IDENTIFICATION OF THE GENES INVOLVED IN PHYTOSIDEROPHORE" SYNTHESIS AND METAL ION UPTAKE IN WHEAT USING RT-PCR

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ABSTRACT

IDENTIFICATION OF THE GENES INVOLVED IN "PHYTOSIDEROPHORE" SYNTHESIS AND METAL ION UPTAKE IN WHEAT USING RT-PCR

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Soils in many agricultural areas have high pH, resulting in low availability of zinc and iron. Plants grown on such soils suffer from either Zn or Fe deficiency or both.

The efficient plant genotypes grown normally in calcerous soils were found to evolve some strategies to acquire the iron which is in insoluble form. Iron efficient graminaceous monocots release iron chelating substances, mugineic acid family phytosiderophores (MAs), in response to iron deficiency stress. Several researchers have suggested that phytosiderophores also can play role in grass Zn nutrition and thus it may be possible that it is the uptake mechanism for Zn efficiency. Several possible genes that take role in phytosiderophore synthesis or found to be induced under iron deficient conditions were identified in several organisms but not on wheat.

In this study, the efficient barley cultivar Tokak-157, efficient wheat cultivar Kıraç-66 and relatively less efficient wheat cultivar BDMM-19 were grown in normal growth conditions for 1 week and transfered to zinc deficient, iron deficient and both zinc and iron deficient nutrient solutions. After growing 1 week on these conditions, plants grown on both zinc and iron deficient nutrient solutions were retransfered to zinc and iron sufficient conditions. Degenerate primers were designed for the conserved regions of previously identified genes that take role in phytosiderophore synthesis or induced under iron deficient conditions and RT-PCRs were performed. The complete open reading frame of IDI-1(Iron deficiency induced-1) gene and the putative gene fragment for SAM-s (S-adenosylmethionine synthetase) were identified.

Key words: Wheat, phytosiderophores, iron deficiency, zinc deficiency, RT-PCR, SAM-synthetase, IDI-1.

ÖZ

BUĞDAYDA "FİTOSİDEROFOR" SENTEZİNDE VE METAL İYON ALIMINDA GÖREV ALAN GENLERİN RT-PCR İLE TANIMLANMASI

Aktaş, Yasemin

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Tarımsal alanların çoğunda topraklar, çinko ve demir elementlerinin düşük çözünürlüğü ve ulaşılabilirliği ile sonuçlanan yüksek pH'a sahiptir. Bu tip topraklarda yetişen bitkiler çinko ve demir eksikliği ile karşılaşırlar. Bu tip topraklarda normal gelişimlerine devam edebilen dayanıklı bitkilerin toprakta düşük çözünürlükte bulunan demiri alabilmek için çeşitli stratejiler geliştirdikleri

tespit edilmiştir. Demir eksikliğine dayanıklı monokotlar, demir stresine cevap olarak topraktaki düşük çözünürlüklü demiri bağlayan mugineik asit ailesine bağlı fitosideroforları salgılarlar. Bir çok araştırıcı fitosidoroforların graminelerin çinko beslenmesinde ve çinko eksikliğine dayanıklılık mekanizmasında da rol oynadıklarını öne sürmüşler. Fitosiderofor sentezinde rol aldığı düşünülen veya demir eksikliği sırasında uyarılan bir çok gen bazı organizmada gösterilmiş ancak henüz bu genler buğdayda araştırılmamıştır.

Bu çalışmada, dayanıklı bir arpa kültüvarı olan Tokak-157, dayanıklı buğday kültüvarı Kıraç-66 ve daha az dayanıklı bir buğday kültüvarı olan BDMM-19 bitkileri normal büyüme koşullarında 1 hafta yetiştirildikten sonra çinko içermeyen, demir içermeyen ve hem çinko hem de demir içermeyen olmak üzere 3 farklı solüsyona aktarılıp yetiştirilmeye devam edilmiştir. 1 hafta sonunda hem çinko hemde demir yönünden eksik ortamda yetiştirilen bitkiler tekrar normal büyüme koşullarına transfer edilmiştir. Daha önce buğday dışındaki diğer bitkilerde fitosiderofor sentezinde rol aldığı bulunmuş genlerin ortak ve korunmuş bölgeleri için dejenere primerler dizayn edilmiş ve bu primerler kullanılarak RT-PCR (ters transkriptaz zincir reaksiyonu) yapılmıştır. Sonuçta ; buğday IDI-1 geni ve buğday SAM-synthetase gen fragmanı bulunmuştur.

Anahtar kelimeler : Buğday, fitosidorofor, demir eksikliği, çinko eksikliği, ters transkriptaz zincir reaksiyonu, SAM-synthetase, IDI-1

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LIST OF ABBREVIATIONS

[γ ^{33}P]-dATP	: [γ ^{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 300 species classified into 22 genera including several other important crops (barley, rye, and triticale) and a number of important forage species (Löve, 1984).

Triticum arose from the cross of two diploid wild grasses to produce tetraploid wheat, which today includes many cultivated durum (pasta or macaroni) wheats (*Triticum turgidum* L. 2*n* = 4x = 48).

Tetraploid wheat later crossed to diploid goat grass (*Triticum tauschii*) and gave rise to hexaploid, or bread wheat (*Triticum aestivum* L. 2*n* = 6x =42).

1.2. The importance of wheat

Wheat *(Triticum aestivum* L.) is the most widely grown and the most important food crop in the world and becomes even more important as the global population increases. To provide sufficient food for the growing world population an enormous increase in food production will be needed.

It is forecasted that, by 2050, the world population will increase from the current level of ≈6 billion to >8 billion people (Source: U.S. Bureau of the Census, International Data Base). Feeding this population will require astonishing increase in food production. It appears more likely that a population /food crisis may be arise not from an exponentially increasing world population, but from a slowdown in the growth of global food production rate.

According to the FAO Statistical Databases, Turkey is among the countries with the highest wheat consumption worldwide. More than 90% of all wheat is directly consumed as food. Turkey is considered among the 10 largest wheat-growing countries in the world. However when the yield is compared to top-level producers like China, India, USA, Russia and major European producers it remains very low. There are certain factors that decrease the yield levels. One of the important factors is micronutrient deficiencies of soils in Turkey.

1.3. Iron (Fe)

Iron (Fe) is taken up as Fe^{3+} and Fe^{2+} by different mechanisms through roots. It is an essential micronutrient with numerous cellular functions; it is required for respiration, photosynthesis and many other cellular functions including DNA synthesis, nitrogen fixation and hormone production.

It is necessary for chlorophyll formation and for oxygen transfer. Also, it is a constituent of electron transport chains both in mitochondria and chloroplasts.

1.4. Zinc (Zn)

Zinc (Zn) is a very important trace element for many biological functions such as development, disease resistance and wound healing. It is taken up by plants as the zinc ion (Zn^{++}) and it is essential for several important enzyme systems in plants. More than 300 enzymes (>100 zinc metalloenzymes, including a large number of NADH dehydrogenases, RNA and DNA polymerases, DNA transcription factors as well as alkaline phosphatase, superoxide dismutase and carbonic anhydrase) require zinc for proper functioning. Several motifs found in transcriptional regulatory proteins are stabilized by zinc, including the Zinc-finger, Zinc-cluster and RING-finger domains (WHO,IPCS, Boston University, AZA, 1996). Zinc is necessary for regulation in expression and affects plant size and maturity.

Zinc also controls the synthesis of indoleacetic acid which is an important plant growth regulator and it is also essential for gene expression and nucleic acid metabolism and it also has many structural roles in biological membranes, cell receptors, enzymes and other proteins.

1.5. Iron (Fe) and Zinc (Zn) deficiency stresses in plants

Soils in many agricultural areas have high pH, resulting in low availability of zinc and iron. Plants grown on such soils suffer from either Zn or Fe deficiency or both.

1.5.1. Iron (Fe) deficiency

Iron deficiency is defined as the condition in which insufficient soluble iron is available for optimal growth of plant.

Ferrous iron, Fe (II), is relatively soluble but it is oxidized by atmospheric oxygen. The solubility of Ferric iron, Fe (III), decreases with increasing pH values due to hydroxylation as $Fe(OH)_3$, polymerization and finally precipitation with inorganic anions. Neilands et al. reported in 1987 that while free Fe (III) is soluble up to 10^{-6} M at pH 3.3, this concentration is only 10^{-17} M at pH 7.

However, plants require between 10^{-4} and 10^{-8} M Fe(III) and thus the solubility of iron ranges lower than required for optimal plant growth in well-aerated soils with pH values above 7 and this situation cause iron deficiency.

Iron deficient plants are characterized by the development of interveinal chlorosis occurring first on the youngest leaves (Figure 1.1.). Interveinal chlorosis is sometimes followed by chlorosis of the veins, so the whole leaf then becomes yellow. In severe cases, the leaves become white with necrotic lesions.

Figure 1.1. The picture of iron deficient wheat (Source: International maize and wheat improvement center, nutrient deficiencies and toxicities in wheat).

This characteristic chlorotic phenotype of iron deficiency in crop plants which are the major sources for iron in food and feed, causes considerable loses of yield. Although iron is not rare in most soils, iron deficiency represents a severe problem for agriculture, since 30% of the arable land worldwide consists of calcareous and thus alkaline soils. This limitation can not be easily overcome by using iron-containing fertilizers because; iron availability is a problem of solubility not of abundance (Guerinot 2001).

Certain plant genotypes are able to grow and yield well under Fe deficient conditions, which has been termed Fe efficiency (FE). In 1988 Kawai et al. and in 1990 Römheld and Marschner classified wheat, barley and oat as iron efficient and rice, sorghum and maize as sensitive to iron deficiency stress.

These efficient plant genotypes grown in calcerous soils were found to evolved some strategies to acquire the iron which is in insoluble form.

1.5.2. Zinc (Zn) deficiency

In calcareous soils, the free Zn^{2+} activity might be as low as 10^{-9}-10^{-11} M, which can be too low to support optimal crop growth (Barber, 1984; Kochian, 1993;Welch, 1995). Therefore, the low Zn availability on these types of soils limits crop production in many countries, including Australia, India, Turkey and the USA (Sillanpaa, 1990).

Characteristic symptoms of Zn deficiency includes chlorosis on young leaves, reduced leaf size (i.e. little leaf), and stunted, thin stems (Figure 1.2.). Under severe Zn deficiency, older leaves show wilting and curling with extensive chlorosis and stunted growth (Marschner, 1995).

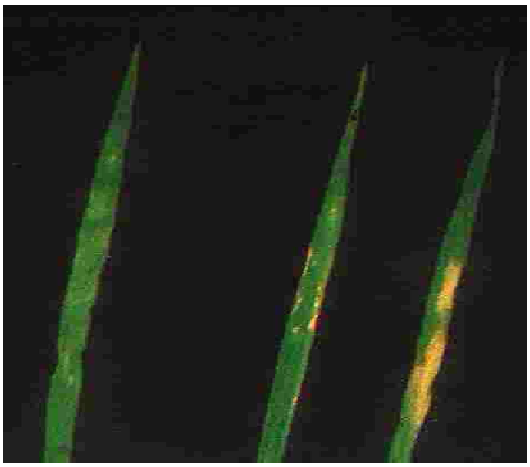

Figure 1.2. The picture of zinc deficient wheat. (Source: International maize and wheat improvement center, nutrient deficiencies and toxicities in wheat)

Certain plant genotypes are able to grow and yield well under Zn deficiency, which has been termed as Zn efficiency (ZE).

Crop species differ in their ability to adapt to zinc deficient soils. According to the classification made by Viets et al. in 1954 and Clark in 1990; among the cereal species, rice, sorghum and maize are classified as zinc deficiency sensitive species, whereas barley, wheat and rye are classified as zinc efficient. There are also large differences in sensitivity to zinc deficiency among the genotypes of a given species.

Wheat is a particular cereal showing great genotypical differences in zinc deficiency. In severely zinc deficient calcareous soils in Turkey, distinct differences in resistance to zinc deficiency has been found between bread and durum wheats and also among genotypes of bread wheat. Based on the study made by Rengel and Graham in 1995, durum wheats are classified as less tolerant to zinc deficiency than bread wheats.

In 1996, Çakmak et.al. classified Turkish bread and durum wheat genotypes according to the zinc efficiency proporties of these genotypes. According to this study, Kıraç-66 was classified as the most zinc efficient bread wheat cultivar and BDMM-19 was classified as the most zinc efficient one among durum wheat cultivars.

1.6. Phytosiderophores (PS)

Phytosiderophores (PS) are nonprotein amino acids that chelate a number of micronutrients, including Fe and Zn and are released from the roots of grasses under iron deficiency as Marschner proposed in 1995. It is widely accepted that PS release and root absorption of Fe(III)-PS are key factors in Fe nutrition in grasses.

From 1986 to date, most steps of the biosynthetic pathways of phytosiderophores from methionine to mugineic acids in iron deficient barley roots have been deduced (Figure 1.3.).

The biosynthesis starts from S-Adenosylmethionine which is converted from L-methionine by SAM (S-Adenosylmethionine) synthase. Then three molecules of S-Adenosylmethionine are integrated into nicotianamine (NA) in one enzymatic step catalyzed by NAS (nicotianamine synthase). NA functions as an iron chelator inside all plant cells.

NAS has been cloned from tomato, barley and rice and its expression in roots is strongly up-regulated by iron availability in strategy-II plants (Herbik et al. *1999*; Higuchi et al. *1999*, *2001*; Ling et al. *1999*). Deamination of NA by NAAT (nicotianamine aminotransferase) leads to deoxymugineic acid (Takahashi et al. *1999*), which is then hydroxylated by the IDS3 protein to form mugineic acid (MA). Both MA and deoxyMA may be further hydroxylated by the IDS2 protein to form further MA derivatives (Mori *1999*; Negishi et al. *2002*).

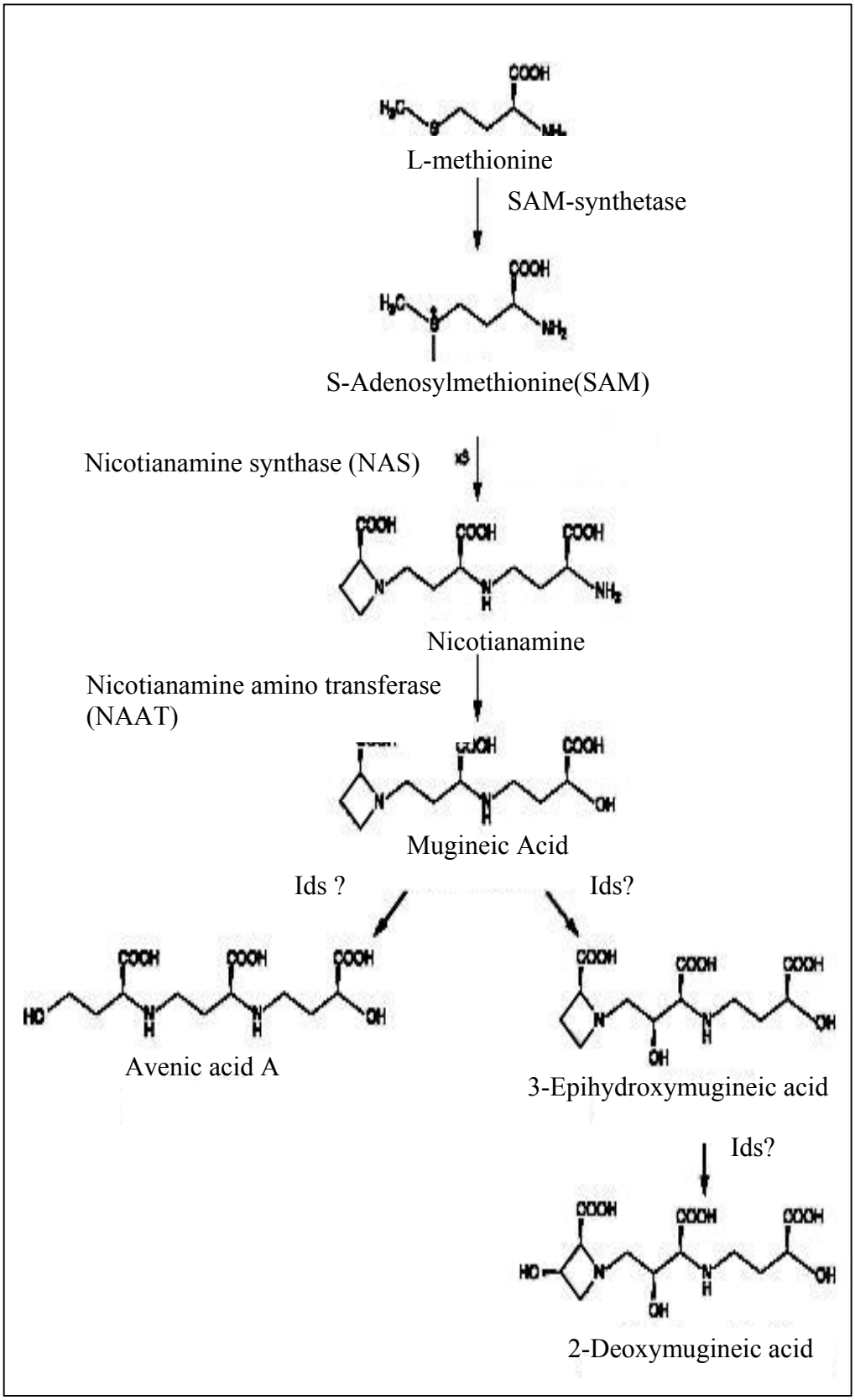

Figure 1.3. Biosynthetic pathways for phytosiderophores

1.7. The role of phytosiderophores in iron uptake

Plants have evolved two separate mechanisms for the acquisition of insoluble iron from the calcareous soils. These two mechanisms were first proposed by Römheld and Marschner in 1986 and termed as strategy-I and strategy-II. Phytosiderophores are the key components of strategy-II.

1.7.1. Strategy-I

Strategy-I is an iron acquisition mechanism used by all higher plants except graminaceous monocots. Under iron deficient conditions non-graminaceous plants release reductans or chelators to the rhizosphere, enhance proton excretion in the rhizosphere, increase their ferric reduction capacity at the root surface and transfer Fe (II) ion through the Fe (II)–transporter in the plasma membrane.

1.7.2. Strategy-II

After Takagi identified mugineic acid (MA) in the root-washings of iron deficient rice in 1976, the strategy-II iron acquisition mechanism was proposed. Strategy-II plants comprise the grasses and thus such important food plants as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), rice *(Oryza sativa*) and maize (*Zea mays*).

Graminaceous monocots release iron chelating substances, mugineic acid family phytosiderophores (MAs), in response to iron deficiency stress. These phytosiderophores solubilize inorganic Fe (III) compounds by chelation and Fe (III)-MAs complexes are taken up through a specific transport system in the root plasma membrane (Figure 1.4.).

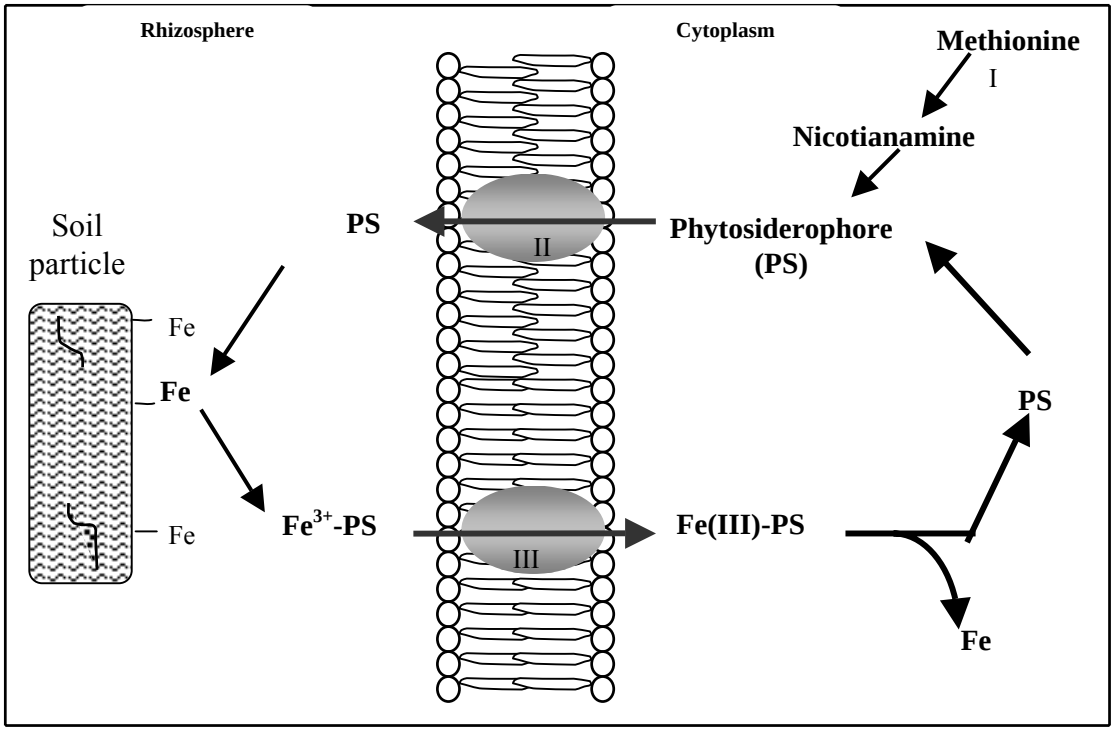

Figure 1.4. Iron uptake mechanism of graminaceous plants. Phytosiderophores are synthesized in the cytoplasm from methionine by the way of nicotianamine (I). An iron deficiency-induced plasma membrane transporter is involved in the release of the PS into the rhizosphere (II). Another iron deficiency-inducible transporter in the plasma membrane recognizes the PS-Iron complex and transports it intact in to the cytoplasm(III).

1.8. The role of phytosiderophores in zinc uptake

It was speculated that two root Zn uptake pathways exist in grasses, one that involves free Zn^{2+}, and a second based on Zn-PS uptake.

With regard to the role of PS in plant Zn nutrition, Zhang *et al*. (1991) reported that Zn deficient graminaceous species released pytosiderophores and thus increased the mobilization of Zn and Fe in soil. Çakmak *et al*. (1996b) reported that Zn-efficient bread wheat genotypes had higher PS release than Zn-inefficient durum wheat genotypes under Zn deficiency stress. In an other study made by Nakai et al in 1992 it was shown that a lower rate of iron transport was associated with the wheat genotype tolerant to zinc deficiency; the suggested physiological deficiency of iron in the shoots was then taken as a possible cause of greater root exudation of phytosiderophores (PS) and thus greater tolerance to zinc deficiency. The role of iron transport to the shoots in the expression of tolerance to zinc deficiency in different wheat genotypes is still unclear.

In another study with bread and durum wheat cultivars, Rengel & Romheld (2000) reported that Zn-efficient bread wheat cultivars released more pytosiderophores than Zn-inefficient cultivars. But there have been several other studies that contradict the role of PS in both Zn nutrition and zinc efficiency. Both Gries *et al*. (1995) and Pedler *et al*. (2000) found no significant Zn-deficiency induced PS release in barley and wheat cultivars, that had been reported by others, in response to Zn deficiency.

Furthermore, in the study by Pedler *et al*. 2000, they found no differences in PS release in barley and wheat cultivars were found unlike previously reported. They did find that Fe deficiency induced a large PS release in all barley and wheat genotypes studied. These researchers suggested that the previous observations of Zn-deficiency induced PS release may be dependent on the growth methods used and might be explained by an induced physiological deficiency of Fe, and not Zn. In a recent review on this topic, Rengel (2001) indicated that further work is needed to arrive at any definitive conclusions about the possible role of PS release in ZE.

As a matter of fact in a recent thesis completed in our Lab., an iron transporter domain was found and sequenced during the screening of the cDNA library of BDMM-19 durum wheat cultivar grown under zinc deficiency stress conditions (Barbaros Yavuz, 2002, Biotechnology MSc. Graduate Program Thesis).

1.9. Hydroponics (Water culture)

In Latin, the word hydroponics means literally "water working". Hydroponics is a technology for growing plants in nutrient solutions (water containing fertilizers) with or without the use of an artificial medium (sand, rock wool etc.) to provide mechanical support (Jensen, 1997) (Figure 1.3). It is also highly productive, conservative of water/land and protective of the environment. Since regulating the aerial and root environment is a major concern in agricultural systems, production

takes place inside the growth chamber, designed to control air, humidity, temperature and light.

Water quality has become a major concern of hydroponics growers, especially where large amounts of water are applied to a restricted volume of growing medium. Plant growth is affected by the interaction of the dissolved chemical elements in the water supply and the chemical properties of the growing medium to which the water is applied. A major advantage of hydroponics, as compared with culture of plants in soil, is the isolation of the crop from the underlying. The costly and time-consuming tasks of soil sterilization and cultivation are unnecessary in hydroponics systems.

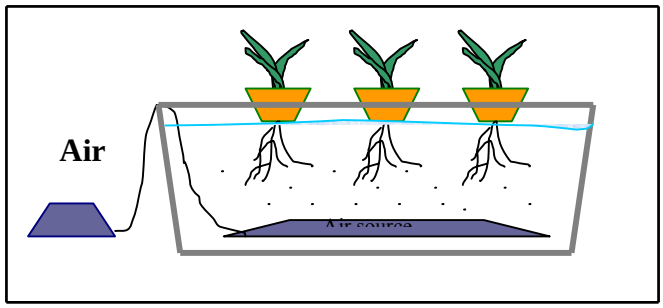

Figure 1.5. A representation of the hydroponic system.

Figure 1.6. The picture of our samples grown hydroponically

In addition to a perfectly balanced diet, hydroponic plants have their food and water delivered directly to their roots. This way, the energy normally used to develop long roots can be redirected to growing more plant, which is a great benefit indeed. With the proper exposure to natural sunlight or supplemental grow lights, the hydroponic plants will grow many times faster, bigger and healthier than those grown in soil.

In a hydroponics system, essential nutrients can be put into 3 categories based on how quickly they are removed from a nutrient solution (Table 1.1). Group 1 elements are actively absorbed by roots and can be removed from solution in a few hours. Group 2 elements have intermediate uptake rates and are usually removed from solution slightly faster than water is removed. Group 3 elements are passively absorbed from solution and often accumulate in solution.

Table 1.1. Approximate uptake rates of the essential plant nutrients (http://milo.usu.edu/cpl/hsapaper.html).

Group 1. Active uptake, fast removal	NO_3, NH_4, P, K, Mn
Group 2. Intermediate uptake	Mg, S, Fe, Zn, Cu, Mo, C
Group3. Passive uptake, slow removal	Ca, B

1.10. RT-PCR

RT-PCR combines cDNA synthesis from RNA templates with PCR to provide a rapid, sensitive method for analyzing gene expression. RT-PCR is used to detect or quantify the expression of messages, often from small amounts of RNA. In addition, the technique is used to analyze differential gene expression or clone cDNAs without constructing a cDNA library. RT-PCR is more sensitive and easier to perform than other RNA analysis techniques.

1.11. Objectives of the study

Our aim was to identify the wheat genes that take role in the phytosiderophore synthesis pathway of other oraganisms such as maize, barley and rice. For this purpose, we selected Kıraç-66 which was classified as the most zinc efficient cultivar among bread wheats and considered to be also iron efficient based on the arguments present in section 1.5.2. deduced from the studies of Çakmak *et al* (1996). BDMM-19 was selected among durum wheats with same reasons. In addition, we have included the barley cultivar Tokak-157 to use as a control since barley is known to excrete more phytosiderophore than wheat and most of the genes that we attempted to identify were already found in barley.

In order to accurately control the concentrations of the elements in the nutrient solution, hydroponics (water culture) system was used to grow the plants.

RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) was used for the identification and comparision of expression levels of the target genes.

CHAPTER II

MATERIALS AND METHODS

2.1. Plant material

The durum wheat cultivar, BDMM-19 (Selçuklu), the bread wheat cultivar KIRAÇ-66 and the barley cultivar TOKAK-157 were obtained from Anatolian Agricultural Research Center, Eskişehir, Turkey.

2.2. Growth conditions

BDMM-19, KIRAÇ-66 and TOKAK-157 seeds were surface sterilized in 20% hypochloric acid solution by shaking for two minutes and then washed twice with double distilled water.

After washing steps, seeds were embedded in trays containing ddH_2O-moistened and $CaSO_4$-saturated perlite. Plates were covered with aluminum foil to create a humid environment for a better germination.

Seeds were stored overnight at 4 ^{o}C for vernalization, and then they were germinated for five days at 25 °C in the dark. The etiolated seedlings were then transferred to 7 L plastic containers (100 seedlings per container) with continuously aerated nutrient solution.

Macronutrient, micronutrient, $ZnSO_4$ and Fe-EDTA stock solutions were prepared separately from each other. The final concentrations of the elements in the nutrient solutions are given in the table 2.1.

Table 2.1. Final concentrations of elements in the nutrient solutions

	Final concentrations of elements in the nutrient solutions			
Elements	+Zn/+Fe	-Zn/+Fe	+Zn/-Fe	-Zn/-Fe
Zn	*1 μM	-	1 μM	-
Fe	*50 μM	50 μM	-	-
Mg	1 mM	1 mM	1 mM	1 mM
K	2 mM	2 mM	2 mM	2 mM
P	0.2 mM	0.2 mM	0.2 mM	0.2 mM
Ca	2 mM	2 mM	2 mM	2 mM
NO_3	4 mM	4 mM	4 mM	4 mM
Cu	0.2 μM	0.2 μM	0.2 μM	0.2 μM
NH_4	0.02 μM	0.02 μM	0.02 μM	0.02 μM
B	1 μM	1 μM	1 μM	1 μM
Mn	0.2 μM	0.2 μM	0.2 μM	0.2 μM

* Optimum concentrations for plant growth.

Double distilled water was used for the preperation of nutrient solutions. Nutrient solutions were changed every three days and the pH of the nutrient solutions were measured and adjusted to pH 6 every day. 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. Light regime begins at 05:00 am and ends at 21:00 pm; dark period begins at 21:00 pm and ends at 05:00 am.

Plants were grown in zinc and iron sufficient nutrient solutions (+Zn/+Fe) for 1 week and then transferred to iron deficient (+Zn/-Fe), zinc deficient (-Zn/+Fe) and both iron and zinc deficient (-Zn/-Fe) conditions to evaulate the expression levels of phytosiderophore synthesis related genes under different conditions. Plants were grown in these conditions for 1 week and 100-300 μg root and shoot tissue were cut off at different time points and stored at -80 °C until use. After 1 week, the plants grown in (-Zn/-Fe) condition were retransferred back to (+Zn/+Fe) condition to detect whether there is a repression in the expression levels of the phytosiderophore related genes' when turning back to normal growth conditions. Again samples were collected as above at different time points. The time points were 0 hour, 3 hours, 24 hours, 3 days and 7 days after the transfer of plants to different nutrient solutions.

The list of the collected plant samples and time points are presented in the tables 2.2, 2.3 and 2.4.

Table 2.2. Zinc and iron concentrations and time points of Kıraç-66 samples

***** Before transfer

Sample Name	Plant Source	Transfered		Time Points (after transfer)
		From	To	
K1	Kıraç-66	+Zn/+Fe		0 hours*****
K2	Kıraç-66	+Zn/+Fe	+Zn/-Fe	3 hours
K3	Kıraç-66	+Zn/+Fe	-Zn/-Fe	3 hours
K4	Kıraç-66	+Zn/+Fe	-Zn/+Fe	3 hours
K5	Kıraç-66	+Zn/+Fe	+Zn/-Fe	24 hours
K6	Kıraç-66	+Zn/+Fe	-Zn/-Fe	24 hours
K7	Kıraç-66	+Zn/+Fe	-Zn/+Fe	24 hours
K8	Kıraç-66	+Zn/+Fe	+Zn/-Fe	3 days
K9	Kıraç-66	+Zn/+Fe	-Zn/-Fe	3 days
K10	Kıraç-66	+Zn/+Fe	-Zn/+Fe	3 days
K11	Kıraç-66	+Zn/+Fe	+Zn/-Fe	7 days
K12	Kıraç-66	+Zn/+Fe	-Zn/-Fe	7 days
K13	Kıraç-66	+Zn/+Fe	-Zn/+Fe	7 days

Sample Name	Plant Source	Transfered				Time Points (after transfer)
		From			To	
K14	Kıraç-66	+Zn/+Fe	-Zn/-Fe	7 days	+Zn/+Fe	3 hours
K15	Kıraç-66	+Zn/+Fe	-Zn/-Fe	7 days	+Zn/+Fe	24 hours
K16	Kıraç-66	+Zn/+Fe	-Zn/-Fe	7 days	+Zn/+Fe	3 days
K17	Kıraç-66	+Zn/+Fe	-Zn/-Fe	7 days	+Zn/+Fe	7 days

Table 2.3. Zinc and iron concentrations and time points of BDMM-19 samples

***** Before transfer

Sample Name	Plant Source	Transfered		Time Points (after transfer)
		From	To	
B1	BDMM-19	+Zn/+Fe		0 hours*****
B2	BDMM-19	+Zn/+Fe	+Zn/-Fe	3 hours
B3	BDMM-19	+Zn/+Fe	-Zn/-Fe	3 hours
B4	BDMM-19	+Zn/+Fe	-Zn/+Fe	3 hours
B5	BDMM-19	+Zn/+Fe	+Zn/-Fe	24 hours
B6	BDMM-19	+Zn/+Fe	-Zn/-Fe	24 hours
B7	BDMM-19	+Zn/+Fe	-Zn/+Fe	24 hours
B8	BDMM-19	+Zn/+Fe	+Zn/-Fe	3 days
B9	BDMM-19	+Zn/+Fe	-Zn/-Fe	3 days
B10	BDMM-19	+Zn/+Fe	-Zn/+Fe	3 days
B11	BDMM-19	+Zn/+Fe	+Zn/-Fe	7 days
B12	BDMM-19	+Zn/+Fe	-Zn/-Fe	7 days
B13	BDMM-19	+Zn/+Fe	-Zn/+Fe	7 days

Sample Name	Plant Source	Transfered				Time Points (after transfer)
		From			To	
B14	BDMM-19	+Zn/+Fe	-Zn/-Fe	7 days	+Zn/+Fe	3 hours
B15	BDMM-19	+Zn/+Fe	-Zn/-Fe	7 days	+Zn/+Fe	24 hours
B16	BDMM-19	+Zn/+Fe	-Zn/-Fe	7 days	+Zn/+Fe	3 days
B17	BDMM-19	+Zn/+Fe	-Zn/-Fe	7 days	+Zn/+Fe	7 days

Table 2.4. Zinc and iron concentrations and time points of TOKAK-157 samples

***** Before transfer

Sample Name	Plant Source	Transfered		Time Points (after transfer)
		From	To	
T1	TOKAK-157	+Zn/+Fe		0 hours*****
T2	TOKAK-157	+Zn/+Fe	+Zn/-Fe	3 hours
T3	TOKAK-157	+Zn/+Fe	-Zn/-Fe	3 hours
T4	TOKAK-157	+Zn/+Fe	-Zn/+Fe	3 hours
T5	TOKAK-157	+Zn/+Fe	+Zn/-Fe	24 hours
T6	TOKAK-157	+Zn/+Fe	-Zn/-Fe	24 hours
T7	TOKAK-157	+Zn/+Fe	-Zn/+Fe	24 hours
T8	TOKAK-157	+Zn/+Fe	+Zn/-Fe	3 days
T9	TOKAK-157	+Zn/+Fe	-Zn/-Fe	3 days
T10	TOKAK-157	+Zn/+Fe	-Zn/+Fe	3 days
T11	TOKAK-157	+Zn/+Fe	+Zn/-Fe	7 days
T12	TOKAK-157	+Zn/+Fe	-Zn/-Fe	7 days
T13	TOKAK-157	+Zn/+Fe	-Zn/+Fe	7 days

Sample Name	Plant Source	Transfered				Time Points (after transfer)
		From			To	
T14	TOKAK-157	+Zn/+Fe	-Zn/-Fe	7 days	+Zn/+Fe	3 hours
T15	TOKAK-157	+Zn/+Fe	-Zn/-Fe	7 days	+Zn/+Fe	24 hours
T16	TOKAK-157	+Zn/+Fe	-Zn/-Fe	7 days	+Zn/+Fe	3 days
T17	TOKAK-157	+Zn/+Fe	-Zn/-Fe	7 days	+Zn/+Fe	7 days

There was also another set of Kıraç-66 plants previously grown in different Zn but constant Fe concentrations for another study. These plants were grown in 0 μM (Zn deficient) and 1.0 μM (Zn sufficient) nutrient solutions for 1 week and then plants grown in 0 μM (Zn deficient) conditions were transfered to 1.0 μM (Zn sufficient) and 100 μM Zn containing solutions and root samples were taken at different time points. Also plants grown in 1.0 μM (Zn sufficient) conditions were transfered to 0 μM and 100 μM Zn containing solutions and root samples were taken again at different time points. The list of plant samples were given in Table 2.5. This set of plant samples were included to the study inorder to investigate the expression levels of phytosiderophore synthesis related genes under different Zn concentrations.

Table 2.5. Zinc concentrations and time points of KIRAÇ-66 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. DNA isolation

DNA isolation was performed from the leaves of Kıraç-66, Tokak-157 and BDMM-19 plants. 2X CTAB (Cetyltrimethylammonium bromide) method was used for DNA isolation. 2X CTAB extraction buffer contains; 2% CTAB (Sigma), 1.4 M NaCl, 100 mM Tris-HCl pH 8, 20 mM EDTA, 0.5% Nabisulfite and 1% 2-mercaptoethanol (added just before use).

Plant tissue sample of 50-100 mg was powdered by mortar and pestle in the presence of liquid nitrogen and transfered to sterile 2 mL tubes. 750 µl 2X CTAB buffer per tube was added and the tubes were incubated 30 min. at 65 °C. Chloroform-isoamylalcohol (24:1) with an amount of 1X volume was added per tube and inverted several times. The tubes were centrifuged at 7,000 rpm at 25 °C for 5 min. The upper phase (~650 µl) was transfered into a fresh 1.5 mL tube and 300 µl 2X CTAB buffer was added to the lower phase. the tubes were centrifuged at 15,000 rpm for 5 min. Aproximately 400 µl of supernatant was collected from upper phase and added into the previously collected upper phase. Isopropylalcohol of 0.6 volume was added to DNA containing aquous phase. Inverted several times and centrifuged for 3-5 min. at 15,000 rpm at 25 °C. Isopopylalcohol was discarded by carefully pouring off. The pellet was washed in 70 % EtOH and air dried. The pellet was dissolved in dd-H_2O stored at 4 °C or at -20°C .

2.4. RNA isolations

Samples, chosen among Tokak-157 and BDMM-19 root tissue samples were used for total RNA isolation.

2.4.1. Precautions

When working with RNA to keep the RNA intact is very important because the degredation of RNA with RNases is a very big problem. So, before working with RNA some precautions should be taken.

First, a room or a place should be seperated from other parts of the labaratuary to use only for RNA works and all equipments; micropipettes, reagents, glass or non-glass equipments, should be cleaned from any Rnase contamination. All the glass equipments were treated with hypo-chromic acid solution and washed with active DEPC treated ddH_20 and stored at 180°C two days before using for isolation. Other non-glass equipments and plastic materials were treated with DEPC treated ddH_20 and autoclaved at 121 ^oC for 1 hour. Also before isolation, all pipettes were cleaned with RNase-off (Applichem). All the solutions used in isolations were prepared by using DEPC treated ddH_20.

2.4.2. Total RNA isolation

Plant root tissue samples in amounts of 50-100 mg were powdered in a mortar in the presence of liquid nitrogen. Then powdered tissue samples were transfered to 2 mL sterile tubes added 1 mL of Trizol Reagent (Invitrogen).

Homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 mL of chloroform per 1 mL of Trizol Reagent was added to each tube. Tubes were shaked vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 minutes. Samples were then centrifuged at 15,000 rpm for 15 minutes at 4 ^{o}C. Following centrifugation, the mixture was separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.

The upper phase was transferred to a fresh sterile 2 mL tube. RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol (0.5 mL isopropyl alcohol was used per 1 mL of initial Trizol Reagent). Samples were incubated 10 minutes at room temperature and then centrifuged at 15,000 rpm for 10 minutes at 4 ^{o}C. The RNA precipitate formed a gel like pellet on the side of the bottom of each tube.

The supernatant was removed and the RNA pellet was washed once with 75% ethanol, adding at least 1 mL of 75% ethanol (per 1 mL of Trizol Reagent used

for the initial homogenization). Sample was mixed by vortexing and centrifuged at 9,000 rpm for 5 minutes at 4 °C.

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 minutes) at the end of the procedure. RNA was dissolved in 50 µL nuclease free water and incubated for 10 minutes at 55-60 °C. Following the incubation, isolated RNA samples were stored at –80 °C.

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.

Concentration determination of RNA samples was achieved according to the equation given below (one absorbance value at 260 nm is considered to be equal to 40µg/mL RNA):

$1A_{260}$ = 40µg/mL RNA

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

2.4.3. mRNA Isolation

mRNAs were isolated from Kıraç-66 root tissue samples. Nearly 150 mg plant tissue sample was powdered in a 2.0 mL tube in the presence of liquid nitrogen by using glass homogenizers. . Powdered tissue was homogenized in 200 μL of Extraction Buffer (4M guanidine thiocyanate, 25mM sodium citrate pH 7.1 and 2% β-Mercaptoethanol). Tubes were shaked for 1-2 minutes and immediately 400 μL of Dilution Buffer (6x SSC, 10 mM Tris-HCl (pH:7.4), 1mM EDTA, 0.25% SDS and 2% β-Mercaptoethanol), preheated at 70 ^{o}C for 5 minutes, was added into each tube and they were mixed throughly by inversion. Then 1 μL of 50 pmol biotinylated oligodT was added. Mixture was incubated at 70 ^{o}C for 5 minutes. After incubation samples were centrifuged at 15.000 rpm for 10 minutes at room temperature. After centrifugation of samples, the mixture was seperated into two phase (RNA remains in the upper phase).

In the mean time, Streptavidin MagneSphere Paramagnetic Particles (SA-PMPs) (Promega) 100 μL per sample were resuspended by gentle rocking. Then the tube was placed on the magnetic stand and SA-PMPs captured on the side facing to the magnet. The storage buffer was poured off. SA-PMPs were resuspended in 100 μL of 0.5x SSC solution. The particules were captured on magnetic stand and solution was poured off. Washing step was repeated twice. After washing SA-PMPs were resuspended with 100 μL of 0.5x SSC solution.

The upper phase was transferred into the washed SA-PMPs. The mixture was incubated for 2 minutes at room temperature. After incubation tubes were placed on magnetic stant and particules were captured and solution was poured off. Particules were resuspended in 0.5 mL of 0.5x SSC solution and washing step was repeated for 3 times.

Captured particules were resuspended in 25 μL nuclease-free water (Promega) and incubated for 2 minutes at room temperature. After incubation tubes were placed on magnetic stand and particules were captured. The eluted mRNAs was transferred to a fresh tube. Elution in 25 μL nuclease-free water was repeated once more. mRNAs were stored at –80 ^{o}C until use.

2.5. RNA gel preparation using phosphate buffer

2.5.1. 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) was added and the volume was adjusted to 10 μL by adding appropriate amount of nuclease free water.

2.5.2. 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 in to the gel tray. 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.

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 12 kb.

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 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 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.

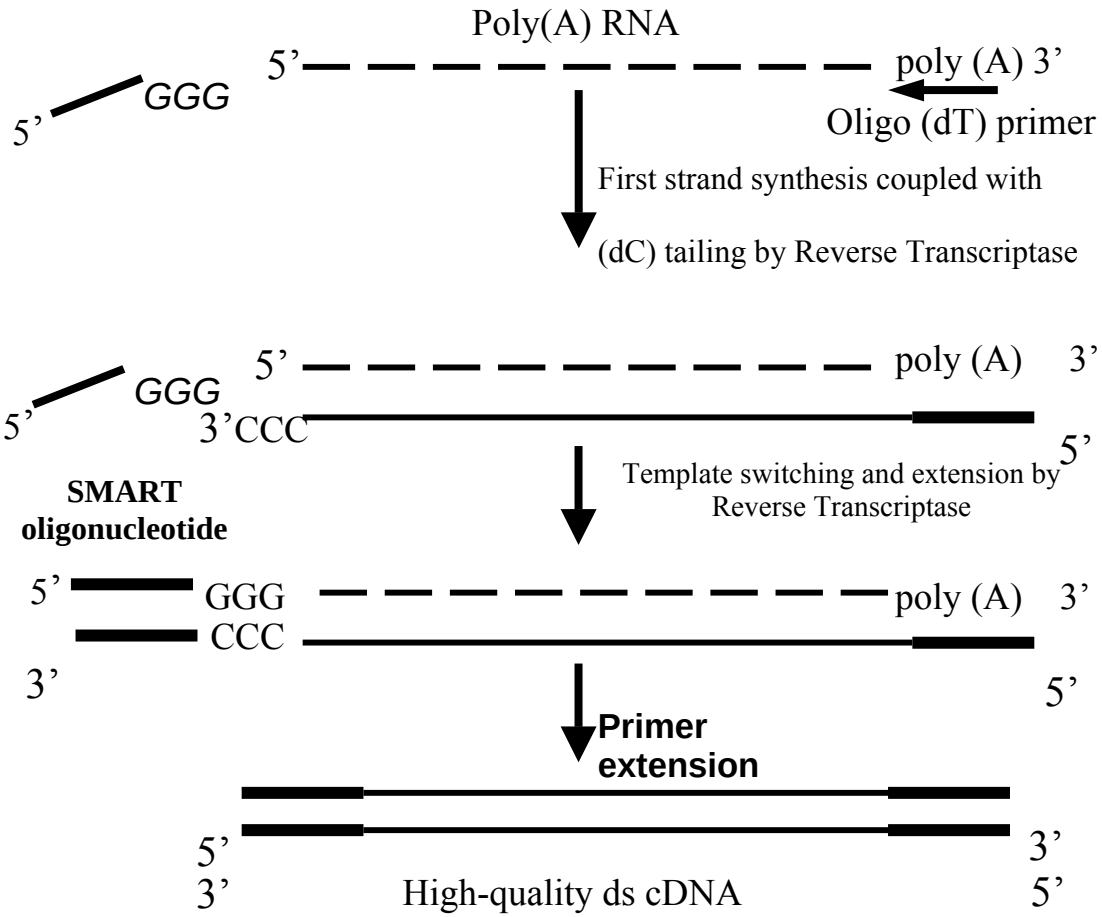

Figure 2.1. Synthesis of ds-cDNA

2.8. Preperation of primers

Primers were designed for RT-PCR. Codehop, Primer 3 and Clustal X programs were used when designing the primers to use in RT-PCR.

2.9. RT-PCR reactions with gene specific primers

The template for RT-PCR can be total RNA or poly(A) RNA. RT-PCR reactions can be primed with random primers, oligo(dT), or a gene-specific primer (GSP) using a reverse transcriptase.

RT-PCR can be carried out either in two-step or one-step formats. In two-step RT-PCR, each step is performed under optimal conditions. cDNA synthesis is performed first in RT buffer and one tenth of the reaction is removed for PCR. In one-step RT-PCR, reverse transcription and PCR take place sequentially in a single tube under conditions optimized for both RT and PCR. Two steps RT-PCR procedure was used. In first step, cDNA was synthesised from RNA templates with oligodT and smart oligo priming and using SuperScript III reverse transcriptase enzyme (Invitrogen). In the second step, PCR reaction was made with spesific primers.

For PCR reaction following components were combined in a 200 µL sterile PCR tube: 2 µL cDNA template, 1X PCR Buffer (MBI) (75 mM Tris-HCl with pH 8.8 at 25^{o}C, 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20), 1.5 mM $MgCl_2$ (MBI), 0.25 mM dNTP mix (MBI), 0.2 pmol forward primer, 0.2 pmol reverse primer, 1.0 unit Taq Polymerase enzyme (MBI), and sterile PCR water up to 25 µL. PCR cycling conditions were; initial denaturation at 94 °C for 3 minutes, 36 cycles of three steps as denaturation at 94 °C for 1 minute, annealing at suitable temperature for selected primer set, for 1 minute and extension at 72 °C for 1 minute and 1 step final extension at 72 °C for 15 minutes.

2.10. Detection of PCR products on agarose gel

The expression levels of PCR products were visualized on 1 % agarose gel with ethidium bromide staining.

2.11. Extraction of PCR products from agarose gels

The bands were cut from the agarose gel and put into 2 mL sterile tubes. Bands were frozen by soaking the tubes into liquid nitrogen and then allowed to melt. The bands were squashed with the help of a sterile tip and the liquid containing PCR product was used as a template for reamplification or direct cloning.

2.12. Cloning of PCR products

2.12.1. Ligation of PCR products to pGEM-T-Easy vector

Band isolated PCR products was cut from agarose gel was ligated to pGEM-T-Easy vector (Promega) in a final volume of 10 μL. Following components were combined; 6 μL recovered PCR products, 5 ng pGEM-T-Easy vector (Promega), 1 x Ligase Buffer (Promega) and 2 units T_4 DNA Ligase enzyme (Promega). Ligation reaction took place at 4°C for 18 hours.

2.12.2. Preparation of *E.coli* competent cells

A single colony of *E.coli* Dh5-α cells was inoculated into 2 ml LB medium. Cells were grown at 37°C with moderate shaking (250 rpm) overnight. 1 mL of overnight grown culture was used to inoculate into 100 ml of LB medium in a sterile 2-liter flask and grown at 37°C, by shaking (250 rpm), until the OD_{590} reaches to 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 4°C at 4000 rpm. Supernatant was poured off and each pellet was resuspended in 10 ml ice-cold $CaCl_2$ (50 mM) solution. Cells were collected by centrifugation as above and supernatant was discarded. Each pellet was suspended in 2 ml of ice-cold $CaCl_2$ (50 mM) solution and stored at 4°C.

2.12.3. 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 containing 5 μL ligation product, 50 μL *E.coli Dh5-α* competent cells. The mixture was then placed on ice for 30 min, then heat shocked at 42 °C for 60 sec. and transfered on ice without shaking. After 2 min of incubation on ice, 150 μL SOC medium was added. Cells were grown at 37°C with moderate shaking and they were spreaded ampicillin (200 μg/L) and inoculated in sterile 2 ml tubes containing 500 μL LB medium with ampicillin and incubated at 37 °C overnight. They were stored in 25% glycerol at -80 °C.

2.13. Confirmation of the clones with inserts

Insert carrying clones were confirmed by PCR using primersat the vector sites flanking insert: 1X PCR Buffer (MBI), 0.25 mM dNTP mix (MBI), 0.8 pmol M13 Forward and Reverse primers (TIB Molecular Biology), 1.2 mM $MgCl_2$ (MBI), 1 unit *Taq* Polymerase enzyme, 1 μL DNA from colonies and sterile PCR water up to 25 μL final volume combined in a sterile PCR tube. PCR was performed as in the followings: after 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 electrophorased on 1.0% agarose gels to detect positive and false positive colonies (colonies that do not carry the expected sized DNA fragment). Positive colonies were selected and used in plasmid isolation step.

2.14. Plasmid isolation for sequencig

Plasmids were isolated using QIAGEN QIAperp Spin Miniprep Kit acording to kit protocol.

Bacterial culture of 2 mL was harvested by centrifugation for 5 min at 15,000 rpm. Supernatant was poured off, the tube was inverted and blotted to on a paper towel to remove excess media. 250 μL of Buffer P1 was added and cell pellet was completely resuspended by vortexing. 250 μL of Buffer P2 was added, then the tube mixed by gentle inversion four times, incubated at RT for approximately 5 minutes. 350 μL of N3 solution was added and mixed by inverting 4 times. Sample tubes were centrifuged at 15,000 rpm for 10 minutes at RT. A spin column, provided by manufacturer, inserted in to collection tube. Cleared lysate, the upperphase was transferred to the spin column, centrifuged at 15,000 rpm for 1 minute at room temperature.

After the centrifugation, lower phase in the collection tube was discarded and collection tube reinserted. 750 μL of column washing solution Buffer PE was added

to spin column, centrifuged at 15,000 rpm for 1 minute 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 the isolated plasmids were stored at – 20 °C.

2.15. Visualization of isolated plasmid

The PCR reaction above was performed on the isolated plasmids again. The products were analysed on the agarose gels to confirm that the plasmids are recombinant.

2.16. Sequencing reactions

600 ng purified pGEM-T Easy (Promega) recombinant clones were combined with 8 pmol T7 primer and sent to Keck DNA sequencing facility at Yale University.

2.17. 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) and BoxShade computer programs (http://www.ch.embnet.org/software/ BOX_form.html).

CHAPTER III

RESULTS AND DISCUSSION

3.1. RNA isolations

mRNAs were isolated from Kıraç-66 root tissue samples and total RNA isolation was performed from Tokak-157 and BDMM-19 root tissue samples according to the procedures presented in sections 2.4.2 and 2.4.3.

3.2. Concentration determination of isolated total RNA samples

Concentrations of the samples were calculated according to the procedure presented in section 2.4.2. Absorbance values and concentrations are listed in Table 3.1.

Table 3.1. The absorbance values and concentrations of the isolated total RNA samples of Tokak-157 andBDMM-19.

Sample Name	**A_{230}**	**A_{260}**	**A_{280}**	**Conc.µg/µL**
T1	0,059	0,021	0,02	0,14
T2	0,094	0,093	0,064	0,619
T3	0,182	0,218	0,146	1,451
T4	0,078	0,096	0,068	0,639
T5	0,1	0,069	0,052	0,459
B1	0,562	0,58	0,386	3,862
B2	0,505	0,105	0,082	0,699
B3	0,176	0,102	0,073	0,679
B4	0,35	0,08	0,058	0,532
B5	0,197	0,049	0,039	0,326
B6	0,116	0,073	0,052	0,486
B7	0,144	0,043	0,033	0,286
B8	0,608	0,12	0,094	0,805

The integrity is very important when working with the RNA. The integrity was confirmed by checking the appearances of the 28 S and 18 S RNA subunits of ribosomal RNA on the RNA gels. As it is seen in Figure 3.1., the 28 S and 18 S RNA subunits of ribosomal RNA appears to be very intact.

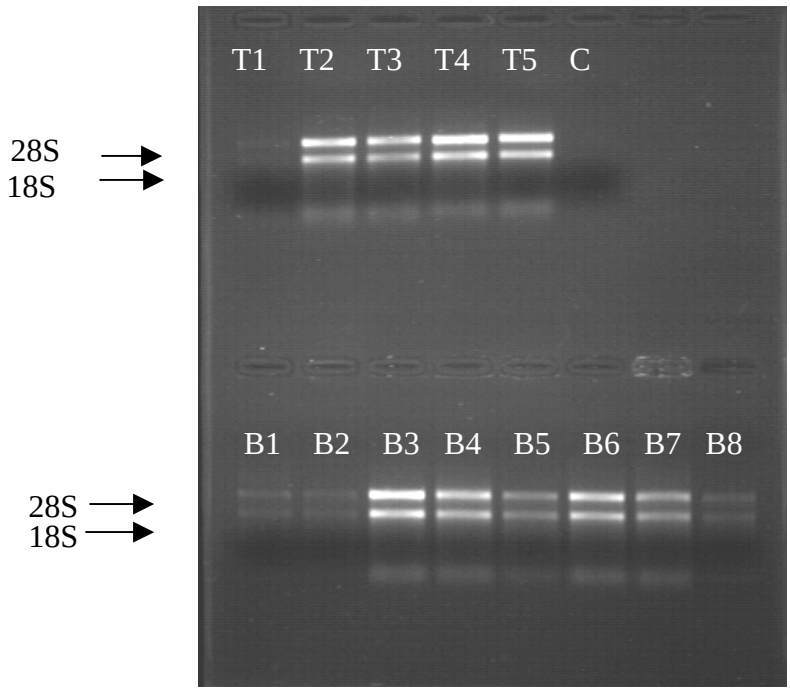

Figure 3.1. 1% RNA gel (with phosphate buffer) containing total RNAs (2 μg) isolated from roots of BDMM/19 and Tokak-157 plants.

3.3. cDNA synthesis reactions

First strand cDNAs were synthesized from Kıraç-66 mRNA and Tokak-157 and BDMM-19 total RNA samples according to the procedures presented in section 2.6. The amount of the total RNA samples used for cDNA synthesis were adjusted according to the band intensities of the RNA gel (Figure 3.1.).

In second strand cDNA synthesis, both Accurase enzyme (Gene Sys. Ltd) which has the ability to synthesize longer products and Taq DNA Polymerase enzymes were used together.

3.4. Primer design

Genes known to take role in phytosiderophore synthesis and genes that were found to be induced in iron deficiency stress were investigated and primers for the conserved regions of these genes were designed by using some bioinformatic programs; *Codehop*, *Primer3* and *Primer Detective*.

3.4.1. S-adenosylmethionine synthetase (SAM synthetase)

SAM synthetase is the first gene of phytosiderophore synthesis pathway. It converts L-methionine to S-adenosyl methionine. SAM synthetase has been identified from many organisms but wheat and expression level studies under iron deficiency stress has been studied but no significant expression difference has been detected altough it is the first enzyme of phytosiderophore synthesis pathway. Degenerate primers for the conserved regions of the previously identified SAM synthetase gene sequeces were designed. Amino acid sequences of the selected genes were written in fasta format and loaded to the *Block Maker* program (Appendix A 1.1.). The blocks generated from the conserved regions of the

sequences (Appendix A 1.2.) were loaded to the Codehop program and degenerate primers were designed (Appendix A 1.3.).

Table 3.2. The sequences of the forward and reverse primers for SAM-synthetase

SAM synthetase forward primer :	5′GAC CAA CAT GGT Gat ggt ntt ygg 3′24bp
SAM synthetase reverse primer :	5′GTC GTT GGT CAC Ggt ytc rtc rtg 3′24bp

3.4.2. Nicotianamine synthase (NAS)

NAS combines three molecules of S-adenosyl methionine to produce one molecules of nicotianamine. Degenerate primers were designed from the conserved regions of the amino acid sequences of the selected NAS genes using Block Maker and Codehop programs as described in section 3.4.1. (Appendix A 1.4, 1.5, and 1.6.)

Table 3.3. The sequences of the forward and reverse primers for NAS

NAS forward primer :	5’GAG GTG GAC GCC ytn tty can ga 3′ 23bp
NAS reverse primer :	5′GGC GGC CAG GAA nac nac rtc rta 3′ 24bp

3.4.3. Nicotianamine aminotransferase (NAAT)

NAAT converts nicotianamine to mugineic acid. Degenerate primers were designed from the conserved regions of the amino acid sequences of the selected NAAT genes using Block Maker and Codehop programs as described in section 3.4.1. (Appendix A 1.7, 1.8, and 1.9.)

Table 3.4 The sequences of the forward and reverse primers for NAAT

NAAT forward primer :	5′GAC CGG CCA Gtt yaa ytg yta 3′21bp
NAAT reverse primer :	5′TTC AGC TTC ACC atn acr aac 3′21bp

3.4.4. Fe deficiency induced gene 3 (FDR-3)

FDR-3 gene was found in *Zea mays* to be induced under iron deficient conditions. The primers were synthesized for *Zea mays* FDR-3 gene nucleotide sequence with *Primer3* program (Appendix A 1.10 and 1.11.).

Table 3.5. The sequences of the forward and reverse primers for FDR-3

FDR-3 forward primer :	5′GCA CCT CGA ATA CGA CCA C 3′ 19 bp
FDR-3 reverse primer :	5′AGG TTC ACC GTC ACC ACT TC 3′ 20 bp

3.4.5. Iron deficiency induced gene-1 (IDI-1)

IDI-1 gene was found to be induced in *Hordeum vulgare* under iron deficient conditions. The primers were synthesized for *Hordeum vulgare* IDI-1 gene nucleotide sequence with *Primer3* program (Appendix A 1.12 and 1.13.).

Table 3.6. The sequences of the forward and reverse primers for IDI-1

IDI-1 forward primer :	5′ATG GAG AAC GAG TTC CAG 3′18 bp
IDI-1 reverse primer :	5′TCA ACG AGC CTC GAC GGT 3′18 bp

3.4.6. Elongation Factor-2 (EF-2)

EF-2 is a constitutively expressed gene. In order to use for concentration determination of cDNAs, primers were designed from wheat EF-2 nucleotide sequence with *Primer Detective* program (Appendix A 1.14 and 1.15.).

Table 3.7. The sequences of the forward and reverse primers for EF-2

EF-2 forward primer :	ATC ACT GAT GGA GCT TTG GTG G 22 bp
EF-2 reverse primer :	GTA AGA GAC GAC CAG ACG TAC 21 bp

3.4.7. Fe(II) related transporter-1 (IRT1)

IRT-1 was found to be induced under iron deficient conditions in *Hordeum vulgare*. IRT-1 primers were designed with *Primer Detective* program from *Hordeum vulgare* IRT-1 nucleotide sequence. (Appendix A 1.16 and 1.17.).

Table 3.8. The sequences of the forward and reverse primers for IRT-1

IRT forward primer :	5′TCA TTA AGT GTT TCG CCT CCG 3′ 21 bp
IRT reverse primer :	5′TTG ATG GAA GCA AAG AGC TGC 3′ 21 bp

3.5. RT-PCR reactions for clonning

3.5.1. S-adenosyl methionine synthetase (SAM synthetase)

RT-PCR reaction was made with spesific primers for SAM synthetase. PCR conditions were; 1 cycle initial denaturation at 94 ^{o}C for 3 min, 36 cycles of denaturation at 94 ^{o}C for1 min, annealing at 55^{o}C for 1 min and extension at 72^{o}C for 1 min and 1 cycle final extension at 72^{o}C for 15 min. The PCR products were electrophorased in 1% agarose gel (Figure 3.2.). The labeled band was cut for cloning steps.

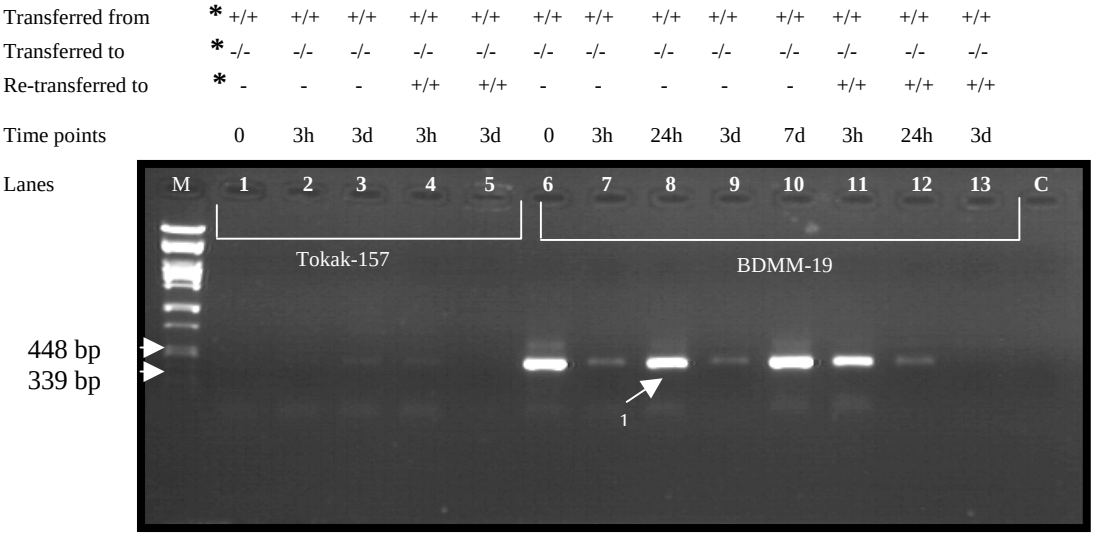

Fig 3.2. The appearance of RT-PCR products of Tokak-157 and BDMM-19 cDNAs (from total RNA) with SAM synthetase primers on 1% agarose gel. The expected product size is 440 bp. The band indicated with an arrow was cut and cloned for sequencing. Plant samples were grown in +Zn/+Fe nutrient solution for 1 week and then transferred to -Zn/-Fe nutrient solution. Root and shoot tissue samples were taken at different time points. After 1 week the samples were retransfered to +Zn/+Fe nutrient solution and again samples were taken at different time points. *****: +/+ indicates +Zn/+Fe and -/- indicates -Zn/-Fe conditions, h: hour, d: day, M: Marker λ/Pst, C: Negative control

3.5.2. Iron deficiency induced gene-1 (IDI-1)

RT-PCR reaction was made with IDI-1 primers. PCR conditions were; 1 cycle initial denaturation at 94 ^{o}C for 3 min, 36 cycles of denaturation at 94^{o}C for1 min, annealing at 55^{o}C for 1 min and extension at 72^{o}C for 1 min and 1 cycle final extension at 72^{o}C for 15 min. The PCR products were electrophorased in 1% agarose gel (Figure 3.3.). The labeled band was cut for cloning.

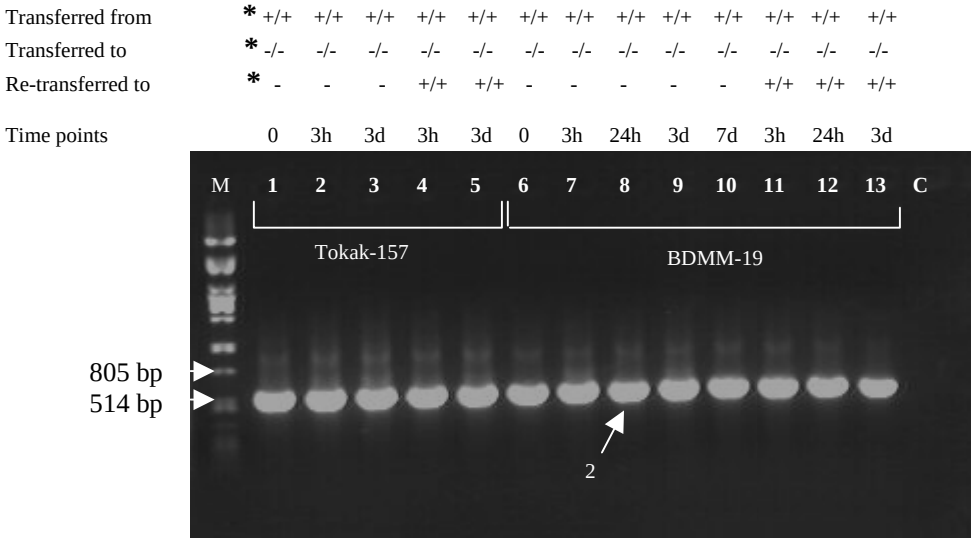

Figure 3.3. The appearance of RT-PCR products of Tokak-157 and BDMM-19 cDNAs (from total RNA) with IDI-1 primers on 1% agarose gel. The expected product size is 590 bp. The band indicated with an arrow was cut and cloned for sequencing. Plant samples were grown in +Zn/+Fe nutrient solution for 1 week and then transferred to -Zn/-Fe nutrient solution. Root and shoot tissue samples were taken at different time points. After 1 week the samples were retransferred to +Zn/+Fe nutrient solution and again samples were taken at different time points. *****: +/+ indicates +Zn/+Fe and -/- indicates -Zn/-Fe conditions. -: no retransfer, h: hour, d: day, M: Marker λ/Pst, C: Negative control

3.5.3. Nicotianamine synthase (NAS)

RT-PCR reaction was made with spesific primers for NAS. PCR conditions were; 1 cycle initial denaturation at 94 ^{o}C for 3 min, 36 cycles of denaturation at 94 ^{o}C for1 min, annealing at 46^{o}C for 1 min and extension at 72^{o}C for 1 min and 1 cycle final extension at 72^{o}C for 15 min. The PCR products were electrophorased in 1% agarose gel (Figure 3.4.). The labeled bands were cut for cloning.

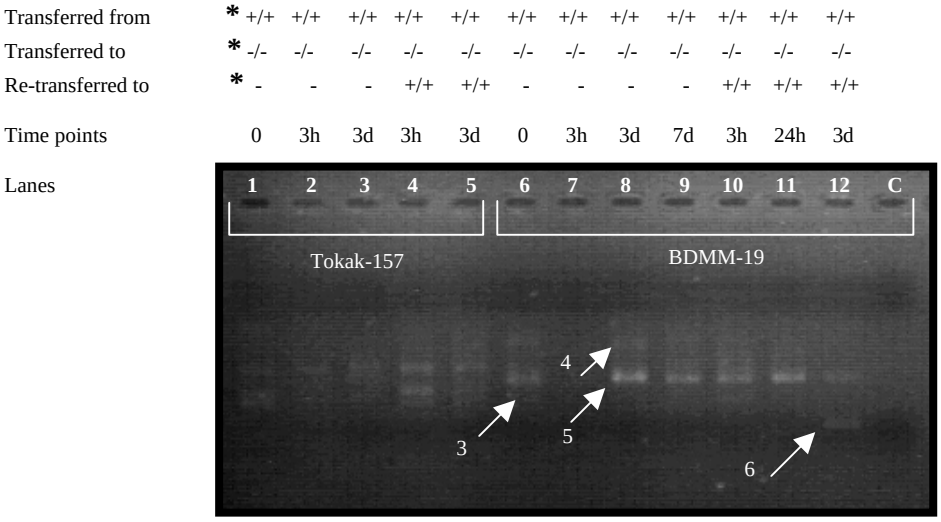

Figure 3.4. The appearance of RT-PCR products of Tokak-157 and BDMM-19 cDNAs (from total RNA) with NAS primers on 1% agarose gel. The expected product size is 510 bp. The bands with different sizes, indicated with arrows, were cut and cloned for sequencing. Plant samples were grown in +Zn/+Fe nutrient solution for 1 week and then transferred to -Zn/-Fe nutrient solution. Root and shoot tissue samples were taken at different time points. After 1 week the samples were retransferred to +Zn/+Fe nutrient solution and again samples were taken at different time points. *****: +/+ indicates +Zn/+Fe and -/- indicates -Zn/-Fe conditions, h: hour, d: day, C: Negative control

3.5.4. Nicotianamine amino transferase (NAAT)

RT-PCR reaction was made with Tokak-157, BDMM-19 and Kıraç-66 cDNAs and NAAT primers. The PCR conditions used for other primers were not worked for NAAT. So, the conditions were changed as; 1 cycle initial denaturation at 94 ^{o}C for 3 min, 36 cycles of denaturation at 94 ^{o}C for1 min, annealing at 47^{o}C for 1 min and extension at 72^{o}C for 4 min and 1 cycle final extension at 72^{o}C for 15 min. The PCR products were electrophorased in 1% agarose gel (Figure 3.5.). The labeled bands were cut for cloning.

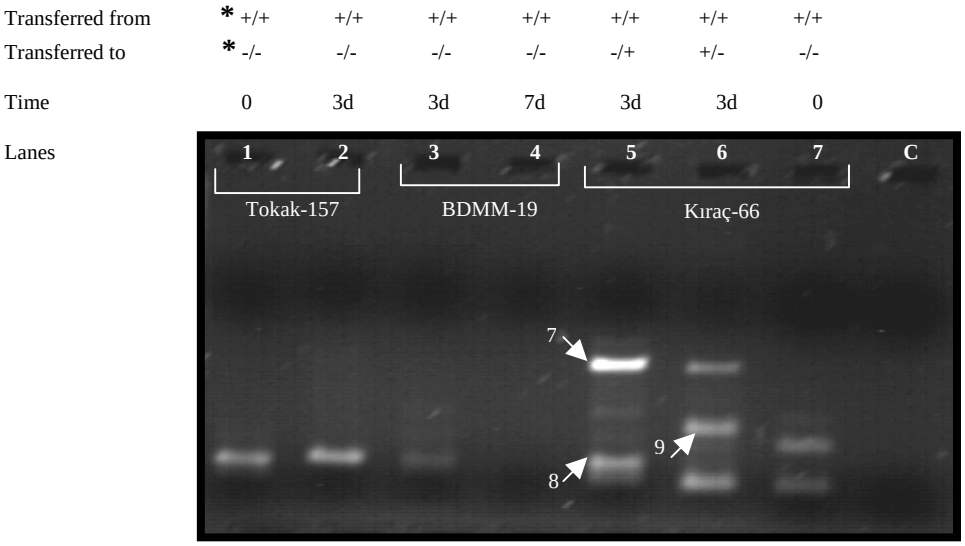

Figure 3.5. The appearance of RT-PCR products with NAAT primers on 1% agarose gel. The expected product size is 530 bp. The bands with different sizes, indicated with arrows, were cut and cloned for sequencing. Plant samples were grown in +Zn/+Fe nutrient solution for 1 week and then transferred to -Zn/-Fe nutrient solution. Root and shoot tissue samples were taken at different time points. *****: +/+ indicates +Zn/+Fe and -/- indicates -Zn/-Fe conditions, h: hour, d: day, C: Negative control

3.5.5. Iron related transporter-1 (IRT-1)

RT-PCR reaction was made with spesific primers for IRT-1. PCR conditions were; 1 cycle initial denaturation at 94 ^{o}C for 3 min, 36 cycles of denaturation at 94 ^{o}C for1 min, annealing at 53^{o}C for 1 min and extension at 72^{o}C for 1 min and 1 cycle final extension at 72^{o}C for 15 min. The PCR products were electrophorased in 1% agarose gel (Figure 3.6.). The labeled bands were cut for cloning.

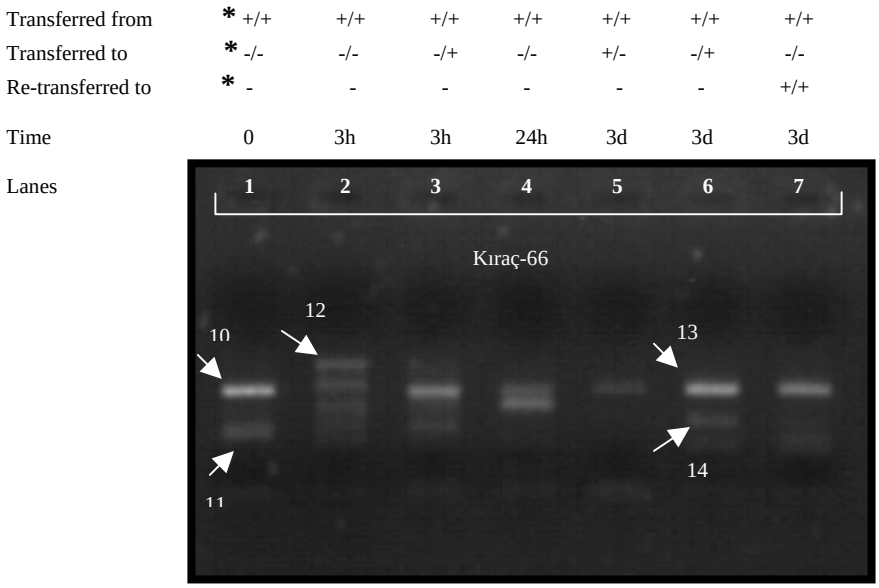

Figure 3.6. The appearance of RT-PCR products with Kıraç-66 cDNAs (from total RNA) and IRT-1 primers on 1% agarose gel. The bands indicated with arrows were cut and cloned for sequencing. Plant samples were grown in +Zn/+Fe nutrient solution for 1 week and then transferred to -Zn/-Fe nutrient solution. Root and shoot tissue samples were taken at different time points. After 1 week the samples were retransferred to +Zn/+Fe nutrient solution and again samples were taken at different time points. *****: +/+ indicates +Zn/+Fe and -/- indicates -Zn/-Fe conditions. -: no retransfer, h: hour, d: day,

3.5.6. Fe deficiency related gene-3 (FDR-3)

RT-PCR reaction was made with spesific primers for FDR-3. PCR conditions were; 1 cycle initial denaturation at 94 ^{o}C for 3 min, 36 cycles of denaturation at 94^{o}C for1 min, annealing at 53^{o}C for 1 min and extension at 72^{o}C for 1 min and 1 cycle final extension at 72^{o}C for 15 min. The PCR products were electrophorased in 1% agarose gel (Figure 3.7.). The labeled band was cut for cloning.

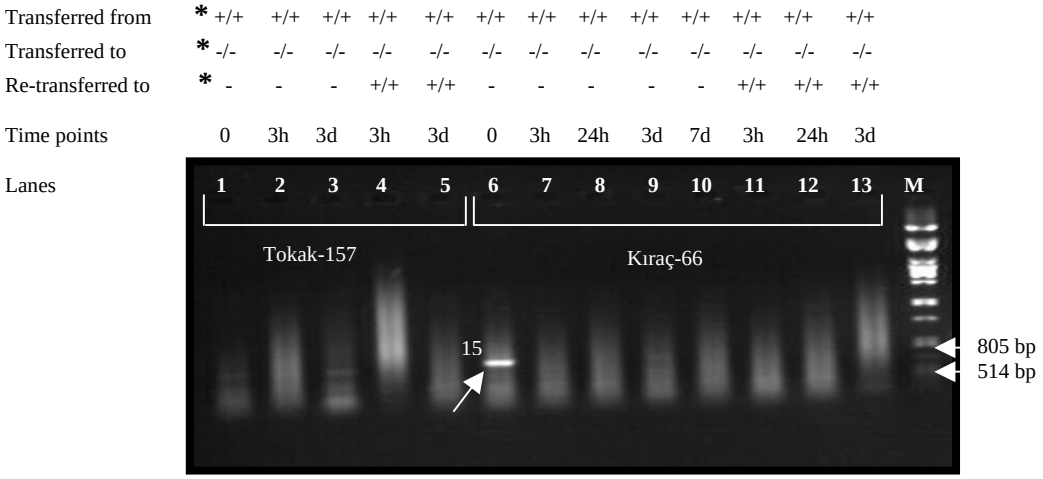

Figure 3.7. The appearance of RT-PCR products with FDR-3 primers on 1% agarose gel. The expected product size is 525bp. The band indicated with an arrow was cut and cloned for sequencing. Plant samples were grown in +Zn/+Fe nutrient solution for 1 week and then transferred to -Zn/-Fe nutrient solution. Root and shoot tissue samples were taken at different time points. After 1 week the samples were retransferred to +Zn/+Fe nutrient solution and again samples were taken at different time points. *****: +/+ indicates +Zn/+Fe and -/- indicates -Zn/-Fe conditions. -: no retransfer, h: hour, d: day, M: Marker λ/Pst.

Table 3.9. The list of cut bands

BAND NUMBER	TARGET GENE	SOURCE ORGANISM
1	SAM-synthetase	BDMM-19
2	IDI-1	BDMM-19
3	NAS	BDMM-19
4	NAS	BDMM-19
5	NAS	BDMM-19
6	NAS	BDMM-19
7	NAAT	KIRAÇ-66
8	NAAT	KIRAÇ-66
9	NAAT	KIRAÇ-66
10	IRT-1	KIRAÇ-66
11	IRT-1	KIRAÇ-66
12	IRT-1	KIRAÇ-66
13	IRT-1	KIRAÇ-66
14	IRT-1	KIRAÇ-66
15	FDR-3	KIRAÇ-66

3.6. Cloning and sequencing of cut bands

Cloning of the fragments was achieved by using *E.coli Dh5-α* competent cells after their ligation into pGEM-T easy vectors and cloned according to the procedure presented in section 2.6. All of the cut bands were cloned but only band with number 1 and 2 were sequenced. Cloning and sequencing results of these two bands are represented in this section. Other cloned bans are going to be sequenced by further studies.

3.6.1. Band number 1 (SAM-synthetase)

Band number 1 belongs to BDMM-19 SAM-synthetase was cloned. Blue and white colonies were selected among SAM synthetase transformants and PCR reaction with M13 primers was made for the selection of recombinant colonies (Figure 3.8).

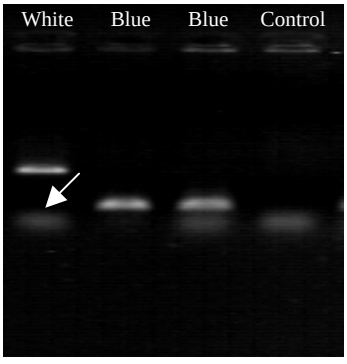

Figure 3.8. The appearance of the results of PCR reaction with M13 primers. It was seen that the white colony carries an insert fragment while blue ones were empty.

The white colony was grown and plasmid isolation was performed according to the protocol written in section 2.7. The absorbance values and the concentration of the isolated recombinant plasmid were measured (Table 3.10.).

Table 3.10. Absorbance values and concentrations of the isolated recombinant plasmid carrying putative SAM-synthetase insert.

A_{230}	A_{260}	A_{280}	Conc.μg/μL
0,031	0,033	0,023	0,275

Plasmids were purified as described in section 2.7. Inserts were custom sequenced, and were read using SP6 primers from one direction. Sequencing reactions of the 600 ng purified pGEM-T Easy (Promega) recombinant clones were performed. Obtained sequences are presented in Table 3.11 and Table 3.12.

Table 3.11. Nucleotide sequence result for SAM-synthetase fragment

```
1 T CGT GCT GGG TAG AGA TGA GGA CGG TGT
GTA CAC GAA CAG GCA CCA TGG CAC CAC CCT
CGT TTA GGT ACT CAA TGG TGA CCT GGG TCT
TTC CGT CAG GCC TGA GCC AGG CAC AGG TGC
CAT TCT TGC GGA CCT CGG TAA GGC GAG CTC
CAA GCT TGG TGG CGA GCA TGT GGG TGA GGG
GCA TCA GCT CAG GAG TCT CAT CAG TGG CGT
AGC CGA ACA TGA TGC CCT GGT CAC CAG CGC
CGA TCT CTT CGG GGC GCT TGG TGA AGT GTC
CGT GAA CAC CCT GGG CAA TGT CAG GGG ATT
GCT GCT CGA TGT TGA CAA GCA CCT TGC AAT
GGT CAG CAT CCA GAC CAA CGT CGT CAG AGA
TGA AGC CGA TGC TGC GGC AGG TGT CGC GCA
CGA TCT TCT CGT AGT CGA CGG TGG CCT TGG
TGG TGA TCT CGC CAA AAA CCA TCA CCA TAA 448
```

Table 3.12. Amino acid sequence result for SAM-synthetase fragment

```
SCWVEMRTVCTRTGTMAPPSFRYSMVTWVFPQAARHRCHF
ADLGKASSKLGGEHVGEGHQLRSLISGVAEHDALVTSADL
FGALGEVSVNTLGNVRGLLLDVDKHLAMVSIQTNVVRDEA
DAAAGVAHDLLVVDGGLGGDLAKNHHH.
```

The obtained nucleotide and amino acid sequences were searched in the NCBI (National Center for Biotechnology Information) database for comparison with available gene sequences. Sequences of bands were searched for sequence similarity in standart nucleotide-nucleotide and protein blast.

The results of standart nucleotide-nucleotide blast are shown in the Table 3.13. and The results of protein blast are shown in the Table 3.14.

Table 3.13. Nucleotide sequences producing significant alignments with the obtained nucleotide sequence of band number 1 (SAM-synthetase).

Band	Blast Hits	Accession Number	Score (Bits)	E Value	Identities
1	Barley mRNA for SAM synhetase complete cds Length = 1353	D63835	664(335)	0,00	416/443 (93%)
	Oryza sativa SAMS2 mRNA,complete cds Length=1736	U82833	448(226)	e-124	391/446 (87%)
	Oryza sativa SAMS1 gene for S-adenosyl methionine synthetase Length = 2183	Z2667	315(159)	3e-84	372/443 (83%)
	Oryza sativa mRNA for(SAMS gene Length = 1594	AJ296743	315(159)	3e-84	372/443 (83%)
	Dendrobium crumenatum Sams mRNA, complete cds Length = 1566	AF420238	276(139)	3e-72	358/431 (83%)
	Zea mays CL2757_5 mRNA sequence Length = 1912	AY109333	266 (134)	3e-69	368/446 (82%)
	Brassica juncea clone MSAMS3 S-adenosylmethionine synthetase mRNA,complete cdsLength = 1459	AF379013	248(125)	6e-64	233/269 (86%)
	Musa acuminata S-adenosyl-L-methionine synthetase homolog mRNA,complete cds Length = 1547	AF004317	246(124)	2e-63	280/332 (84%)
	Pinus contorta sams2 mRNA, complete cds Length = 1418	AF187821	196(99)	2e-48	360/447 (80%)
	Carica papaya SAMS mRNA, complete cds Length = 1886	AF531479	172(87)	3e-41	357/447 (79%)
	Phaseolus lunatus SAMS mRNA for,complete cds Length = 1659	AB062358	168(85)	5e-40	331/413 (80%)
	A.thaliana Sadenosylmethionine synthetase gene, complete cds Length = 2559	M55077	157(79)	2e-36	355/447 (79%)
	A.thaliana DNA for S-adenosylmethionine synthetase gene sam-1Length = 2559	X53323	157(79)	2e-36	355/447 (79%)
	L.esculentum S-adenosyl-L-methionine synthetase mRNA, complete CDS Length = 1479	Z24741	151(76)	1e-34	292/364 (80%)
	Zea mays methionine adenosyltransferase mRNA, partial cds Length = 760	AF439721	147(74)	2e-33	227/278 (81%)
	Nicotiana tabacum S-adenosyl-L-methionine synthetase(SAMS) mRNA,complete cdsLength = 1636	AF127243	147(74)	2e-33	227/278 (81%)
	Arabidopsis thaliana S-adenosylmethionine synthase 2 (At4g01850/T7B11_11) mRNA, complete cds Length = 1182	BT000575	145(73)	7e-33	220/269 (81%)

Table 3.14. Amino acid sequences producing significant alignments with the obtained amino acid sequence of band number 1 (SAM-synthetase)

Band	Blast Hits	Accession Number	Score (Bits)	E Value	Identities
1	S-adenosylmethionine synthetase [Hordeum vulgare] Length = 394	BAA09895	304 (779)	4e-83	147/149 (98%)
	S-adenosyl-L-methionine synthetase [Oryza sativa] Length = 394	AAC05590	291 (746)	3e-79	140/149 (93%)
	S-adenosyl-L-methionine synthetase [Dendrobium crumenatum]Length = 395	AAL16064	288 (736)	4e-78	136/149 (91%)
	S-adenosylmethionine synthase 2 [Arabidopsis thaliana] Length = 393	AAL61934	287(734)	7e-78	137/149 (91%)
	S-adenosylmethionine synthetase [Brassica juncea] Length = 393	AAK71235	286(732)	1e-77	137/149 (91%)
	S-adenosylmethionine synthetase [Pinus contorta] Length = 393	AAG17036	286 (732)	1e-77	139/149 (93%)
	S-adenosyl-L-methionine synthetase [Elaeagnus umbellata]Length = 393	AAK29409	286(731)	2e-77	137/149 (91%)
	S-adenosylmethionine synthetase [Oryza sativa]Length = 396	CAC82203	283(725)	7e-77	136/149 (91%)

The alignment of the nucleotide sequence of the cloned SAM-synthetase fragment and *Hordeum vulgare* SAM-synthetase sequence is shown in Table 3.15.

Table 3.15. The alignment of the nucleotide sequence of the cloned SAM-synthetase fragment and *Hordeum vulgare* SAM-synthetase sequence

```
>gi|960356|dbj|D63835.1|BLYSAS  Barley  mRNA  for  S-adenosylmethionine  synthetase,
complete cds
Length = 1353 Score = 664 bits (335), Expect = 0.0
Identities = 416/443 (93%) Strand = Plus / Minus


Query:5   tgctgggtagagatgaggacggtgtgtacacgaacaggcaccatggcaccaccctcgttt 64
          |||||||| |||||||||||||||| |||||||| ||||||||||||||||||||||||
Sbjct:650 tgctgggtggagatgaggacggtgtgcacacgaacgggcaccatggcaccaccctcgttt 591


Query: 65 aggtactcaatggtgacctgggtctttccgtcaggcctgagccaggcacaggtgccattc 124
          |||||||||||||||||||||||||||||| ||||||||||||||| ||||||||||||
Sbjct:590 aggtactcaatggtgacctgggtctttccatcaggcctgagccaggcgcaggtgccattc 531


Query: 125 ttgcggacctcggtaaggcgagctccaagcttggtggcgagcatgtgggtgaggggcatc 184
           ||||||||||||||| |||||||||| |||||||||| ||||||||||||||||||||
Sbjct: 530 ttgcggacctcggtgaggcgagctccgagcttggtggcaagcatgtgggtgaggggcatc 471


Query: 185 agctcaggagtctcatcagtggcgtagccgaacatgatgccctggtcaccagcgccgatc 244
           |||||||| |||||||||||||||||| |||||||||||||||||| ||||||| |
Sbjct: 470 agctcaggggtctcatcagtggcgtagccaaacatgatgccctggtcaccggcgccgacc 411


Query: 245 tcttcggggcgcttggtgaagtgtccgtgaacaccctgggcaatgtcaggggattgctgc 304
           ||||| || ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 410 tcttctggacgcttggtgaagtgtccgtgaacaccctgggcaatgtcaggggattgctgc 351


Query: 305 tcgatgttgacaagcaccttgcaatggtcagcatccagaccaacgtcgtcagagatgaag 364
           |||||||||||| |||||||||||||||| ||||| ||||| ||||||||||||||||
Sbjct: 350 tcgatgttgacgagcaccttgcaatggtcggcatcgagaccgacgtcgtcagagatgaag 291


Query: 365 ccgatgctgcggcaggtgtcgcgcacgatcttctcgtagtcgacggtggccttggtggtg 424
           |||||| |||||||||||||||| |||||||| ||||| ||||||||||||||||||
Sbjct: 290 ccgatgtcacggcaggtgtcgcgcacaatcttctcatagtcaacggtggccttggtggtg 231


Query: 425 atctcgccaaaaaccatcaccat 447
           |||||||| || ||||| |||||
Sbjct: 230 atctcgccgaagaccatgaccat 208
```

3.6.2. Band number 2 (IDI-1)

Band number 2 belongs to BDMM-19 IDI-1 was cloned. Blue and white colonies were selected among IDI-1 transformants and PCR reaction with M13 primers was made for the selection of recombinant colonies (Figure 3.9)

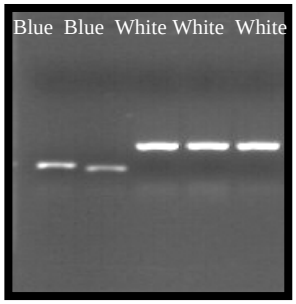

Figure 3.9. The appearance of the results of PCR reaction with M13 primers. It was seen that the white colonies carry an insert fragment while blue ones were empty.

Plasmid isolations were performed from the grown white colonies according to the protocol written in section 2.7. The absorbance values and the concentrations were measured (Table 3.16.).

Table 3.16. Absorbance values and concentrations of the isolated recombinant plasmid carrying putative IDI-1 insert.

A_{230}	A_{260}	A_{280}	Conc.μg/μL
0,039	0,037	0,035	0,308

Plasmids were purified as described in section 2.7. Inserts were custom sequenced, and were read using SP6 primers from one direction. Sequencing reactions of the 600 ng purified pGEM-T Easy (Promega) recombinant clones were performed. Obtained sequences are presented in Table 3.17 and Table 3.18.

Table 3.17. Nucleotide sequence result for IDI-1 fragment

```
1ATG GAG AAC GAG TTC CAG GAT GGC AAG GAG
 GAG GTC ATC CAA GCA TGG CTA CAT GGN ATG
 ACA GTG AAG AGG ATC AGA GGC TTC CTC ACC
 ACC GTG AGC CCA AAG AGT TCA TTC CTC TTG
 CCA AAC TTT CAG AAT TAG GTG TTG TAA GCT
 GGA ACC TAA ATG CTG ATA ACT GGG AGA AAG
 ACG AGA ATC TCA AGA AAA TCC GTG AGG CCA
 GGG GAT ACT CCT ATG TGG ACA TCT GCG ACG
 TAT GTC CTG AGA AAT TGC CAA ACT ATG AGG
 CCA AGC TGA AGA ATT TCT TTG AAG AGC ACT
 TGC ATA CTG ATG AGG AGA TAC GCT ATT GTC
 TTG AGG GCA GTG GAT ACT TTG ATG TGA GGG
 ACC AAA ATG AAC AGT GGA TCC GTA TAG CAG
 TTA AGA AAG GTG GCA TGA TTG TTT TGC CTG
 CAG GAA TGT ATC ACC GCT TTA CAT TGG ATA
 GTG ACA ACT ACA TCA AGG CAA TGC GGC TCT
 TTG TGG GAG AGC CTA TCT GGA CGC CTT ACA
 ATC GTC CCC ATG ACC ATC TCC CAG CTA GAA
 AGG AGT ATG TCG ACA AGA TTA TCA ACA GAG
 GTG GGA ACC AAA CCG TCG AGG CTC GTT GA 600
```

Table 3.18. Amino acid sequence result for IDI-1 fragment

```
MENEFQDGKEEVIQAWLHGMTVKRIRGFLTTVSPKSSFL
LPNFQNVLAGTMLITGRKTRISRKSVRPGDTPMWTSATY
VLRNCQTMRPSRISLKSTCILMRRYAIVLRAVDTLCEGP
KTVDPYSSERCMIVLPAGMYHRFTLDSDNYIKAMRLFVG
EPIWTPYNRPHDHLPARKEYVDKIINRGGNQTVEAR
```

The obtained nucleotide and amino acid sequences were searched in the NCBI (National Center for Biotechnology Information) database for comparison with available gene sequences. Sequences of bands were searched for sequence similarity in standart nucleotide-nucleotide and protein blast.

The results of standart nucleotide-nucleotide blast are shown in the Table 3.19. and the results of protein blast are shown in the Table 3.20.

Table 3.19. Sequences producing significant alignments with the obtained sequence of band number 2 (IDI-1)

Band	Blast Hits	Accession Number	Score (Bits)	E Value	Identities
2	Hordeum vulgare IDI1 mRNA, complete cds Length = 997	AB02559	1009 (509)	0.0	577/597 (96%)
	Oryza sativa submergence induced protein 2A mRNA, complete cds Length = 980	AF06833	678(342)	0.0	518/574 (90%)
	Zea mays PCO075536 mRNA sequence Leng = 1097	AY10374	561(283)	e-158	513/587 (87%)
	Oryza sativa (japonica cultivar-group) cDNA clone:001-036-C08, full insert sequence Length = 612	AK06165	406 (205)	e-111	259/277 (93%)
	Oryza sativa submergence induced protein 2 (sip2) mRNA, complete cds Length = 872	AF05020	361(182)	8e-98	457/546 (83%)
	Arabidopsis thaliana clone 2738 mRNA, complete sequence Length = 991	AY08675	56.0(28)	7e-06	67/80 (83%)
	Musa acuminata submergence induced protein like protein mRNA,partial cds Length = 532	AF41412	56.0(28)	7e-06	109/136 (80%)

Table 3.20. Amino acid sequences producing significant alignments with the obtained amino acid sequence of band number 2 (IDI-1).

Band	Blast Hits	Accession Number	Score (Bits)	E Value	Identities
2	iron-deficiency induced gene [Hordeum vulgare]Length = 198	BAB61039	383(983)	e-106	182/194 (93%)
	submergence induced protein 2A [Oryza sativa]Length = 198	AAC19375	365(937)	e-101	169/194 (87%)
	Putative probable submergence induced, nickel-binding protein 2A[Oryza sativa (japonica cultivar-group)] Length = 254	AAN06863	35(919)	e-101	167/186 (89%)
	putative zinc finger protein ID1 [Oryza sativa (japonica cultivar-group)]Length = 230	AAP53793	329 (843)	8e-91	150/178 (84%)
	submergence induced protein 2A [Arabidopsis thaliana] Length = 199	AAM63805	311(797)	1e-85	139/174 (79%)
	putative heat shock protein [Oryza sativa (japonica cultivar-group)]Length = 184	AAP53794	219(559)	2e-57	94/154 (61%)

The alignment of the nucleotide sequence of cloned IDI-1 fragment and *Hordeum vulgare* IDI-1 sequence is shown in Table 3.21.

Table 3.21. The alignment of the nucleotide sequence of cloned IDI-1 fragment and *Hordeum vulgare* IDI-1 sequence.

```
>gi|14522833|dbj|AB025597.1|   Hordeum vulgare IDI1 mRNA, complete cds
 Length = 997 Score = 1013 bits (511), Expect = 0.0
 Identities = 579/599 (96%), Gaps = 2/599 (0%)
 Strand = Plus / Plus

Query: 2   atggagaacgagttccaggatggcaaggaggaggtcatccaagcatggctacatggnatg 61
           ||||||||||||||||||||||||||||| |||||||||||||||| ||||||| |||
Sbjct: 80  atggagaacgagttccaggatggcaaggagcaggtcatccaagcatgg-tacatgg-atg 137


Query: 62  acagtgaagaggatcagaggcttcctcaccaccgtgagcccaaagagttcattcctcttg 121
           ||||||||||||| ||||||||||||| ||||||||||||||||||||||||||||||
Sbjct: 138 acagtgaagaggaccagaggcttcctcatcaccgtgagcccaaagagttcattcctcttg 197


Query: 122 ccaaactttcagaattaggtgttgtaagctggaacctaaatgctgataactgggagaaag 181
           | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 198 caaaactttcagaattaggtgttgtaagctggaacctaaatgctgataactgggagaaag 257


Query: 182 acgagaatctcaagaaaatccgtgaggccaggggatactcctatgtggacatctgcgacg 241
           | |||||||||||||||||||||||||||||||||||| |||||||||||| ||||| |
Sbjct: 258 atgagaatctcaagaaaatccgtgaggccaggggatactcttatgtggacatttgcgatg 317


Query: 242 tatgtcctgagaaattgccaaactatgaggccaagctgaagaatttctttgaagagcact 301
           ||||||| ||||| |||||||||||| |||||||||||||||||||||||||||| ||||
Sbjct: 318 tatgtccggagaagttgccaaactacgaggccaagctgaagaatttctttgaagaacact 377


Query: 302 tgcatactgatgaggagatacgctattgtcttgagggcagtggatactttgatgtgaggg 361
           |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 378 tgcatactgatgaagagatacgctattgtcttgagggcagtggatactttgatgtgaggg 437


Query: 362 accaaaatgaacagtggatccgtatagcagttaagaaaggtggcatgattgttttgcctg 421
           ||||||| |||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct: 438 accaaaacgaacagtggatccgtatagcagttaagaaaggcggcatgattgttttgcctg 497


Query: 422 caggaatgtatcaccgctttacattggatagtgacaactacatcaaggcaatgcggctct 481
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 498 caggaatgtatcaccgctttacattggatagtgacaactacatcaaggcaatgcggctct 557


Query: 482 ttgtgggagagcctatctggacgccttacaatcgtccccatgaccatctcccagctagaa 541
           |||||||||||||| |||||||||| |||||||| |||||||||||||||||||||||
Sbjct: 558 ttgtgggagagcccatctggacgccgtacaatcgccccccatgaccatctcccagctagaa 617


Query: 542 aggagtatgtcgacaagattatcaacagaggtgggaaccaaaccgtcgaggctcgttga 600
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 618 aggagtatgtcgacaagattatcaacagaggtgggaaccaaaccgtcgaggctcgttga 676
```

3.7. RT-PCR reactions for the evaulation of expression levels of the genes

3.7.1. RT-PCR with 18S

In order to confirm that our BDMM-19 and TOKAK-157 cDNA samples' concentrations are identical RT-PCR reaction was made with 18S ribozomal RNA primers. PCR conditions were; 1 cycle initial denaturation at 94 ^{o}C for 3 min, 36 cycles of denaturation at 94^{o}C for1 min, annealing at 53^{o}C for 1 min and extension at 72^{o}C for 1 min and 1 cycle final extension at 72^{o}C for 15 min. The PCR products were electrophorased in 1% agarose gel. The concentrations of the samples can be determined from the expression levels of 18S ribozomal RNA gene in figure 3.10.

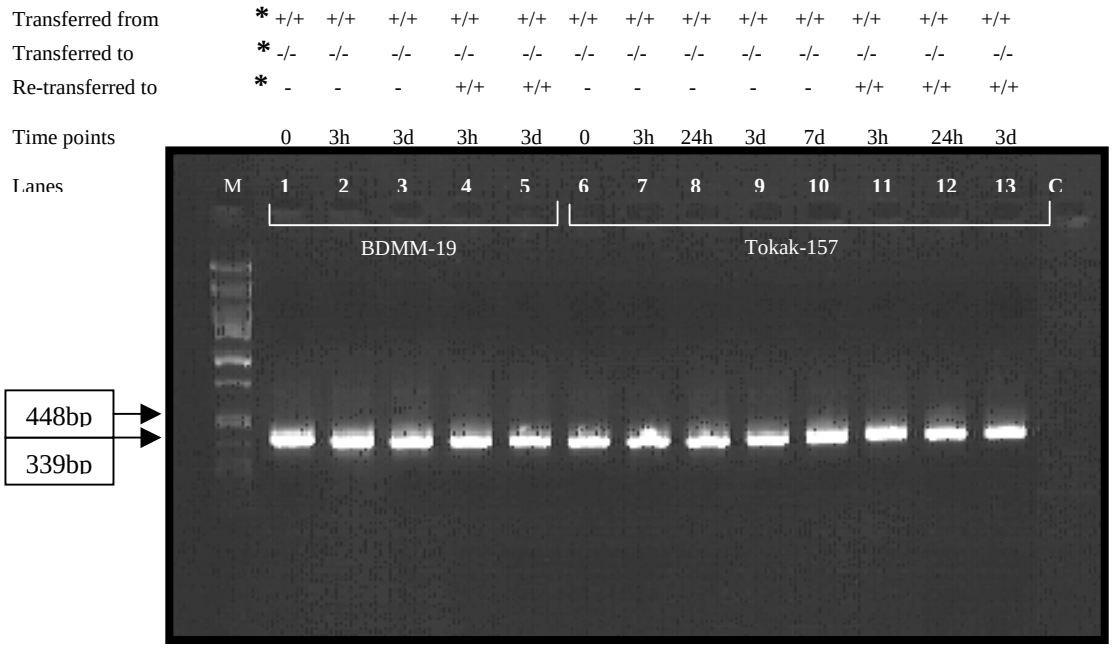

Figure 3.10. 1% DNA gel containing 18S RT-PCR products. The product size is 406bp. *****: +/+ indicates +Zn/+Fe and -/- indicates -Zn/-Fe conditions. -: no retransfer, h: hour, d: day, M: Marker λ/Pst, C: Negative control

3.7.2. RT-PCR with EF-2

Because EF-2 is a constitutively expressed gene it was used to control the concentrations of the samples. RT-PCR was made with Tokak-157, BDMM-19 and Kıraç-66 samples. PCR conditions were; 1 cycle initial denaturation at 94 ^{o}C for 3 min, 36 cycles of denaturation at 94^{o}C for1 min, annealing at 56^{o}C for 1 min and extension at 72^{o}C for 1 min and 1 cycle final extension at 72^{o}C for 15 min. The PCR products were electrophorased in 1% agarose gel. It was seen that the concentrations of the samples were very close to each other (Figure 3.11, 3.12 and 3.13).

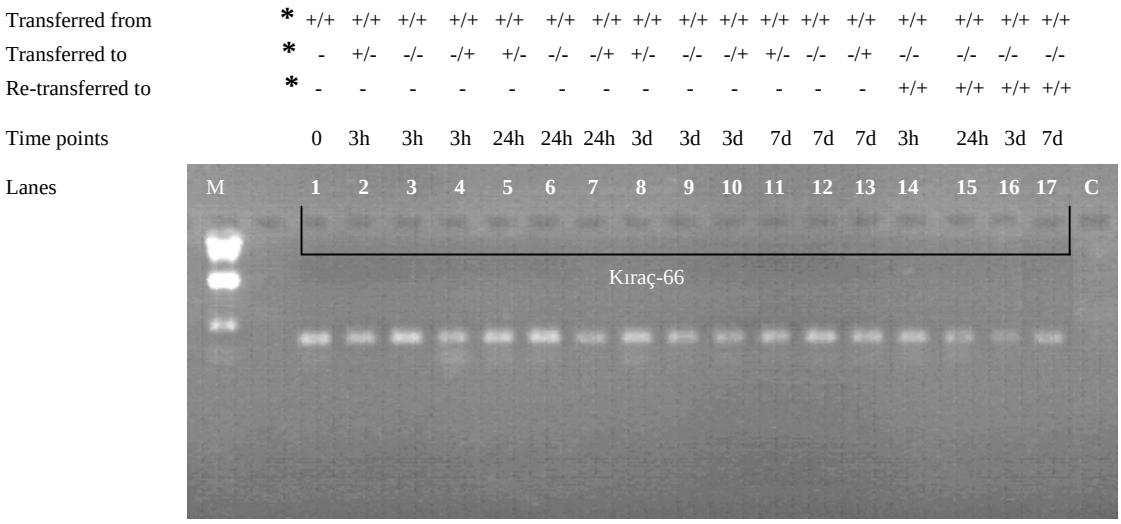

Figure 3.11. The appearance of RT-PCR products of Tokak-157 and BDMM-19 samples (from total RNA) with EF-2 primers on 1% agarose gel. . *****: +/+ indicates +Zn/+Fe and -/- indicates -Zn/-Fe conditions. -: no retransfer, h: hour, d: day, M: Marker λ/Pst, C: Negative control.

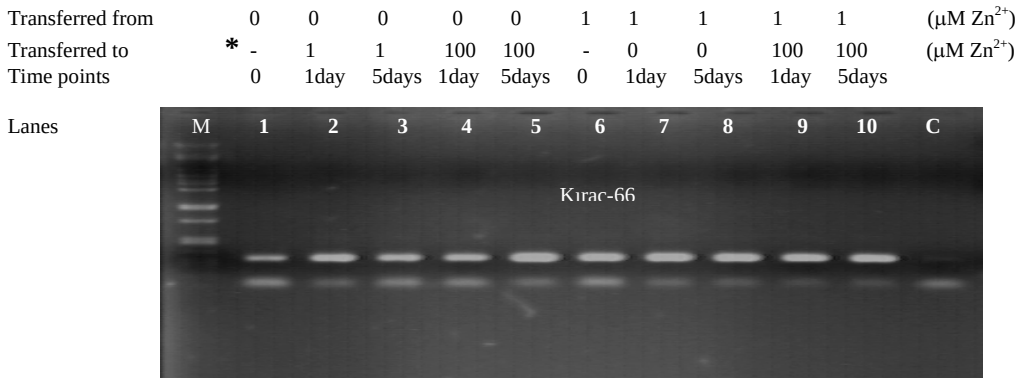

Figure 3.12 The appearance of RT-PCR products of Kıraç-66 samples with EF-2 primers (from total RNA) on 1% agarose gel. It was seen that the concentrations of the samples were very close to each other. Plant samples were grown in +Zn/+Fe nutrient solution for 1 week and then transferred to -Zn/-Fe nutrient solution. Root and shoot tissue samples were taken at different time points. After 1 week the samples were retransferred to +Zn/+Fe nutrient solution and again samples were taken at different time points. *****: +/+ indicates +Zn/+Fe and -/- indicates -Zn/-Fe conditions. -: no retransfer, h: hour, d: day, M: Marker λ/Pst, C: Negative control.

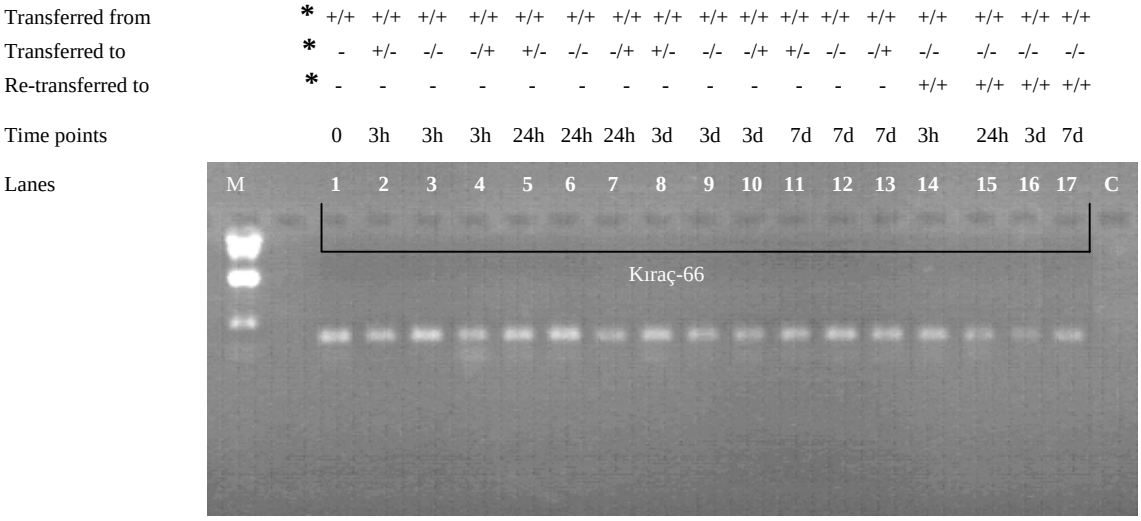

Figure 3.13. The appearance of RT-PCR products of Kıraç-66 samples (from mRNA) with EF-2 primers on 1% agarose gel. The plants were grown in 0μM Zn and 1μM Zn containing nutrient solutions seperately. The Fe concentrations of the solutions were same. And after 1 week the plants were transfered to 0, 1 and 100 μM Zn containing solutions and samples were taken at different time points after the transfers. ***** -: no retransfer, M: Marker λ/Pst, C: Negative control.

3.7.3. RT-PCR with SAM synthetase

After the confirmation of the concentrations of the samples with 18S and EF-2 reactions, RT-PCRs with other primers were tried in order to evaluate the expression levels. First started with SAM-synthetase (Figure 3.14., 3.15 and 3.16).

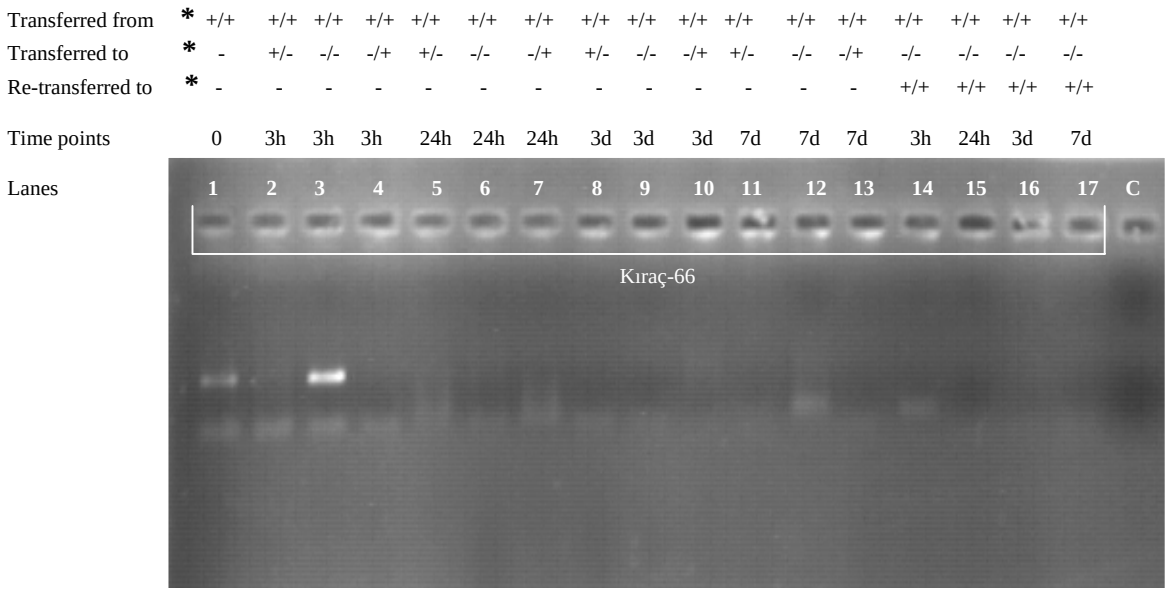

Figure 3.14. The appearance of RT-PCR products of Kıraç-66 (from mRNA) samples with SAM-synthetase primers on 1% agarose gel. *****: +/+ indicates +Zn/+Fe and -/- indicates -Zn/-Fe conditions. -: no retransfer, h: hour, d: day, C: Negative control.

Because SAM-synthetase was the first gene involved in the biosynthesis of phytosiderophores, the expression level was supposed to be induced in iron and zinc deficient conditions but the expression of SAM-synthetase was only induced in lanes 1 and 3 (Figure 3.14). The experiments should be optimized to assess the expression levels of SAM-synthetase with wheat SAM-synthetase primers to be designed with the sequences obtained in this study.

These results can suggest that Kıraç-66 may use another pathway for the synthesis of L-methionine which is the first step of phytosiderophore synthesis.

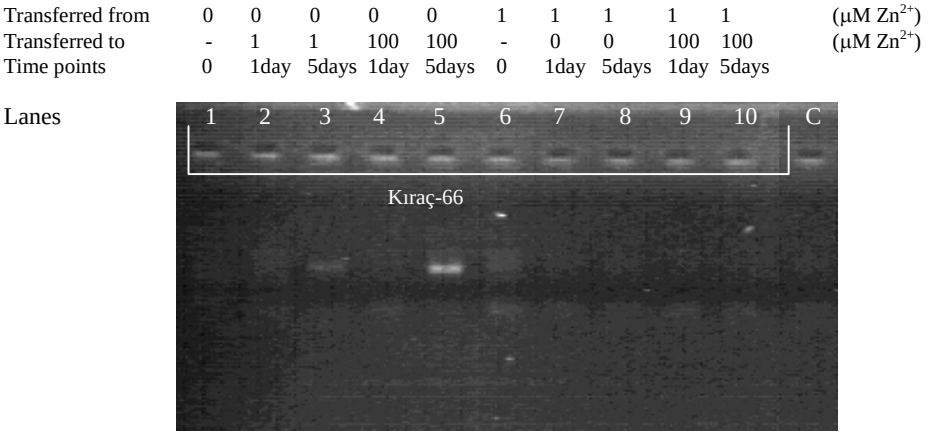

Figure 3.15. The appearance of RT-PCR products of Kıraç-66 samples (from total RNA) with SAM-synthetase primers on 1% agarose gel. ***** -: no transfer, C: Negative control.

The second set of Kıraç-66 samples comprises Kıraç-66 plants grown at solutions containing different Zn but constant iron concentrations. In the plants which were grown in Zn deficient conditions for 1 week the SAM-synthetase gene expression was not detected (Figure 3.15 Lane 1). 1 day after the transfer of plants to 1μM Zn containing solutions (Zn sufficient), the small induction in the expression of SAM-synthetase gene was seen (Figure 3.15 Lane 2) and higher induction was detected 5 days after the transfer of plants to 1μM Zn containing solutions (Figure 3.15 Lane 3). Also, the induction was seen 5 days after the transfer of the samples from 0μM Zn to 100μM Zn containing solutions (Figure 3.15 Lane 5).

In the plants which were grown in 1μM Zn containing solutions (Zn sufficient) for 1 week the SAM-synthetase gene expression was detected (Figure 3.15 Lane 6). But the expression level decrease was detected in the same plants after 1 day and 5 day of transfer to no Zn (Figure 3.15 Lane 7 and 8) and excess amount of Zn containing solutions (Figure 3.15 Lane 9 and 10).

From these results it was seen that SAM-synthetase is expressed in the Zn sufficient conditions (1μM) not in Zn deficient (no Zn) and Zn excess (100μM) conditions. It may be speculated that for detectible level expression of SAM-synthetase requires the presence of Zn since Zn is very vital for the transcription processes and it is necessary for the transcription of SAM-synthetase as well.

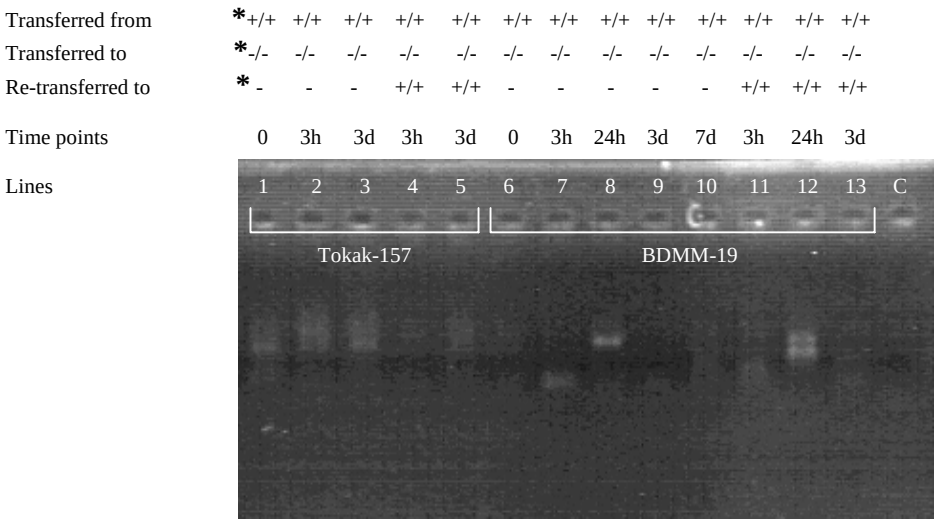

Figure 3.16. The appearance of RT-PCR products of Tokak-157 and BDMM-19 samples (from total RNA) with SAM-synthetase primers on 1% agarose gel. *****: +/+ indicates +Zn/+Fe and -/- indicates -Zn/-Fe conditions. -: no retransfer, h: hour, d: day, C: Negative control.

The expression of SAM-synthetase and the induction is seen in Tokak-157 samples with the increasing Fe and Zn deficiency treatment time (Figure 3.16 Lanes 1, 2, 3). In BDMM-19 samples the detectable level of expression is seen only 24 hours after Fe and Zn deficiency tratment (Figure 3.16 Lane 8). This may be because of BDMM-19, durum wheat cultivar, being less zinc efficient compared to Tokak-157, barley cultivar.

3.7.4. RT-PCR with IDI-1

IDI-1 gene had been found to be induced under iron deficient conditions in barley. As expressed in section 3.6.2.. we have cloned the IDI-1 gene from wheat samples (BDMM-19) and the homology anlaysis revealed that the sequence is 96% identical to barley IDI-1 gene. So, the RT-PCR with IDI-1 primers were made to evaluate the expression levels (Figure 3.17, 3.18 and 3.19).

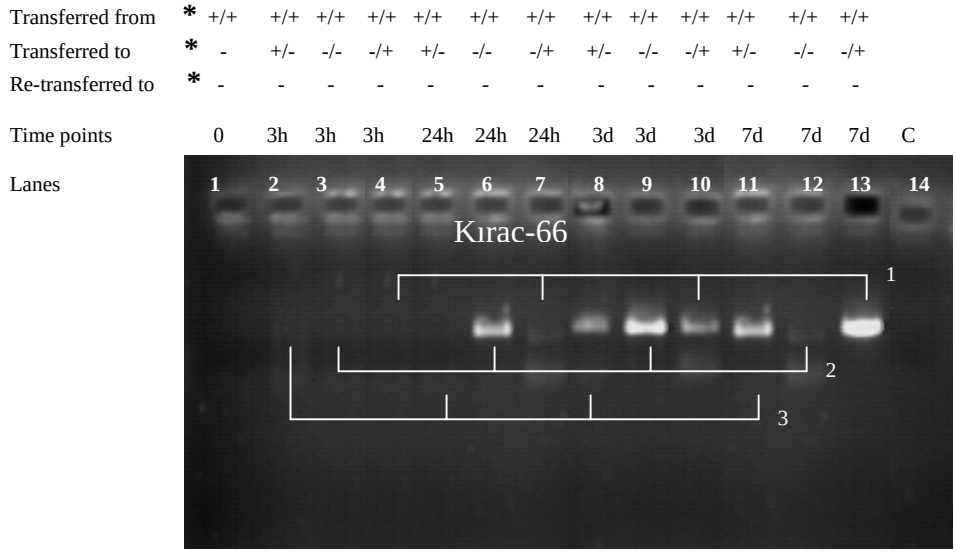

Figure 3.17. The appearance of RT-PCR products of Kıraç-66 samples (frommRNA) with IDI-1 primers on 1% agarose gel. *****:+/+ indicates +Zn/+Fe and -/- indicates -Zn/-Fe conditions. -: no retransfer, h: hour, d: day, C: Negative control. The number 1-3 are refered to the zinc deficient, zinc and iron deficient, iron deficient conditions, respectively.

In Zn deficient conditions of the Kıraç-66 plants at the 3rd day, the IDI-1 expression level began to be induced (Figure 3.17 Group 1). Same induction was observed in the iron deficient condition at the same time point. (Figure 3.17 Group 3). In the both Fe and Zn deficient conditions, induction of IDI-1 started earlier, after 24 hours of transfer. (Figure 3.17 Group 2).

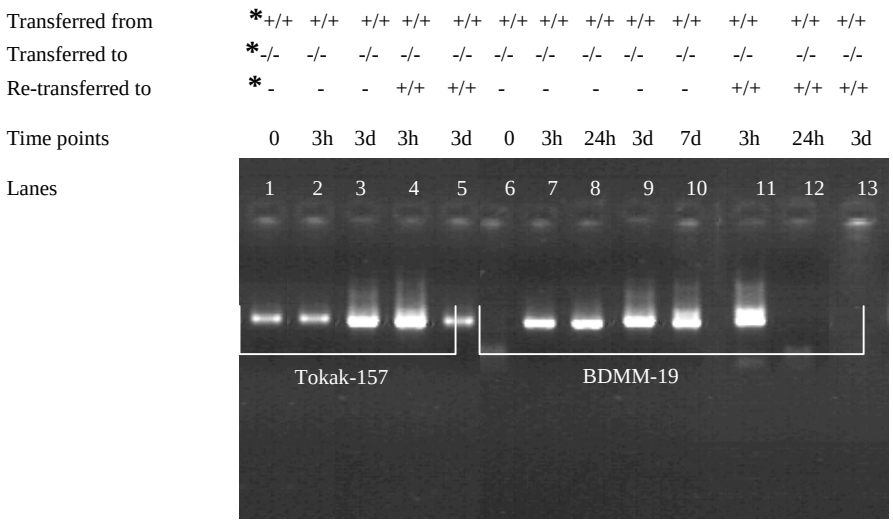

Figure 3.18. The appearance of RT-PCR products of Tokak-157 and BDMM-19 samples (from total RNA) with IDI-1 primers on 1% agarose gel. *****: +/+ indicates +Zn/+Fe and -/- indicates -Zn/-Fe conditions. -: no retransfer, h: hour, d: day.

In Tokak-157 samples the induction can be seen 3 days after the transfer of samples to Fe and Zn deficient condition and the decrease in the expression level can be seen in the samples retransfered to Fe and Zn sufficient conditions (Figure 3.18 Lanes 3, 4 and 5). In BDMM-19 samples, the induction was seen after 3 days of transfer of plants to Fe and Zn deficient conditions and the expression was diminished 24 hours after the retransfer to Zn and Fe sufficient conditions. (Figure 3.18 Lanes 9, 10 and 11).

These results may suggest us that the expression of IDI-1 gene, previously identified in iron deficiency in *Zea mays,* is induced under Zn and Fe deficient conditions in wheat and barley cultivars.

3.7.5. RT-PCR with NAAT

NAAT had been found to induce under iron deficient conditions in various organisms but the expression levels in wheat had not been worked. So, RT-PCR was made with the spesific primers for NAAT to investigate the expression changes of this gene.

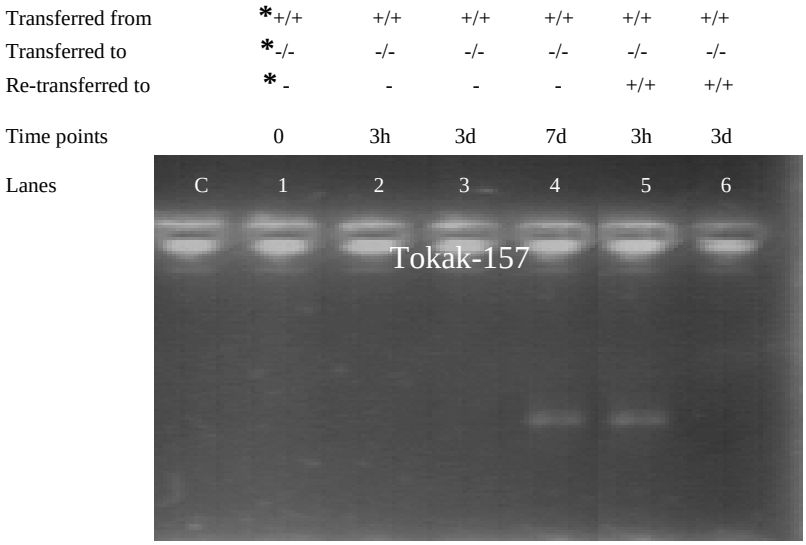

Figure 3.19. The appearance of RT-PCR products of Tokak-157 (from total RNA) samples with NAAT primers on 1% agarose gel. *****: +/+ indicates +Zn/+Fe and -/- indicates -Zn/-Fe conditions. -: no retransfer, h: hour, d: day, C: Negative control

NAAT had been found to be induced under iron deficient conditions in barley. The NAAT expression in the barley cultivar Tokak-157 samples, that we use as a control, is induced after 7 days of treatment with Fe and Zn deficiency (Figure 3.19). Resuply of Fe and Zn after 3 hours of treatment did not suppress the expression of NAAT, however after 3 days deficiency induced expression diminished. (Figure 3.19).

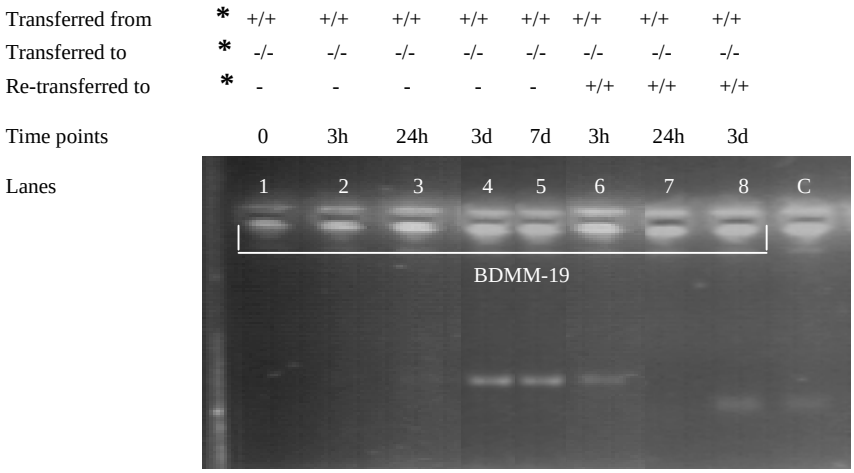

Figure 3.20. The appearance of RT-PCR products of BDMM-19 (from total RNA) samples with NAAT primers on 1% agarose gel. *****: +/+ indicates +Zn/+Fe and -/- indicates -Zn/-Fe conditions. -: no retransfer, h: hour, d: day, C: Negative control

In BDMM-19 samples the induction in NAAT expression can be seen after 3 days of treatment with Fe and Zn deficiency and the expression decrease can be seen after the transfer of plants to Fe and Zn sufficient conditions (Figure 3.20).

These results suggest that the NAAT gene, functions in phytosiderophore synthesis, is also induced in Fe and Zn deficiency treated wheat cultivars supporting the idea that the phytosiderophore synthesis is activated in Fe and Zn deficiency tolerant wheat cultivars facing with these two deficiency conditions.

3.7.6. RT-PCR with IRT-1

IRT-1 gene had been found to be induced under Fe deficient conditions in *Arabidopsis thaliana* and thought to take role as a membrane transporter. But both in barley and wheat the expression levels had not be studied. So, the RT-PCR was made with primers spesific for IRT-1.

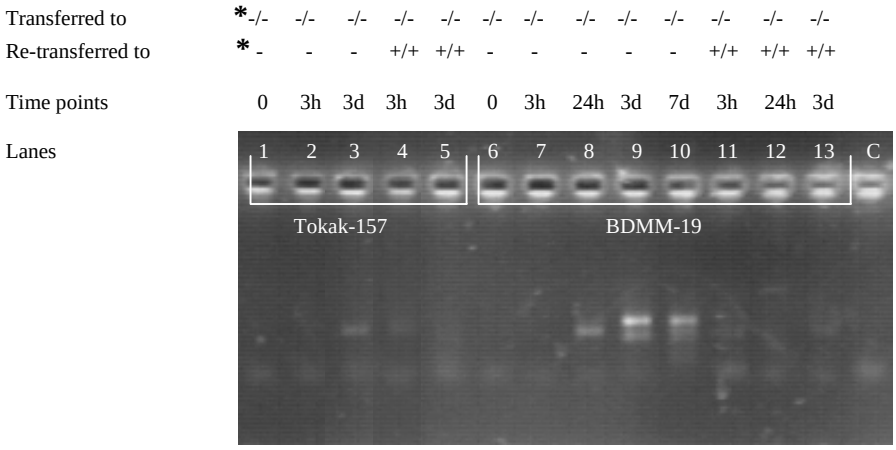

Figure 3.21. The appearance of RT-PCR products of Tokak-157 and BDMM-19 samples (from total RNA) with IRT-1 primers on 1% agarose gel. *****: +/+ indicates +Zn/+Fe and -/- indicates -Zn/-Fe conditions. -: no retransfer, h: hour, d: day.

It can be seen that the induction in IRT-1 expression begins 3 days after treatment with Fe and Zn deficient condition in Tokak-157 and after 24 hours in BDMM-19 samples (Figure 3.21 Lane 3 and 8). The expression decrease can be seen 3 hours after the retransfer of plants to Fe and Zn sufficient condition both in Tokak-157 and BDMM-19 samples. (Figure 3.21 Lanes 4 and 11). Also in BDMM-19 samples with increasing deficiency treatment another band was seen (Figure 3.21 Lanes 9 and 10).

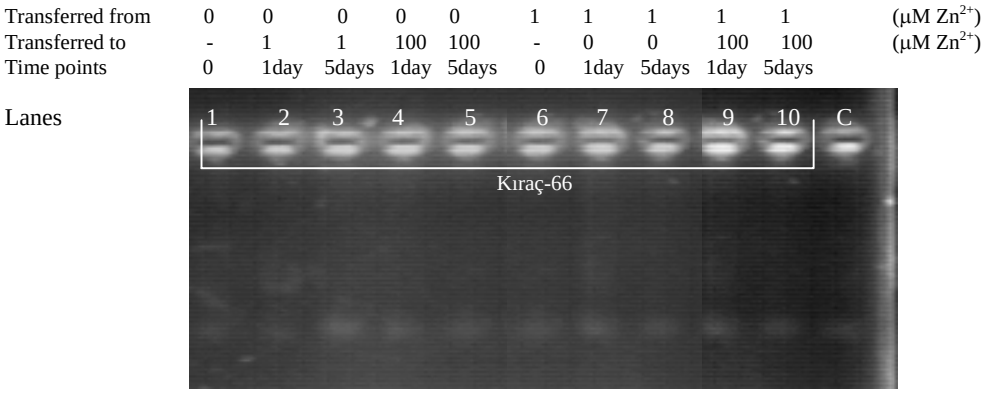

Figure 3.22. The appearance of RT-PCR products of Kıraç-66 samples (from total RNA) with IRT-1 primers on 1% agarose gel. ***** -: no transfer, C: Negative control.

In Kıraç-66 samples grown in different Zn concentrations but Fe sufficient conditions there was not any IRT-1 gene expression, suggesting that the expression induction seen in figure 3.21 was only because of Fe deficient conditions not Zn.

These results may suggest that IRT-1 gene which was found to be induced in Fe deficiency treated *Arabidopsis thaliana*, the strategy-1 plant, is also induced in Fe deficiency tolerant barley and wheat cultivars and it is possible that both strategy-I and strategy-II plants can use same transporters even their uptake mechanisms were different. Also it can be deduced that the IRT-1 expression is not altered by Zn deficiency treatment.

CHAPTER IV

CONCLUSION

In this study, the zinc and iron efficient barley genotype Tokak-157, efficient wheat genotype Kıraç-66 and relatively less efficient wheat genotype BDMM-19 were grown in zinc and iron deficient and sufficient conditions and several transfers were carried out between these conditions. Degenerate primers were designed for the conserved regions of previously identified genes that take role in phytosiderophore synthesis or genes induced under iron deficient conditions and RT-PCRs were performed. Several RT-PCR products were cloned. Two of them were sequenced and homology analyses were carried out.

The fragment belongs to BDMM-19 SAM-synthetase RT-PCR product was cloned and sequenced. The sequenced fragment showed very high homology to other SAM-synthetase genes (93% to *Hordeum vulgare* SAM-synthetase) which supports the finding that the isolated gene is wheat SAM-synthetase.

Also, the fragment belongs to BDMM-19 IDI-1 RT-PCR product was cloned and sequenced. The sequenced fragment showed very high homology to other IDI-1 genes (96% to *Hordeum vulgare* IDI-1). The sequencing results revealed that the isolated fragment represents the entire open reading frame of the wheat IDI-1 gene.

When comparing the expression levels of the genes it was seen that SAM-synthetase expression was not induced in Kıraç-66 samples whereas the induction was seen in Tokak-157 and BDMM-19 samples.

The other cloned gene IDI-1 showed induction in all samples grown in Fe and Zn deficient conditions supporting the results observed in *Hordeum vulgare* by Yamaguchi,H., Nakanishi,H., Nishizawa,N.K. and Mori,S. in 2000.

Also the expression levels of NAAT and IRT-1 was investigated and NAAT was found to be induced also in wheat cultivars treated with Fe and Zn deficiency stress, supporting previous results found in other plants. The *Arabidopsis thaliana* IRT-1 gene, thought to be a membrane located transporter functions in strategy-I plants, is also induced in iron deficiency treated barley and wheat cultivars but not in Zn deficiency treated plant samples in our study. These findings are very important because they suggest that both strategy-I and strategy-II plants can use same Fe transporters even though their Fe uptake mechanisms may be different.

As a conclusion this thesis is the first molecular study investigating the genes that take role in iron and zinc efficiency mechanisms of the wheat. The gene fragment belongs to SAM-synthetase and the entire open reading frame of IDI-1 gene were cloned and sequenced in wheat for the first time and expression levels of these two genes were evaluated.

As a future perspective, the PCR products of other genes especially NAAT and IRT-1 will be cloned and sequenced. The complete gene sequences of the newly identified wheat genes, SAM-synthetase will be cloned.

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APPENDIX A

PRIMERS RELATED SEQUENCES

Table A.1. The SAM synthetase amino acid sequences in fasta format

>*Oryza sativa* S-adenosylmethionine synthetase 1 (P46611)

MAALDTFLFTSESVNEGHPDKLCDQVSDAVLDACLAEDPDSKVACETCTKTNMVMVFGEITTKANVDYEKIVRET
CRNIGFVSADVGLDADHCKVLVNIEQQSPDIAQGVHGHFTKRPEEIGAGDQGHMFGYATDETPELMPLSHVLATKL
GARLTEVRKNGTCAWLRPDGKTQVTVEYRNESGARVPVRVHTVLISTQHDETVTNDEIAADLKEHVIKPVIPEQYL
DEKTIFHLNPSGRFVIGGPHGDAGLTGRKIIIDTYGGWGAHGGGAFSGKDPTKVDRSGAYVARQAAKSIVASGLAR
RCIVQVSYAIGVPEPLSVFVDTYGTGRIPDKEILKIVKENFDFRPGMIIINLDLKKGGNGRYLKTAAYGHFGRDDPDF
TWEVVKPLKWE KPSA

>*Lycopersicon esculentum* S-adenosylmethionine synthetase 1 (CAA80865.1)

METFLFTSES VNEGHPDKLC DQISDAVLDA CLEQDPESKV ACETCTKTNL VMVFGEITTKAIVDYEKIVR
DTCRNIGFVS DDVGLDADNC KVLVYIEQQS PDIAQGVHGH LTKRPEEIGA GDQGHMFGYA TDETPELMPL
SHVLATKLGA RLTEVRKNGT CAWLRPDGKTQVTVEYSNDNGAMVPIRVHT VLISTQHDET VTNDEIARDL
KEHVIKPVIP EKYLDENTIF HLNPSGRFVIGGPHGDAGLT GRKIIIDTYG GWGAHGGGAF SGKDPTKVDR
SGAYIVRQAA KSIVASGLARRCIVQVSYAI GVPEPLSVFV DTYGTGKIPD REILKIVKEN FDFRPGMMSI
NLDLKRGGNR RFLKTAAYGH FGRDDPDFTW EVVKPLKWEK PQD

>*Aradopsis thaliana* S-adenosylmethionine synthetase 2 (P17562)

METFLFTSES VNEGHPDKLC DQISDAVLDA CLEQDPDSKV ACETCTKTNM VMVFGEITTKATIDYEKIVR
DTCRSIGFIS DDVGLDADKC KVLVNIEQQS PDIAQGVHGH FTKRPEDIGAGDQGHMFGYA TDETPELMPL
SHVLATKIGRLTEVRKNGT CRWLRPDGKT QVTVEYYNDNGAMVPVRVHT VLISTQHDET VTNDEIARDL
KEHVIKPIIP EKYLDDKTIF HLNPSGRFVIGGPHGDAGLT GRKIIIDTYG GWGAHGGGAF SGKDPTKVDR
SGAYIVRQAA KSVVANGMARRALVQVSYAI GVPEPLSVFV DTYGTGLIPD KEILKIVKET FDFRPGMMTI
NLDLKRGGNGRFQKTAAYGH FGRDDPDFTW EVVKPLKWDK PQA

>*Aradopsis thaliana* S-adenosylmethionine synthetase 1 (NM_100131)

METFLFTSES VNEGHPDKLC DQISDAVLDA CLEQDPDSKV ACETCTKTNM VMVFGEITTKATVDYEKIVR
DTCRAIGFVS DDVGLDADKC KVLVNIEQQS PDIAQGVHGH FTKCPEDIGAGDQGHMFGYA TDETPELMPL
SHVLATKLGA RLTEVRKNGT CAWLRPDGKT QVTVEYYNDKGAMVPIRVHT VLISTQHDET VTNDEIARDL
KEHVIKPVIP EKYLDEKTIF HLNPSGRFVIGGPHGDAGLT GRKIIIDTYG GWGAHGGGAF SGKDPTKVDR
SGAYIVRQAAVVANGMARRALVQVSYAI GVPEPLSVFV DTYETGLIPD KEILKIVKES FDFRPGMMTI
NLDLKRGGNGRFLKTAAYGH FGRDDPDFTW EVVKPLKWDK PQA

Table A.2. The blocks of conserved regions generated with *Block Maker* program

block A, width = 38

Aradopsis1	(0)	21	DQISDAVLDACLEQDPDSKVACETCTKTNMVMVFGEIT
Lycopersicon	(0)	21	DQISDAVLDACLEQDPESKVACETCTKTNLVMVFGEIT
Oryza	(0)	24	DQVSDAVLDACLAEDPDSKVACETCTKTNMVMVFGEIT
Aradopsis 2	(0)	82	DQISDAVLDACLEQDPDSKVACETCTKTNMVMVFGEIT

Block B, width = 30

Aradopsis 1	(0)	178	NDNGAMVPVRVHTVLISTQHDETVTNDEIA
Lycopersicon	0)	178	NDNGAMVPIRVHTVLISTQHDETVTNDEIA
Oryza	(0)	181	NESGARVPVRVHTVLISTQHDETVTNDEIA
Aradopsis 2	(0)	239	NDKGAMVPIRVHTVLISTQHDETVTNDEIA

Table A.3. The result of *Codehop* degenerate primer design program

Block A Oligos

A C E T C T K T N M V M V F

GACCAACATGGTGatggtnttygg -3' Core: degen=4 len=11 Clamp: score=80, len=15 temp= 60.7
GAGACCAACATGGTGatggtnttyggyat -3' Core: degen=8 len=11 Clamp: score=84, len=20 temp= 61.3
GAGACCTGCACCAAGACCaayatggtnatg -3' Core: degen=8 len=12 Clamp: score=80, len=18 temp= 61.3
GAGACCTGCACCAAGACCaayatggtnat -3' Core: degen=8 len=11 Clamp: score=80, len=18 temp= 61.3
GCGAGACCTGCACCAAGacnaayatggt -3' Core: degen=8 len=11 Clamp: score=81, len=17 temp= 62.0

Complement of Block A Oligos

A C E T C T K T N M V M V F
tgnttrtaccaCTACCACAAG -5' Core: degen=8 len=11 Clamp: score=86, len=10 temp= 43.2
trtaccantaCCACAAG -5' Core: degen=8 len=11 Clamp: score=91, len=7 temp= 6.7
trtaccantaccACAAG -5' Core: degen=8 len=12 Clamp: score=83, len=5 temp= 6.7
taccantaccaCAAG -5' Core: degen=4 len=11 Clamp: score=74, len=4 temp= 6.7

Block B Oligos

H T V L I S T Q H D E T V T N D E I A R
CGACGAGACCGTGACCaaygaygarat -3' Core: degen=8 len=11 Clamp: score=79, len=16 temp= 61.9
GCTGATCTCCACCCAGcaygaygarac -3' Core: degen=8 len=11 Clamp: score=77, len=16 temp= 61.0
CGTGCTGATCTCCACCcarcaygayga -3' Core: degen=8 len=11 Clamp: score=72, len=16 temp= 61.9
CACacngtnytnat -3' Core: degen=128 len=11 Clamp: score=79, len=3 temp=-65.6

Complement of Block B Oligos

T Q H D E T V T N D E I A
gtygtrctrctCTGGCACTGGTTGCTGC -5' Core: degen=8 len=11Clamp: score=80, len=17 temp=62.8
gtrctrctytgGCACTGGTTGCTGCTC **-5' Core: degen=8 len=11 Clamp: score=79, len=13 temp= 60.7**
ttrctrctytaGCGGGCC -5' Core: degen=8 len=11 Clamp: score=68, len=7 temp= 38.4

The primers written in bold were chosen as forward and reverse primers.

Table A.4. The NAS protein sequences in fasta format

>*Oryza sativa* nicotianamine synthase 3 (AB023819)
MTVEVEAVTMAKEEQPEEEEVIEKLVEKITGLAAAIGKLPSLSPSPEVNLFTELVMTCIPPSSVDVEQLGAEAQMR
GRLIRLCADAEGHLEAHYSDVLAAHDNPLDHLALFPYFNNYIQLAQLEYALLARHLPAAPPPSRLAFLGSGPLPSSLVL
AARHLPAASFHNYDICADANRRASRLVRADRDLSARMAFHTSDVAHVTTDLAAYDVVFLAALVGMAAEEKARMVE
HLGKHMAPGAALVVRTAHGARGFLYPVVDPEEIRRGGFDVLAVHHPEGEVINSVIIARNRPWPGPALEGG
DAHAHGHGAVVSRPCQRCEMEARAHQKMEDMSAMEKLPSS
> *Oryza sativa* nicotianamine synthase 1 (BQ619012)
MEAQNQEVAALVEKIAGLHAAISKLPSLSPSAEVDALFTDLVTACVPASPVDVAKLGPEAQAMREELIRLCSAAEGHL
EAHYADMLAAFDNPLDHLARFPYYGNYVNLSKLEYDLLVRYVPGIAPTRVAFVGSGPLPFSSLVLAAHHLPDAVFDN
YDRCGAANERARRLFRGADEGLGARMAFHTADVATLTGELGAYDVVFLAALVGMAAEEKAGVIAHLGAHMADGA
ALVVRTAHGARGFLYPIVDPEDVRRGGFDVLAVCHPEDEVINSVIVARKVGAAAAAAAAARRDELADSRGVVLPVVGP
PSTCCKVEASAVEKAEEFAANKELSV
> *Hordeum vulgare* nicotianamine synthase 5-1 (AB011267)
MEAENGEVAALVEKITGLHAAISKLPALSPSPQVDALFTELVAACVPSSVDVTKLGPEAQEMRQDLIRLCSAAEGLLE
AHYSDMLTALDSPLDHLGRFPYFDNYVNLSKLEHDLLAGHWFSSLFLATYHLPDTRFDNYDRCSVANGRAMKLVGA
ADEGVRSRMAFHTAEVTDLTAELGAYDVVFLAALVGMTSKEKADAIAHLGKHMADGAVLRARSAHGARAFLYPVV
ELDDVGRGGFQVLAVHHPAGDEVFNSFIVARKVKMSA
>*Hordeum vulgare* nicotianamine synthase 1 (AF136941)
MDAQNKEVAALIEKIAGIQAAIAELPSLSPSPEVDRLFTDLVTACVPPSPVDVTKLSPEHQRMREALIRLCSAAEGKLEA
HYADLLATFDNPLDHLGLFPYYSNYVNLSRLEYELLARHVPGIAPARVAFVGSGPLPFSSLVLAAHHLPETQFDNYDL
CGAANERARKLFGATADGVGARMSFHTADVADLTQELGAYDVVFLAALVGMAAEEKAKVIAHLGAHMVEGASLV
VRSARPRGFLYPIVDPEDIRRGGFEVLAVHHPEGEVINSVIVARKAVEAQLSGPQNGDAHARGAVPLVSPPCNFSTKME
ASALEKSEELTAKELAF
> *Hordeum vulgare* nicotianamine synthase 3 (AB011264)
MAAQNNNKDVAALVEKITGLHAAIAKLPSLSPSPDVDALFTELVTACVPPSPVDVTKLGPEAQEMREGLIRLCSEAEG
KLEAHYSDMLAAFDNPLDHLGIFPYYSNYINLSKLEYELLARYVRRHRPARVAFIGSGPLPFSSFVLAARHLPDTMFDN
YDLCGAANDRASKLFRADTDVGARMSFHTADVADLASELAKYDVVFLAALVGMAAEDKAKVIAHLGAHMADGAA
LVVRSAHGARGFLYPIVDPQDIGRGGFEVLAVCHPDDDVVNSVIIAQKSKEVHADGLGSARGAGRQYARGTVPVVSP
PCRFGEMVADVTQNHKRDEFANAEVAF
> *Hordeum vulgare* nicotianamine synthase 2 (AF136942)
MAAQNNQEVDALVEKITGLHAAIAKLPSLSPSPDVDALFTELVTACVPPSPVDVTKLGPEAQEMREGLIRLCSEAEGK
LEAHYSDMLAAFDKPLDHLGMFPYYNNYINLSKLEYELLARYVPGGYRPARVAFIGSGPLPFSSFVLAARHLPDTMFD
NYDLCGAANDRASKLFRADRDVGARMSFHTADVADLAGELAKYDVVFLAALVGMAAEDKAKVIAHLGAHMADGA
ALVVRSAHGARGFLYPIVDPQDIGRGGFEVLAVCHPDDDVVNSVIIAQKSKDVHADGLGSGRGAGGQYARGTVPVVS
PPCRFGEMVADVTQNHKRDEFANAEVAF
> *Hordeum vulgare* nicotianamine synthase 4 (AB011266)
MDGQSEEVDALVQKITGLHAAIAKLPSLSPSPDVDALFTDLVTACVPPSPVDVTKLAPEAQAMREGLIRLCSEAEGKLE
AHYSDMLAAFDNPLDHLGVFPYYSNYINLSKLEYELLARYVPGRHRPARVAFIGSGPLPFSSYVLAARHLPDTVFDNY
DLCGAANDRATRLFRADKDVGARMSFHTADVADLTDELATYDVVFLAALVGMAAEDKAKVIAHLGAHMADGAAL
VARHGARGFLYPIVDPQDIGRGGFEVLAVCHPDDDVVNSVIIAQKSNDVHEYGLGSGRGGRYARGTVVPVVSPPCRF
GEMVADVTQKREEFANAEVAF
> *Hordeum vulgare* nicotianamine synthase 5-2 (AB011268)
MEAENGEVAALVEKITGLHAAISKLPALSPSPQVDALFTELVAACVPSSPVDVTKLGPEAQEMRQDLIRLCSAAEGLLE
AHYSDMLTALDSPLDHLGRFPYFDNYVNLSKLEHDLLAGHVAAPARVAFIGSGPLPFSSLFLATYHLPDTRFDNYDRC
SVANGRAMKLVGAADEGVRSRMAFHTAEVTDLTAELGAYDVVFLAALVGMTSKEKADAIAHLGKHMADGAVLVR
EALHGARAFLYPVVELDDVGRGGFQVLAVHHPAGDEVFNSFIVARKVKMSA
>*Hordeum vulgare* nicotianamine synthase 6 (AB011269)
MDAQNKEVDALVQKITGLHAAIAKLPSLSPSPDVDALFTDLVTACVPPSPVDVTKLGSEAQEMREGLIRLCSEAEGKL
EAHYSDMLAAFDNPLDHLGMFPYYSNYINLSKLEYELLARYVPGGIARPAVAFIGSGPLPFSSYVLAARHLPDAMFDN
YDLCSAANDRASKLFRADKDVGARMSFHTADVADLTRELAAYDVVFLAALVGMAAEDKAKVIPHLGAHMADGAA
LVVRSAQARGFLYPIVDPQDIGRGGFEVLAVCHPDDDVVNSVIIAHKSKDVHANERPNGRGGQYRGAVPVVSPPCRF
GEMVADVTHKREEFTNAEVAF
> *Hordeum vulgare* nicotianamine synthase 7 (AB019525)
MDAQSKEVDALVQKITGLHAAIAKLPSLSPSPDVDALFTDLVTACVPPSPVDVTKLAPEAQAMREGLIRLCSEAEGKL
EAHYSDMLAAFDNPLDHLGVFPYYSNYINLSKLEYELLARYVPGGIAPARVAFIGSGPLPFSSYVLAARHLPDTVFDNY
VPVRAANDRATRLFRADKDVGARMSFHTADVADLTDELATYDVVFLAALVGMAAEDKGQGDPHLGAHMADGAAL
VRSAHGARGFLYPIVDPQDIGRGGFEVLAVCHPDDDVVNSVIIAQKSKDMFANGPRNGCGGRYARGTVPVVSPPCRF
GEMVADVTQKREEFAKAEVAF
> *Hordeum vulgare* nicotianamine synthase 1a (AB010086)
MDAQNKEVDALVQKITGLHAAIAKLPSLSPSPDVDALFTDLVTACVPPSPVDVTKLGSEAQEMREGLIRLCSEAEGKL
EAHYSDMLAAFDNPLDHLGMFPYYSNYINLSKLEYELLARYVPGRHRPARVAFIGSGPLPFSSYVLAARHLPDAMFDN
YDLCSAANDRASKLFRADKDVGARMSFHTADVADLTGELAAYDVVFLAALVGMAAEDKTKVIAHLGAHMADGAA
LVVRSAHGHVGFLYPIVDPQDIGRGGFEVLAVCHPDDDVVNSVIIAHKSKDVHANERPNGVVDSTRGAVPVVSPPCRF
GEMVADVTHKREEFTNAEVAF

Table A.5. The blocks of conserved regions generated with *Block Maker* program

Block A, width = 55

H1	15	AGIQAAIAELPSLSPSPEVDRLFTDLVTACVPPSPVDVTKLSPEHQRMREALIR
H1a	15	ITGLHAAIAKLPSLSPSPDVDALFTDLVTACVPPSPVDVTKLGSEAQEMREGLIR
H2	16	ITGLHAAIAKLPSLSPSPDVDALFTELVTACVPPSPVDVTKLGPEAQEMREGLIR
H3	17	ITGLHAAIAKLPSLSPSPDVDALFTELVTACVPPSPVDVTKLGPEAQEMREGLIR
H4	15	ITGLHAAIAKLPSLSPSPDVDALFTDLVTACVPPSPVDVTKLAPEAQAMREGLIR
H5-1	15	ITGLHAAISKLPALSPSPQVDALFTELVAACVPSSPVDVTKLGPEAQEMRQDLIR
H5-2	15	ITGLHAAISKLPALSPSPQVDALFTELVAACVPSSPVDVTKLGPEAQEMRQDLIR
H6	15	ITGLHAAIAKLPSLSPSPDVDALFTDLVTACVPPSPVDVTKLGSEAQEMREGLIR
H7	15	ITGLHAAIAKLPSLSPSPDVDALFTDLVTACVPPSPVDVTKLAPEAQAMREGLIR
O1	15	IAGLHAAISKLPSLSPSAEVDALFTDLVTACVPASPVDVAKLGPEAQAMREELIR
O3	29	ITGLAAAIGKLPSLSPSPEVNALFTELVMTCIPPSSVDVEQLGAEAQDMRGRLIR

Block B, width = 49

H1	(51)	173	TADGVGARMSFHTADVADLTQELGAYDVVFLAALVGMAAEEKAKVIAHL
H1a	(51)	173	ADKDVGARMSFHTADVADLTGELAAYDVVFLAALVGMAAEDKTKVIAHL
H2	(51)	174	ADRDVGARMSFHTADVADLAGELAKYDVVFLAALVGMAAEDKAKVIAHL
H3	(50)	174	ADTDVGARMSFHTADVADLASELAKYDVVFLAALVGMAAEDKAKVIAHL
H4	(51)	173	ADKDVGARMSFHTADVADLTDELATYDVVFLAALVGMAAEDKAKVIAHL
H5-1	(34)	156	ADEGVRSRMAFHTAEVTDLTAELGAYDVVFLAALVGMTSKEKADAIAHL
H5-2	(49)	171	ADEGVRSRMAFHTAEVTDLTAELGAYDVVFLAALVGMTSKEKADAIAHL
H6	(51)	173	ADKDVGARMSFHTADVADLTRELAAYDVVFLAALVGMAAEDKAKVIPHL
H7	(51)	173	ADKDVGARMSFHTADVADLTDELATYDVVFLAALVGMAAEDKGQGDPHL
O1	(51)	173	ADEGLGARMAFHTADVATLTGELGAYDVVFLAALVGMAAEEKAGVIAHL
O3	(51)	187	ADRDLSARMAFHTSDVAHVTTDLAAYDVVFLAALVGMAAEEKARMVEHL

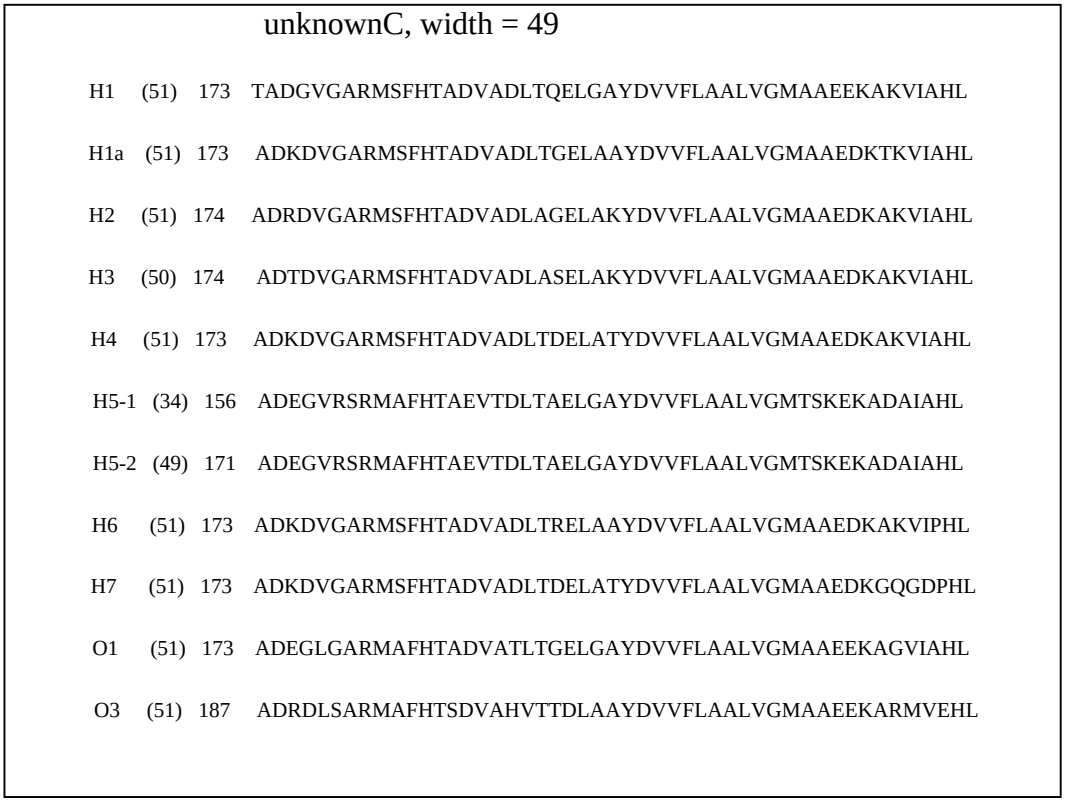
unknownC, width = 49
H1 (51) 173 TADGVGARMSFHTADVADLTQELGAYDVVFLAALVGMAAEEKAKVIAHL
H1a (51) 173 ADKDVGARMSFHTADVADLTGELAAYDVVFLAALVGMAAEDKTKVIAHL
H2 (51) 174 ADRDVGARMSFHTADVADLAGELAKYDVVFLAALVGMAAEDKAKVIAHL
H3 (50) 174 ADTDVGARMSFHTADVADLASELAKYDVVFLAALVGMAAEDKAKVIAHL
H4 (51) 173 ADKDVGARMSFHTADVADLTDELATYDVVFLAALVGMAAEDKAKVIAHL
H5-1 (34) 156 ADEGVRSRMAFHTAEVTDLTAELGAYDVVFLAALVGMTSKEKADAIAHL
H5-2 (49) 171 ADEGVRSRMAFHTAEVTDLTAELGAYDVVFLAALVGMTSKEKADAIAHL
H6 (51) 173 ADKDVGARMSFHTADVADLTRELAAYDVVFLAALVGMAAEDKAKVIPHL
H7 (51) 173 ADKDVGARMSFHTADVADLTDELATYDVVFLAALVGMAAEDKGQGDPHL
O1 (51) 173 ADEGLGARMAFHTADVATLTGELGAYDVVFLAALVGMAAEEKAGVIAHL
O3 (51) 187 ADRDLSARMAFHTSDVAHVTTDLAAYDVVFLAALVGMAAEEKARMVEHL

Table A.6. The result of Codehop degenerate primer design program

Block A Oligos

L P S L S P S P D V D A L F T D L V T A C V P P S P V D V T K L G P E A Q E M R E G L I R
CACCGACCTGGTGACGrcntgyrtncc -3' Core: degen=128 len=11 Clamp: score=73, len=16 temp= 57.9
TGGACGCCCTGTTCacnganytngt -3' Core: degen=128 len=11 Clamp: score=79, len=14 temp= 57.3
GGTGGACGCCCTGttyacnganyt -3' Core: degen=64 len=11 Clamp: score=73, len=13 temp= 56.0
GAGGTGGACGCCytnttyacnga -3' Core: degen=64 len=11 Clamp: score=74, len=12 temp= 62.4

Complement of Block A Oligos

P S P D V D A L F T D L V T A C V P P S P V D V T K L G P E A Q E M R E G L I R
aartgnctnraCCACTGCCGGACG -5' Core: degen=64 len=11 Clamp: score=73, len=13 temp= 56.7
tgnctnrancaCTGCCGGACGCA -5' Core: degen=128 len=11 Clamp: score=72, len=12 temp= 55.5
canctrcanykGTTCGACCCGGGGC -5' Core: degen=128 len=11 Clamp: score=74, len=14 temp= 60.9

Block B Oligos

I N L S K L E Y E L L A R H V P
CAACCTGTCCAAGCTGgaryaygmnyt -3' Core: degen=128 len=11 Clamp: score=72, len=16 temp= 58.2

Complement of Block B Oligos

I N L S K L E Y E L L A R H V P
ctyrtrcknraCGACCGGGCCG -5' Core: degen=128 len=11 Clamp: score=69, len=11 temp= 58.0

Block C Oligos

A D K D V G A R M S F H T A D V A D L T G E L A A Y D V V F L A A L V G M

GGCGCCCGGATGdsnttycayac -3' Core: degen=96 len=11 Clamp: score=82, len=12 temp= 57.1
GGCGCCCGGATgdsnttycaya -3' Core: degen=96 len=11 Clamp: score=78, len=11 temp= 57.1
GGCGCCCGGatgdsnttyca -3' Core: degen=48 len=11 Clamp: score=68, len=9 temp= 57.1

Complement of Block unknownC Oligos

A A Y D V V F L A A L V G M AAEDKAKVIAHL

atrctrcancanAAGGACCGGCGG -5' Core: degen=64 len=12 Clamp: score=85, len=12 temp= 63.1
ctrcancanaarGACCGGCGGGACCA -5' Core: degen=64 len=12 Clamp: score=80, len=14 temp= 60.3

The primers written in bold were chosen as forward and reverse primers.

Table A.7. The NAAT protein sequences in fasta format

>***Hordem vulgare*** **nicotianamine aminotransferase A (D88273)**
MVHQSNGHGEAAAAAANGKSNGHAAAANGKSNGHAAAAAVEWNFARGKDGILATTGAKNSIRAIRY
KISASVEESGPRPVLPLAHGDPSVFPAFRTAVEAEDAVAAALRTGQFNCYAAGVGLPAARSAVAEHLSQ
GVPYKLSADDVFLTAGGTQAIEVIIPVLAQTAGANILLPRPGYPNYEARAAFNKLEVRHFDLIPDKWEIDI
DSLESIADKNTTAMVIINPNNPCGSVYSYDHLAKVAEVARKLGILVIADEVYGKLVLGSAPFIPMGVFGH
IAPVLSIGSLSKSWIVPGWRLGWVAVYDPTKILEKTKISTSITNYLNVSTDPATFVQEALPKILENTKADFF
KRIIGLLKESSEICYREIKENKYITCPHKPEGSMFVMVKLNLHLLEEIHDDIDFCCKLAKEESVILCPGSVL
GMENWVRITFACVPSSLQDGLERVKSFCQRNKKKNSINGC
>***Hordem vulgare*** **nicotianamine aminotransferase B (AB005788)**
MATVRQSDGVAANGLAVAAAANGKSNGHGVAAAVNGKSNGHGVDADANGKSNGHGVAADANGKS
NGHAEATANGHGEATANGKTNGHRESNGHAEAADANGESNEHAEDSAANGESNGHAAAAAEEEEAV
EWNFAGAKDGVLAATGANMSIRAIRYKISASVQEKGPRPVLPLAHGDPSVFPAFRTAVEAEDAVAAAL
RTGQFNCYPAGVGLPAARSAVAEHLSQGVPYMLSADDVFLTAGGTQAIEVIIPVLAQTAGANILLPRPG
YPNYEARAAFNRLEVRHFDLIPDKGWEIDIDSLESIADKNTTAMVIINPNNPCGSVYSYDHLSKVAEVAK
RLGILVIADEVYGKLVLGSAPFIPMGVFGHITPVLSIGSLSKSWIVPGWRLGWVAVYDPRKILQETKISTSI
TNYLNVSTDPATFIQAALPQILENTKEDFFKAIIGLLKESSEICYKQIKENKYITCPHKPEGSMFVMVKLNL
HLLEEIDDDIDFCCKLAKEESVILCPGSVLGMANWVRITFACVPSSLQDGLGRIKSFCQRNKKRNSSDDC
>***Hordem vulgare*** **nicotianamine aminotransferase AB (AB024006)**
MATVRQSDGVAANGLAVAAAANGKSNGHGVAAAVNGKSNGHGVDADANGKSNGHGVAADANGKS
NGHAEATANGHGEATANGKTNGHRESNGHAEAADANGENEHAEDSAANGESNGHAAAAAEEEEAVE
WNFAGAKDGVLAATGANMSIRAIRYKISASVQEKGPRPVLPLAHGDPSVFPAFRTAVEAEDAVAAALR
TGQFNCYPAGVGLPAARSAVAEHLSQGVPYMLSADDVFLTAGGTQAIEVIIPVLAQTAGANILLPRPGYP
NYEARAAFNRLEVRHFDLIPDKGWEIDIDSLESIADKNTTAMVIINPNNPCGSVYSYDHLSKVAEAKRLGI
LVIADEVYGKLVLGSAPFIPMGVFGHITPVLSIGSLSKSWIVPGWRLGWVAVYDPRKILQETKISTSITNY
LNVSTDPATFIQAALPQILENTKEDFFKAIIGLLKESSEICYKQIKENKYITCPHKPEGSMFVMVKLNLHLL
EEIDDDIDFCCKLAKEESVILCPGSVLGMANWVRITFACVPSSLQDGLGRIKSFCQRNKKRNSSDDC

Table A.8. The blocks of conserved regions generated with *Block Maker* program

Block A , width = 34

horNAATA	(0)	85	HGDPSVFPAFRTAVEAEDAVAAALRTGQFNCYAA
horNAATAB	(0)	175	HGDPSVFPAFRTAVEAEDAVAAALRTGQFNCYPA
horNAATB	(0)	175	HGDPSVFPAFRTAVEAEDAVAAALRTGQFNCYPA

Block B, width = 30

horNAATA	(0)	242	VAEVARKLGILVIADEVYGKLVLGSAPFIP
horNAATAB	(0)	332	VAEVAKRLGILVIADEVYGKLVLGSAPFIP
horNAATB	(0)	332	VAEVAKRLGILVIADEVYGKLVLGSAPFIP

TableA.9. The result of Codehop degenerate primer design program

Block A Oligos

V A A A L R T G Q F N C Y P A G V G L P A A R S
GACCGGCCAGTTCaaytgytaysc -3' Core: degen=16 len=11 Clamp: score=82, len=15 temp= 62.3
GACCGGCCAGttyaaytgyta -3' Core: degen=8 len=11 Clamp: score=80, len=13 temp= 61.9
TGCGGACCGGCCArttyaaytgyt -3' Core: degen=16 len=11 Clamp: score=81, len=13 temp= 61.7
TGCGGACCGGCcarttyaaytg -3' Core: degen=8 len=11 Clamp: score=74, len=11 temp= 61.7

Complement of Block A Oligos

A V E A E D A V A A A L R T G Q F N C Y P A G V G L P A A R S
ctycgnctyctGCGGCACCGGC -5' Core: degen=16 len=11 Clamp: score=79, len=11 temp= 60.5
cgnctyctrcgGCACCGGCGG -5' Core: degen=16 len=11 Clamp: score=77, len=10 temp= 60.5
ctyctrcgncaCCGGCGGCGG -5' Core: degen=16 len=11 Clamp: score=77, len=10 temp= 60.5

Block B Oligos

C P H K P E G S M F V M V K L N L H L L E E I H D D I D F C C K L A K E E S
CCCGAGGGCTCCATGttygtnatggt -3' Core: degen=8 len=11 Clamp: score=79, len=15 temp= 62.1
CCCGAGGGCTCCATgttygtnatgg -3' Core: degen=8 len=11 Clamp: score=76, len=14 temp= 62.1
CCCGAGGGCTCCatgttygtnatg -3' Core: degen=8 len=12 Clamp: score=68, len=12 temp= 62.1
CCCGAGGGCTCCatgttygtnat -3' Core: degen=8 len=11 Clamp: score=68, len=12 temp= 62.1

Complement of Block B Oligos

E G S M F V M V K L N L H L L E E I H D D I D F C C K L A K E E S
caarcantaCCACTTCGACTT -5' Core: degen=8 len=9 Clamp: score=86, len=12 temp= 61.8
acaarcantaccACTTCGACTTGGACGT -5' Core: degen=8 len=12 Clamp: score=82, len=16 temp= 61.8
caarcantaccaCTTCGACTTGGACGT -5' Core: degen=8 len=12 Clamp: score=80, len=15 temp= 61.8
aarcantaccaCTTCGACTTGGACGT -5' Core: degen=8 len=11 Clamp: score=80, len=15 temp= 61.8

The primers written in bold were chosen as forward and reverse primers.

Table A.10. The open reading frame of the Zea mays FDR-3 gene

Zea mays **FDR-3 (Fe-deficient related) (AF262623)**

```
atgcaaacca caacggcaac gaccccgctg gcggacaggc tgcgccgcta cacgccggcg
ctggccagcc tggcacgcgc cctgtacgac gcccgcggcg cgctgcatgg caccgtgcgg
gccgtgcccg agggaccgcc gcgcaccggc aagaccgcgc agttgaccgt cgcttgcgac
cagggcgagc tgcacgtcca cgtcgacgcc gacgccgcct tcgaggcgat cgccctcgaa
ccggaggcag cgtgccgcgc cgccgtggcc agcctgtacc tggccggccc gctggcggcg
ctggcgcgcc atggcgccac gcggccggcg gtacgggatg tgcgcctcgc cgcgccgtcc
gcctcgcgcg ccggcgtgct gcacctcgaa tacgaccacg acggcgcccc caccggggcc
gccaccgggg ccacgacggg cgccaccgtg gccggcgtgt ccgccgcgct ggccgccgcg
ctggcggagc gcatcggccc gcgcggccgc ggcgtcctga ccccggcgct ggcggcgctg
gccctgccga cccgcctgcg cctgcgcacc cgccacgcca cgcccgcgct gctgcgcacg
ctgcgtccgg gcgacgtgct gctcgggtgg ccagcggccc cgggcttcgc gccgcacgcg
acgctcggcc aggcgaccct gctgtggggc gccgccaacg gtcacgccgt ccatgcgcac
gcgcgcatcg attcccgcaa cgtcatcctg gagaggagtc cctacgccat gaaccacgat
cccgacctgt ccctgcgcgc cgcgccggat gccgcctcct ccccgctcga cgtcagcgac
gtcgaactgc cggtccacat cgaagtggtg acggtgaacc tgccgatcgg gcagatcgcc
gccctgcagc cgggctatat cctcgcgttg ccggtcgcgc tggccgacgc cgacatccgg
ctcgtggcgc acggccagac cctggccttc ggggagctcg tcgccatcgg cgaccagctc
ggcctgcaca tccgccgtat cgcgaacgcc gatgaacgcc gcgcctga
```

Table A.11. The primer3 output of FDR-3

Primer 3 output for FDR-3

OLIGO	start	len	tm	gc%	
LEFT PRIMER	381	19	59.10	57.89	gcacctcgaatacgaccac
RIGHT PRIMER	881	20	60.01	55.00	aggttcaccgtcaccacttc

SEQUENCE SIZE: 1068
INCLUDED REGION SIZE: 1068
PRODUCT SIZE: 502 bp

```
atgcaaacca caacggcaac gaccccgctg gcggacaggc tgcgccgcta cacgccggcg

ctggccagcc tggcacgcgc cctgtacgac gcccgcggcg cgctgcatgg caccgtgcgg

gccgtgcccg agggaccgcc gcgcaccggc aagaccgcgc agttgaccgt cgcttgcgac

cagggcgagc tgcacgtcca cgtcgacgcc gacgccgcct tcgaggcgat cgccctcgaa

ccggaggcag cgtgccgcgc cgccgtggcc agcctgtacc tggccggccc gctggcggcg

ctggcgcgcc atggcgccac gcggccggcg gtacgggatg tgcgcctcgc cgcgccgtcc

gcctcgcgcg ccggcgtgct gcacctcgaa tacgaccacg acggcgcccc caccggggcc
                     >>>>>>>>>>>>>>>>>>>>
gccaccgggg ccacgacggg cgccaccgtg gccggcgtgt ccgccgcgct ggccgccgcg

ctggcggagc gcatcggccc gcgcggccgc ggcgtcctga ccccggcgct ggcggcgctg

gccctgccga cccgcctgcg cctgcgcacc cgccacgcca cgcccgcgct gctgcgcacg

ctgcgtccgg gcgacgtgct gctcgggtgg ccagcggccc cgggcttcgc gccgcacgcg

acgctcggcc aggcgaccct gctgtggggc gccgccaacg gtcacgccgt ccatgcgcac

gcgcgcatcg attcccgcaa cgtcatcctg gagaggagtc cctacgccat gaaccacgat

cccgacctgt ccctgcgcgc cgcgccggat gccgcctcct ccccgctcga cgtcagcgac

gtcgaactgc cggtccacat cgaagtggtg acggtgaacc tgccgatcgg gcagatcgcc
                     <<<<<<<<<<<<<<<<<<<<
gccctgcagc cgggctatat cctcgcgttg ccggtcgcgc tggccgacgc cgacatccgg

ctcgtggcgc acggccagac cctggccttc ggggagctcg tcgccatcgg cgaccagctc

ggcctgcaca tccgccgtat cgcgaacgcc gatgaacgcc gcgcctga
```

Table A.12. The nucleotide sequence of *Hordeum vulgare* IDI-1

***Hordeum vulgare* IDI(iron deficiency induced)-1 (AB025597)**

tccgagcttt tccgacgaga ggaaggaagg aaagcagagg agagagcagt
tgcgcgggag actgcgaggg gccgccgcca tggagaacga gttccagga
tggcaaggag caggtcatcc aagcatggta catggatgac agtgaagagg
accagaggct tcctcatcac cgtgagccca aagagttcat tcctcttgca
aaactttcag aattaggtgt tgtaagctgg aacctaaatg ctgataactg
gagaaagatg agaatctcaa gaaaatccgt gaggccaggg gatactctta
tgtggacatt tgcgatgtat gtccggagaa gttgccaaac tacgaggcca
agctgaagaa tttctttgaa gaacacttgc atactgatga agagatacgc
tattgtcttg agggcagtgg atactttgat gtgagggacc aaaacgaaca
gtggatccgt atagcagtta agaaaggcgg catgattgtt ttgcctgcag
gaatgtatca ccgctttaca ttggatagtg acaactacat caaggcaatg
cggctctttg tgggagagcc catctggacg ccgtacaatc gcccccatga
ccatctccca gctagaaagg agtatgtcga caagattatc aacagaggtg
ggaaccaaac cgtcgaggct cgttgatggc ttctacagtg ttccgcaacg
agtgatcttc tgtatgtatc tacatatcac accaaaagtt actgaataag
atgtgtgtga ttggctttcg ccgtgtactc gtaccagcat cgatcatgta
tcacttgtgt ggtagtctgc accgttaccc gctcgaaatc tttcctggaa
cttcttcgcc cggcaatgat gcctgtattg aataataatg atccagtgtc
agcaacggtg taacgaaaac agatgcatgc tcgtctaaat ctgtgagaaa
tgttgtgcca ttattggctg aaactatgca tgtgtgcatg aaaaaaa

Table A.13. The primer3 output of IDI-1

Primer 3 output for IDI-1

OLIGO	start	len	tm	gc%	
LEFT PRIMER	1	18	59.10	57.89	atggagaacgagttccag
RIGHT PRIMER	881	18	60.01	55.00	tcaacgagcctcgacggt

SEQUENCE SIZE: 594
INCLUDED REGION SIZE: 594
PRODUCT SIZE: 594 bp

```
atggagaacg agttccagga tggcaaggag caggtcatcc aagcatggta
>>>>>>>>>>>>>>>>>>>
catggatgac agtgaagagg accagaggct tcctcatcac cgtgagccca

aagagttcat tcctcttgca aaactttcag aattaggtgt tgtaagctgg

aacctaaatg ctgataactg gagaaagatg agaatctcaa gaaaatccgt

gaggccaggg gatactctta tgtggacatt tgcgatgtat gtccggagaa

gttgccaaac tacgaggcca agctgaagaa tttctttgaa gaacacttgc

atactgatga agagatacgc tattgtcttg agggcagtgg atactttgat

gtgagggacc aaaacgaaca gtggatccgt atagcagtta agaaaggcgg

catgattgtt ttgcctgcag gaatgtatca ccgctttaca ttggatagtg

acaactacat caaggcaatg cggctctttg tgggagagcc catctggacg

ccgtacaatc gcccccatga ccatctccca gctagaaagg agtatgtcga

caagattatc aacagaggtg ggaaccaaac cgtcgaggctcg ttga
                               <<<<<<<<<<<<<<<
```

Table A.14. The nucleotide sequence of *Triticum aestivum* elongation factor-2 (EF-2)

***Triticum aestivum* elongation factor-2 (EF-2) (AF005085)**

```
tgttcttcgt atcactgatg gagctttggt ggttgttgac tgtattgagg
gtgtctgtgt gcagactgaa actgtgctgc gccaagctct tggtgagagg
attaggccag tccttactgt gaacaagatg gacagatgct tccttgagct
tcaggtggaa ggtgaggaag catatcagac tttctcccgt gttatcgaga
atgccaatgt catcatggca acatatgaag atgtgctcct tggtgatgtc
caagtgtacc cagaaaaggg gactgttgca ttctctgctg gtctgcatgg
gtgggctttc acccttacaa actttgctaa gatgtatgcc tccaactttg
gagttgatga ggcaaagatg atg
```

Table A.15. The Primer detective results for wheat EF-2 gene

Sequence : EF2WHEAT (373 bp)
Primer Length: 18-22 bp
Target region length: 100-373 bp
Primer GC% range: 45-55%
Melt. Temperature: 76-83.5 ^{o}C
Searching base #: 1-339
3’ end homologies filtered: Yes
Anti-sense primers displayed in TRANSFORMED form (3’-5’)

	GC%	Tm	Product Size
Sn 11= **ATCACTGATGGAGCTTTGGTGG**	50		
		80.3	289 bp
Asn 278= **CGTAAGAGACGACCAGACGTAC**	54.5		
Sn 29= GTGGTTGTTGACTGTATTGA	40		
		80.4	317 bp
Asn 326= CGATTCTACATACGGAGGTT	45		
Sn 41= TGTATTGAGGGTGTCTGTGTGC	50		
		80.4	284 bp
Asn 303= CCCGAAAGTGGGAATGTTTGAA	45		

Table A.16. The Primer detective results for barley IRT-1 gene

Sequence : IRT-1 BARLEY (1329 bp)
Primer Length: 18-22 bp
Target region length: 100-373 bp
Primer GC% range: 45-55%
Melt. Temperature: 76-83.5 ^{o}C
Searching base #: 1-339
3’ end homologies filtered: Yes
Anti-sense primers displayed in TRANSFORMED form (3’-5’)

	GC%	Tm	Product Size
Sn 256= **TCATTAAGTGTTTCGCCTCCG**	47.6		
		79.5	437 bp
Asn 672= **GCAGCT CTT TGCTTCCATCAA**	47.6		