of distribution in northern Israel and southern Syria. Morphologically it is polymorphic for glume hairiness and spike color (Nevo et al., 2002).

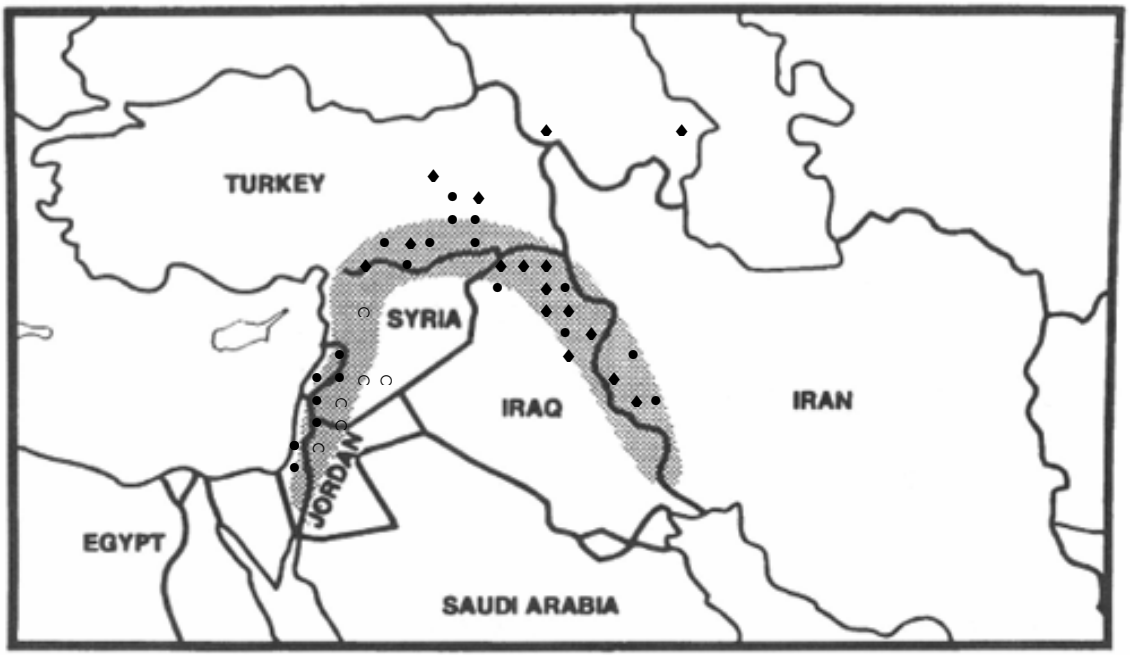

Figure 1.5 Distribution of wild tetraploid wheat: (•, o) wild emmer wheat, *Triticum turgidum* ssp. *dicoccoides* (*Triticum dicoccoides*); (♦) wild Thimopheevi's wheat, *Triticum timopheevi* ssp. *araraticum* (*Triticum araraticum*) (Zohary and Hopf, 1993) (Nevo et al., 2002)

1.3.5 *Triticum* species, A Genetic Resource for Wheat Improvement

The presence of wild- and less-advanced wheats are being used as a potential genetic resource in order to improve cultivated modern wheats. For instance, several disease resistance genes in the A genome of wild diploid wheat species have been transferred into other susceptible wheat species (Hussien et al., 1997). *Triticum turgidum* ssp. *dicoccoides* was observed to show a high heat resistance among *Triticum* species, especially the wild diploid wheats, *T. monococcum* ssp. *boeticum* and *T. urartu* are considered. Wild tetraploid wheats also show high variation in sensitivity to rust diseases. (Cakmak et al., 1999; Rekika et al. 1997).

Cakmak et al. found that tetraploid wheats, including wild and primitive species, showed higher sensitivity to Zn deficiency when compared to diploid and hexaploid wheat species. The shoot dry weight of tetraploid wheats was higher with Zn supply, but tended to be lower under Zn-deficient conditions. Among the subspecies of tetraploid wheats, although there was a little difference in the severity of Zn deficiency symptoms, a greater range of Zn efficiency was detected. It was observed that there is no relation between Zn efficiency and Zn content and concentration in seeds. For instance, Zn-inefficient wild tetraploid wheats (*Triticum dicoccoides*) exhibiting higher seed Zn concentrations and contents suggest that internal mobility and retranslocation of Zn is limited in this species. The reason for the high sensitivity of tetraploid wheats to Zn deficiency might be related to slow release of PS from roots and low Zn uptake capacity of roots under Zn-deficient conditions. The absence of D genome in tetraploid wheat might play a role for its low Zn efficiency. It was found that the transfer of the whole D genome from *Ae. tauschii* to tetraploid wheat significantly increased Zn tolerance. Therefore, D genome can be used to improve Zn efficiency of wheats subjected to Zn deficiency. The relative higher sensitivity to Zn deficiency of tetraploid wheats compared to diploid wheats suggests that either the existence of suppressor genes for Zn efficiency in the B genome or modification in the A genome of tetraploids occurred during evolution (Cakmak et al., 1999).

Different accessions of *Triticum dicoccoides* were studied for seed Zn and Fe levels and they have been found to be promising for improvement of Zn and Fe concentrations of wheat seeds. *Triticum dicoccoides* showed the highest variation in Zn concentration, from 14 to 190 mg kg^{-1} DW for Zn in seeds with an average of 61 mg kg^{-1}, which was reflected in high Zn content values, reaching up to 7 μg per seed. The positive relation between the seed Zn concentration and seed Zn content indicates that high concentration was not caused by a concentration effect due to small seed size. Two different *Triticum dicoccoides* chromosome substitution lines were examined in order to investigate the localization of genes affecting high levels of seed Zn in these wheat species. It was found that 6B, 6A and 5B substitution lines had higher levels of Zn and Fe but not other nutrients. Thus, genes contributing high seed Zn levels might be located in 6B, 6A and 5B chromosomes. It was found that the gene(s) for high protein concentration is (are) located very close to centromere of 6B. If the genes responsible for high Zn and high protein content are linked on chromosome 6B, selection for high

seed Zn levels may lead to simultaneous selection of high levels of seed protein. On the other hand, the genes controlling tolerance to Zn deficiency do not contribute for enhanced seed concentration of Zn because *Triticum dicoccoides* are known to be susceptible to Zn deficiency although they possess high seed Zn levels (Cakmak et al., 2004).

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant material

Wild emmer wheats (*Triticum dicoccoides* accessions) used in this experiment were obtained from Institute of Plant Sciences & Genetics in Agriculture, The Hebrew University of Jerusalem.

2.1.2 Chemicals

Please refer to Appendix A.1 for the detailed list of chemicals and kits that were used during this study.

2.1.3 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. Please refer to Appendix A.2 for the recipes of the solutions.

2.1.4 Equipment

Please refer to Appendix A.3 for the detailed list of equipments that were used during this study.

2.2 Methods

2.2.1 Plant Growth Conditions and Zinc Treatments

2.2.1.1 Greenhouse Experiments

The soil used in this study was Zn deficient (0.1 mg Zn kg^{-1} soil) and obtained from Eskişehir -Central Anatolia. The main characteristics of the soil were: pH 8.04, $CaCO_3$ 14.9 %, organic matter 0.69 %, salt 0.08 % and soil texture was 60.6 % clay. Plants grown under greenhouse conditions were sown in plastic pots containing 1700 g soil. Pots were washed with diluted HCl and then rinsed several times in order to eliminate any trace amounts of contaminating elements. About 15 seeds were sown in each pot and after emergence the seedlings were reduced to 10 per pot. Plants were supplied either with Zn (control plants) or without Zn (stressed plants). Zn was only applied to control plants at a rate of 5 mg kg^{-1} soil in the form of $ZnSO_4.7H_2O$. Other than Zn application, all plants were supplied with 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 FeEDTA ($C_{10}H_{12}FeN_2NaO_8$). All nutrients were mixed thoroughly with soil before sowing. The treatments were performed in triplicate and the pots were randomized every 5-6 days. After the initial application of nutrients, plants were watered daily with deionized water. Shoots of 35-day old plants were harvested as Zn-deficiency symptoms became apparent. Harvested shoots were dried at 70ºC for determination of shoot dry matter production and element concentration in the whole shoot.

2.2.1.2 Nutrient Solution Experiments

Seeds were germinated in perlite containing saturated $CaSO_4$. After six days, seedlings were transferred to 2.5 L plastic pots containing continuously aerated nutrient solution. The composition of the nutrient solution, including macro- and micronutrients was as follows: 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 $(NH_4)_6Mo_7O_{24}$. Instead of –Zn vs. +Zn treatments in the greenhouse experiments, plants were supplied with 5 different doses of Zn in the form of $ZnSO_4$ as follows: -Zn (severe Zn deficiency), 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 supply). Plants were grown 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}). Nutrient solutions were refreshed every 3-4 days.

After the growth for 14 days, as the symptoms of Zn-deficiency became severe on plants, shoots and roots were harvested separately, frozen in liquid nitrogen immediately and stored at -80 º C. At harvest, roots were rinsed with deionized water thoroughly in order to eliminate adsorbed Zn and other elements on the root surface. Right before freezing, roots were briefly dried on a sterile filter paper in order to avoid excess water.

2.2.2 Dry Matter Production and Zinc Tolerance Index

Plants dried at 70 ºC were weighed for determination of dry matter production. The Zn tolerance index was calculated as the ratio of dry matter production at -Zn to the dry matter production at +Zn as follows:

Zn Tolerance Index = (Dry Weight at -Zn/Dry Weight at +Zn) x 100

2.2.3 Zinc Concentration and Content

The dried shoot samples from the greenhouse experiment were ground and approximately 0.2 g ground samples were ashed at 500 º C for 12 hours for calculation of element concentration. The ashed samples were then dissolved in 3.3 % HNO_3 (v/v). The concentration of the elements was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian, Australia) at proper emission wavelengths regarding to corresponding elements. During the element analysis, reference plant materials which were supplied from the National Institute of Standards and Technology (Gaithersburg, USA) were used as positive controls in order to check certified element values. Since the amount of Zn in seeds has an important role in Zn efficiency, the concentration of Zn in all seeds were also analyzed. Content of the elements were calculated as follows:

Content = Dry Weight x Concentration

2.2.4 Total RNA Isolation

200 mg shoot and 300 mg root samples were ground in 1.5 ml Trizol® reagent (without adding liquid nitrogen) using autoclaved mortars and pestles. 1 ml of homogenized sample was transferred to a microtube using a wide-bore pipette tip. Homogenized samples were kept on ice while other samples were being ground with Trizol®. All the processed samples were then incubated at room temperature for 10 minutes. 0.4 ml chloroform was added to each sample and the tubes were shaken vigorously and incubated at room temperature for 5 minutes. The samples were centrifuged at 11,000 x g for 15 minutes at 4 ºC. The upper aqueous phase containing the RNA was transferred to a clean microtube. After chloroform extraction, 0.5 ml isopropanol was added to precipitate RNA. The samples were then incubated at room temperature for 10 min and centrifuged at 11,000 x g for 10 min at 4ºC. The supernatant was discarded and the RNA pellet was washed with 1 ml 75 % ethanol. The samples were briefly vortexed and centrifuged at 7,500 x g for 5 min at 4 ºC. The

supernatants were discarded and the RNA pellets were briefly air-dried. The pellets were then dissolved in 40/50 µl diethyl pyrocarbonate (DEPC)-treated H_2O and incubated at 55 ºC water bath for 10 minutes to improve suspension. NanoDrop spectrophotometer was used in order to measure the quantification of RNA samples at 260 nm wavelength. The samples were then stored either at -20 ºC for short-term or at -80 ºC for long-term use.

2.2.5 DNase I Treatment

RNA was treated with DNase I to remove any contaminating chromosomal DNA. 5 µg of total RNA were treated with 5 units of RNase-free DNase I in 1X Reaction Buffer containing $MgCl_2$. The reactions were then incubated at 37 ºC for 30 minutes. For DNaseI clean-up, 5 µl of 25 mM EDTA was added and the samples were incubated at 65 ºC for 10 minutes. RNA was then precipitated by ethanol precipitation. 0.1 volumes of 3 M NaOAc at pH 5.2 and 2 volumes of cold 100 % ethanol was added to the samples and incubated at -80 ºC for 1 hour. The supernatant was discarded after the centrifugation and at 4 ºC and the pellet was washed with 0.5 ml 70 % cold ethanol. The pellets were allowed to air-dry for about 10 minutes and the RNA was quantified by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer. The samples were either stored at -20 ºC for short-term or -80 ºC for long-term use.

2.2.6 First Strand cDNA Synthesis

First strand cDNA synthesis was performed using the Omniscript reverse transcription kit. The reactions were carried out in a total volume of 20 µl containing 1X Buffer RT, 0.5 mM dNTP mix, 0.5 µg oligo$(dT)_{12-18}$ primer, 10 u RNaseOUT™ Recombinant Ribonuclease Inhibitor, 3 µg DNase I-treated RNA sample and 4 u Omniscript Reverse Transcriptase. OligodT primers used in reverse transcription reaction were purchased from Invitrogen (0.5 µg /µl). Utilization of oligodT primers

enabled the selection of mRNAs to be converted into cDNAs. The reactions were incubated at 37 ºC for 60 minutes and then stored at -20 ºC.

2.2.7 Primer Design

2.2.7.1 Primer Design for ZIP transporter mRNA

Based on the literature search for determination of currently present ZIP transporters in wheat, National Center for Biotechnology Information (NCBI) webpage supplied one ZIP mRNA complete coding sequence (Accession number: AY864924) which was amplified from *Triticum aestivum* (bread wheat). The sequence was submitted in 2005 and contains 1083 basepairs (bp). The reader is referred to Appendix B for detailed information about the sequence.

Two different primer pairs were designed to target this ZIP transporter mRNA sequence in *Triticum dicoccoides*. P1-ZIP primers were designed inside this sequence which excludes that start and stop codon sites. To conduct further functional analyses, ZIP mRNA sequence was blasted against the wheat EST database in order to find 5' and 3' untranslated (UTR) regions of the transcript. Obtained ESTs were aligned and UTR-ZIP primers were designed to cover UTR regions in order to obtain full length sequences. Table 2.1 lists the detailed information of P1-ZIP and UTR-ZIP primers.

Table 2.1 Primers designed for *T. aestivum* ZIP mRNA complete coding sequence

Name	Orientation	Sequence	Amplicon Size (bp)
P1-ZIP	Forward	5'-ATCATACCTTGCAGGCGCTT-3'	~1069
	Reverse	5'-TGGCAAGCATGGACATCATC-3'	
UTR-ZIP	Forward	5'-CACACATAGTCAGCAATTCAGC-3'	~1166
	Reverse	5'-GCTTCAGCACGAAATACAAGTG-3'	

2.2.7.2 Primer Design for Quantitative Real-time PCR

Two different primer pairs, which target inside of ZIP mRNA coding sequence, were designed. Table 2.2 provides information about primer pairs for quantitative Real-time PCR analysis. RT1 primer pair was designed to target TM4 which is known to be the most conserved region among ZIP transporters. Alternatively, RT2 primer pair was targeted to the cytoplasmic region of the transmembrane protein, between TM4 and TM5. The prediction of the transmembrane helices were determined utilizing TMHMM server v. 2.0 of Center For Biological Sequence Analysis (CBS). Please refer to Appendix C for detailed information about the structure of the predicted transmembrane ZIP transporter protein.

Table 2.2 Quantitative RT-PCR primers designed for *T. aestivum* ZIP mRNA

Name	Orientation	Sequence	Amplicon Size (bp)
RT1	Forward	5'-CCTTTTGCGGGACTTGTGG-3'	66
	Reverse	5'AGCAGCCAGCGAGTCTATC-3'	
RT2	Forward	5'-AACATCGACATACCCGGAGA-3'	88
	Reverse	5'-CAACAATTGCGTCACCATGT-3'	

2.2.8 PCR Amplification of ZIP Transporter cDNA

Polymerase chain reactions (PCR) using P1-ZIP and UTR-ZIP primers were either carried out in a total volume of 20 µl or 50 µl. The reactions were performed with concentrations of the reagents as follows: 1X *Taq* Buffer without $MgSO_4$, 0.2 mM dNTP mix, 1 µM primers, 1.5 mM $MgSO_4$, 2 µL first strand cDNA, and 0.4 u / 20 µl of *Taq* DNA Polymerase. After amplification, PCR products were run on 0.8 % agarose gel in 0.5X TBE buffer at 100 V for about 40 minutes. The bands were stained with ethidium bromide visualized under UV light. GeneRuler™ 1 kb DNA ladder, GeneRuler™ High Range DNA ladder or GeneRuler™ DNA ladder Mix were used to determine the size of cDNA fragment bands.

Table 2.3 Thermocycle Conditions for P1-ZIP and UTR-ZIP PCR

Cycle for P1 - ZIP PCR		
Cycles	**Temperature (ºC)**	**Duration**
1	95	2:00
30	94	1:00
	56	1:00
	72	1:30
1	72	7:00
	4	∞

Cycle for UTR - ZIP PCR		
Cycles	**Temperature (ºC)**	**Duration**
1	95	2:00
30	94	1:00
	61.5	1:00
	72	1:30
1	72	7:00
	4	∞

2.2.9 Gel Extraction

The agarose gel fragments, both amplified with P1-ZIP and UTR-ZIP primers with expected molecular weights, were excised with a clean scalpel. The bands of interests were purified using Qiaquick® Gel Extraction Kit following the manufacturer's protocol. cDNA was eluted in 30-40 µL of Elution Buffer and quantified by measuring the absorbance of samples at 260 nm using a NanoDrop spectrophotometer. The samples were stored at -20 ºC.

2.2.10 Ligation to Vector

The PCR products were ligated to the pGEM®-T Vector System I (Appendix D) in 10-µL reactions. 3:1 (insert:vector) ratios were prepared in order to obtain proper ligation via T-A cloning. Ligation reactions were performed following manufacturer's protocol. The reactions were either incubated at room temperature for 1 hour or overnight at 4 ºC to maximize the number of transformants as indicated by the manufacturer's protocol.

2.2.11 Preparation of Electrocompetent Cells

20 ml of DH5α cell culture (*E.coli* strain) were grown overnight at 37 ºC with shaking at 200 rpm. Overnight cultures were inoculated in 1 lt LB Broth next morning. The culture were let to grow at 37 ºC with shaking at 200 rpm until OD_{600} is around 0.7. As OD_{600} reaches around 0.7, the culture were cooled down to 4 ºC and centrifuged at 2,500 x g for 15 minutes at 4 ºC. Supernatant was discarded and pellet was washed with 500 ml cold sterile water. Centrifugation at 2,500 x g for 15 minutes at 4 ºC and washing with 500 ml cold sterile water steps were repeated once more. The culture was again centrifuged at 2,500 x g for 15 minutes at 4 ºC and the cells were suspended in 20 ml 10 % glycerol. The suspension were then centrifuged at 2,500 x g for 15 minutes at 4 ºC and the culture was resuspended in 2 ml 10 % glycerol. 50 µl cultures were prepared as aliquots and these electrocompetent cells were immediately frozen in liquid nitrogen and stored at -80 ºC for further use.

2.2.12 Transformation

1 µl of ligation reaction was gently added into 50 µl electrocompetent DH5α strain of *Escherichia coli* and kept on ice for exactly 1 minute. The mixture then put into cold electroporation quvette and pulsed. 550 µl of SOC medium was pipetted into the quvette and transformed cells were transferred into a fresh microtube. The transformation reactions were incubated at 37 ºC for 1 hour with shaking at 140 rpm. After incubation, cells were centrifuged at 5,000 rpm for about 3 minutes. Excess supernatant was discarded and the cells were suspended in 200-µL SOC medium. Transformation reactions were then plated on LB plates containing 100 µg/mL ampicillin, 10 nmol of IPTG, and 1 µg of X-Gal and the plates were incubated at 37 ºC for 12 hours.

2.2.13 Colony Selection

The vector allows for blue/white selection by utilizing of *LacZ* gene and positive (which are supposed to appear as white colonies) clones were selected and replicas of these clones were prepared.

2.2.14 Colony PCR

Same set of primers, which were previously used to amplify the fragments with the expected sizes, were used in colony PCR reaction in order to confirm that the transformation is correct positive. Before the addition of PCR reagents, bacterial colony was inoculated into 8 µl deionized water and incubated at 95 °C for 8 minutes for lysis.

2.2.15 Preparation of Glycerol Stocks of Transformants

Glycerol stocks of transformants were prepared in 15% glycerol and kept at -80ºC.

2.2.16 Plasmid Isolation

Selected white colonies were inoculated in 5 mL of LB medium containing 100 µg/mL ampicillin and incubated overnight (12-16 hours) at 37 ºC with shaking at 270 rpm. After incubation the bacterial cells were centrifuged at 8,500 x *g* for 3 minutes at room temperature. Plasmids were isolated using Diaper® Spin Miniprep Kit (Qiagen) following the manufacturer's protocol. DNA was eluted in 30-40 µL Elution Buffer and quantified by measuring the absorbance of samples at 260 nm using a NanoDrop spectrophotometer. The samples were stored at -20 ºC.

2.2.17 Sequencing

According to manufacturer's protocol, 400 ng (1/10th of vector size) vector was used as template for sequencing reaction. 5 pmol of M13 primers and 8 µl of Premix were used in a total volume of 20 µl reaction. Since the sequence product using either forward of reverse primer would provide a sequence of approximately a 500 bp-long fragment, both forward and reverse primers were used for a given template. By this way, both ends of the total sequence were obtained in order to cover ~1000 bp-long total ZIP sequence. First, 400 ng vector, 5 pmol of either M13 primer (i.e. M13 forward and M13 reverse primer reactions were carried out in separate reaction tubes) and deionized water were mixed and the reaction was incubated for 2 minutes at 95 ºC for initial denaturation. Then, 8 µl of Premix was added into the reaction and thermocycle conditions of the sequencing reaction were carried out as indicated in Table 2.4:

Table 2.4 Thermocycle Conditions for Sequencing Reaction using M13 Primers

Sequencing Reaction using M13 Primers		
Cycles	**Temperature (ºC)**	**Duration**
30	95	1:00
	50	0:20
	60	2:00
1	4	∞

The PCR products were cleaned up by ethanol precipitation. 2 µl of 7.5 M ammonium acetate and 55 µl of 100% cold ethanol was added into the PCR products and mixed. The reactions were centrifuged at 13,000 rpm for 30 minutes for precipitation. Supernatant was discarded and the pellet was washed with 70 % cold ethanol. The pellet then briefly air-dried and dissolved in 10 µl loading buffer supplied by the kit. The reaction was then sequenced.

Selected plasmids were also sent to Mclab for commercially sequencing in order to double check the sequences.

2.2.18 Sequence Analysis

The sequences obtained were first subjected to the VecScreen algorithm (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) in order to eliminate the contaminating vector sequences. BLASTN, BLASTX, and TBLASTX algorithms (Altschul et al., 1997) were used to analyze the DNA sequences. Obtained sequences were also compared with currently existing *T. aestivum* ZIP mRNA coding sequence present in NCBI webpage.

Protein secondary structure prediction was done using the PELE Protein Structure Prediction Tool available at Biology WorkBench (http://workbench.sdsc.edu). This tool utilizes seven different algorithms, and each algorithm assigns a structure to each amino acid as α-helix, β-strand or coil. This tool also provides a "joint prediction," which incorporates the predictions made by the other algorithms and uses a "winner takes all" procedure for each amino acid prediction to assign the structure.

3 RESULTS

3.1 Physiological Data Analysis

3.1.1 Screening Experiment

In order to select extreme genotypes, which show either high or low tolerance to Zn deficiency, screening experiment was performed using different wild tetraploid wheat accessions (*Triticum dicoccoides*). Initial screening experiment was done in Sakarya where severe Zn deficiency in soil is present. According to this preliminary screening experiment, six extreme wild wheat accessions were selected since they showed extreme responses among other wild wheats. The selected accessions were grown in greenhouse with +Zn and –Zn conditions to confirm their response in Zn deficiency. Dry matter production and Zn tolerance indices were calculated using these plants grown in Zn-deficient soil under controlled greenhouse conditions.

3.1.2. Plant Growth, Dry Matter Production and Zn Tolerance Index

3.1.2.1 Greenhouse Experiment

Different types of wheats differ considerably in their ability to tolerate low levels of available Zn. A wide range of variation in response to Zn deficiency was detected both among different wild wheats and between wild and modern tetraploid wheats as well. Generally, the initial response of the plants to Zn deficiency was reduced shoot growth and the emergence of light green to white chlorotic and necrotic

streaks on either side of the young leaves since Zn is relatively immobile under conditions of deficiency. With the duration of time, the development of interveinal chlorosis and necrotic patches on the bases and sheats of the old leaves was detected as characteristic symptoms for Zn deficiency. Necrotic spots appeared on the upper surface of the leaves later join each other to form brown necrotic patches. Moreover, it was observed that leaves totally collapsed as Zn deficiency becomes more severe.

Tolerance to Zn deficiency was determined by considering tolerance index and leaf symptoms. The experiment carried out in the controlled greenhouse conditions revealed that three of the wild wheat accessions show higher tolerance when compared to other three wild wheats. *T. durum* wheats which were included in this experiment as indicator plants also showed difference in their responses to Zn deficiency. The most susceptible genotype was from *T. durum* cv. Ç-1252. According Zn tolerance index, *T. dicoccoides* accessions of 18-60, 24-39, MM 5/4 and MM5/2 were detected as tolerant whereas 33-48 and 19-36 were recorded as susceptible accessions. It is important to note that although dry matter production of 18-60 was not significantly affected by Zn deficiency, leaf symptoms of chlorosis and necrosis was the most pronounced among other accessions (see Fig. 3.1). Therefore, 18-60 was determined to be susceptible to Zn deficiency. The relative leaf symptoms, dry matter production and Zn tolerance index of the plants are given in Table 3.1.

Table 3.1 Shoot dry matter production and Zn tolerance index of 6 *T. dicoccoides* and 3 *T. durum* wheat genotypes grown for 35 days under greenhouse conditions with (+Zn: 5 mg Zn kg^{-1}) and without (-Zn) Zn application. Zn tolerance index was calculated as the ratio of dry weight of –Zn to that of +Zn. The data represent mean and standard deviations of three independent replications.

Genotypes	Leaf Symptoms *	Dry Matter Production		Zn Tolerance Index
		-Zn	Control	
		(g plant-1)		(%)
T.dicoccoides				
18-60	5	0.30 ± 0.06	0.53 ± 0.11	57
MM 5/4	2	0.36 ± 0.05	0.64 ± 0.03	57
24-39	1	0.37 ± 0.01	0.68 ± 0.06	55
MM 5/2	2	0.32 ± 0.02	0.66 ± 0.12	49
19-36	3	0.19 ± 0.02	0.51 ± 0.05	37
33-48	5	0.14 ± 0.05	0.43 ± 0.12	33
Mean		0.31	0.60	48
T.durum				
Balcalı 85	4	0.29 ± 0.04	0.62 ± 0.04	47
Meram	4	0.33 ± 0.13	0.91 ± 0.17	36
Ç 1252	5	0.14 ± 0.01	0.73 ± 0.08	19
Mean		0.31	0.77	34

Table 3.1: * Severity of leaf symptoms of Zn deficiency developed as chlorosis and necrosis 1 (slight) to 5 (severe)

The average decreases in shoot dry matter production due to Zn deficiency were 48

% in *T. dicoccoides* and 34 % in *T. durum*. Shoot tolerance index, expressed as the ratio of shoot dry weight without Zn supply to the shoot dry weight at Zn treatment, showed a considerable difference between the most and least affected accessions. For example, Zn tolerance index of MM5/4 was 57 % whereas 33-48 had 33 % tolerance index with severe leaf symptoms. Figures 3.1 and 3.2 illustrate pre-harvest pictures of the most tolerant (MM5/4) and susceptible accessions (33-48) among wild wheats, respectively.

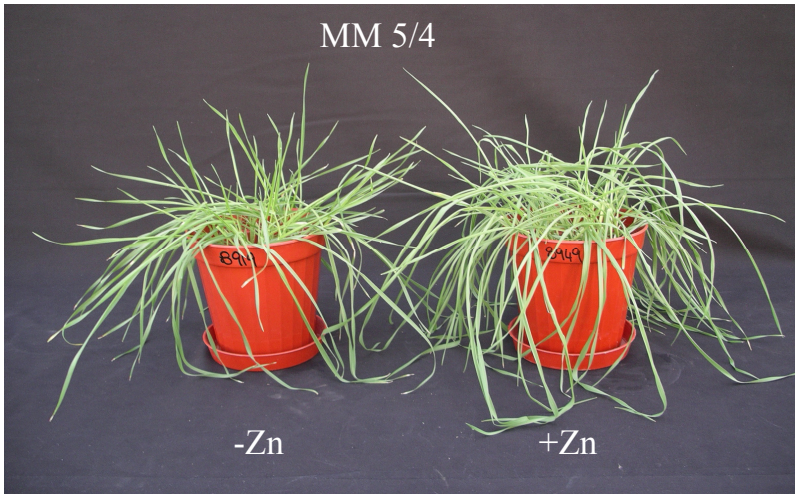

Figure 3.1 Pre-harvest picture of the most tolerant accession, MM 5/4, to Zn deficiency

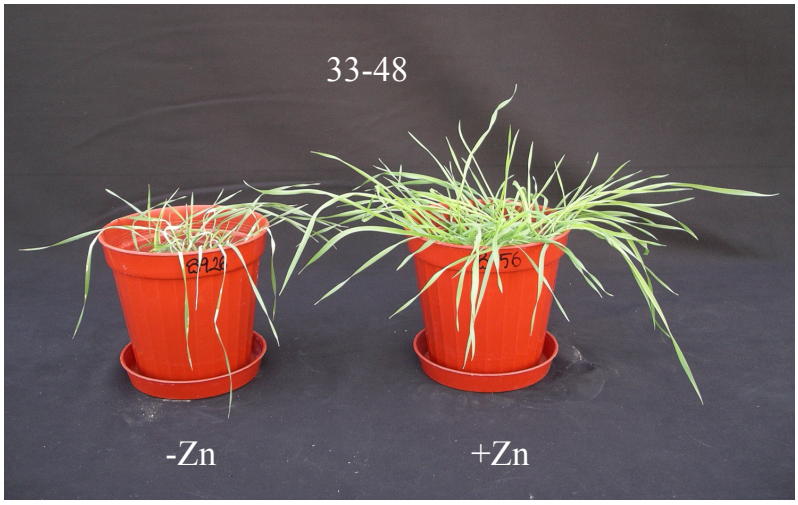

Figure 3.2 Pre-harvest picture of the most susceptible accession, 33-48, to Zn deficiency

Based on the results of the greenhouse screening experiment, all of the cultivars were selected for further nutrient solution experiments since they either show tolerance or susceptibility to Zn deficiency. Shoots of these plants, grown in the greenhouse conditions, were only used for physiological analysis.

3.1.2.2 Nutrient Solution Experiment

Nutrient solution experiment was performed with the application of five different Zn concentrations and the plant materials obtained from hydroponics were used to isolate RNA. Shoots and roots of the plants were harvested after 18 days, as deficiency symptoms became severe yet plants were not totally collapsed. When compared to greenhouse experiment, the response of plants in the hydroponic experiment was similar but it appeared earlier than in greenhouse plants, as expected. This is probably due to hydroponic culture effect since the elements are more mobile in nutrient solution than in soil.

The application of different concentrations of Zn revealed that as the degree of Zn deficiency stress increased the severity of symptoms also increased. Therefore, there was a linear relationship between the shoot growth and the application of Zn, i.e. shoot growth of plants decreased at Zn-limited conditions. Thus, it was observed that the shoot growth increased with the following Zn applications: -Zn (severe Zn deficiency) $< 10^{-8}$ M Zn (moderate Zn deficiency) $< 10^{-7}$ M Zn (slight Zn deficiency) $<10^{-6}$ M Zn (adequate Zn supply). However, since 10^{-4} M Zn is a toxic dose, shoot growth was detected to be repressed in plants treated with 10^{-4} M Zn. In compatible with the greenhouse experiment, reduced shoot growth, interveinal chlorosis and necrosis were the typical symptoms appeared under Zn deficiency in nutrient solution experiment.

The same genotypes were detected to behave similarly to Zn deficiency as observed in greenhouse conditions. Figures 3.3 and 3.4 illustrate pre-harvest pictures of two different wild wheat accessions: MM 5/4 and 33-48, as the most tolerant and susceptible genotypes to Zn deficiency, respectively.

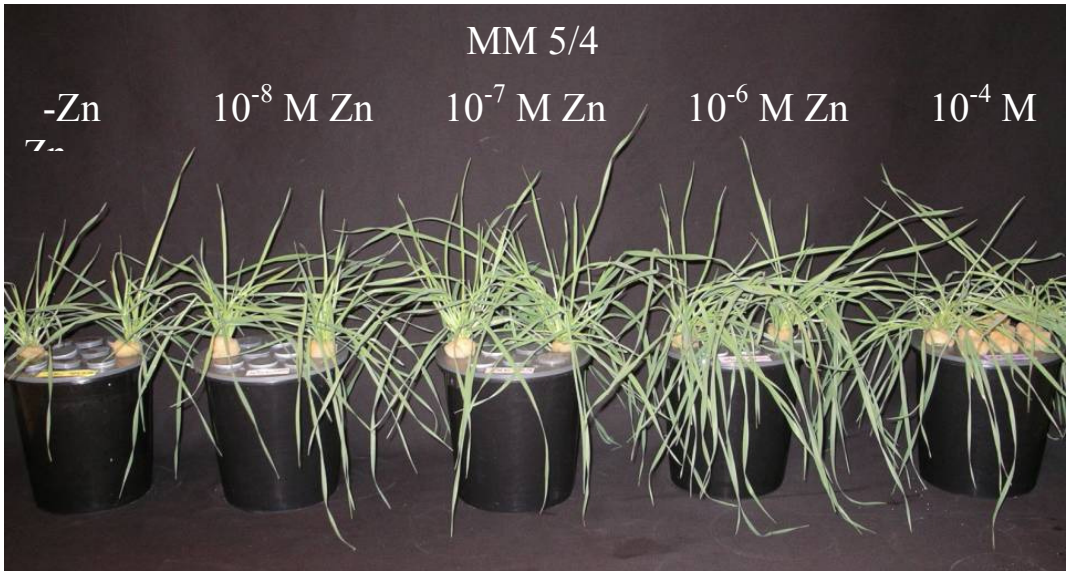

Figure 3.3 Pre-harvest picture of *T. dicoccoides* MM 5/4 as showing relative tolerance to Zn deficiency

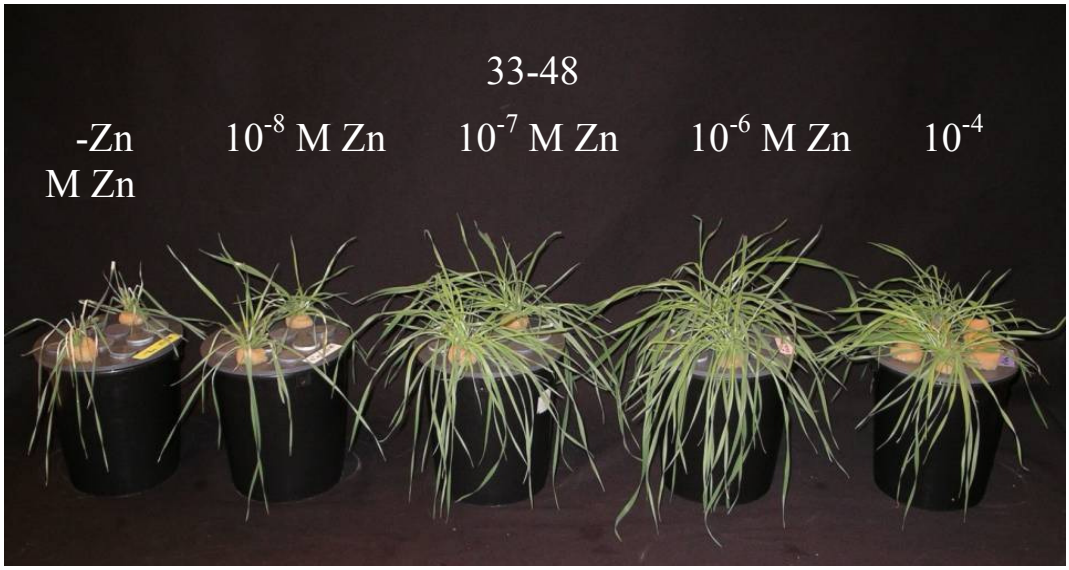

Figure 3.4 Pre-harvest picture of *T. dicoccoides* 33-48 as showing relative susceptibility to Zn deficiency

Elevated concentrations of Zn might be present in soils due to pollution caused by industry, however it is not likely to be important for most agricultural land throughout the world. Although elevated concentrations of Zn is not a natural

phenomenon, toxic dose of Zn in the form of 10^{-4} M $ZnSO_4.7H_2O$ was included in this experiment in order to observe plant response to toxic Zn which may contribute to understanding of pathway(s) involved in metal transport. In all plants, this toxic dose caused a reduction in growth together with brown patches on the leaves. Figure 3.5 clearly shows the effect of Zn toxicity.

Figure 3.5 Zn toxicity symptoms on leaves of *T. dicoccoides* MM 5/4 at 10^{-4} M Zn concentration

3.1.3 Element Analysis

Element analysis was performed using plant shoots in ICP/OES. Generally, both in –Zn and +Zn conditions, *T. dicoccoides* were recorded to contain higher Zn concentrations than *T. durum* wheats. Table 3.2 represents the shoot concentrations of Zn and Fe and the genotypes are ordered in decreasing Zn tolerance indices, expressed as Zn efficiency.

Table 3.2 Shoot Zn and Fe concentrations of wild and modern tetraploid wheats, ordered by decreasing Zn deficiency tolerance index (i.e., Zn efficiency). Plants were grown for 35 days in greenhouse on a Zn-deficient soil supplied with (+5 mg Zn kg^{-1} soil) and without Zn (-Zn).

Genotype	Zn		Fe		Zn Efficiency
	(-) Zn	(+) Zn	(-) Zn	(+) Fe	
	($mg\ kg^{-1}$)		($mg\ kg^{-1}$)		(%)
T.dicoccoides					
18-60	7.0 ± 0.1	77 ± 13.6	145 ± 11.9	57 ± 4.3	57
MM 5/4	6.0 ± 0.5	56 ± 2.2	90 ± 12.5	52 ± 1.8	57
24-39	5.9 ± 0.2	64 ± 3.3	87 ± 14.7	50 ± 4.1	55
MM 5/2	5.6 ± 0.5	56 ± 4.8	104 ± 16.6	54 ± 4.0	49
19-36	6.6 ± 1.1	77 ± 7.4	190 ± 11.8	75 ± 2.3	37
33-48	7.0 ± 0.4	74 ± 7.7	119 ± 14.6	78 ± 8.9	33
T.durum					
Balcalı 85	5.3 ± 0.1	51 ± 2.5	121 ± 2.5	49 ± 1.8	47
Meram	5.3 ± 0.1	68 ± 2.2	113 ± 12.3	59 ± 1.3	36
Ç 1252	5.4 ± 0.4	76 ± 4.9	141 ± 17.0	51 ± 1.4	19

According to Table 3.2, Zn concentration is not proportional to Zn tolerance indices since an increase in Zn tolerance was not reflected by the increase in Zn concentration. Although Zn concentration in different wild wheat accessions show variation to a certain extent, tolerant genotypes can not be differentiated from the susceptible ones by increased Zn concentrations. However, in all wheat genotypes, irrespective of tolerance and susceptibility, Zn deficiency caused approximately 10-fold decrease in shoot Zn concentration when compared to their control plants. In control conditions, where adequate Zn was supplied, two of the susceptible wild wheat accessions, 33-48 and 19-36, had approximately 10 ppm higher Zn concentrations (74 ppm and 77 ppm, respectively) than two of the tolerant wild wheat accessions, MM 5/4 and MM 5/2, both of which contained 56 ppm shoot Zn concentrations.

In order to figure out whether the amount of elements in plants differ between each other, contents of the elements were calculated by multiplying dry weights and concentration. Table 3.3 illustrates the shoot Zn and Fe contents where the genotypes are ordered by decreasing Zn tolerance indices, expressed as Zn efficiency.

Table 3.3 Shoot Zn and Fe contents of wild and modern tetraploid wheats, ordered by decreasing Zn deficiency tolerance index (i.e., Zn efficiency). Plants were grown for 35 days in greenhouse on a Zn-deficient soil supplied with (+5 mg Zn kg^{-1} soil) and without Zn (-Zn).

Genotype	Zn		Fe		Zn
	(-) Zn	(+) Zn	(-) Zn	(+) Zn	Efficiency
	($\mu g\ plant^{-1}$)		($\mu g\ plant^{-1}$)		(%)
T.dicoccoides					
18-60	2.12 ± 0.43	41 ± 10	44 ± 12	30 ± 5	57
MM 5/4	2.19 ± 0.36	36 ± 1	33 ± 10	33 ± 2	57
24-39	2.19 ± 0.14	43 ± 5	32 ± 6	34 ± 3	55
MM 5/2	1.81 ± 0.13	37 ± 7	34 ± 8	35 ± 3	49
19-36	1.23 ± 0.08	40 ± 6	35 ± 4	39 ± 4	37
33-48	1 ± 0.3	32 ± 9	17 ± 4	34 ± 13	33
T.durum					
Balcalı 85	1.55 ± 0.2	32 ± 2	36 ± 5	31 ± 1	47
Meram	1.73 ± 0.72	62 ± 11	38 ± 20	53 ± 11	36
Ç 1252	0.74 ± 0.08	56 ± 8	19 ± 3	37 ± 5	19

As Table 3.3 illustrates, although susceptible wild wheat accessions incorporate higher shoot Zn concentrations than tolerant ones, the reduced dry weight is reflected in the reduced Zn and Fe contents under Zn deficiency conditions. This suggests that high shoot Zn concentration is not a determinant factor in Zn tolerance indices. However, total Zn amount significantly differs between tolerant and susceptible genotypes under Zn deficiency.

3.2 ZIP Sequence Analysis

3.2.1 RNA Isolation

Please refer to Appendix E for the quality of isolated RNA.

3.2.2 PCR Amplification and Gel Extraction using UTR-ZIP Primers

PCR amplification using UTR-ZIP primer also gave promising results since the amplified fragments from different samples were about 1200 kb, as expected. Figure 3.4 shows the gradient-PCR picture which demonstrates that either 56 °C or 61.5 °C as annealing temperature, regardless of $MgCl_2$ concentration, provided proper amplification with the desired molecular weight, which is around 1200 bp. Figure 3.6 serves as an example from different samples. The fragments with desired molecular weights were extracted from gels to be purified.

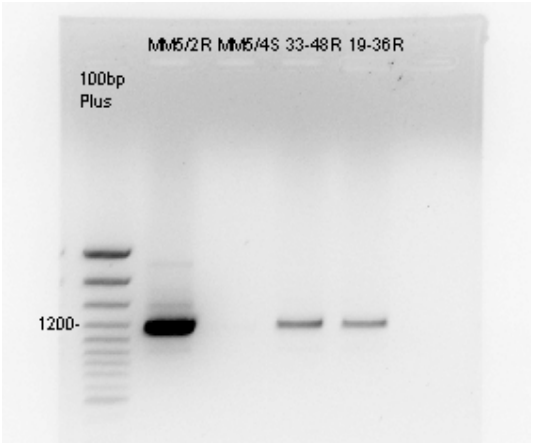

Figure 3.6 PCR gel picture using UTR-ZIP primers. Expected bands were obtained from the root cDNA samples of MM 5/2, 33-48 and 19-36 was used as a template. Proper amplification was not obtained from any of the shoot samples. 1 kb marker was used in order to detect molecular weights.

3.2.3 PCR Amplification and Gel Extraction using P1-ZIP Primers

By reverse-transcription reaction, RNA was converted into cDNA and initial amplification using P1-ZIP primers was performed. The concentration of RNAs were generally measured around 1.5 µg/µl. P1-ZIP primers were designed inside of the *T. aestivum* ZIP mRNA coding sequence and possibly succeeded to amplify the fragment band of interest since the amplified fragment appeared around 1 kb. Figure 3.7 illustrates P1-ZIP PCR products using root samples of MM 5/2, MM 5/4 and 24-39 as templates. It was observed that increasing Zn application tends to decrease in ZIP transcript which suggests that as plants suffer from Zn deficiency ZIP transcription might have been induced in order to increase the utilization of available Zn in soil. However, for accurate quantification of the transcript quantitative real-time PCR experiments were performed as well. Fragment bands appeared around 1 kb were excised from the gel in order to purify the fragment.

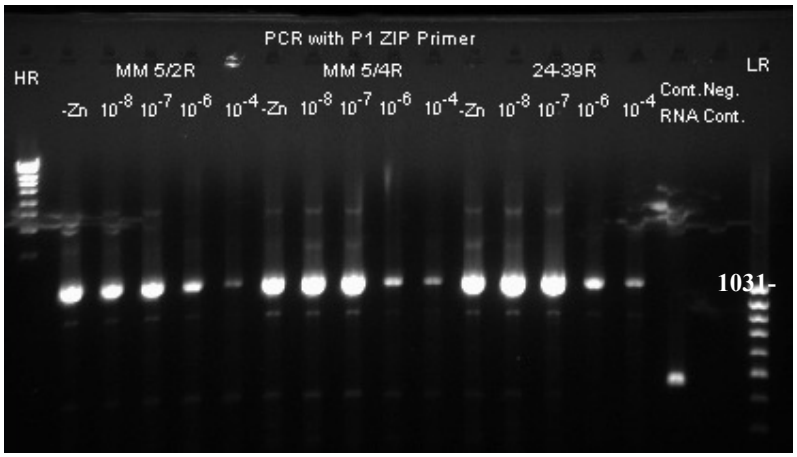

Figure 3.7 PCR gel picture using P1-ZIP primers. HR and LR represent high-range and low-range molecular markers, respectively.

3.2.4 Ligation, Transformation and Plasmid Screening

The extracted bands of interest, either amplified with P1-ZIP or UTR-ZIP primers, were ligated into pGEM®-T Vector via T-A cloning system and transformed to

either *E. coli* TOP10F' or DH5α cells. 3'-T overhangs found in pGEM®–T Easy vector enhance the efficiency of ligation. Multiple cloning region of this vector contains a *lacZ* region which encodes for the enzyme *β*-galactosidase degrading X-gal to yield blue colony formation. Therefore, insertional inactivation of this gene allows for direct identification by blue-white selection. Positive colonies which produce incomplete *β*-galactosidase protein appear as white and were selected for further analysis. After selecting white colonies, colony PCR reactions were also performed in order to check and confirm that the white colonies truly contain the vectors with the fragment band of interest. Products of the reactions were separated in 0.8 % agarose gels at 100V for 35 minutes, as illustrated in Figure 3.8. Colony-PCR amplification of the bands with desired molecular weights confirmed that ZIP cDNA candidates (either amplified with P1-ZIP or UTR-ZIP primers) were cloned into DH5α cells.

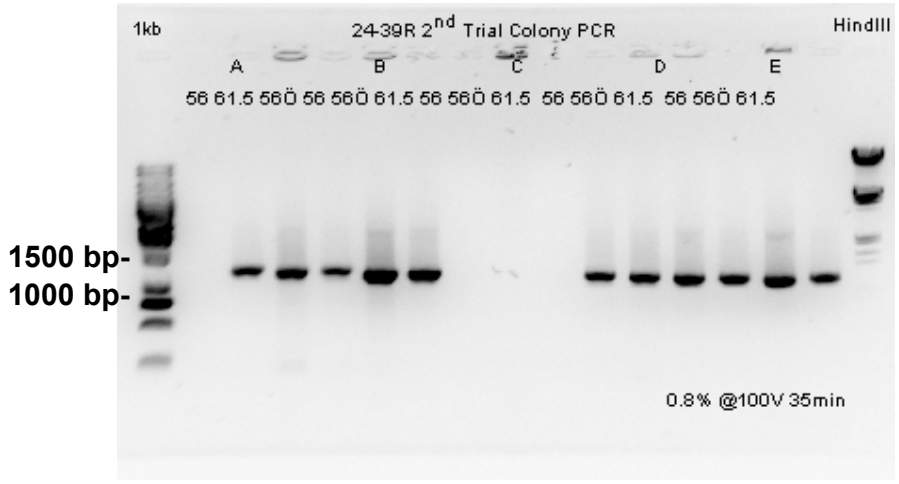

Figure 3.8 UTR-ZIP Colony-PCR gel picture using root sample of 24-39. The letters stands for different colonies. 1 kb and Lambda/EcoRI Hind III molecular markers

The clones which harbor the cDNAs of interest were selected for the sequencing analysis. The colonies that are supposed to possess ligated vectors were grown overnight in LB-Amp Broth with shaking at 200 rpm. Plasmid isolation was performed to these colonies for the following sequence analysis. To determine concentration and purity of plasmid DNA, optical densities were measured at 260 nm using Nanodrop spectrophotometer. Additionally, glycerol stocks of these colonies were prepared for long-term storage and use.

3.2.5 Sequence Analysis

Isolated plasmids were sequenced twice using M13 forward and reverse primers. Obtained sequences were first exposed to VecScreen algorithm of NCBI in order to remove the vector contamination. The insert sequences were then compared to the nucleotide and protein sequences available at the Entrez nucleotide and protein databases using the BLASTN, BLASTX, and TBLASTX algorithms. Alignments with an E Value < 0.0001 were considered to be significant. All of the sequenced bands hit to *T. aestivum* ZIP transporter at first glance, however, a detailed sequence comparison was also conducted in order to find any polymorphisms if available.

3.2.5.1 Sequence Analysis of UTR-ZIP Amplified Fragments

Biology Workbench tools were used to analyze the cloned sequences. Four root samples were succeeded to be sequenced which are; Balcali 85 root, MM 5/4 root, 24-39 root, 33-48 root and 19-36 root samples.

3.2.5.1.1 UTR-ZIP Sequence Amplified from MM 5/4 Root Sample

Full length ZIP1 clone of MM5/4 root sample was confirmed with alignment analysis via CLUSTALW. Alignment of MM 5/4 root UTR-ZIP amplified fragment with *Triticum aestivum* ZIP mRNA complete coding sequence gave full match except for two single nucleotide mutations (see Appendix F.1). One of these nucleotide changes was reflected at the amino acid level which converted methionine to isoleucine. Figure 3.9 illustrates ClustalW alignment of *T. aestivum* ZIP mRNA with cloned MM5/4 ZIP sequence.

```
T.a._ZIP        MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
MM54R_UTR       MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
                ************************************************************

T.a._ZIP        GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
MM54R_UTR       GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGIVHILPAAFDGLTSPCIYKGGGDR
                ***********************************:************************

T.a._ZIP        NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
MM54R_UTR       NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
                ************************************************************

T.a._ZIP        GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
MM54R_UTR       GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
                ************************************************************

T.a._ZIP        ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
MM54R_UTR       ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
                ************************************************************

T.a._ZIP        IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
MM54R_UTR       IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
                ************************************************************
```

Figure 3.9 Amino Acid Alignment of *T. aestivum* ZIP with MM5/4 root ZIP sequence

3.2.5.1.2 UTR-ZIP Sequence Amplified from 24-39 Root Sample

The full length ZIP sequence of 24-39 root sample could not be obtained due to sequencing inefficiency. Consensus sequence was not found by overlapping opposite sequences from M13F and M13R due to poor sequence quality. Thus, this inner region whose sequence could not be obtained is indicated with dots. Two differences occurred in the nucleotide sequence is not reflected in the amino acid sequence (Appendix F2).

```
T.a._ZIP        MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
2439R_UTR       MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
                ************************************************************

T.a._ZIP        GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
2439R_UTR       GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
                ************************************************************

T.a._ZIP        NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
2439R_UTR       NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
                ************************************************************

T.a._ZIP        GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
2439R_UTR       GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
                ************************************************************

T.a._ZIP        ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
2439R_UTR       ALSFHQFFEGIGLGGCIVQANF--------------------------------------
                **********************

T.a._ZIP        IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
2439R_UTR       ------------------------------------------------------------
```

Figure 3.10 Amino Acid Alignment of *T. aestivum* ZIP with 24-39 root ZIP sequence

3.2.5.1.3 UTR-ZIP Sequence Amplified from 33-48 Root Sample

Full length ZIP1 clone from 33-48, *T. dicoccoides*, root sample was found to be 100% identical to that of *T. aestivum*.

```
T.a._ZIP        MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
3348R_UTR       MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
                ************************************************************

T.a._ZIP        GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
3348R_UTR       GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
                ************************************************************

T.a._ZIP        NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
3348R_UTR       NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
                ************************************************************

T.a._ZIP        GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
3348R_UTR       GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
                ************************************************************

T.a._ZIP        ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
3348R_UTR       ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
                ************************************************************

T.a._ZIP        IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
3348R_UTR       IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
                ************************************************************
```

Figure 3.11 Amino Acid Alignment of *T. aestivum* ZIP with 33-48 root ZIP sequence

3.2.5.1.4 UTR-ZIP Sequence Amplified from 19-36 Root Sample

Alignment of 19-36 root ZIP1 with *T. aestivum* ZIP mRNA complete coding sequence gave full match except for one nucleotide difference (Appendix F.4).

```
T.a._ZIP        MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
1936R_UTR       MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
                ************************************************************

T.a._ZIP        GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
1936R_UTR       GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
                ************************************************************

T.a._ZIP        NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
1936R_UTR       NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
                ************************************************************

T.a._ZIP        GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
1936R_UTR       GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
                ************************************************************

T.a._ZIP        ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
1936R_UTR       ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
                ************************************************************

T.a._ZIP        IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
1936R_UTR       IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
                ************************************************************
```

Figure 3.12 Amino Acid Alignment of *T. aestivum* ZIP with 19-36 root ZIP sequence

3.2.5.1.5 UTR-ZIP Sequence Amplified from Balcali 85 Root Sample

Alignment of Balcali 85 root ZIP1 with *T. aestivum* ZIP mRNA complete coding sequence gave full match except for one nucleotide difference (Appendix F.5) which resulted in elimination of one histidine residue.

Figure 3.13 Amino Acid Alignment of *T. aestivum* ZIP with Balcali 85 root ZIP sequence

3.2.5.2 Sequence Analysis of P1-ZIP Amplified Fragments

Biology Workbench tools were used to analyze the cloned sequences. Four root and one shoot samples were succeeded to be sequenced which are; Balcali 85 root, C-1252 root, MM 5/2 root, 33-48 root and MM 5/4 shoot. Although there might be slight changes in between the nucleotide sequences, none of the samples resulted in any changes in the amino acid sequence in P1-ZIP amplification reactions.

3.2.5.2.1 P1-ZIP Sequence Amplified from MM 5/4 Shoot Sample

MM5/4 was the only accession from which ZIP sequence was identified in shoots. Alignment of MM 5/4 shoot P1-ZIP amplified fragment with *Triticum aestivum* ZIP mRNA complete coding sequence gave full match except for two single nucleotide deletions observed in MM 5/4 shoot sequence (Appendix F.6). However, since P1-ZIP

primers targeted inside of full length ZIP mRNA, full length MM 5/4 sequence could not be obtained.

```
T.a._ZIP      MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
MM54S_P1      ------TLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
                    ************************************************************

T.a._ZIP      GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
MM54S_P1      GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
              ************************************************************

T.a._ZIP      NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
MM54S_P1      NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
              ************************************************************

T.a._ZIP      GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
MM54S_P1      GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
              ************************************************************

T.a._ZIP      ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAP--VGIVLGIAISSSYNVHSST
MM54S_P1      ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTARGHRARDCDIVLCALYCLHYGS
              ************************************    .   .*.:.: * :* .:

T.a._ZIP      AFIIEG-VFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLA
MM54S_P1      LQLSLGRDFNLHVPG--------GPSSKRFQ-PKATDKYKASADDISCTFPRCRDDVHAC
                :  *  **   .*        .  :* *: **   : * .     . *  .      .

T.a._ZIP      IWA
MM54S_P1      ---
```

Figure 3.14 Amino Acid Alignment of *T. aestivum* ZIP with MM 5/4 shoot P1-ZIP amplified fragment

3.2.5.2.2 P1-ZIP Sequence Amplified from MM 5/2 Root Sample

Alignment of MM 5/2 root P1-ZIP amplified fragment with *Triticum aestivum* ZIP mRNA complete coding sequence gave full match. However, the full-length sequence could not be obtained since P1-ZIP primers targeted inside of the ZIP mRNA.

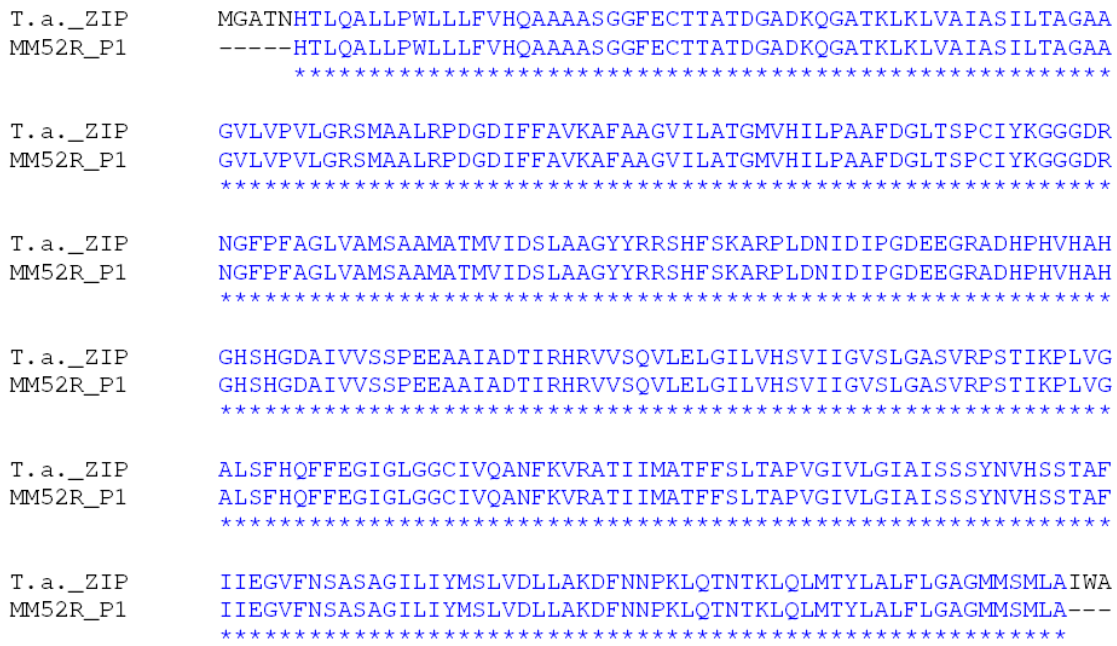

Figure 3.15 Amino Acid Alignment of *T. aestivum* ZIP with MM 5/2 root P1-ZIP amplified fragment

3.2.5.2.3 P1-ZIP Sequence Amplified from C-1252 Root Sample

Alignment of C-1252 root P1-ZIP amplified fragment with *Triticum aestivum* ZIP mRNA complete coding sequence gave full match. However, since P1-ZIP primers targeted inside of full length ZIP mRNA, full length C-1252 sequence could not be obtained.

```
T.a._ZIP        MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
C1252R_P1       ----YHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
                     ************************************************************

T.a._ZIP        GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
C1252R_P1       GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
                ************************************************************

T.a._ZIP        NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
C1252R_P1       NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
                ************************************************************

T.a._ZIP        GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
C1252R_P1       GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
                ************************************************************

T.a._ZIP        ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
C1252R_P1       ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
                ************************************************************

T.a._ZIP        IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
C1252R_P1       IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLA---
                ************************************************************
```

Figure 3.16 Amino Acid Alignment of *T. aestivum* ZIP with C-1252 shoot P1-ZIP amplified fragment

3.3 Quantitative Real-time PCR Analysis of ZIP Protein

With the utilization of TMHMM server, two different primer pairs were targeted to amplify ZIP mRNA sequence. One of which, RT1 primer pair, was designed to target TM4 since this region is defined as the most conserved region among ZIP transporters, whereas the other pair, RT2, was targeted to the cytoplasmic region located in between TM4 and TM5. PCR reaction using RT1 primer pair resulted in more than one melting curve which suggests that RT1 primer is not specific to one region, thereby allows for the amplification of more than one fragment. The utilization of melting curves in quantitative real-time PCR (QRT-PCR) provides information about any bias found in quantification of the gene that might come from multiple band amplifications. Since RT1 primer pair caused multiple amplification, it was eliminated to be used in QRT-PCR experiment. The amplification of multiple bands might due to the fact that RT1 primer pair was targeted to amplify the most conserved region of ZIP proteins. Amplification of multiple bands suggested that there are more than one ZIP-like transcripts which harbor the same conserved sequence -but differ in length- that

can be amplified with the same primer pair. Unlike RT1 primer pair, one single fragment was amplified with RT2 primers. The amplification of a single fragment using RT2 primer pair suggested that this region might be variable among different ZIPs. Figure 3.17 represents the gel picture of RT1-PCR and RT2-PCR products run on 1.5 % agarose gel at 100V for 50 minutes.

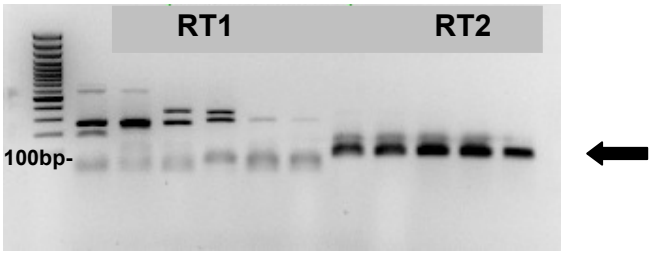

Figure 3.17 Gel picture of QRT-PCR using RT1 and RT2 primers

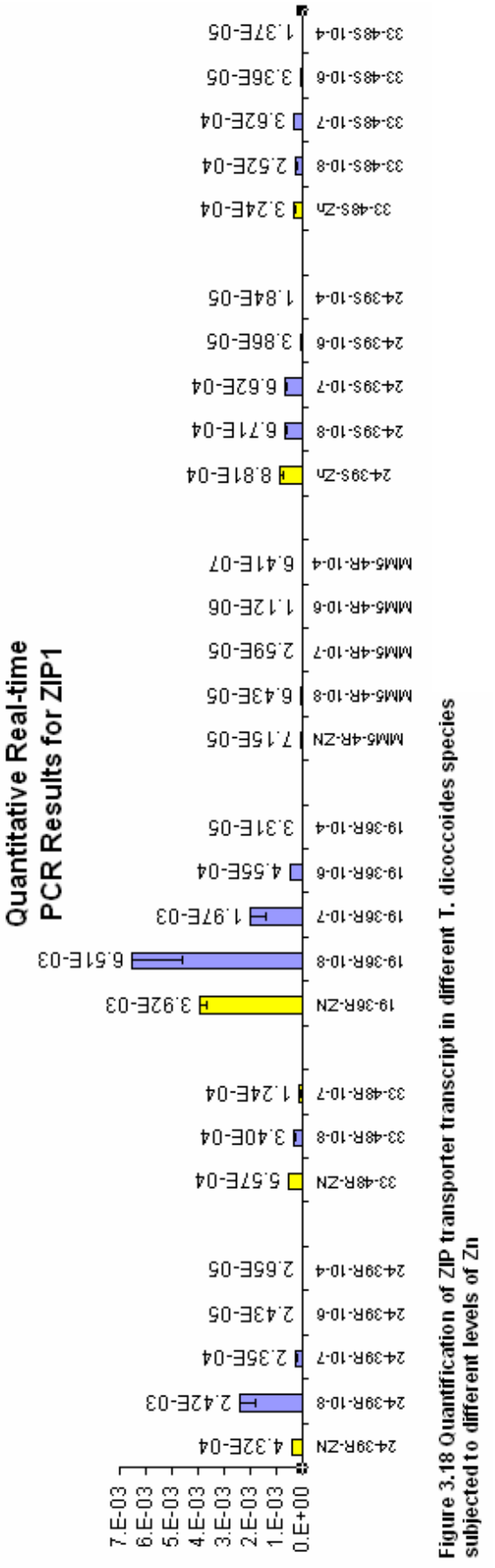

Figure 3.18 Quantification of ZIP transporter transcript in different T. dicoccoides species subjected to different levels of Zn

Since specific amplification was achieved by RT2 primers, all QRT-PCR data are obtained by using this primer set. QRT-PCR results revealed that ZIP transcript levels are elevated with decreasing Zn supply in all accessions. It was observed that ZIP transcript accumulation was lower in the root of MM5/4, which is the most tolerant to Zn deficiency, than that of 19-36, which is the susceptible genotype (Fig. 3.18). This indicates susceptible accession, 19-36, may sense Zn deficiency stress earlier than that of tolerant, MM 5/4, so that the response of root cells of 19-36 are much quicker than the response of MM 5/4 root cells.

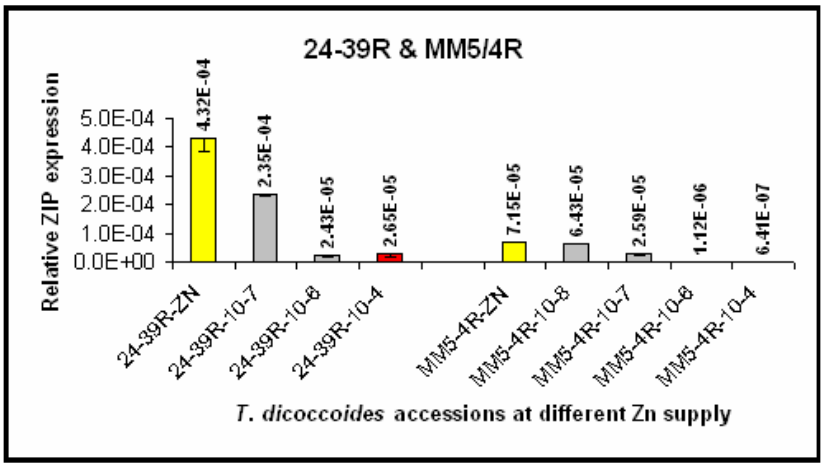

Figure 3.19 Relative expression of ZIP transporter in root samples of 24-39 and MM5/4

The relative ZIP expression of two Zn-deficiency tolerant genotypes, 24-39 and MM 5/4, showed difference in response to Zn deficiency (Fig. 3.19). The relative low expression of ZIP in MM 5/4 roots suggested that this accession did not sense the Zn deficiency stress as pronounced as for 24-39 roots. Moreover, the time of harvest may be a determinant factor.

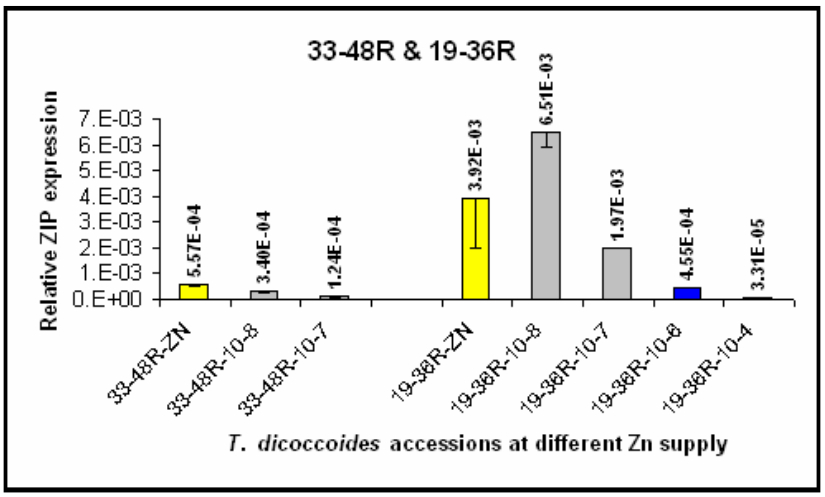

Figure 3.20 Relative expression of ZIP transporter in root samples of 33-48 and 19-36

When the root ZIP expression of the two susceptible wild wheat accessions are compared, it was observed that 33-48 expressed more less amount of ZIP than 19-36. In compatible with the greenhouse screening experiment, one might suggest that relative high ZIP expression in 19-36 roots would increase Zn tolerance to some degree. On the other hand, since 33-48 was almost dying, it is reasonable not to observe elevated levels of ZIP which might rescue the plant from Zn-deficiency stress to some extent.

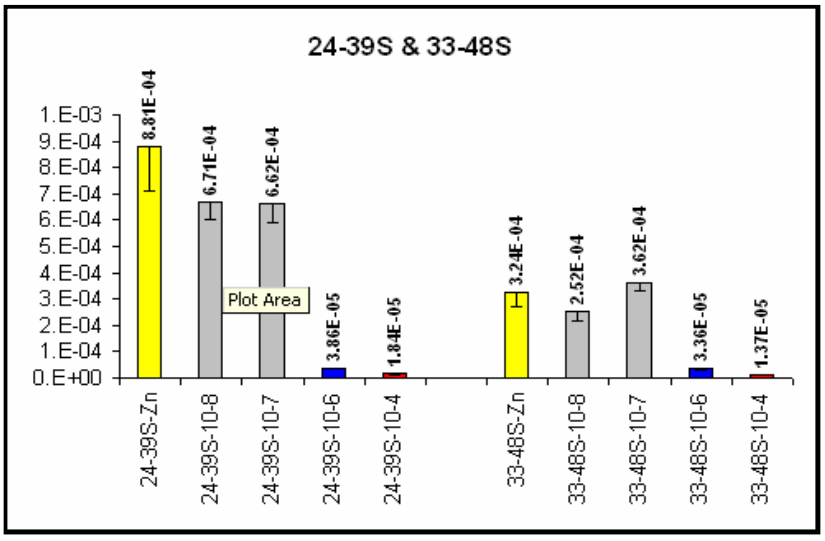

Figure 3.21 Relative expression of ZIP transporter in shoot samples of 24-39 and 33-48

Considerable amount of ZIP expression was detected in the shoot tissue as well. However, shoot ZIP transcripts were only obtained from 24-39 and 33-48 samples. The presence of ZIP expression in shoots suggests that this family of ZIP transporter may play a role somewhere from roots to shoots. Thus, it may not be root specific.

Some metal ions, such as Fe, were recorded to compensate Zn in some cellular processes. However, the absence of ZIP transcript at 10^{-4} M Zn concentration suggests that this ZIP metal transporter found in *T. dicoccoides* species is Zn-specific.

4 DISCUSSION

The aim of this study was to identify and characterize a cDNA encoding a ZIP transporter protein in six different *Triticum dicoccoides* (wild emmer) accessions subjected to varying Zn treatments. The initial part of this study was comprised of greenhouse and nutrient solution experiments conducted for the purposes of screening, obtaining growth data and fresh sample collection for molecular studies. After RNA was collected from plant roots and shoots grown in the hydroponic solution, regular cloning techniques were performed to identify ZIP homologs in wild emmer wheat.

Screening experiments revealed that there is a considerable variation among *Triticum dicoccoides* accessions in response to Zn deficiency. Within the wild species used in this study, three wild emmer wheat accessions (MM 5/4, MM 5/2 and 24-39) were found to show higher tolerance to Zn deficiency whereas two of the accessions were detected as sensitive (19-36 and 33-48). Under Zn deficient conditions the sensitive and tolerant genotypes were more or less same in their shoot Zn concentration. This result indicates that the genotypes possibly differ in their Zn utilization capacity at cellular level. There are several physiological mechanisms involved in differential utilization of Zn in the cell, such as better solubility and mobility in shoot tissue and less compartmentation of Zn in vacuoles and/or cell wall (Erenoglu et al., 1996, 2002; Rengel 2001).

Induction of Fe uptake under Zn deficiency was previously reported in different bread and durum wheat genotypes. It was reported that advanced Zn deficiency triggered Fe uptake in all wheat genotypes studied, irrespective of their susceptibility to Zn deficiency. However, except for the early stages, Zn deficiency caused lower rate of Fe transport to shoots in Zn-efficient (tolerant to Zn deficiency) genotypes in comparison to Zn-inefficient (susceptible to Zn deficiency) genotypes (Rengel and Römheld, 2000). As illustrated in Table 3.2, Zn deficiency induced Fe concentration of

plants. Moreover, 19-36 and 33-48, which were relatively more sensitive to Zn deficiency had higher shoot Fe concentrations in comparison to MM 5/4 and 24-39, which were more tolerant. This result is in well agreement with the idea that uptake and accumulation of Fe is stimulated with the severity of Zn deficiency (Cakmak, 2000). Alternatively, high Fe accumulation in Zn-deficient plants might be a concentration effect and with the aid of time-course dependent kinetics study for Fe uptake and transport to shoots, the reason behind increased shoot Fe concentration would be clarified.

The difference between susceptible and tolerant genotypes regarding shoot Zn concentration is very small, around 1 mg kg^{-1} (Table 3.2). As indicated above, Fe was also more accumulated in susceptible genotypes than in tolerant genotypes. The fact that the susceptible wild wheat accessions contained higher amounts of element concentrations might be due to concentration effect. Since their tolerance indices are lower, the elements found in the shoot of plants appeared as more concentrated. From these observations, one can argue that internal utilization of Zn in tolerant accessions, such as MM 5/4, might be greater than in the susceptible ones, such as 19-36 since the latter had relatively higher shoot Zn concentrations. Alternatively, content instead of concentration can be discussed as physiological parameter involved in expression of high Zn efficiency because tolerant accessions resulted in higher shoot Zn contents due to higher tolerance indices.

The sequence of *Triticum aestivum* Zn transporter ZIP mRNA found in the literature (NCBI Locus: AY864924) was targeted in order to identify and characterize ZIP homologs from wild emmer wheat. Two different primers were designed, one of which covered the full length cDNA, and the other amplified a fragment which was located in the open reading frame (ORF) region. Full length cDNAs targeting a ZIP transporter protein were identified from the following accessions: MM 5/4 root sample, 24-39 root sample, 33-48 root sample, 19-36 root sample and Balcali 85 root sample (cultivated durum wheat). Using P1-ZIP primers, a partial cDNA encoding a ZIP transporter protein were also identified from the following samples: MM 5/4 shoot sample, MM 5/2 root sample and C-1252 root sample. The failure of obtaining a full length ZIP cDNA sequence from shoot samples may be related to tissue-specific UTRs located at 5' and 3' regions, out of ORFs. Primers targeting full length ZIP mRNA

designed by overlapping available wheat ESTs which were obtained from grain and root samples. Therefore, ZIP mRNA, which was already amplified an ORF with P1-ZIP primers, may fail to be amplified by UTR primers since shoot UTRs might differ from root UTRs of ZIP transporter.

Identification of ZIP homologs obtained from wild emmer wheat accessions resulted in a few polymorphic sites both at nucleotide and amino acid level. For instance, a cDNA encoding a ZIP transporter obtained from MM 5/4 root sample revealed that one of the two nucleotide polymorphisms altered methionine (M) to isoleucine (I) (Appendix F.1). This might be an error but methionine and isoleucine are both nonpolar amino acids. Full length cDNA cloned from Balcali 85 also resulted in one amino acid change from methionine to arginine, which are also similar amino acids, which in turn, would probably have not distorted functionality of the transporter. However, in root sample of Balcali 85, the partial cDNA had no polymorphism (Appendix F.5). There were also SNPs in other samples at the nucleotide level but they did not cause any amino acid change. From sequence analyses, no significant polymorphism, which may alter protein functionality, was detected in any of the samples.

In order to monitor relative ZIP expression of these six wild emmers, QRT-PCR experiments were performed (Figs. 3.18, 3.19, 3.20, 3.21). It was observed that one of the most susceptible accessions, 19-36, showed a relative greater ZIP expression in comparison to all other accessions. Interestingly, one of the most tolerant accessions, MM 5/4, showed the least ZIP expression level relatively. These results might suggest that the expression level of ZIP transporter mRNA may reflect the level of stress by which the plant was affected rather than a cause of Zn deficiency response. In this context, 19-36 is said to be very susceptible since it was affected more dramatically by Zn deficiency so that it increased ZIP mRNA expression to relieve the stress. It is also reasonable to observe low ZIP expression levels in MM 5/4 since it is the most tolerant accession that did not require to increase ZIP levels. However, these suggestions are not valid to all tolerant and susceptible accessions. Tables 3.19 and 3.20 illustrate that 24-39 and 33-48 showed higher and lower, respectively, amounts of ZIP expression in comparison to MM 5/4 and 19-36. Thus, a consensus in Zn deficiency responses either for tolerant or susceptible genotypes was not achieved.

The interaction of elements affects their uptake by roots. For instance high concentration of one element may cause plant to suffer from deficiency of another element by causing antagonistic effect during uptake. It was previously recorded that Cd can induce Fe or Zn deficiency at physiological levels and thus affects expression of genes associated with Fe or Zn deficiency in plants (Siedlecka and Baszynsky, 1993). Relatively high soil Zn application contributed to reduction of Cd content in durum wheats where Zn and Cd act antagonistically to each other (Choudhary et al., 1995; Kokeli et al., 2004). Moreover, IRT1 which is a ZIP protein from *Arabidopsis thaliana* was shown to be a metal transporter with broad range substrate specificity as it transports Zn, Mn, Cd and Co (Cohen et al., 1998; Korshunova et al., 1999). In this study, at 10^{-4} M Zn, transport of other elements, such as Fe, might probably have been prevented due to high level of Zn concentration in growth medium for plant roots. Since some of the ZIP transporters are capable of transporting several divalent cations unspecifically, the expression of a ZIP protein transporting divalent cations other than Zn was also expected to be high under Zn toxicity. However QRT-PCR results in this study showed that ZIP expression levels were not upregulated at toxic Zn level. The fact that ZIP expression was not induced at toxic Zn levels suggests that ZIP transporter identified in this study is much more likely to be Zn-specific.

It is important to note that QRT-PCR gives some idea about transcript level which does not necessarily reflect the amount of the protein. Post-regulation mechanisms found in eukaryotes altering the function of the transcript may take place under Zn deficiency. Alternatively, various pathways, such as internal utilization mechanisms, should also be considered in explaining Zn deficiency response.

5 CONCLUSION

This study clearly showed that there is a great variation between different wild emmer (*Triticum dicoccoides*) wheats in response to Zn deficiency. The existence of variation and the genetic importance of wild emmer throughout the evolution of wheat make these wild species interesting to work on. To our knowledge, this is the first study that a cDNA encoding a ZIP transporter, similar to *AtZIP1*, was identified from the roots of *Triticum dicoccoides* subjected to Zn deficiency. There are few SNPs present both at nucleotide and amino acid level identified from wild emmer wheats when compared to cultivated wheat. Full length ZIP cDNAs were only identified from roots but not shoots. This is most likely due to tissue specificity of UTR regions, where primers were used to target.

Verification of ZIP transporter sequence in wild emmer requires further investigation at the protein level via functional analyses and reverse genetic methods. Complementation assays using mutant yeast strains defective in Zn and Fe uptake will be performed in order understand the function of the protein. However, our current data suggests that this ZIP transporter is Zn-specific since ZIP expression was decreased at 10^{-4} M Zn.

QRT-PCR results showed that the expression of ZIP transporter was induced as Zn deficiency stress was elevated. However, it seems that reduction in Zn supply beyond 10^{-7} M Zn does not cause a significant increase in ZIP expression suggesting that 10^{-7} M Zn is the critical dosage (Figs. 3.18-3.21) This result is interesting and should studied in more detail in future studies. Additionally, the most tolerant accession, MM 5/4 resulted in the lowest ZIP transporter whereas the susceptible, 19-36, gave the highest ZIP expression. This data suggests that QRT-PCR measurement may reflect the results caused by Zn deficiency stress rather than the reason of the response. In order to enlarge the scope of this experiment, time-dependent analysis of ZIP transporters will be monitored.

6 REFERENCES

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APPENDIX A

Equipment

Autoclave sterilizers:	Hirayama, Japan (HiClave HV-110)
	Nüve, Turkey (OT 032)
Centrifuges:	Eppendorf, Germany (Centrifuge 5415C, 5415D)
Cold room:	Alarko Carrier, Turkey
Electronic balances:	Schimadzu, Japan (Libror EB-3200HU)
	Sartorius, Germany (BP221S)
Electrophoresis equipment:	Labnet International, USA (Gel XL Ultra V-2™)
Freezers:	Bosch, Turkey
	Thermo Electron Corporation, USA (Thermo Forma 917)
Gel documentation system:	Bio-Rad Laboratories, USA (Universal Hood II)
Heating block:	Fisher, France (Bioblock Scientific™)
Heating magnetic stirrer:	VELP Scientifica, Italy (ARE)
Incubator:	Memmert®, Germany (D06059 Modell 300)
Incubator shaker:	New Brunswick Scientific, USA (Innova® 4330)
Laboratory bottles:	SCHOTT DURAN®, Germany
Laminar flow cabinets:	Heraeus Instruments, Germany (HERAsafe® HS12)
Micropipettes:	Gilson, USA (Pipetman® P)
	Eppendorf, Germany (Research®)
Microwave:	Vestel, Turkey

pH meter:	Windaus Labortechnik, Germany (TitroLine alpha)
Growth Chambers:	DigiTech, Turkey
Refrigerators:	Bosch, Turkey
Software:	Invitrogen Corporation, USA (Vector NTI 9.1.0)
	Technelysium Pty. Ltd., Australia (Chromas 2.31)
Spectrophotometer:	NanoDrop Technologies, USA (ND-1000)
Thermal cycler:	GMI, USA (MJ Research PTC-100)
Vortex mixer:	VELP Scientifica, Italy (ZX3)
Water bath:	Techne, UK (Refrigerated Bath RB-5A)
Water purification system:	Millipore, USA (Milli-Q Academic)

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)$_{12-18}$ Primer:

Invitrogen, USA (18418-012)

Oligonucleotides:

Integrated DNA Technologies Inc. USA

İontek, Turkey

Pfu DNA Polymerase (recombinant):

Fermentas, Canada (EP0502)

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)

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

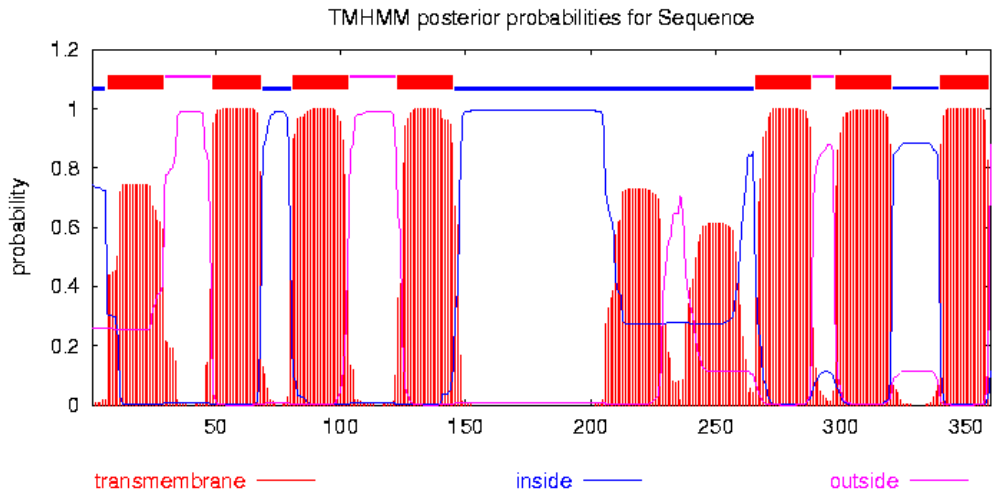
```
LOCUS       AY864924 1083 bp mRNA linear PLN 01-FEB-2005
DEFINITION  Triticum aestivum zinc transporter ZIP mRNA, complete cds.
ACCESSION   AY864924
VERSION     AY864924.1  GI:58221592
KEYWORDS    .
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta;
Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae;
BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 1083)
  AUTHORS   Zhao,Y.L., Zhou,R.H. and Jia,J.Z.
  TITLE     Diversity, evolution and gene expression of zinc
transporters from
            wheat
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1083)
  AUTHORS   Zhao,Y.L., Zhou,R.H. and Jia,J.Z.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-DEC-2004) Key Laboratory of Crop Germplasm &
            Biotechnology, Institute of Crop Germplasm Resources,
CAAS, 12
            Southern Zhongguanchun Street, Beijing 100081, China
FEATURES             Location/Qualifiers
     source          1..1083
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /db_xref="taxon:4565"
     CDS             1..1083
                     /codon_start=1
                     /product="zinc transporter ZIP"
                     /protein_id="AAW68439.1"
                     /db_xref="GI:58221593"
/translation="MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGAT
KLKLVAIASILTAGAAGVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILP
AAFDGLTSPCIYKGGGDRNGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLD
NIDIPGDEEGRADHPHVHAHGHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVH
SVIIGVSLGASVRPSTIKPLVGALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSL
TAPVGIVLGIAISSSYNVHSSTAFIIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQT
NTKLQLMTYLALFLGAGMMSMLAIWA"

ORIGIN
        1 atgggcgcca ccaatcatac cttgcaggcg cttctcccat ggctcctcct
gtttgtgcac
       61 caggccgcgg cggccagcgg cgggttcgag tgcacgaccg ccacggacgg
ggcggacaag
      121 cagggcgcga cgaagctgaa gctggtcgcc atcgcgtcca tcctcaccgc
cggggcggct
```

```
      181 ggcgtgctgg tgccggtgct cggacgctcc atggccgcgc tgcgccccga
cggcgacatc
      241 ttcttcgcgg tcaaggcgtt cgccgctggc gtcatccttg ccactggcat
ggtgcacatc
      301 ctgccggcgg cgtttgacgg gctcacctcc ccgtgcatct acaaaggtgg
cggggacagg
      361 aacggcttcc cttttgcggg acttgtggcc atgtctgcag ccatggccac
aatggtgata
      421 gactcgctgg ctgctgggta ctaccgccgg tctcacttca gcaaggcacg
cccacttgac
      481 aacatcgaca tacccggaga tgaggaaggg agggccgatc atccacatgt
gcacgcgcat
      541 ggccattcac atggtgacgc aattgttgtc agctcaccgg aggaggctgc
catagctgac
      601 acaatccggc acagggtggt atctcaggtt ctagagctgg gaatcttggt
gcattcagtg
      661 ataattggtg tgtcattagg agcatctgtg aggccatcca ccatcaagcc
tctggtcggt
      721 gccctcagct tccatcaatt ctttgaaggc ataggcttgg gtggttgcat
tgtacaggct
      781 aatttcaagg taagggcaac catcatcatg gcaacgtttt tctccctgac
cgcacccgtg
      841 ggcatcgtgc tagggattgc gatatcgtct agctataatg tgcatagctc
tactgccttc
      901 attattgagg gagtcttcaa ctcagcctcg gcagggattt taatctacat
gtccctggtg
      961 gaccttctag caaaagattt caataaccca aagctacaga caaatacaaa
gcttcagctg
     1021 atgacatatc ttgcactttt cctaggtgca gggatgatgt ccatgcttgc
catatgggca
     1081 tag
```

APPENDIX C

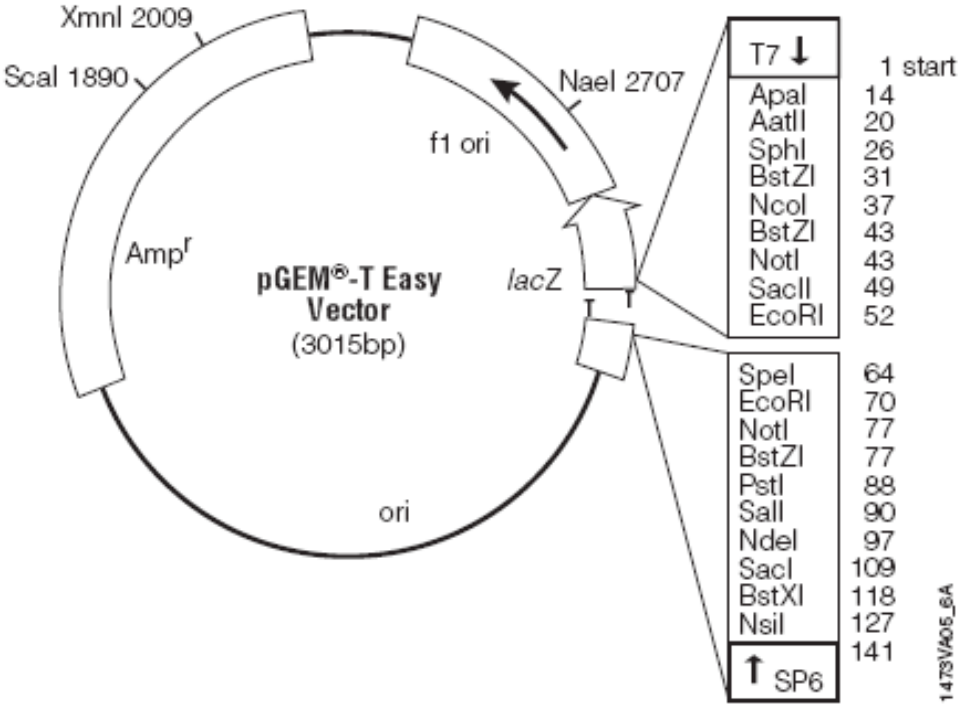
TMHMM Analysis of ZIP Transporter




```
# Sequence Length: 360
# Sequence Number of predicted TMHs:  7
# Sequence Exp number of AAs in TMHs: 175.27868
# Sequence Exp number, first 60 AAs:  28.70506
# Sequence Total prob of N-in:        0.74171
# Sequence POSSIBLE N-term signal sequence
Sequence        TMHMM2.0        inside       1     6
Sequence        TMHMM2.0        TMhelix      7    29
Sequence        TMHMM2.0        outside     30    48
Sequence        TMHMM2.0        TMhelix     49    68
Sequence        TMHMM2.0        inside      69    80
Sequence        TMHMM2.0        TMhelix     81   103
Sequence        TMHMM2.0        outside    104   122
Sequence        TMHMM2.0        TMhelix    123   145
Sequence        TMHMM2.0        inside     146   265
Sequence        TMHMM2.0        TMhelix    266   288
Sequence        TMHMM2.0        outside    289   297
Sequence        TMHMM2.0        TMhelix    298   320
Sequence        TMHMM2.0        inside     321   339
Sequence        TMHMM2.0        TMhelix    340   359
Sequence        TMHMM2.0        outside    360   360
```

APPENDIX D

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

APPENDIX E

Gel Picture of DNase and non-DNase treated RNA samples

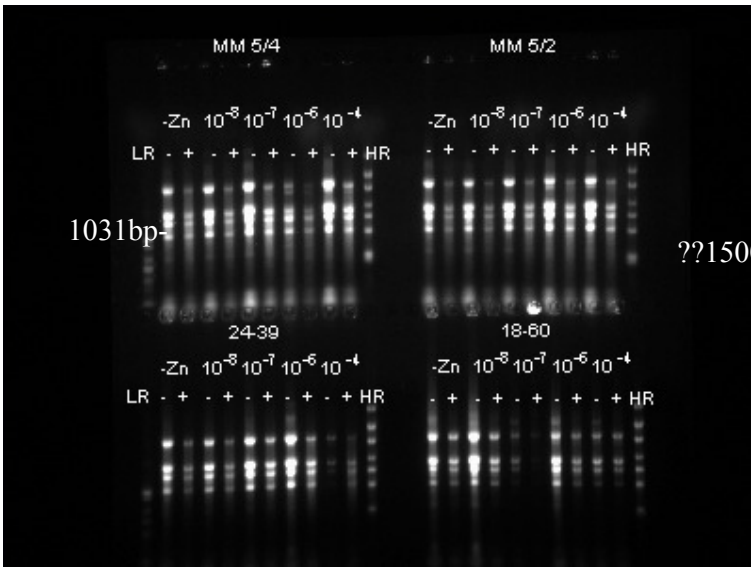


Figure E.1 Gel picture of RNA isolated from the shoots of MM 5/4, MM 5/2, 24-39 and 18-60 which are supplied with 5 different Zn concentrations. "+" denotes for DNase-treated samples whereas "-" stands for non-DNase treated samples. HR and LR represent high-range and low-range molecular markers, respectively.

Gel Picture of PCR reaction using WRKY primers

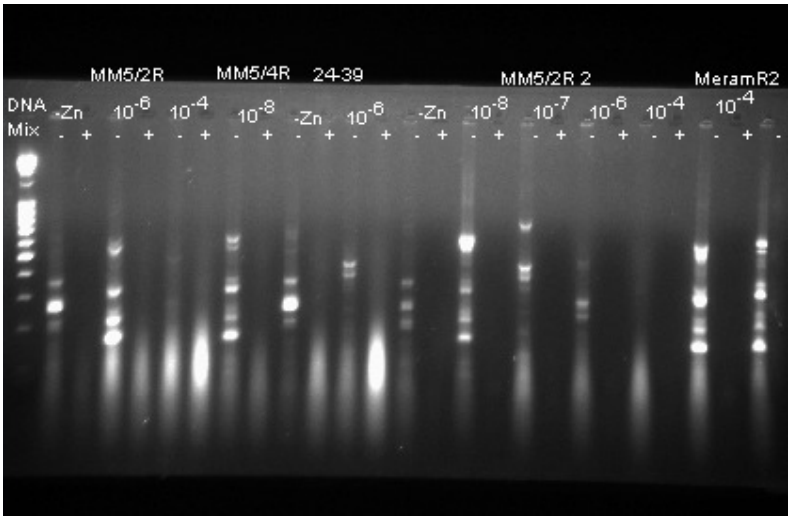


Figure E.2 PCR gel picture using WRKY primers. "+" denotes for DNase-treated whereas "-" stands for non-DNase treated samples. No amplification detected in DNase-treated samples confirmed that DNase-treated RNA samples are free of DNA contamination.

APPENDIX F

2) UTR-ZIP Sequence Amplified from MM 5/4 Root Sample

ATGGGCGCCACCAATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGT
GCACCAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGTACGACCGCCACGGACGG
GGCGGACAAGCAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCT
CACCGCCGGGGCGGCTGGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCG
CTGCGCCCCGACGGCGACATCTTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCA
TCCTTGCCACTGGCATAGTGCACATCCTGCCGGCGGCGTTTGACGGGCTCACCTC
CCCGTGCATCTACAAAGGTGGCGGGGACAGGAACGGCTTCCCTTTTGCGGGACTT
GTGGCCATGTCTGCAGCCATGGCCACAATGGTGATAGACTCGCTGGCTGCTGGGT
ACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGACAACATCGACATACC
CGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCATGGCCATTC
ACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGACACA
ATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAG
TGATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCT
GGTCGGTGCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGC
ATTGTACAGGCTAATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCT
CCCTGACCGCACCCGTGGGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAA
TGTGCATAGCTCTACTGCCTTCATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAG
GGATTTTAATCTACATGTCCCTGGTGGACCTTCTAGCAAAAGATTTCAATAACCC
AAAGCTACAGACAAATACAAAGCTTCAGCTGATGACATATCTTGCACTTTTCCTA
GGTGCAGGGATGATGTCCATGCTTGCCATATGGGCATAG

Figure F.1.1: ZIP1 transporter protein sequenced from root sample of MM5/4

```
MM54R_UTR       ATGGGCGCCACCAATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCAC
T.a._ZIP        ATGGGCGCCACCAATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCAC
                ************************************************************

MM54R_UTR       CAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGTACGACCGCCACGGACGGGGCGGACAAG
T.a._ZIP        CAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACAAG
                ******************************** ***************************

MM54R_UTR       CAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGCGGCT
T.a._ZIP        CAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGCGGCT
                ************************************************************

MM54R_UTR       GGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGACGGCGACATC
T.a._ZIP        GGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGACGGCGACATC
                ************************************************************

MM54R_UTR       TTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTGGCATAGTGCACATC
T.a._ZIP        TTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTGGCATGGTGCACATC
                ************************************************** *********

MM54R_UTR       CTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTACAAAGGTGGCGGGGACAGG
T.a._ZIP        CTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTACAAAGGTGGCGGGGACAGG
                ************************************************************

MM54R_UTR       AACGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCTGCAGCCATGGCCACAATGGTGATA
T.a._ZIP        AACGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCTGCAGCCATGGCCACAATGGTGATA
                ************************************************************

MM54R_UTR       GACTCGCTGGCTGCTGGGTACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGAC
T.a._ZIP        GACTCGCTGGCTGCTGGGTACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGAC
                ************************************************************

MM54R_UTR       AACATCGACATACCCGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCAT
T.a._ZIP        AACATCGACATACCCGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCAT
                ************************************************************

MM54R_UTR       GGCCATTCACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGAC
T.a._ZIP        GGCCATTCACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGAC
                ************************************************************

MM54R_UTR       ACAATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTG
T.a._ZIP        ACAATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTG
                ************************************************************

MM54R_UTR       ATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGT
T.a._ZIP        ATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGT
                ************************************************************

MM54R_UTR       GCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCT
T.a._ZIP        GCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCT
                ************************************************************

MM54R_UTR       AATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCTCCCTGACCGCACCCGTG
T.a._ZIP        AATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCTCCCTGACCGCACCCGTG
                ************************************************************

MM54R_UTR       GGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAATGTGCATAGCTCTACTGCCTTC
T.a._ZIP        GGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAATGTGCATAGCTCTACTGCCTTC
                ************************************************************

MM54R_UTR       ATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAGGGATTTTAATCTACATGTCCCTGGTG
T.a._ZIP        ATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAGGGATTTTAATCTACATGTCCCTGGTG
                ************************************************************

MM54R_UTR       GACCTTCTAGCAAAAGATTTCAATAACCCAAAGCTACAGACAAATACAAAGCTTCAGCTG
T.a._ZIP        GACCTTCTAGCAAAAGATTTCAATAACCCAAAGCTACAGACAAATACAAAGCTTCAGCTG
                ************************************************************

MM54R_UTR       ATGACATATCTTGCACTTTTCCTAGGTGCAGGGATGATGTCCATGCTTGCCATATGGGCA
T.a._ZIP        ATGACATATCTTGCACTTTTCCTAGGTGCAGGGATGATGTCCATGCTTGCCATATGGGCA
                ************************************************************

MM54R_UTR       TAG
T.a._ZIP        TAG
                ***
```

Figure F.1.2: Nucleotide alignment of *T. aestivum* ZIP1 with MM5/4 ZIP root sequence

```
T.a._ZIP        MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
MM54R_UTR       MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
                ************************************************************

T.a._ZIP        GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
MM54R_UTR       GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGIVHILPAAFDGLTSPCIYKGGGDR
                ************************************:***********************

T.a._ZIP        NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
MM54R_UTR       NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
                ************************************************************

T.a._ZIP        GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
MM54R_UTR       GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
                ************************************************************

T.a._ZIP        ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
MM54R_UTR       ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
                ************************************************************

T.a._ZIP        IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
MM54R_UTR       IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
                ************************************************************
```

3) UTR-ZIP Sequence Amplified from 24-39 Root Sample

ATGGGCGCCACCAATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGT
GCACCAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGG
GGCGGACAAGCAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCT
CACCGCCGGGGCGGCTGGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCG
CTGCGCCCCGACGGCGACATCTTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCA
TCCTTGCCACTGGCATGGTGCACATCCTGCCGGCGGCGTTTGACGGGCTCACCTC
CCCGTGCATCTACAAAGGTGGCGGGGACAGGAACGGCTTCCCTTTTGCGGGACTT
GTGGCCATGTCTGCAGCCATGGCCACAATGGTGATAGACTC**A**CTGGCTGCTGGGT
ACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGACAACATCGACATACC
CGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCATGGCCATTC
ACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGACACA
ATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAG
TGATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCT
GGTCGGTGCCCTCAGCTT**T**CATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGC
ATTGTACAGGCTAATTTC.....TAGCAACAGATTTCAATAACCCAAAGCTAC
AGACAAATACAAAGCTTCAGCTGATGACATATCTTGCACTTTTCCTAGGTGCAGG
GATGATGTCCATGCTTGCCATATGGGCATAG

Figure F.2.1: 902 bp-long ZIP sequence obtained from UTR-ZIP PCR reaction using 24-39 root sample as template

```
2439R_UTR       ATGGGCGCCACCAATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCAC
T.a._ZIP        ATGGGCGCCACCAATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCAC
                ************************************************************

2439R_UTR       CAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACAAG
T.a._ZIP        CAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACAAG
                ************************************************************

2439R_UTR       CAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGCGGCT
T.a._ZIP        CAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGCGGCT
                ************************************************************

2439R_UTR       GGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGACGGCGACATC
T.a._ZIP        GGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGACGGCGACATC
                ************************************************************

2439R_UTR       TTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTGGCATGGTGCACATC
T.a._ZIP        TTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTGGCATGGTGCACATC
                ************************************************************

2439R_UTR       CTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTACAAAGGTGGCGGGGACAGG
T.a._ZIP        CTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTACAAAGGTGGCGGGGACAGG
                ************************************************************

2439R_UTR       AACGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCTGCAGCCATGGCCACAATGGTGATA
T.a._ZIP        AACGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCTGCAGCCATGGCCACAATGGTGATA
                ************************************************************

2439R_UTR       GACTCACTGGCTGCTGGGTACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGAC
T.a._ZIP        GACTCGCTGGCTGCTGGGTACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGAC
                ***** ********************************************************

2439R_UTR       AACATCGACATACCCGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCAT
T.a._ZIP        AACATCGACATACCCGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCAT
                ************************************************************

2439R_UTR       GGCCATTCACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGAC
T.a._ZIP        GGCCATTCACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGAC
                ************************************************************

2439R_UTR       ACAATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTG
T.a._ZIP        ACAATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTG
                ************************************************************

2439R_UTR       ATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGT
T.a._ZIP        ATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGT
                ************************************************************

2439R_UTR       GCCCTCAGCTTTCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCT
T.a._ZIP        GCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCT
                *********** ************************************************

2439R_UTR       AATTTC------------------------------------------------------
T.a._ZIP        AATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCTCCCTGACCGCACCCGTG
                ******

2439R_UTR       ------------------------------------------------------------
T.a._ZIP        GGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAATGTGCATAGCTCTACTGCCTTC


2439R_UTR       ------------------------------------------------------------
T.a._ZIP        ATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAGGGATTTTAATCTACATGTCCCTGGTG


2439R_UTR       ------------------------------------------------------------
T.a._ZIP        GACCTTCTAGCAAAAGATTTCAATAACCCAAAGCTACAGACAAATACAAAGCTTCAGCTG

2439R_UTR       ------------------------------------------------------------
T.a._ZIP        ATGACATATCTTGCACTTTTCCTAGGTGCAGGGATGATGTCCATGCTTGCCATATGGGCA


2439R_UTR       ---
T.a._ZIP        TAG
```

Figure F.2.2: Nucleotide alignment of *T.aestivum* ZIP1 with 24-39 root ZIP sequence

```
T.a._ZIP        MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
2439R_UTR       MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
                ************************************************************

T.a._ZIP        GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
2439R_UTR       GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
                ************************************************************

T.a._ZIP        NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
2439R_UTR       NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
                ************************************************************

T.a._ZIP        GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
2439R_UTR       GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
                ************************************************************

T.a._ZIP        ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
2439R_UTR       ALSFHQFFEGIGLGGCIVQANF--------------------------------------
                **********************

T.a._ZIP        IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
2439R_UTR       ------------------------------------------------------------
```

4) UTR-ZIP Sequence Amplified from 33-48 Root Sample

ATGGGCGCCACCAATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGT
GCACCAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGG
GGCGGACAAGCAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCT
CACCGCCGGGGCGGCTGGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCG
CTGCGCCCCGACGGCGACATCTTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCA
TCCTTGCCACTGGCATGGTGCACATCCTGCCGGCGGCGTTTGACGGGCTCACCTC
CCCGTGCATCTACAAAGGTGGCGGGGACAGGAACGGCTTCCCTTTTGCGGGACTT
GTGGCCATGTCTGCAGCCATGGCCACAATGGTGATAGACTCGCTGGCTGCTGGGT
ACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGACAACATCGACATACC
CGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCATGGCCATTC
ACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGACACA
ATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAG
TGATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCT
GGTCGGTGCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGC
ATTGTACAGGCTAATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCT
CCCTGACCGCACCCGTGGGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAA
TGTGCATAGCTCTACTGCCTTCATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAG
GGATTTTAATCTACATGTCCCTGGTGGACCTTCTAGCAAAAGATTTCAATAACCC
AAAGCTACAGACAAATACAAAGCTTCAGCTGATGACATATCTTGCACTTTTCCTA
GGTGCAGGGATGATGTCCATGCTTGCCATATGGGCATAG

Figure F.3.1: Full length ZIP sequence obtained from 33-48 root sample

Figure F.3.2: Nucleotide alignment of *T.aestivum* ZIP1 with 33-48 root ZIP sequence

```
T.a._ZIP        MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
3348R_UTR       MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
                ************************************************************

T.a._ZIP        GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
3348R_UTR       GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
                ************************************************************

T.a._ZIP        NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
3348R_UTR       NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
                ************************************************************

T.a._ZIP        GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
3348R_UTR       GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
                ************************************************************

T.a._ZIP        ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
3348R_UTR       ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
                ************************************************************

T.a._ZIP        IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
3348R_UTR       IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
                ************************************************************
```

5) UTR-ZIP Sequence Amplified from 19-36 Root Sample

ATGGGCGCCACCAATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGT
GCACCAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGG
GGCGGACAAGCAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCT
CACCGCCGGGGCGGCTGGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCG
CTGCGCCCCGACGGCGACATCTTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCA
TCCTTGCCACTGGCATGGTGCACATCCTGCCGGCGGCGTTTGACGGGCTCACCTC
CCCGTGCATCTACAAAGGTGGCGGGGACAGGAA**T**GGCTTCCCTTTTGCGGGACTT
GTGGCCATGTCTGCAGCCATGGCCACAATGGTGATAGACTCGCTGGCTGCTGGGT
ACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGACAACATCGACATACC
CGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCATGGCCATTC
ACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGACACA
ATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAG
TGATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCT
GGTCGGTGCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGC
ATTGTACAGGCTAATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCT
CCCTGACCGCACCCGTGGGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAA
TGTGCATAGCTCTACTGCCTTCATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAG
GGATTTTAATCTACATGTCCCTGGTGGACCTTCTAGCAAAAGATTTCAATAACCC
AAAGCTACAGACAAATACAAAGCTTCAGCTGATGACATATCTTGCACTTTTCCTA
GGTGCAGGGATGATGTCCATGCTTGCCATATGGGCATAG

Figure F.4.1: Full length ZIP sequence obtained from 19-36 root sample

```
1936R_UTR       ATGGGCGCCACCAATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCAC
T.a._ZIP        ATGGGCGCCACCAATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCAC
                ************************************************************

1936R_UTR       CAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACAAG
T.a._ZIP        CAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACAAG
                ************************************************************

1936R_UTR       CAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGCGGCT
T.a._ZIP        CAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGCGGCT
                ************************************************************

1936R_UTR       GGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGACGGCGACATC
T.a._ZIP        GGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGACGGCGACATC
                ************************************************************

1936R_UTR       TTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTGGCATGGTGCACATC
T.a._ZIP        TTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTGGCATGGTGCACATC
                ************************************************************

1936R_UTR       CTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTACAAAGGTGGCGGGGACAGG
T.a._ZIP        CTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTACAAAGGTGGCGGGGACAGG
                ************************************************************

1936R_UTR       AATGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCTGCAGCCATGGCCACAATGGTGATA
T.a._ZIP        AACGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCTGCAGCCATGGCCACAATGGTGATA
                ** ********************************************************

1936R_UTR       GACTCGCTGGCTGCTGGGTACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGAC
T.a._ZIP        GACTCGCTGGCTGCTGGGTACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGAC
                ************************************************************

1936R_UTR       AACATCGACATACCCGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCAT
T.a._ZIP        AACATCGACATACCCGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCAT
                ************************************************************

1936R_UTR       GGCCATTCACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGAC
T.a._ZIP        GGCCATTCACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGAC
                ************************************************************

1936R_UTR       ACAATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTG
T.a._ZIP        ACAATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTG
                ************************************************************

1936R_UTR       ATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGT
T.a._ZIP        ATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGT
                ************************************************************

1936R_UTR       GCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCT
T.a._ZIP        GCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCT
                ************************************************************

1936R_UTR       AATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCTCCCTGACCGCACCCGTG
T.a._ZIP        AATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCTCCCTGACCGCACCCGTG
                ************************************************************

1936R_UTR       GGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAATGTGCATAGCTCTACTGCCTTC
T.a._ZIP        GGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAATGTGCATAGCTCTACTGCCTTC
                ************************************************************

1936R_UTR       ATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAGGGATTTTAATCTACATGTCCCTGGTG
T.a._ZIP        ATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAGGGATTTTAATCTACATGTCCCTGGTG
                ************************************************************

1936R_UTR       GACCTTCTAGCAAAAGATTTCAATAACCCAAAGCTACAGACAAATACAAAGCTTCAGCTG
T.a._ZIP        GACCTTCTAGCAAAAGATTTCAATAACCCAAAGCTACAGACAAATACAAAGCTTCAGCTG
                ************************************************************

1936R_UTR       ATGACATATCTTGCACTTTTCCTAGGTGCAGGGATGATGTCCATGCTTGCCATATGGGCA
T.a._ZIP        ATGACATATCTTGCACTTTTCCTAGGTGCAGGGATGATGTCCATGCTTGCCATATGGGCA
                ************************************************************

1936R_UTR       TAG
T.a._ZIP        TAG
                ***
```

Figure F.4.2: Nucleotide alignment of *T.aestivum* ZIP1 with 19-36 root ZIP sequence

```
T.a._ZIP        MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
1936R_UTR       MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
                ************************************************************

T.a._ZIP        GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
1936R_UTR       GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
                ************************************************************

T.a._ZIP        NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
1936R_UTR       NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
                ************************************************************

T.a._ZIP        GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
1936R_UTR       GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
                ************************************************************

T.a._ZIP        ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
1936R_UTR       ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
                ************************************************************

T.a._ZIP        IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
1936R_UTR       IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
                ************************************************************
```

6) UTR-ZIP Sequence Amplified from Balcali 85 Root Sample

ATGGGCGCCACCAATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGT
GCACCAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGG
GGCGGACAAGCAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCT
CACCGCCGGGGCGGCTGGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCG
CTGCGCCCCGACGGCGACATCTTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCA
TCCTTGCCACTGGCATGGTGCACATCCTGCCGGCGGCGTTTGACGGGCTCACCTC
CCCGTGCATCTACAAAGGTGGCGGGGACAGGAACGGCTTCCCTTTTGCGGGACTT
GTGGCCATGTCTGCAGCCATGGCCACAATGGTGATAGACTCGCTGGCTGCTGGGT
ACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGACAACATCGACATACC
CGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCATGGCC**G**TTC
ACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGACACA
ATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAG
TGATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCT
GGTCGGTGCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGC
ATTGTACAGGCTAATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCT
CCCTGACCGCACCCGTGGGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAA
TGTGCATAGCTCTACTGCCTTCATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAG
GGATTTTAATCTACATGTCCCTGGTGGACCTTCTAGCAAAAGATTTCAATAACCC
AAAGCTACAGACAAATACAAAGCTTCAGCTGATGACATATCTTGCACTTTTCCTA
GGTGCAGGGATGATGTCCATGCTTGCCATATGGGCATAG

Figure F.5.1: Full length ZIP sequence obtained from Balcali 85 root sample

```
BalR_UTR      ATGGGCGCCACCAATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCAC
T.a._ZIP      ATGGGCGCCACCAATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCAC
              ************************************************************

BalR_UTR      CAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACAAG
T.a._ZIP      CAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACAAG
              ************************************************************

BalR_UTR      CAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGCGGCT
T.a._ZIP      CAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGCGGCT
              ************************************************************

BalR_UTR      GGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGACGGCGACATC
T.a._ZIP      GGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGACGGCGACATC
              ************************************************************

BalR_UTR      TTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTGGCATGGTGCACATC
T.a._ZIP      TTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTGGCATGGTGCACATC
              ************************************************************

BalR_UTR      CTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTACAAAGGTGGCGGGGACAGG
T.a._ZIP      CTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTACAAAGGTGGCGGGGACAGG
              ************************************************************

BalR_UTR      AACGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCTGCAGCCATGGCCACAATGGTGATA
T.a._ZIP      AACGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCTGCAGCCATGGCCACAATGGTGATA
              ************************************************************

BalR_UTR      GACTCGCTGGCTGCTGGGTACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGAC
T.a._ZIP      GACTCGCTGGCTGCTGGGTACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGAC
              ************************************************************

BalR_UTR      AACATCGACATACCCGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCAT
T.a._ZIP      AACATCGACATACCCGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCAT
              ************************************************************

BalR_UTR      GGCCGTTCACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGAC
T.a._ZIP      GGCCATTCACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGAC
              **** ********************************************************

BalR_UTR      ACAATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTG
T.a._ZIP      ACAATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTG
              ************************************************************

BalR_UTR      ATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGT
T.a._ZIP      ATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGT
              ************************************************************

BalR_UTR      GCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCT
T.a._ZIP      GCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCT
              ************************************************************

BalR_UTR      AATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCTCCCTGACCGCACCCGTG
T.a._ZIP      AATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCTCCCTGACCGCACCCGTG
              ************************************************************

BalR_UTR      GGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAATGTGCATAGCTCTACTGCCTTC
T.a._ZIP      GGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAATGTGCATAGCTCTACTGCCTTC
              ************************************************************

BalR_UTR      ATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAGGGATTTTAATCTACATGTCCCTGGTG
T.a._ZIP      ATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAGGGATTTTAATCTACATGTCCCTGGTG
              ************************************************************

BalR_UTR      GACCTTCTAGCAAAAGATTTCAATAACCCAAAGCTACAGACAAATACAAAGCTTCAGCTG
T.a._ZIP      GACCTTCTAGCAAAAGATTTCAATAACCCAAAGCTACAGACAAATACAAAGCTTCAGCTG
              ************************************************************

BalR_UTR      ATGACATATCTTGCACTTTTCCTAGGTGCAGGGATGATGTCCATGCTTGCCATATGGGCA
T.a._ZIP      ATGACATATCTTGCACTTTTCCTAGGTGCAGGGATGATGTCCATGCTTGCCATATGGGCA
              ************************************************************

BalR_UTR      TAG
T.a._ZIP      TAG
              ***
```

Figure F.5.2: Nucleotide alignment of *T.aestivum* ZIP1 with Balcali 85 root ZIP sequence

ATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCACCAGGCCG
CGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACA
AGCAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGC
CGGGGCGGCTGGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTG
CGCCCCGACGGCGACATCTTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCAT
CCTTGCCACTGGCATGGTGCACATCCTGCCGGCGGCGTTTGACGGGCTCACCT
CCCCGTGCATCTACAAAGGTGGCGGGGACAGGAACGGCTTCCCTTTTGCGGGA
CTTGTGGCCATGTCTGCAGCCATGGCCACAATGGTGATAGACTCGCTGGCTGC
TGGGTACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGACAACATCG
ACATACCCGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCAT
GGCCATTCACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCAT
AGCTGACACAATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTT
GGTGCATTCAGTGATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCAC
CATCAAGCCTCTGGTCGGTGCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGG
CTTGGGTGGTTGCATTGTACAGGCTAATTTCAAGGT

Figure F.5.3: P1-ZIP sequence obtained from Balcali 85 root sample

```
T.a._ZIP        ATGGGCGCCACCAATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCAC
Bal85R_P1       ------------ATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCAC
                            **********************************************

T.a._ZIP        CAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACAAG
Bal85R_P1       CAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACAAG
                ************************************************************

T.a._ZIP        CAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGCGGCT
Bal85R_P1       CAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGCGGCT
                ************************************************************

T.a._ZIP        GGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGACGGCGACATC
Bal85R_P1       GGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGACGGCGACATC
                ************************************************************

T.a._ZIP        TTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTGGCATGGTGCACATC
Bal85R_P1       TTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTGGCATGGTGCACATC
                ************************************************************

T.a._ZIP        CTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTACAAAGGTGGCGGGGACAGG
Bal85R_P1       CTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTACAAAGGTGGCGGGGACAGG
                ************************************************************

T.a._ZIP        AACGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCTGCAGCCATGGCCACAATGGTGATA
Bal85R_P1       AACGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCTGCAGCCATGGCCACAATGGTGATA
                ************************************************************

T.a._ZIP        GACTCGCTGGCTGCTGGGTACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGAC
Bal85R_P1       GACTCGCTGGCTGCTGGGTACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGAC
                ************************************************************

T.a._ZIP        AACATCGACATACCCGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCAT
Bal85R_P1       AACATCGACATACCCGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCAT
                ************************************************************

T.a._ZIP        GGCCATTCACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGAC
Bal85R_P1       GGCCATTCACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGAC
                ************************************************************

T.a._ZIP        ACAATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTG
Bal85R_P1       ACAATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTG
                ************************************************************

T.a._ZIP        ATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGT
Bal85R_P1       ATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGT
                ************************************************************

T.a._ZIP        GCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCT
Bal85R_P1       GCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCT
                ************************************************************

T.a._ZIP        AATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCTCCCTGACCGCACCCGTG
Bal85R_P1       AATTTCAAGGT-----------------------------------------------
                ***********

T.a._ZIP        GGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAATGTGCATAGCTCTACTGCCTTC
Bal85R_P1       ------------------------------------------------------------


T.a._ZIP        ATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAGGGATTTTAATCTACATGTCCCTGGTG
Bal85R_P1       ------------------------------------------------------------


T.a._ZIP        GACCTTCTAGCAAAAGATTTCAATAACCCAAAGCTACAGACAAATACAAAGCTTCAGCTG
Bal85R_P1       ------------------------------------------------------------


T.a._ZIP        ATGACATATCTTGCACTTTTCCTAGGTGCAGGGATGATGTCCATGCTTGCCATATGGGCA
Bal85R_P1       ------------------------------------------------------------


T.a._ZIP        TAG
Bal85R_P1       ---
```

Figure F.5.4: Nucleotide alignment of *T.aestivum* ZIP1 with Balcali 85 ZIP sequence obtained from P1-PCR

```
T.a._ZIP      MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
BalR_UTR      MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
              ************************************************************

T.a._ZIP      GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
BalR_UTR      GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
              ************************************************************

T.a._ZIP      NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
BalR_UTR      NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
              ************************************************************

T.a._ZIP      GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
BalR_UTR      GRSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
              *:************************************************************

T.a._ZIP      ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
BalR_UTR      ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
              ************************************************************

T.a._ZIP      IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
BalR_UTR      IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
              ************************************************************
```

7) P1-ZIP Sequence Amplified from MM 5/4 Shoot Sample

TACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCACCAGGCCGCGGCGG
CCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACAAGCAGGGCG
CGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGCGGCTGG
CGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGACGGCGAC
ATCTTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTGGCATGGT
GCACATCCTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTACAAAGGT
GGCGGGGACAGGAACGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCTGCAGCCA
TGGCCACAATGGTGATAGACTCGCTGGCTGCTGGGTACTACCGCCGGTCTCACTT
CAGCAAGGCACGCCCACTTGACAACATCGACATACCCGGAGATGAGGAAGGGAG
GGCCGATCATCCACATGTGCACGCGCATGGCCATTCACATGGTGACGCAATTGTT
GTCAGCTCACCGGAGGAGGCTGCCATAGCTGACACAATCCGGCACAGGGTGGTA
TCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTGATAATTGGTGTGTCATT
AGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGTGCCCTCAGCTTC
CATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCTAATTTCA
AGGTAAGGGCAACCATCATCATGGCAACGTTTTTCTCCCTGACCGCCCGTGGGCA
TCGTGCTAGGGATTGCGATATCGTCTAGCTATAATGTGCATAGCTCTACTGCCTTC
ATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAGGGATTTTAATCTACATGTCCCT
GGTGGACCTTCTAGCAAAAGATTTCAATAACCCAAAGCTACAGACAAATACAAA
GCTTCAGCTGATGACATATCTTGCACTTTTCCTAGGTGCAGGGATGATGTCCATGC
TTGCCA

Figure F.6.1: P1-ZIP sequence obtained from MM5/4 shoot sample

```
MM5/4S_P1       -----------------TACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCAC
T.a._ZIP        ATGGGCGCCACCAATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCAC
                                 ****************************************

MM5/4S_P1       CAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACAAG
T.a._ZIP        CAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACAAG
                ************************************************************

MM5/4S_P1       CAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGCGGCT
T.a._ZIP        CAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGCGGCT
                ************************************************************

MM5/4S_P1       GGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGACGGCGACATC
T.a._ZIP        GGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGACGGCGACATC
                ************************************************************

MM5/4S_P1       TTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTGGCATGGTGCACATC
T.a._ZIP        TTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTGGCATGGTGCACATC
                ************************************************************

MM5/4S_P1       CTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTACAAAGGTGGCGGGGACAGG
T.a._ZIP        CTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTACAAAGGTGGCGGGGACAGG
                ************************************************************

MM5/4S_P1       AACGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCTGCAGCCATGGCCACAATGGTGATA
T.a._ZIP        AACGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCTGCAGCCATGGCCACAATGGTGATA
                ************************************************************

MM5/4S_P1       GACTCGCTGGCTGCTGGGTACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGAC
T.a._ZIP        GACTCGCTGGCTGCTGGGTACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGAC
                ************************************************************

MM5/4S_P1       AACATCGACATACCCGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCAT
T.a._ZIP        AACATCGACATACCCGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCAT
                ************************************************************

MM5/4S_P1       GGCCATTCACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGAC
T.a._ZIP        GGCCATTCACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGAC
                ************************************************************

MM5/4S_P1       ACAATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTG
T.a._ZIP        ACAATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTG
                ************************************************************

MM5/4S_P1       ATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGT
T.a._ZIP        ATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGT
                ************************************************************

MM5/4S_P1       GCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCT
T.a._ZIP        GCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCT
                ************************************************************

MM5/4S_P1       AATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCTCCCTGACCGC--CCGTG
T.a._ZIP        AATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCTCCCTGACCGCACCCGTG
                ****************************************************  *****

MM5/4S_P1       GGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAATGTGCATAGCTCTACTGCCTTC
T.a._ZIP        GGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAATGTGCATAGCTCTACTGCCTTC
                ************************************************************

MM5/4S_P1       ATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAGGGATTTTAATCTACATGTCCCTGGTG
T.a._ZIP        ATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAGGGATTTTAATCTACATGTCCCTGGTG
                ************************************************************

MM5/4S_P1       GACCTTCTAGCAAAAGATTTCAATAACCCAAAGCTACAGACAAATACAAAGCTTCAGCTG
T.a._ZIP        GACCTTCTAGCAAAAGATTTCAATAACCCAAAGCTACAGACAAATACAAAGCTTCAGCTG
                ************************************************************

MM5/4S_P1       ATGACATATCTTGCACTTTTCCTAGGTGCAGGGATGATGTCCATGCTTGCCA--------
T.a._ZIP        ATGACATATCTTGCACTTTTCCTAGGTGCAGGGATGATGTCCATGCTTGCCATATGGGCA
                ************************************************

MM5/4S_P1       ---
T.a._ZIP        TAG
```

Figure F.6.2: Nucleotide alignment of *T.aestivum* ZIP1 with MM5/4 obtained from P1-PCR from shoots

```
T.a._ZIP      MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
MM54S_P1      ------TLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
                    ************************************************************

T.a._ZIP      GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
MM54S_P1      GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
              ************************************************************

T.a._ZIP      NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
MM54S_P1      NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
              ************************************************************

T.a._ZIP      GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
MM54S_P1      GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
              ************************************************************

T.a._ZIP      ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAP--VGIVLGIAISSSYNVHSST
MM54S_P1      ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTARGHRARDCDIVLCALYCLHYGS
              ************************************    .   .*.:.: * :* .:

T.a._ZIP      AFIIEG-VFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLA
MM54S_P1      LQLSLGRDFNLHVPG--------GPSSKRFQ-PKATDKYKASADDISCTFPRCRDDVHAC
                :  *  **   .*       .  :* *: **   : * .     . *  .      .

T.a._ZIP      IWA
MM54S_P1      ---
```

8) P1-ZIP Sequence Amplified from MM 5/2 Root Sample

```
T.a._ZIP      MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
MM52R_P1      -----HTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
                   ************************************************************

T.a._ZIP      GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
MM52R_P1      GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
              ************************************************************

T.a._ZIP      NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
MM52R_P1      NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
              ************************************************************

T.a._ZIP      GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
MM52R_P1      GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
              ************************************************************

T.a._ZIP      ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
MM52R_P1      ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
              ************************************************************

T.a._ZIP      IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
MM52R_P1      IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLA---
              ************************************************************
```

Figure F.7.1: P1-ZIP sequence obtained from MM5/2 root sample

```
MM5/2R_P1       -------------ATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCAC
T.a._ZIP        ATGGGCGCCACCAATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCAC
                             *********************************************

MM5/2R_P1       CAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACAAG
T.a._ZIP        CAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACAAG
                ************************************************************

MM5/2R_P1       CAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGCGGCT
T.a._ZIP        CAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGCGGCT
                ************************************************************

MM5/2R_P1       GGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGACGGCGACATC
T.a._ZIP        GGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGACGGCGACATC
                ************************************************************

MM5/2R_P1       TTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTGGCATGGTGCACATC
T.a._ZIP        TTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTGGCATGGTGCACATC
                ************************************************************

MM5/2R_P1       CTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTACAAAGGTGGCGGGGACAGG
T.a._ZIP        CTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTACAAAGGTGGCGGGGACAGG
                ************************************************************

MM5/2R_P1       AACGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCTGCAGCCATGGCCACAATGGTGATA
T.a._ZIP        AACGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCTGCAGCCATGGCCACAATGGTGATA
                ************************************************************

MM5/2R_P1       GACTCGCTGGCTGCTGGGTACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGAC
T.a._ZIP        GACTCGCTGGCTGCTGGGTACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGAC
                ************************************************************

MM5/2R_P1       AACATCGACATACCCGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCAT
T.a._ZIP        AACATCGACATACCCGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCAT
                ************************************************************

MM5/2R_P1       GGCCATTCACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGAC
T.a._ZIP        GGCCATTCACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGAC
                ************************************************************

MM5/2R_P1       ACAATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTG
T.a._ZIP        ACAATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTG
                ************************************************************

MM5/2R_P1       ATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGT
T.a._ZIP        ATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGT
                ************************************************************

MM5/2R_P1       GCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCT
T.a._ZIP        GCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCT
                ************************************************************

MM5/2R_P1       AATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCTCCCTGACCGCACCCGTG
T.a._ZIP        AATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCTCCCTGACCGCACCCGTG
                ************************************************************

MM5/2R_P1       GGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAATGTGCATAGCTCTACTGCCTTC
T.a._ZIP        GGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAATGTGCATAGCTCTACTGCCTTC
                ************************************************************

MM5/2R_P1       ATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAGGGATTTTAATCTACATGTCCCTGGTG
T.a._ZIP        ATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAGGGATTTTAATCTACATGTCCCTGGTG
                ************************************************************

MM5/2R_P1       GACCTTCTAGCAAAAGATTTCAATAACCCAAAGCTACAGACAAATACAAAGCTTCAGCTG
T.a._ZIP        GACCTTCTAGCAAAAGATTTCAATAACCCAAAGCTACAGACAAATACAAAGCTTCAGCTG
                ************************************************************

MM5/2R_P1       ATGACATATCTTGCACTTTTCCTAGGTGCAGGGATGATGTCCATGCTTGCCA--------
T.a._ZIP        ATGACATATCTTGCACTTTTCCTAGGTGCAGGGATGATGTCCATGCTTGCCATATGGGCA
                ************************************************

MM5/2R_P1       ---
T.a._ZIP        TAG
```

Figure F.7.2: Nucleotide alignment of *T.aestivum* ZIP1 with MM5/2 obtained from P1-PCR

9) P1-ZIP Sequence Amplified from C-1252 Root Sample

TATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCACCAGGCCGC
GGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACAAGCA
GGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGC
GGCTGGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGAC
GGCGACATCTTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTG
GCATGGTGCACATCCTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTA
CAAAGGTGGCGGGGACAGGAACGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCT
GCAGCCATGGCCACAATGGTGATAGACTCGCTGGCTGCTGGGTACTACCGCCGGT
CTCACTTCAGCAAGGCACGCCCACTTGACAACATCGACATACCCGGAGATGAGG
AAGGGAGGGCCGATCATCCACATGTGCACGCGCATGGCCATTCACATGGTGACG
CAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGACACAATCCGGCACAG
GGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTGATAATTGGT
GTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGTGCCC
TCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCT
AATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCTCCCTGACCGCAC
CCGTGGGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAATGTGCATAGCTC
TACTGCCTTCATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAGGGATTTTAATCT
ACATGTCCCTGGTGGACCTTCTAGCAAAAGATTTCAATAACCCAAAGCTACAGAC
AAATACAAAGCTTCAGCTGATGACATATCTTGCACTTTTCCTAGGTGCAGGGATG
ATGTCCATGCTTGCCAA

Figure F.8.1: P1-ZIP sequence obtained from C-1252 root sample

```
C1252R_P1       ------------TATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCAC
T.a._ZIP        ATGGGCGCCACCAATCATACCTTGCAGGCGCTTCTCCCATGGCTCCTCCTGTTTGTGCAC
                             *********************************************

C1252R_P1       CAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACAAG
T.a._ZIP        CAGGCCGCGGCGGCCAGCGGCGGGTTCGAGTGCACGACCGCCACGGACGGGGCGGACAAG
                ************************************************************

C1252R_P1       CAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGCGGCT
T.a._ZIP        CAGGGCGCGACGAAGCTGAAGCTGGTCGCCATCGCGTCCATCCTCACCGCCGGGGCGGCT
                ************************************************************

C1252R_P1       GGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGACGGCGACATC
T.a._ZIP        GGCGTGCTGGTGCCGGTGCTCGGACGCTCCATGGCCGCGCTGCGCCCCGACGGCGACATC
                ************************************************************

C1252R_P1       TTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTGGCATGGTGCACATC
T.a._ZIP        TTCTTCGCGGTCAAGGCGTTCGCCGCTGGCGTCATCCTTGCCACTGGCATGGTGCACATC
                ************************************************************

C1252R_P1       CTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTACAAAGGTGGCGGGGACAGG
T.a._ZIP        CTGCCGGCGGCGTTTGACGGGCTCACCTCCCCGTGCATCTACAAAGGTGGCGGGGACAGG
                ************************************************************

C1252R_P1       AACGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCTGCAGCCATGGCCACAATGGTGATA
T.a._ZIP        AACGGCTTCCCTTTTGCGGGACTTGTGGCCATGTCTGCAGCCATGGCCACAATGGTGATA
                ************************************************************

C1252R_P1       GACTCGCTGGCTGCTGGGTACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGAC
T.a._ZIP        GACTCGCTGGCTGCTGGGTACTACCGCCGGTCTCACTTCAGCAAGGCACGCCCACTTGAC
                ************************************************************

C1252R_P1       AACATCGACATACCCGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCAT
T.a._ZIP        AACATCGACATACCCGGAGATGAGGAAGGGAGGGCCGATCATCCACATGTGCACGCGCAT
                ************************************************************

C1252R_P1       GGCCATTCACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGAC
T.a._ZIP        GGCCATTCACATGGTGACGCAATTGTTGTCAGCTCACCGGAGGAGGCTGCCATAGCTGAC
                ************************************************************

C1252R_P1       ACAATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTG
T.a._ZIP        ACAATCCGGCACAGGGTGGTATCTCAGGTTCTAGAGCTGGGAATCTTGGTGCATTCAGTG
                ************************************************************

C1252R_P1       ATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGT
T.a._ZIP        ATAATTGGTGTGTCATTAGGAGCATCTGTGAGGCCATCCACCATCAAGCCTCTGGTCGGT
                ************************************************************

C1252R_P1       GCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCT
T.a._ZIP        GCCCTCAGCTTCCATCAATTCTTTGAAGGCATAGGCTTGGGTGGTTGCATTGTACAGGCT
                ************************************************************

C1252R_P1       AATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCTCCCTGACCGCACCCGTG
T.a._ZIP        AATTTCAAGGTAAGGGCAACCATCATCATGGCAACGTTTTTCTCCCTGACCGCACCCGTG
                ************************************************************

C1252R_P1       GGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAATGTGCATAGCTCTACTGCCTTC
T.a._ZIP        GGCATCGTGCTAGGGATTGCGATATCGTCTAGCTATAATGTGCATAGCTCTACTGCCTTC
                ************************************************************

C1252R_P1       ATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAGGGATTTTAATCTACATGTCCCTGGTG
T.a._ZIP        ATTATTGAGGGAGTCTTCAACTCAGCCTCGGCAGGGATTTTAATCTACATGTCCCTGGTG
                ************************************************************

C1252R_P1       GACCTTCTAGCAAAAGATTTCAATAACCCAAAGCTACAGACAAATACAAAGCTTCAGCTG
T.a._ZIP        GACCTTCTAGCAAAAGATTTCAATAACCCAAAGCTACAGACAAATACAAAGCTTCAGCTG
                ************************************************************

C1252R_P1       ATGACATATCTTGCACTTTTCCTAGGTGCAGGGATGATGTCCATGCTTGCCAA-------
T.a._ZIP        ATGACATATCTTGCACTTTTCCTAGGTGCAGGGATGATGTCCATGCTTGCCATATGGGCA
                ***************************************************

C1252R_P1       ---
T.a._ZIP        TAG
```

Figure F.8.2: Nucleotide alignment of *T.aestivum* ZIP1 with C-1252 obtained from P1-PCR

```
T.a._ZIP        MGATNHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
C1252R_P1       ----YHTLQALLPWLLLFVHQAAAASGGFECTTATDGADKQGATKLKLVAIASILTAGAA
                     ************************************************************

T.a._ZIP        GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
C1252R_P1       GVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILPAAFDGLTSPCIYKGGGDR
                ************************************************************

T.a._ZIP        NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
C1252R_P1       NGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLDNIDIPGDEEGRADHPHVHAH
                ************************************************************

T.a._ZIP        GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
C1252R_P1       GHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVHSVIIGVSLGASVRPSTIKPLVG
                ************************************************************

T.a._ZIP        ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
C1252R_P1       ALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSLTAPVGIVLGIAISSSYNVHSSTAF
                ************************************************************

T.a._ZIP        IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLAIWA
C1252R_P1       IIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQTNTKLQLMTYLALFLGAGMMSMLA---
                ************************************************************
```