# INVESTIGATION OF WHEAT GENES INVOLVED IN ZINC EFFICIENCY MECHANISM USING DIFFERENTIAL DISPLAY TECHNIQUE

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## ABSTRACT

### INVESTIGATION OF WHEAT GENES INVOLVED IN ZINC EFFICIENCY MECHANISM USING DIFFERENTIAL DISPLAY TECHNIQUE

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Zinc is a metal involved in structure of many enzymes, in the growth and differentiation of plants. Wheat is one of the most consumed cereals. Some wheat cultivars can't deal with zinc deficiency and this situation not only reduces grain yield but also weakens the resistance of cereals to diseases and impairs the

nutritional quality of the grain. Some wheat cultivars are not affected by zinc deficiency.

In this study, 'differential display', used for determination differentially expressed genes between two samples, was performed. The most zinc efficient bread wheat cultivar Kıraç-66 was grown in hydroponics medium and samples were taken at different time periods. RNA isolations were done and differential display technique was performed. After examining the results, differentially expressed bands were selected and sequenced. DNA sequence analysis were done in available databases which showed that three of the bands were fragments of putative zinc transporters.

In this study we have found threee putative gene fragments using differential display technique on zinc efficient plants grown under differeing zinc concentrations. These fragments showed homology with zinc transporter, ABC transporter and ADH (Alcohol Dehydrogenase). It is known that all of these three genes are involved in zinc efficiency mechanism. Further studies will be conducted on these gene fragments.

**Key words:** Zinc efficieny, Zinc deficiency, Differential Display, Bread wheat, Hydroponics, ABC transporter, Zinc transporter, Alcohol Dehydrogenase

# ÖZ

## ÇİNKO EKSİKLİĞİNE DAYANIKLILIK MEKANİZMASINDA ROL ALAN BUĞDAY GENLERİNİN FARKLILIK GÖSTERİMİ YÖSTERİMİ YÖNTEMİ İLE ARAŞTIRILMASI

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Çinko birçok enzimin yapısına katılan, bitkilerin büyümesinde ve farklılaşmasında rol alan bir metaldir. Buğday dünyada en çok tüketilen tahıl ürünlerinden birisidir. Bazı buğday türleri çinko eksikliğine karşı direnç gösterememektedir ve bu durum ürün veriminde kayıplara yol açmakla birlikte

bitkilerde hastalık dirençliliğinin azalmasına ve ürün kalitesinin düşmesine de yol açmaktadır. Bazı buğday türleri ise çinko eksikliğinden etkilenmemektedir.

Bu çalışmada iki farklı örnek arasındaki farklı düzeyde ifade olmuş genlerin tanımlanmasında kullanılan 'farklılık gösterimi' yöntemi kullanıldı. Çinko eksikliğine karşı en dirençli olan Kıraç-66 ekmeklik buğday türü hidroponik besi ortamında farklı çinko konsantrasyonlarında büyütüldü ve farklı zaman aralıklarında bitkilerden örnekler alındı. Bitkilerden RNA izolasyonları yapıldı ve farklılık gösterimi yöntemi uygulandı. Sonuçlar incelendikten sonra farklı ifade olan bantlar seçildi ve sekans analizi yapıldı. DNA sekans analizleri sonucunda seçilen üç bantın mevcut veritabanlarında çinko alımında görevli muhtemel genlere ait parçalar oldukları tespit edildi.

Bu çalışmada farklı çinko konsantrasyonlarında büyütülen çinko eksikliğine dayanıklı bitkiler üzerine farklılık gösterimi yöntemi uygulanarak üç muhtemel gen parçası bulundu. Bu parçalar çinko taşıyıcısı, ABC taşıyıcı ve ADH (alkol dehidrogenaz) genleri ile benzerlik gösterdi. Bu üç genin çinko eksikliğine dayanıklılık mekanizmasında görev aldıkları biliniyor. Bu genler üzerine ileri çalışmalar uygulanacak.

**Anahtar kelimeler:** Çinko eksikliği, Çinko dayanıklılığı, Farklılık gösterimi, Ekmeklik buğday, Hidroponik, ABC taşıyıcı, Çinko taşıyıcısı, Alkol Dehidrogenaz

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| | |
|---|---|
| [$\gamma$ $^{33}$P]-dATP | : [ $\gamma$ $^{33}$P ]-deoxyadenosine triphosphate |
| µg | : Microgram |
| µl | : Microliter |
| bp | : base pair |
| ds | : double stranded |
| kb | : kilobase |
| min | : minute |
| mM | : Milimolar |
| ng | : Nanogram |
| PAGE | : Polyacrylamide Gel Electrophoresis |
| PCR | : Polymerase Chain Reaction |
| pmol | : Pico mole |
| mRNA | : Messenger RNA |
| rDNA | : Ribosomal DNA |
| cDNA | : Complementer DNA |
| Rpm | : Rotation per minute |
| TEMED | : N, N, N, N'-Tetramethylethylene diamine |
| UV | : Ultra Violet |

| | |
|---|---|
| v/v | : volume/volume |
| w/v | : weight/volume |

# CHAPTER I

# INTRODUCTION

## 1.1. Wheat

Wheat belongs to the tribe *Triticeae* comprising some 300 species classified into 22 genera including several other important crops (barley, rye, and triticale) and a number of important forage species (Löve, 1984).

The proportion of wheat, maize and rice consumed in diets of people in developing countries comprises about 90% of the average diet (Graham and Welch, 1994). Wheat consumption in developing countries has been growing at a rate of 4.8% over the last three decades (1961-90). Growth of wheat will slow to around 2.1% from 1990 to 2005, but consumption of wheat in developing countries will grow 3.0% per year, in the meantime, it reaches 1.0% per year in industrialized countries. As a result, developing countries will probably consume 3/5 of the

world's wheat by 2005. (CIMMYT Economics Program database).

(http://www.agnic.org/pmp/1997/whf9707.html)

Turkey is one of the major wheat producing countries in the world. In the 1992/1993 period, Turkey produced about 20 million tons of wheat.

## 1.2. Zinc

### 1.2.1. Zinc properties

Zinc ($Zn^{2+}$) is an essential catalytic component of over 300 enzymes, including alkaline phosphatase, alcohol dehydrogenase, Cu-Zn superoxide dismutase, and carbonic anhydrase. Zinc also plays a critical structural role in many proteins. For example, several motifs found in transcriptional regulatory proteins are stabilized by Zn, including the Zn finger, Zn cluster, and RING finger domains. Proteins containing these domains are very common; the yeast genome sequencing project has revealed that almost 2% of all yeast gene products contain these types of Zn binding domains.

Zinc is essential for gene expression and nucleic acid metabolism. It also means that it has important roles in plant growth and differentiation. Many of enzymes' catalytic site uses its ligand binding feature.

In xylem, zinc presents as both free divalent form and bound to organic acids. Zinc may be also present as an organic ligand in both the soil solution and within the plant tissues.

Zinc is one of the essential micronutrients. However, it is toxic at over optimal conditions. Therefore, its concentration must be balanced carefully. Two main proteins are responsible for metal ion storage and detoxification: ferritins and metallothioneins. The ferritins are a class of ubiquitous multimeric iron storage proteins, able to sequester several thousands of iron atoms per molecule. In plants, these proteins are located in plastids and coded by a small gene family**.** Their expression is regulated and can be activated by various environmental signals. One of these environmental signals is excess iron. Although it is not documented in plants, animal ferritins are able to accumulate metals other than iron within their metal cores, especiellay Cu, Zn, Cd, Pb, Be and Al.

Metallothioneins are small proteins. They have been identified in various organisms. They sequester excess amount of certain metal ions, most commonly $Zn^{2+}$, $Cu^{+}$, $Cd^{2+}$, by co-ordination of these metals with cysteine residues organized as CysXCyc or CysXXCys repeats.

Zinc efficiency is defined as the ability of a species or cultivar to grow and yield better on a Zn deficient soil than other species or cultivars. Zinc deficient plants don't have this ability.

### 1.2.2. The effects of zinc on plants

Zinc deficiency is probably the most widespread micronutrient deficiency in cereals, especially in wheat, limiting grain yield, for example in Australia, India and Turkey. In a global study initiated by FAO and carried out in 30 countries, it was estimated that about 30% of the agricultural soils of the world are Zn deficient (Cakmak et al., 1996). Approximately 40% of the world's population suffer from micronutrient deficiencies including zinc deficiency (Graham and Welch, 1994).

The normal range of Zn in most plants is between 20 to 100 ppm. Under the level of 15 ppm, zinc deficiency symptoms take place.

Zn deficiency has become a serious agricultural problem. It is associated with high pH calcareous soils and sandy, highly leached soils, which cause reduced Zn availability and low total Zn content, respectively (Swietlik, 1989). Also nearly 45% (4.5 million ha) of the wheat producing area in Turkey is located on highly calcerous soils in Central Anatolia (Cakmak et. al, 1996). Severe soil erosion can

also cause a loss of organic matter accentuating zinc deficiencies. Conditions that tend to promote zinc deficiencies in plants include: loss of soil organic matter, soils leveled for irrigation, severe soil erosion, high pH soils (> 7.5), excessive phosphorus fertilizer applications, cool, wet weather.

Based on the analysis of 298 soil samples, Turkish soils were also classified as Zn deficient (About 14 Mha of cropped land in Turkey are known to be Zn deficient, particularly cereal growing areas of Anatolia) (Cakmak et al., 1999).

The effects of zinc deficiency are well documented in humans and may be severe, ranging from impaired neuropsychological functions, growth retardation and stunting, impaired reproduction, immune disorders, dermatitis, impaired wound healing, lethargy, loss of appetite, loss of hair. In addition to slowing growth and development, zinc deficiency can impair reproduction, immunity, the senses of taste and vision, and cognition. Diarrhea is a significant cause of retarded growth and death in the developing world.

In plants, zinc deficiency symptoms appear first on the younger leaves, because zinc is not translocated from older to younger plant tissue. In broadleaf crops, zinc deficiency results in shortening of internodes (rosetting) and a decrease in leaf size. When a severe zinc deficiency occurs, finally the older leaves may turn gray or brown and die. Zinc deficiency decreases crop yield.

Zinc is essential for maintaining membrane structure and functions. In zinc deficient plants leakage of solutes like potassium, sugars and amino acids from roots is increased. These effects of zinc deficiency result in excessive generation of toxic oxygen radicals. In zinc deficient tissues the detoxification of oxygen species doesn't work properly (Cakmak and Marschner, 1988b) and it causes breakdown of membrane phospholipids (Cakmak and Marschner, 1988a). Zinc plays a crucial role in the signal transduction pathway which controls the genes encoding high affinity phosphorus transporters in plant roots and zinc deficient plants lose control over the phosphate uptake mechanism and it causes phosphorus toxicity (Langridge, Huang, Smith, Barker and Graham, 2000).

There is also another factor causing zinc deficiency: phytic acid. It is storage form of phosphorus in seeds. Phytic acid severely limits zinc and iron bioavailability and it causes micronutrient deficiency.

In the presence of high zinc concentrations, zinc sensitive grasses have been shown to develop phosphorus (P) deficiency symptoms although P tissue concentrations appeared normal (Plenderleith, 1984). Similarly, across a wide variety of crop species grown under excess zinc, symptoms were similar to those for P deficiency but P tissue concentrations did not decrease (Boawn and Rasmussen, 1971). These findings suggest that zinc toxicity may result in P deficiency caused not by effects on P uptake but by effects on P metabolism.

Under zinc deficient conditions, shoot dry matter production decreases after 14 days and more after 20 days (Cakmak et al., 1996).

### 1.2.3. Zinc efficiency in wheat

Plant species in general and genotypes of wheat in particular greatly differ in their ability to acquire Zn from soils or to utilize Zn internally. The comparision of zinc efficiency within cereal species could be shown in this order: rye>triticale>barley>bread wheat>oat>durum wheat (Cakmak et al., 1998; Ekiz et al., 1998). Also bread wheat and durum wheat show differences in their efficiencies. Kirac-66 is the most efficient cultivar among bread wheats. BDMM-19 is the most efficient cultivar among durum wheats. (Marschner et al., 1993) Zinc deficiency symptoms become severe in durum wheats, but in bread wheat symptoms appear moderately (Cakmak et al., 1996; Marschner et al., 1996). Zinc deficiency symptoms on wheat leaves was shown in figure below.

**Figure 1.1.** Appearance of zinc deficiency symptoms on wheat leaves

### 1.3. Correcting micronutrient deficiency

The three traditional methods of correcting micronutrient deficiencies in the field are by 1: soil application at or before planting, 2: foliar application post-emergence, and 3: application of the micronutrient to the seed at the time of planting. Zinc deficiencies are most commonly corrected by application of the zinc fertilizer to the soil. While foliar applications of zinc are often beneficial, soil applications remain effective much longer. It was found that foliar application on tomato corrected zinc deficiency and increased plant growth and green fruit biomass when it was applied at 0.35 mM zinc concentration (Kaya et al., 2002). Also Erenoglu et al., 2002 found that, because after foliar zinc application, zinc was easily translocated from old leaves to young leaves and roots, zinc transport is more mobile in pholem, while zinc don't very mobile in xylem. Also it was showed that shoot zinc concentration was 2 fold greater with foliar application of zinc than with soil zinc application (Haslett et al., 2001). Seed applications of zinc, although successful on some plants, have not been shown to produce a yield response on crops.

## 1.4. Zinc uptake mechanism in plants

### 1.4.1. Zinc transporters

Zinc is taken up as a divalent cation ($Zn^{2+}$). It is also taken up as $ZnOH^{+}$ at high pH. Inside cells, zinc is neither oxidized nor reduced; thus, the essential role of zinc in cells is based largely on its behavior as a divalent cation that has a strong tendency to form stable tetrahedral complexes.

Studies of zinc uptake in plants mainly have been focused on hyper accumulators, i.e., plants that can grow in soils containing high levels of zinc and accumulate high concentrations of zinc in their shoots. Certain populations of *Thlaspi caerulescens* can tolerate up to 40,000 μg of Zn/g tissue in their shoots whereas the normal zinc concentration for most plants is between 20 and 100 μg of Zn/g tissue (Marschner 1995).

It has been thought that there are two major factors that control the zinc concentration in plant tissue. The first one is the membrane potential which allows high cation accumulation, second one is the low solubility of zinc phosphates at the pH of the cytoplasm (Cogliatti, 1998).

Two families of transporters, the ZIP (Zrt-, Irt-like Protein) and CDF (Cation Diffusion Facilitator) families, have been found to play a number of important roles in zinc transport. The characterized members of each group have been implicated in the transport of metal ions, frequently zinc, across lipid bilayer membranes. This remarkable conservation of function suggests that other, as yet uncharacterized members of the family, will also be involved in metal ion transport. Many of the ZIP family transporters are involved in cellular zinc uptake and at least one member, the Zrt3 transporter of *S. cerevisiae*, transports stored zinc out of an intracellular compartment during adaptation to zinc deficiency (Gaither and Eide, 2001)

The ZIP proteins (first identified in Arabidopsis) contain eight potential transmembrane domains, numbered I-VIII. *ZIP 1*, *ZIP 2* and *ZIP 3* each have a potential signal sequence and are predicted to be plasma membrane proteins; *ZIP 4* contains a potential chloroplast targeting sequence. A potential metal binding motif, containing multiple histidine residues, is found in the variable regions of almost all of the fully sequenced members of this family including *IRT 1*, *IRT 2*, *ZIP 1*, *ZIP 4*, *ZRT 1* and *ZRT 2*. In contrast, *ZIP 2* and *ZIP 3* contain only a single histidine in this variable region. Studies of *ZRT 1* suggest that the histidine-rich motif is located on the intracellular face of the plasma membrane and also indicate that it is essential for transporter function. Three of the transmembrane domains (II, IV and V) contain a histidine residue that is fully conserved among all family members. These histidines are predicted to lie on the polar face of amphipathic helices, suggesting a possible role for these residues in substrate transport through the membrane. Furthermore,

conserved acidic amino acids present in the transmembrane domains of several of the ZIP proteins also may be important for substrate movement. The ZIP genes of Arabidopsis represent four members of a rapidly growing family of eukaryotic proteins. To date, six of the family members have been implicated in metal ion transport. These results demonstrate that the ZIP transporter family plays roles in metal ion metabolism in a diverse array of eukaryotic organisms. The *ZIP* family is structurally distinct from other metal ion transporters such as the *CDF* family, which includes the recently identified mammalian zinc effluxers P-type ATPases.

In contrast, CDF family members mediate zinc efflux out of cells or facilitate zinc transport into intracellular compartments for detoxification and/or storage. The activity of many of these transporters is regulated in response to zinc through transcriptional and post-transcriptional mechanisms to maintain zinc homeostasis at both the cellular and organismal levels. The ZAT1 gene, encoding a protein with similarity to Znt1 identified in Arabidopsis and it is a member of CDF transporter family. When ZAT1 is overexpressed, Zn accumulates only in roots, but not in shoots and it is suggested that ZAT1 might be involved in vacuolar sequestration of Zn (Zaal,1999).

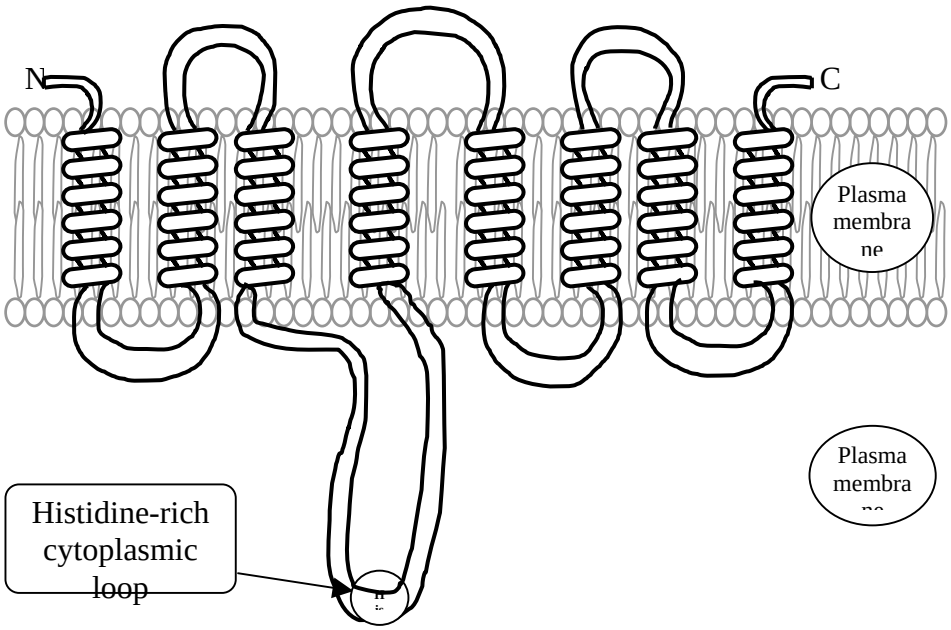



**Figure 1.2.** Predicted protein structure of the members of the ZIP family of micronutrient transporters. The notable structural features include eight membrane-spanning domains and a cytoplasmic loop of variable length situated between the third and forth transmembrane helices. This cytoplasmic domain contains histidine repeats that may function in metal binding (Eide, 1998).

**Table 1.1.** Currently identified ZIP micronutrient transporter genes (Source:Eide., 1998)

| Gene | Species | Speculative transport function | Localization |
|---|---|---|---|
| IRT 1 | *A. thaliana* | $Fe^{2+}$ uptake | Roots |
| IRT 1 | *P. sativum* | $Fe^{2+}/Zn^{2+}/Cd^{2+}$ uptake | Roots |
| ZIP 1 | *A. thaliana* | $Zn^{2+}$ uptake | Roots |
| ZIP 2 | *A. thaliana* | $Zn^{2+}$ uptake | ? |
| ZIP 3 | *A. thaliana* | $Zn^{2+}$ uptake | Roots and shoots |
| ZIP 4 | *A. thaliana* | $Zn^{2+}$ uptake | Roots and shoots |
| ZNT 1 | *T. caerulescens* | $Zn^{2+}/Cd^{2+}$ uptake | Roots and shoots |

### 1.4.2. Metal chelating agents (Phytosiderophores)

*Graminaceous* species synthesize phytosiderophores. These organic compounds belong to the mugineic acid family. They are secreted into the rhizosphere and they bind to ferric iron ($Fe^{3+}$) and phytosiderophore-$Fe^{3+}$ complex is recognized by specific transporters and transported across the root plasma membrane by an uptake system that is not characterized at the molecular level. But many studies showed that these compounds chelate not only ferric iron but also zinc.

Actually it is thought that phytosiderophores don't chelate zinc directly. The mechanism takes place *via* iron deficiency. When zinc is free in soil, zinc specific transporters become active and zinc is transported into roots. In calcerous soils, zinc is not available. When free zinc level decreases, plants use iron deficiency-induced phytosiderophore strategy to acquire zinc. In this case, iron is not sent to shoots from roots and iron deficiency takes place. This situation results in release of phytosiderophores. These compounds chelate iron and transfer it into roots, also zinc is transported by this complex across root plasma membrane (Wiren, Marschner, Römheld, 1996). The zinc uptake mechanism was shown schematically below.

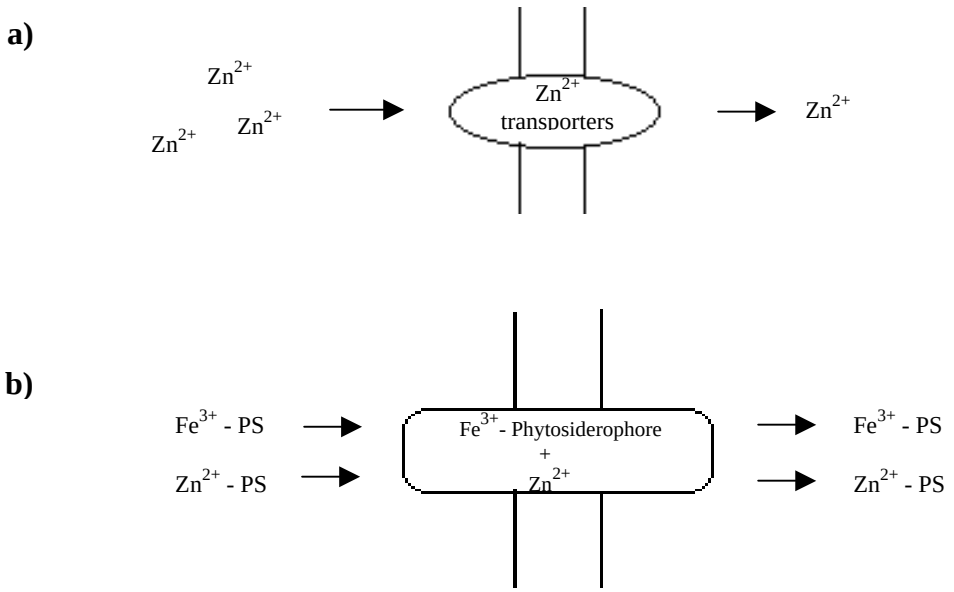



**Figure 1.3.** Zinc uptake strategies **a)** strategy 1 when free zinc is available **b)** strategy 2 when free zinc is not available

### 1.5.Differential Display

Differential Display method used in this study was developed first by Drs. Arthur Pardee and Peng Liang in 1992 to allow rapid, accurate and sensitive detection of altered gene expression (Science. 1992, 257:967; U.S. Patent 5,262,311). Differential Display conditions were optimized in our laboratory. The procedure of DD was summarized below:

- RNA extraction
- cDNA construction
- PCR amplification
- Separation on polyacrylamide gel
- Cutting of bands of interest
- Re-amplification by PCR of bands
- Separation on agarose gels
- Cutting of re-amplified products
- Cloning into vector and sequence

### 1.6. RT-PCR

RT-PCR (reverse transcriptase polymerase chain reaction) is one of the most sensitive techniques for mRNA detection and quantitation detection. The technique consists of two parts: Synthesis of cDNA from RNA by reverse transcription (RT) and amplification of a specific cDNA by polymerase chain reaction (PCR).

### 1.7. Hydroponics (Water culture)

A hydroponics system offers a sustainable growing method that recycles water, requires far less herbicides and pesticides and uses compact, hygienic and renewable growing media. Hydroponics system techniques yield bigger, faster

growing, healthier plants without disturbing the environment. Also a hydroponics system can be set up anywhere for 1 plant to 1000 plants. In soil, plants need a great deal of time and energy growing a large root system to find food and water. Additionally, nutrients in soil must be broken down first before they can be used, even if they are present. With hydroponics, because nutrients and water are delivered directly to the roots, the plant can spend its energy on producing more vegetation, larger fruit, flowers and vegetables. This efficiency allows plants to grow up to two times faster and with larger yields than with conventional soil gardening methods.

Because keeping zinc concentration in determined level is crucial for our experiment and in this system we can control all the nutrition concentrations, we chose hydroponics system for growing plants.

**Figure 1.4.** Appearance of growth plants in hydroponic medium in growing chamber.

### 1.8. Objectives of the study

When zinc is deficient in soil, some wheat cultivars suffer from this deficiency, but somehow other cultivars called zinc efficient cope this. Our aim is to determine the zinc uptake mechanism in wheat. Because it is the most efficient cultivar among bread wheats and Kıraç-66 plants were used in our experiments.

Different genes are expressed or expressions of certain genes are induced/reduced in response to different conditions. For instance, when we compare plants grown in zinc efficient and deficient conditions, different expressions of transporter genes or genes responsible for uptake mechanisms should be odserved.

So the best way to investigate how plants take up zinc at zinc deficient condition is to analyze expression levels of genes.

In our study, we have tried to search the changes of mRNA levels of genes related with zinc uptake and transport. Differential display is one of the most efficient techniques to examine changes in mRNA levels. Because accurate nutrient concentration is crucial, hydroponics system was set up instead of soil.

Despite the fact that zinc deficiency has severe outcomes not enough studies have been conducted. The purpose of this study is to contribute to the presently meager information regarding genes playing role in zinc efficiency in Anatolian wheat cultivars and our results will have a basic scientific quality.

# CHAPTER II

# MATERIALS AND METHODS

## 2.1. Plant materials

The bread wheat cultivar Kıraç-66 which is efficient to zinc deficiency, containing 75 individuals, was obtained from Anatolian Agricultural Research Center, Eskisehir, Turkey.

## 2.2. Growth conditions

Seeds of Kıraç-66 were surface sterilized in 20% hypochloric acid solution by shaking for two minutes, then washing twice with double distilled water. Then, the seeds were embedded in autoclaved perlite moistened with double distilled water

and solid $CaSO_4$ was sprinkled on them. Containers were covered with aluminum foil to create a humid environment for a better germination. Seeds were stored at 4 $^oC$ overnight for vernalization, then they were germinated for five days at 25 °C in dark.

The etiolated seedlings were then transferred to 7.0 L plastic vessels (100 seedlings per 7L) with continuously aerated nutrient solution. Double distilled water was used for preparing nutrient solutions. The nutrient solutions were exchanged every three days with freshly prepared ones. Plants were harvested at different growth time periods. Plants were grown in a growth chamber under controlled environmental conditions: 16/8 hours light/dark regime, 20/15 °C day/night temperature, 65% relative humidity and continuous aeration of nutrient solution.

Macronutrient, micronutrient, $ZnSO_4$ and Fe-EDTA stock solutions were prepared separately from each other. Macronutrient solution contains 0.88 mM $K_2SO_4$, 0.25 mM $KH_2PO_4$, 1.0 mM $MgSO_4$, 2.0 mM $Ca(NO_3)_2$. Micronutrient solution contains 1 μM $H_3BO_3$, 0.5 μM $MnSO_4$, 0.2 μM $CuSO_4$. Fe-EDTA concentration was 50 mM. Zinc was supplied as $ZnSO_4$ at a concentration of 0 μM for zinc deficient growth condition, 0.4 μM and 1.0 μM for zinc sufficient growth condition. Plants were grown 12 days, then transfers were performed. Growth conditions and the time points of the samples harvested are presented in the following tables (Table 2.1. and Table 2.2.)

**Table 2.1.** List of collected plant samples refered as Group 1set.

| Zinc Concentrations in Hydroponic Solution | | Time Points (after transfer) |
|---|---|---|
| Plants Transferred ($Zn^{2+}$) | | |
| from | to | |
| 0 μM | - | 0 |
| 0 μM | 1.0 μM | 5hours |
| 0 μM | 1.0 μM | 24hours |
| 0 μM | 1.0 μM | 3days |
| 0 μM | 1.0 μM | 7days |
| 1.0 μM | | 0 |
| 1.0 μM | 0 μM | 5hours |
| 1.0 μM | 0 μM | 24hours |
| 1.0 μM | 0 μM | 3days |
| 1.0 μM | 0 μM | 7days |
| 0.4 μM | - | 0 |
| 0 μM | 0.4 μM | 5hours |
| 0 μM | 0.4 μM | 24hours |
| 0 μM | 0.4 μM | 3days |
| 0 μM | 0.4 μM | 7days |

**Table 2.2.** List of group 2 collected plant samples

| Zinc Concentrations in Hydroponic Solution | | Time Points (after transfer) |
|---|---|---|
| Plants Transferred ($Zn^{2+}$) | | |
| from | to | |
| 0 μM | - | 0 |
| 0 μM | 1.0 μM | 1 day |
| 0 μM | 1.0 μM | 5 days |
| 0 μM | 100 μM | 1 day |
| 0 μM | 100 μM | 5 days |
| 1.0 μM | | 0 |
| 1.0 μM | 0 μM | 1 days |
| 1.0 μM | 0 μM | 5 days |
| 1.0 μM | 100 μM | 1 day |
| 1.0 μM | 100 μM | 5 days |

### 2.3. Total RNA isolation

To maintain intactness of RNAs avoid RNase contamination is crucial. All the glass equipments were treated with hypo-chromic acid solution and washed with active DEPC treated dd$H_20$ before use. Other non-glass equipments and plastic materials were treated with DEPC treated dd$H_20$ and autoclaved. Non-plastic materials were stored at 180°C for two days before RNA isolation. All pipets and bench were cleaned with RNase-off before isolation. All the solutions used in isolations were prepared by using DEPC treated dd$H_20$. Isolated total RNA was also redissolved in DEPC treated dd$H_20$ in order to avoid degradation by RNases after isolation. Aliquots were prepared from samples and kept in -80°C until use. Total RNAs of group 1 plants were isolated, according to detailed procedure presented in the following section.

#### 2.3.1. Homogenization

The plant root tissue (50-100 mg) from Kıraç-66 was powdered in a 2.0 mL tube in the presence of liquid nitrogen. Powdered tissue was homogenized in 700 μL of Trizol Reagent (Gibco BRL/Life Technologies) using 1mL flow rounded tips.

### 2.3.2. Phase separation

Homogenized samples were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Chloroform of 0.14 mL added to each tube. Tubes were shaked vigorously by hand for 15 sec and incubated at room temperature for 2 to 3 min. Samples were centrifuged at 15,000 rpm for 15 min at 4 $^{o}$C. Following the centrifugation step, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase (RNA remains exclusively in the aqueous phase).

### 2.3.3. RNA precipitation

The upper phase was transferred to a fresh tube. RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. Isopropyl alcohol of 0.35 mL was used per 700 μL of Trizol Reagent added during the initial homogenization. Samples were centrifuged at 15,000 rpm for 10 min at 4 $^{o}$C after 10 min of incubation at room temperature. The RNA precipitate, often invisible before centrifugation, formed a gel like pellet on the side and bottom of the tube.

### 2.3.4. RNA wash

The supernatant was removed. RNA pellet was washed once with 75% ethanol, adding at least 700 μL of 75% ethanol per 700 μL of Trizol Reagent used for the initial homogenization. Sample was mixed by vortexing and centrifuged at 9,000 rpm for 5 min at 4 °C.

### 2.3.5. Redissolving the RNA

Since it is important not to let the RNA pellet dry completely, as this will greatly decrease its solubility, the RNA pellet was dried briefly (10–15 min) at the end of the procedure. RNA was dissolved in DEPC-treated sterile water and incubated for 10 min at 55-60 °C. Following the incubation, tubes were stored at –80 °C.

### 2.3.6. Concentration determination of the isolated RNA samples

RNA samples were diluted 1/100 in 0.5 mL of double distilled water and absorbance values were measured at 230 nm, 260 nm and 280 nm in Shimadzu UV-1601 spectrophotometer. RNA absorbs UV light at 260 nm, but it is also required to

know the absorbance values of proteins at 280 nm and phenolic compounds at 230 nm in order to evaluate the purity of RNA samples.

Concentrations of RNA samples were determined according to the equation given below:

$1A_{260}$ = 40μg/mL RNA

Conc.of RNA (mg/μL) = $A_{260}$ value x dilution factor x 40 mg/mL RNA/$A_{260}$

**2.4. mRNA isolation**

Because roots are so important for zinc uptake and efficiency mechanism, roots of group 2 plants were used for mRNA isolation.

**2.4.1. Sample preparation**

Kıraç-66 plant root tissue (150 mg) samples were powdered in a 2.0 mL tube in the presence of liquid nitrogen. Powdered tissue was homogenized in 200 μL of Extraction/BME Buffer (4 M guanidine thiocyanate, 25 mM sodium citrate pH 7.1 and β-Mercaptoethanol with final concentration of 2%). Tubes were shaked for 1-2

minutes and immediately 400 μL of Dilution Buffer preheated at 70 $^{o}$C for 5 min (6X SSC, 10 mM Tris-HCl (pH:7.4), 1 mM EDTA, 0.25% SDS and β-Mercaptoethanol with final concentration of 2%), was added into each tube and they were mixed throughly by inversion. Then 1 μL of 50 pmol biotinlated oligo dT probes was added. Mixture was incubated at 70 $^{o}$C for 5 min. After incubation, samples were centrifuged at 15,000 rpm for 10 min at room temperature.

### 2.4.2. Preparation of streptavidin-beads

Streptavidin MagneSphere Paramagnetic Particles (SA-PMPs) (Promega), as 100 μL per sample were added into 1.5 mL tube. Then the tube was placed on the magnetic stand and let SA-PMPs to accumulate on the site facing to the magnet. The storage buffer was poured off. SA-PMPs were resuspended in 100 μL of 0.5X SSC solution and washed off. This washing was repeated twice. SA-PMPs were resuspended with 100 μL of 0.5x SSC solution (4.38 g sodium chloride, 2.20 g sodium citrate, pH: 7).

### 2.4.3. Phase seperation

After the centrifugation of samples, aquous and organic phases were seperated. RNA remains in the upper phase.

### 2.4.4. Washing captured mRNAs

The upper phase was transferred onto ready to use SA-PMPs. The mixture was incubated for 2 minutes at room temperature. After incubation, tubes were placed on magnetic stand and the particles were captured and the wash solution was poured off. Beads were resuspended in 0.5 mL of 0.5X SSC solution and washing step was repeated 3 times.

### 2.4.5. Elution of mRNAs

Captured beads were resuspended in 25 μL nuclease-free water and incubated for 2 minutes at room temperature. After incubation tubes were placed on magnetic stand and particles were captured. The liquid containing eluted mRNA was transferred to a fresh tube. This step was repeated twice. mRNAs were stored at –80 $^{o}$C until use.

## 2.5. RNA gels

### 2.5.1. RNA gel preparation using MOPS buffer

#### 2.5.1.1. Sample preparation for RNA gel

To 4 μg total RNA 2 μL of loading dye (50% glycerol, 1 mM EDTA (pH:8.0), 0.25% bromophenol blue, 0.25% xylene cyanol FF) and 1 μL of 0.5 μg/mL Ethidium-bromide onto the samples was added, the volume was adjusted to 10 μL by adding appropriate amount of sterile PCR water. All of the mixture was loaded onto the gels. Gel photographs were taken by Vilber Lourmat Gel Documentation System, France.

#### 2.5.1.2. RNA gel preparation

All of the electrophoresis apparatus, trays and combs were washed several times with DEPC-treated water, then cleaned with RNAse-OFF (Applichem) solution to inactivate contaminating RNases due to handling. Agarose (0.6 g) (Quantum Biotechnologies) was dissolved in 36 mL DEPC-treated double distilled

water, when cooled down to about 60 °C, 5 mL 10X MOPS (0.4 M MOPS, 0.1 M sodium acetate, 0.01 M EDTA, solutions were mixed, pH adjusted to 7.0, filtered, covered with aluminum foil and stored at RT), 9 mL formaldehyde (Sigma Chemical Co.) were added to agarose solution, mixed and poured off to the gel cassette. Gel was electrophoresed in 1X MOPS buffer at 80 voltages for 2 hours.

### 2.5.2. RNA gel preparation using phosphate buffer

#### 2.5.2.1. Sample preparation for RNA gel

Sample preparation was the same as above except Ethidium-bromide was included in the gel.

#### 2.5.2.2. RNA gel preparation

All the electrophoresis apparatus was washed several times with DEPC-treated water, then cleaned with RNAse-OFF (Applichem) solution to inactivate contaminating RNases due to handling. Agarose 0.5 g (Quantum Biotechnologies) melted by boiling in 50 mL, 10 mM Sodium Phosphate buffer (1.340 g/L $Na_2HPO_4.7H_2O$ and 0.689 g/L $NaH_2PO_4.H_2O$, pH 6.8). When cooled down to about

60 °C, 3 μL of Ethidium-bromide was added and it was poured off into the gel tray. Because of the rising acidity during electrophoresis and it causes degredation of RNAs, gel buffer (10 mM sodium phosphate) was changed every 20 minutes.

**2.6. Synthesis of first strand cDNA**

Following components were combined in a 200 μL sterile PCR tube: 10 pmol cDNA synthesis primer (Gibco-BRL), 10 pmol SmartOligo (Gibco-BRL), 4 μg total RNA or 4 μL of mRNA isolates, 0.5 mM dNTP each, sterile double distilled water up to 12 μL. The reaction mixture was incubated at 65 °C for 5 min and quickly chilled on ice. To the mix above the following components were added in the following order; 0.01 M DTT (Gibco BRL), 1.5 U RNase inhibitor (Ambion), 1X First Strand Buffer (Gibco-BRL). Contents of the tube were briefly centrifuged and incubated at 42 °C for 2 min. Finally, 2 U of Superscript II Reverse transcriptase enzyme (Gibco-BRL) was added. Reaction was carried out at 42 °C for 1 hour and stopped by incubating at 70 °C for 15 min.

To check the efficiency of cDNA construction, RT-PCR was performed. For this purpose, constitutively expressed 18S which amplified 18S subunit rRNA and Elongation Factor 2 (EF2) were used. Because 18S primer amplifies rRNA this primer set was used to check cDNA efficiency constructed with total RNA. EF2

primer set was used to check efficiency of cDNAs constructed with mRNA. Sequences of 18S and EF2 primers were given below.

18S F 5’ ATG GCC GTT CTT AGT TGG TG 3’

18S R 5’ AAC ACT TCA CCG GAC CAT TC 3’

EF2 F 5’ ATC ACT GAT GGA GCT TTG GTG G 3’

EF2 R 5’ CAT GCA GAC CAG CAG AGA ATG C 3’

### 2.7. Synthesis of second strand cDNA

Double stranded cDNA was constructed by using 2 different methods. In our initial experiments we produced ds-cDNA by PCR using *Taq* DNA polymerase enzyme which has potential to amplify bands that have sizes < 1.5-2 kb. In the second method ds-cDNA was constructed by PCR using *Accurase* (DNA amp) enzyme, allowing to synthesis of longer products up to 12kb.

#### 2.7.1. Synthesis of second strand cDNA using *Taq* DNA polymerase

Following components were combined in a 200 μL sterile PCR tube: 1X PCR Buffer (Roche), 1.5 mM $MgCl_2$ (Roche), 0.2 mM dNTP each, 0.8 pmol PCR

primer (Gibco BRL), 1.0 U *Taq* DNA Polymerase, 2 μL first strand reaction product and volume of the reaction was brought to 50 μL by adding appropriate amount of sterile PCR water. PCR cycling conditions were; initial denaturation at 94 °C for 2 min, 35 cycles of three steps as denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1.5 min. Products were stored at –35 $^{o}$C until use.

### 2.7.2. Synthesis of second strand cDNA by long PCR

Following components were combined in a 200 μL sterile PCR tube: 1X Long Buffer (DNA amp), 1.2 mM MgAoc (DNA amp), 0.2 mM dNTP each (DNA amp), 40 pmol PCR primer (Gibco BRL), 1.25 u *Accurase* (DNA amp), 2 μL first strand reaction product, sterile distilled water to a volume of 50 μL. PCR conditions were 94°C for 2 min as initial denaturation, 10 cycles of 94°C for 20 sec, 60°C for 30 sec and 68°C for 15 min followed by 25 cycles of 94°C for 20 sec, 60°C for 30 sec and 68°C for 15 min + 20 sec and 1 cycle of final extension at 68°C for 7 min. Products were stored at – 35°C until use.

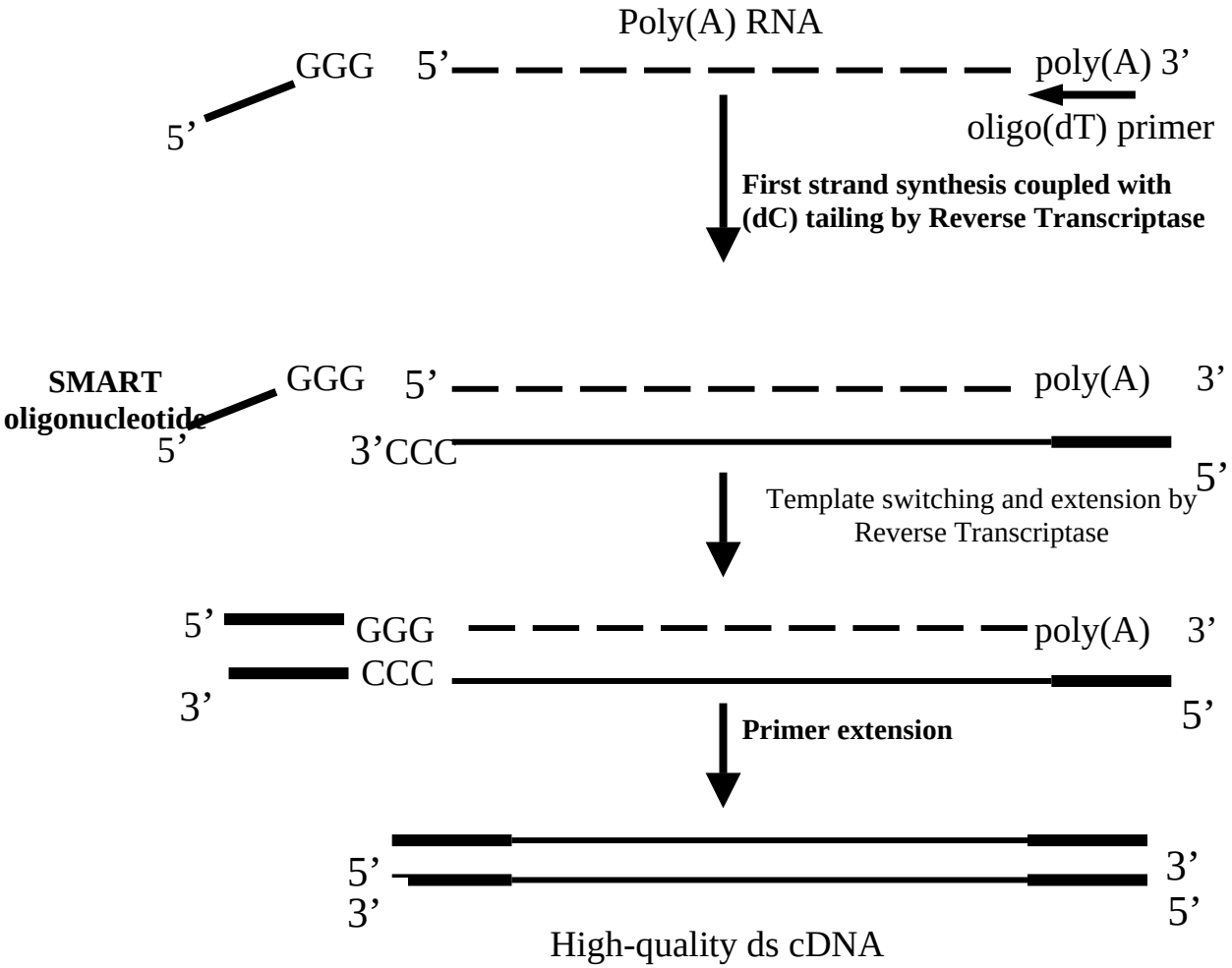



**Figure 2.1.** Synthesis of ds-cDNA

### 2.8. Differential Display

Initially, differential display (DD) reactions were performed using ss-cDNA constructed by SuperScript II RNase H reverse transcriptase and regular *Taq* DNA polymerase (MBI-Fermentas) which has potential to amplify bands that have sizes <

1.5-2 kb. Therefore in our initial experiments cDNA fragments were 1.5- 2 kb in length. In our further experiments we performed DD reactions using ds-cDNA constructed by using SuperScript II RNase H reverse transcriptase and *Accurase* enzyme. First strand cDNA was synthesized by using SuperScipt II RNase H Reverse transcriptase (Gibco-BRL) that can generate cDNA up to 12.3 kb, therefore by using *Accurase*, synthesis of larger ds-cDNA was achieved. All PCR reactions for DD were performed in MJ Research PTC 100 type thermocycler. The experiments were carried out by using custom P and T primer combinations. Sequences of the primers used are listed in the Table 2.3. P primers were designed to anchor the 5′ region of the mRNA whereas T primers were designed to anchor the 3′ (polyA) region of the mRNA.

**Table 2.3.** Sequences of the primers used in differential display analysis

| Primer Name | Primer Sequence |
|---|---|
| P1 | AAT AAC CCT CAC TAA ATG GGG A |
| P2 | ATTAACCCTCACTAAATCGGTCATAG |
| P4 | ATTAACCCTCACTAAATGCTGGTAG |
| T1 | CAT TAT GCT GAG TGA TAT $CT_{(9)}$ AA |
| T2 | CAT TAT GCT GAG TGA TATC$T_{(9)}$ AC |
| T3 | CAT TAT GCT GAG TGA TAT $CT_{(9)}$ AG |
| T4 | CAT TAT GCT GAG TGA TAT $CT_{(9)}$ CA |
| T5 | CAT TAT GCT GAG TGA TAT $CT_{(9)}$ CC |
| T6 | CAT TAT GCT GAG TGA TAT $CT_{(9)}$ CG |
| T7 | CAT TAT GCT GAG TGA TAT $CT_{(9)}$ GA |
| T8 | CAT TAT GCT GAG TGA TAT $CT_{(9)}$ GC |
| T9 | CAT TAT GCT GAG TGA TAT $CT_{(9)}$ GG |

### 2.8.1. Differential Display using single stranded cDNA

Same cDNA synthesis procedure was used when performing DD with ss-cDNA and RT-PCR.

### 2.8.1.1. Synthesis of first strand cDNA for RT-PCR

Total RNA of 2 μg and 2 μmol Oligo-$dT_{18}$ and sterile distilled water up to 5 μl were combined in a 200 μl sterile PCR tube. The mixture is incubated at 70$^{o}$C for 3 min., then immediately chilled on ice. After 2 min incubation on ice following components were added: 2 μl of 5X First Strand Buffer (Gibco-BRL), 0.1 mM dNTP each. Contents of the tube were briefly centrifuged and incubated at 42 °C for 2 min. Finally, 2 U of Superscript II reverse transcriptase enzyme (Gibco BRL) was added. Reaction was carried out at 42 °C for 1 h and stopped by incubating at 70 °C for 15 min. Samples were diluted 1/5 and stored at -35$^{0}$C until use.

### 2.8.2. Differential Display using ds-cDNA

PCR reactions were performed by incorporating radioactively labeled nucleotide [($\alpha$-$^{33}$P)-dATP]. In a 200 μL sterile PCR tube following components were mixed ; 1X PCR Buffer (75 mM Tris-HCl with pH 8.8 at 25$^{o}$C, 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20), 0.25 mM dNTP mix (DNA Amp), 1.5 mM $MgCl_2$ (DNA Amp), 1U of *Taq* DNA polymerase, 15 pmol forward primer, 15 pmol reverse primer and sterile distilled water up to 20 μl reaction volume were mixed in a 200 μl PCR tube. PCR cycling conditions were 94 °C for 2 min as denaturation,

35 cycles of three steps as denaturation at 94 °C for 1 min, annealing at 60°C for 1 min and extension at 72 °C for 1 min and 1 cycle of 5 min extension at 72°C. The reactions were terminated by adding 4 μL of stop solution (95% formamide, 20 mM EDTA, 0.25% bromophenol blue and 0.025% xylene cyanol).

### 2.9. Visualizing

#### 2.9.1. Visualizing on autoradiography

Differential display products were denatured at 94 °C before loading on the denaturing gel. Samples were loaded onto 6 % denaturing polyacrylamide gel (5.7% acrylamide, 0.3% N, N'methylene-bis-acrylamide, 8M urea, 1X TBE (90mM Tris base, 90mM Boric acid, 2mM EDTA)). For 60 mL of gel solution, 650μL of 10% APS (ammoniumpersulfate) and 25μL of TEMED (N, N, N', N'-Tetraethyl ethylene diamine) were added and gel was poured into sigmacoat treated sequencing gel plate sandwich immediately. Radioactively labeled differential display products were electrophoresed at a constant power of 60 Watt (Biometra High Voltage Power Supply, Pack P30, Germany), electrophoresis let until the xylene cyanol dye migrates 10 cm after the bromophenol blue dye goes out of the gel. After completion of electrophoresis, the gel was transferred on Whatman paper (3MM),

and covered with stretch film . The gel left on the gel dryer (Savant SGD 2000) and dried at 78°C for 30-40 min . After drying, the gel was exposed to X-ray film. For a time period (2-3 days) depending on the half-life of the radioactive material, films were developed at METU Health Center.

### 2.9.2. Visualizing by silver staining

Samples were loaded onto 10% non-denaturing polyacrylamide gel (9.7% acrylamide, 0.3% N, N'methylene-bis-acrylamide 1X TBE (90 mM Tris base, 90 mM Boric acid, 2 mM EDTA)). For 100 mL of gel solution, 1.08 mL of 10% APS (ammoniumpersulfate) and 41 μL of TEMED (N, N, N', N'-Tetraethyl ethylene diamine) were added into gel mixture and the gel was immediately poured into plate sandwich (one of the plate was bind silane treated and the other plate was sigma coat treated). The plate gel adhered *via* bind silane was transferred to a plastic tray and immersed into the fixing solution (an aqueous solution of 10% (v/v) ethanol and 5% (v/v) acetic acid) for 6 min. The fixing solution was replaced with staining solution (an aqueous solution of 1% $AgNO_3$) and the gel was incubated for 20 min. The gel was rinsed twice with double distilled water and then was replaced in developing solution (an aqueous solution of 1.5% (w/v) NaOH and 0.1% (v/v) formaldehyde (should be added prior to use). After the bands become visible, the gel was soaked into stop solution (an aqueous solution of 0.75% (w/v) $Na_2CO_3$) for 20 min to stop

further development with dark background. At the end of the silver staining procedure, the gel was photographed.

### 2.10. RT-PCR

Primers were designed for conserved region of a gene of interest comparing other organisms using Primer 3 program. (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/). Primers were designed for ZAT (zinc arabidopsis transporter) gene. Sequence of primers were shown below.

| | | | | |
|---|---|---|---|---|
| ZAT Forward Primer: | 5' | TGGTGTWATGATTGG | 3' | Tm: 53.4 |
| ZAT Reverse Primer: | 5' | CCCACTGTGATAGCCCAKA | 3' | Tm: 52.3 |

(W referred to A/T and K referred to G/T)

#### 2.10.1. PCR amplification

Following components were combined in a 200 µL sterile PCR tube: 1X PCR Buffer (Roche), 1.5 mM $MgCl_2$ (Roche), 0.2 mM dNTP each (DNA amp), 0.2 pmol forward primer, 5 pmol reverse primer, 1.0 U Taq Polymerase enzyme, and sterile PCR water up to 25 µL. PCR cycling conditions were; initial denaturation at

94 °C for 2 min, 35 cycles of three steps as denaturation at 94 °C for 1 min, annealing temperature for amplification of ZAT (Zinc Arabidopsis Transporter) primer 49 $^{o}$C, for 1 min and extension at 72 °C for 15 min. Products were electrophoresed in 1% agarose gel.

### 2.11. Cloning of differentially expressed bands

Differentially expressed bands detected in differential display and analysis were cloned for sequencing. As an initial step differentially expressed bands were cut form sequencing gel and dissolved in 15 μL distilled water, dissolved products were reamplified by PCR

### 2.11.1 Reamplification of differentially expressed fragments

Reactions were carried out in 50 μL of final volume and following componenents were mixed; 5 μl redissolved product, 1X PCR Buffer, 0.25 mM dNTP each, 1,5 mM $MgCl_2$, 1U of *Taq* DNA polymerase (MBI Fermentas), 10 pmol forward primer and reverse primers each, sterile distilled water making up to 50 μl volume. PCR cycling conditions were 94°C for 2 min as initial denaturation, 25

cycles of three steps as denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72 °C for 2 min and final extension at 72°C for 5 min. Re-amplified bands were loaded on 1.0% agarose gel. Re-amplified bands were cut and redissolved in 20 µl distilled water for cloning.

### 2.11.2 Ligation of re-amplified fragments to pGEM-T-Easy vector

Redissolved DNA that was cut from agarose gel was ligated to pGEM-T-Easy vector (Promega) with a final volume of 10 µL in a PCR tube. Following components were combined; 6 µL redissolved DNA , 5 ng pGEM-T-Easy vector (Promega), 1X Ligase Buffer ( Promega) and 2 units of $T_4$ DNA Ligase enzyme (Promega). Reaction mixture was incubated at 4°C overnight (approximately 18 hours).

### 2.11.3 Preperation of *E.coli* competent cells

A single colony of *E.coli* Dh5-α cells was inoculated into 2 mL LB medium. Cells were incubated at 37°C with moderate shaking (250rpm) overnight. 1 mL of overnight grown culture was inoculated into 100 mL of LB medium in a sterile 2 L flask and grown at 37°C, shaking (250rpm), to an $OD_{590}$ of 0.375. Culture was

aliquated into two 50 mL prechilled tubes and leaved on ice for 30 min. Cells were centrifuged for 10 min at 4000 rpm at 4 $^{o}$C. Supernatant was poured off and each pellet was resuspended in 20 mL ice-cold $CaCl_2$ (50 mM) solution and leaved on ice for 30 min. Cells were centrifuged for 10 min at 4000 rpm at 4 $^{o}$C and supernatant was discarded. Each pellet was resuspended in 2 mL of ice-cold $CaCl_2$ (50 mM) solution and store at 4 $^{o}$C until use.

### 2.11.4 Transformation of *E.coli* competent cells with ligation products

*E.coli* Dh5-α competent cells were transformed with ligation products according to the following procedure; In a sterile 2 mL tube the followings were combined; 5 μL ligation product, 0.02 M β-Mercaptoethanol and 50 μL *E.coli* Dh5-α competent cells. Mixture was placed on ice for 30 min, then heat shocked at 42 °C for 45 sec. SOC Medium was added upto 200 μL.

Mixture was incubated at 37 °C for 45 min and quickly chilled on ice for 2 min. Mixture was spread on plates as 50 μL/plate (plates contain LB Agar). Plates were incubated at 37 °C overnight. After the incubation, white colonies were selected among grown colonies and these colonies were transferred to 100 μL LB medium containing sterile 2 mL tubes. Selected colonies were PCR amplified.

### 2.11.5 PCR amplification of colonies

1X PCR Buffer, 0.2 mM dNTP each, 0.8 pmol M13 Forward and Reverse primers (TIB Molecular Biology), 1.2 mM $MgCl_2$, 1 U *Taq* DNA Polymerase, 1 μL of selected colonies and sterile PCR water up to 25 μL final volume combined in a sterile PCR tube. PCR cycling conditions were initial denaturation at 94°C for 2 minutes followed by 30 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min.

PCR products were run on 1.0% agarose gel to detect positive and false positive colonies. Insert containing recombinant cells were selected and used in plasmid isolation step.

## 2.12. Plasmid isolation from colonies

Plasmids were isolated using QIAGEN QIAperp Spin Miniprep Kit according to its protocol.

Overnight grown bacterial culture of 2 mL was harvested by centrifugation for 5 min at 15.000 rpm. Supernatant was poured off, tube was inverted and blotted on a paper towel to remove excess media. 250 μL of Buffer P1 was added to tube

and cell pellet was completely resuspended by vortexing. After adding 250 μL of Buffer P2, the tube mixed by gently inverting four times, incubated at RT for approximately 5 min. N3 solution of 350 μL was added and mixed by inverting 4 times. Sample tubes were centrifuged at 15,000 rpm for 10 min at RT. A spin column, provided by the manufacturer, inserted in to collection tube. Cleared lysate, the upper phase was transferred to the spin column and centrifuged at 15,000 rpm for 1 min at room temperature.

After the centrifugation, lower phase in the collection tube was discarded and collection tube reinserted. Column Washing solution Buffer PE of 750 μL was added to spin column, centrifuged at 15,000 rpm for 1 min at RT, flowthrough discarded and the collection tube reinserted again and to remove residual wash buffer completely additional 1 min centrifugation was involved. The contents of spin column was transferred to a new sterile 1.5 mL tube, the plasmid DNA was eluted by adding 50 μL of Buffer EB (elution buffer) and centrifugating at 15,000 rpm for 1 min. Spin column assembly was removed and harvest stored at –20 °C.

### 2.12.1 Visualization of isolated plasmid

In order to determine DNA integration to the plasmid and to be informed about the size of the insert, plasmid DNA was digested with *Eco*RI restriction enzyme. 5 μL purified plasmid, 1X NE Buffer (New England Biolabs), 1 U *Eco*RI enzyme (New England Biolabs) and PCR water were combined in a PCR tube with a final volume of 10 μL. Mixture was incubated at 37 °C overnight (approximately 18 hours). After the incubation samples were run on 1.0% agarose gel.

## 2.13. Sequencing reactions

Plasmids were purified as described in section 2.12. Inserts were custom sequenced and they were read using SP6 primers. Sequencing reactions of the 600 ng purified pGEM-T easy (Promega) recombinant clones were performed and the sequences were read on ABI prism-310 Genetic Analyzer.

## 2.14. Sequence and homology analysis

NCBI databanks were searched for finding homolog sequences that are available. Sequence analysis was performed using Editseq 4.0 (DNA Star Inc.)

expert sequence analysis software. The sequence alignments were obtained using ClustalX 1.81 (Jeanmougin et al., 1998) computer programs.

**2.15. Gene walking**

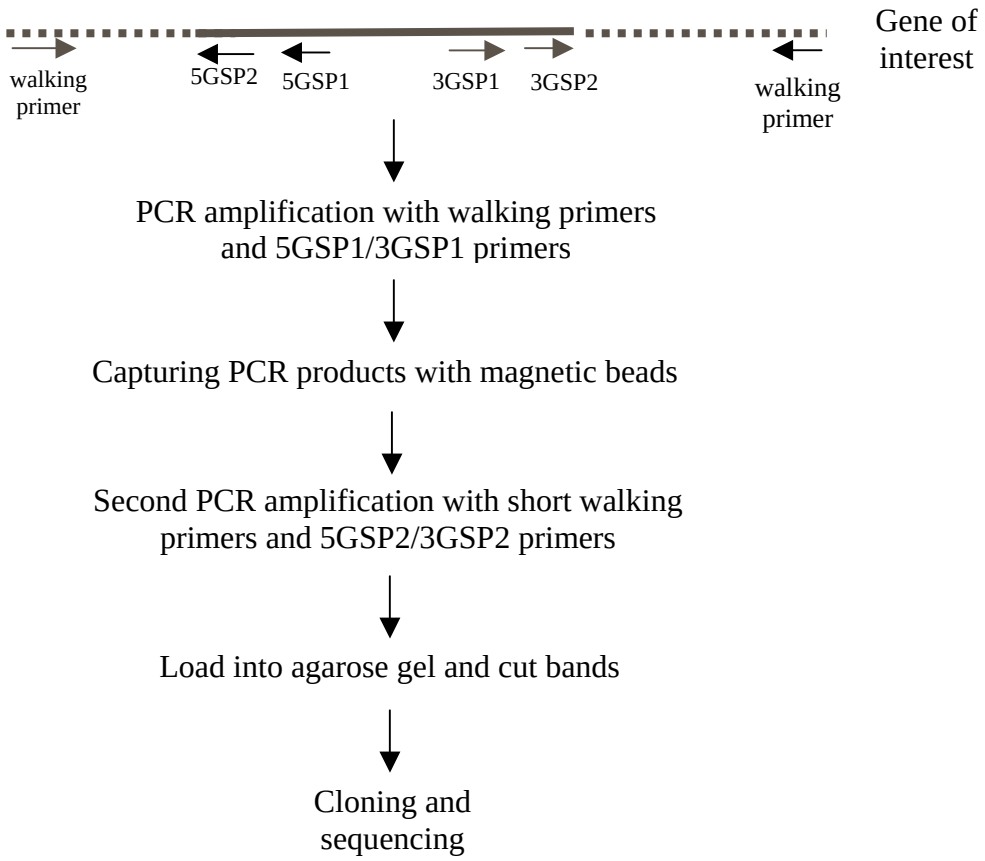
After sequencing, gene walking procedure was performed to determine the whole sequence of bands which had sequence homology with genes in the gene bank.

For this purpose, gene specific and arbitrary walking primers were designed. Sequences of walking primers and gene specific primers were listed in Table 2.4.

**Table 2.4.** List of primer sequences used in gene walking

| Primer Name | Primer Sequence |
|---|---|
| MT1-5GSP1 | Biotin- GAGGTAAATAGTTGAGAGGC |
| MT1-5GSP2 | TTATAAGCCCCTATCTTTCT |
| MT1-3GSP1 | Biotin- AGATCTTTCCCCAACACATG |
| MT1-3GSP2 | TGCATAATGGTAATTAAGTA |
| MT5-3GSP2 | Biotin- CAATGGTCTCTTGGTCTGAAT |
| MT5-3GSP2 | TCATCTTCAAACTGTGTTCC |
| MT5-3GSP2 | Biotin- GGAGAAACTGGCTCTGGTAA |
| MT5-3GSP2 | CACAACCTCGTCGAGTAGCA |
| MT8-3GSP2 | Biotin- TCATTAGATGATCTTGGCTA |
| MT8-3GSP2 | GAATCAATTGAACCTTGCAG |
| MT8-3GSP2 | Biotin- AGTCAAATCCTTCTACCTCT |
| MT8-3GSP2 | AGAATACAAACCAACTCAGT |
| WP1 | CTAATACGACTCACTATAGGGNNNATGC |
| WP2 | CTAATACGACTCACTATAGGGNNNGATC |
| WP3 | CTAATACGACTCACTATAGGGNNNTAGC |
| WP4 | CTAATACGACTCACTATAGGGNNNTAGC |
| Short WP | CTAATACGACTCACTATAGGG |

Gene walking procedure was showed below. Experiments were performed to the end of 5' genes, determination of 3' end of the gene will be performed later.




### 2.15.1. PCR amplification

Following components were combined in a 200 µL sterile PCR tube: 1X PCR Buffer, 1.5 mM $Mg(OAc)_2$, 0.2 mM dNTP mix, 1.0 pmol walking primer, 1.0 pmol gene specific primer, 0.5 U *Taq* DNA Polymerase enzyme, 0.5 U *Accurase* enzyme and sterile PCR water up to 50 µL. PCR cycling conditions were; initial denaturation at 94 °C for 2 min, 30 cycles of three steps as denaturation at 94 °C for

1 min, annealing temperature 47 $^{o}$C for 4 min and extension at 72 °C for 10 min. Products were electrophoresed in 1% agarose gel.

# CHAPTER III

# RESULTS

## 3.1. Total RNA isolation for differential display analysis

Intactness of RNA is one of the most important criteria in differential display (DD). Therefore, isolation of pure, and intact high quality RNA is so crucial for the rest of the experiments. As DD is based on comparing the mRNA levels, the concentrations of samples must be determined very carefully and they should be equally aliquoted as strarting materials. Since DD analysis compares expression levels indirectly, the mRNA concentrations after dilutions are also factorized on agarose gels for the correct similar intensities.

**Table 3.1.** Concentrations and absorbance values of total RNAs isolated from roots of Kıraç-66.

| Sample No: | Transferred from (μM $Zn^{2+}$) | Transferred to (μM $Zn^{2+}$) | Time Points | $A_{230}$ (nm) | $A_{260}$ (nm) | $A_{280}$ (nm) | Concentration (μg/μL) |
|---|---|---|---|---|---|---|---|
| 1 | 0 | 1 | 0 | 0.598 | 0.165 | 0.135 | 0.825 |
| 2 | 0 | 1 | 5 hours | 0.559 | 0.157 | 0.128 | 0.785 |
| 3 | 0 | 1 | 24 hours | 0.895 | 0.482 | 0.447 | 2.410 |
| 4 | 0 | 1 | 3 days | 0.586 | 0.234 | 0.187 | 1.170 |
| 5 | 0 | 1 | 7 days | 0.451 | 0.167 | 0.126 | 0.835 |
| 6 | 1 | 0 | 0 | 0.315 | 0.115 | 0.091 | 0.575 |
| 7 | 1 | 0 | 5 hours | 0.898 | 0.181 | 0.137 | 0.905 |
| 8 | 1 | 0 | 24 hours | 0.504 | 0.193 | 0.153 | 0.965 |
| 9 | 1 | 0 | 3 days | 0.417 | 0.142 | 0.113 | 0.700 |
| 10 | 1 | 0 | 7 days | 0.277 | 0.115 | 0.091 | 0.575 |
| 11 | 0 | 0.4 | 0 | 1.316 | 0.272 | 0.219 | 1.36 |
| 12 | 0 | 0.4 | 5 hours | 0.459 | 0.174 | 0.136 | 0.870 |
| 13 | 0 | 0.4 | 24 hours | 0.944 | 0.727 | 0.693 | 3.635 |
| 14 | 0 | 0.4 | 3 days | 0.907 | 0.247 | 0.187 | 1.235 |
| 15 | 0 | 0.4 | 7 days | 0.506 | 0.196 | 0.135 | 0.980 |

Integrity of RNA was verified by electrophoresing the samples on 1% MOPS-agarose gel (Figure 3.1.) and 1% sodium phosphate-agarose RNA gel (Figure 3.2.) by the appearances of the ribosomal RNA subunits. It is a common practice that, if the RNA subunits appear non-degraded on the agarose gels, then it is considered that rest of the mRNAs are intact as well.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Transferred from | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0.4 | 0 | 0 | 0 | 0 | ($\mu M\ Zn^{2+}$) |
| Transferred to | - | 1 | 1 | 1 | 1 | - | 0 | 0 | 0 | 0 | - | 0.4 | 0.4 | 0.4 | 0.4 | ($\mu M\ Zn^{2+}$) |
| Time points | 0 | 5h | 24h | 3d | 7d | 0 | 5h | 24h | 3d | 7d | 0 | 5h | 24h | 3d | 7d | |

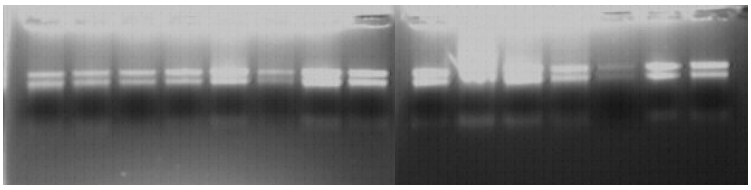


**Figure 3.1.** Appearance of total RNA samples (5μg) isolated from root tissues on 1% MOPS-agarose RNA gel.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Transferred from | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0.4 | 0 | 0 | 0 | 0 | ($\mu M\ Zn^{2+}$) |
| Transferred to | - | 1 | 1 | 1 | 1 | - | 0 | 0 | 0 | 0 | - | 0.4 | 0.4 | 0.4 | 0.4 | ($\mu M\ Zn^{2+}$) |
| Time points | 0 | 5h | 24h | 3d | 7d | 0 | 5h | 24h | 3d | 7d | 0 | 5h | 24h | 3d | 7d | |

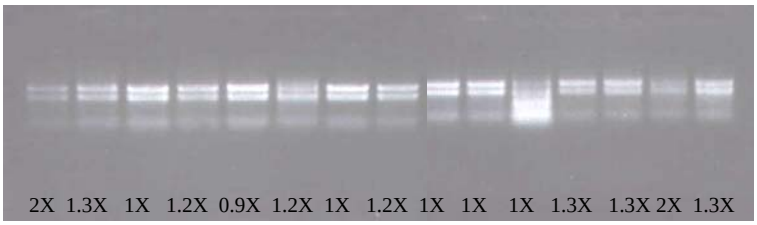



**Figure 3.2.** Appearance of total RNA samples (5μg) isolated from root tissues on 1% sodium phosphate-agarose RNA gel

Because the sample lane in 11 in Figure 3.2. degraded, total RNA isolation of the sample was repeated. When constructing cDNA, we took same amount of total RNA. For this purpose, we referred the amount of RNA used for cDNA construction as X (2 μL of total RNA) and other samples were prepared on this base. For example sample number 1 was referred 2X meant 4 μL of total RNA was used.

### 3.2. Synthesis of cDNA

Because when cDNA construction was performed with *Accurase* (DNA amp) enzyme, longer cDNA fragments could be obtained we preferred to use the smart cDNA synthesis method by using *Accurase* enzyme. After concentration determination of total RNAs, we performed RT-PCR by using primers which were designed on constitutively expressed genes. For this purpose 18S primers and Elongation Factor 2 (EF2) primers were used (Figure 3.3., Figure 3.4. and Figure 3.5.)

| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Transferred from | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0.4 | 0 | 0 | 0 | 0 | ($\mu M\ Zn^{2+}$) |
| Transferred to | - | 1 | 1 | 1 | 1 | - | 0 | 0 | 0 | 0 | - | 0.4 | 0.4 | 0.4 | 0.4 | ($\mu M\ Zn^{2+}$) |
| Time points | 0 | 5h | 24h | 3d | 7d | 0 | 5h | 24h | 3d | 7d | 0 | 5h | 24h | 3d | 7d | |

406 bp →

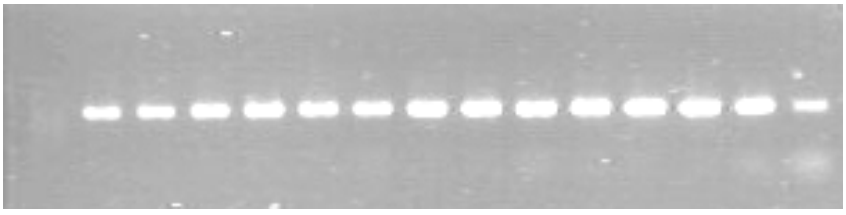


**Figure 3.3.** Appearance of PCR products of group 1 plants performed with 18S primers on 1% agarose gel.

PCR amplification of the sample lane in1 (no zinc at time 0) could not be obtained. The reason probably was due to handling during PCR reaction. Because PCR amplification was performed using EF2 primer set and all of the samples were amplified.

| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **C** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Transferred from | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0.4 | 0 | 0 | 0 | 0 | | ($\mu M\ Zn^{2+}$) |
| Transferred to | - | 1 | 1 | 1 | 1 | - | 0 | 0 | 0 | 0 | - | 0.4 | 0.4 | 0.4 | 0.4 | | ($\mu M\ Zn^{2+}$) |
| Time points | 0 | 5h | 24h | 3d | 7d | 0 | 5h | 24h | 3d | 7d | 0 | 5h | 24h | 3d | 7d | | |

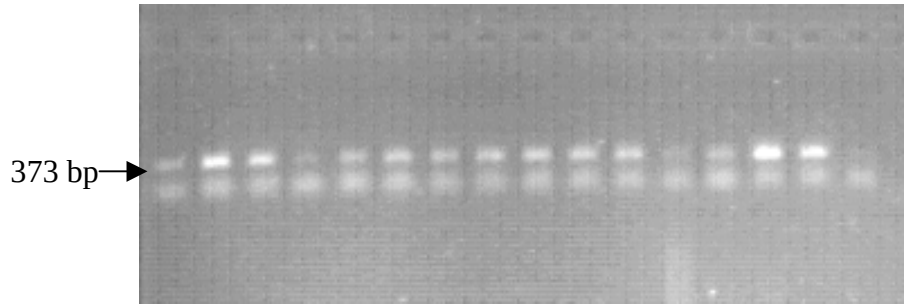



**Figure 3.4.** Appearance of PCR products of group 1 plants performed with EF2 primers on 1% agarose gel. **C:** Negative Control (water)

| | **M** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **C** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Transferred from | | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | | ($\mu M\ Zn^{2+}$) |
| Transferred to | | - | 1 | 1 | 100 | 100 | - | 0 | 0 | 100 | 100 | | ($\mu M\ Zn^{2+}$) |
| Time points | | 0 | 1days | 5days | 1days | 5days | 0 | 1days | 5days | 1days | 5days | | |

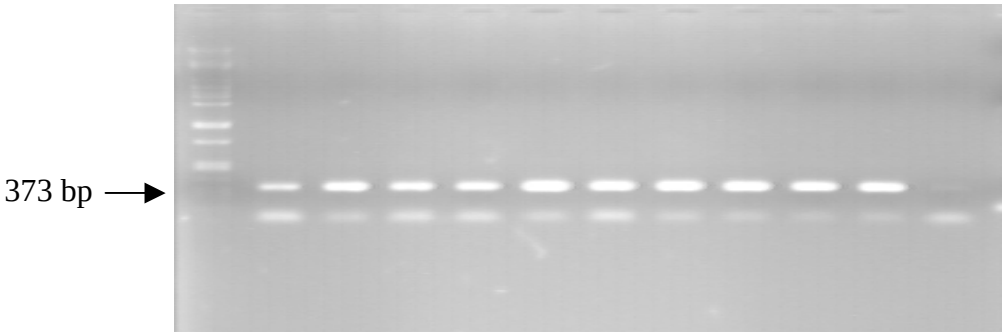



**Figure 3.5.** Appearance of PCR products of group 2 plants performed with EF2 primers on 1% agarose gel. **M:** λ/Pst1, **C:** Negative Control (water)

The reason that PCR reactions with EF2 primers carried out was to check all the cDNAs synthesized with the same efficiency. Because EF2 is a constitutively expressed gene and it is supposed to present the same level amplification in all

samples. We have varied cDNA amounts in order to achieve same levels of amplification by PCR. From then on, we have used the same cDNA amounts for our experiments. If cDNA was needed to be synthesized, all the above experiments were repeated

### 3.3. Optimization of DD analysis

Samples were loaded onto gels twice (Figures 3.7., 3.8., 3.9.) PCR product performed with 1 μL cDNA was loaded into the first lane and PCR product performed with 2 μL cDNA was loaded into the second lane, so that it could be possible to see PCR reactions were carried out correctly.

We performed DD reactions using both first strand cDNAs and second strand cDNAs and differentially expressed bands were observed in reactions using both first strand and second strand cDNAs.

### 3.4. Assesment of results obtained from DD analysis

The PCR products of DD were analyzed as described in the Materials and Methods section. We have tried 20 primer combinations for DD. Many

differentially expressed bands were cut of for cloning and sequencing.

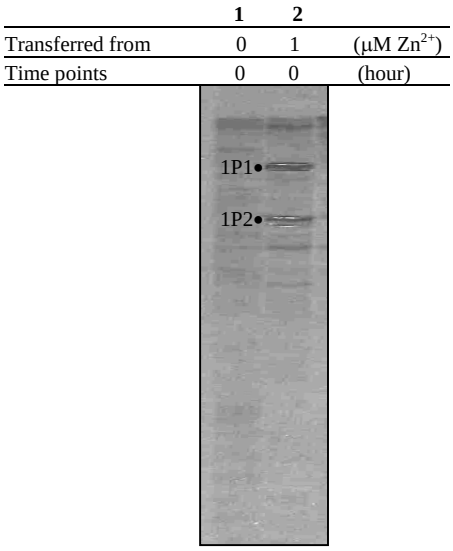



**Figure 3.6.** mRNA Differential Display autoradiograph performed using ss-cDNA of wheat total RNA using P1 and T2 primers.

We cut of two bands. Both of the 1P1 and 1P2 bands were observed only in the samples grown at 1 μM zinc concentration.

| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Transferred from | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | R | C | ($\mu M\ Zn^{2+}$) |
| Transferred to | - | - | 1 | 1 | 1 | 1 | - | - | 0 | 0 | 0 | 0 | | | ($\mu M\ Zn^{2+}$) |
| Time points | 0 | 0 | 5h | 24h | 3d | 7d | 0 | 0 | 5h | 24h | 3d | 7d | | | |

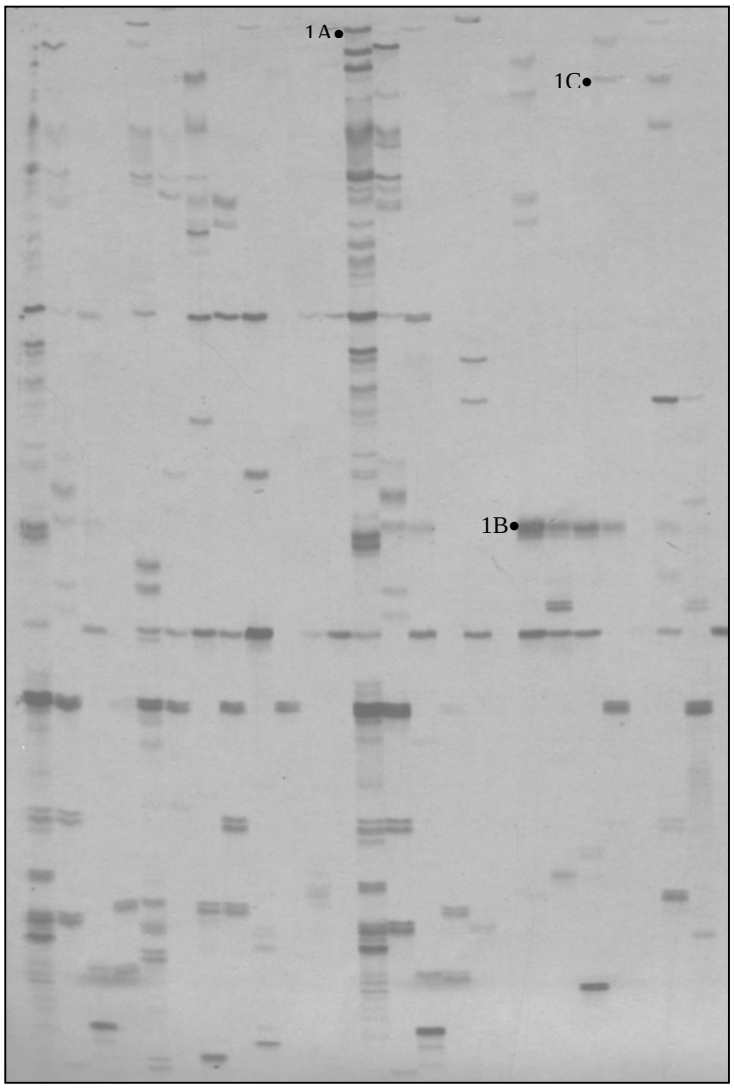



**Figure 3.7.** mRNA Differential Display autoradiograph performed using ss-cDNA of wheat total RNA using P2 and T1 primers. **R:** Total RNA, **C:** Negative Control (water)( To check the concentration verification, every sample was loaded twice, sample in first lane was done with 1 μL cDNA, sample in second lane was done with 2 μL cDNA). To check DNA contamination in RNA samples, total RNA was also loaded.

Band of 1A in lane 7 was observed only in sample grown at no zinc concentration and it could be responsible for zinc uptake mechanism. Band of 1B in lane 10 was detected in sample transferred from 1 μM to no zinc solution at 24 hours and also at 3 days. It also could be related with zinc uptake mechanism. Because its expression induced only when zinc is deficient. Band of 1C in lane 11 was detected in sample transferred from 1 μM to no zinc solution at 3 days and 7 days. It was not observed when zinc is efficient in nutrient solution but it appeared when zinc was absent, so this band also might be related with zinc efficiency mechanism.

| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Transferred from | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | R | C | ($\mu M\ Zn^{2+}$) |
| Transferred to | - | - | 1 | 1 | 1 | 1 | - | - | 0 | 0 | 0 | 0 | | | ($\mu M\ Zn^{2+}$) |
| Time points | 0 | 0 | 5h | 24h | 3d | 7d | 0 | 0 | 5h | 24h | 3d | 7d | | | |

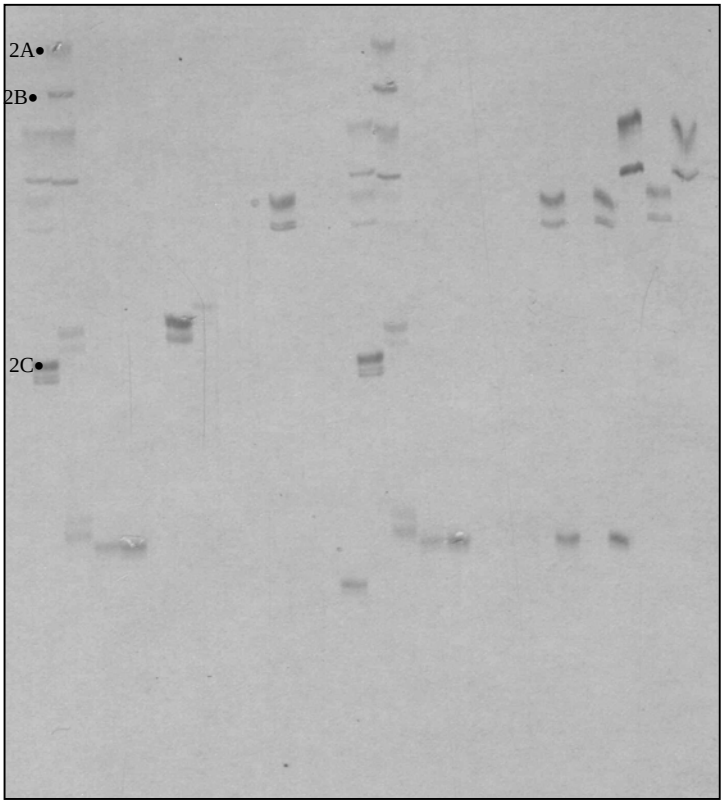



**Figure 3.8.** mRNA Differential Display autoradiograph performed using ss-cDNA of wheat total RNA using P2 and T2 primers. **R**: Total RNA, **C:** Negative Control ( To check the concentration verification, every sample was loaded twice, sample in first lane was done with 1 μL cDNA, sample in second lane was done with 2 μL cDNA)

Bands of 2A, 2B and 2C in lane 1 were only observed in sample grown at no zinc concentration. It was thought that if all of these bands expressed under zinc stress condition, they were possibly responsible for zinc efficiency mechanism.

| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Transferred from | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | R | C | ($\mu M\ Zn^{2+}$) |
| Transferred to | - | - | 1 | 1 | 1 | 1 | - | - | 0 | 0 | 0 | 0 | | | ($\mu M\ Zn^{2+}$) |
| Time points | 0 | 0 | 5h | 24h | 3d | 7d | 0 | 0 | 5h | 24h | 3d | 7d | | | |

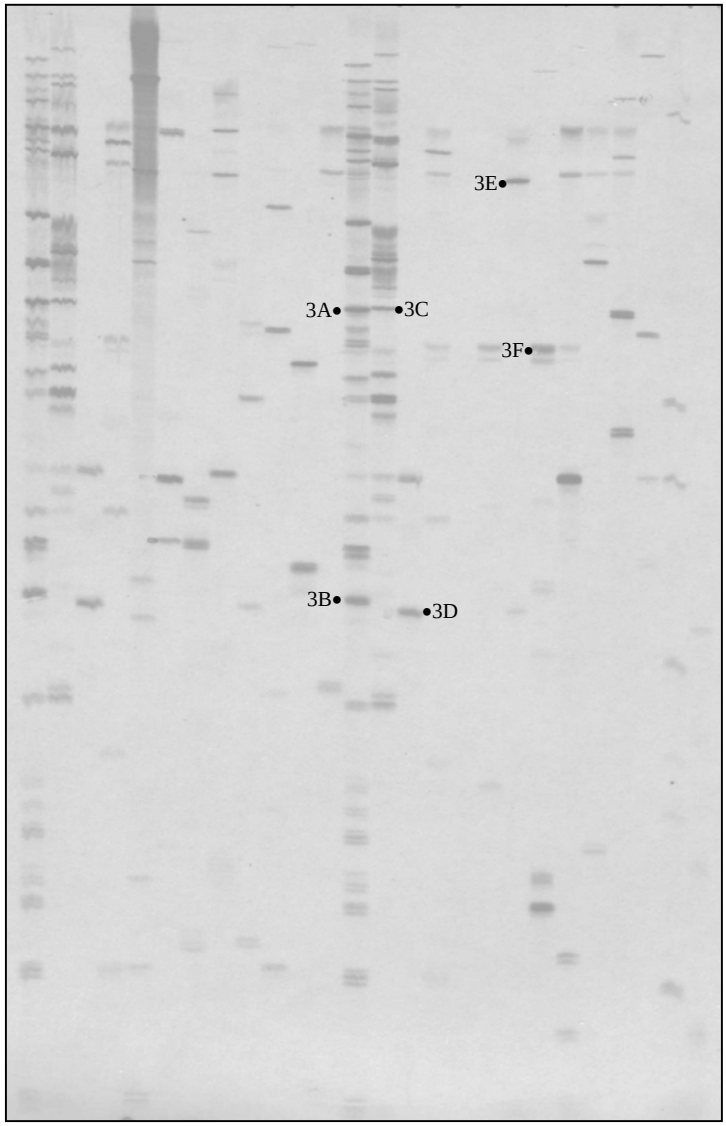



**Figure 3.9.** mRNA differential display autoradiograph performed using ss-cDNA of wheat total RNA using P2 and T3 primers. **R:** Total RNA, **C:** Negative control ( To check the concentration verification, every sample was loaded twice, sample in first lane was done with 1 μL cDNA, sample in second lane was done with 2 μL cDNA)

Bands of 3A, 3B and 3C in lane 7 were detected only in samples grown at no zinc concentration and it is possible that they have role in zinc efficiency mechanism. Band of 3D in lane 8 was detected in sample growth at 1 μM zinc concentration and it possibly related with zinc mechanism because presence of zinc affected its expression and it could be detected only when zinc was at optimum level . Also band of 3E in lane 10 was detected in sample transferred from 1 μM zinc to no zinc concentration at 24 hours, band of 3F in lane 10 was detected in sample transferred from 1 M zinc to no zinc concentration at 3 days. Both of the bands were not observed when zinc was at optimum level, they were appeared when zinc was deficient. So they could be related with zinc efficiency mechanism.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Transferred from | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | ($\mu M\ Zn^{2+}$) |
| Transferred to | - | 1 | 1 | 100 | 100 | - | 0 | 0 | 100 | 100 | ($\mu M\ Zn^{2+}$) |
| Time points | 0 | 1days | 5days | 1days | 5days | 0 | 1days | 5days | 1days | 5days | |

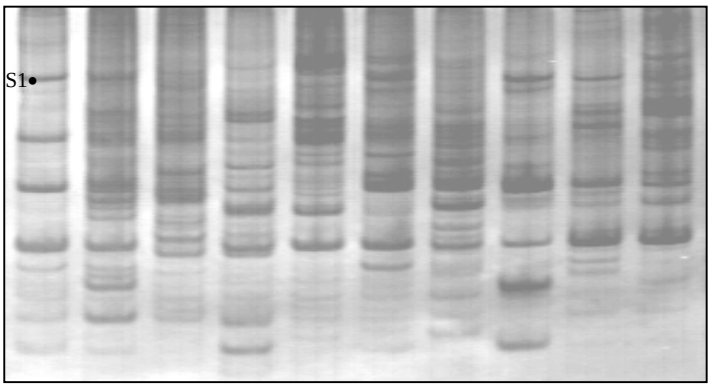



**Figure 3.10.** Appearance of silver stained mRNA Differential Display performed using ds-cDNA of wheat mRNA using P4 and T1 primers.

Band of S1 in lane 1 was observed only in sample growth at no zinc concentration and it could be responsible for zinc uptake mechanism.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Transferred from | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | (μM $Zn^{2+}$) |
| Transferred to | - | 1 | 1 | 100 | 100 | - | 0 | 0 | 100 | 100 | (μM $Zn^{2+}$) |
| Time points | 0 | 1days | 5days | 1days | 5days | 0 | 1days | 5days | 1days | 5days | |

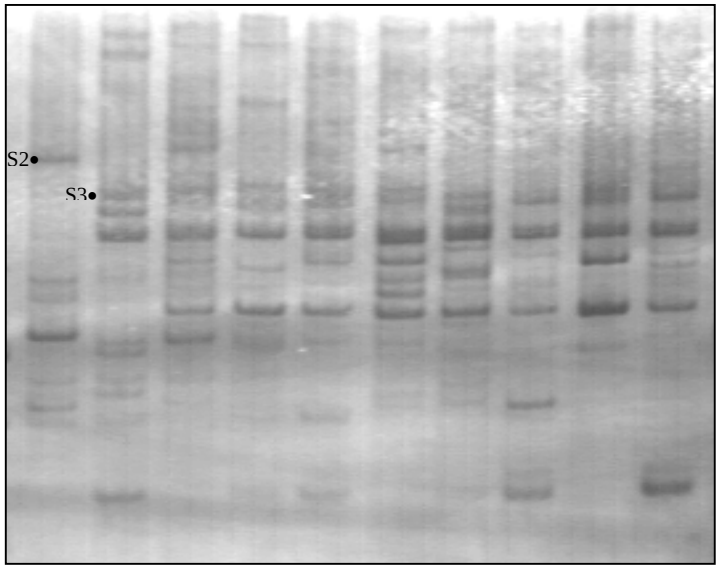



**Figure 3.11.** Appearance of silver stained mRNA Differential Display performed using ds-cDNA of wheat mRNA using P4 and T3 primers

Band of S2 in lane 1 was detected in sample growth at no zinc condition. Because it was expressed only under zinc deficient condition it could play a role in zinc efficiency mechanism. Band of S3 in lane 2 was seen in sample transferred from no zinc to 1 μM zinc condition. So its expression could be induced by zinc and it also might be involved in zinc efficiency mechanism.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Transferred from | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | ($\mu$M $Zn^{2+}$) |
| Transferred to | - | 1 | 1 | 100 | 100 | - | 0 | 0 | 100 | 100 | ($\mu$M $Zn^{2+}$) |
| Time points | 0 | 1days | 5days | 1days | 5days | 0 | 1days | 5days | 1days | 5days | |

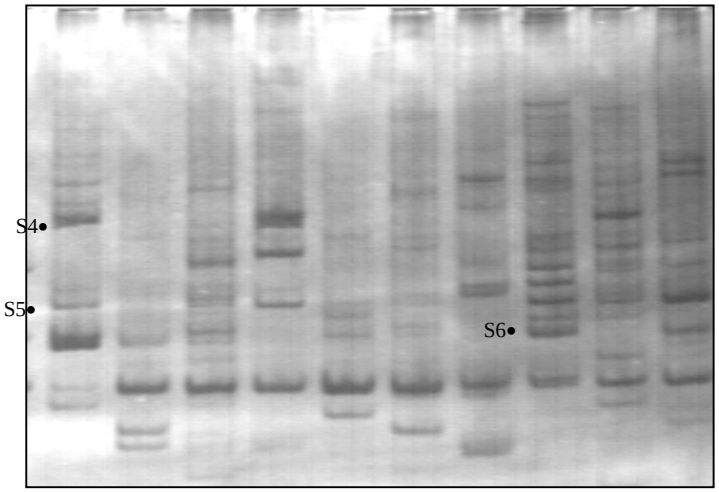



**Figure 3.12.** Appearance of silver stained mRNA Differential Display performed using ds-cDNA of wheat mRNA using P4 and T4 primers

Bands of S4 and S5 in lane 1 were only detected in sample growth in no zinc condition. So it was highly possible that these bands were related to the zinc uptake mechanism. Band of S6 in lane 8 was seen in sample transferred from 1 M to no zinc concentration. It was thought that the band's expression was induced in zinc deficient condition and it could be related to zinc transport.

### 3.5. Reamplifications of selected bands

2 bands from the P1/T2 primer combination, 3 bands from the P2/T1 primer

combination 3 bands from the P2/T2 primer combination and 6 bands from the P2/T3 primer combinations were chosen to be cloned. The selected fragments were precisely cut from DNA denaturing gels and re-amplified using the appropriate primer sets. Re-amplified fragments can be seen in Figure 3.13. and 3.14.

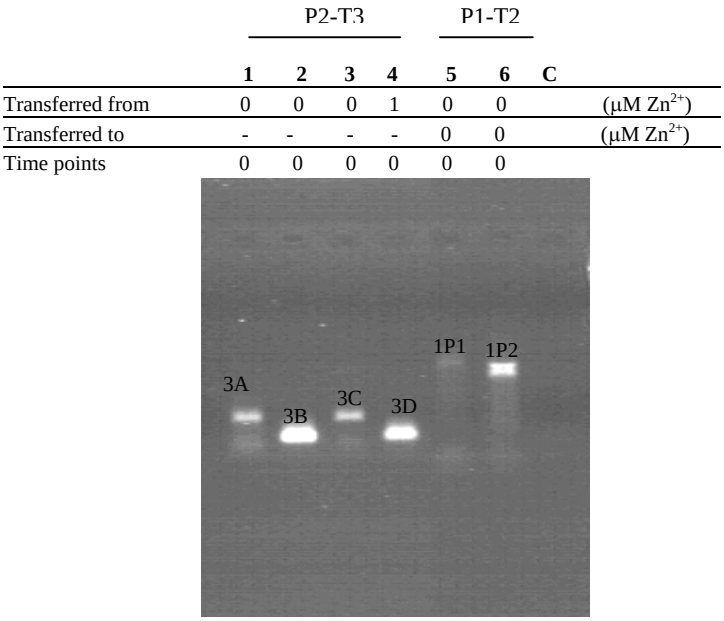



**Figure 3.13.** Appearance of reamplifications of selected DD bands on 1% agarose gel. **C:** Negative Control (Water)

| | P2-T1 | | | P2-T2 | | | P2-T3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **C** | |
| Transferred from | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | | ($\mu M\ Zn^{2+}$) |
| Transferred to | - | 0 | 0 | - | - | - | 0 | 0 | | ($\mu M\ Zn^{2+}$) |
| Time points | 0 | 24h | 3d | 0 | 0 | 0 | 24h | 24h | | |

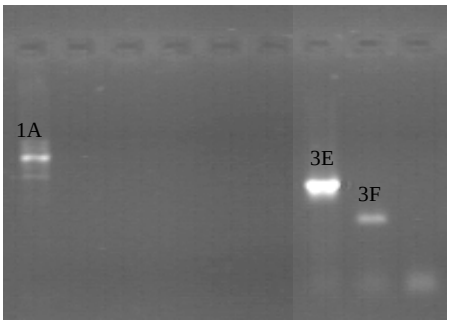



**Figure 3.14.** Appearance of reamplifications of selected DD bands on 1% agarose gel **C:** Negative Control (Water)

5 of the selected bands could not be reamplified. It could be because of degredation of the bands during recovering of the bands.

### 3.6 Cloning and sequencing of differentially expressed fragments

Cloning of the fragments was achieved by ligation into pGEM-T Easy vector and transforming into *E.coli Dh5-α* cells. 2 of 14 selected bands could not be cloned. PCR amplification from the selected colonies was performed and 7 of 12 were observed to be carrying the expected size inserts using primers flanking the pGEM-T Easy promoter region. Plasmids were isolated from these colonies and

prepared for sequencing. In order to confirm the isolation of plasmids carrying the correct inserts were also electrophoresed.

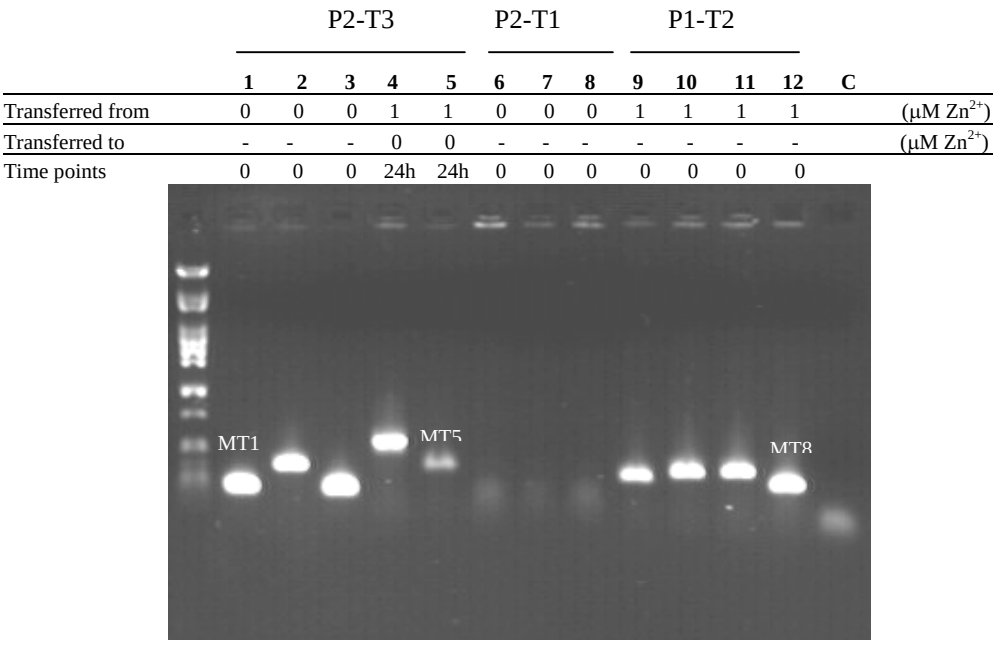



**Figure 3.15.** Appearance of PCR products performed with selected colonies carrying the fragments of putative genes on 1% agarose gel. **C**: Negative Control (Water)

Because samples seen in lanes 6, 7, 8 could not been cloned and in lane 10 and 11 had unexpected inserts in length, they were not sent for sequencing.

### 3.7. Results of the obtained sequences

The obtained sequences were searched in the NCBI (National Center for Biotechnology Information) database for comparison with available genes and DNA sequences. The sequences of bands seen on Figure 3.15. in lane 2, 3, 4 and 9 contained stop codons and they did not give any significant similarity between the sequences in the NCBI Gene Bank. The remining DNA sequences of bands (Table 3.2.) were translated to amino acid sequences (Table 3.3.) (without any stop codons) using Editseq 4.0 (DNA Star Inc.) expert sequence analysis software and Amino acid sequences were searched in the NCBI (National Center for Biotechnology Information) database for comparison with available protein sequences. The result of Blast search for sequence similarities are shown in Table3.4., Table 3.5., Table 3.6. and Table 3.7.

**Table 3.2.** DNA sequences of colonies

**(MT1)**

AGAAAGATAGGGGCTTATAATTTTGCCTCTCAACTATTTACCTCAAGGGTAATG
GCAACAATAATAACTCATGATCACCTACATCCGAGTGGATATATGTGTCTAGAT
CTTTCCCCAACACATGACTCTTGCATAATGGTAATTAAGTA

**(MT5)**

GGAACACAGTTTGAAGATGATTCAGACCAAGAGACCATTGATGCAAAAGATAT
TCTTAAAAGGGAGCTCCAGGATGAACGGAAAACCCTTCCAATCTATAAATTCAG
AGATGAACTGCTCAAGGCTGTTGATGAATATCAGGTTATTGTCATAGTGGGAGA
AACTGGCTCTGGTAAAACGACACAAATACCTCAATATCTTCATGAAGCTGGATA
CACAGCAAGAGGAAAGGTTGCTTGTACACAACCTCGTCGAGTAGCAG

**(MT8)**

CTGCAAGGTTCAATTGATTCTAGCCAAGATCATCTAATGAGCAGTGCAAATGGT
CAGAATAACAGTGGGAATAATGGTGCAATTCCCAAGGTGAACCCCGCAAGTTC
ATTACAGTCAAATCCTTCTACCTCTTTCCCTTCCCAGGTACCTATATCATCCAAT
AACAACATGATGCCTGCCCTTCAGAATACAAACCAACTCAGT

**Table 3.3.** Aminoacid sequences of colonies

**(MT1)**

| |
|---|
| RKIGAYNFASQLFTSRVMATIITHDHLHPSGYMCLDLSPTHDSCIMVIK |

**(MT5)**

| |
|---|
| GTQFEDDSDQETIDAKDILKRELQDERKTLPIYKFRDELLKAVDEYQVIVIVGETGSGKTTQIPQYLHEA GYTARGKVACTQPRRVA |

**(MT8)**

| |
|---|
| LQGSIDSSQDHLMSSANGQNNSGNNGAIPKVNPASSLQSNPSTSFPSQVPISSNNNMMPALQNTNQLS |

**Table 3.4.** Related amino acid sequences with sequenced colonies

| Band | Blast Hits | Accession Number | Score (Bits) | E Value | Identities |
|---|---|---|---|---|---|
| MT1 | zinc transporter [Thlaspi caerulescens] | AAK69428 | 25 | 38 | 10/23 (43%) |
| | zinc transporter [Thlaspi caerulescens] | AAK69428 | 17.6 | 38 | 5/10 (50%) |
| | F-box protein family [Arabidopsis thaliana] | NP_195911 | 26.09 | 8.8 | 7/7 (100%) |
| | expressed protein [Arabidopsis thaliana | NP_179336 | 26 | 16 | 8/13 (61%) |
| | Hypothetical protein [Oryza sativa (japonica cultivar-group)] | AAO16992 | 26 | 16 | 8/10 (80%) |
| | disease resistance protein (TIR-NBS-LRR class), putative [Arabidopsis thaliana] | NP_176590 | 24 | 51 | 15/29 (51%) |
| | resistant specific protein-2 [Vigna radiata] | BAC22500 | 24 | 69 | 7/9 (77%) |
| | hypothetical protein AT4g30990 [imported] - Arabidopsis thaliana | H85362 | 24 | 69 | 8/12 (66%) |
| | unknown protein [Arabidopsis thaliana] | BAC43622 | 24 | 69 | 9/13 (69%) |
| | expressed protein [Arabidopsis thaliana] | NP_191743 | 24 | 69 | 9/13 (69%) |
| | expressed protein [Arabidopsis thaliana] | NP_193885 | 24 | 69 | 7/10 (70%) |
| | PRL2 protein - Arabidopsis thaliana (fragment) | S49821 | 24 | 92 | 12/20 (60%) |

continued

| Band | Blast Hits | Accession Number | Score (Bits) | E Value | Identities |
|---|---|---|---|---|---|
| MT5 | unnamed protein product [Oryza sativa (japonica cultivar-group)] | BAA96166 | 81 | 4e-16 | 35/66 (53%) |
| | hypothetical protein F3I3.40 - Arabidopsis thaliana | T10542 | 70 | 6e-13 | 30/70 (42%) |
| | ABC-type transport protein T18B16.180 - Arabidopsis thaliana | T04442 | 32 | 09 | 10/15 (66%) |
| | ABC transporter family protein [Arabidopsis thaliana] | NP_194759.1 | 30 | 09 | 10/15 (66%) |
| | ABC transporter family protein [Arabidopsis thaliana] | NP_850781 | 30 | 22 | 20/56 (35%) |
| | ABC transporter-like protein [Arabidopsis thaliana] | BAB11402 | 30 | 22 | 20/56 (35%) |
| | ABC transporter family protein [Arabidopsis thaliana] | NP_191473 | 30 | 22 | 12/15 (80%) |
| | ABC transporter-like protein - Arabidopsis thaliana | T47796 | 30 | 22 | 12/15 (80%) |
| | ABC transporter family protein [Arabidopsis thaliana] | NP_568169 | 29 | 22 | 20/56 (35%) |
| | MDR-like ABC transporter [Oryza sativa (japonica cultivar-group)] | CAD59587 | 29 | 39 | 10/13 (76%) |
| | Multidrug resistance protein 1 homolog [Triticum aestivum] | BAB85651 | 29 | 39 | 10/13 (76%) |
| | Putative multidrug resistance protein 1 homolog [Oryza sativa (japonica cultivar-group)] | BAC10738 | 30 | 39 | 10/13 (76%) |
| | GTP-binding protein - related [Arabidopsis thaliana] | NP_187182 | 30 | 29 | 10/15 (66%) |
| | Zinc finger (C3HC4-type RING finger) protein family [Arabidopsis thaliana] | NP_563717 | 30 | 22 | 16/39 (41%) |
| | Resistance gene analog NBS7 [Helianthus annuus] | AAL07542 | 30 | 29 | 16/33 (48%) |

continued

| Band | Blast Hits | Accession Number | Score (Bits) | E Value | Identities |
|---|---|---|---|---|---|
| MT8 | unknown protein [Oryza sativa] | AAF34437 T18548 | 146 | 1e-35 | 51/68 (75%) |
| | flax rust resistance protein M – flax | | 29 | 2.6 | 13/31 (41%) |
| | bZIP DNA-binding protein HBF-1 – soybean | T07154 | 29 | 3.5 | 9/18 (50%) |
| | bZIP transcription factor [Nicotiana tabacum] | AAL27150 | 29 | 3.5 | 9/18 (50%) |
| | leucine-rich repeat transmembrane protein kinase, putative [Arabidopsis thaliana] | NP_569046 | 29 | 4.6 | 15/28 (53%) |
| | leucine rich repeat protein family [Arabidopsis thaliana] | NP_181808 | 28 | 8.4 | 15/28 (53%) |
| | zinc finger (C3HC4-type RING finger) protein family [Arabidopsis thaliana] | NP_175727 | 28 | 6.2 | 15/29 (51%) |
| | protein kinase-related [Arabidopsis thaliana] | NP_190153 | 27 | 11 | 9/13 (69%) |
| | disease resistance protein-related [Arabidopsis thaliana] | NP_680723 | 27 | 15 | 9/13 (69%) |
| | disease resistance protein homolog F24J7.80 -Arabidopsis thaliana | T06145 | 27 | 15 | 9/13 (69%) |
| | putative pathogenesis-related protein [Oryza sativa (japonica cultivar group)] | BAC57321 | 27 | 15 | 13/27 (48%) |

Translated clone MT1 revealed 43% homology with zinc transporter gene [*Thlaspi caerulescens*]. It is supposed that zinc transporter genes are expressed or overexpressed under zinc deficient conditions, in order to plant can extract maximum amount of zinc from soil. Our result supports this mechanism. Because MT1 was obtained at condition of no zinc concentration.

The other band giving aminoacid homology was clone MT5. It showed 80% homology with ABC transporter . This result is very logical. ABC transporters belong to ATP-Binding Cassette (ABC) superfamily. These transporters have active import transport systems components of ferric iron uptake transporter and manganese/zinc/iron chelate transporter. Also they have active export transport systems components of drug/siderophore exporter-3. This band was observed at samples transferred from 1μM zinc to no zinc at 24h. condition. Also this band was seen in sample grown in no zinc concentration and 1μM zinc to no zinc at 5h condition with less band intensity. It means ABC transporter gene was expressed at no zinc concentration, not at 1μM zinc concentration. Root does not need to take more zinc, because there is enough zinc in environment. But when the plant transferred to solution having no zinc concentration, plant expresses more zinc transporter genes to uptake more zinc.

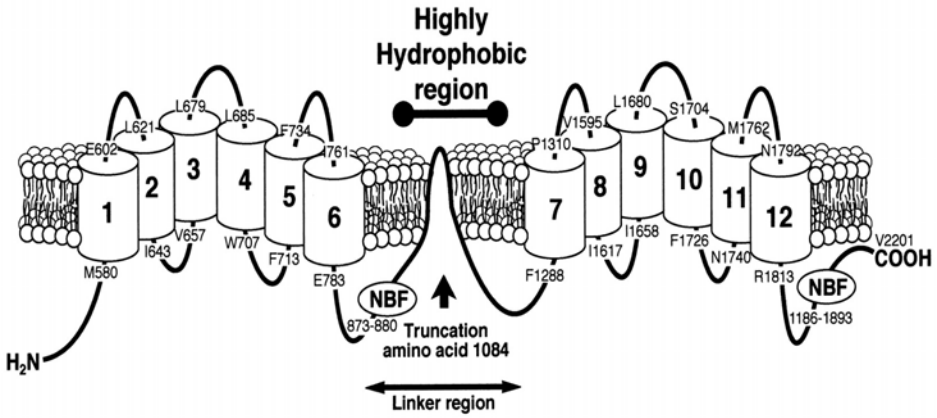



**Figure 1.3.** Protein structure of ABC transporter family.

Translated clone MT8 has 75% homology with ADH (Alcohol Dehydrogenase). Zinc-containing ADH's are dimeric or tetrameric enzymes that bind two atoms of zinc per subunit. One of the zinc atom is essential for catalytic activity while the other is not. Both zinc atoms are coordinated by either cysteine or histidine residues; the catalytic zinc is coordinated by two cysteines and one histidine. Zinc-containing ADH's are found in bacteria, mammals, plants, and in fungi. In most species there are more than one isozyme (for example, human have at least six isozymes, yeast have three, etc.). A number of other zinc-dependent dehydrogenases are closely related to zinc ADH and are included in this family.

**Table3.5.** Alignments of amino acid sequence of clone MT1 generated with ClustalX (1.8) software.

6.....THDHL.....SGY.....
8.....HDHL....PS...Y.....
2.....TI.THDH.....D....PTHD.....
4. .....TIITHDH.....
3.....VMA...II...HDH.....
13..... ...H..HLH.....GY...CL...L..PT...D.....
1 RKIGAYNFASQLFTSRVMATIITHDHLHPSGYMCLDLSPTHDSCIMVIK
12.....PSGY...CLDLS.....
10.....HPS...Y...CLDL..P.....
11.....HPS...Y...CLDL..P.....
9.....CLDLS..T.....CI.....
5.....LDLS.....DSCIMV.....

1.Clone MT1
2. zinc transporter [Thlaspi caerulescens]
3. zinc transporter [Thlaspi caerulescens]
4. F-box protein family [Arabidopsis thaliana]
5. expressed protein [Arabidopsis thaliana]
6. Hypothetical protein [Oryza sativa (japonica cultivar-group)]
7. disease resistance protein (TIR-NBS-LRR class), putative [Arabidopsis thaliana]
8. resistant specific protein-2 [Vigna radiata]
9. hypothetical protein AT4g30990 [imported] - Arabidopsis thaliana
10. unknown protein [Arabidopsis thaliana]
11.expressed protein [Arabidopsis thaliana]
12.Expressed protein [Arabidopsis thaliana]

**Table 3.6.** Alignments of amino acid sequence of clone MT5 generated with ClustalX (1.8) software.

```
3.....IL..RELQD.....LPIY....FR..ELLK...V...E....QVIVIVGETGSGKTT
4.....QVIVIVGE...GSGKTT
5.....QVIVIVGE...GSGKTT
1 GTQFEDDSDQETIDAKDILKRELQDERKTLPIYKFRDELLKAVDEYQVIVIVGETGSGKTT
6.....TLPIYKFRDELL...AVDE.....VI..IVG...TGSGKTT
7.....TLPIYKFRDELL...AVDE.....VI..IVG...TGSGKTT
10.....TLPIYKFRD.....L...AVDE.....VI..IVG...TGSGKTT
8.....I..IVG...TGSGKTT
9.....I..IVG...TGSGKTT
12.....IVIVGE.....SGKTT
11.....IVGETGSGKTT
13.....IVGETGSGKTT
14.....IVGETGSGKTT
2 .....LPIYKFR.....LL.....Y..VIV.VGETGSGKTT
16..... ..... DE...TLP....R..ELLKAV....E…QV.VIV.....
15.....EDDSDQ.. ..IDA ..DIL..RELQDE.....L..... .....FRDE.....
```

```
3  QI-QYLEAG.....
4  .....
1  QIPQYLHEAGYTARGKVACTQPRRVA
6  .....
7  .....
10 .....
8  ...I.....
9  ...I.....
12 ...I.....
11 ...I.....
13 ...I.....
14 ...I.....
2  QIPQYL...EAGY.....GKV...CTQ.....
16 .....
15. .....
```

1. Clone MT5
2. Unnamed protein product [Oryza sativa (japonica cultivar-group)]
3. Hypothetical protein F3I3.40 - Arabidopsis thaliana
4. ABC-type transport protein T18B16.180 - Arabidopsis thaliana
5. ABC transporter family protein [Arabidopsis thaliana]
6. ABC transporter family protein [Arabidopsis thaliana]
7. ABC transporter-like protein [Arabidopsis thaliana]
8. ABC transporter family protein [Arabidopsis thaliana]
9. ABC transporter-like protein - Arabidopsis thaliana
10. ABC transporter family protein [Arabidopsis thaliana]
11. MDR-like ABC transporter [Oryza sativa (japonica cultivar-group)]
12. Multidrug resistance protein 1 homolog [Triticum aestivum]
13. Putative multidrug resistance protein 1 homolog [Oryza sativa (japonica cultivar-group)]
14. GTP-binding protein - related [Arabidopsis thaliana]
15. Zinc finger (C3HC4-type RING finger) protein family [Arabidopsis thaliana]
16. Resistance gene analog NBS7 [Helianthus annuus]

**Table 3.7.** Alignments of amino acid sequence of clone MT8 generated with ClustalX (1.8) software.

```
1. LQGSIDSSQDHLMSSANGQNNSGNNGAIPKVNPASSLQSNPSTSFPSQVPISSNNNMMPALQNTNQLS
12.....LMSSANGQN...SGNNGA.....
6.. .....SGNNG...IP...V...P...SS..QSNP.....
4.....IP...VNPASSL..S...PSTSF.....
5.....IP...VNPASSL..S...PSTSF.....
3.....VNPASS.QSNPS...SFPS.....
7.....P..SSLQSNPS...S.....QVPISS.....
2  LQGSIDS...QDH..MSSANG.....SGNN...AIPKVN.....SSLQS....PSTSFPS..VP.SSNNNMMPA..QNTNQLS...
9.....SSNNNMMP...LQN.....
10.....ISSN.....MMP...LQ.....
11.. .....ISSN.....MMP...LQ.....
8... .....SLQ...NPS.....FP..Q.P.....NNNM....PAL.....
```

1.Clone MT8
2. adh 1-2 protein region [Oryza sativa]
3. flax rust resistance protein M – flax
4. bZIP DNA-binding protein HBF-1 – soybean
5. bZIP transcription factor [Nicotiana tabacum]
6. leucine-rich repeat transmembrane protein kinase, putative [Arabidopsis thaliana]
7. leucine rich repeat protein family [Arabidopsis thaliana]
8. zinc finger (C3HC4-type RING finger) protein family [Arabidopsis thaliana]
9. protein kinase-related [Arabidopsis thaliana]
10. disease resistance protein-related [Arabidopsis thaliana]
11. disease resistance protein homolog F24J7.80 -Arabidopsis thaliana
12. putative pathogenesis-related protein [Oryza sativa (japonica cultivar-group)]

The translated forms of sequences were searched for homologies in available protein motif libraries using the MOTIF (http://motif.genome.ad.jp) program. Significant protein motif similarities with ABC transporter protein were found for MT5. Motif search result was shown in the Table 3.8.

**Table 3.8**.: Protein motif search of MT5 protein result ABC transporter transmembrane region (homologous region with ABC transporter was written bold).

| **Protein sequence of MT5:** |
| --- |
| GTQFEDDSDQETIDAKDILKRELQDERKTLPIYK**FRDELLKAVDEYQVIVIV GETGSGKTTQIPQYLHEAGYTARGKVACT**QPRRVA |

The region having ABC transporter transmembrane domain was written bold.

### 3.8. RT-PCR

#### 3.8.1. Primer design

NCBI gene bank was searched for important genes involved in zinc transport and/or zinc uptake in plant. All plant species having selected gene fragment (Zinc Arabidopsis Transporter) were aligned using Clustal X program (Appendix B). Primers were designed for the conserved regions at 5' and 3' ends using Codehop program.

### 3.8.2. PCR amplification

Products obtained with PCR amplification performed with ZAT primers were electrophoresed and their sizes were at 255 bp (Figure 3.16.). As template second strand cDNAs constructed with mRNAs of group 2 plants were used.

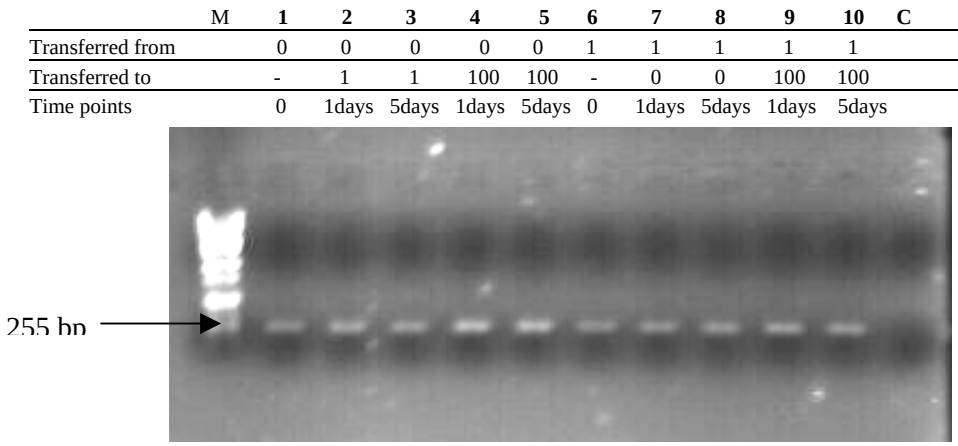



**Figure 3.16.** Appearance of PCR amplifications on first strand cDNAs using ZAT (Zinc Arabidopsis Transporter) primers on 1% agarose gel. M: Marker λ/Pst1, C: Negative Control (Water)

Zaal (1999) showed that ZAT gene was constitutively expressed under all zinc conditions. We aslo obtained the same results. When we performed RT-PCR with ZAT primers, we observed that ZAT gene was constitutively expressed.

### 3.9. Gene walking

3 of the sequenced bands gave significant similarity between the sequences in the gene bank. To determine the whole gene sequence we did gene walking. Reactions were done using biotinlated gene specific MT primers and arbitrary WP primers. In this thesis, we used primers (Table 2.4.) which amplifies through 5' end of gene. Both genomic DNA and cDNA were used seperately as template in PCR reactions (Figure 3.17. and Figure 3.18.). The experiments related to the 3' end amplification will be conducted later.

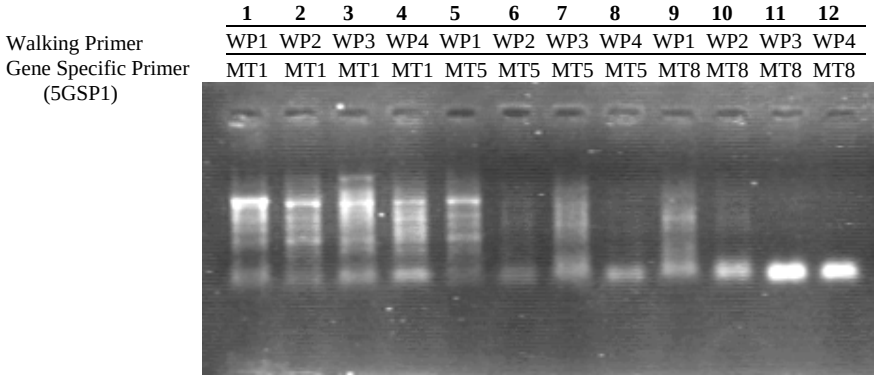



**Figure 3.17.** Appearance of PCR products done by gene walking primers on 1% agarose gel. PCR reactions were performed using Kıraç-66 DNA as template.

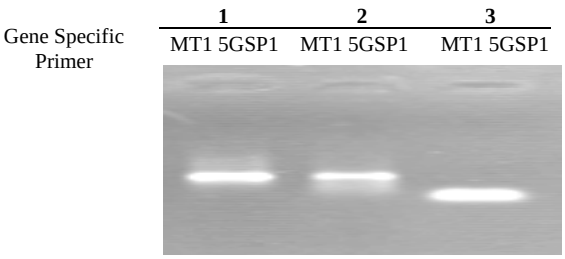



**Figure 3.18.** Appearance of PCR products done by gene specific walking primers and PCR primer on cDNAs

After first PCR , all WP1, WP2; WP3 and WP4 PCR products were combined and they were captured using magnetic beads. Then, we did second PCR on these samples (Figure 3.19.). When doing secong PCR with samples on DNA as primers non-biotinlated MT 5GSP2 primers and short walking primer were used and we obtained the same bands with the first PCR results.

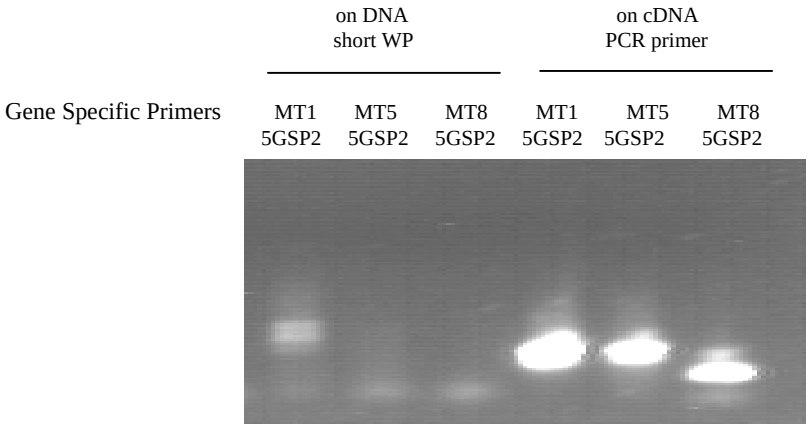



**Figure 3.19.** Appearance of second PCR products on 1% agarose gel.

# CHAPTER IV

# DISCUSSION AND CONCLUSION

Zinc deficiency is one of the major problems causing decrease of grain yield and economic loss. Under low zinc stress conditions, plants overcome the stress by genetic means. Some cultivars of wheat are more efficient then others. Moreover, zinc deficient nutrition results in some developmental abnormalities in children.

In summary, it is thought that there are four major factors playing roles in zinc efficiency: Zinc bioavailability, zinc uptake and translocation, subcellular zinc compartmentation and biochemical zinc utilization (Hacisalihoglu, 2003). Zinc bioavailability is affected by some chemical, physical and biological factors. Low pH increases zinc bioavailabilty. Thus, we grown our plants hydroponically and we maintained pH at 6-6,5 level. Mycorrhizae formation increases zinc uptake, but using hydroponics medium this formation was prevented.

Also iron plaques outside roots affects zinc bioavailability (Zhang, X. et al., 1998). The experiments on rice roots showed that zinc uptake was increased when the amount of iron plaque increased to 12.1 g/kg root dry weight, because zinc was adsorbed on iron plaque and also phytosiderophores could mobilize more zinc. But when the iron plaque was thicker zinc uptake decreased, because zinc concentration in plaque was diluted.

It was showed that there are two root uptake systems for zinc: High affinity transport and low affinity transport (Hacisalihoglu et al., 2001). Low affinity transport system is active when the zinc concentration is at μM levels. But when the zinc concentration decreases at pM levels high affinity transport system is activated in order to uptake very little amount of zinc. In our experiments, we supplied zinc at μM levels and we thought that low affinity transport system was active, but at condition of no zinc high affinity transport system should be involved.

In this study, we attempt to determine genes involved in zinc efficiency in wheat using differential display technology by comparing mRNA levels at different zinc concentrations.

Zinc efficient 'Kıraç-66' bread wheat cultivar seedlings were grown at different zinc concentrations. RT-PCRs were carried out using 20 DD primer

combination sets. Among many, 7 bands were selected for cloning and sequencing. Three clones showed great homology with genes known to be involved in zinc uptake and transport. Gene walking experiments were initiated for these genes to obtain sequences of the rest of the open reading frame.

The clone MT1 with 43% homology (10bp of clone MT1/23bp of zinc transportre gene) with zinc transporter in *Thlaspi caerulescens* was obtained from the roots of the plants grown in the absence of zinc condition and not detected in any other sample. This result supportes that this putative zinc transporter might be responsible for zinc uptake from nutrition medium. Thus the gene fragment we cloned may be a putative zinc transporter responsible for zinc uptake and induced only in the absence of zinc, furthermore it may be involved in high affinity uptake mechanism.

The clone MT5 showed 80% homology (20bp of clone MT5/56bp of ABC transporter gene) wit ABC transporter protein. It seems that ABC transporters have an important role for zinc uptake. In various bacteria, it was showed that several ABC transporters are involved in zinc uptake (Hazzlet et al., 2003, Janulczykr et al., 2003). In Staphylococcus aureus, ABC transporters are regulated by Zur (zinc responsive regulatory element) repressors (Lindsay, J., Foster, S., 2001). It has been showed that ABC transporters are involved in nutrient uptake in many organisms (Appendix A). It was found that ABC transporters were involved in cytocrome c

biogenesis (accession number AAC32374 and AAC34369) and heme transport (accession number CAA56116) in wheat but there is not any document for their role in zinc uptake in wheat. Also wheat heme and cytocrome c ABC transporters and clone MT5 did not have any significant homology (Appendix D). ABC transporters consist of two transmembrane domains and two nucleotide binding domains (Yamaguchi, Nishizawa, Nakanishi, Mori, 2002). According to protein motif search, clone MT5 has ABC transmembrane domain. We have observed that this putative transporter was induced when the plants were transferred from 1 μM $Zn^{2+}$ to no zinc medium. It means that when zinc concentration is at normal level, plant doesn't need ABC transporter like protein, but when the zinc deficiency appears in the environment, plant starts to express ABC transporter like protein to acquire zinc.

Zinc containing alcohol dehydrogenase (ADH) activity is induced in roots when there are flood (Brown et al, 1978). ADH expression is induced under anaerobic conditions to oxidize ethanol. Actually ADH amino acid sequence was found in *Triticum aestivum* but there is no significant homology between ADH gene and clone MT8 (Appendix E). Instead our clone showed homology with ADH of *Oryza sativa.* The clone MT8 might be a different gene of *Triticum aestivum* ADH. ADH is only active in the presence of zinc. We have also found the induced band having homology to ADH (MT8) (75% homology: 51bp of clone MT8/68bp of ADH) at condition of 1 μM zinc concentration.

ZAT (Zinc Arabidopsis Transporter) gene is one of the members of CDF family transporters. Zaal (1999) showed that the ZAT (Zinc Arabidopsis Transporter) gene (accession number AF072858) was constitutively expressed and also in our experiments we have showed for the first time that ZAT gene was constitutively expressed in *Triticum aestivum* too. Zaal also showed that when ZAT expression is overexpressed in *Arabidopsis thaliana* under toxic level of zinc, zinc concentrations of roots were increased but there was no any induction in shoot zinc concentration. Because of this reason it is thought that ZAT gene is involved in vacuolar sequestration of zinc

.

Gene walking experiments were performed through 5' end of the genes. When PCR reactions were carried out on cDNAs, we obtained PCR products around 300 bp in length for MT1 and MT5, 200 bp in length for MT8 (Figure 3.19.). The clone MT1 and *Thlaspi caerulescens* zinc transporter (accession number AAK69428) showed homology starting from 198th amino acid of the zinc transporter at 5' end of the gene. Thus, we might amplify the gene fragment very close to the 5' end.

```
Clone MT1:           20  TIITHDHLHPSGYMCLDLSPTHD   42
                         T+ THDH         D  PTHD
Zinc Transporter:   198  TVTTHDH---------D--PTHD   209
(Thlaspi caeurelescens)
(Accession no: AAK69428)
```

The clone MT5 and *Arabidopsis thaliana* ABC transporter showed homology starting from 152nd amino acid of the ABC transporter at 5' end of the gene. The length of the transporter is 456 bp from 5' end and we obtained about 300 bp PCR product. Again, we might amplify our clone very close to the 5' end.

```
Clone MT5:          29  TLPIY-KFRD----------------
                        TLPI+ KFRD
ABC Transporter: 152  TLPIFLKFRDVTYKVVIKKLTSSVEK

Clone MT5:              ELLK----AVD--EYQVIVIVGETGSGKTT   61
                        E+L     +V+  E  V+ ++G +GSGKTT
ABC Transporter:        EILTGISGSVNPGE--VLALMGPSGSGKTT  205
```

The clone MT8 and *Oryza sativa* ADH gene (accession number AAF34437) showed homology starting from the 683rd amino acid of the ADH at 5' end of the gene but we could obtain PCR product which was about 200 bp in length. To obtain the 5' end of the open reading frame more trials needs to be conducted.

```
Clone MT8:1   LQGSIDSSQDHLMSSANGQNNSGNNGAIPKVNPASSLQSN 40
              LQGS++S QDH MSSANG   SGN+ AIPKVN  +SLQS
ADH:      683 LQGSMNSRQDHPMSSANGPYTSGNSAAIPKVNSTTSLQST 723
(Oryza sativa)
(accession no: AAF34437)

Clone MT8:41 PSTSFPSQVPISSNNNMMPALQNTNQLS  68
              PSTSFPS VP +SNNNMMPA QNTNQLS
ADH:      724  PSTSFPSPVPTTSNNNMMPAPQNTNQLS 750
```

It is believed that ZIP genes are involved in zinc uptake but it is not the key parameter in zinc efficiency mechanism. Also transport of zinc to shoots is not the

key mechanism, because there are no significant differences in shoot zinc concentration, when different plant cultivars grown under zinc sufficient and deficient conditions. Because of these reasons it was suggested that zinc efficiency mechanism is actually a shoot mediated process and biochemical zinc utilization is the major factor in zinc efficiency (Hacisalihoglu et al., 2003). The activity of several shoot localized zinc requiring enzymes is the most important factor involved in zinc efficiency mechanism, such as carbonic anhydrase (CA) and Cu/Zn superoxide dismutase (SOD) enzymes. Rengel (1995) showed that CA activity is higher in zinc efficient wheat genotypes than in zinc deficient genotypes under zinc deficient conditions and this high CA activity help to maintain higher photosynthesis rates and dry matter production. Also Hacisalihoglu (2003) was showed that Cu/Zn SOD gene expression was higher in zinc efficient wheat genotypes than zinc deficient wheat genotypes under zinc deficient condition. Alcohol dehydrogenase (ADH) is one of the zinc requiring enzyme and clone MT8 which showed homology with ADH may be one of the important components involved in zinc efficiency mechanism.

We are suggesting that the SOD and CA transcriptional inductions in the zinc deficient conditions should be used as control when evaluating the roles of other putative genes found to be involved in the zinc efficiency mechanisms.

Future projects:

There were diferentially expressed other bands which were cut out and stored for further analysis. All of these bands should be re-amplified, cloned and sequenced.

We should design primers for DNA sequences based on the obtained in this stıdy in order to study the level of induction more relialy. The induction of these gene fragments should be shown.

Gene walking to 5’ and 3’ ends of the genes should be completed .

We have found 3 putative zinc transporter gene fragments induced in determined conditions. It would be necessary to show whether these genes are also induced in leaves. Because not only uptake but also transport of zinc within plant is important for efficiency mechanism.

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# APPENDIX A

## ATB-Binding Cassette, ABC Transporter-Type Profile

taken directly from
http://www.genome.ad.jp/dbget- bin/www_bget?prosdoc+PDOC00185)

ABC transporters belong to the ATP-Binding Cassette (ABC) superfamily which uses the hydrolysis of ATP to energize diverse biological systems. ABC transporters are minimally constituted of two conserved regions: a highly conserved ATP binding cassette (ABC) and a less conserved transmembrane domain (TMD). These regions can be found on the same protein or on two different ones. Most ABC transporters function as a dimer and therefore are constituted of four domains, two ABC modules and two TMDs.

ABC transporters are involved in the export or import of a wide variety of substrates ranging from small ions to macromolecules. The major function of ABC import systems is to provide essential nutrients to bacteria. They are found only in prokaryotes and their four constitutive domains are usually encoded by independent

polypeptides (two ABC proteins and two TMD proteins). Prokaryotic importers require additional extracytoplasmic binding proteins (one or more per systems) for function. In contrast, export systems are involved in the extrusion of noxious substances, the export of extracellular toxins and the targeting of membrane components. They are found in all living organisms and in general the TMD is fused to the ABC module in a variety of combinations . Some eukaryotic exporters encode the four domains on the same polypeptide chain.

The ABC module (approximately two hundred amino acid residues) is known to bind and hydrolyze ATP, thereby coupling transport to ATP hydrolysis in a large number of biological processes. The cassette is duplicated in several subfamilies. Its primary sequence is highly conserved, displaying a typical phosphate-binding loop: Walker A and a magnesium binding site: Walker B. Besides these two regions, three other conserved motifs are present in the ABC cassette: the switch region which contains a histidine loop, postulated to polarize the attaching water molecule for hydrolysis, the signature conserved motif (LSGGQ) specific to the ABC transporter, and the Q-motif (between Walker A and the signature), which interacts with the gamma phosphate through a water bond. The Walker A, Walker B, Q-loop and switch region form the nucleotide binding site.

The 3D structure of a monomeric ABC module adopts a stubby L-shape with two distinct arms. ArmI (mainly beta-strand) contains Walker A and Walker B. The

important residues for ATP hydrolysis and/or binding are located in the P-loop. The ATP-binding pocket is located at the extremity of armI. The perpendicular armII contains mostly the alpha helical subdomain with the signature motif. It only seems to be required for structural integrity of the ABC module. ArmII is in direct contact with the TMD. The hinge between armI and armII contains both the histidine loop and the Q-loop, making contact with the gamma phosphate of the ATP molecule.

ATP hydrolysis leads to a conformational change that could facilitate ADP release. In the dimer the two ABC cassettes contact each other through hydrophobic interactions at the antiparallel beta-sheet of armI by a two-fold axis.

Proteins known to belong to this family are classified in several functional subfamilies depending on the substrate used. All different types of transporters with a functional attribution are listed below (references are only provided for recently determined sequences).

**In prokaryotes:**

Active import transport systems components:

- Carbohydrate uptake transporter.
- Cobalt uptake transporter (cbiO).
- Ferric iron uptake transporter.
- Hydrophobic amino acid uptake transporter.
- Iron Chelate uptake transporter.
- Manganese/Zinc/Iron chelate uptake transporter.
- Molybdate uptake transporter.
- Nitrate/Nitrite/Cyanate uptake transporter.
- Peptide/Opine/Nickel uptake transporter.
- Phosphate uptake transporter.
- Phosphonate uptake transporter.
- Polyamine/Opine/Phosphonate uptake transporter.

Quaternary amine uptake transporter.

- Sulfate uptake transporter.
- Taurine uptake tranporter (tauB).
- Thiamin uptake transporter (thiamin/thiamin pyrophosphate) (thiQ/yabJ).
- Vitamine B12 uptake tranporter (btuC).

Active export transport systems components:

- Capsular polysaccharide exporter (kpsT).
- Drug exporter-1: daunorubicin/doxorubicin (drrA); oleandomycin (oleC4).
- Drug resistance ATPase-1.
- Drug/siderophore exporter-3.
- Glucan exporter: Beta-(1,2)-glucan export (chvA/ndvA).
- Lipid A exporter (msbA).
- Lantibiotic exporter: hemolysin/bacteriocin (cylB).
- Lipooligosaccharide exporter (nodulation protein nodI from Rhizobium).
- Lipopolysaccharide exporter (rbfA).
- Micrococin B17 exporter (mcbF).
- Micrococin J25 exporter (mcjD).
- Peptide -2 exporter: competence factor (comA/comB).
- Peptide-3 exporter: modified cyclic peptide (syrD.
- Protein-1 exporter: hemolysin (hlyB).
- Protein-2 exporter: colicin V(cvaB).
- S-layer protein exporter (rsaD/sapD).
- Techoic Acid Exporter (tagH).

**In eukaryotes:**

- ALDP, a peroxisomal protein involved in X-linked adrenoleukodystrophy.
- Antigen peptide transporters 1 (TAP1, PSF1, RING4, HAM-1, mtp1) and 2 (TAP2, PSF2, RING11, HAM-2, mtp2), which are involved in the transport of antigens from the cytoplasm toa membrane-bound compartment for association with MHC class I molecules.
- Cystic fibrosis transmembrane conductance regulator (CFTR), which is most probably involved in the transport of chloride ions.
- Drosophila proteins white (w) and brown (bw), which are involved in the import of ommatidium screening pigments.
- Fungal elongation factor 3 (EF-3).
- Multidrug transporters (Mdr1) (P-glycoprotein), a family of closely related proteins which extrude a wide variety of drugs out of the cell.
- 70 Kd peroxisomal membrane protein (PMP70).
- Sulfonylurea receptor, a putative subunit of the B-cell ATP-sensitive potassium channel.

## APPENDIX B

Multiple sequence alignment of *Hordeum vulgare* ZAT mRNA and *Arabidopsis thaliana* mRNA using CLUSTAL X (1.81). The sequences where primers were designed were written bold.

Hor referred to *Hordeum vulgare,* Ara referred to *Arabidopsis thaliana*.

```
Hor  ------------------------------------------------------------
Ara  AAAAAGGGTTAGGTGGATCTCTCATGAAATCGGTGGAGCTCCCCCATATCTTTTTTAAAT

Hor  ------------------------------------------------------------
Ara  AGCTTTCTTCTTTTGGCCCCTTACCCAGAGATAGATTCTTATATACTACTGAGAATCTTC

Hor  ------------------------------------------------------------
Ara  AATTTCTGCAACTTTTGTATCTCTTCCTGATCACCAAATAAGAAAACTCTTGTGGTTCTA

Hor  ------------------------------------------------------------
Ara  TTGAATTGGGTTTCCACTATCTTTATTGTAGAGATGGAGTCTTCAAGTCCCCACCATAGT

Hor  ------------------------------------------------------------
Ara  CACATTGTTGAGGTTAATGTTGGAAAATCTGATGAAGAGAGAATAATTGTGGCGAGTAAA

Hor  ------------------------------------------------------------
Ara  GTCTGTGGAGAAGCACCATGTGGGTTTTCAGATTCTAAGAATGCTTCCGGGGATGCTCAC
```

```
Hor  ------------------------------------------------------------
Ara  GAACGCTCTGCTTCTATGCGGAAGCTTTGTATCGCCGTCGTGCTGTGTCTAGTGTTCATG

Hor  ------------------------------------------------------------
Ara  AGTGTTGAAGTTGTTGGTGGGATTAAAGCCAATAGTTTAGCTATATTAACCGATGCAGCT


Hor  ------------------------------------------------------------
Ara  CATTTGCTCTCTGACGTTGCTGCCTTTGCTATCTCCCTCTTCTCATTGTGGGCTGCTGGC

Hor  ------------------------------------------------------------
Ara  TGGGAAGCGACTCCTAGGCAGACTTACGGGTTCTTCAGGATTGAGATTTTGGGTGCTCTT

Hor  ------------------------------------------------------------
Ara  GTATCTATCCAGCTCATTTGGTTGCTCACGGGTATTCTGGTTTATGAAGCGATTATCAGA

Hor  ------------------------------------------------------------
Ara  ATTGTTACAGAGACCAGTGAGGTTAATGGATTCCTCATGTTTCTGGTTGCTGCCTTTGGT

Hor  ------------------------------------------------------------
Ara  CTAGTGGTGAACATCATAATGGCTGTTCTGCTAGGGCATGATCATGGTCCACAGTCATGG

Hor  ------------------------------------------------------------
Ara  ACATGGGCATGGCACGGCCATGACCATCACAATCATAGCCATGGGGTGACTGTTACCACT

Hor  ------------------------------------------------------------
Ara  CATCACCATCATCACGATCATGAACATGGCCATAGTCATGGTCATGGAGAGGACAAGCAT

Hor  ------------------------------------------------------------
Ara  CATGCTCATGGGGATGTTACTGAGCAATTGTTGGACAAATCGAAGACTCAAGTCGCAGCA

Hor  ------------------------------------------------------------
Ara  AAAGAGAAAAGAAAGAGAAACATCAATCTCCAAGGAGCTTATCTGCATGTCCTTGGGGAT

                          Forward primer ⟶
Hor  ------GAGAGCATTGGTGTAATGATTGGAGGGGCTCTCATCTGGTACAAGCCCGAATGG
Ara  TCCATCCAGAGTGTTGGTGTTATGATTGGAGGAGCTATCATTTGGTACAATCCGGAATGG
            ****  ******* *********** *** **** ******** ** ******

Hor  AAGATTATTGATCTCATATGCACCCTCATCTTCTCTGTGATTGTACTGTTCACCACAATC
Ara  AAGATAGTGGATCTGATCTGCACACTTGCCTTTTCGGTTATTGTCCTAGGAACAACCATC
     *****  * ***** ** ***** **   *** ** ** ***** **    ** ** ***
```


Hor AAGATGATTCGGAACATACTTGAAGTCCTTATGGAGAGCACGCCCCGCGAGATCGATGCC
Ara AACATGATTCGCAACATTCTAGAAGTATTGATGGAGAGTACACCCAGAGAGATTGACGCC
Hor ACCAGGCTTGAGACTGGTCTCCGTGAGATGGAAGGTGTGATTGCTGTCCATGAGCTGCAC
Ara ACAAAGCTCGAAAAGGGTTTGCTCGAAATGGAAGAAGTGGTGGCTGTTCATGAGCTCCAC
Reverse primer
Hor ATCTGGGCTATCACAGTGGGGAAGGTGCTCTTGGCATGCCATGTGACGATCACGCAGGAT
Ara ATATGGGCTATCACAGTGGGAAAAGTGCTATTGGCTTGCCATGTCAATATCAGACCAGAA
Hor GTGGATGCTGATAAAATGCTTGACAAGGTCATTGGGTACATCAAGGCAGAGTACAACATC
Ara GCAGATGCAGATATGGTGCTCAACAAGGTAATTGATTACATCCGCAGGGAGTACAACATT
Hor AGTCATGTGACCATTCAGATTGAGCGAGAGTAAGGCACATGTCAGGTAGTTGGGGATAAA
Ara AGTCATGTCACGATACAAATCGAGCGCTAA-AAGCTAAGTAAGATCTGATGAAGGGTTTT
Hor GGCATTAACGGTTTATCATCT-TAATGCGGTTAATGTTAGCTTTGCACACGCAAAGGCGT
Ara TGTATCAGCA--TTCTCATTAACAATAAAATCAATAAAGTTTCTACTCGTGCC-------
Hor TGCAGGTTCATCTAGCTGTTGCCTTTGGTGCTGGAGAAGATATTATATGTACGCGTTTTC
Ara ------------------------------------------------------------
Hor ATTAGCCCCTTAGTTTTATGAACTATTAATCGGGGGATGTAGTCGTTTGTATTCTCCCAT
Ara ------------------------------------------------------------
Hor GGATGCAATTCCAGACATTTTTGAGCCCCTGTGAGT
Ara ----------------------------------

## APPENDIX C

Multible amino acid sequence alignment of Clone MT1 and Zinc transporter gene of *Thlaspi caerulescens* using Clustal X program

```
mt1  ------------------------------------------------------------
zinc MESSSHIIEVNGGRSDEERRAVASKVCGEAPCGFSDAKNVSGDTKERNASMRKLCIAVVL

mt1  ------------------------------------------------------------
zinc CLVFMSVEIVGGIKANSLAIMTGAAHLLSDVAAFAISLFALWAAGWEATPRQTYGFFRIE

mt1  ------------------------------RKIGAYN-FASQLF---TSRVMATIITHDH
zinc ILGALVSIQLIWLLTGILVYEAISRLLTETSEVNGFLMFAVATFGLLVNIIMAVMLGHDH
                                    ::..:  **   *   .. :**.:: ***

mt1  LHPSGY-------------MCLDLSPTHDS-------------------------CIMV
zinc GHSHGHGHDHENHSHGVTVTTHDHDPTHDHDHDHDHDDGHGHSHGEDNQDEAHGDVTEQL
      *. *:                * .****                              :

mt1  IK------------------------------------------------------------
zinc LEKPKQEKEKKKRNINLQGAYLHVLGDSIQSVGVMIGGAAIWYNPKWKIIDLICTLAFSV
     ::

mt1  ------------------------------------------------------------
zinc IVLGTTINMIRNILEVLMESTPREIDATKLEKGLLEMEEVVAVHELHIWAITVGKVLLAC

mt1  ----------------------------------
zinc HVNVTPQADADMVLNKVVDYIRREYNISHVTVQIER
```

Multible amino acid sequence alignment of Clone MT5 and *Arabidopsis thaliana* ABC transporter gene using Clustal X program.

```
mt5  ------------------------------------------------------------
ABC  MIENYWTSFCGNHHTSSNCTVRFLQICFVSALFNGIIGSLDLVLGIWVLRENHSKPLILW

mt5  ------------------------------------------------------------
ABC  LVILIQGFTCCLSVNNAVFGDELAVRTILDVLLLPGSVLLLLSAYKGYRFDESGESSLYE

mt5  ------------------------------------------------------------
ABC  PLNAGDSNGFSEKADFDNRVSQFAKAGLFSTLSFWWLNSLIKRGNVKDLEEEDIPELRKE

mt5  ------------------------------------------------------------
ABC  ERAETCYSLFEENLIEQKRRLGSSCQPSILKVTVLCVWRELLTSGFFAFMKIVAVSAGPL

mt5  ------------------------------------------------------------
ABC  LLNAFILVAEGNASFRYEGLVLAVLLFFSKMIESLSQRQWYFRCRIVGLRVRSLLTAAIN

mt5  ------------------------------------------------------------
ABC  KKQLRLNNSSRLIHSGSEIMNYATVDAYRIGEFPYWFHQLWTTSFQLLIALGILFHSVGV

mt5  ------------------------------------------------------------
ABC  ATFSALAVIILTVLCNAPIAKLQNKFQSELMTSQDERLKACNESLVNMKVLKLYAWESHF

mt5  ------------------------------------------------------------
ABC KKVIEKLRNIELKSLKAVQMRKAYNAVLFWSSPVFVSAATFATCYFLDIPLRASNVFTFV

mt5  ------------------------------------------------------------
ABC  ATLRLVQDPVRMIPDVIGVTIQAKVAFSRIATFLEAPELQGGERRRKQRSEGNQNAIIIK

mt5  ------------------------------------------------------------
ABC  SASFSWEEKGSTKPNLRNVSLEVKFGEKVAVCGEVGSGKSTLLAAILGETPCVSGTIDFY

mt5  ------------------------------------------------------------
ABC  GTIAYVSQTAWIQTGTIRDNILFGGVMDEHRYRETIQKSSLDKDLELLPDGDQTEIGERG

mt5  ------------------------------------------------------------
ABC  VNLSGGQKQRIQLARALYQDADIYLLDDPFSAVDAHTASSLFQEYVMDALAGKAVLLVTH
```

```
mt5 ------------------------------------------------------------
ABC QVDFLPAFDSVLLMSDGEITEADTYQELLARSRDFQDLVNAHRETAGSERVVAVENPTKP


mt5  ------------------------------------------------------------
ABC  VKEINRVISSQSKVLKPSRLIKQEEREKGDTGLRPYIQYMNQNKGYIFFFIASLAQVTFA


mt5  ------------------------------------------------------------
ABC  VGQILQNSWMAANVDNPQVSTLKLILVYLLIGLCSVLCLMVRSVCVVIMCMKSSASLFSQ


mt5  ------------------------------------------------------------
ABC  LLNSLFRAPMSFYDSTPLGRILSRVSSDLSIVDLDVPFGLIFVVASSVNTGCSLGVLAIV


mt5  ------------------------------------------------------------
ABC  TWQVLFVSVPMVYLAFRLQKYYFQTAKELMRINGTTRSYVANHLAESVAGAITIRAFDEE


mt5  ------------------------------------------------------------
ABC  ERFFKKSLTLIDTNASPFFHSFAANEWLIQRLETVSAIVLASTAFCMILLPTGTFSSGFI


mt5  ------------GTQFEDDSD---------QETIDAKDILK---RELQDERK---TLPIY
ABC  GMALSYGLSLNMGLVYSVQNQCYLANWIISVERLNQYTHLTPEAPEVIEETRPPVNWPVT
                  *  :. :.:          * ::    *.    *: :* :   . *:

mt5  ----------KFRDE---LLKAVD-EYQ---VIVIVGETGSGKTTQIP---QYLHEAG--
ABC  GRVEISDLQIRYRRESPLVLKGISCTFEGGHKIGIVGRTGSGKTTLISALFRLVEPVGGK
               ::* *   :**.:.  ::    * ***.******* *.   : :. .*

mt5  YTARG----KVACTQPR-RVA---------------------------------------
ABC  IVVDGVDISKIGVHDLRSRFGIIPQDPTLFNGTVRFNLDPLCQHSDAEIWEVLGKCQLKE
      .. *    *:.  : * *..

mt5  ------------------------------------------------------------
ABC  VVQEKENGLDSLVVEDGSNWSMGQRQLFCLGRAVLRRSRVLVLDEATASIDNATDLILQK


mt5  ------------------------------------------------------------
ABC  TIRREFADCTVITVAHRIPTVMDCTMVLSISDGRIVEYDEPMKLMKDENSLFGKLVKEYW


mt5  ---------
ABC  SHYNSADSR
```

Multible amino acid sequence alignment of Clone MT8 and *Oryza sativa* ADH1-ADH2 region using Clustal X program

```
mt   ------------------------------------------------------------
adh  MVPSGPPNPMGPGQPVGAASLLRTSSSLLSGGQQGMGSGGGMLPSQSPFSSLVSPRTQFG

mt   ------------------------------------------------------------
adh  ANGLLGGGSNVSSLLNRPFGNGGHMLGPGSMPGGGGLPMNTLQQQRGGLDGAGDLVGAGG

mt   ------------------------------------------------------------
adh  SDSLSFPSSSQVSLGNQLGSDNLHPPPQHQQQQQHLDAMQDLQHQHQHQQQLPMSYNQQQ

mt   ------------------------------------------------------------
adh  LPPQPPQQPQATVKLENGGSTGGVKLEPQMGQPDQNSTAQMMRNASNVKIEPPQLQALRS

mt   ------------------------------------------------------------
adh  LSAVKMEQQSSDPSAFLQQQQQQQHLLQLTKQNPQAAAAAQLNLLQQQRILQMQQQQQQQ

mt   ------------------------------------------------------------
adh  QQILKNLPLQRNQLQQQQQQQQQQQQLLRQQSLNMRTPGKSAPYEPGTCAKRLTHYMYHQ

mt   ------------------------------------------------------------
adh  QNRPQDNNIEYWRNFVNEYFSPNAKKRWCVSLYGSGRQTTGVFPQDVWHCEICNRKPGRG

mt   ------------------------------------------------------------
adh  FETTVEVLPRLCQIKYASGTLEELLYVDMPRESQNASGQIVLDYTKAIQESVFEQLRVVR

mt   ------------------------------------------------------------
adh  EGHLRIVFNPDLKIASWEFCARRHEELIPRRSIIPQVSQLGAVVQKYQSAVQNSTNLSTQ

mt   ------------------------------------------------------------
adh  DMQNNCNSFVACARQLAKALEVPLVNDLGYTKRYVRCLQIAEVVNCMKDLIDYSRQNGSG

mt   ------------------------------------------------------------
adh  PIASLHSFPRRTSSGGVNPQQSQQQQPEEQQSIPQSSNQSGQNAAPMTGVQASASANADV

mt   ----------------------LQGSIDSSQDHLMSSANGQNNSGNNGAIPKVNPASSLQ
adh  TSNNSLSCAPSTSAPSPSVVGLLQGSMNSRQDHPMSSANGPYTSGNSAAIPKVNSTTSLQ
                           ****::* *** ******  .***..******.::***
```

```
mt   SNPSTSFPSQVPISSNNNMMPALQNTNQLS-----------------------------
adh  STPSTSFPSPVPTTSNNNMMPAPQNTNQLSSPTASSNLPPMQPPATRPQEPDPNESQSSV
     *.******* ** :******** *******

mt   ------------------------------------------------------------
adh  QRILQDLMMSPQMNGVGQLGNDMKRPNGLTSSVNGVNCLVGNAVTNNSGMGGMGFGAMGG

mt   ------------------------------------------------------------
adh  LGPNHAASGLRTAIANNAMAISGRMGMNHSAHDLSQLGQLQQQQQHQHQHQHQQQQQQ


mt   --------------------------------
adh  QQQQQHDLGNQLLSGLRAANSFNNLQYDWKPSQ
```

## APPENDIX D

Multible nucleotide sequence alignment of Clone MT1 and *Thlaspi caerulescens* zinc transporter gene using Clustal X program.

```
MT1  ------------------------------------------------------------
Zinc GCCCAACGGAGAGACCGAGAGAGCAAAGGAAGGGAAGGGACCCAGAAAAGATGGAGTCTT

MT1  ------------------------------------------------------------
Zinc CAAGTCACATCATTGAGGTTAATGGAGGAAGATCTGATGAAGAAAGAAGGGCTGTGGCAA

MT1  ------------------------------------------------------------
Zinc GTAAAGTCTGTGGAGAGGCACCGTGTGGGTTCTCAGATGCCAAGAATGTTTCAGGGGATA

MT1  ------------------------------------------------------------
Zinc CCAAAGAACGCAATGCTTCTATGCGGAAGCTCTGTATCGCGGTGGTGTTATGTCTTGTGT

MT1  ------------------------------------------------------------
Zinc TCATGAGCGTTGAAATCGTTGGTGGAATCAAAGCCAATAGTTTGGCTATAATGACAGGTG

MT1  ------------------------------------------------------------
Zinc CAGCACATTTGCTCTCTGACGTTGCTGCCTTTGCCATCTCTCTCTTCGCCTTGTGGGCTG

MT1  ------------------------------------------------------------
Zinc CTGGTTGGGAAGCGACGCCGAGGCAGACTTACGGGTTCTTCAGGATTGAGATCTTGGGAG

MT1  --------------------------------AGAAAGATAGGGGCTTATAATTTTGCCT
Zinc CTCTTGTGTCTATCCAGCTCATTTGGTTGCTTACTGGGATCTTGGTTTATGAAGCCATAT
                                       *    ***   ** **** *       *
```

```
MT1  CTCAACTATTTACCTCAAGGGTAATGGCAACAATA-ATAACTCATGATCAC-----CTAC
Zinc CAAGGCTTCTTAC--CGAGACCAGTGAGGTTAATGGATTCCTTATGTTTGCTGTTGCTAC
     *    **  ****  * **   * **     ***  **  ** *** *  *     ****

MT1  ATCCGA---GTGGATATATGTGTCTAGATCTTT--CCCCAACACATGACTCTTG-CATAA
Zinc GTTTGGTCTGCTGGTGAATATCATAATGGCTGTGATGCTTGGGCATGATCATGGTCATAG
      *  *    *  * *  ** *    *   ** *    *     *****   * * ****

MT1  TGGTAATTAAGTA-----------------------------------------------
Zinc TCATGGTCATGGTCATGACCATGAAAACCATAGCCATGGGGTGACTGTTACCACTCATGA
     *  *  * * *

MT1  ------------------------------------------------------------
Zinc TCATGATCCCACTCATGATCATGATCATGATCATGATCACGATGATGGTCATGGTCATAG


MT1  ------------------------------------------------------------
Zinc TCATGGAGAGGACAATCAGGATGAAGCTCATGGAGACGTTACTGAGCAGCTGTTGGAGAA


MT1  ------------------------------------------------------------
Zinc ACCAAAGCAGGAGAAAGAGAAAAAGAAAAGGAACATCAATTTGCAAGGAGCTTATCTTCA


MT1  ------------------------------------------------------------
Zinc TGTTCTTGGTGATTCAATCCAGAGCGTTGGTGTTATGATTGGAGGAGCTGCCATTTGGTA


MT1  ------------------------------------------------------------
Zinc CAACCCGAAATGGAAGATAATTGATCTGATCTGCACTCTTGCCTTTTCGGTTATCGTCTT


MT1  ------------------------------------------------------------
Zinc GGGGACAACCATCAACATGATTCGAAACATTCTTGAAGTGTTGATGGAGAGTACGCCTAG


MT1  ------------------------------------------------------------
Zinc AGAGATTGACGCTACAAAGCTCGAAAAGGGTTTGCTCGAAATGGAAGAAGTGGTGGCTGT


MT1  ------------------------------------------------------------
Zinc TCATGAGCTTCACATTTGGGCTATCACAGTGGGAAAGGTTTTACTTGCTTGCCATGTCAA


MT1  ------------------------------------------------------------
Zinc TGTTACACCACAAGCAGATGCAGATATGGTGCTCAACAAGGTGGTTGATTACATCCGCAG


MT1  ------------------------------------------------------------
Zinc AGAGTACAATATCAGTCATGTAACCGTACAAATCGAGCGCTGAAAGCAAAGCAAAGCAAA
```

```
MT1  ------------------------------------------------------------
Zinc AGCTAAGATCTTGATGTGTTTTTTGTATCAGCATTCTCATTATCAATAATTTTTTCTACC


MT1  ----------------------------------
Zinc CTTTTTGCAAAAAAAAAAAAAAAAAAAAAAAAAA
```

## APPENDIX E

Multible nucleotide sequence alignment of Clone MT5 and *Arabidopsis thaliana* ABC transporter gene using Clustal X program.

```
mt5  ------------------------------------------------------------
ABC ATGATAGAAAATTATTGGACTTCCTTCTGCGGGAATCACCACACATCATCAAACTGCACT

mt5  ------------------------------------------------------------
ABC  GTTCGATTTCTACAGATATGTTTCGGTATCACACTCTCATTCCTAACCCTCTGTATTTGC

mt5  ------------------------------------------------------------
ABC  TTGTTTCACAAGGAACCTCCCAAAAGGATTCATCAATTCTTTTGTCTGCGACTAGTGTCT

mt5  ------------------------------------------------------------
ABC  GCTCTATTCAACGGAATCATCGGATCTCTAGATTTGGTTTTAGGGATTTGGGTTTTACGA

mt5  ------------------------------------------------------------
ABC GAGAATCACAGTAAGCCCTTAATCCTCTGGCTGGTCATTCTGATTCAAGGGTTTACATGG

mt5  ------------------------------------------------------------
ABC  TTATTCATTAACTTAATCATTTGCGTTAGAGGAACAAGAATCCGCAAGTCTTCGTTGAGA

mt5  ------------------------------------------------------------
ABC  TTGTTATCTATCTTCTCCTTCTTTTATGGTTTGGTCTCAAGTTGTTTATCTGTGAACAAT

mt5  ------------------------------------------------------------
ABC  GCTGTGTTTGGAGATGAATTAGCAGTTAGGACAATTCTTGATGTTTTGTTACTTCCTGGG
```

```
mt5  ------------------------------------------------------------
ABC  TCAGTTTTGTTACTCTTAAGTGCTTATAAAGGCTATAGATTTGATGAATCTGGTGAGAGT

mt5  ------------------------------------------------------------
ABC  AGTTTATACGAGCCTCTTAATGCTGGTGATTCAAATGGGTTTAGCGAAAAAGCCGATTTT

mt5  ------------------------------------------------------------
ABC  GATAACCGGGTTAGCCAGTTTGCTAAAGCAGGATTGTTCAGTACGTTATCATTCTGGTGG

mt5  ------------------------------------------------------------
ABC  TTGAATTCTTTGATAAAGAGAGGAAATGTGAAAGACTTGGAGGAAGAAGATATACCCGAG

mt5  ------------------------------------------------------------
ABC  CTTCGAAAGGAAGAGCGTGCAGAGACGTGTTATTCCTTGTTTGAGGAAAATCTCATTGAG

mt5  ------------------------------------------------------------
ABC  CAGAAGAGAAGATTAGGGAGTTCTTGTCAACCTTCGATCTTGAAAGTTACTGTTCTTTGT



mt5  ------------------------------------------------------------
ABC  GTCTGGAGAGAGCTTTTGACCTCTGGTTTCTTTGCTTTTATGAAGATTGTTGCTGTTTCA

mt5  ------------------------------------------------------------
ABC  GCTGGTCCTTTGCTTCTGAATGCTTTCATTTTGGTTGCTGAAGGCAATGCAAGCTTTAGA

mt5  ------------------------------------------------------------
ABC  TATGAAGGGCTTGTGTTGGCTGTGTTGCTGTTCTTCTCAAAGATGATAGAGTCTTTGTCA

mt5  ------------------------------------------------------------
ABC  CAGAGACAATGGTATTTCAGATGTAGAATTGTCGGTTTACGCGTGAGGTCTCTTCTAACT

mt5  ------------------------------------------------------------
ABC  GCAGCTATAAACAAGAAGCAGCTGAGATTGAATAACTCTTCAAGACTGATTCATTCTGGC

mt5  ------------------------------------------------------------
ABC  AGCGAGATTATGAACTACGCTACTGTTGATGCTTACAGAATTGGGGAGTTTCCGTATTGG

mt5  ------------------------------------------------------------
ABC  TTTCACCAGCTTTGGACAACAAGCTTTCAGCTACTAATCGCGCTTGGGATTCTCTTTCAT
```

```
mt5  ------------------------------------------------------------
ABC TCTGTGGGAGTTGCTACGTTTTCAGCTCTAGCTGTGATAATACTTACTGTTTTATGCAAC

mt5  ------------------------------------------------------------
ABC  GCTCCTATCGCAAAACTTCAGAACAAGTTTCAAAGCGAACTCATGACCTCTCAGGACGAG

mt5  ------------------------------------------------------------
ABC  AGGCTGAAGGCCTGCAATGAGTCTCTTGTCAACATGAAGGTCTTGAAGCTCTATGCGTGG

mt5  ------------------------------------------------------------
ABC  GAATCACATTTCAAGAAGGTTATTGAAAAGCTTAGGAACATTGAGTTGAAATCTTTGAAG

mt5  ------------------------------------------------------------
ABC  GCGGTTCAGATGAGAAAGGCTTATAATGCGGTTCTGTTCTGGTCATCACCGGTGTTTGTC

mt5  ------------------------------------------------------------
ABC  TCTGCTGCAACTTTTGCCACTTGTTATTTCCTAGACATTCCATTGAGAGCAAGTAACGTT

mt5  ------------------------------------------------------------
ABC  TTCACTTTTGTGGCAACCCTGCGTCTGGTCCAAGATCCAGTAAGAATGATCCCTGATGTT

mt5  ------------------------------------------------------------
ABC  ATTGGAGTGACAATTCAGGCTAAAGTTGCCTTCAGTCGTATTGCAACATTTCTAGAAGCT

mt5  ------------------------------------------------------------
ABC  CCTGAGCTTCAAGGTGGGGAGAGGCGGAGAAAGCAGAGATCCGAAGGCAATCAGAACGCG

mt5  ------------------------------------------------------------
ABC  ATTATTATTAAATCTGCTAGCTTTTCTTGGGAGGAGAAAGGTTCAACAAAACCAAACTTG

mt5  ------------------------------------------------------------
ABC  AGAAATGTGAGTCTTGAGGTTAAGTTTGGTGAGAAAGTGGCTGTTTGTGGTGAGGTTGGC

mt5  ------------------------------------------------------------
ABC  TCTGGCAAGTCAACACTTTTAGCTGCCATTCTCGGTGAAACTCCATGTGTCTCAGGAACA

mt5  ------------------------------------------------------------
ABC  ATCGATTTTTATGGTACCATAGCCTATGTTTCTCAAACAGCATGGATCCAAACGGGGACA
```

```
mt5  ------------------------------------------------------------
ABC  ATAAGAGATAACATCCTCTTTGGAGGTGTGATGGATGAACACCGTTACCGTGAGACAATT


mt5  -----------------AAGGAACAC-AGTTTGAAGATGATTCAGACCAAGAGACCATT
ABC  CAAAAATCTAGCCTAGACAAAGATCTTGAGCTCTTACCTGATGGAGACCAGACTGAGATT
                      ** ** *   ** *   *  ****  ******       ***

mt5  GATGCAAAAGATATTCTTAAAAGGGAGCTCCAGGATGAACGGAA--AACCCTTCCAATCT
ABC  GGTGAAAGAGGTGTAAATCTAAGTGGAGGACAGAAACAGAGGATTCAACTCGCTCGTGCG
     * ** ** ** * *   *  *** *     *** *  *  ***   *** *   *   *

mt5  ATAAATTCAGAGATGAACTGCTCAAGGCT-GTTGATGAATATCAGGTTATTGT----CAT
ABC  CTATACCAAGATGCAGACATTTATCTCCTTGATGATCCATTTAGTGCTGTTGATGCACAC
      ** *   ***     **   *     ** * ****  ** *   * * ***     **

mt5  AGTGGGAGAAACTGGCTCTGGTAAAACGACACAAATACCTCAAT----ATCTTCATGAAG
ABC  ACTGCTTCAAGTCTGTTCCAAGAATATGTTATGGATGCTCTCGCGGGAAAAGCTGTGTTG
     * **    **    * **    ** * *  *   ** *          *      **  *

mt5  CTGGATACACAGCAAGAGGAAAGGTTGCTTGTACACAACCTCGTCGAGTAGCAG------
ABC  TTGGTTACACATCAAGTGGATTTCTTGCCTGCTTTTGATTCTGTTTTGTTGATGTCAGAT
      *** ****** **** ***    **** **      *    **   ** *  *

mt5   ------------------------------------------------------------
ABC  GGAGAAATCACTGAAGCTGATACATACCAAGAACTCCTAGCTAGAAGCAGAGACTTTCAA


mt5  ------------------------------------------------------------
ABC  GATCTAGTGAACGCTCACAGAGAAACCGCTGGTTCAGAAAGAGTGGTTGCGGTAGAAAAC


mt5  ------------------------------------------------------------
ABC  CCCACCAAACCGGTGAAGGAAATCAACAGAGTCATTTCATCTCAATCCAAGGTCTTGAAA


mt5  ------------------------------------------------------------
ABC  CCGAGTCGTTTGATCAAACAGGAAGAGCGAGAGAAGGGAGACACGGGATTGAGACCGTAC


mt5  ------------------------------------------------------------
ABC  ATACAGTACATGAATCAGAACAAAGGTTACATATTCTTCTTCATCGCGAGCTTAGCTCAG


mt5  ------------------------------------------------------------
ABC  GTCACATTTGCAGTTGGACAGATTCTTCAGAACTCTTGGATGGCTGCAAACGTAGATAAC


mt5  ------------------------------------------------------------
ABC  CCTCAAGTTAGCACTTTGAAGTTGATCTTGGTCTACTTACTGATTGGATTGTGCTCAGTT
```

```
mt5  ------------------------------------------------------------
ABC  CTCTGCTTGATGGTTAGATCTGTCTGTGTCGTGATTATGTGCATGAAGTCATCAGCTTCA

mt5  ------------------------------------------------------------
ABC  TTGTTTTCTCAGCTTCTTAACTCTCTTTTTAGAGCACCTATGTCATTTTATGACTCCACA

mt5  ------------------------------------------------------------
ABC  CCTCTTGGACGGATTCTTAGCAGGGTCTCATCTGACTTGAGCATTGTAGACCTTGACGTT

mt5  ------------------------------------------------------------
ABC  CCTTTTGGTCTGATCTTTGTGGTTGCGTCTTCGGTAAACACAGGTTGTAGTCTTGGAGTG

mt5  ------------------------------------------------------------
ABC  TTAGCTATTGTTACTTGGCAAGTCTTGTTTGTATCTGTTCCCATGGTTTATCTAGCTTTT

mt5  ------------------------------------------------------------
ABC  CGTTTACAGAAGTACTACTTCCAAACAGCTAAAGAGTTGATGCGGATCAATGGCACGACA

mt5  ------------------------------------------------------------
ABC  AGATCTTATGTGGCGAATCATTTAGCAGAATCAGTAGCAGGAGCAATAACAATAAGAGCA

mt5  ------------------------------------------------------------
ABC  TTTGATGAAGAAGAGAGGTTTTTCAAGAAAAGTCTCACACTCATTGATACAAACGCCAGT

mt5  ------------------------------------------------------------
ABC  CCTTTCTTTCATAGCTTTGCAGCGAACGAATGGCTGATCCAGCGGCTTGAAACCGTTAGC

mt5  ------------------------------------------------------------
ABC  GCCATTGTTCTCGCCTCCACTGCTTTCTGCATGATTTTGCTTCCCACAGGAACATTTAGC

mt5  ------------------------------------------------------------
ABC  TCTGGGTTCATCGGTATGGCGCTATCTTATGGTTTATCTTTGAATATGGGACTTGTTTAC

mt5  ------------------------------------------------------------
ABC  TCTGTTCAGAACCAATGTTACTTAGCTAACTGGATCATTTCGGTTGAGAGACTTAATCAG

mt5  ------------------------------------------------------------
ABC  TACACACATTTAACACCTGAGGCTCCTGAAGTAATAGAAGAGACTCGACCACCGGTTAAT
```

```
mt5  ------------------------------------------------------------
ABC  TGGCCGGTCACAGGTCGAGTCGAAATCTCGGATTTGCAGATAAGATACAGAAGAGAATCC

mt5  ------------------------------------------------------------
ABC  CCACTGGTTCTAAAAGGAATCAGCTGCACATTTGAAGGAGGACACAAGATTGGAATTGTT

mt5  ------------------------------------------------------------
ABC  GGCCGAACCGGTAGTGGGAAGACAACTCTGATCAGTGCTCTGTTCAGACTTGTTGAGCCT

mt5  ------------------------------------------------------------
ABC  GTTGGAGGAAAGATTGTCGTTGACGGTGTTGACATCTCCAAAATTGGAGTTCATGATCTG

mt5  ------------------------------------------------------------
ABC  AGATCAAGGTTTGGGATTATACCTCAAGATCCAACTCTCTTCAATGGAACAGTGAGATTT

mt5  ------------------------------------------------------------
ABC  AATCTGGATCCTTTGTGTCAGCATTCAGATGCTGAGATTTGGGAGGTTCTTGGCAAGTGT

mt5  ------------------------------------------------------------
ABC  CAACTAAAAGAAGTGGTTCAAGAAAAAGAGAACGGCTTAGATTCATTAGTTGTGGAGGAT

mt5  ------------------------------------------------------------
ABC  GGATCAAATTGGAGCATGGGACAGAGACAGTTGTTCTGTTTAGGCAGAGCAGTTTTGAGA

mt5  ------------------------------------------------------------
ABC  AGAAGCAGAGTATTAGTCCTGGACGAAGCCACGGCATCGATAGATAATGCAACAGATTTG

mt5  ------------------------------------------------------------
ABC  ATACTTCAGAAAACAATCAGGCGAGAATTTGCAGATTGCACTGTCATTACAGTTGCTCAC

mt5  ------------------------------------------------------------
ABC  CGTATCCCTACCGTTATGGATTGTACAATGGTTCTCTCCATCAGCGACGGACGGATTGTG

mt5  ------------------------------------------------------------
ABC  GAGTATGATGAGCCAATGAAGTTGATGAAGGATGAGAACTCTTTGTTCGGAAAGCTTGTG

mt5 ----------------------------------------
ABC AAAGAGTATTGGTCTCATTACAACTCGGCTGACTCACGTTGA
```

## APPENDIX F

Multible sequence alignment of clone MT5 and ABC transporter genes found in wheat using CLUSTAL X (1.81).

**Clone:** Clone MT5
**1:** ABC transporter CcbB [Triticum aestivum] (Accession number AAC32374)
**2:** ABC transporter CcbB [Triticum aestivum](Accession number AAC64369)
**3:** Putative heme ABC transporter membrane domain [Triticum aestivum] (Accession number CAA56116)
**4:** probable transport protein 240 - wheat mitochondrion (Accession S62088)
**5:** Putative ABC transporter [Triticum monococcum](Accession number AAL74187)
**6:** Putative ABC transporter [Triticum monococcum] (Accession number AAL74186)

```
5               ------MGREPRGNRAPLLDHGETARVPSDLEEGSNVQAANVGFCRVIKLAKPDAWKLIF
6               MVREMRINTAPRGNRVPLLNNGETSRILSDLEEGSNVQAANVGFCRVIKLAKHDAGKLVF
1               ------------------------------------------------------------
2               ------------------------------------------------------------
3               ------------------------------------------------------------
4               ------------------------------------------------------------
clone           ------------------------------------------------------------


5               ATTALIIASLSNLLVPKYGGKIIDIVSRDVRLPEDRAQALADVNGTILYIVLIVVTGSAC
6               ATIALLVASLSNLLVPKYGGKIIDIVSRDVQRPEDKAQALADVNGTILYIVLIVVTGSVC
1               ------------------------------------------------------------
2               ------------------------------------------------------------
3               ------------------------------------------------------------
4               ------------------------------------------------------------
clone           ------------------------------------------------------------
```

```
5             TALRAWVFNSASERVVARLRHDLFSHLINQEIAFFDVTRTGELLSRLSEDTQIIKNAATT
6             TALRAWLFNSASERVVARLRQDLFSHLVNQEIAFFDVTRTGELLSRLSEDTQIIKNAATT
1             -----------------MRRLFLEQFYKQIFSSTPIT----------------------
2             -----------------MRRLFLEQFYKQIFSSTPIT----------------------
3             -------------MSVSLLQPYFFMSKTKSYAQILIG----------------------
4             -------------MSVSLLQPYFFMSKTKSYAQILIG----------------------
clone         ------------------------------------------------------------


5             NLSEALRNLTTTAIGLGFMFSTSWKLTLLSLAIVPVISAVVRKFGRFLRELSHQTQAAAA
6             NLSEALRNLTTTAIGLGFMFSTSWKLTLLALVIVPVISVAVRKFGRFLRELSHQTQAAAA
1             ----------------------SFFLFLLYIVVTPLMIGFEKDF----------------
2             ----------------------SFFLFLLYIVVTPLMIGFEKDF----------------
3             ----------------------SRLFLTAMAIHLSLRVAPPDLQ----------------
4             ----------------------SWLFLTAMAIYLSLWVAPPDLQ----------------
clone         --------------------------------GTQFEDDSDQE----------------
                                                :     .

5             VASSIAEESFGAIRTVRAFAQEPHEISRYSGKVNETLKLGLKQATVVGLFSGGLNAASTL
6             VASSIAEESFGAIRTVRAFAQEPHEISRYGGKVNETLKLGLKQAKVVGLFSGGLNAASTL
1             ----LCHFHLGLIWISLLFSFLSEPFFRND-----------KESGTLELY----------
2             ----LCHFHLGLIWISLLFSFLSEPFFRND-----------KESGTLELY----------
3             -----QGGNSRISYVHVPAARMSIVIYIAT---------------AINSS----------
4             -----QGGNSRILYVHVPVAWMSIVIYIAT---------------AINSF----------
clone         -----TIDAKDILKRELQDERKTLPIYKFR-----------------DEL----------
                              .  :

5             SVVIVVIYGAKLTINGYMTTGALTSFILYSLTVGSSVSALSGLYTTVMKASGSSRRVFQL
6             SVVVVVIYGANLTINGYMTTGSLTSFILYSLTVGSSVSALSGLYTTVMKASGASRRVFQL
1             ----------------YLSAYCLPKILLLQL-VGHWVIQISCVFC-----------AFPM
2             ----------------YLSAYCLPKILLLQL-VGHWVIQISCVFC-----------AFPM
3             -----------------LFPLTKHPLFLRSSGTGTEIGAFSTLFTLVTGG----FRGRPM
4             -----------------LFLLTKHPLFLRSSGTGTEIGAFFTLFTLVTGG----FWGRPM
clone         -----------------LKAVDEYQVIVIVGETGSGKTTQIPQYL---------------
                              :       .::    .*         :

5             LDRISSMKNSGDKCPKNENDGEVELDDVWFAYPSRPSHLILKGITLKLAPGSKVALVGPS
6             LDRVSSMTNTGDKCPKIENEGEVELDDVWFAYPSRPSHMILKGITLKLAPGSKVALVGPS
1             LQLLYQFDRSG-------------------------------------------------
2             LQLLYQFDRSG-------------------------------------------------
3             WGTFRVWDARLTS-----------------------------------------------
4             WGTFWVWDARLTS-----------------------------------------------
clone         -------HEAG-------------------------------------------------


5             GGGKTTIANLIERFYDPIKGRILLNGVPLVEISHQYLHQMVSIVSQEPTLFNCSIEENIA
6             GGGKTTIANLIERFYDPLKGRILLNGVPLVEISHQYLHQKVSIVSQEPTLFNCSIEENIA
1             -----------MDWLNILLGSLVLT------------------------LLCGIHSCLA
2             -----------MDWLNILLGSLVLT------------------------LLCGIHSCLA
3             ----------VFILFLIYLGALRFQKLP---------------VEPAPISIRAGPIDIP
4             ----------VFILFFIYLGALCFQKLS---------------VELASILICVGLIDIP
clone         ---------------YTARGK---------------------------VACTQPRRVA
                            *                                        :.

5             YGLEGKASSADVENAAKMANAHDFICGFPDQYKTIVGERGIRLSGGQKQRVAIARALLMN
6             YGLEGKASSADVENAAKMANAHDFICSFPDQYKTVVGERGIRLSGGQKQRVAIARALLMN
1             LGITSSSGWNSLQNLTTLP-----------------------------------------
2             LGITSSSGWNSLQNLTTLP-----------------------------------------
3             IIKSPVNWWNTSHQPGSISRSG--------------------------------------
4             IIKFSVNWWNTLHQPGSISRFG--------------------------------------
clone         ------------------------------------------------------------
```

```
5             PRVLLLDEATSALDAESEHLVQDAMDSLMRGRTVLVIAHRLSTVKTADTVAVISEGQIVE
6             PRVLLLDEATSALDAESEYLVQDAMDSLMKGRTVLVIAHRLSTVKSADTVAVISEGQIVE
1             -TLLPLTVFCTSIETEGFHVL------LLIGYFFLFVS----------------------
2             -TLLPLTVFCTSIETEGFHVL------LLIGYFFLFVS----------------------
3             -TSIHVPMPIPILSNFANFPFSTRILFVLETRLPIPSFP---------------------
4             -TSIHVSMLIPILSNFANFLFFTCILFVLETRLLILSFL---------------------
clone         ------------------------------------------------------------


5              SGTHDELLGRDGIYTALVKRQLQLPKFEGTANGTDEVEP-VDGH
6              RGTHDELLERDGIYTALVKRQLQLPKFEGTANGTAEIEPSSNGQ
1              ------------LYPILVSISLQD--------------------
2              ------------LYPILVSISLQD--------------------
3              ------------ESPLTEEIEAREGIPLKT--------------
4              ------------ESSLTEEIEAREGIPLKT--------------
clone          ----------------------------------------
```

## APPENDIX G

Multible amino acid sequence alignment of clone MT8 and ADH gene found in wheat using CLUSTAL X (1.81).

ADH: Alcohol dehydrogenase (EC 1.1.1.1) - wheat (cv. Millewa)(Accession no A61024)
Clone: Clone MT8

```
ADH             MATAGKVIECKAAVAWEAGKPLSIEEVEVAPPHAMEVRVKILYTALCHTDVYFWEAKGQT
clone           ------------------------------------------------------------


ADH             PVFPRILGHEAGGIVESVGEGVTELVPGDHVLPVFTGECKDCAHCKSEESNLCDLLRINV
clone           ------------------------------------------------------------


ADH             DRGVMIGDGQSRFTINGKPIFHFVGTSTFSEYTVIHVGCLAKINPEAPLDKVCVLSCGIS
clone           ------------------------------------------------------------


ADH             TGLGATLNVAKPKKGSTVAIFGLGAVGLAAMEGARMAGASRIIGVDLNPAKYEQAKKFGC
clone           ------------------------------LQGS----------ID--------------
                                              ::*:          :*

ADH             TDFVNPKDHTKPVQEVLVEMTNGGVDRAVECTGHIDAMIAAFECVHDGWGVAVLVGVPHK
clone           ----SSQDH-------LMSSANGQNN-----SG------------NNG-------AIP--
                     ..:**       *:. :**  :     :*            ::*       .:*

ADH             EAVFKTYPMNFLNERTLKGTFFGNYKPRTDLPEVVEMYMRKELELEKFITHSVPFSQINT
clone           ----KVNPASSLQS-----------NPSTSFPS-----------------QVPISSNNN
                    *. * . *:.           :* *.:*.                 .**:*. *.

ADH              AFDLMLKGEGLRCIMRMDQ
clone            MMPALQNTNQLS-------
                    :  : : : *
```