

**COMPLEMENTATION STUDIES TO IDENTIFY GENES WITH
ROLES IN ZINC EFFICIENCY IN BARLEY**

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COMPLEMENTATION STUDIES TO IDENTIFY GENES WITH ROLES IN ZINC EFFICIENCY IN BARLEY

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ABSTRACT

COMPLEMENTATION STUDIES TO IDENTIFY GENES WITH ROLES IN ZINC EFFICIENCY IN BARLEY

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Zinc (Zn) is an essential micronutrient for the growth and development of all organisms. Zinc deficiency is a widespread micronutrient disorder worldwide, which reduces crop yields and the nutritive value of the grain. Understanding the process of zinc absorption and translocation in crop is essential for this purpose. Zinc is taken up by plants and translocated within plants through high-affinity zinc transporter proteins embedded in the plasma membrane. The Zn transporters are induced under Zn deficiency, and it is speculated that the expression levels of some of zinc

transporters are critical for improved tolerance to low zinc. A number of Zn transporters have been cloned from higher plants including rice and Arabidopsis, but little has been done in barley and wheat. This project aims to investigate genes involved in zinc efficiency mechanism by complementation analysis in yeast, which is double mutant of zinc-transporters, using cDNA expression library from a most zinc efficient barley cultivar, Tokak-157.

Key words: Zinc efficiency, zinc deficiency, zinc transporters, cDNA library, barley, complementation analysis.

ÖZ

ARPADA ÇİNKO YETERLİLİĞİNDE ROL ALAN GENLERİN TAMAMLAMA ANALİZLERİYLE BELİRLENMESİ

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Çinko (Zn) bütün organizmaların büyümesi ve gelişmesi için gerekli olan önemli bir microelementtir. Topraktaki çinko eksikliği ise ülkemizde ve dünyada yaygın olan bir sorundur. Bu eksiklik bitkisel üretimi sınırlayarak önemli ekonomik kayıplara neden olmaktadır. Bu yüzden, bitkilerde çinko alımı ve taşınımının mekanizmasını anlamak büyük önem taşımaktadır. Çinko bitkilerde hücre zarına gömülü olarak bulunan taşıyıcı proteinler yardımıyla alınır ve bitki içinde taşınır. Bu taşıyıcı proteinler çinko eksikliğinde artmakta ve bu proteinlerin ekspresyon düzeylerinin inko eksikliğine tolerans geliştirmekte önemli olduğu

düşünülmektedir. Bu projede arpada çinko yeterliliğini sağlayan mekanizmada rol oynayan genlerin belirlenmesi amaçlanmaktadır. Bu amaçla arpadan (Tokak-157) cDNA ekspresyon kütüphanesi oluşturulmuş ve bu kütüphane, çinko taşıyıcıları mutasyona uğratılmış mayada tamamlama analizi yöntemi kullanılarak araştırılmıştır.

Anahtar kelimeler: Çinko eksikliği, çinko eksikliğine dayanıklılık, çinko taşıyıcıları, cDNA kütüphanesi, arpa, tamamlama analizi,

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TABLE OF CONTENTS

PLAGIARISM	iii
ABSTRACT.....	iv
ÖZ.....	vi
ACKNOWLEDGEMENTS.....	viii
TABLE OF CONTENTS.....	ix
LIST OF TABLES.....	xiv
LIST OF FIGURES.....	xv
LIST OF ABBREVIATIONS.....	xvii
CHAPTERS	
1. INTRODUCTION.....	1
1.1 Barley.....	1
1.2 Zinc.....	1
1.2.1 Properties of zinc	1
1.2.2 The effects of zinc on plants.....	2
1.2.3 Hyperaccumulator plants.....	7
1.3 Correcting micronutrient deficiency.....	7
1.4 Zinc uptake mechanism in plants.....	8
1.4.1 Zinc transporters.....	8
1.4.2 Metal chelating agents (Phytosiderophores).....	11
1.5 Zinc uptake mechanisms in yeast.....	11

1.5.1 Intracellular zinc transport.....	14
1.6 Functional complementation analysis.....	15
1.7 cDNA library.....	17
1.8 Aim of the study.....	18
2. MATERIALS AND METHODS	19
2.1 Plant materials	19
2.2 Growth conditions.....	19
2.3 Collection of samples.....	20
2.4 RNA isolation.....	21
2.4.1 Precautions	21
2.4.2 Total RNA isolation.....	21
2.4.3 mRNA isolations.....	22
2.5 Preparation of agarose gels	24
2.5.1 Preparation of RNA gels.....	24
2.5.2 Sample preparation for RNA gels.....	25
2.5.3 Preparation of DNA gels.....	25
2.6 cDNA synthesis.....	25
2.6.1 Synthesis of first strand cDNA.....	25
2.6.2 Synthesis of second strand cDNA	27
2.6.3 Processing the ends of cDNA.....	27
2.6.3.1 Blunting the cDNA termini	27
2.6.3.2 Ligating EcoRI adaptors.....	28
2.6.3.3 Phosphorylating EcoRI ends	28
2.6.3.4 Digesting with XhoI	28
2.6.4 PCR amplification with actin primer	29

2.6.5	Size fractionation of cDNA	29
2.7	Preparation of vector for library construction	30
2.7.1	Choice of vector for transformation.....	30
2.7.2	<i>E.coli</i> competent cell preparation for transformation	31
2.7.3	Preparation of growth medium	32
2.7.4	Transformation of competent cells with plasmid pUE2.....	32
2.7.5	Isolation of plasmid.....	32
2.7.6	Restriction digestion of pUE2.....	33
2.7.7	Purification of plasmid from gel	34
2.7.8	Dephosphorylation of plasmid	34
2.7.9	DNA purification and concentration.....	38
2.8	Construction and characterization of cDNA library	39
2.8.1	Ligation of plasmid pUE2 with cDNA	39
2.8.2	Transformation of the ligation product into <i>E.coli</i>	39
2.8.3	Determination of cDNA library titer.....	40
2.8.4	Determination of transformation efficiency.....	40
2.8.5	Amplification of the library	40
2.8.6	Collection of the library from plates	41
2.8.7	Plasmid isolation	41
2.8.8	Qualifying cDNA library.....	41
2.9	Complementation analysis.....	41
2.9.1	Preparation of yeast growth medium and solutions.....	41
2.9.2	Growing of <i>S. cerevisiae</i> strains.....	42
2.9.3	Transformation of yeast cells with plasmid.....	42
2.9.4	Growing transformants in selective medium	44

2.9.5 Isolation of plasmids from yeast.....	44
2.9.6 Re-transformation of isolated plasmid into <i>E.coli</i>	45
2.9.7 Isolation of plasmids.....	46
2.9.8 Visualization of isolated plasmid	46
2.9.9 Double digestion of plasmid with <i>EcoRI</i> and <i>XhoI</i>	46
3. RESULTS AND DISCUSSION.....	47
3.1 Collection of plant samples	47
3.2 Total RNA isolation.....	47
3.3 mRNA isolations.....	51
3.4 Visualization of cDNA.....	52
3.5 Control of cDNA <i>via</i> PCR with actin primers.....	53
3.6 Size fractionation of cDNA.....	54
3.7 Isolation of plasmid pUE2.....	55
3.8 Linearization of Plasmid pUE2 with <i>EcoRI</i> and <i>XhoI</i>	55
3.9 Determining the titer cDNA library.....	56
3.10 Determining transformation efficiency.....	57
3.11 Qualifying the cDNA library.....	57
3.12 Yeast cultivation.....	58
3.13 Results of the transformation.....	60
3.14 Screening of the library.....	62
3.15 Sequence analysis.....	64
4. CONCLUSION.....	66
REFERENCES.....	68

APPENDICES

Appendix A: Sequence of the plasmid pUE2.....	75
Appendix B: Sequences of the primers designed for pUE2.....	79
Appendix C: Results of multiple sequence alignment.....	80

LIST OF TABLES

TABLE

Table 2.1	Growth supplements added to SD medium and their concentrations.....	43
Table 3.1	Concentrations of total RNA samples isolated from barley.....	51

LIST OF FIGURES

FIGURE

Figure 1.1	Worldwide barley production.....	2
Figure 1.2.	Geographic distribution of severe and moderate Zn deficient soils in the world.....	4
Figure1.3	Causes of zinc deficiency.....	5
Figure1.4	The picture of zinc deficient wheat	6
Figure1.5	Predicted protein structure of the members of the ZIP family of micronutrient transporters.....	10
Figure1.6	CDF transporters.....	10
Figure1.7	Model of zinc transport and its regulation in <i>S.cerevisiae</i> ...	13
Figure1.8	A model of zinc-responsive post-translational inactivation of Zrt1.....	14
Figure1.9	ZHY3 mutant strain of <i>S. cerevisiae</i> lacking ZRT1 and ZRT2 transporters.....	16
Figure 2.1	A representation of the hydroponics system	20
Figure 2.2	Schematic representation of mRNA isolation.....	23
Figure 2.3	cDNA synthesis flowchart.....	26
Figure 2.4	Map of pUE2.....	31
Figure 2.5	Schematic representation of dephosphorylation.....	36
Figure 2.6	Restriction sites of enzymes <i>EcoRI</i> and <i>XhoI</i> on pUE2.....	37
Figure 3.1	An example of comparison of the length of roots of barley grown in Zn(-) and Zn(+) mediums.....	48
Figure 3.2	Discoloration and necrosis of the older leaves of barley grown in Zn(-) medium.....	49

Figure 3.3	Appearances of total RNA isolates from barley grown in zinc deficient medium.....	50
Figure 3.4	The length of synthesized cDNA.....	53
Figure 3.5	Actin PCR result.....	54
Figure 3.6	Vector preparation.....	55
Figure 3.7	Appearance of cut plasmid on gel.....	56
Figure 3.8	Appearance of digested plasmid containing cDNA fragments.....	57
Figure 3.9	2 days growth of ZHY3	59
Figure 3.10	Control, untransformed ZHY3 cells were spreaded onto -URA minimal medium	61
Figure 3.11	ZHY3 cells that are transformed with pUE2 and so can grow on -URA minimal medium.....	61
Figure 3.12	Screening of the library.....	63
Figure 3.13	Isolated plasmid DNA from putatively positive clones that were amplified in <i>E.coli</i>	64
Figure 3.14	Appearance of digested putative recombinant plasmid.....	65

LIST OF ABBREVIATIONS

μg	: Microgram
μl	: Microliter
bp	: base pair
ds	: double stranded
kb	: kilobase
min	: minute
mM	: Milimolar
ng	: Nanogram
PCR	: Polymerase Chain Reaction
pmol	: Pico mole
mRNA	: Messenger RNA
cDNA	: Complementer DNA
Rpm	: Rotation per minute
UV	: Ultra Violet
v/v	: volume/volume
w/v	: weight/volume

CHAPTER I

INTRODUCTION

1.1 Barley

Barley (*Hordeum vulgare*) belongs to the tribe Triticeae and family Poaceae. It is an annual diploid species with $2n = 14$ chromosomes.

Barley is one of the earliest cereals cultivated and has been known to man for more than 10,000 years. In ancient times it was imported for making bread and beer and also used for medicinal purposes. Today it's no longer used for bread making (wheat was replaced with it). Half of the world's barley production is used as an animal feed. A large part of the remainder used for malting and is a key ingredient in beer and whiskey production. Also it is an ingredient of breakfast cereals. A small amount of barley is also used in soups and stews, particularly in Eastern Europe, and also as a medicinal herb (Duke and Ayensu, 1985).

Barley was grown in about 100 countries worldwide in 2005 and Figure 1.1 shows top ten barley producers including Turkey.

1.2 Zinc

1.2.1 Properties of zinc

Zinc ($^{65}\text{Zn}^{+2}$) is a very important trace element for many biological functions and it plays an amazing number of critical roles in all organisms such as development,

Top Ten Barley Producers — 2005
(million metric ton)

Russia	16.7
Canada	12.1
Germany	11.7
France	10.4
Ukraine	9.3
Turkey	9.0
Australia	6.6
United Kingdom	5.5
USA	4.6
Spain	4.4
World Total	138

Figure 1.1 Worldwide barley production (Source: UN Food & Agriculture Organization FAO)

disease resistance and wound healing. Zinc is an essential catalytic component of over 300 enzymes, including alkaline phosphatase, alcohol dehydrogenase, Cu-Zn superoxide dismutase, and carbonic anhydrase. Zinc also plays a critical structural role in many proteins. For example, several motifs found in transcriptional regulatory proteins are stabilized by zinc, including the zinc finger, zinc cluster, and RING finger domains.

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, has many structural roles in biological membranes, cell receptors and other proteins.

1.2.2 The effects of zinc on plants

Zn is an essential element for plants and other organisms and is involved in many cellular processes, including activation of enzymes, protein synthesis, and membrane stability (Welch *et al.*, 1982; Marschner, 1986).

Zn deficiency, defined as the condition in which insufficient Zn is available for optimal growth, may cause not only reduction in crop yield, but also weakens the resistance of cereals to diseases and impairs the nutritional quality of the grain. It is probably the most widespread micronutrient deficiency in cereals limiting the grain yield and affecting 30 % of cultivated world's soils in Australia, India, Turkey and USA (Cakmak *et al.*, 1996a, Sillanpaa, 1990-Figure 1.2). Zn deficiency is common in a wide range of soil types, including high pH calcareous soils, sandy soils, and high phosphorus-containing fertilized soils (Marschner, 1995). Many plant species such as bean, maize, wheat, rice and tomatoes are considered less tolerant to Zn deficiency stress and exhibit significant yield losses due to Zn deficiency compared to more tolerant species such as peas, carrots, and rye (Chapman, 1966).

Additionally, 40% of world's population suffers from micronutrient deficiencies (the so-called "hidden hunger"), including Zn deficiency (ZD) (Bouis, 1996; Graham and Welch, 1996). High consumption of cereal-based foods with low zinc content is considered to be one of the major reasons for the widespread occurrence of zinc deficiency in humans, especially in developing countries. This is because of the plants grown in zinc deficient soils, have increased levels of phytic acid which is the main storage form of phosphorus in cereal grains (75% of total P is stored as phytic acid) (Raboy *et al.*, 1991). As phytic acid has high Zn binding and complexing ability, it hampers zinc's biological availability in diets (Welch, 1993) which is a very efficient chelator of zinc. Therefore, the biological availability of zinc decreases in such plants.

Zinc efficiency (ZE) is defined as the ability of a species or cultivar to grow and yield better on a Zn deficient soil than other species or cultivars. Zinc deficient plants don't have this ability. In most crops typical leaf Zn concentration required for adequate growth approximates 15–20 mg Zn kg⁻¹ dry weight (Marschner, 1995). Among the

cereal species, Zn efficiency was found to decline in the order; rye >triticale > barley > bread wheat > oat > durum wheat (Cakmak *et al.*,1998; Ekiz *et al.*,1998).

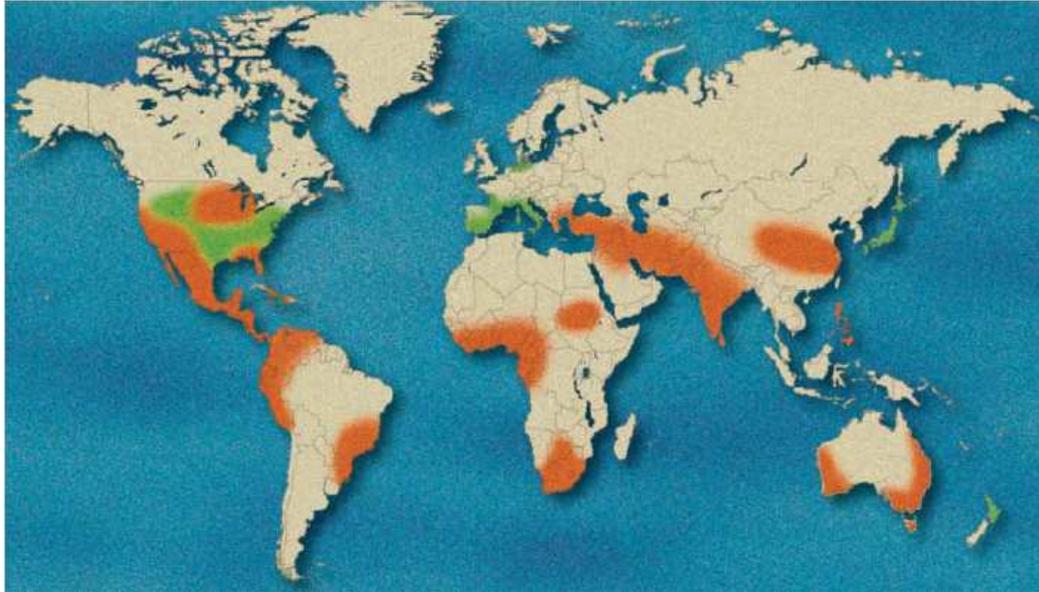


Figure 1.2 Geographic distribution of severe- (red areas) and moderate- (green areas) Zn deficient soils in the world (Source: Alloway, 2001).

Several mechanisms have been proposed to explain Zn efficiency in crop plants, including increased Zn uptake, increased Zn bioavailability in the rhizosphere due to release of root exudates, and more efficient internal Zn use (Rengel, 1999).

In Zn-deficient plants, a number of critical cellular functions and processes are impaired, leading to severe reductions in growth and development (Brown *et al.*, 1993). The adverse effects of Zn deficiency occur more distinctly in meristematic tissues, where intensive cell division and elongation take place. There is a high specific requirement for Zn in meristematic tissues, especially for maintenance of protein synthesis (Kitagishi *et al.*, 1987; Cakmak *et al.*,1989). The causes of zinc deficiency can be summarized as in Figure 1.3.

More than 300 enzymes are Zn dependent (Coleman, 1992). Zinc plays catalytic, co-catalytic, and structural roles in these enzymes. The enzyme copper-zinc superoxide dismutase (CuZn-SOD) is required for protecting plant cells against toxic superoxide

radical and activity of this enzyme is very low in plants under Zn-deficient conditions (Cakmak and Marschner, 1988b,c; 1993). Measurement of CuZn-SOD is suggested as a tool for screening cereals for Zn efficiency.

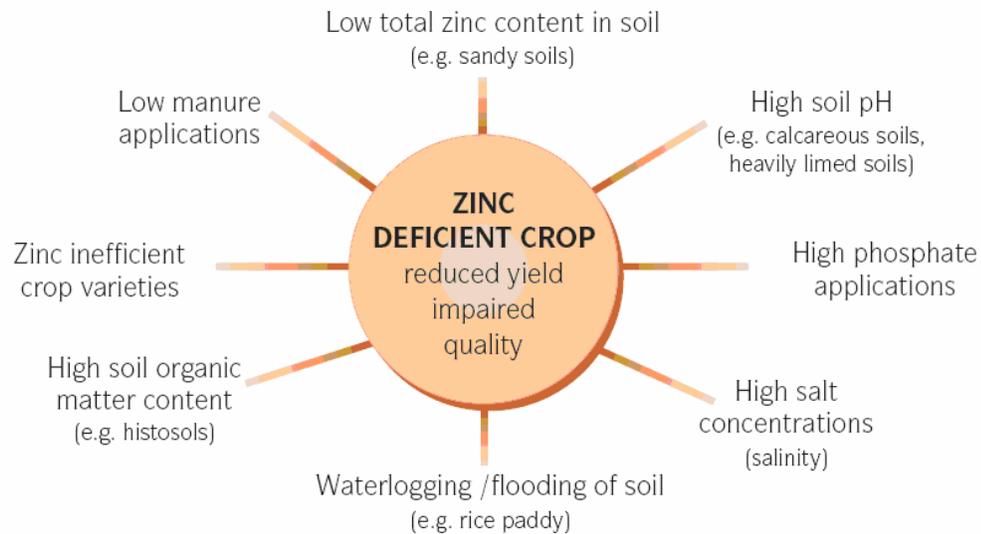


Figure 1.3 Causes of zinc deficiency (Source: Alloway 2003)

Zinc plays a decisive role in DNA and RNA metabolism, chromatin structure, and gene expression (Vallee and Falchuk, 1993). Several Zn-containing proteins are known to be involved in DNA replication and gene transcription processes (Klug and Rhodes, 1987). The structural and functional integrity of cellular membranes is greatly affected by the lack of Zn, which plays both structural and protective roles in membrane integrity (Welch *et al.*, 1982). Zinc can stabilize membranes by binding to phospholipids and sulphhydryl groups of cell membranes, thereby protecting these compounds against oxidative damage (Marschner, 1995; Cakmak and Marschner, 1988b,c; Welch and Norwell, 1993; Rengel, 1995a). When these compounds undergo oxidative damage, membrane integrity of Zn-deficient plant cells is impaired, and leakage of ions from Zn-deficient root cells is increased (Welch rhizosphere, Zn deficient plants may be susceptible to root diseases such as *Fusarium e t al.*, 1982).

Due to the increased leakage of carbon containing compounds in the rhizosphere, Zn deficient plants may be susceptible to root diseases such as *Fusarium graminearum* (Sparrow and Graham, 1988), *Gaeumannomyces graminis* (Brennan, 1992), and *Rhizoctonia solani* (Thongbai *et al.*, 1993). Zinc deficiency also affects metabolism of phytohormones, especially indoleacetic acid (IAA). The concentration of IAA is reduced by Zn deficiency, and this reduction is accompanied by decreases in shoot elongation (Skoog, 1940; Cakmak *et al.*, 1989).

Characteristic symptoms of Zn deficiency includes chlorosis on young leaves, reduced leaf size (i.e. little leaf), and stunted, thin stems (Figure 1.4). Under severe Zn deficiency, older leaves show wilting and curling with extensive chlorosis and stunted growth (Marschner, 1995).



Figure 1.4 The picture of zinc deficient wheat (Source: Reynolds *et al.*, 2001). Chlorosis of the leaves is apparent as patches of yellow areas on the leaves.

1.2.3 Hyperaccumulator plants

Despite its importance as a nutritional element, excess amount of Zn in the soil limits plant growth and is considered toxic. However, plant species growing in high concentrations of heavy metals, such as Zn, Cu, Ni, Cd, Mg, etc. have been reported for several centuries (Brown *et al.*, 1995; Taiz and Zeiger, 1998). Plants growing in high

concentrations of these metals have been proposed to be used for decontamination of soils containing heavy metal, a process called phytoremediation. Phytoremediation is the most economical and environmental friendly way of removing hazardous heavy metals from soil. Plants that are able to grow in high concentrations of heavy metals and capable of accumulating them are called hyperaccumulators (Taiz and Zeiger, 1998). Over 200 terrestrial species have been reported as accumulating various heavy metals (Baker and Brooks, 1989). One of the best known hyperaccumulator plant species is *Thlaspi caerulescens*, which can accumulate up to 3% Zn in dry shoots without showing any sign of toxicity.

1.3 Correcting zinc deficiency

Micronutrient deficiencies can be corrected by traditional methods like; soil application at or before planting, foliar application post-emergence, and application of the micronutrient to the seed at the time of planting. Zinc deficiencies are most commonly corrected by application of the zinc fertilizer to the soil. While foliar applications of zinc are often beneficial, soil applications remain effective much longer. Seed applications of zinc, although successful on some plants, have not been shown to produce a yield response on crops.

However, correction of Zn deficiency *via* fertilization is not always successful due to agronomic and economic factors. Some of those factors include reduced availability of zinc due to topsoil drying, subsoil constraints, disease interactions, and cost of fertilizer in developing countries; Zn fertilizers may be unavailable or unaffordable in developing countries (Graham and Rengel, 1993). Because of the widespread problems of Zn deficiency and difficulties in alleviating it *via* fertilization, an alternative may be identification of Zn efficient genotypes (Hacisaglihoglu *et al.*, 2001) and their molecular and genetic analysis. Also, the genes involved in zinc efficiency mechanism can be identify by using functional genomics techniques and transgenic plants can be generated that are normally zinc inefficient and such a research can be helpful to creat transgenic plants with enhanced mineral content.

1.4 Zinc uptake mechanisms in plants

1.4.1 Zinc transporters

Although zinc is an essential micronutrient, it is toxic at over optimal concentrations. Therefore, its concentration must be balanced carefully. Because zinc is a hydrophilic, highly charged species, it cannot cross biological membranes by passive diffusion, but rather it must be transported. It is taken up from the soil solution as a divalent cation (Zn^{2+}). It is also taken up as $ZnOH^+$ at high pH. Inside cells, zinc is neither oxidized nor reduced; thus, the role of zinc in cells is based on its behavior as a divalent cation that has a strong tendency to form tetrahedral complexes (Berg and Shi, 1996).

In eukaryotes two families of transporters, the ZIP (ZRT, IRT-like Protein) and CDF (Cation Diffusion Facilitator) families, have been found to play a number of important roles in zinc transport. The ZIP family plays role in zinc uptake, transporting it from outside the cell into the cytoplasm. The second group, CDF family, transports zinc in the direction opposite to that of the ZIP proteins, promoting zinc efflux or compartmentalization by pumping zinc from the cytoplasm out of the cell.

The ZIP (ZRT; Zinc-regulated transporters, IRT; Iron-regulated transporter-Like Protein) proteins (first identified in Arabidopsis) contain eight potential transmembrane domains, numbered I-VIII. The majority of ZIP proteins share a similar predicted topology where the amino and carboxy termini are extracytoplasmic (Figure 1.5). Another feature shared by many of the ZIP proteins is a long loop region located between transmembrane domains III and IV. This region is referred to as the 'variable region' because both its length and sequence shows little conservation among the family members (Guerinot 2000). One feature of the variable region that is shared by several of the ZIP proteins is the presence of many histidine residues. The function of this motif is unknown, however its conservation in many of the ZIP proteins suggests a role in metal ion transport or its regulation. The transport function is eliminated when the conserved histidines or certain adjacent residues are replaced by mutation (Rogers et al., 2000). Finally, the variable region is predicted to be cytoplasmic and this

location has been confirmed for Zrt1 of *Saccharomyces cerevisiae* (Gitan and Eide 2000).

In the *Arabidopsis thaliana* genome, a large number of cation transporters potentially involved in metal ion homeostasis have been identified (Maser *et al.*, 2001). Several members of the 15 Zinc-regulated transporters, Iron-regulated transporter-like Protein (ZIP) gene family (Guerinot, 2000) have been characterized and shown to be involved in metal uptake and transport in plants (Eide *et al.*, 1996; Korshunova *et al.*, 1999).

In contrast to ZIP family, CDF family members mediate zinc efflux out of cells or facilitate zinc transport into intracellular compartments for detoxification and/or storage. Most CDF proteins share a similar topology where both the amino and carboxy are cytoplasmic (Figure 1.6). The activity of many of these transporters is regulated in response to zinc through transcriptional and post-transcriptional mechanisms to maintain zinc homeostasis at both the cellular and organismal levels. The ZAT1 gene of *Arabidopsis* is a member of CDF transporter family. When ZAT1 is overexpressed, Zn accumulates only in roots, but not in shoots and it is suggested that ZAT1 might be involved in vacuolar sequestration of Zn (Zaal, 1999).

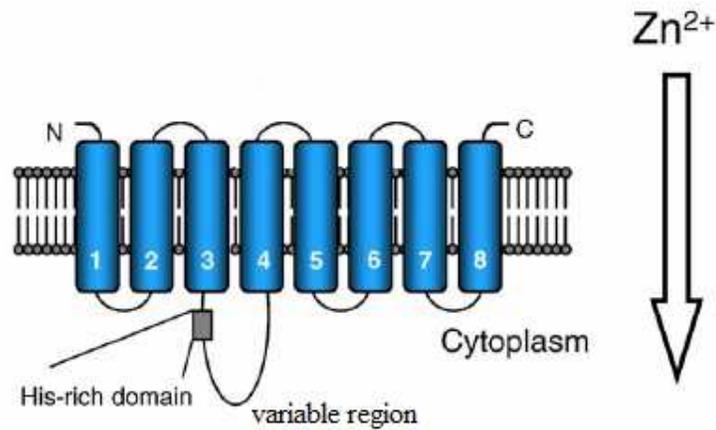


Figure 1.5 Predicted protein structure of the members of the ZIP family of micronutrient transporters. The notable structural features include eight membrane-spanning domains and a cytoplasmic loop of variable length situated between the third and fourth transmembrane helices. This cytoplasmic domain contains histidine repeats that may function in metal binding (Gaither *et al.*, 2001).

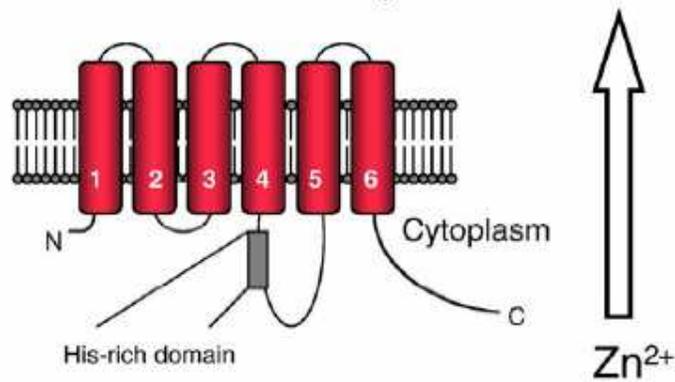


Figure 1.6 CDF transporters are predicted to have six transmembrane domains (I–VI). Conserved polar or charged residues within transmembrane domains I, II, and V. The locations of histidine-rich regions are potentially involved in metal binding (Gaither *et al.*, 2001).

1.4.2 Metal chelating agents (Phytosiderophores)

Phytosiderophores are organic compounds that are released into the rhizosphere and they bind to ferric iron (Fe^{3+}). Phytosiderophore- Fe^{3+} complex is recognized by specific transporters and transported across the root plasma membrane by an uptake system. But many studies showed that these compounds chelate not only ferric iron but also zinc.

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

1.5 Zinc uptake mechanisms in yeast

Zinc is estimated to be required for the function of 3% of the yeast proteome, and therefore cells must possess mechanisms to obtain sufficient quantities of this important nutrient (Eide, 2003).

Kinetic studies of zinc uptake by cells grown with different amounts of zinc in the medium suggested the presence of at least two uptake systems. One system has a high affinity for zinc and is active in zinc-limited cells (Zhao and Eide 1996a). The second system has a lower affinity for zinc and its activity is detectable in zinc-replete cells (Zhao and Eide 1996b). The *ZRT1* and *ZRT2* genes encode the transporter proteins of the high and low affinity systems, respectively (Zhao and Eide 1996a, b). *ZRT1* and *ZRT2* are the members of the ZIP family of metal ion transporters and they share 44% sequence identity and 67% similarity. They each contain eight potential transmembrane domains and have a similar predicted membrane topology in which the

amino- and carboxy-terminal ends of the protein are located on the outside surface of the plasma membrane (Figure 1.5). The high affinity system is induced more than 30-fold in zinc-limited cells resulting from increased transcription of the *ZRT1* gene (Zhao and Eide 1996a).

Zinc uptake in *Saccharomyces cerevisiae* is controlled at the transcriptional level in response to intracellular zinc levels. Regulation of these genes in response to zinc is mediated by the product of the *ZAP1* gene (Zhao and Eide 1997). *ZAP1* encodes a transcriptional activator. Zap1 protein was also found to regulate its own transcription through a positive autoregulatory mechanism. Zap1 binds to zinc-responsive elements (ZREs) in the promoters of the *ZRT1*, *ZRT2*, and *ZAP1* genes (Zhao *et al.* 1998).

As cells enter the initial phases of zinc limitation, their first response is to increase the activity of the Zrt2 transporter. As zinc limitation becomes more severe, the Zrt1 transporter is induced to provide high affinity uptake activity for zinc acquisition (Figure 1.7). Finally, increased expression of the *ZAP1* gene, allowing for maximum expression of its target genes, would only be needed under conditions of extreme zinc-limitation.

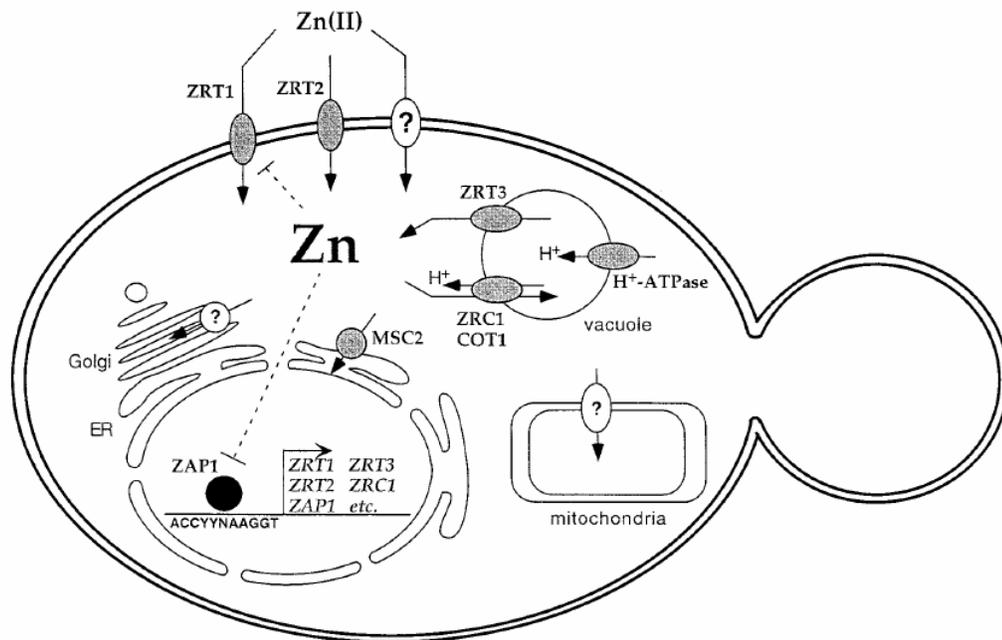


Figure 1.7 Model of zinc transport and its regulation in *S. cerevisiae*. Proteins involved in zinc transport are shown in *gray* and the Zap1 regulatory protein is in *black*. Solid arrows indicate transport steps and dashed lines indicate regulatory interactions. Transporters that are anticipated to exist but have not been identified are indicated by the question marks (Gaither and Eide, 2001).

A second mechanism in *S.cerevisiae* regulates zinc transporter activity at a post - translational level. In zinc-limited cells, Zrt1 is a stable plasma membrane protein. Exposure to high levels of extracellular zinc triggers a rapid loss of Zrt1 uptake activity and protein. This inactivation occurs through zinc-induced endocytosis of the protein and its subsequent degradation in the vacuole (Figure 1.8). Therefore, this system prevents the overaccumulation of this potentially toxic metal. (Gaither and Eide, 2001).

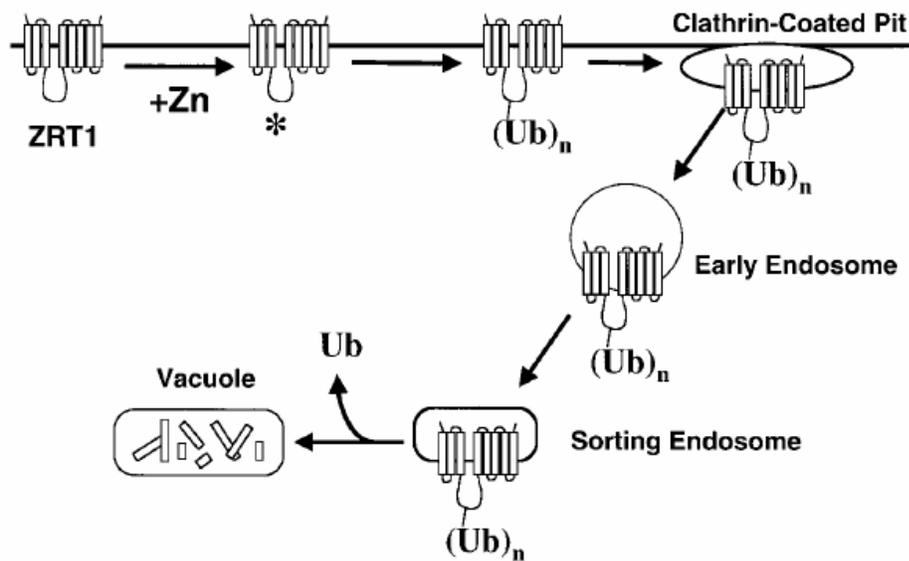


Figure 1.8 A model of zinc-responsive post-translational inactivation of Zrt1. High zinc causes the ubiquitination of Zrt1 which results in the protein's migration into clathrin-coated pits and subsequent endocytosis. Zrt1 then passes through the endocytic pathway to the vacuole where it is degraded by vacuolar proteases. The model proposes that zinc alters the conformation of Zrt1 (indicated by the asterisk) to make it a better substrate for ubiquitination (Gaither and Eide, 2001).

1.5.1 Intracellular zinc transport

Once zinc is taken up across the plasma membrane, some of the metal must be transported into organelles such as the mitochondria and compartments of the secretory system to serve as a cofactor of zinc-dependent proteins found within those compartments. The vacuole function in the storage and detoxification of zinc (Ramsay and Gadd, 1997). Two potential intracellular zinc transporters have been identified in *S. cerevisiae* that transport zinc into vacuole. These transporters are members of the CDF family, Zrc1, Cot1 (Figure 1.7). More recently, a gene in yeast has identified, ZRT3, that also plays a role in vacuolar zinc transport (MacDiarmid *et al.* 2000). Although distantly related to Zrt1 and Zrt2, Zrt3 is a potential transport protein that is a member of the ZIP family. Like the ZRT1 and

ZRT2 genes, ZRT3 is a ZAP1 target gene and is upregulated in zinc-limited cells. Zinc sequestered within the vacuole serves as a storage pool of zinc that can be mobilized under zinc-deficient conditions for use by the cell (Eide,2006).

1.6 Functional complementation analysis

The brewer's yeast *Saccharomyces cerevisiae* is a eukaryotic, unicellular microorganism of the fungus kingdom. As simple free-living cells, yeast are convenient tool for studying fundamental processes in eukaryotic genetics, cell biology and molecular biology. Much of our understanding of zinc transport and its regulation comes from studies of the yeast *S. cerevisiae*. Complementation analysis is also an important tool while working on zinc transporters. For this purpose a mutant 'ZHY3' was generated by Prof. David Eide from University of Wisconsin. The yeast strains ZHY3(*MAT α ade6 can1 his3 leu2 trp1ura3 zrt1::LEU2 zrt2::HIS3*) was derived from its parent strain DY1457 (*MAT α ade6 can1 his3 leu2 trp1 ura3*) by mutating *zrt1* and *zrt2* genes. So, this mutant lacks both high and low affinity zinc uptake systems and is extremely sensitive to zinc limitation because of its reliance on less efficient uptake pathways (Figure 1.9).

By using complementation analysis in ZHY3 many zinc transporter genes were identified up to date. ZIP1, ZIP2, and ZIP3 genes of *Arabidopsis* were isolated by functional expression cloning in a *zrt1 zrt2* mutant yeast strain; expression of these genes in yeast restored zinc-limited growth to this mutant and biochemical analysis of metal uptake has demonstrated that these genes encode zinc transporters (Grotz *et al.*,1998). ZNT1 is another gene that was isolated from zinc hyperaccumulator *Thalassia caerulea* by using the yeast Zn transport-deficient double mutant ZHY3 (Pence *et al.*,2000).

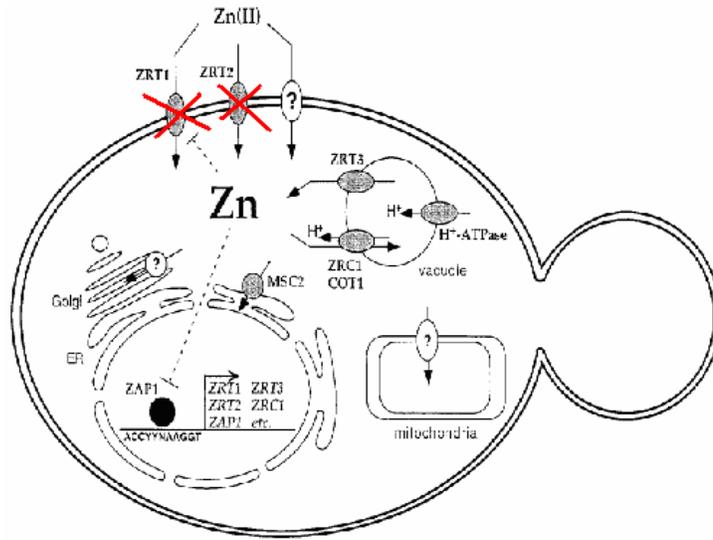


Figure 1.9 ZHY3 mutant strain of *S. cerevisiae* lacking ZRT1 and ZRT2 transporters (Original figure is from Gaither and Eide, 2001, this is modified form).

However, the strain lacking both the high and low affinity systems, the *zrt1zrt2* double mutant, is still viable in normal zinc conditions. Undoubtedly, these cells are obtaining zinc, and this uptake may represent the activity of a third system for zinc uptake. The identity of this third system is suggested by earlier studies in which zinc uptake in yeast attributed to a “divalent cation uptake system” that was also capable of transporting magnesium, cobalt, manganese, and nickel (Fuhrmann *et al.*, 1968).

1.7 cDNA libraries

In eukaryotic genomes, the entire sequence of genes is often very large due to presence of introns. Only about 1 to 10% of the total DNA in plant or animal cell is expressed or is functional genetically. The advantage of a cDNA library is that only the exons are templates for the creation of DNA to collect the preferred genes. A cDNA library is the set of all the mRNAs contained within a cell. It represents only the expressed genes so, would be proportionately much smaller and hence much simpler to handle and construct.

Such a library has several uses. A cDNA of a eukaryotic organism can be cloned into a prokaryotic organism and expressed there. The complete cDNA library of an organism represent the actively translated proteins in the condition that the organism is in total of the. Also, comparison of cDNA sequence between libraries constructed from cells derived from different organisms can provide insight into the genetic and evolutionary relationship between organisms through the similarity of their cDNA. cDNA libraries can be screened by complementation analysis or probes may be designed to hybridize with the gene of interest.

cDNA libraries are prepared by first isolating total RNA from plant cells. Plants can be grown under certain conditions so gene expression under this conditions can be examined. Then, poly(A)⁺ mRNA is isolated from total RNA. It is then converted into a single-stranded cDNA copy of the message using the enzyme reverse transcriptase. This single-stranded cDNA copy is then hybridized with a primer and DNA Polymerase is used to synthesise the complimentary strand forming double stranded cDNA. The cDNA fragments can be inserted into an appropriate plasmid (phage or cosmid) vector for maintenance and cloning. The population of recombinant vectors will represent the complete set of expressed genes in the cell from which the mRNA was isolated.

1.8 Aim of the study

When zinc is deficient in soil, some barley cultivars suffer from this deficiency, but somehow other cultivars called zinc efficient cope with this. Our aim is to investigate the genes involved in zinc efficiency mechanism in barley. As Tokak-157 plants are the most efficient cultivar among barley, we used it in our experiments. In our study, we have tried to investigate the genes by using complementation analysis technique, in which zinc-transport ability of mutant yeast should be restored by the gene coming from cDNA library. So, our aim was to construct a representative, intact cDNA library. All of the genes expressed (at the time of harvesting) by barley grown in zinc (-) conditions should be represented within this cDNA library.

The molecular and functional analysis are needed to be understood the mechanism underlying the expression of these genes/proteins that provide efficiency. Despite the fact that zinc deficiency has severe outcomes there is not enough studies that are focused on the molecular mechanism behind zinc efficiency.

CHAPTER II

MATERIALS AND METHODS

2.1 Plant materials

The barley cultivar Tokak-157, which is efficient to zinc deficiency, was obtained from Anatolian Agricultural Research Center, Eskişehir, Turkey.

2.2 Growth conditions

Seeds of Tokak-157 were surface sterilized in 20% hypochloric acid solution by shaking for two minutes, then washing under tap water and rinsing twice with double distilled water. Then, the seeds were embedded into autoclaved perlite moistened with double distilled water. Containers were covered with aluminum foil to create a humid environment for better germination. Seeds were stored at 4 °C for 2 days for vernalization, then they were germinated for five days at 25 °C in dark.

The germinated seedlings were then transferred to 7 L plastic vessels in continuously aerated nutrient solution as in the figure 2.1. One set of the plants were grown in medium containing sufficient amount of zinc (10 µM) for normal plant growth while the other set of plants were grown in zinc deficient medium (no zinc) to stimulate expression of genes playing role in zinc efficiency. In order to reduce the possible zinc contamination from water and other nutrient solutions, double distilled water was used.

The nutrient solutions were changed every three days with freshly prepared ones. Plants were grown in a growth chamber under controlled environmental conditions: 16/8 hours light/dark, 20/15 °C day/night temperature, 65% relative humidity.

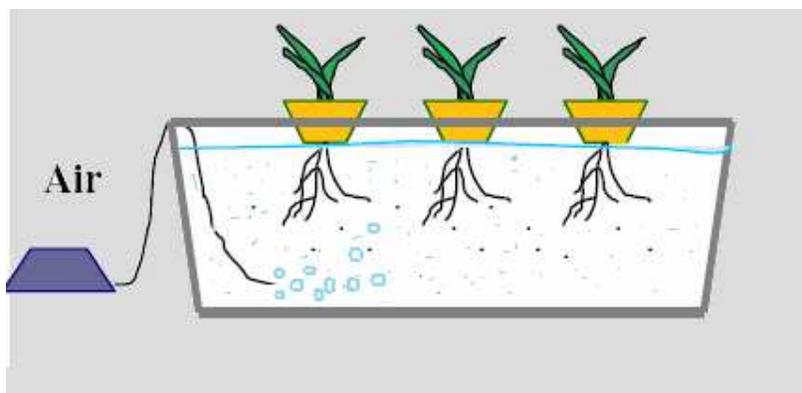


Figure 2.1 A representation of the hydroponic system

Macronutrient, micronutrient, ZnSO_4 and Fe-EDTA stock solutions were prepared separately with ddH₂O. Macronutrient solution contains, 0.88 mM K_2SO_4 , 0.25 mM KH_2PO_4 , 1.0 mM MgSO_4 , 2.0 mM $\text{Ca}(\text{NO}_3)_2$. Micronutrient solution contains 1 μM H_3BO_3 , 0.5 μM MnSO_4 , 0.2 μM CuSO_4 . Fe-EDTA concentration was 50 mM.

2.3 Collection of plant samples

Roots and leaves of plant samples were harvested on 20th day then 25th day, 30th day, 35th day from plants both growing in zinc(-) and zinc(+) solutions. They were stored at -80 °C separately. On the times of harvesting some of the plants grown in zinc(-) solution had showed Zn deficiency symptoms such as chlorosis on young leaves, reduced leaf size etc.

2.4 RNA isolations

Roots and leaves of plants, grown in Zn deficient medium were harvested at the time points given in section 2.3, were used for RNA isolation.

2.4.1 Precautions

When working with RNA to keep the RNA intact is very important because it is much more prone to degradation than DNA. Thus, before working with RNA some precautions should be taken. All of the glass equipments were treated with hypochromic acid solution and washed with active DEPC (diethylpyrocarbonate)- treated ddH₂O and autoclaved at 121° C for 30 minutes. Other non-glass equipments and plastic materials were treated with active ddH₂O and autoclaved at 121° C for 1 hour. Before isolation, micropipettes and bench were cleaned with RNase-off (Applichem). Also DEPC-treated ddH₂O was used for preparation of solutions and buffers.

2.4.2 Total RNA isolation

Plant tissue sample of 50-100 mg was powdered by mortar and pestle in the presence of liquid nitrogen. Frozen tissue samples were crushed using the chilled mortar and pestle. Occasionally liquid nitrogen was added to mortar to keep the sample submerged and frozen. Tissue sample was grinded for 5 minutes until sample becomes powdery and transferred into 2 mL microfuge tube.

For each 50-100 mg of sample, 1mL of Trizol Reagent (Invitrogen) was added incubated for 5 minutes at room temperature. Then 0.2 mL of chloroform was added per 1mL of Trizol Reagent, shaken by hand for 15 seconds vigorous and incubated at room temperature for 2-3 minutes. Then samples were centrifugated at 12000 rpm for 30 minutes at 4° C. Following the centrifugation step, the mixture separated into a lower red phase, an interphase, and a colorless upper aqueous phase (RNA remains in this aqueous phase).

The upper aqueous phase was transferred to another eppendorf tube. Iso-propanol (500

mL, half of the Trizol Reagent) was used to precipitate RNA. Samples were incubated for 10 minutes at room temperature and centrifugated at 12,000 rpm for 30 minutes at 4° C. The supernatant was decanted. (The pellet at the bottom is the total RNA). The tube was let stand upside down on clean paper towels. 1 mL of cold 75% ethanol (per 1 mL Trizol Reagent) was added. Then samples were centrifugated at 12,000 rpm for 30 minutes at 4° C. Supernatant was decanted and pellet was kept. The pellet was air dried horizontally on clean paper towels nearly for 1 hour. Approximately 40 µL of RNase-free water was added and the sample was incubated in a 65° C water bath for 10-15 minutes. Then samples were taken on ice and stored at -80° C.

RNA degradation was checked on agarose gel prepared with 1% phosphate buffer as described in section 2.5.1. Concentration of isolated total RNA samples was directly measured on Nano-Drop D-1000 UV Spectrophotometer at 260 nm. Nucleic acids, DNA and RNA, absorb at 260 nm, proteins absorb at 280 nm and phenolic compounds at 230. A ratio 260nm/280nm of 1.8-1.9 indicates pure DNA. A ratio of 1.9-2.0 indicates pure RNA. The ratio will be low if there is a lot of protein absorbing at 280 nm.

2.4.3 mRNA isolation

All of the total RNA samples, isolated from roots and leaves of plants grown in zinc(-) medium and harvested on 20th day then 25th day, 30th day, 35th day, were mixed. mRNA isolations was done by using Dynabeads Oligo (dT), which helps to pick mRNAs from their poly(A) tail, according to the procedure below (Figure 2.2)

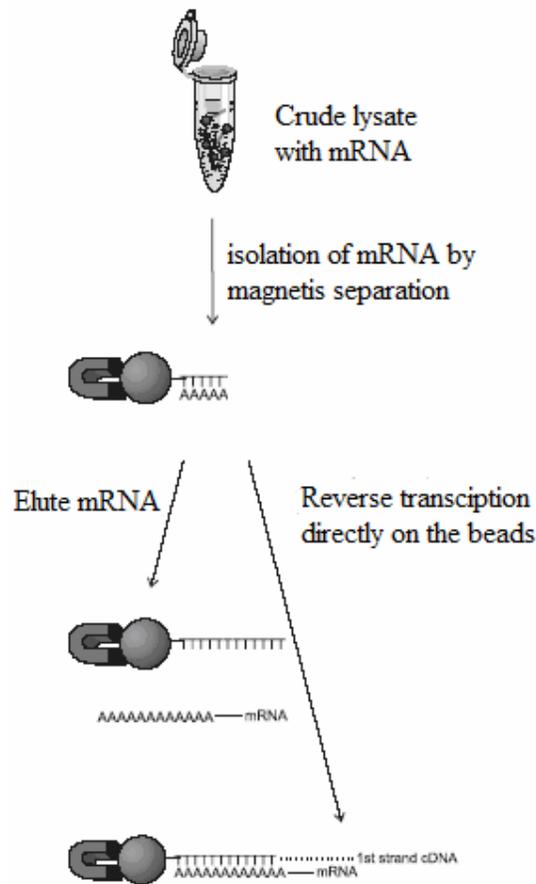


Figure 2.2 Schematic representation of mRNA isolation

The volume of ~ 75 µg RNA was adjusted to 100 µl with distilled DEPC-treated water. Optimal hybridisation conditions was obtained in Binding Buffer added in a 1:1 ratio relative to sample volume. Total RNA samples were heated to 65°C for 2 minutes to disrupt secondary structures forming at 3' end and placed on ice. In the meantime, 200 mL of resuspended Dynabeads Oligo (dT)₂₅ were removed from the stock tube suspension and dispensed into a 1.5 ml eppendorf tube and the vial was placed on the DYNAL MPC-DYNAL Crop.-(Magnetic Particle Concentrator). After 30 seconds the supernatant was pipetted off and the Dynabeads Oligo (dT)₂₅ were washed once by removing the tube from the magnet, resuspending DynabeadsOligo (dT)₂₅ in 100 µL Binding Buffer (20 mM Tris-HCl pH 7.5, 1.0 M LiCl, 2 mM EDTA) and placing the tube back on the magnet. After 30 seconds the supernatant was pipetted off and the tube was removed from the magnet.

Binding Buffer (100 μ L) was added to the Dynabeads Oligo (dT)₂₅. The total RNA was added to the Dynabeads Oligo (dT)₂₅ in Binding Buffer, mixed thoroughly and annealed for 3-5 minutes at room temperature. Then the tube was placed on the magnet for at least 30 seconds or until solution is clear and the supernatant was removed. The tube was removed from the magnet and washed twice with 200 μ L Washing Buffer B (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA) using the magnet. It is important to remove all of the supernatant between each washing step. mRNA was eluted from beads by using 5 μ L DEPC-treated water or 10mM Tris-HCl. The tube was immediately placed on the magnet and the eluted mRNA was quickly transferred to a new RNase-free tube. mRNA isolations was done several times in order to obtain the necessary amount, which is 5 μ g, for cDNA synthesis. Their concentrations were again determined by using Nano-Drop D-1000 UV Spectrophotometer at 260 nm.

2.5 Preparation of agarose gels

2.5.1 Preparation of gels for RNA

All of the electrophoresis apparatus, trays and combs were washed several times with DEPC-treated water, then cleaned with RNase-OFF (Applichem) solution to inactivate contaminating Rnases. To prepare 1 % gel, 0.5 g of Agarose (Applichem) was melted by boiling in 50 mL, 10 mM Sodium Phosphate buffer (for 10 X buffer; 1.340 g/L Na₂HPO₄.7H₂O and 0.689 g/L NaH₂PO₄.H₂O, pH 6.8). When cooled down to about 60 °C, 13 μ L of Ethidium-bromide added and it was poured off in to the gel tray.

2.5.2 Sample preparation for RNA gels

To 2 μL RNA sample 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.

2.5.3 Preparation of DNA gels

To prepare 1 % gel, 0.5 g of Agarose (Applichem) was melted by boiling in 50 mL, 0.5 X TBE buffer (for 10 X buffer; 107.78 g Tris, 7.44 gr EDTA- Na_2 salt and and 55 g boric acid was dissolved in 1L dH_2O). When cooled down to about 60 °, 13 μL of ethidium bromide was added (final concentration should be 0.5 $\mu\text{g}/\text{ml}$) and it was poured off in to the gel tray.

2.6 cDNA synthesis

cDNA synthesis was performed by using ZAP-cDNA Synthesis Kit according to the manufacturer's procedure. Figure 2.3 shows the basic steps in cDNA synthesis.

2.6.1 Synthesis of first strand cDNA

The final volume of the first-strand synthesis reaction should be 50 μL . The volume of added reagents and enzymes is 12.5 μL , thus the mRNA template and DEPC-treated water should be added in a combined volume of 37.5 μL . mRNA (36 μL) was obtained from the mRNA isolation procedure and 1.5 μL DEPC-treated water added to complete volume to 37.5 μL . In an RNase-free microcentrifuge tube, following reagents were added in order; 5 μL of 10 X first-strand buffer, 3 μL of first-strand methyl nucleotide mixture, 2 μL of linker-primer (1.4 $\mu\text{g}/\mu\text{l}$), 1.5 μL of DEPC-treated water and 1 μL of RNase Block Ribonuclease Inhibitor (40 U/ μl). Poly(A) RNA (36 μL) was incubated at 65°C for 10 mins.

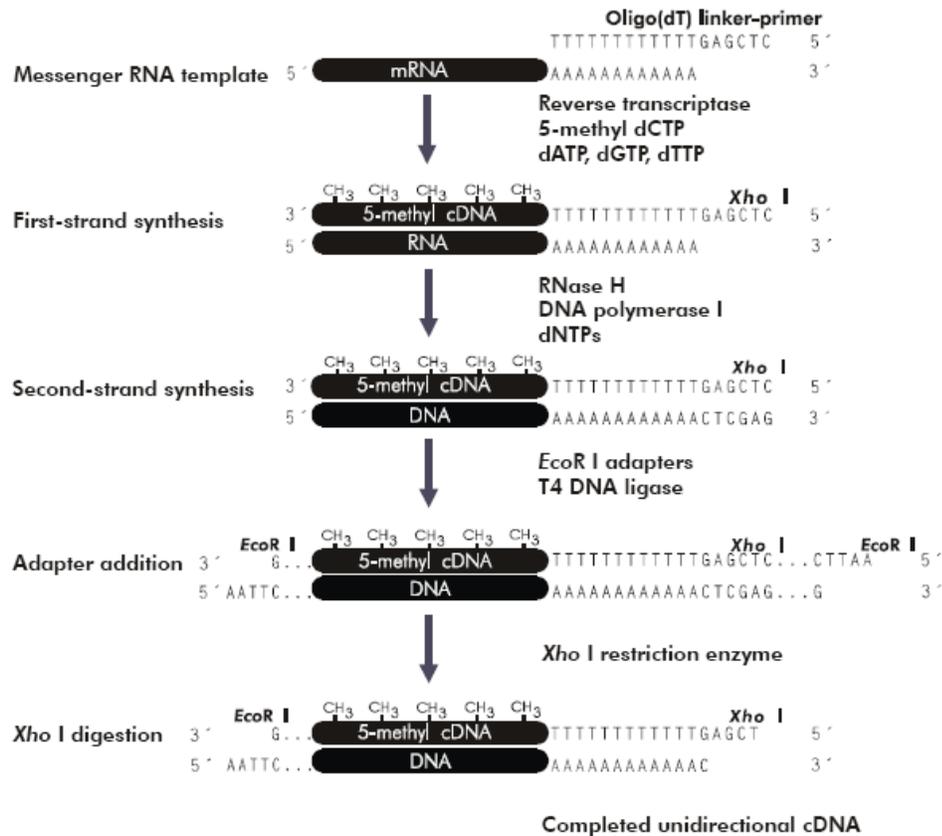


Figure 2.3 cDNA synthesis flowchart

Approximately 5 μg of poly(A) RNA in 36 μL was added to reaction mixture and mixed gently. The primer was allowed to anneal to the template for 10 minutes at room temperature. Then 1.5 μL of StrataScript RT (50 U/ μL) was added to the first-strand synthesis reaction. The final volume of the first-strand synthesis reaction was brought up to 50 μL . The sample was spun down gently in a microcentrifuge. The first-strand synthesis reactions was incubated at 42°C for one hour. After 1 hour, it was removed from water bath and placed on ice.

2.6.2 Synthesis of second strand cDNA

All nonenzymatic second-strand components were centrifuged briefly before placing the tubes on ice. It is important that all reagents must be kept below 16°C when the

DNA polymerase I is added. Onto 50 μL first-strand synthesis reaction the following components were added; 20 μL of 10 \times second-strand buffer, 6 μL of second-strand dNTP mixture, 111 μL of sterile distilled water, 2 μL of RNase H (1.5 U/ μL), 11 μL of DNA polymerase I (9.0 U/ μL).

The reaction mixture was spun in a microcentrifuge and incubated for 2.5 hours at 16°C. Temperatures above 16°C can cause the formation of hairpin structures, which are unclonable and interfere with the efficient insertion of correctly synthesized cDNA into the prepared vector. Following 2.5 hours it was placed on ice.

2.6.3 Processing the ends of cDNA

2.6.3.1 Blunting the cDNA termini

Blunting dNTP mix (23 μL) and 2 μL of cloned Pfu DNA polymerase (2.5 U/ μL) was added to second strand reaction. The mixture was spun in a microcentrifuge and incubated at 72°C for 30 minutes. Then 200 μL of phenol–chloroform [1:1 (v/v)] was added. The reaction was spun in a microcentrifuge at maximum speed for 2 minutes at room temperature and the upper aqueous layer, containing the cDNA, was transferred to a new tube. An equal volume of chloroform was added and the mixture was mixed by vortex then spun in a microcentrifuge at maximum speed for 2 minutes at room temperature and the upper aqueous layer, containing the cDNA, was transferred to a new tube again.

cDNA was precipitated overnight at -20°C by adding, 20 μL of 3 M sodium acetate and 400 μL of 100% (v/v) ethanol, to the saved aqueous layer. It was centrifuged in a microcentrifuge at maximum speed for 60 minutes at 4°C. Then the supernatant was discarded carefully and the pellet was washed with 500 μL of 70% (v/v) ethanol. It was spun in a microcentrifuge at maximum speed for 2 minutes at room temperature. The pellet was air-dried until all of the ethanol completely remove.

2.6.3.2 Ligating the EcoRI adapters

The pellet was resuspended in 9 μL of *EcoRI* adapters and incubated at 4°C for at least 30 minutes to allow the cDNA to resuspend. The following components were added to the tube containing the blunted cDNA and the *EcoR* I adapters; 1 μL of 10X ligase buffer, 1 μL of 10 mM rATP, 1 μL of T4 DNA ligase (4 U/ μL). The reaction was spun down in a microcentrifuge and incubated at 4°C for 2 days. In the morning, the ligase was inactivated by placing the tubes in 70°C for 30 minutes.

2.6.3.3 Phosphorylating the EcoRI ends

After the ligase was heat inactivated, the reaction was spun in a microcentrifuge for 2 seconds and cooled at room temperature for 5 minutes. The adapter ends was phosphorylated by adding 1 μL of 10 X ligase buffer, 2 μL of 10 mM rATP, 5 μL of sterile water and 2 μL of T4 polynucleotide kinase (5 U/ μL). The reaction was incubated for 30 minutes at 37 °C then the kinase was heat inactivated for 30 minutes at 70°C. It was spun in a microcentrifuge for 2 seconds and allowed equilibrate to room temperature for 5 minutes.

2.6.3.4 Digesting with XhoI

Xho I buffer supplement (28 μL) and 3 μL of *Xho* I (40 U/ μL) was added to the reaction mixture and incubated for 1.5 hours at 37 °C then 5 μL of 10 X STE buffer and 125 μL of 100% (v/v) ethanol was added to the microcentrifuge tube. The reaction was precipitated overnight at -20 °C. Following precipitation, the reaction was spun in a microcentrifuge at maximum speed for 60 minutes at 4°C. The supernatant was discarded, the pellet was dried completely and resuspended in 14 μL of 1 X STE buffer. (10 X STE buffer: 100 mM NaCl, 10 mM Tris-HCl pH:8.0, 1mM EDTA).

2.6.4 PCR amplification with actin primers

cDNA was tested for the presence of actin gene by using actin gene specific primers. For PCR reaction following components were combined in a 200 μL sterile PCR tube; 0.5 μL cDNA template, 4 μL 10 X PCR buffer (GeneMark), 2.4 μL 25mM MgCl_2 (GeneMark), 0.5 μL dNTP (10mM each –Qiagen), 1 μL forward primer (5' AAT GGT CAA GGC TGG TTT CGC 3'), 1 μL reverse primer (5' CTG CGC CTC ATC ACC AAC ATA 3') 1 μL *Taq DNA polymerase* (5u/ μL) (GeneMark) and the volume was completed to 40 μL with sterile PCR water. PCR cycling conditions were; initial denaturation at 94 °C for 3 minutes, 34 cycles of three steps as denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, and extension at 72 °C for 1 minute.

PCR product was visualized on 1% agarose gel with ethidium bromide staining prepared as in section 2.5.3.

2.6.5 Size fractionation of cDNA

It was done to eliminate cDNA products smaller than 400 bp as we wanted to obtain full length cDNA which is probably longer than 400 bp. One of the columns in Qiagen PCR Purification Kit was used, the cotton in it was removed and the column was packed with 1 mL CL-2B sepharose. The column was placed into a 1,5 mL microfuge tube, then 2 eppendorf tubes (1.5 mL) were placed inside 15mL falcon tube and the eppendorf containing column was placed on the assembly.

The system was centrifuged (DENLEY BS400) at 1500 rpm for 2 minutes. The buffer was poured and than 1mL 1X STE buffer was put onto column to pack it tightly and the falcon tube was centrifuged at 1500rpm for 2 mins. (Before this the centrifuge was cooled by pouring liquid nitrogen inside). This step was repeated once more. The falcon tube was centrifuged once more without buffer at 1500 rpm for 1 minutes. cDNA was added onto the column and centrifuged at 1500 rpm for 5 mins. Microfuge tube under the column was replaced with a new DEPC-treated one and 30 μL ddH₂O

was added to the column for elution. Then centrifuged again and cDNA was collected. Elution was done 3 more times with 30 μ L dd H₂O and cDNA was collected into separate tubes. Then concentrations of eluted cDNA samples were measured by using Nano-Drop D-1000 UV Spectrophotometer at 260 nm.

2.7 Preparation of vector for library construction

2.7.1 Choice of vector for transformation

The vector that will be used in complementation analysis should be specifically designed for this purpose. It is a shuttle vector that can multiply both in yeast and *E.coli* and also an expression vector containing *EcoRI* (at N-terminus end) and *XhoI* (at C-terminus) cloning sites.

Grotz *et al.* (1998) and Pence *et al.* (2000) used plasmid pFL61 (Minet *et al.*,1992) in their studies for complementation analysis. But this vector doesn't contain *XhoI* restriction enzyme site so, it is not suitable for cloning of our cDNA that have *EcoRI* adaptor at 5' end and *XhoI* site at 3' end. Eckhardt *et al.* (2001), derived vectors from pFL61 called pUE1 and pUE2. Among these pUE2 (sequence is provided in Appendix A) is suitable for our purposes (Figure 2.4). It was provided kindly from Dr.Ulrich Eckhardt from Humboldt-University of Berlin.

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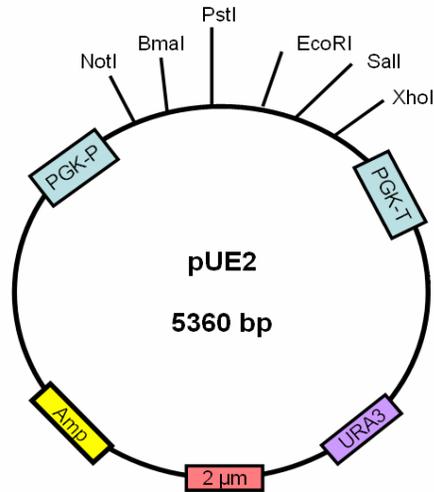


Figure 2.4 Map of pUE2

2.7.2 *E.coli* competent cell preparation for transformation

Z- Competent *E.coli* Transformation Buffer Set (Zymo Research) was used. fresh overnight *E.coli* (DH 5α) culture (0.5 mL) was inoculated into 50 mL SOB prepared by dissolving 20 g tryptone, 5 g yeast extract , 0.58 g NaCl , 0.186 g KCl in 1L dH₂O then autoclaved at 121°C for 30 minutes. After autoclaving 10 mL 1M MgCl₂ and MgSO₄ was added. Shaking vigorously at 20 °C-25 °C , the bacteria were grown until A₆₀₀ reaches 0.4 to 0.6. It was measured by using Shimadzu UV-1601 spectrophotometer.

Then the cells were pelleted at 2500g for 6 minutes at 4 °C. Supernatant was removed and cells were resuspended in 5 mL ice-cold 1 X Wash Buffer, prepared by adding 2.5 mL of Dilution Buffer to 2.5 mL of 2 X Wash Buffer provided by the manufacturer. Cells were re-pelleted and again supernatant was removed completely. The cells were gently resuspended in 5 mL ice cold 1 X Competent buffer, prepared by adding 2.5 mL of Dilution Buffer to 2.5 mL of 2 X Competent Buffer. (They should be freshly prepared). Then 0.5 mL aliquots were stored at – 80 °C.

2.7.3 Preparation of growth medium

Ampicilin solution was prepared by dissolving 500 mg ampicilin (AppliChem) in 10 mL dH₂O and filter sterilized by using MN Sterilizer (0.22 µm CA-Macherey-Nagel) and stored at - 20 °C. LB was prepared by dissolving 10g tryptone, 5 g yeast extract 10g NaCl in 1L d H₂O and LB-agar by adding 15 g agar to them and aoutoclaved at 121 °C for 30 minutes.

2.7.4 Transformation of the competent cells with plasmid pUE2

An aliquot of frozen competent cell was thawed on ice, 3 µL of plasmid DNA was added gently onto 200 µL competent cells. Then 1 µL DMSO (Dimethyl sulfoxide) was added and incubated on ice for about 30 minutes with occasional gentle mixing. After 30 minutes, it was put into 42 °C water bath for 45 seconds for heat shock then transferred to ice. The SOC medium was prepared by adding 10 mL of 2 M sterile glucose onto 1L SOB, was added to complete the volume to 1 mL and incubated in shaker for 1 hour.

After 1 hour 100 µL of the cells were spread onto ampicilin containing (50 µg/mL) LB agar plates and grown overnight at 37 °C in incubator.

One single colony was picked and grown in 20 mL LB containing ampicilin (50 µg/mL) overnight in the shaker at 37 °C 250 rpm.

2.7.5 Isolation of plasmid

Plasmid isolation from transformed *E.coli* cells was done with QIAprep Spin Miniprep Kit (Qiagen) according to the procedure below.

The bacterial cells were pelleted in microcentrifuge (CLP 3410) at 10.000 rpm for 5 minutes. The pelleted cells were resuspended in 250 µl Buffer P1 (containing RnaseH)

vortexing or pipetting up and down until no cell clumps remain. Buffer P2 (250 µl) was added and mixed thoroughly by inverting the tubes 4–6 times. Buffer N3 (350 µl) was added and mixed immediately and thoroughly by inverting the tube 4–6 times. Centrifuged for 10 min at 13,000 rpm in microcentrifuge. The supernatants were applied to the QIAprep spin column and centrifuged for 30–60 seconds, the flow-through was discarded. QIAprep spin column was washed by adding 0.5 mL Buffer PB and centrifuged for 30–60 seconds, the flow-through was discarded. QIAprep spin column was washed again by adding 0.75 mL Buffer PE and centrifuged for 30–60 seconds, the flow-through was discarded, and centrifuged for an additional 5 min to remove residual wash buffer. The QIAprep columns were placed in clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl Buffer EB (10 mM Tris-Cl, pH 8.5) was added, stood for 1 min and centrifuged for 1 minute. Concentrations of the plasmids were measured on NaNo-Drop ND-1000 UV Spectrophotometer.

2.7.6 Restriction digestion of pUE2

Isolated plasmid pUE2 was double digested from *EcoRI* and *XhoI* restriction sites to insert cDNA to that sites. R Buffer was chosen for restriction reaction as both enzymes work in this enzyme. 10 µL of plasmid DNA (400ng/ µL) was cut in 2 µL of 1X Buffer R(Fermentas), 1.5 µL of *EcoRI* restriction enzyme (10u / µL-Fermentas), 1.5 µL of *XhoI* restriction enzyme (10u / µL-Fermentas) and the volume was completed to 20 µL by adding 5 µL dH₂O. Digestion reaction was hold at 37 °C in 2.5 hours and 80 °C for 10 minutes for inactivation of enzymes.

After the reaction, double digested plasmid was loaded into 0.8 % agarose gel to visualize cut and uncut plasmids and to separate linearized plasmid from the insert between enzyme sites.

2.7.7 Purification of linearized pUE2 vector

The large band containing double digested plasmid thr linearized vector which was excised from the gel after and purified from gel by using Gel Elution Kit (GeneMark) according to the procedure below.

The gel slice was placed into a 1.5 ml clean sterile microcentrifuge tube and equal volume of Binding Solution was added to the gel slice, and incubated for 15 minutes at 60°C to dissolve agarose. Spin Column was inserted into a collection tube.

After the gel slice was completely melted, the DNA/agarose solution was transferred to spin column and centrifuged for 1 minute at top speed (12-14,000 x g). The filtrate was discarded and 700 mL of Wash Solution was added and centrifuged again for 1 min at top speed. This step was repeated for one more time. The filtrate was discarded then centrifuged for 2 min at top speed to remove residual trace of ethanol. The spin column was transferred into a new microcentrifuge tube and incubated at 45-60 °C oven for 5 min to evaporate all of the ethanol. 30 ml of preheated (at 60°C) elution solution (10mM Tris buffer, pH 8.5) was added to elute the DNA by centrifugation for 1 minute and the eluted DNA was stored at -20°C. Concentrations of 2 eluted samples were measured with Nano-Drop ND-1000 UV Spectrophotometer.

2.7.8 Dephosphorylation of 5' ends of the plasmid

Dephosphorylation was done prior to ligation in order to remove the 5' phosphate group from the ends, to prevent unwanted ligation because; ligation requires a 5' phosphate group and a 3' hydroxyl group on the ends of the compatible DNA fragments for efficient cloning. Figure 2.5 shows a schematic representation of dephosphorylation.

If the plasmid vector being used was linearized with a single restriction enzyme (generating either a blunt or overhanging end), then dephosphorylation of the vector is a prerequisite to reduce religated vector background. To verify double digestion may be

difficult to observe on the agarose gel since DNA piece between the restricted regions are very small, also the remaining vector size difference between the single and double digested forms. However, if the vector was cut with two different restriction enzymes that leave incompatible ends (this does not include two different enzymes that both leave blunt ends), then dephosphorylation may be omitted. However, it is still necessary to dephosphorylate the vector, since we cannot be certain by inspecting the digested plasmids on the gel if both enzymes cut the plasmid to completion. The presence of a small amount of singly cut plasmid vector in the subsequent ligation reaction can increase background by causing ligation of the plasmid without an insert and this decreases the quality of the library. In pUE2 the restriction sites for EcoRI and XhoI is present as shown in Figure 2.6. When both enzymes digest the plasmid, approximately a 28 bp insert should be separated from the plasmid. So, it is necessary to dephosphorylate plasmid prior to ligation.

Before dephosphorylation procedure, the picomoles of end of linear double-stranded plasmid was calculated according to formula below, in order to determine the amount of enzyme will be used. (0.01 u enzyme is used for 1 pmol ends.)

$$(\mu\text{g plasmid DNA} / \text{kb size of plasmid DNA}) \times 3.04 = \text{pmol of ends}$$

45 μL of plasmid with the concentration 35 ng/ μL (1.575 μg) was used.

$$(1.575 \mu\text{g plasmid DNA} / 5.36) \times 3.04 = 0.893 \text{ pmol of ends} = \sim 0.9 \text{ pmol of ends.}$$

So, nearly 0.01 u enzyme was used.

Plasmid digested with *HindIII*

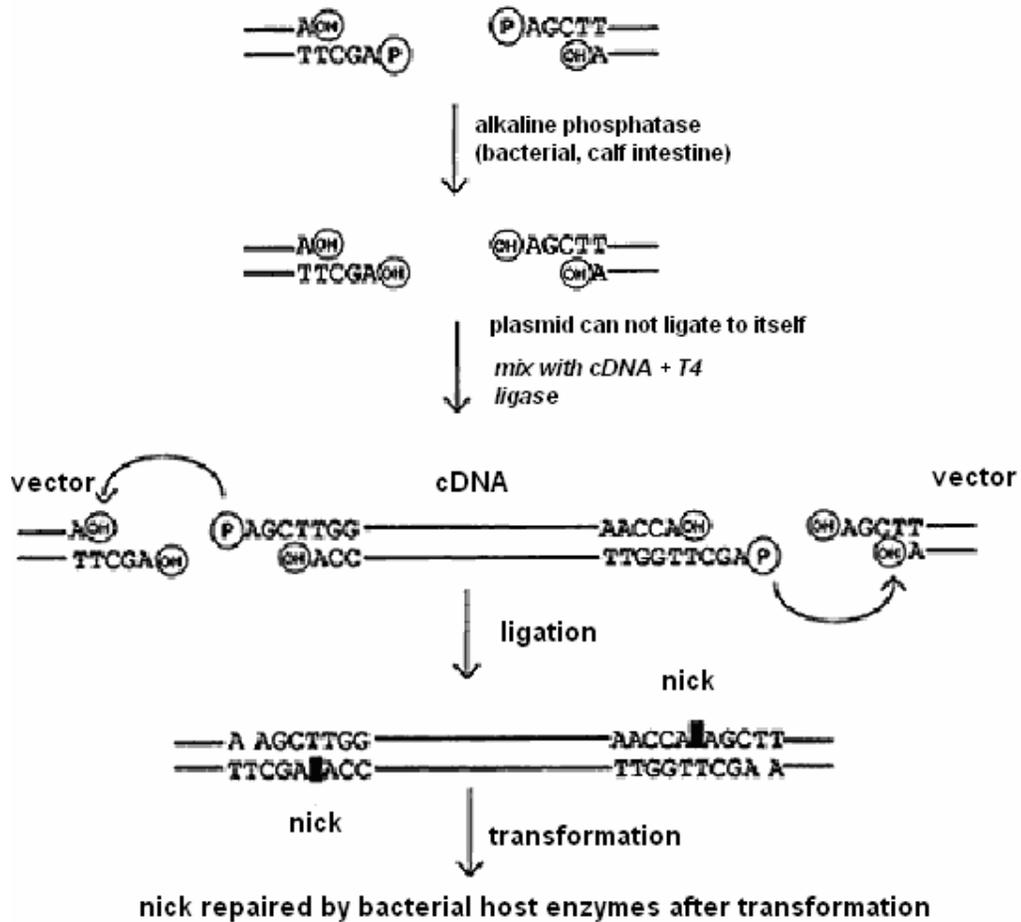


Figure 2.5 Schematic representation of dephosphorylation. Phosphatase treatment eliminated phosphate groups at 5' ends and prevents self-ligation of plasmid without an insert.

Concentrator Kit (Zymo Research) according to the procedure provided by the manufacturer. 2 volumes of DNA binding buffer was added to mix and the mixture were loaded to the column then centrifuged at maximum speed for 5-10 seconds, the filtrate was discarded and 200 μL wash buffer was added and centrifuged at max speed for 5-10 second, filtrate was discarded and centrifugation was repeated once more. The column was transferred to a new eppendorf tube and DNA was eluted with 20 μL dH_2O then a second elution was done with 10 μL dH_2O . The concentrations of the elutions were measured on NanoDrop ND-1000 UV Spectrophotometer at 260nm.

2.8 Construction and characterization of cDNA library

2.8.1 Ligation of plasmid pUE2 with cDNA

In ligation reaction vector:insert DNA molar ratio 1:3 works well and the following formula helps to determine the amounts of insert and vector accordingly.

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng insert}$$

210 ng of vector was used and average insert size was taken 2 kb and the amount of insert was calculated according to the formula above;

$$\frac{210 \times 2}{5.36} \times \frac{3}{1} = \sim 235 \text{ ng insert}$$

1 μL of cDNA ($\sim 290\text{ng}/\mu\text{L}$) and 7 μL vector (30 $\text{ng}/\mu\text{L}$), 10 μL Ligase Buffer (2X), 0.5 μL Ligase (1u) was added and the volume was completed to 20 μL with dH_2O and incubated at 4°C overnight.

2.8.2 Transformation of the ligation product into *E.coli*

Transformation was done according to the procedure in Section 2.7.4. 10 μL ligation product was used to transform 300 μL competent cells. Transformation product (1 μL) was diluted to 500 μL with SOC and 100 μL of this solution was plated onto ampicillin containing (80 $\mu\text{g}/\mu\text{L}$) agar plates, grown overnight at 37 $^{\circ}\text{C}$. Then the colonies were counted.

2.8.3 Determining the cDNA library titer

The titer of cDNA was calculated according to the formula ;

$$\text{cfu} / \text{mL} = \frac{\text{Colonies on plate} \times \text{dilution factor}}{\text{volume plated (mL)}}$$

$$\text{cfu} / \text{mL} = \frac{1600 \times 500}{0.1} = 8.10^6 \text{ cfu} / \text{mL}$$

2.8.4 Determining transformation efficiency

In ligation reaction ~290 ng plasmid DNA was used. Total volume of ligation reaction was 20 μL so the concentration of DNA become 14.5 ng/ μL . I used 10 μL of this ligation reaction in transformation which means 145 ng DNA was used. 0.145 μg DNA yield 8.10^6 colonies so, 1 μg DNA yield was $0.55 \cdot 10^8$ cfu / μg (Transformation efficiencies around 1.10^8 cfu / μg is good for cDNA libraries.)

2.8.5 Amplification of the library

cDNA library must be amplified in order to obtain enough plasmid DNA for yeast transformation. Growing transformants on solid medium instead of liquid culture minimizes uneven amplification of the various clones.

Before starting, large LB- agar plates containing 80 µg/µL ampicillin were prepared. 3 µL of transformed cells were diluted to 100 µL with SOC medium and plated onto plates. All of the transformed cells were diluted in this way and plated onto 310 large plates (R=14 cm). The cells were grown overnight at 37 °C then transferred to 4 °C.

2.8.6 Collection of the library from plates

All the plates were inspected if there was a visible contamination of mold or other contaminants. If there is contamination the contaminants must be excised using a sterile razor blade prior to beginning harvest of library transformants. 2 mL of LB was poured on to the plates and shaken with the help of glass beads until all colonies were re-suspended (1-2 min). Using a sterile pipette, all of the colonies were collected into a falcon tubes.

2.8.7 Plasmid isolation

Plasmids of the cells were isolated by using Qiagen Plasmid Isolation Kit as described in Section 2.7.5.

2.8.8 Qualifying cDNA library

It is important to qualify the library to determine its success. To do so, 10 µL of the plasmid was double digested with the EcoRI and XhoI just like in the Section 2.7.6 and then separated on 1% agarose gel to see the inserts. The quality of the library was quite good.

2.9 Complementation analysis

2.9.1 Preparation of yeast growth media

YPD is rich medium used to grow yeast cells. It is prepared by dissolving 10 g yeast extract, 20 g peptone and 20 g glucose in 1 L dH₂O and autoclaved at 121 ° C for 15 minutes. To prepare solid medium 20 g agar was added to the medium.

Synthetic dextrose medium minimal medium (SD) contains 6.7 g yeast nitrogen base without amino acids, 20 g glucose, 20 g agar - for solid media - dissolved in 1 L dH₂O and autoclaved at 121° C for 15 minutes. To prepare selective medium for selection of transformants, various growth supplements have been added to SD medium with the amount indicated on the table 2.1 (Rose *et al.*,1990).

2.9.2 Growing of *S. cerevisiae* strains

The yeast strains ZHY3 (*MAT α ade6 can1 his3 leu2 trp1ura3 zrt1::LEU2 zrt2::HIS3*) and its parent strain DY1457 (*MAT α ade6 can1 his3 leu2 trp1ura3*) was provided by Prof. David Eide form University of Wisconsin, Nutritional Sciences. ZHY3 is a special mutant strain that can not grow under zinc deplete conditions while DY1457 can grow both in zinc deplete and replete growth medium. To amplify both of them rich media were used. They were inoculated into YPD medium either solid or liquid media and incubated overnight in incubater or shaker at 30 ° C , respectively.

2.9.3 Transformation of yeast cells with pUE2

A method with high efficiency is needed to transform library into yeast cells. The following procedure uses lithium acetate (LiOAc) to increase efficiency. A single yeast colony was picked and inoculated into 10 mL YPD and grown overnighth at 30 °C in the shaker at top speed. Next day, A₆₀₀ was measured and appropriate dilution was made to make final A₆₀₀ is 0.2 into 50 ml of media (in a sterile 250 ml) and incubated at 30°C,

by shaking at 200 rpm, until $A_{600} = 1.0$ (5-7 hours). The culture was transferred (using sterile technique) to sterile 15 mL falcon tubes, and spun in clinical centrifuge at 3000 rpm, for 2 min. The media was dumped off carefully and 10 ml sterile water was added. The cells were resuspended gently and pelleted by centrifugation at 3000 rpm for 2 min. The water was poured off and 5 mL of 1 X LiOAc/TE solution [made freshly from 10 X filter sterile stocks; 10X TE (0.1 M Tris-HCl, 0.01 M EDTA, pH 7.5) and 10X LiOAc (1M LiOAc)] was added to resuspend the cells and pelleted at 3000 rpm, 2 minutes. Then LiOAc/TE was poured off and 0.25 ml of LiOAc/TE solution was added.

Cells were resuspended again gently and for each transformation 50 μ L aliquot of cells was prepared to sterile eppendorf tubes. Also there was a control tube that will get no DNA. 1 μ g plasmid DNA, 30 μ g single-stranded carrier DNA (Salmon Sperm DNA, Applichem) and DMSO to 10% (~ 6 μ L)/50 μ L of cells was added and mixed gently. 300 μ L 40% PEG (40% PEG 4000, 1X TE, 1X LiAc, made freshly from sterile 50% PEG stock, and sterile 10 X TE and 10 X LiAc) was added and mixed by inverting two or three times. In the presence of PEG yeast cells are more fragile and shouldn't vortex. Then incubated at 30^oC for 30 minutes. After this step it was incubated at 42^oC for exactly 15 min. Lastly, 200-300 μ L bulk of the cells were plated onto plates containing selective minimal medium and grown at 30^oC for 2-3 days.

2.9.4 Growing transformants on selective medium

Selective medium was prepared by adding all of the components except URA in Table 2.1 into SD as plasmid containing cells can produce uracil, so -URA plates were used to select transformants. The other selection is done to determine zinc efficient clones. To do so, low-zinc medium containing 50 μ M ZnSO₄ and high-Zn medium containing 1 mM ZnSO₄ was used. Both medium contain 1 mM EDTA to provide buffering for the concentration of free metal ions. ZHY3 can not grow on low-Zn medium unless it contains a gene to restore zinc transport activity.

Table 2.1 Growth suppliments added to SD medium and their concentration

Constituent	Final concentration in the medium (mg/liter)
Adenine	20
Uracil	20
L- Tryptophan	20
L- Histidine	20
L- Arginine	20
L- Methionine	20
L- Tyrosine	30
L- Leucine	100
L- Isoleucine	30
L- Lysine	30
L- Phenylalanine	50
L- Glutamis acid	100
L- Aspartic acid	100
L- Valine	150
L- Threonin	200
L- Serine	400
Casamino acids	1000

2.9.5 Isolation of plasmids from yeast

The plasmids of the obtained colonies were isolated by using USB Yeast Plasmid Isolation Kit according to the manufacturer's procedure. The colonies were grown in 3mL liquid drop-out medium, to maintain selection for the plasmid, at 30 °C overnight in the shaker. The cells were transferred to 2 mL microcentrifuge tubes and centrifuged at 7,500 rpm for 3 minutes, supernatant was poured off and the cells were washed with 1 mL ddH₂O (re-suspended and centrifuged at 7,500 rpm for 2–3 minutes, supernatant was removed). Cells were resuspended in 100 µL Spheroplast Buffer, 4 µl Enzyme Solution was added and incubated at 37°C for 1/2 to 2 hours. Mixture was centrifuged at 7.500 rpm for 4 minutes and supernatant was removed very carefully. Lysis Solution (150 µL) was added to the spheroplast pellet but was not re-suspended. Using the tip pellet was grasped and transferred to a fresh 0.5 mL microcentrifuge tube. The remaining lysis buffer was also transferred and pellet was re-suspended. The mixture was incubated at 95°C for 10 minutes and chilled on ice. Precipitation Solution (50 µL) was added and mixed gently by inverting the tube 3–5 times. A white fluffy precipitated and incubated on ice for 10–20 minutes. Tubes were centrifuged at 7,500 rpm for 4 minutes and supernatant was transferred to a fresh 0.5 mL microcentrifuge tube. Pellet was discarded and the supernatant was centrifuged at 14,000 rpm for 2 minutes to remove residual precipitate. To the supernatant, an equal volume of chilled 100% isopropanol was added, mixed by inversion and incubated at -80 °C for at least 1 hour. Tubes were centrifuged at 14,000 rpm for 20 minutes at 4°C and supernatant was removed. The pellet was washed once with an equal volume of 70% ethanol, centrifuged at 14,000 rpm for 5 minutes, dried and re-suspended in 13 µL of ddH₂O.

2.9.6 Re-transformation of isolated plasmid into *E.coli*

Transformation was done according to procedure in Section 2.7.4 and then one transformed colony was picked from each of the plates and were grown overnight (in shaker at 37 °C) in LB medium, containing ampicilin.

2.9.7 Isolation of plasmids

Plasmids were isolated as in the section 2.7.5.

2.9.8 Visualization of the plasmid

Concentrations of the plasmids were measured by using Nano-drop UV spectrophotometere and they were loaded into 1% agarose gel as described in the section 2.5.3.

2.9.9 Double digestion of the plasmid with EcoRI and XhoI

Isolated plasmids were digested as in Section 2.7.6. Then visualized on 1 % agarose gel.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Collection of plant samples

The plant samples were collected at 20th day then 25th day, 30th day, 35th day from both grown in zinc (-) and zinc (+) mediums. Some of the plants that were grown in zinc (-) medium showed distinct Zn deficiency symptoms like; longer root length compared to normal one - nearly 25 % longer- (Figure 3.1). In all of the zinc deficient plants discoloration and necrosis of the older leaves were apparent around 20th day (Figure 3.2-A, B, C).

3.2 Total RNA isolations

Total RNA from the roots and leaves of plant samples were isolated as described in Section 2.4.2. They were loaded into 1% RNA gel to check their integrity (Figure 3.3). RNA samples were intact and they seem very bright as they are too concentrated. Their concentrations are listed on Table 3.1.

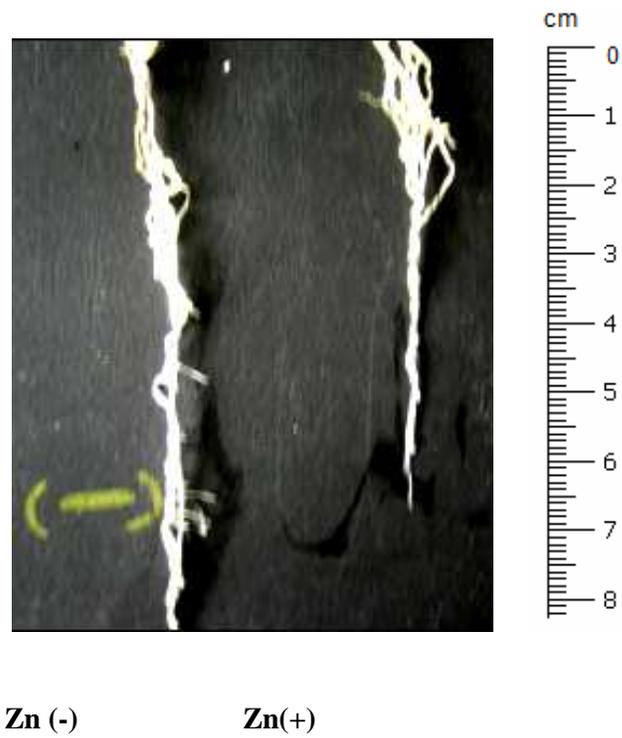


Figure 3.1 An example of comparison of the length of roots of barley grown in Zn(-) and Zn(+) mediums.



A



B



C

Figure 3.2 A, B, C Discoloration and necrosis of the older leaves of barley grown in Zn(-) medium (25 days old).

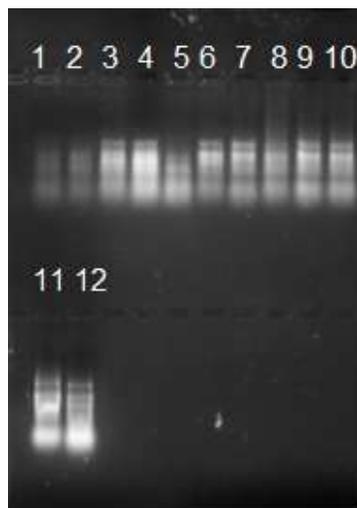


Figure 3.3 Appearances of total RNA isolates from barley grown in zinc deficient medium, by using trizol method. **Lane 1, 2:** Root samples from 20 days old plant, **Lane 3, 4:** Leaf samples of 20 days old plant, **Lane 5:** Root sample of 25 days old plant, **Lane 6:** Leaf sample of 25 days old plant, **Lane 7:** Root sample of 30 days old plant, **Lane 8:** Leaf sample of 30 days old plant, **Lane 9, 10:** Root samples of 35 days old plant, **Lane 11, 12:** Leaf samples of 35 days old plants.

The appearances of the RNA samples on the gel, integrity of the bands and the concentration values were satisfactory. Then, all of the samples were mixed and concentration of the total RNA mixture was measured 996 ng/ μL in 100 μL .

Table 3.1 Concentrations of total RNA samples isolated from barley. Numbers refer to the samples as indicated in Figure 3.3

Sample	Concentration (ng/ μ L)
1	876 ng/ μ L
2	979 ng/ μ L
3	1003 ng/ μ L
4	1140 ng/ μ L
5	1066 ng/ μ L
6	2021 ng/ μ L
7	775 ng/ μ L
8	955 ng/ μ L
9	1572 ng/ μ L
10	1857 ng/ μ L
11	1168 ng/ μ L
12	1221 ng/ μ L

3.3 mRNA isolations

For the cDNA synthesis reaction, nearly 5 μ g poly(A) RNA is needed. In order to obtain this amount, mRNA isolation was done several times from the total RNA. It was a critical step since, we wanted a cDNA to represent all the messages. Five different sets of isolations were performed, either 5 μ L Tris-HCl (10mM) or PCR grade H₂O was used for the elution of mRNA from Dynabeads Oligo (dT)₂₅. Because we wanted to increase the efficiency of elution. Their concentrations were measured and the results are listed below. The ones indicated in bold were used for cDNA synthesis reaction as their concentrations were higher compared to other samples.

- First mRNA isolation; **1. elution, concentration = 104 ng/ μ l**
2. elution, concentration = 58 ng/ μ l
- Second mRNA isolation; **1. elution, concentration = 53 ng/ μ l**
2. elution, concentration = 42 ng/ μ l
- Third mRNA isolation; **1. elution, concentration = 73 ng/ μ l**
2. elution, concentration = 52 ng/ μ l
- Fourth mRNA isolation; **1. elution, concentration = 596 ng/ μ l**
2. elution, concentration = 155 ng/ μ l
3. elution, concentration = 55 ng/ μ l
4. elution, concentration = 28 ng/ μ l
- Fifth mRNA isolation; **1. elution, concentration = 99 ng/ μ l**
2. elution, concentration = 61 ng/ μ l
3. elution, concentration = 18 ng/ μ l
4. elution, concentration = 12 ng/ μ l

PCR grade ddH₂O was used in the elutions of samples performed in the fourth and fifth mRNA isolations and it was much more efficient compare to 10 mM Tris-HCl solution.

Finally, the mRNA samples written in bold were mixed together and concentration was measured and found as 5,02 μ g in nearly 36 μ L (as each of the elutions was \sim 4 μ L). This amount is enough to start cDNA synthesis reaction.

3.4 Visualization of cDNA

cDNA was synthesized using ZAP-cDNA Synthesis Kit and it was directly loaded onto 1% agarose gel to assess the longest fragments of reverse transcribed messages by comparing with molecular weight marker (Figure 3.4).

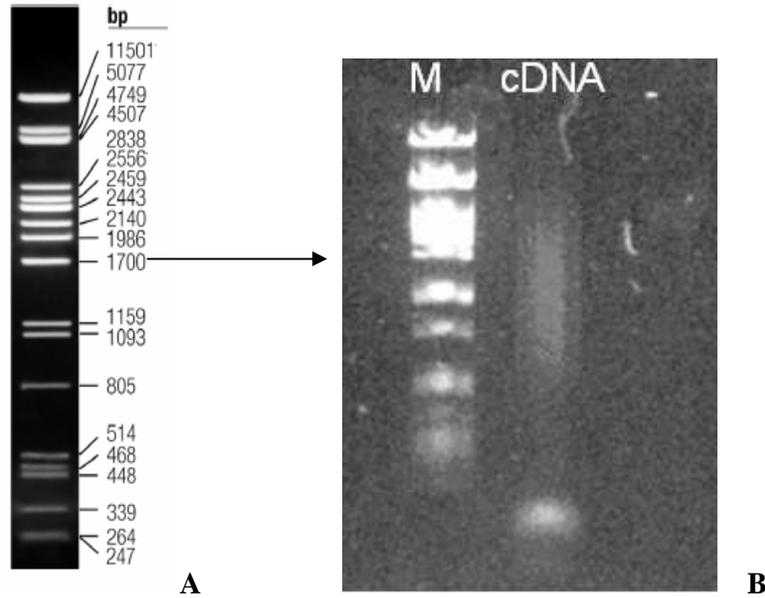


Figure 3.4 The length of synthesized cDNA. **A.** Appearance of λ / PstI marker, **B.** 4 80 volts for 60 minutes.

3.5 Control of cDNA via PCR with actin primers

Actin gene is one of the abundant and constitutively expressed gene in wheat, based on our routine analysis with differentially treated samples. So, by performing PCR reaction with actin gene specific primers (sequences are provided in the section 2.6.4) we can control cDNA. The product of the reaction was loaded onto the agarose gel. The size of the product expected to appear in between the band of 400-500bp (target size is 450 bp). The figure 3.5 shows the successful expression of actin fragment.

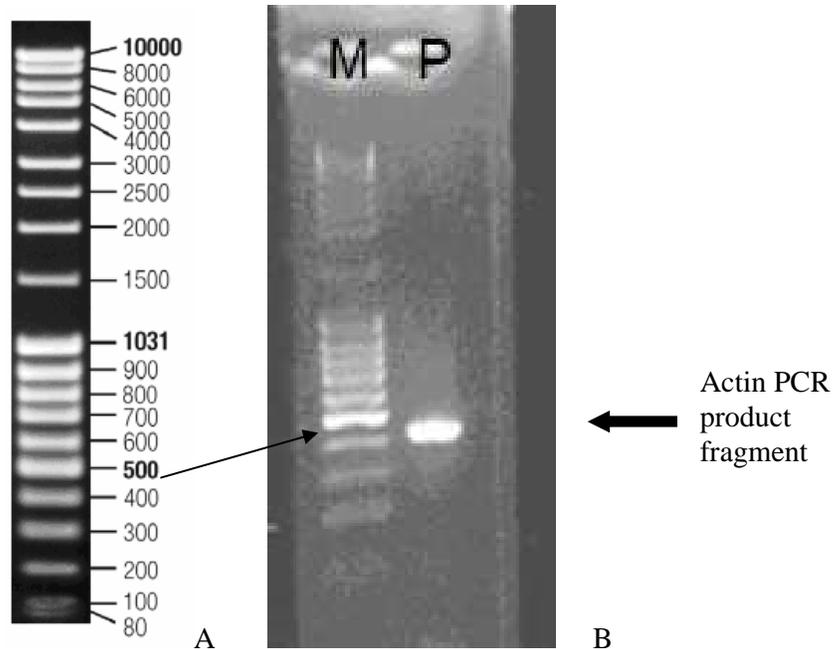


Figure 3.5 Actin PCR result. **A.** DNA Mass Ruler, **B.** Actin PCR of cDNA. M; 4 μ L Marker (MassRuler) P; PCR product 40 μ L with 5 μ L dye was loaded into % 2 agarose gel.

3.6 Size fractionation of cDNA

Size selection of cDNA was performed to eliminate cDNA fragments smaller than 400 bp as we wanted to obtain full length cDNA as much as possible. The longer cDNA fragments were selected on CL-2B sepharose column. cDNA was eluted four times with PCR grade ddH₂O.

The concentrations of eluted four cDNA samples were measured and found to be 16 ng/ μ L, 630 ng/ μ L, 519 ng/ μ L, 390 ng/ μ L in 4 sequential cDNA elutes. Then all of the eluted material was mixed and, concentration was measured again resulting 292 ng/ μ L (volume 120 μ L).

3.7 Isolation of plasmid pUE2

pUE2 vector was prepared to be used to construct the cDNA library. The first step in preparation was isolation of plasmid from *E.coli* cells transformed with this plasmid. Isolation was done according to the procedure in section 2.7.5. at the end of the procedure there were 4 different microfuge tubes containing different amounts of plasmid. Their concentrations were measured; **1-** 95 ng / μL , **2-** 396 ng / μL , **3-** 400 ng / μL , **4-** 169 ng / μL . Among them, the third one was selected for the next steps as it contains more plasmid compare to others.

3.8 Linearization of Plasmid pUE2 with *EcoRI* and *XhoI*

For ligation, the plasmid pUE2 was linearized with the enzymes *EcoRI* and *XhoI* as the cDNA fragments have *EcoRI* and *XhoI* adapters on the 5' to 3' directions, respectively. Digestion reaction was performed as described in Section 2.7.6 then cut and uncut plasmids were loaded onto gel to compare them and to be sure about if the digestion reaction worked properly (Figure 3.6).

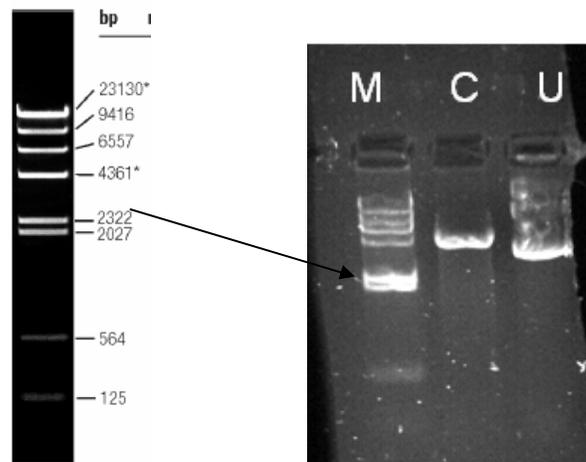


Figure 3.6 Vector preparation **C:** Cut plasmid 3 μL (1 μL cut plasmid ,1 μL of dH_2O and 1 μL of dye), **U:** Uncut plasmid 3 μL (1 μL uncut plasmid ,1 μL of dH_2O and 1 μL of dye), **M:** Marker λ / *HindIII* 5 μL were loaded on 0.8 % agarose gel.

The plasmid was cut well so all of them was loaded onto the agarose gel to separate the cut plasmid from the insert between cut regions (Figure 3.7). The size of the removed multiple cloning site is about 30 bp which was not expected to be observed on the gel.

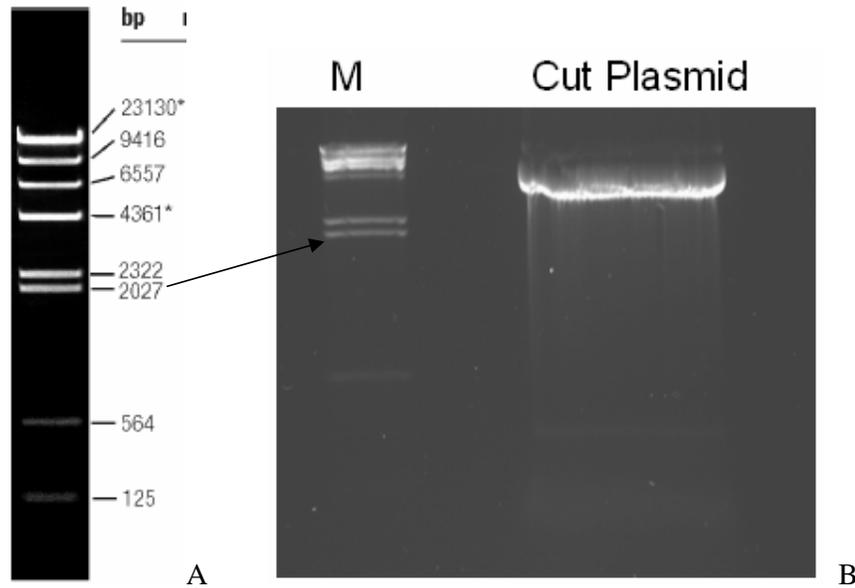


Figure 3.7 Appearance of cut plasmid on gel. **A**- λ /HindIII marker, **B**-Appearance of cut plasmid on 0.8 % agarose gel. 20 μ L digested plasmid + 5 μ L dye was loaded into second well, M is λ /HindIII marker.

3.9 Determining the titer cDNA library

The titer of cDNA was calculated according to the formula below and the number of colonies were ~1600 colonies

$$\text{cfu / mL} = \frac{\text{Colonies on plate} \times \text{dilution factor}}{\text{volume plated (mL)}}$$

$$\text{cfu/ mL} = \frac{1600 \times 500}{0.1} = 8.10^6 \text{ cfu / mL}$$

3.10 Determining transformation efficiency

In ligation reaction ~290 ng plasmid DNA was used. Total volume of ligation reaction was 20 μ L so the concentration of DNA become 14.5 ng/ μ L. I used 10 μ L of this ligation reaction in transformation which means 145 ng DNA was used. 0.145 μ g DNA yield 8.10^6 colonies so, 1 μ g DNA yield was $0.55 \cdot 10^8$ cfu / μ g. (Transformation efficiencies around 1.10^8 cfu / μ g is good for cDNA libraries.)

3.11 Qualifying the cDNA Library

It is important to qualify the library to determine if it is representative or not. After colonies were collected from plates, their plasmids were isolated and concentration was found to be 103 ng/ μ L, 10 μ L of it was double digested with the *Eco*RI and *Xho*I as in section 2.7.6. The smear on the figure 3.8 of the lane next to the marker indicates the high quality of the library.

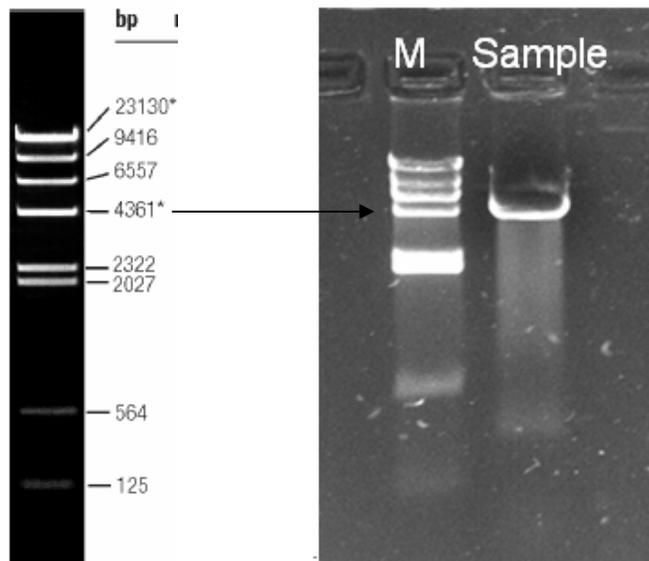


Figure 3.8. Appearance of digested plasmid containing cDNA fragments, on 1% agarose gel. **M:** marker λ / HindIII 5 μ L was loaded, **Sample:** 20 μ L digestion product + 5 μ L dye was loaded.

3.12 Yeast cultivation

S. cerevisiae strains ZHY3 (*MAT α ade6 can1 his3 leu2 trp1ura3 zrt1::LEU2 zrt2::HIS3*) and its parent strain DY1457 (*MAT α ade6 can1 his3 leu2 trp1ura3*) was grown on rich medium for amplification. However, to see the differences in their growth, SD plates containing different amounts of zinc (2mM, 650μM, 50μM ZnSO₄ all contain 1mM EDTA) were used. In the plate containing 2mM ZnSO₄, both strains grew well (Figure 3.9-A) as its zinc concentration is normal for growth of both strains. In the plate containing 650μM ZnSO₄, DY1457 grew well but ZHY3's growth was detectably regressed (Figure 3.9-B). In this plate, zinc concentration started to be limiting for ZHY3 but it is still enough for growth. The plate containing 50μM ZnSO₄, DY1457 shows the same limit of growth in that with 2 mM ZnSO₄ plates (as it has the high and low affinity zinc transporters). The mechanism works as; firstly, low affinity zinc transporter (ZRT1) is activated to supply zinc and when the zinc limitation became severe, high affinity zinc transporter (ZRT2) activated to provide more zinc. However, both of the zinc transporters of ZHY3 are mutated and can not function properly so, it can not provide zinc from the medium when zinc concentration is very low. This makes it very sensitive to zinc limitation and it can not grow efficiently on zinc-limiting medium (Grotz et al., 1998). Only inoculated cells had survived until the 100mM zinc stored in their vacuole (Simm *et al.*, 2007) are used up. This regressed growth of ZHY3 is obvious in Figure 3.9-C.

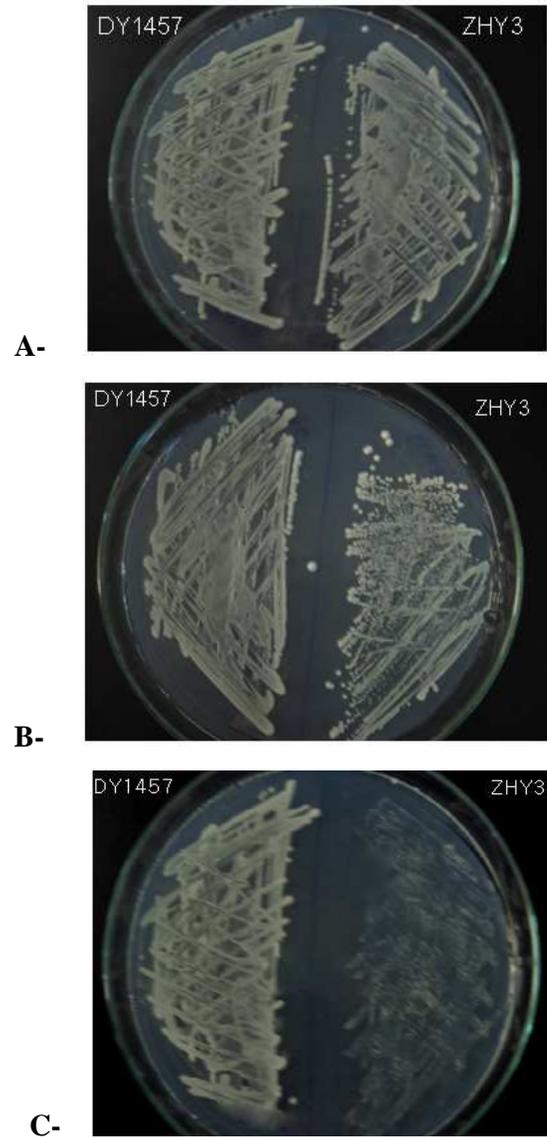


Figure 3.9 2 days growth of ZHY3 (zinc deficient double mutant) and DY1457 (ZHY3's parent strain) in medium with different zinc concentrations. Growth of DY1457 and ZHY3 on SD medium, **A**-containing 2mM ZnSO₄ (sufficient zinc concentration), **B**- 650µM ZnSO₄ (allowable concentration for ZHY3 mutant, **C**- 50µM ZnSO₄

3.13 Results of the transformation

ZHY3 cells transformed with the library were spreaded onto 90 large plates (R= 14cm) containing minimal medium with 1mM EDTA, 50 μ M ZnSO₄ and lacking uracil (-URA). ZHY3 has mutated uracil synthesizing gene so, its growth on uracil (-) medium is dependent on the presence of the vector pUE2 containing this gene. To prove the plasmid dependence of cells for growth, ZHY3 cells were directly spreaded onto minimal medium lacking uracil, without transformng them with pUE2 (the medium contains 2 mM ZnSO₄ and 1mM EDTA which is adequate for efficient growth of ZHY3). As expected, ZHY3 cells couldn't grow on this medium (Figure 3.10) showing that transformation with pUE2 is vital for them to grow on uracil lacking medium under normal zinc concentration.

To control effectiveness of transformation and calculate its efficiency transformed ZHY3 cells were spreaded onto a plate containing minimal medium (without uracil) with 2mM ZnSO₄ and 1mM EDTA. This is a selective medium for the transformants containing plasmid so, they can effectively grow on this uracil lacking, zinc sufficient medium (Figure 3.11). There were nearly 2500 colonies on the plate. By extrapolation, the transformation efficiency is nearly 225.000 as there were 90 plates. In the previous studies, Grotz *et al.*, 1998 and Pence *et al.*, 2000 found 270.000 and 350.000 respectively. The number of colonies is less than these numbers but it was still adequate to proceed further.

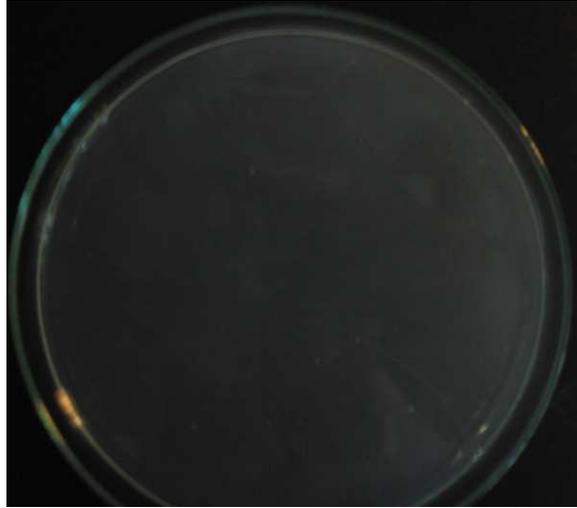


Figure 3.10 Control, untransformed ZHY3 cells were spreaded onto -URA minimal medium (2mM ZnSO₄ and 1mM EDTA), no growth was detected.



Figure 3.11 ZHY3 cells that were transformed with pUE2 and so can grow on -URA minimal medium (2mM ZnSO₄ and 1mM EDTA)

3.14 Screening of the library

The transformed ZHY3 cells were grown on minimal medium (-URA) containing 50 μ M ZnSO₄ and 1mM EDTA as a chelator. They were grown 2 days at 30 °C. It was indicated that if the cells were kept at 30 °C a long time, they started to grow but the mechanism behind this is unclear (Prof. David Eide's personal statement). So, the plates were screened immediately for growth of yeasts. Actually, we investigated the presence of colonies that grew drastically better than the background cells. A slight growth was observed on some plates but this was normal as cells can sustain for some period by using zinc inside the vacuole for survival. We have found 2 large colonies on separate plates among the 90 plates (Figure 3.12 A-B). They are considered containing candidate zinc efficiency genes for further evaluation. The plasmids were isolated from these 2 colonies for sequencing. For which, the colonies were amplified in liquid minimal medium and some of the yeast cells were inoculated onto plates containing zinc limiting (50 μ M ZnSO₄) minimal medium and their growth were observed (Figure 3.12 C-D). The plasmids of the cells in liquid medium were isolated and re-transformed again into *E.coli*. The transformed cells were grown in LB medium containing ampicillin and then their plasmids were isolated (concentrations were measured as 366 ng/ μ L and 389 ng/ μ L) and visualized on 1% agarose gel (Figure 3.13). The smear seen on gel, on top of the plasmid, was probably coming from genomic DNA.

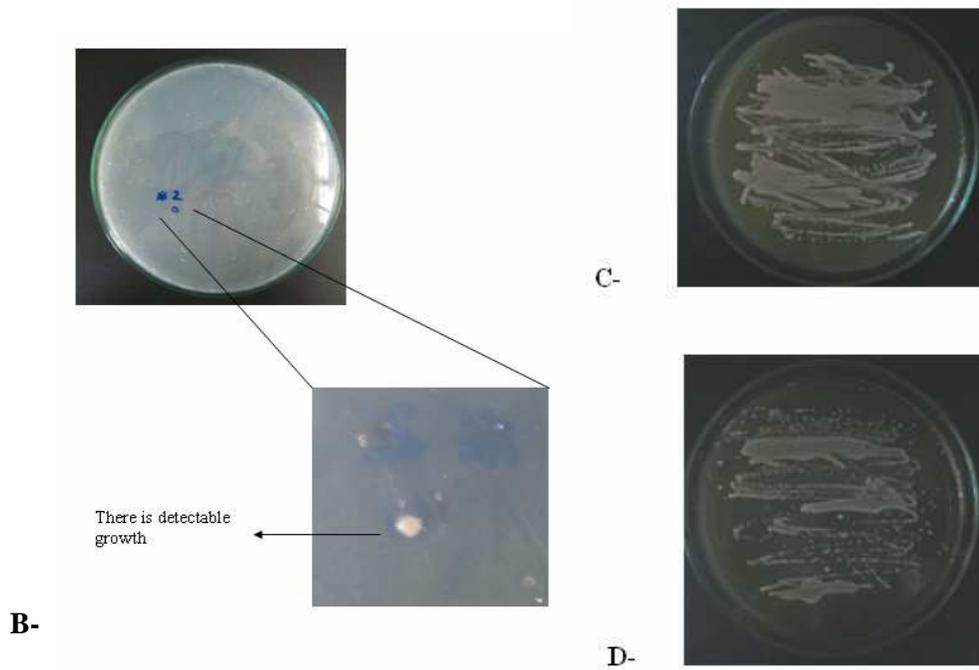
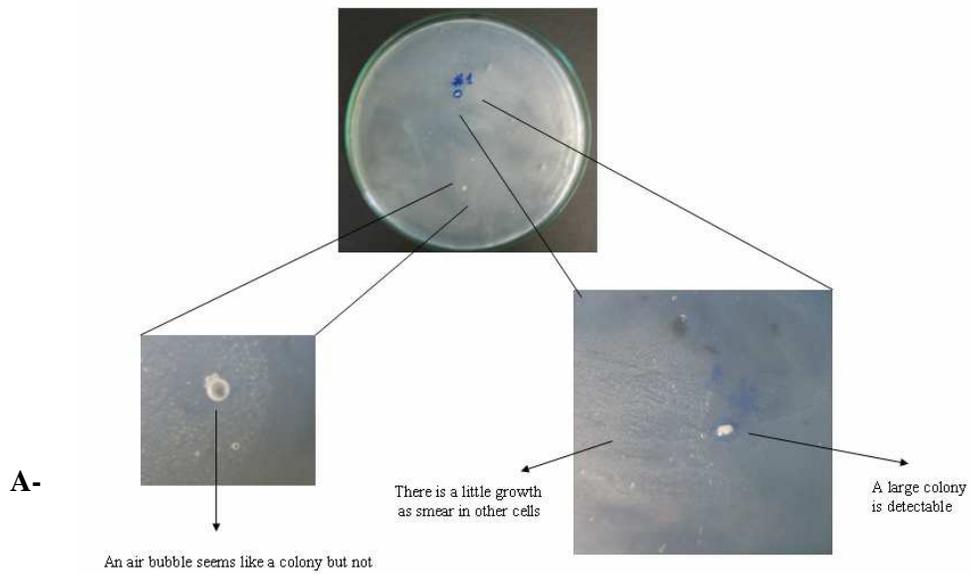


Figure 3.12 Screening of the library **A-** One colony obtained which can grow on zinc-deficient minimal medium, marked as #1, **B-** The other colony obtained which can grow on zinc-deficient minimal medium, marked as # 2, **C,D-** Growth of the colonies #1 and #2, respectively, on zinc limiting minimal medium inoculated after amplified in liquid medium.

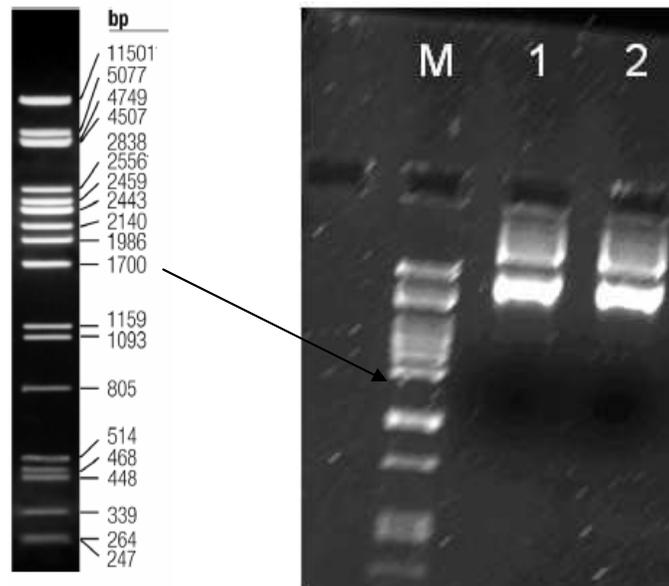


Figure 3.13 Isolated plasmid DNA from putatively positive clones that were amplified in *E.coli*. **M:** λ PstI marker, **Lane 1:** contains the plasmid isolated from colony #1, **Lane 2:** contains the plasmid isolated from colony #2

3.15 Sequence analysis

Sequence analysis of the plasmids were performed by Ref-Gen with the primers specifically designed for the vector pUE2 (sequences are provided in Appendix B). Results of the sequence analysis are listed in Appendix C. Unfortunately, it seems that the plasmids don't contain inserts. To be sure about that there is no gene on the vectors, they were digested with *EcoRI* and *XhoI* as describe in the section 2.7.6 and loaded onto agarose gel (Figure 3.14). However, we couldn't see any insert on the gel, only the plasmids were present meaning that our results were false positive.

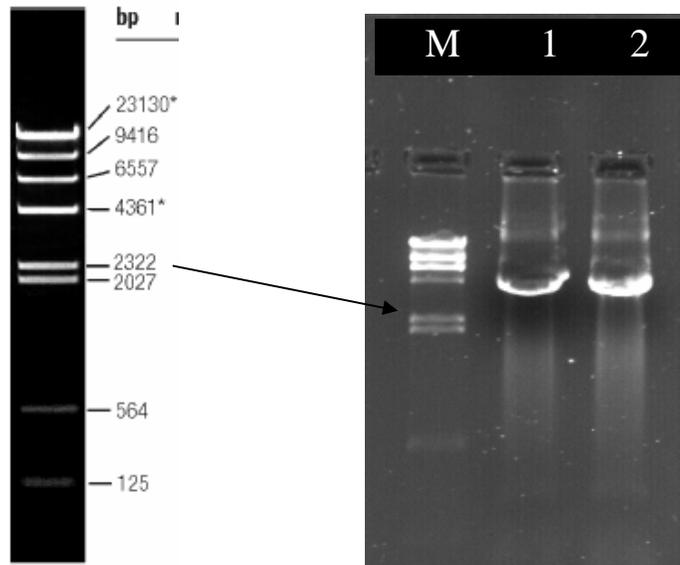


Figure 3.14 Appearance of digested putative recombinant plasmid on 1% agarose gel. **M:** λ HindIII marker, **Lane 1:** cut plasmids isolated from colony #1, **Lane 2:** cut plasmids isolated from colony #2

Growing of the two colonies may be explained as; there could be a back mutation in yeast that one of ZRT1 or ZRT2 (or both of them) genes might have regained its function again or the activity of the third transported which was mentioned in Section 1.6 (which is uncharacterized yet) may be increased so that yeast can obtain necessary amount of zinc for growth. Another possibility is, the amount of zinc stored in vacuole was high so that it can use this stock. More specifically, the activity of genes ZRC1 or COT1, that transport zinc into vacuole, may be increased so, more zinc accumulation occur in vacuole and the cell used the zinc in vacuole to sustain. Alternatively, activity of ZAP1 gene was increased, and as its product is the activator of transcription of ZRC1 and COT1, they were expressed much more and stored more zinc in vacuole. Another possibility is that, although the amount is low, zinc can be accumulated in mitochondria (regulated independently of vacuolar zinc storage) (Simm *et al.*, 2007) and the cells may be using zinc in it.

CHAPTER IV

CONCLUSION

Low levels of zinc in plants is responsible for the high incidence of zinc deficiency in humans, especially in developing countries. World's growing population is affected by zinc deficiency in two ways; decreased grain yield cause less food production to feed the population and low levels of zinc in plants used as food source, cause zinc deficiency in humans which can lead to severe health problems.

In this study zinc efficient Tokak-157 plants were used to construct cDNA library. We used both the roots and leaves of the plant grown in zinc deficient hydroponic medium. The reason for this is, we don't know exactly where the putative zinc efficiency genes are located.

Our aim was to construct cDNA library, which is not very easy and extensive care should be taken to obtain an intact, representative library to screen *via* complementation analysis for finding genes putatively involved in efficiency mechanism when grown in the extremely low zinc medium. However, it is not an easy process as you do screening in living organisms, there may be mutations, inactivation of genes that can effect screening process so, false positive results raise.

We were investigating putative zinc transporter genes that provide zinc efficiency in barley Tokak-157 cultivar. However, the clones we have obtained didn't contain genes, in other words they seemed to be false positive.

Although our results did not provide the necessary information about the zinc efficiency mechanism, it is the first study done on barley to investigate zinc efficiency genes by using zinc-transport complementation in mutant yeast. To investigate the genes function in zinc efficiency mechanism, other techniques may be used such as differential display, differential screening etc. But the results of them also needed to be proven by real-time PCR analysis. Also the function of candidate zinc transporter genes that were found by using other techniques can be proved by using complementation analysis.

If we would have found candidate gene/genes for zinc-efficiency mechanism, we had to check the expression levels with real-time PCR by using total RNAs isolated from plants grown in zinc (+) conditions as control.

Complementation analysis is done in our lab for the first time and the necessary information about the key points of the technique is gained that can be useful in further studies.

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APPENDIX A: Sequence of the plasmid pUE2

323 - 1146: PGK promoter, 1234 - 1487: PGK terminator, 1748 - 2551: URA3, approximately 2900 - 3400: 2 μ m replication origin, 4300 - 5160: Amp-r

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AGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACCTCTCAAGGATCTT
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CATACTCTTCCTTTTCAATATTATTGAAGCATTATCAGGGTTATTGTCTCA
TGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTC
CGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTA
TCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTC

APPENDIX C: Results of multiple sequence alignment

Multiple sequence alignments of the plasmid pUE2 with the sequences resulted from sequence analysis by using Clustal X program.

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pUE2      TGCAAGAAATAGATATTTGGTCTTTTCTAAATTCGTAGTTTTCAAGTTCTTAGATGCTTTTC 1080
f-1      -----

pUE2      TTTTTCTCTTTTTACAGATCATCAAGGAAGTAATTATCTACTTTTTACAACAAATATAA 1140
f-1      -----TATACATGATCTG--AACATG---AG 21
                *** * * * **** *

pUE2      AAACCAGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGATATCAAGC 1200
f-1      ATATTCCTCGGTCGCTCT-GTTTTAGTGGATCCCCCGGGCTGCTTGAATTCGATATCTGGC 80
                * *   *** ***** * ***** ***** **

pUE2      TTATCGATACCGTC-GACCTCGAGGGGGGGCCGGCCCATCAATTTTTT-CTTTTCTCT 1258
f-1      TTATCGATACCGCCCGACCTCGAGGGGGGGCCG---CGATCAATTTTTTCTTTTCTCT 136
                ***** * ***** * ***** *****

pUE2      TTCCCCATCCTTTACGCTAAAATAATAGTTTATTTTATTTTGAATATTTTATTTAT 1318
f-1      TTCCCCATCCTTTACGCTAAAATAATAGTTTATTTTATTTTGAATATTTTATTTAT 196
                *****

pUE2      ATACGTATATATAGACTATTATTTATCTTTTAATAGATTATTAAGATTTTTATTA 1378
f-1      ATACGTATATATAGACTATTATTTATCTTTTAAT-GATGATTAAGATTTTTATTA 255
                *****

pUE2      AAAATTCGCTCCTCTTTTTAATGCCTTTATGCAGTTTTTTTTCCCATTCGATATTTCTAT 1438
f-1      AAAATTCGCTCCTCTTTT-AATGCCTTTATGCAGTCTTTTTTCCCGTTCTATTTTCTAT 314
                *****

pUE2      GTTCGGGTTTCAGCGTATTTTAAAGTTAATAACTCGAAAATTCGCGTTCGTTAAAGCTTG 1498
f-1      GTTCGGGCTCAGCGAAAATTTAAAGTGAATAACTCGAAAATTCGCGTTCGTTAAAGTTTG 374
                *****

pUE2      GCGTAATCATGGTCATAGCTGTTTCCCTGTGTGAAATGTTATCCGCTCACAATCCACAC 1558
f-1      GAATACTCATGGCCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAAACT 433
                * ** *****

pUE2      AACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAAACT 1618
f-1      AACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGAGCCTAATGAGGGAGCTAACGC 493
                *****

pUE2      ACATTAATTGCGTTCGCGTCACTGCCCCGTTTTCCAGTCGGGAAACCTGTCGTGCCAGCAG 1678
f-1      ACATTAATTGCGTTCGAGCTCACTGCCCCGTTTTCCAGTCGGGAAACCTGTCGTGCCAGCAG 553
                *****

pUE2      ATCTGAGCTTTTTCTTTCCAATTTTTTTTTTTGTGAGTTAGTATACATGCATTTACTT 1738
f-1      ATCTG-GATTTTGCCTTTGCAATTTTATTGG--GTGAGTCTAGTATACATGCATTTACTT 610
                *****
    
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pUE2 ATAATACAGTTTTTTAGTTTTGCTGGCCGCATCTTCTCAAATATGCTTCCCAGCCTGCTT 1798
f-1 ATAATACAGTATTTGAGTTTTGCTGGCCGCATCTTCTCAAATATGCTTCCCAGCCTGCTT 670
***** ** *****

pUE2 TTCTGTAACGTTCCACCTCTACCTTAGCATCCCTTCCCTTTGCAAAATAGTCCTCTTCCAA 1858
f-1 TTCTGTAACGTTCCACCTCTACCTTAGCATCCCTTCCCTTTGCAAAATAGTCCTCT-CCTA 729
***** ***** ** *

pUE2 CAATAATAATGTCAGATCCTGTAGAGACCACATCATCCACGGTCTATACTGTTGACCCA 1918
f-1 CCATAATAATGTCAGATCCTGCAAAGACCACATCATCCACGGTCTATACTGCTGACCCA 789
* ***** * ***** *****

pUE2 ATGCG-TCTCCCTTGTATCTAAACCCACACCGGGTGCATAAT-CAACCAATCGTAACC 1976
f-1 ATGCGGTCTCCATAGTCATCTAA-CCACACCGGGCGTCATAATTCAACCCATCGTAACC 848
***** ** * ***** ***** ***** ***** *****

pUE2 TTCATCTCTCCACCCATGTCTCTTTGAGCAATAAAGCCGATAACAAAATCTTTGTGCGCT 2036
f-1 TTGCACCTCTCCACC-ATGT-TCCTTGTAGCAATACACCCGATGACAAA-TCCTTGTGCGCT 905
** ***** ** * ***** * ***** ***** *****

pUE2 CTTGCAATGTCAACAGTACCCTT-----ATCCTTTGTTACTTCTTCTGCCGCCCTGCTTC 2091
f-1 CTTCCACAAGACA-CAGTGCCCTTCTGTATATTCTCCAGAAGATAGGGAGCCCTTGCATGC 964
**** * * * * * ***** ** * * * * * * * * * * *

pUE2 AAACCGCTAACAAATACCTGGGCCACCACACCGTGTGCGTATATTCTCCAGTAGATAGGG 2151
f-1 AATTCAGCTAAAATCTAAGGGCCCA--AGTCCCTTGGTACATGTTCTGCGGCCCTGCTTCG 1022
** * * * * * ***** * * * * * ** * * * * *

pUE2 AGCCCTTGCATGACAATTCTGCTAACATCAAAAGGCCTCTAGGTATTGTAATGTCTGCC 2211
f-1 AACGTT-----ACAACCCGGCC--CACCA-----CCCGTGGGCACTCG-GATGCCCGCC 1068
* * * * * ***** * * * * * ** * * * * * * * * * * *

pUE2 CATTCTGCTATTCTGTATACACCCGAGACTGCAATTTGACTGTATTACCAATGTCA 2271
f-1 CATT--GCTATTG---ATA-ACCCATAGCACGCATTAACCTTCCAGTCCAAATCTTTTC 1122
**** ***** ** * * * * * * * * * * *

pUE2 GCAAAATTTCTGTCTTCGAAGAGTAAAAAATGTAAGTGGCGGATAATGCCCTTAGCGGC 2331
f-1 GAAAAAATG--ACTCGCGAAAGCATA---TCGTTTCAGGACCTGAAATCCGATC--CTGT 1175
* *

pUE2 TTAAGTGTGCCCTCCATGGAAAAATCAGTCAAGATATCCACATGTGTTTTTAGTAAACAA 2391
f-1 ATAAACGGCCATTCCATCGGAAC-TGGGCCACCACAGT----- 1212
*** * * * * * * * * * * * * * * * *

pUE2 ATTTTGGGACCTAATGCTTCAACTAACTCCAGTAATTCCTTGGTGTACGAACATCCAAT 2451
f-1 -----

pUE2 TGCAAGAAATAGATATTTGGTCTTTTTCTAATTCGTAGTTTTCAAGTTCCTTAGATGCTTTC 1080
F-2 -----

pUE2 TTTTTCTCTTTTTTACAGATCATCAAGGAAGTAATTATCTACTTTTTTACAACAAATATAA 1140
F-2 -----TATACATGATCTG-AACATG---AG 21
*** * * * **** *

pUE2 AAACCAGCGGCGCTCTAGAACTAGTGGATCCCCGGGCTGCAGGAATTCGATATCAAGC 1200
F-2 ATATTCTCGGTGCTCT-GTTTTAGTGGATCCCCGGGCTGCTTGAATTCGATATCTGGC 80
* * *** ***** * ***** ***** **

pUE2 TTATCGATACCGTC-GACCTCGAGGGGGGGCCGGCCGCCATCAATTTTTT-CTTTTCTCT 1258
F-2 TTATCGATACCGCCGACCTCGAGGGGGGGCCG---CGATCAATTTTTTCTTTTCTCT 136
***** * ***** * ***** *****

pUE2 TTCCCCATCCTTTACGCTAAAATAATAGTTTATTTTTATTTTTTGAATTTTTTTATTTAT 1318
F-2 TTCCCCATCCTTTACGCTAAAATAATAGTTTATTTTTATTTTTTGAATTTTTTTATTTAT 196

pUE2 ATACGTATATATAGACTATTATTTATCTTTTAATAGATTATTAAGATTTTTTATTAATAA 1378
F-2 ATACGTATATATAGACTATTATTTATCTTTTAAT-GATGATTAAGATTTTTTATTAATAA 255
***** *** *****

pUE2 AAATTCGCTCCTCTTTTTAATGCCTTTATGCAGTTTTTTTTTCCCAITTCGATATTTCTAT 1438
F-2 AAATTCGCTCCTCTTTTT-AATGCCTTTATGCAGTCTTTTTTTCCCGTTCATTTTTCTAT 314
***** ***** ***** ** *****

pUE2 GTTCGGGTTTCAGCGTATTTTAAAGTTTAAATAACTCGAAAATTCTGCGTTCGTTAAAGCTTG 1498
F-2 GTTCGGGCTCAGCGAAATTTAAAGTGTAAATAACTCGAAAATTCTGCGTTCGTTAAAGTTTG 374
***** ***** * ***** ***** *****

pUE2 GCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATCCACAC 1558
F-2 GAATACTCATGGCCATAACTGTTGCCTGAGTGAAGTTGTTATCCGCTCCCAATCCA-AC 433
* ** ***** ** * ** * ** * ** * ** * ** * ** * ** * ** *

pUE2 AACATACGAGCCGGAAGCATAAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTC 1618
F-2 AACATACGAGCCGGAAGCATAAAAGTGTAAAGCCTGGGGAGCCTAATGAGGGAGCTAACGC 493
***** ***** ***** *

pUE2 ACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCAG 1678
F-2 ACATTAATTGCGTTGAGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCAG 553
***** ***** ***** ***** ***** ** *****

pUE2 ATCTGAGCTTTTTCTTTCCAATTTTTTTTTTTTGTGAGTTTAGTATACATGCATTTACTT 1738
F-2 ATCTG-GATTTTGTCTTTGCAATTTTATTTGG--GTGAGTCTAGTATACATGCATTTACTT 610
***** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *

pUE2 ATAATACAGTTTTTTAGTTTTGCTGGCCGCATCTTCTCAAATATGCTTCCCAGCCTGCTT 1798
F-2 ATAATACAGTATTTGAGTTTTGCTGGCCGCATCTTCTCAAATATGCTTCCCAGCCTGCTT 670
***** ** ***** ***** ***** ***** *****

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pUE2      TTCTGTAACGTTACCCCTCTACCTTAGCATCCCTTCCCTTTGCAAATAGTCTCTTCCAA 1858
F-2      TTCTGTAACGTTACCCCTCTACCTTAGCATCCCTTCCCTTTGCAAATAGTCTCT-CCTA 729
          ***** ** *

pUE2      CAATAATAATGTCAGATCCTGTAGAGACCACATCATCCACGGTCTTATACTGTTGACCCA 1918
F-2      CCATAATAATGTCAGATCCTGCAAAGACCACATCATCCACGGTCTTATACTGTTGACCCA 789
          * *****

pUE2      ATGCG-TCTCCCTTGTCTCTAAACCCACACCGGGTGTCTATAAT-CAACCAATCGTAACC 1976
F-2      ATGCGGTCTCCATAGTCTCTAA-CCACACCGGGCGTCTATAATCAACCCATCGTAACC 848
          *****

pUE2      TTCATCTCTTCCACCCATGTCTCTTTGAGCAATAAAGCCGATAACAAAATCTTTGTCGCT 2036
F-2      TTGCACCTTCCACC-ATGT-TCCTTGAGCAATACACCCGATGACAAA-TCCTTGACGCT 905
          ** *****

pUE2      CTCGCAATGTCAACAGTACCCCTT-----ATCCTTTGTTACTTCTTCTGCCGCTGCTTC 2091
F-2      CTTCCACAAGACA-CAGTGCCTTTCGTATATTTCTCCAGAAGATAGGGAGCCCTTGCATGC 964
          **** * * * * * ** * * * * *

pUE2      AAACCGCTAACAATACCTGGGCCACCCACACCGTGTGCGTATATTTCTCCAGTAGATAGGG 2151
F-2      AATTTCAGCTAAAATCTAAGGGCCCA--AGTCTTGGTACATGTTCTGCGCCTGCTTCG 1022
          ** * * * * * * * * * *

pUE2      AGCCCTTGCATGACAATTCGCTAACATCAAAGCCCTCTAGGTATTCGTAATGTCTGCC 2211
F-2      AACGTT-----ACAACCCGGCC--CACCA-----CCCGTGGGCACTCG-GATGCCCGCC 1068
          * * * * * * * * * * * * * * *

pUE2      CATTCTGCTATTCTGTATACACCCGCAGAGTACTGCAATTTGACTGTATTACCAATGTCA 2271
F-2      CATT--GCTATTG--ATA-ACCCATAGCAGCATTAACTTCCAGTCCAAATTTCTTTTC 1122
          **** * * * * * * * * * * * * * *

pUE2      GCAAAATTTCTGTCTTCGAAGAGTAAAAAATGTACTTGGCGGATAATGCCTTTAGCGGC 2331
F-2      GAAAAAATG--ACTCGCGGAAAGCATA---TCGTTCAGGACCTGAAATCCGATC--CTGT 1175
          * * * * * * * * * * * * * * *

pUE2      TTAACCTGTGCCCTCCATGGAAAAATCAGTCAAGATATCCACATGTGTTTTTAGTAAACAA 2391
F-2      ATAAACGGCCATTCCATCGGAAC-TGGGCCACCCACAGT----- 1212
          *** * * * * * * * * *

pUE2      ATTTTGGGACCTAATGCTTCAACTAACTCCAGTAATTCCTTGGTGGTACGAACATCCAAT 2451
F-2      -----

pUE2      GAAGCACACAAGTTTGTGCTTTTCGTGCATGATATTAATAGCTTGGCAGCAACAGGA 2511
F-2      -----

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