MOLECULAR CHARACTERIZATION AND EXPRESSION PROFILING OF Cd-RESPONSIVE GENES IN *Triticum durum*

by

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ABSTRACT

Cadmium (Cd) is a toxic heavy metal which has detrimental effects both in plants and human. There is a lack of knowledge on the molecular mechanisms of Cd toxicity in crop plants. The objective of this study was to identify and clone expressed Cd-responsive genes from two *Triticum durum* cvs. Balcalı-85 and Balcalı-2000 using mRNA differential display technique. We identified 10 cDNA clones whose level of expression significantly changed upon Cd exposure and thus isolated for further characterization. Differential expression patterns of cDNA clones were verified by Northern blot and Real-Time PCR analyses. Analysis of putative protein sequences of these clones revealed that BO2 encodes an *HCBT*-like defense response protein, and BO4 encodes *NADH dehydrogenase*, which is known to be involved in stress mechanism in plant systems. Four of these cDNA clones, BO5, BO6, BO8, and BO10, showed significant homology to the receptor kinase proteins. Identification of receptor kinase proteins to an abiotic stress factor, Cd stress, is noted to be the first in literature. One of the cDNA clones, BO11, found to have significant homology to *PsaC* gene, encoding a photosystemI 8 kDa subunit. This result is in well agreement with the previous physiological studies indicating that Cd has detrimental effects on photosystemI and II in plants. The cDNA clones BO3, BO7, and BO9 found to be novel sequences for which sequence comparison showed no homology in GenBank. These results suggest that Cd has various effects on gene expression and alters expression of different genes. Our findings also demonstrate that mRNA differential display technique is useful to understand molecular mechanisms affected by Cd.

Keywords: Cd-responsive genes, durum wheat, functional genomics, mRNA differential display

ÖZET

Kadmiyum (Cd), bitkilere ve insanlara, hücre düzeyinde zararlı etkileri olan ağır bir metaldir**.** Tahıllarda, Cd toksisitesinin moleküler mekanizmaları hakkında yeterli bilgi bulunmamaktadır. Bu çalışmanın amacı, mRNA differential display tekniğiyle *Triticum durum* buğday çeşitleri Balcalı-2000 ve Balcalı-85'te Cd'a bağlı olarak ifadeleri değişen genleri belirlemek ve klonlamaktır. Ekspresyonları farklılık gösteren cDNA parçalarını doğrulamak için Northern Blot ve Real-time PCR analizleri yapılmıştır. Ekspresyon seviyeleri önemli bir şekilde değişen 10 tamamlayıcı DNA (cDNA) parçası tanımlanmış ve diğer analizler için izole edilmiştir. Bu klonların putatif protein analizleri, tanımlanmış bantlardan BO2'nin *HCBT*-benzeri savunma proteinini ve BO4'ün bitkilerde stres mekanizmasında rol oynadığı bilinen *NADH dehidrojenaz* enzimini kodladığını göstermiştir. BO5, BO6, BO8 ve BO10 klonları ise reseptör kinaz proteinine önemli bir benzerlik göstermiştir. Literatürde ilk defa bir reseptör kinaz proteini abiyotik stres faktörlerinden Cd stresiyle ilişkilendirilmiştir. BO11 klonu ise fotosistem I'in 8 kDa alt birimini kodlayan *PsaC* geniyle önemli bir benzerlik göstermiştir. Bu bulgu, Cd' un fotosistem I ve II'ye zararlı etkilerini gösteren fizyolojik çalışmalarla örtüşmektedir. BO3, BO7 ve BO9 klonları ise özgündür ve bu klonlara GenBank veritabanında rastlanmamıştır. Sonuçlar Cd' un gen ekspresyonu üzerinde çok yönlü etkisi olduğunu ve farklı genlerin ekspresyonunu etkilediğini belirtmiştir. Ayrıca, bu bulgular mRNA DD yönteminin Cd'un etkilediği moleküler mekanizmaları anlamaktaki faydasını göstermiştir.

To my family with all my heart

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ABBREVIATIONS

ABA: Abscisic acid

Amp: Ampicillin

ATP: Adenosine triphosphate

ATPase: Adenosine triphosphatase

AtPCS: *A. thaliana* phytochelatin synthase

BLAST: Basic local alignment search tool

Cd: Cadmium

cDNA: Complementary DNA

CDF: Cation diffusion facilitator

CePCS: *C. elegans* phytochelatin synthase

cv: Cultivar

mRNA DD: mRNA differential display

Ec: Early Cys-labelled

Et-Br: Ethidium bromide

GADPH: Glyceraldehyde-3-phosphate dehydrogenase

GP: Glycoprotein

GSH: Glutathione

HMT: Heavy metal tolerance factor

HMW: High molecular weight

HSP: Heat shock protein

IPTG: Isopropyl β-D-Thiogalactopyranoside

LB: Luria bertani

LMW: Low molecular weight

MAPK: Mitogen-activated protein kinase

MT: Metallothionein

NADH: Nicotinamide adenine dinucleotide

NRAMP: Natural resistance-associated macrophage protein

PC: Phytochelatin

PCR: Polymerase chain reaction

ROS: Reactive oxygen species

RT: Reverse transcription

SpPCS: *S. pombe* phytochelatin synthase

TaPCS1: *T. aestivum* phytochelatin synthase 1

X-Gal: 5-brom-4-chloro-3-indolyl-beta-D-galactopyranoside

ZIP: Zinc/Iron permease

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1 INTRODUCTION

Cadmium (Cd) is a non-essential heavy metal and represents an important environmental pollutant. Especially in highly industrialized regions, Cd pollution is widespread through the activities of metal-processing industries, waste incinerators, cement factories, urban traffic and as a by-product of phosphate fertilizers (Sanitá di Toppi and Gabrielli, 1999). As a result of such antropogenic activities, Cd is accumulated in soils and enters the food chain. Humans are exposed to Cd via ingestion of Cd-contaminated food (Waalkes et al., 1992). Durum wheat, flax, sunflowers and potatoes can take up Cd from slightly Cd-contaminated soils, and these Cd concentrations are above the acceptable levels for human intake (Grant et al., 1998). Even at very low concentrations, Cd is very dangerous for human health, since it is a suspected carcinogen (Clemens et al., 1999) and induces tumor development in lungs and prostate (International Agency of Cancer Research, 1993). Besides its carcinogenic effects, accumulation of Cd in liver, kidney and, lung has detrimental effects on kidney and liver and leads to development of respiratory diseases and neurological disorders (Pinot et al., 2000).

Cadmium also has damaging effects at cellular level. These include oxidation and structural alterations of proteins, lipids, and DNA (Yiin et al., 2000). It has been proposed that Cd binds to sulphydryl groups of proteins and leads to disruption of structure and as a result inhibition of activity of that protein (Hall, 2002). In addition, Cd exposure may stimulate the formation of free radicals and reactive oxygen species (ROS), resulting in oxidative stress (Risso-de Faverney et al., 2001).

Despite the growing knowledge of Cd toxicity and detoxification mechanisms in plant systems, information on genetic mechanisms involved in differential heavy metal

tolerance between plant species and among the genotypes of a given species is still limited. There is a growing interest in understanding the genetic mechanisms underlying the differential tolerance to Cd, between plant genotypes. Closely related genotypes are valuable tools for studying differential heavy metal tolerance. Identification of genotypes that greatly differ in their tolerance to Cd toxicity sheds light on genetic differences between these genotypes.

Durum wheat grain is a primary energy source for human beings particularly for the people living in the developing world. For a better human nutrition and health, identification of wheat genotypes with high ability to inhibit Cd uptake by the roots and/or repress Cd translocation to edible parts of the plants, like seeds, via detoxification in the roots is of great importance. To our knowledge, there is no study in the literature dealing with Cd-induced alterations in transcriptome of durum wheat genotypes that differ greatly in tolerance to Cd.

The objective of this study was to identify, clone and characterize the differentially expressed Cd-responsive genes in two durum wheat cultivars. In a parallel study conducted by Kokturk et al. (unpublished data, 2006), two durum wheat genotypes, Balcalı-85 and Balcalı-2000 were found to display differences in tolerance to Cd toxicity. In this study, these two cultivars were used in screening for genes that alter their expression levels upon three different doses of Cd-treatments, by a genomics tool called mRNA differential display. The harmful effects of Cd in plant systems, a better understanding of plant response mechanisms to Cd stress, and identification of Cd- responsive genes by using mRNA differential display method constitute the main topics of the overview.

2 OVERVIEW

2.1 Effects of Cd toxicity in higher plants

Roots are first sites exposed to damaging effects of Cd in plants. Cadmium enters the roots through the cortical tissue. Cadmium ions are retained in the roots in most cases, and only small amounts are transported to the shoots (Sanitá di Toppi and Gabbrielli, 1999). Cadmium has been reported to alter the lipid composition and fluidity of cell membrane (Quartacci et al., 2001), and therefore change the membrane permeability. In rice seedlings, Cd has been reported to alter RNA levels and inhibit activity of ribonuclease (Shah and Dubey, 1995). Cadmium also reduces nitrate uptake from the soil and its retranslocation to shoots, through inhibition of nitrate reductase activity in the shoots (Hernandez et al., 1996). Another important effect of Cd is on photosynthesis by induction of Fe(II) deficiency resulting from suppression of Fe(III) reductase activity (Alcantara et al., 1994).

Cadmium interferes with photosynthesis by damaging the photosynthetic apparatus. Its harmful effects are particularly on light harvesting complex II (Krupa, 1988) and photosystems I and II (Siedlecka and Baszynsky, 1993). It has been demonstrated that Cd reduced total concentration of chlorophyll and carotenoid, and increased the non-photochemical quenching in *Brassica napus* plants (Larsson et al., 1998). Furthermore, Cd interacts with water balance through inhibition of stomatal opening. Cadmium exerts this effect via interfering with movements of K^+, Ca^{2+}, and abscisic acid into guard cells (Barcelo and Poschenrieder, 1990).

Cadmium was found to induce oxidative stress (Hendry et al., 1992), by either inhibition of antioxidative enzymes or inducing generation of ROS (Risso-de Faverney et al., 2001). For instance, Cd was found to increase lipid peroxidation, enhance activity of lipoxygenase and reduce the activity of superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase and dehydroascorbate reductase in sunflower (Gallego et al., 1996). In another study, Cd was shown to induce lipid peroxidation, decrease catalase activity, and increase guaiacol peroxidase and ascorbate peroxidase activity in *Phaseolus aureus* (Shaw, 1995). In pea, Cd remarkably enhanced lipid peroxidation. *Daucus carota* roots gave a different response to Cd treatment and no lipid peroxidation was detected (Sanitá di Toppi et al., 1998). Responses of plants to oxidative stress are different most probably due to the differences in duration and concentration of Cd supplied and the amount of available thiolic compounds either existing in plants or induced by Cd exposure. Thiols have been reported to have high antioxidative potential and to counteract oxidative stress (Pichorner et al., 1993).

Cadmium can also replace several cofactors, such as Ca^{2+}, $Zn^{2+}$and, Fe^{2+} which are required for the proper functioning of essential enzymes (Stohs et al., 2000). Displacement of essential elements results in inhibition of activity or disruption of structure of those proteins. Based on this knowledge, it can be proposed that excess Cd in the cells can replace Zn ions in the 'zinc fingers' in transcription factors and consequently can interfere with the transcription and signal transduction mechanisms.

2.2 Response of higher plants to cadmium stress

Plants protect themselves from damaging effects of Cd by adapting cellular mechanisms involving reduction in root uptake of Cd or detoxification of Cd at cellular level. As it has been reported in a review by Hall (2002), most of these response mechanisms are primarily based on the avoidance of excessive accumulation of Cd within the cell rather than synthesizing compounds that can resist harmful effects of the heavy

metal. Plant species which can prevent onset of heavy metal stress via avoiding heavy metal build up inside the cells are designated to be 'tolerant to heavy metals'. Heavy metal tolerance is the ability of plants to survive in a soil that is toxic to other plants. Tolerant plants exhibit various strategies to prevent heavy metal build-up in the cells. These include immobilization of toxic metals by means of cell walls, secretion of extracellular exudates, complexing Cd in growth medium, and prevention of heavy metal uptake through the action of plasma membrane.

At root level, the most effective mechanism against Cd stress can be the immobilization of Cd by cell wall (Sanitá di Toppi and Gabbrielli, 1999). It has been found that when bush bean plants are exposed to Cd, most of the heavy metal is bound by pectic sides and histidyl groups of the cell wall (Leita et al., 1996). However, the contribution of cell wall to metal tolerance has been controversial. There are several papers in literature reporting that heavy metal binding property of cell wall is not a critical mechanism in heavy metal detoxification. According to Hall (2002), it is hard to explain metal-specific tolerance with such a broad mechanism.

Another heavy metal tolerance mechanism is complexion of heavy metals by root exudates in growth medium. The role of organic acid secretion from roots in detoxification of highly toxic Al has been clearly shown in buckwheat (Ma et al., 1997). Although there is no example for a role of root secretions in Cd tolerance in literature, it is possible that there might be such compounds released from roots and involved in detoxification of Cd in rhizosphere.

Integrity of cell membrane is very crucial for the survival of the cell. Therefore, mechanisms which maintain structural and functional integrity of cell membrane, protect membrane against the damaging attack of Cd, and finally repair membrane after damage should result in a great contribution to metal tolerance at plasma membrane level. Besides maintenance of plasma membrane integrity, plasma membrane might play an active role in metal homeostasis either by prevention of metal entry into the cell or efflux of toxic metal (Hall, 2002). However, in literature there are only a few examples for prevention of metal

uptake or efflux by plasma membrane. Although, there is no clear evidence for function of efflux in heavy metal tolerance, it has been reported that there are several metal transporter protein families in plants. These include the heavy metal CPx-ATPases, the Nramps, and the CDF (cation diffusion facilitator) family (Williams et al., 2000) and the ZIP protein family (Guerinot, 2000). In *Arabidopsis thaliana*, inhibition of *AtNramp3* gene slightly increased Cd resistance, whereas overexpression of this gene resulted in Cd hypersensitivity (Thomine et al., 2000). This research has indicated that metal transporters should be involved in metal uptake and homeostasis, and could play a critical role in metal tolerance. However, further research is required for elucidation of the cellular location, specificity and transporting properties of these proteins.

In addition to detoxification processes which aim to prevent onset of stress, plants have evolved a variety of mechanisms to tolerate the harmful effects of Cd inside the cells. These include synthesis of ligands which are able to chelate Cd heavy metal and sequestration of the Cd-complexes in a safe compartment inside the cell. There are two major heavy metal binding ligands in plant systems: phytochelatins (PCs) and metallothioneins (MTs).

2.2.1 Phytochelatins

2.2.1.1 Structure and Biosynthesis of PCs

Phytochelatins (PCs) are composed of several repeats of $(\gamma\text{-Glu-Cys})_n$ dipeptides, where n is in the range of 2-11, followed by a terminal glycine to have a final form of $(\gamma\text{-Glu-Cys})_n$–Gly (Zenk, 1996). Phytochelatins have been first identified in *Schizosaccharomyces pombe*, and called cadystins (Kondo et al., 1983). Phytochelatins have also been identified in several plant species, fungi and marine diatoms (Cobbett, 2000). In addition to classical form of PCs with terminal glycine residue, some other sub-forms of PCs have been identified in some plant species (Cobbett and Goldsbrough, 2002).

All forms of PCs identified so far, have been reported to have high metal binding affinity, especially for Cd, and to facilitate the vacuolar compartmentalization of heavy metals.

Biosynthesis of PCs depends on the presence of glutathione (GSH: γGluCysGly). Several physiological, biochemical, and genetic studies reported the crucial role of GSH as a substrate in PC biosynthesis (Cobbett, 2000). GSH-deficient *S. pombe* and *A. thaliana* mutants were found to be PC deficient and to display Cd hypersensitivity (Vatamaniuk et al., 1999; Ha et al., 1999). The enzyme which synthesizes PCs from GSH, PC synthase, was first partially purified by Grill et al. (1989). This enzyme catalyzes the transfer of a (γ-Glu-Cys) unit from one thiol peptide to another or to a previously synthesized PC. The gene encoding this enzyme was first identified in *A. thaliana*, and designated as *AtPCS1*. Cadmium-sensitive, *cad1*, mutants were unable to synthesize PCs, although GSH, which is required for PC biosynthesis, was present in wild-type. These mutants contained a low level of PC synthase activity and were deficient in their ability to form Cd-binding complexes (Howden et al., 1995).

Three research groups have simultaneously and separately isolated PC synthase genes, in three different organisms, *A. thaliana*, *S. pombe* and *T. aestivum*. These genes are designated *AtPCS1*, *SpPCS*, and *TaPCS1*, respectively. Vatamaniuk et al. (1999) reported that they have cloned *AtPCS1* gene isolated from *A. thaliana*, and expressed it in *S. cerevisiae*. Phytochelatin synthase encoded by this gene was a 55-kDa soluble protein and conferred increased Cd-tolerance to *S. cerevisiae*. In addition, transformed yeast has been shown to accumulate more PC upon Cd exposure. Ha et al. (1999) identified a gene, *SpPCS1*, which is similar to the PC synthase gene in *A. thaliana*, in the genome of *S. pombe*. Deletion mutants of that gene displayed Cd-hypersensitivity and PC deficiency, like *Arabidopsis cad1* mutants. This result corroborated the analogous function of the two genes in the different organisms. Clemens et al. (1999) expressed a wheat cDNA, *TaPCS1*, in *S. cerevisiae* and demonstrated that this plant gene conferred cellular Cd tolerance in yeast cells. The PC synthase encoded by this gene was ~55kDa protein which was found to be homologous to PC synthase protein family members identified previously. These data provided molecular evidence for the model that PCs play a crucial role in metal tolerance.

A second PC synthase gene has been identified in *Arabidopsis* genome which has significant homology to *CAD1/AtPCS1* (Ha et al., 1999). This result was surprising, since the studies so far have suggested that there exists a single active PC synthase in plants. For instance, in *cad1* mutants, PCs were absent after prolonged exposure to Cd. Nevertheless, *AtPCS2* is expressed in most plant tissues at a low level. In a PC synthase knockout strain of *S. pombe, AtPCS2* gene was demonstrated to encode for a functional PC synthase (Cazale and Clemens, 2001). This enzyme should confer selective advantage to the organism it is expressed, hence is preserved through evolution. Physiological function of this protein has not been defined yet.

Another study by Clemens et al. (2001) suggested a role for phytochelatins in metal tolerance in certain animals. A functional PC synthase gene was cloned and characterized in *C. elegans*. *CePCS* was shown to rescue Cd sensitive and PC deficient *S. pombe* strain. *CePCS* expression enhanced PC synthase activity in this strain (Clemens et al., 2001). These results demonstrated that *CePCS* gene encodes a protein which has a similarity to PC synthases identified in plant species.

2.2.1.2 Properties of PC synthase enzymes

The PC synthase enzyme is self-regulated. It starts to catalyze PC synthesis immediately after the cell is exposed to Cd. Product of this reaction, PC continues to be synthesized unless further Cd is provided (Sanitá di Toppi and Gabbrielli, 1999).

Through the history of the studies, there has been a dilemma on the models for the PC synthase activation. Early models suggested that there should have been a direct interaction between the enzyme and the metal for the activation of the enzyme. However, these models could not explain the ability of this enzyme to be activated by a wide range of metals. A study by Vatamaniuk et al. (2000) proposed an alternative route for activation of this enzyme and overturned the previous models. It was shown that direct metal binding to this enzyme is not responsible for catalytic activation. Instead, metal ions were shown to

play a role in enzyme activation as a part of the substrate. Therefore, any metal ions that have the ability to form thiolate bonds with GSH can activate PC synthase. These data indicate that the enzyme requires GSH or GSH-like peptides with blocked thiol groups for activity.

The N-terminal domain, preserved among species, is assumed to be the catalytic domain. In a study by Howden et al. (1995), *cad1-5* mutants were shown to produce a truncated PC synthase which lacks 9 of the 10 Cys residues in the C-terminal domain. These mutants were least sensitive to Cd, and produced highest level of PC in comparison to other *cad1* mutants with truncations in different regions of the protein. The C-terminal domain is proposed to function in bringing GSH-metal complexes in proximity of to the catalytic domain. In this way, it enhances the activity of this enzyme.

2.2.1.3 Compartmentalization of Cd-PC complexes

Cd-PC complexes are preferentially sequestered to vacuole in plants and yeast. Molecular mechanism underlying heavy metal detoxification has been clearly demonstrated in *S. pombe*. In this organism, low molecular weight (LMW) Cd-PC complexes are transported into vacuole by means of a MgATP dependent transporter protein called heavy metal tolerance factor 1 (HMT1). HMT1 is a 90.5kDa vacuolar ABC type ATP-binding cassette transporter. High molecular weight (HMW) Cd-PC-S^{2-} complex containing acid labile sulfide is also taken up by vacuolar transporter vesicles, but with lower efficiency. HMW complex has higher Cd binding capacity than LMW complex. PC biosynthesis and vacuolar sequestration of Cd-PC complexes are summarized in Fig. 2.1.

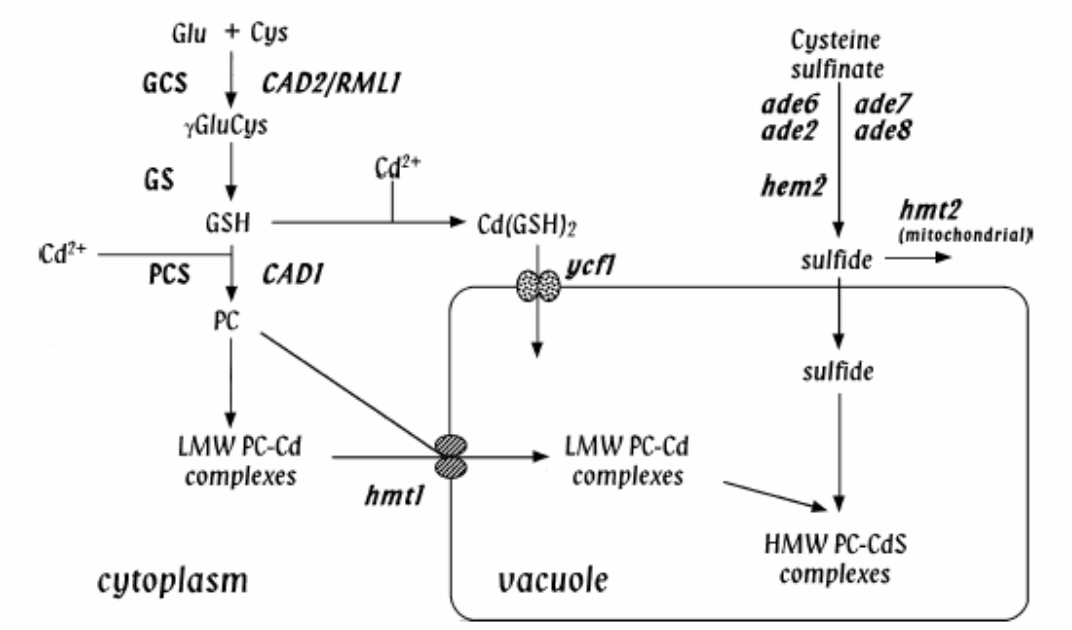

Fig. 2.1 Genes contributing to PC biosynthesis and vacuolar sequestration of PC-Cd complexes (Cobbett and Goldsbrough, 2002).

Previously it was shown that *hmt1* mutants were unable to transport LMW Cd-PC complexes into the vacuole and form HMW Cd-PC complexes (Ortiz et al., 1995).

In Cd tolerant plant species, minimum 90% of Cd is accumulated as Cd-PC complex in vacuole. A plant gene encoding for a vacuolar transporter homologous to HMT1 has not yet been identified. However, an ATP dependent, proton gradient independent activity, able to transport Cd-PC complexes has been characterized in vacuolar membrane vesicles isolated from oat roots (Salt et al., 1995).

2.2.2 Metallothioneins (MTs)

2.2.2.1 Structure of MTs

Metallothioneins (MTs) constitute a group of gene encoded, cysteine-rich (about 30%) peptides with high metal binding ability (Kägi, 1993). MTs bind a variety of Group IB and IIB metals via the large number of cysteins in their structure. The metals found in cysteine thiolate metal clusters include Zn^{2+}, Cu^{2+}, Cd^{2+}, and Hg^{2+}.

The first metallothionein was discovered as a metal binding peptide in horse kidney by Margoshes and Vallee in 1957. Since their identification, MTs have been subject to intensive research, and MT genes and proteins have been found throughout the animal and plant kingdoms. The most common characteristics of MTs identified so far, is the presence of two Cys-rich domains in their structure. Metallothioneins are usually classified into two groups based on the arrangement of the cysteine residues (Cherian et al., 1993). The most distinguishing characteristics of Class I MTs is the presence of cysteine residues that align strictly with a mammalian renal MT. Class II MTs also possess cysteines, however these cysteins can not be strictly aligned with Class I MTs. This type of MT is widespread in plants and fungi. After this classification, MTs which do not conform to these two categories were identified and designated as Class III MT and Class IV MTs (Robinson et al., 1993).

It has been suggested that the different classes of MTs identified in plant systems might differ in function, too (Cobbett and Goldsbrough, 2002).

2.2.2.2 Early Cys-labelled (Ec) protein

Early Cys-labelled protein was isolated from mature dry wheat embryos as a Zn binding protein and constitutes the first characterized Class II MT in higher plants (Lane et

al., 1987). Afterwards, MT genes have been identified in *Arabidopsis* (Murphy et al., 1997). Expression of wheat Ec MT is restricted to developing seeds. Ec MT genes are found as a single copy and located on the long arm of the 1A, 1B and 1D genomes of hexaploid wheat (Kawashima et al., 1992).

Ec MT genes contain promoter sequences with homology to ABA response elements on the 5'-flanking region like other plant genes. Expression of Ec MT gene is regulated at transcriptional level by ABA (White et al., 1995). Addition of ABA to germination medium increased the Ec MT mRNA levels, whereas addition of Zn^{2+}did not do the same effect. Ec mRNA level increases during maturation of embryos and the highest level is reached at early stages of embryogenesis, shortly after the beginning of the rapid cell division and decreases during early germination (Kawashima et al., 1992). This embryo-specific expression of Ec MT gene is proposed to be a mechanism for storing zinc that is required during germination. This property of plant metallothioneins can be exploited to manipulate metal concentrations in seeds (Lucca et al., 2001).

2.2.2.3 Expression profile of plant MTs

Plant MT gene expression has been detected in a variety of plant tissue types by several research groups. Detection of MT gene expression in trichomes (Foley and Singh, 1994) suggested that MTs may play a role in metal delivery to these specialized cells, in which the specific metal binding enzymes are highly expressed. Another group suggested that high level of MT gene expression is required in trichomes to detoxify toxic metals, like Cd, that accumulate in trichomes at high levels (Salt et al., 1995). MT transcripts were also detected in phloem of *Arabidopsis* (Garcia-Hernandez et al., 1998). This observation suggests a function for MTs in metal ion transport. Nevertheless, MTs could not be found among phloem exudate proteins identified until now. Difficulties in identifying MTs in plant tissues might arise from instability of these proteins in the presence of oxygen.

MT gene expression is effected by several environmental factors. MT genes were shown to be induced by various stress factors, such as metal exposure, heat shock, cold stress (Reid and Ross, 1997), sucrose deprivation (Hsieh et al., 1995), viral infection and wounding (Choi et al., 1996), leaf senescence (Clemens, 2001) in a variety of organisms. According to these observations, it can be proposed that MTs might also be synthesized as part of a general stress response, besides its expression as a specific response to high metal accumulation.

Although MT gene expression is found in several plant tissues, the exact function of MTs remains to be elucidated by future studies.

2.3 Differential Display

As mentioned previously**,** despite the growing knowledge regarding heavy metal toxicity and detoxification mechanisms in higher plants, there is still limited information about the cellular mechanisms of heavy metal stress-induced signaling.

In recent years, several research groups have focused their attention on the identification and isolation of genes to investigate the effects of Cd on cellular mechanisms. For instance, Suzuki et al. (2001) screened for differentially expressed genes in *Arabidopsis* populations after Cd exposure. For this purpose, they adopted differential display (DD), which has been reported to be a powerful technique to rapidly identify and isolate genes that are differentially expressed between two cellular populations, or within a single cell type under altered conditions (Ito et al., 1994). This method was first described by Liang and Pardee in 1992 as a method for analyzing gene expression in eukaryotic cells and tissues. Since its introduction, this technique has been extensively used to study changes in transcript levels induced by development, cellular factors, and environmental factors. Despites its drawbacks, such as low reproducibility of fingerprints and formation of non-specific products and ‘false positives’, it is simple, powerful and requires small amounts of

starting material. One of the most important advantages of this technique is that it can simultaneously screen for both up-regulated and down-regulated transcripts in multiple cell populations (Leslie et al., 2000). Employing this genomic tool enable Suzuki et al. (2001) to identify 31 genes whose expression is changed upon Cd treatment in *Arabidopsis*. By further characterization of the genes, they were classified into three functional groups. One third of the identified genes were elucidated to encode for proteins which function in signal transduction pathways including transcription factors, protein kinases and calmodulin-related proteins. As a result, it was proposed that plants respond to Cd by activating signal transduction pathways, including protein phosphorylation pathway. Second third of the identified genes were found to encode for proteins, which have a direct role in detoxification of Cd, such as metal binding proteins, chaperones, and abiotic stress response proteins. The final third of the genes identified encoded for Cu related proteins (Stohs and Bagchi, 1995). Copper is known to be involved in ROS production and is a cofactor in oxidative enzyme. This result suggests that Cd also plays an important role in production of ROS and generates oxidative stress in plant systems.

mRNA differential display technique was applied to identify and isolate genes whose expression is altered in response to Cd, in the Antarctic fish, *Chionodraco hamatus* (Carginale et al., 2002). Employing this technique, 12 cDNA bands were shown to be differentially expressed in Cd treated fish and among these bands, 7 fragment bands were further characterized. Of the 7 fragment bands, three were found to have no significant match to gene sequences previously identified, whereas the remaining 4 had significant homology to previously identified genes in data-bases. Out of these 4 genes, 2 were found to be up-regulated, whereas the other two were down-regulated by Cd. Up-regulated group included a gene encoding for a heat shock protein, HSP70 and a gene encoding for chorionic glycoprotein GP49. Both of these proteins were proposed to have a protective role towards the harmful effect of the metal toxicity. Down-regulated genes included the transferrin gene and a gene encoding for a protein homologous to T2K found in mammalian cells. Down regulation of transferrin gene in Cd treated fish revealed that Cd may also affect absorption of other essential metals, like iron. T2K protein has been established as an anti-apoptotic agent in mammalian cells, hence down-regulation of T2K-

like protein in Cd treated fish may provide an evidence for the apoptotic effect of this metal.

Differential display technique was also successful in identification of Cd-responsive genes in another organism, liverwort, *Lunularia cruciata* (Basile et al, 2005). Four differentially expressed genes were further characterized and classified into two groups. Up-regulated gene was encoding for cystathionine γ-synthase (CGS), an enzyme which functions in cysteine biosynthesis and known to be responsible for the enhanced Cd tolerance in *Arabidopsis* (Dominguez- Solis et al., 2004). Down-regulated group included a gene encoding for a tyrosine phosphatase, a gene encoding for methyl transferase, and a gene homologous to EST 408 cDNA clone, with unknown function. Tyrosine phosphatase plays important roles in signal transduction pathways and has been reported to inactivate MAPKs (mitogen-activated protein kinases) (Keyse and Emslie, 1992), that are activated in response to abiotic stress factors such as, Cu and Cd ions (Jonak et al., 2004). Based on this information, they concluded that; (1) down-regulation of tyrosine phosphatase gene might enhance MAPK activity and results in strengthening of cell against the harmful effects of Cd, (2) methyl transferases function in alteration of gene expression through methylation of DNA, and (3) down-regulation of methyl transferase gene by Cd might result in hypomethylation of DNA and enhancement of transcriptional activity, and hence induces over-expression of stress-induced proteins, such as metallothioneins (Riggio et al., 2003).

In summary, the differential display technique is a powerful tool for identification of genes which play essential roles in various metabolic processes in several organisms, including plants. We have adopted mRNA DD genomics tool to identify Cd-responsive genes and perform comparative gene analysis in *Triticum durum***.**

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

In the experiments, the two durum wheat cultivars (*Triticum durum* cv. Balcalı-2000 and Balcalı-85) were used. All plant materials were kindly provided by Çukurova University.

3.1.2 Chemicals

All chemicals were obtained from Merck (Germany), SIGMA (US), Fluka (Switzerland), and Riedel de Häen (Germany).

3.1.3 Growth Media, Buffers and Solutions

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

3.1.4 Equipment

Equipments used in this research is listed in Appendix C.

3.2 Methods

3.2.1 Plant growth conditions and cadmium treatments

Seeds of two durum wheat genotypes (*Triticum durum* cvs. Balcalı-2000 and Balcalı-85) were surface sterilized with 1% NaOCl for 5 minutes and then rinsed with distilled water (dH_2O). Seeds were germinated in perlite moistened with saturated $CaSO_4$. After 5 days, seedlings were transferred to continuously aerated nutrient solutions composed of the following macro and micronutrients: 0.88 mM K_2SO_4, 2 mM $Ca(NO_3)_2$, 0.2 mM KH_2PO_4, 1.0 mM $MgSO_4$, 0.1 mM KCl, 100 μM Fe-EDTA, 1.0 μM H_3BO_3, 1.0 μM $ZnSO_4$, 1.0 μM $MnSO_4$, 0.2 μM $CuSO_4$, and 0.02 μM $(NH_4)_6Mo_7O_{24}$.

Plants were grown for 7 days in a growth chamber under controlled conditions (light/dark regime 16/8 h, temperature 24/22ºC, relative humidity 60/70%, and photon flux density of 600-700 μmol m^{-2} s^{-1}). Nutrient solutions were renewed every 3 days.

On completion of 7 days, plants were treated with varying levels of Cd (0, 2, 10, and 30 μM) in the form of $CdSO_4$ for 7 days. Nutrient solutions of the plants were renewed every 3 days. When the symptoms of increasing Cd treatments became severe on the plants, 0.2 g leaf tissue was collected from each pot, immediately frozen in liquid nitrogen and stored at -80ºC until analysis.

3.2.2 Total RNA isolation

Two hundred mg leaf tissue was ground with 1.5 ml Trizol® reagent (Invitrogen) (without adding liquid nitrogen). Using a wide bore pipette tip, 1 ml of liquid was taken into an eppendorf tube, which was kept on ice while processing the other samples. After processing all the samples, they were incubated at room temperature for 10 minutes, 0.4 ml chloroform was added and the tubes were shaken and incubated at room temperature for 5 minutes. Then, samples were centrifuged at 12,000 rpm for 15 minutes at 4ºC. The upper layer containing RNA was transferred to a fresh tube. After chloroform extraction, 0.5 ml isopropanol was added to precipitate RNA. Samples were then incubated at room temperature for 10 min and spun at 12,000 rpm for 10 min at 4ºC. The RNA pellet was washed with 1 ml 75% ethanol after centrifugation. Samples were mixed by vortexing and spun at 7,500 rpm for 5 min at 4ºC. The RNA pellet was dried at room temperature for 10 minutes and placed in 20-50 µl formamide, depending on the size of the pellet, and allowed to sit in the 55ºC water bath for an hour to improve suspension.

3.2.3 Dnase I treatment

RNA was treated with Dnase I (Fermentas) to remove contaminating chromosomal DNA. 50 µg of total RNA were treated with 10 units of Dnase I in 10 mM Tris-HCl pH 7.4, 1.5 mM $MgCl_2$, and incubated at 37ºC for 30 minutes. The supernatant was ethanol precipitated with 0.3 M sodium acetate. The pellet was air dried and resuspended in 50 µl formamide.

3.2.4 cDNA synthesis and mRNA differential display

The RNA was quantified on a spectrophotometer and visualized on a 1% agarose gel to ensure high quality. To synthesize first-strand cDNA, 3 µg total RNA from the cultivars

with and without Cd treatment was independently reverse transcribed using the Omniscript reverse transcription kit (Qiagen). OligodT primers used in reverse transcription reaction were purchased from Invitrogen (0.5 μg /μl).

Amplification of cDNA fragments were performed in 20 μl PCR reactions, using 72 possible combinations of arbitrary forward (designed as "P") and reverse (designed as "T") primer pairs. The "P" and "T" primer pairs used in mRNA differential display PCR reactions were purchased from Integrated DNA Technologies, Inc. (USA). The sequences of "P" and "T" primers are listed in Table 3.1.

Table 3.1

Primers used in mRNA differential display

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

Each reaction mixture contained 2 μl first strand cDNA, 2 μl 10X PCR buffer (without $MgCl_2$), 2.5 mM $MgCl_2$, 50 μM dNTP mix, 2 μCi [α-^{33}P]dATP, 1 μM of P primer , 1 μM of T primer, 1.25 unit Taq DNA polymerase (Promega). Reactions were carried out

in a DNA thermocycler GeneAmp PCR System 9700 (PE Applied Biosystems) with the following conditions:

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

3.2.5 Polyacrylamide gel electrophoresis (PAGE)

The PCR products obtained from first strand cDNAs synthesized from control and Cd treated plants were size-fractionated in denaturing 6% polyacrylamide/8 M urea gels. Gels were prepared using 10X TBE buffer and Acrylamide:Bisacrylamide mix (29:1) and pre-run in 0.5X TBE buffer at 180 V for ~45 minutes. After cleaning the wells, to get rid of excess urea, PCR samples were mixed with 6X loading dye (including sucrose), and loaded into the wells using a microliter syringe. Gels were run at 185 V, approximately for 3 hours.

3.2.6 Autoradiography

After electrophoresis, gels were placed on two sheets of Whatman 3MM filter paper and saran wrap was put on the gels carefully, avoiding air-trapping between the gel and the wrap. Then, gels were dried at 76ºC using E-C corp. gel dryer apparatus for approximately 50 minutes. Dried gels were exposed to X-ray films (Kodak) and the cassettes were incubated at -80ºC over night. The films were developed using developer and fixer solutions (Kodak).

3.2.7 DNA extraction from polyacrylamide gels

Differentially expressed fragment bands were excised from dried gels and eluted either using QIAEX II polyacrylamide gel extraction kit (Qiagen) or by incubating gel pieces in 40 µl of 10 mM Tricine, pH 9.5 containing 0.2 mM EDTA, at 100ºC for 5 minutes.

3.2.8 Reamplification of differentially expressed cDNA fragment bands

Eluted fragments were reamplified in 50 µl PCR reactions using the same sets of primers. PCR conditions were similar to those used for mRNA differential display with a few exceptions. The first modification was the removal of 3 initial rounds for non-specific annealing and the second was the omission of [α-^{33}P]dATP.

3.2.9 Agarose gel electrophoresis

PCR products of the reamplification reactions were size-fractionated by agarose gel electrophoresis Gels were prepared at 1% concentration using 1X TBE buffer and were run

in 0.5X TBE buffer at 100 mV of constant voltage for 50 minutes. In order to determine the size of cDNA fragment bands, intensity of each band was compared with GeneRuler ™1 kb DNA ladder (Fermentas)

3.2.10 DNA extraction from agarose gels

Reamplified cDNA bands were excised from the gels and then purified using Qiaquick® gel-extraction kit (Qiagen).

3.2.11 Cloning

Basic procedures were carried out according to Sambrook et al., 2001.

3.2.11.1 Subcloning

PGEM®-T Easy Vector System I (Promega) was used to subclone the reamplified cDNA fragments bands. *E. coli* (strain DH5α) competent cells were transformed with the recombinant plasmids. Positive clones were selected and used for plasmid purification.

3.2.11.2 Ligation

Reamplified differentially expressed cDNA fragment bands were ligated to PGEM®-T Easy vector (Promega) according to the specified amount (3:1 insert/vector ratio) in the pGEM –T Easy kit protocol. Ligation reaction was incubated at room temperature for 1 hour. Positive and negative controls of ligations were also used.

3.2.11.3 Chemically competent cell preparation

Single colony of *E. coli* DH5α strain was inoculated in 50 ml LB and grown over night in a shaking incubator at 37ºC, 250 rpm. 4 ml from overnight culture was transfered into 400 ml LB in sterile 2L flask and grown at similar conditions. When the OD_{590} of the cells reached ~0.375, culture was taken into 50 ml prechilled, sterile falcon tubes and left on ice for 5-10 minutes. The tubes were spun at 2700 rpm for 7 minutes at 4ºC. Supernatants were discarded and the pellets were gently resuspended in 10 ml ice-cold $CaCl_2$ solution, containing 60 mM $CaCl_2$, 15% glycerol and 10 mM PIPES (pH 7.0). The tubes were spun at 1800 rpm for 5 minutes at 4ºC. Supernatants were discarded and the pellets were resuspended in 10 ml ice-cold $CaCl_2$ solution, and kept on ice for 30 minutes. Again, the tubes were spun at 1800 rpm for 5 minutes at 4ºC. Supernatants were discarded and the pellets were resuspended in 2 ml ice-cold $CaCl_2$ solution. 200 µl aliquots were dispensed into prechilled, sterile eppendorf tubes, immediately frozen in liquid nitrogen, and stored at -80ºC. Competency of the cells was checked by transforming 5-10 ng plasmids.

3.2.11.4 Transformation

DH5α strain of *E. coli* was used for transformation. 5 µl of ligation reaction was mixed with 200 µl chemically competent cells according to the manufacturer's protocol. Since the vector contains Ampicillin resistance and *LacZ* genes, 100 µl of transformation culture was plated on LB agar/Amp/IPTG/X-Gal plate and incubated over night at 37ºC. The *pUC 18* was transformed to competent cells as a positive control. Uncut plasmid was transformed as a negative control.

3.2.11.5 Colony selection

On the basis of blue/white selection, positive clones were selected and replicas of these clones were prepared.

3.2.11.6 Colony PCR

Same set of primers, that were previously used to detect differential expressed Cd-induced genes, was used in colony PCR reaction to confirm that the transformation is correct positive.

3.2.11.7 Preparation of glycerol stocks of transformants

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

3.2.11.8 Plasmid isolation

One colony of positive transformant was inoculated in 5 ml of Luria Bertani (LB) broth containing 100 µg/ml ampicillin in sterile culture tubes. Cells were incubated in a shaker incubator (270 rpm) at 37ºC overnight (12-16 hours). QIQprep® spin miniprep kit (Qiagen) was used for plasmid isolation from the over night culture. Isolated plasmids were checked by agarose gel electrophoresis and their concentrations were determined by absorption spectroscopy.

3.2.11.9 Restriction enzyme digestion

Purified plasmids containing the differentially expressed cDNAs were digested with EcoRI restriction enzyme (Promega) for a double check of the transformation and results were analysed by agarose gel electrophoresis. These digests were also used for preparation of probes which would be used in northern blot analysis.

3.2.11.10 DNA sequence analysis

Of 18 clones, 10 clones were selected for the sequence analysis based on differential expression banding patterns. Sequence analyses were commercially provided by Iontek Company (Istanbul).

The cloned cDNA sequences were first exposed to 'VecScreen' algorithm (http://www.ncbi.nlmn.nih.gov/) to remove vector contamination. The BLAST algorithm (Altschul et al., 1990; http://www.ncbi.nlm.nih.gov) was used to analyze the DNA sequences. The identified cDNA sequences were compared with the known genes available in GenBank.

3.2.12 Northern blot analysis

Northern Blot analysis was performed according to the manufacturer's instructions (Gene images random prime labelling kit, Amersham Biosciences) to confirm whether the identified fragment bands are differentially expressed.

3.2.12.1 Preparation of the probe

The differentially expressed cDNA fragment bands were labeled using Gene images random prime labeling kit (Amersham Biosciences) according to the manufacturer's instructions and used as probes for northern blot hybridization.

3.2.12.2 Blotting , hybridization, and detection

Total RNA (25 μg) of control and Cd-treated durum wheat genotypes were prepared in solutions containing 1X MOPS buffer, 2.2M formaldehyde, 50% (v/v) formamide and were size-fractioned on a denaturing, 1.2% agarose gel containing 1X MOPS buffer, 0.66M formaldehyde and Et-Br (0.5 μg/ ml). The Et-Br stained rRNA in the different samples serves as a visual indicator of equal loading. The RNA was blotted onto nitrocellulose membrane (Schleicher&Schuell) and fixed by baking the filter at 80ºC in a vacuum oven for 2 hours. Membrane was hybridized over night at 60ºC with probe in hybridization buffer. The membrane was then washed twice with 1X SSC, 0.1 % (w/v) SDS at 60ºC for at least 15 minutes, followed by a second wash in 0.5X SSC, 0.1% (w/v) SDS at 60ºC for 15 minutes. After stringency wash, blots were incubated in anti-fluorescein-HRP conjugate solution. With a final wash with 0.1% (v/v) Tween-20 in buffer A, unincorporated antibodies were removed. Finally, the blots were incubated in detection reagent and exposed to ECL films. After 2 minutes incubation, the films were developed.

3.2.13 Real-Time Quantitative PCR with the SYBR Green I dye-based detection system

First-strand cDNA was synthesized from total RNA isolated from leaf tissue from the resistant and susceptible cultivars, as described above. Gene-specific primers were designed from cloned cDNA fragments BO4 and BO10 by using Vector NTI (Vector NTI

Advance™ 9, Invitrogen) program. The primers were purchased from IONTEK Company (Istanbul) and are listed in Table 3.2. Quantification of amplified cDNAs was performed with gene specific primers using Real Time PCR.

Table 3.2 Gene-specific primers designed for cDNAs BO4, and BO10

BO4_F	5'- GCCGATCTTAGTATTGGTGTTT- 3'
BO4_R	5'-AACCCTCACTAAATGCTGGTAGA- 3'
BO10_F	5' –TGATTCATTATGCTGAGTGATATCTT- 3'
BO10_R	5' –CGCACACGAGTCCACATA- 3'

Diluted cDNA samples (400ng) were used as templates in PCR reactions containing 25 µl of Quantitect™ SYBR® Green PCR master mix (Qiagen) and 1 µM of each primer. All reactions were set up in duplicate and subjected to real-time PCR analysis with the iCyclerİQ™ (BIO-RAD) and the SYBR Green I dye-based detection system. No-template controls were included in each PCR plate to ensure purity of reagents and minimal carryover contamination. PCR conditions are presented below:

1. Denaturation :	T = 94ºC	0:04:00 min
2. Denaturation :	T = 94ºC	0:00:30 sec
3. Annealing :	T = 52ºC	0:01:00 min
4. Extension :	T = 72ºC	0:00:30 sec
5. GOTO 4 Repeat cycle 35 times		
6. Final extension :	T = 72ºC	0:08:00 min

The threshold cycle (Ct) values of the duplicate PCRs were averaged and used for quantification of transcript. Quantification of BO4 and BO10 gene expressions was performed using Relative Standard Curve method (Giuletti et al., 2001). GADPH was chosen as a housekeeping gene, since its expression was found to be constant in different experimental conditions (i.e. same in control and Cd-treated samples) with pre-experiments. Means of the Ct values of duplicates were normalized by GADPH. GADPH

forward and reverse primers used in Real-Time PCR reactions were purchased from Qiagen (Qiagen Inc., Valencia, CA).

4 RESULTS

4.1 Plant Growth

Increasing Cd applications resulted in significant reduction in shoot and root growth in both cultivars, Balcalı-85 and Balcalı-2000. The retardation in growth was more severe in Balcalı-2000 when compared to Balcalı-85 (Figs. 4.1 and 4.2). Additionally, Balcalı-2000 displayed more severe toxicity symptoms on the leaves compared to Balcalı-85. Cd toxicity led to the development of necrotic patches on the bases and sheaths of the old leaves. With the duration of time, the leaf symptoms on old leaves of Balcalı-2000 became more severe and later on the old leaves totally collapsed. Middle-aged leaves also became necrotic (Fig. 4.3). Leaf samples collected from the plants were used in further molecular analysis.

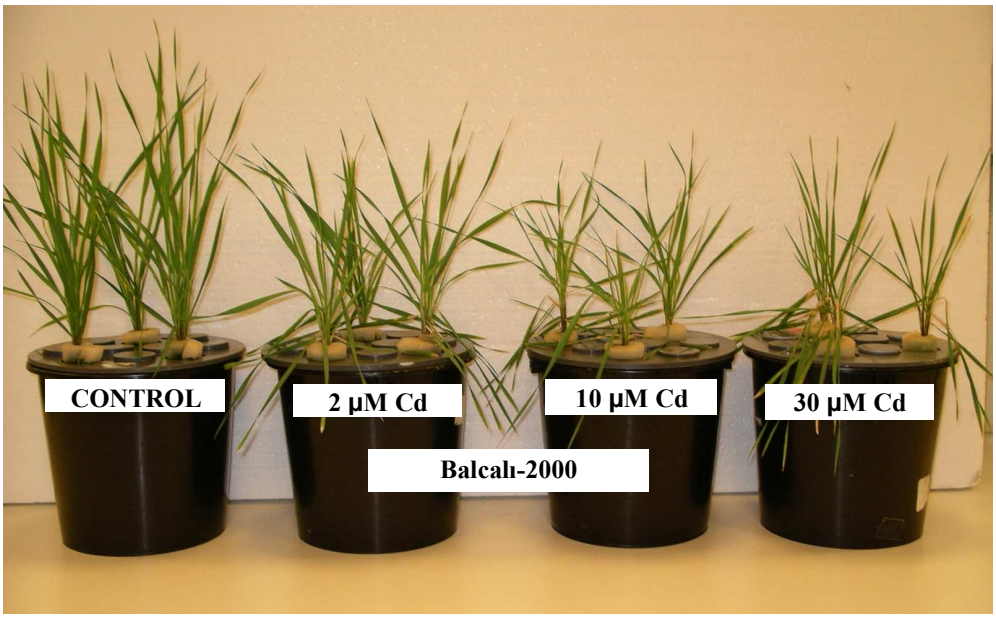

Fig. 4.1 Shoot growth of *Triticum durum* cv. Balcalı-2000 as affected by increasing supply of Cd.

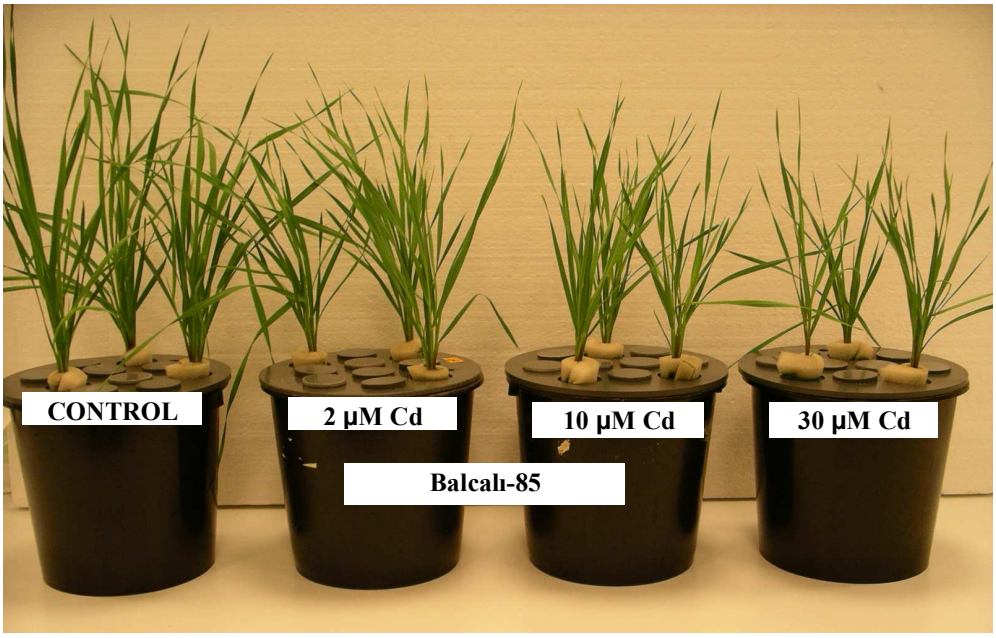

Fig. 4.2 Shoot growth of *Triticum durum* cv. Balcalı-85 as affected by increasing supply of Cd.

Fig. 4.3 Cadmium toxicity symptoms on leaves of *Triticum durum* cv. Balcalı-2000.

4.2 Identification of Cd-responsive genes by mRNA DD

The mRNA DD technique was performed to identify and isolate genes whose expression was changed in response to varying degrees of cadmium application. Equal amounts of cDNAs were synthesized from total RNA isolated from plants that had been exposed to 0, 2, 10, and 30 μM concentrations of Cd. Isolated RNA were treated with Dnase I to eliminate genomic DNA contamination. Nine 5'-arbitrary primers were used for PCR amplification in combination with the eight 3'-oligo(dT) primers (Table 3.1). These primer pairs were used to amplify cDNAs from control and Cd treated plant samples.

A total of 18 cDNA fragments were found to be differentially expressed. Only 10 cDNAs whose levels of expression were significantly altered by Cd treatment were selected for further investigation. These fragments were amplified and amplified products were labelled with α-^{33}P. The radioactively labeled PCR products were size-fractionated in 6%

polyacrylamide/8M urea gels and visualized by autoradiography. The results of the differential display experiments are depicted in Figs. 4.4-4.9. The fragment bands are designated as BO2, BO3, BO4, BO5, BO6, BO7, BO8, BO9, BO10, and BO11.

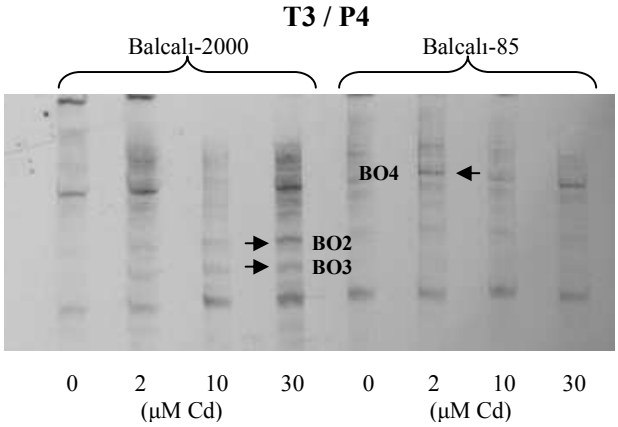

Fig. 4.4 Autoradiograph of differentially expressed cDNA bands amplified with T3/P4 primer combination upon exposure to different levels of Cd.

The fragments, BO2 and BO3 were found to be up-regulated by 30 µM Cd in Balcalı-2000. Additionally, Cd, at all doses applied, was shown to up-regulate cDNA designated as BO4 in Balcalı-85 with the same primer combination. However, this cDNA was not found in Balcalı-2000.

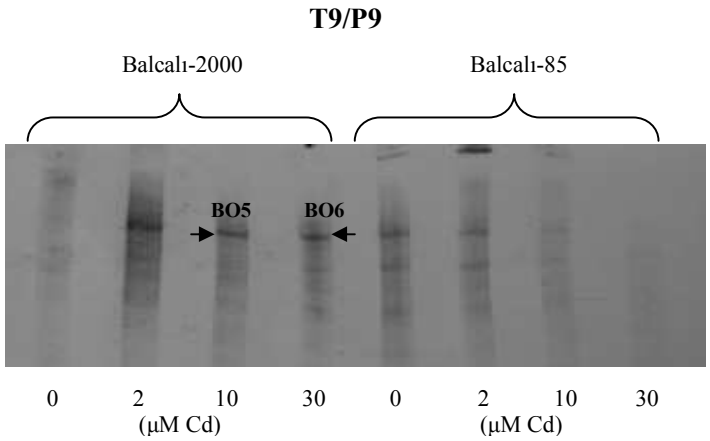

Fig. 4.5 Autoradiograph of differentially expressed cDNA bands amplified with T9/P9 primer combination upon exposure to different levels of Cd.

cDNAs, designated as BO5 and BO6, were found to be up-regulated at all levels of Cd, except control lane, in Balcalı-2000. However, the same effect was not observed in Balcalı-85. Hence the differential expression of these genes was considered to be significant and they were used for further characterization.

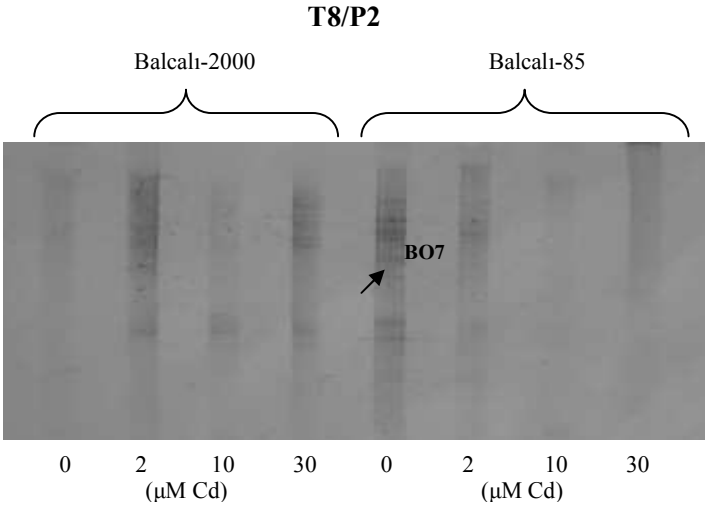

Fig. 4.6 Autoradiograph of differentially expressed cDNA band amplified with T8/P2 primer combination upon exposure to different levels of Cd.

cDNA fragment band, BO7 was found to be only fragment band present in control lane in Balcalı-85. In other words, this band was down-regulated in Cd-treated durum wheat cv. Balcalı-85. BO7 was however absent in all lanes of Cd-sensitive Balcalı-2000. Hence, we further characterized this differentially expressed cDNA.

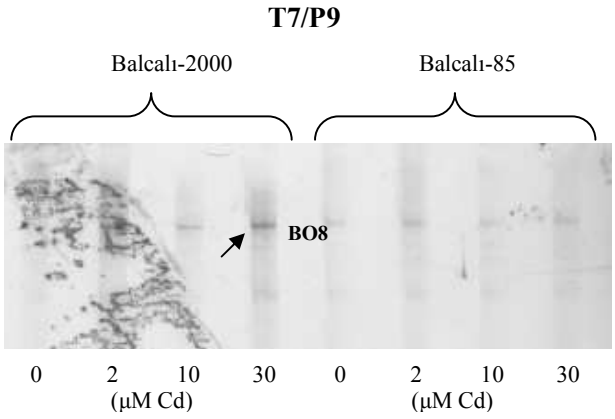

Fig. 4.7 Autoradiograph of differentially expressed cDNA band amplified with T7/P9 primer combination upon exposure to different levels of Cd.

A gradual increase was observed in expression level of BO8 with increasing Cd concentration in Balcalı-2000. In particular, with 30 µM Cd treatment, this effect was more pronounced in Balcalı-2000. However, there was no significant difference in expression level of this gene in Balcalı-85. Hence, differentially expressed cDNA fragment band, BO8, was also used for further analysis.

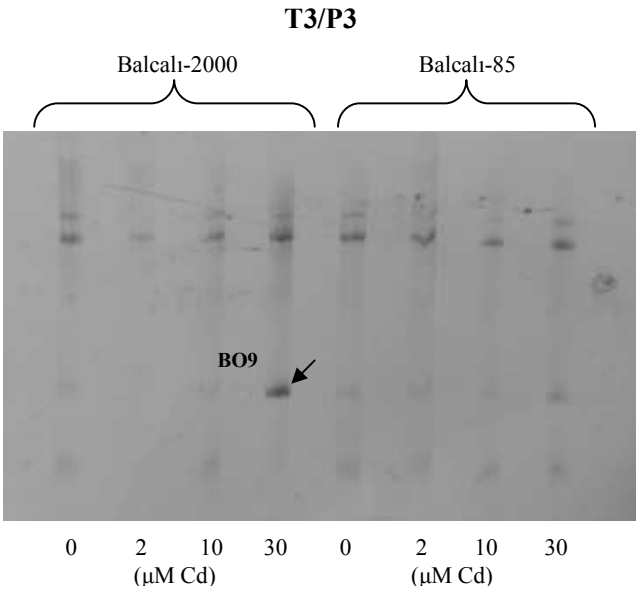

Fig. 4.8 Autoradiograph of differentially expressed cDNA bands amplified with T3/P3 primer combination upon exposure to different levels of Cd.

cDNA fragment, BO9 was found to be up-regulated in Balcalı-2000 treated with the highest Cd application (30μM). However, this effect was not detected in Balcalı-85. This result led us to elucidate this cDNA band in more detail.

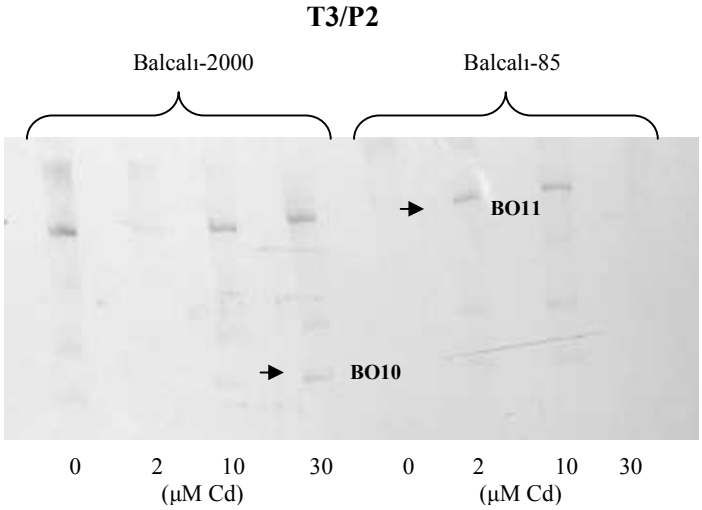

Fig. 4.9 Autoradiograph of differentially expressed cDNA bands amplified with T3/P2 primer combination upon exposure to different levels of Cd.

cDNA fragment, BO10 was found to be up-regulated in Balcalı-2000 in 30 μM Cd application. However, we were not able to detect any fragment bands in Balcalı-85. We also found that there was one up-regulated cDNA, BO11, in Balcalı-85 exposed to both 2 and 10 μM Cd. However, this cDNA band was absent in Balcalı-2000. These differentially expressed bands were selected for further analysis.

The differentially expressed cDNA bands were identified and eluted from the polyacrylamide gels. Eluted cDNA samples were reamplified with corresponding primer sets and size separated in 1% agarose gels. Amplified PCR products were extracted from the gels and cloned. To prevent non-specific amplification, first two initial rounds of the PCR cycle were excluded in reamplification reactions.

4.3 Subcloning of Cd-responsive genes in *E.coli* with PGEM-T Easy vector

Reamplified fragment bands were ligated into pGEM®–T Easy vector and transformed to *E. coli* DH5α cells. pGEM®–T Easy vector has 3'-T overhangs which enhance efficiency of ligation. Multiple cloning region of this vector contains a *lacZ* region which encodes for the enzyme *β*-galactosidase and insertional inactivation of this gene allows direct identification by blue-white selection. Positive colonies which produce incomplete *β*-galactosidase protein were white and selected for further analysis. To check and confirm that the white colonies truly contain the vectors with differentially expressed cDNAs, colony PCR reactions were performed. Products of the reactions were separated in 1% agarose gels (Figs. 4.10-4.13).

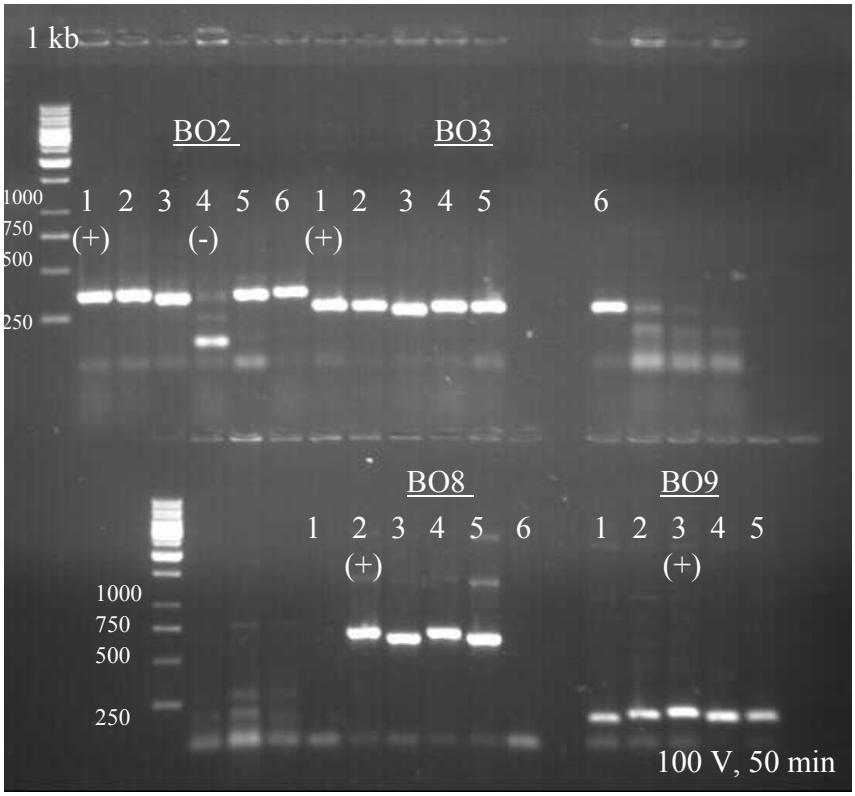

Fig. 4.10 Colony PCR analysis of clones BO2, BO3, BO8, and BO9

The clones which harbor the differentially expressed cDNAs have been indicated to be positive clones and selected for the sequencing analysis. The lanes with negative signs were the clones which lack the inserts of interest.

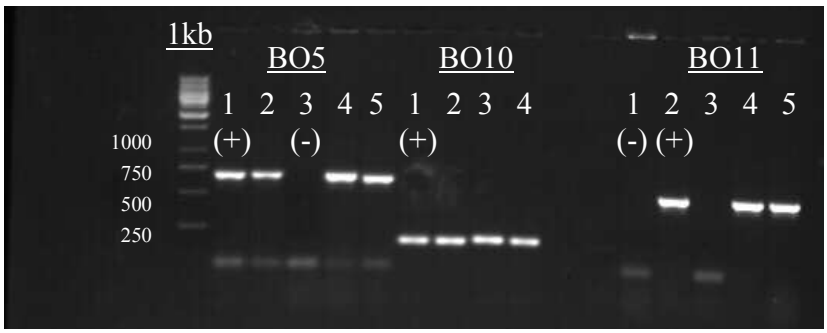

Fig. 4.11 Colony PCR analysis of clones BO5, BO10, and BO11

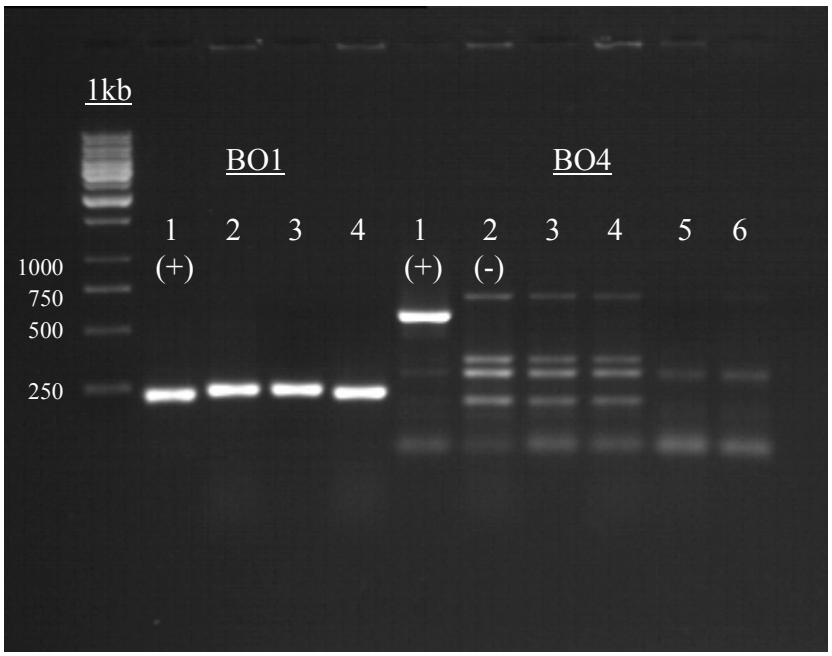

Fig. 4.12 Colony PCR analysis of clones BO1, and BO4

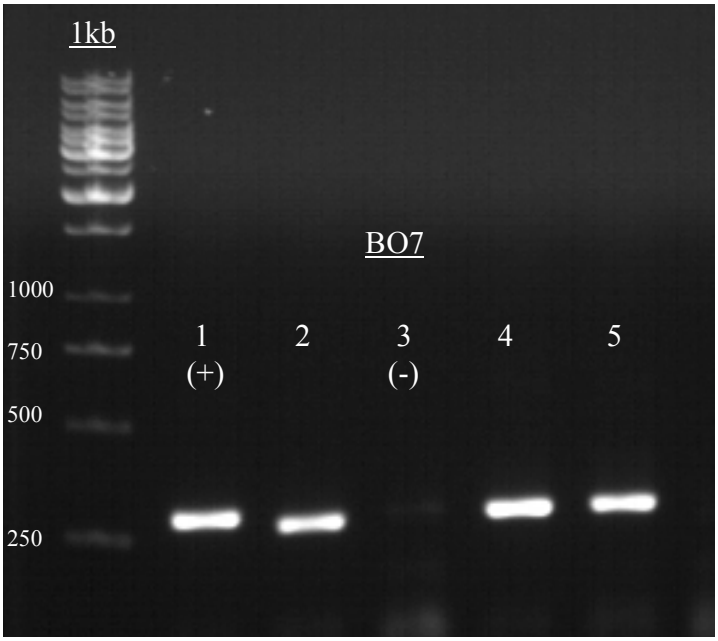

Fig. 4.13 Colony PCR analysis of clone BO7

After selection of positive clones, vectors harboring the inserts were isolated from these colonies for the following sequence analysis. Additionally, glycerol stocks of these colonies were prepared for long-term storage for further studies. Isolated plasmids were run on 1% agarose gels at 100V for 50 minutes to check whether the plasmids were isolated successfully (Figs 4.14-4.16). To determine concentration and purity of plasmid DNA, optical densities were measured at 260 and 280 nm using spectrophotometer (Varian, Australia)

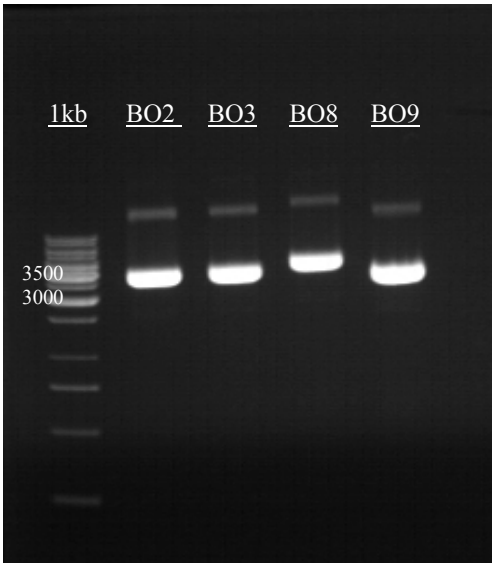

Fig. 4.14 Agarose gel analysis of minipreps for BO2, BO3, BO8, BO9

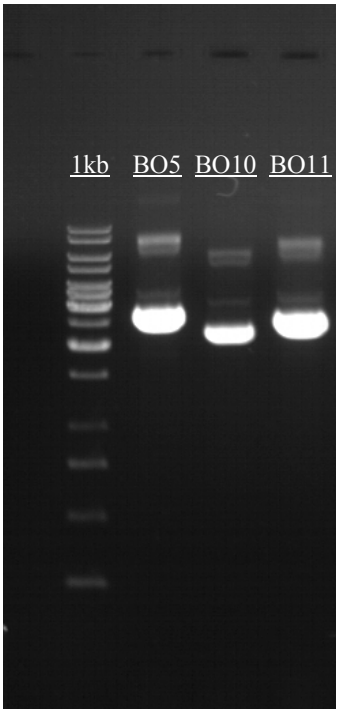

Fig. 4.15 Agarose gel analysis of minipreps for BO5, BO10, BO11

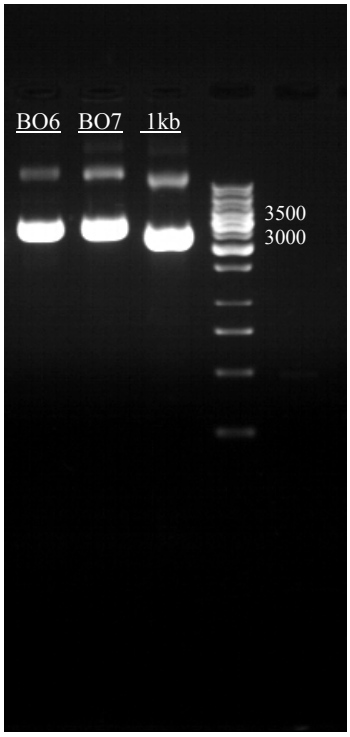

Fig. 4.16 Agarose gel analysis of minipreps for BO4, BO6, BO7

4.4 Sequencing and characterization of cDNA clones

The differentially expressed positive cDNA clones were sequenced using pUC/M13 forward primer. The nucleotide sequences obtained in this study were compared against NCBI (http://www.ncbi.nlm.nih.gov/) and EMBL (http://www.embl-heidelberg.de/) to identify homology with the known or putatively annotated genes. Results of this analysis are presented in Table 4.1.

Table 4.1. BLASTN search results of Cd-regulated cDNAs isolated by differential display

Clone	Sequence Homology	Effect of cadmium	E-value	NCBI accession no.
BO2	HCBT-like defense response protein gene	+	2e-7	AF446141
BO3	Unknown	+		
BO4	NADH dehydrogenase sub-unit A gene	+	6e-80	HVU010976
BO5	Receptor kinase gene	+	3e-21	AF509776
BO6	Receptor kinase gene	+	1e-23	AF509776
BO7	Unknown	-		
BO8	Receptor kinase gene	+	9e-28	AF509776
BO9	Unknown	+		
BO10	Receptor kinase gene	+	1e-60	AY368673
BO11	Photosystem I 8kDa sub-unit gene	+	2e-93	X13158

Nucleotide sequence sizes of the differentially expressed cDNAs and the primer combinations used in the mRNA DD PCR reactions are listed in Table 4.2.

Table 4.2 Sizes of the differentially expressed cDNA fragment bands and primer combinations used in mRNA DD.

Clone	Size of PCR product (bp)	Primers used in PCR
BO2	328	T3/P4
BO3	387	T3/P4
BO4	610	T3/P4
BO5	726	T9/P9
BO6	728	T9/P9
BO7	327	T8/P2
BO8	729	T7/P9
BO9	233	T3/P3
BO10	222	T3/P2
BO11	585	T3/P2

Among these ten differentially expressed cDNA clones, BO4 and BO10 were selected for Northern blot and Real Time PCR analysis. The primary reason for the selection of these two for further analysis was that there was a significant homology to genes which encode for proteins that are essential for Cd stress mechanisms in plant cells.

4.5 Isolation of differentially expressed cDNA fragments from the subcloning vector

Since our subcloning vector (pGEM-Teasy) has two *Eco*RI recognition sites, selected differentially expressed cDNA fragments were isolated from the vectors by digesting with *Eco*RI. Inserts were checked after isolation from 1% agarose gels for probe synthesis which was used in northern blot analysis (Fig. 4.17).

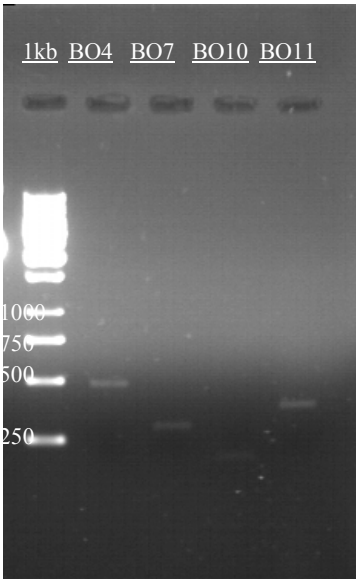

Fig. 4.17 Agarose gel showing digests, BO4 and BO10, to be used in probe synthesis for northern blot analysis.

4.6 Northern blot analysis

Northern blot analysis was carried out to affirm that the banding patterns of identified cDNA fragments represent the true differences in gene expression and are not due to non-specific amplification in PCR reactions. In other words, to eliminate the probability that the genes determined to be differentially expressed are false positives, northern blot analysis was performed. Using total RNA isolated from control and Cd-treated plant samples of *T. durum* and fluorescently-labelled cDNA as probes for hybridization, we found that the cDNA clone, BO4 was up-regulated in Balcalı-85 with the 2 μM Cd application when compared to control. Et-Br stained rRNA was used as a loading control (Fig. 4.18).

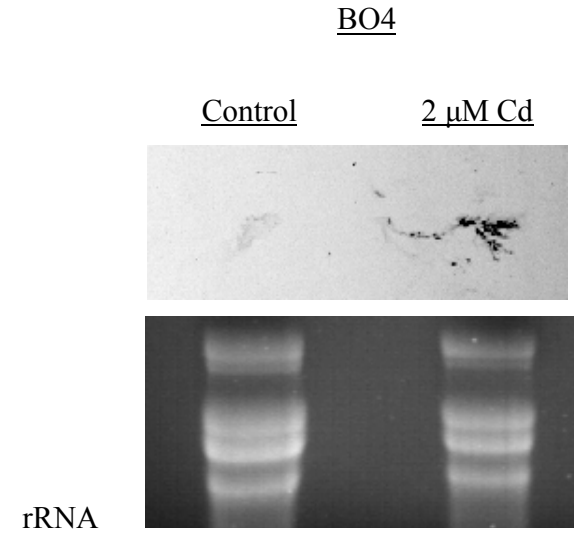

Fig. 4.18 Northern blot analysis of total RNA from control and 2 µM Cd treated plants. Total RNA isolated from control and Cd-treated *T. durum* plants was subjected to northern blot hybridization with fluorescently-labelled cDNA probes obtained from the BO4 cDNA fragment bands. The Ethidium Bromide-stained ribosomal RNA (rRNA) in the different samples served as an indicator of equal loading.

4.7 Real Time PCR analysis of differentially expressed genes

Real time PCR was performed to quantify the expression levels of cDNA clones whose expression levels were altered significantly with varying degree of Cd application. The two cDNA clones (BO4 and BO10), involved in Cd response mechanism were selected for confirmation of differential expression. cDNA clone, BO4 showed significant homology to *NADH dehydrogenase* subunit A, and BO10 displayed significant homology to a receptor kinase gene with E values of $6e^{-80}$, and $1e^{-60}$, respectively. Gene-specific primers BO4_F and BO4_R (Table 3.2) were used in Real-Time PCR reactions to quantify expression of cDNA, BO4, identified in 0 and 2 µM Cd treated plant samples of Balcalı-85. Gene-specific primers BO10_F and BO10_R (Table 3.2) were used in Real-Time PCR reactions to quantify expression of cDNA, BO10, identified in 0 and 30 µM Cd treated plant samples of Balcalı-2000. Ct (Threshold cycle) vs PCR Base Line Subtracted CF RFU graphs of BO4 and BO10 are shown in Figs. 4.19-4.20.

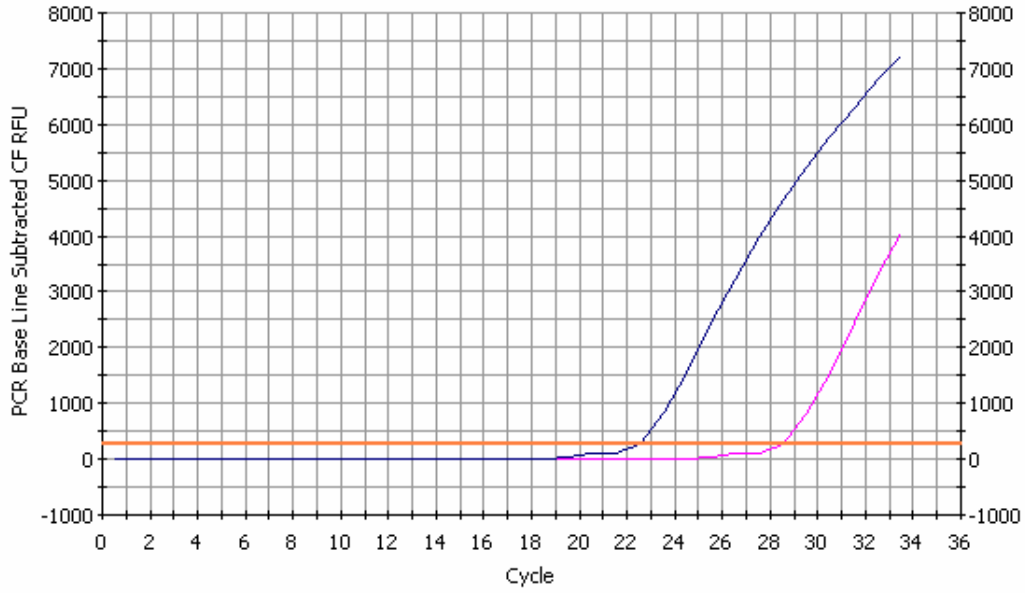

Fig. 4.19 Real Time PCR analysis of differently expressed BO4 gene from Balcalı-85. Ct vs PCR Base Line Subtracted CF RFU graph of BO4 cDNA clone. Amplification of BO4 starts at an earlier cycle (Mean Ct = 22.25) in Balcalı-85 treated with 2 µM Cd (Dark blue line). In control plants, amplification of BO4 starts later (Mean Ct = 28.4) (Pink line).

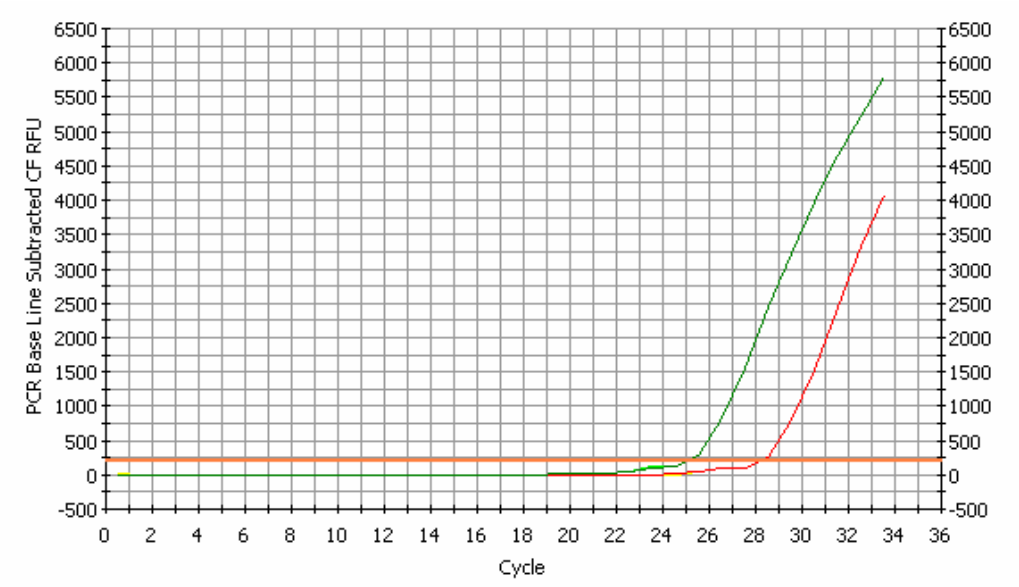

Fig. 4.20 Real Time PCR analysis of differently expressed BO10 gene from Balcalı-2000. Ct vs PCR Base Line Subtracted CF RFU graph of BO10 cDNA clone. Amplification of BO10 starts at an earlier cycle (Mean Ct = 25.2) in Balcalı-2000 treated with 30 µM Cd (Green line). In control plants, amplification of BO10 starts later (Mean Ct = 28.2) (Red line).

Serial dilutions of cDNA from control and Cd-treated plants were amplified using GADPH primers to construct a standard curve (Fig. 4.21).

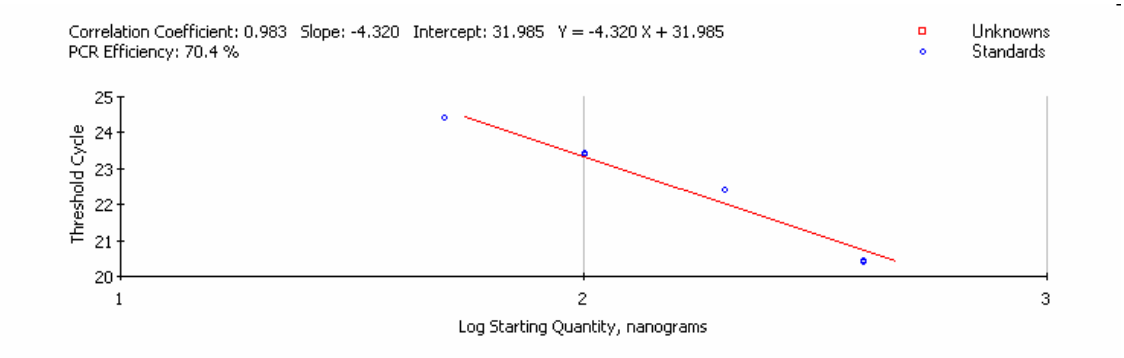

Fig. 4.21 Standard curve for GADPH.

Since, amplification of GADPH started at same cycles in both Balcalı-85 and Balcalı-2000, with or without Cd application, the standard curve depicted in Fig. 4.21 was utilized for quantification of BO4 and BO10 genes expression using Relative Standard Curve method (Giuletti et al., 2001). Results are depicted in graphs below (Figs. 4.22-4.23).

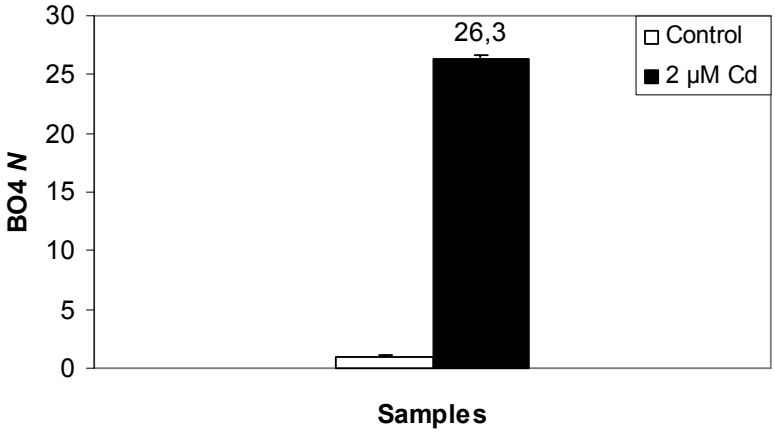

Fig. 4.22 Expression patterns of BO4 gene in Balcalı-85 with 0 and 2 µM Cd application monitored by Real-Time Quantitative PCR. The subscript N at the vertical axis indicates relative (to the calibrator, control, taken as 1.00) transcript level of target gene, BO4, normalized by GADPH. Mean fold difference (Cd-treated/Control) in target-gene transcript level is indicated above the Cd-treated sample bar in the graph. Standard deviations of the mean (of two replicate PCR reactions) are indicated by vertical lines on the control and Cd-treated sample bars.

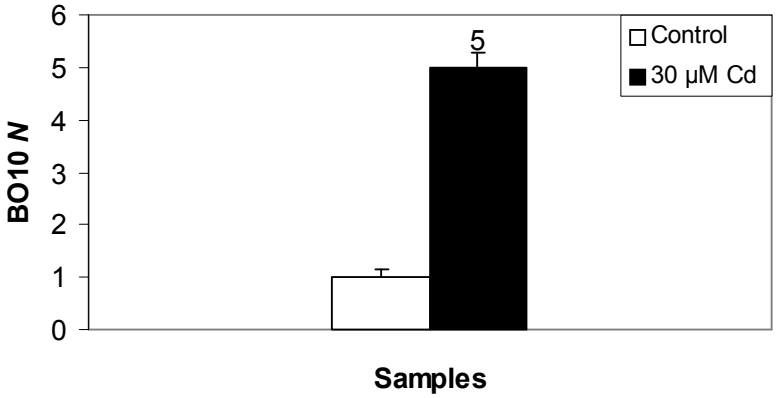

Fig. 4.23 Expression patterns of BO10 gene in Balcalı-2000 with 0 and 30 µM Cd application monitored by Real-Time Quantitative PCR. The subscript N at the vertical axis indicates relative (to the calibrator, control, taken as 1.00) transcript level of target gene, BO10, normalized by GADPH. Mean fold difference (Cd-treated/Control) in target-gene transcript level is indicated above the Cd-treated sample bar in the graph. Standard deviations of the mean (of two replicate PCR reactions) are indicated by vertical lines on the control and Cd-treated sample bars.

As shown in graphs above, Real-Time PCR data confirm the differentially expressed genes BO4, and BO10. The expression level of BO4 gene identified in Balcalı-85 treated with 2 µM Cd was approximately 26.3-fold greater compared to control. In Balcalı-2000, the expression level of BO10 was 5-fold greater than that of control when the highest level of Cd applied.

5 DISCUSSION

Durum wheat is generally more sensitive to Cd and accumulates more Cd than bread wheat (Hart et al., 1998) Therefore, it is important to study genetic variation i)in tolerance to Cd toxicity, and ii) root uptake and shoot accumulation capacity for Cd between durum wheat cultivars. Additionally, availability of durum wheat genotypes with different level of tolerance to this heavy metal, will give opportunity for researchers who are interested in understanding the underlying physiological and cellular mechanisms of which plants develop to cope with this metal. Until now, numerous studies have reported the physiological effects of this heavy metal on plant systems (Krupa, 1988, Siedlecka and Baszynsky, 1993, Barcelo and Poschenrieder, 1990). However, Cd stress-dependent molecular mechanisms have not been examined in detail, particularly by comparing two plant genotypes differing greatly in tolerance to Cd toxicity.

Here, we aimed to identify genes whose expression may be influenced by Cd exposure in two durum wheat cultivars, which show differential Cd tolerance. For the identification and isolation of differentially expressed genes, mRNA differential display technique was used. This method has several advantages over other gene identification methods. First of all, it is a rapid method to identify genes that are differentially expressed between two cellular populations, or within a single cell type under altered conditions (Liang and Pardee, 1992; Ito et al., 1994). Secondly, it is based on simple, well established, and widely used techniques. Another important advantage of this technique is that it can simultaneously screen for both up-regulated and down-regulated transcripts in multiple cell populations. Additionally, it is a very sensitive method that it can detect rare mRNA transcripts. Furthermore, small amount of RNA is needed for this technique (Lievens et al.,

2001). Besides its advantages, it has, however, a few drawbacks, such as low reproducibility of fingerprints and formation of non-specific products and 'false positives'.

mRNA differential display method was performed to compare changes in gene expression patterns of two durum wheat genotypes. We focused our attention to the effects of Cd on transcriptome. For this purpose 4 different doses of Cd (no Cd, 2 μM Cd, 10 μM, 30 μM) were applied to plants grown in hydroponic culture, and alterations in gene expression in response to Cd applications were studied. From this examination, 10 cDNAs were identified whose levels of expression were altered significantly after Cd application in comparison to that of control plants with no Cd treatment. These cDNA fragment bands were cloned, sequenced, and identified. The homology search of genes against the NCBI and EMBL Sequence Data-bases was performed. The BLAST algorithm (Altschul et al., 1997; http://www.ncbi.nlm.nih.gov) was used to analyze the DNA sequences. Of these 10 cDNA fragments, two (BO4 and BO10) were successfuly validated by Northern blot and Real-Time PCR analysis.

The first gene identified in our study is BO2 and is up-regulated by 30 μM Cd application in Cd-sensitive durum wheat genotype, Balcalı-2000. BLASTN search revealed that it has significant homology to a gene identified in *A. tauschii* which encodes for a *HCBT*-like putative defense response protein (Brooks et al., 2002). *HCBT* catalyzes the biosynthesis of unique dianthramide phytoalexins (Yang et al., 1998). Fungal pathogen elicitors induce transcription of *HCBT* and, dianthramide synthesis is enhanced to resist fungal pathogens. Hence, accumulation of these biological compounds is essential for the resistance of plants to fungal pathogens. Identification of a gene with great homology to *HCBT*-like disease resistance protein in our study indicates that biotic and abiotic stress resistance proteins share common motifs. This data also provides evidence for higher level of genetic complexity associated with plant resistance to abiotic stress factors.

Another gene identified in our analysis is BO4, and is up-regulated by 2 μM Cd application in Cd-tolerant durum wheat genotype, Balcalı-85. It was found to have significant homology to a gene encoding for a *NADH dehydrogenase* subunit A. The

physiological function of this enzyme is unclear, but it is known to play important role in responses to various plant stress mechanisms (Geisler et al., 2004). Recently, *NADH dehydrogenase* gene expression upon Cd exposure was also reported in a marine alga *Nannochloropsis oculata* (Kim et al., 2005). Hence induction of *NADH dehydrogenase* gene appears to be involved in Cd tolerance. Northern blot analysis and Real-Time PCR results also confirmed that this gene is up-regulated approximately 26.3-fold in Balcalı-85 treated with 2 μM Cd in comparison to control plants.

Other genes shown to be up-regulated by Cd in Balcalı-2000 with the 10 μM Cd application are BO5 and BO6. Sequence analysis indicated significant homology to the barley stem-rust resistance gene, *Rpg1*, which encodes for a receptor kinase. This gene is a novel type of plant disease-resistance gene and was first identified in *H. vulgare* (Brueggeman et al., 2002). The *Rpg1* gene encodes for a receptor kinase-like protein with two tandem protein kinase domains, which is a novel structure for a plant disease-resistance gene. Homolog of this protein is also present in rice and wheat (Brueggeman R., 2002). The *Rpg1* gene product has an N-terminal domain which does not resemble any of the previously identified receptor-kinases. Identification of this type of kinase in response to an abiotic stress factor, Cd stress, is noted to be the first in literature. This result suggests a common feature between Cd and disease responses. This finding also supports the hypothesis that signaling is mediated through a protein phosphorylation cascade in response to both biotic and abiotic stress factors.

Another gene whose expression is enhanced in Balcalı-2000 with 30 μM Cd application is BO8. This gene also has significant homology to *Rpg1* gene which encodes for receptor kinase, mentioned above. Obtaining the same result with another primer set affirms that this gene is surely differentially expressed in response to varying levels of Cd.

BO10 is also identified to be an up-regulated gene in Balcalı-2000 with the 30 μM Cd application. This gene matches significantly with a gene located on HMW-glutenin locus in *T. turgidum*. It encodes for a protein receptor kinase. It has been reported that this receptor kinase protein possess Leucine Rich- Repeat (LRR) region which is a fingerprint

for one group of plant-resistance genes (Kong et al., 2003). This LRR receptor kinase showed significant homology to the A2 class (Wang et al., 1998) of the *Xa21* gene family of rice (Song et al., 1997) that confers resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae*. Our result suggests that this motif also present in receptor kinases which are induced in response to an abiotic stress factor, Cd. It also confirms that plants respond rapidly to Cd by activating signal transduction pathways which may include a protein phosphorylation cascade. Real-Time PCR data also validated that this gene is up-regulated approximately 5-fold in Balcalı-2000 with the highest level of Cd application in comparison to control plants.

BO11, identified as an up-regulated gene in Balcalı-85 with the 2 μM Cd application has significant homology to *PsaC* gene. This gene encodes for photosystem I 8 kDa sub-unit. This result is not surprising, because one of the heavy metal tolerance mechanisms developed by plants is activation or modification of plant metabolism to allow adequate functioning of metabolic pathways and thus rapid repair of damaged cell structures. Cd is shown to damage photosystems I and II in plants (Siedlecka and Baszynsky, 1993). To alleviate the harmful effect of the metal on photosynthetic apparatus, plants induce expression of *PsaC* gene which encodes for a subunit of photosystem I. Plants respond in such a way in order to overcompensate for decrease in photosynthesis rate. Photosynthesis is essential for proper growth of plants. Therefore, induction of structural genes upon Cd exposure may be interpreted as a response to allow adequeate functioning of photosynthetic machinery.

Expression of the remaining genes BO3, BO7, and BO9 was also altered in response to Cd application, in our study. Unfortunately, the functions of these genes are unknown. These genes did not have significant homology to previously identified genes in data-bank.

6 CONCLUSION

Using the mRNA differential display technique, 10 genes were identified whose expression is affected by Cd application. Our data suggest that Cd alters expression of different genes, either by induction or repression. In other words, this metal has multiple effects at gene level. Even though the results of this work do not explain all possible alterations in gene expression induced by Cd, they shed new light on the changes in response to Cd at gene level. Additionally, this report is the first to identify, clone, and sequence Cd-responsive genes in *T. durum*. The way Cd affects gene expression is still unclear. However, it is apparent that more than one mechanism is responsible for gene activation/inhibition and Cd induces damage to cellular mechanisms through a complex network of biochemical events. For example, Cd at high concentrations can induce Fe or Zn deficiency at physiological levels and thus affects expression of genes associated with Fe or Zn deficiency in plants (Siedlecka and Baszynsky, 1993)

Northern blot and Real-Time PCR analysis demonstrated that the pattern of bands obtained represented true differences in gene expression and are not artifacts of the PCR amplification process.

The differentially expressed bands were cloned into PGEM®-T Easy and stocked in glycerol for future studies, such as 5'- 3' RACE, and functional expression studies. Further functional analysis of identified genes will bring out the molecular mechanisms of heavy metal responses of higher plants.

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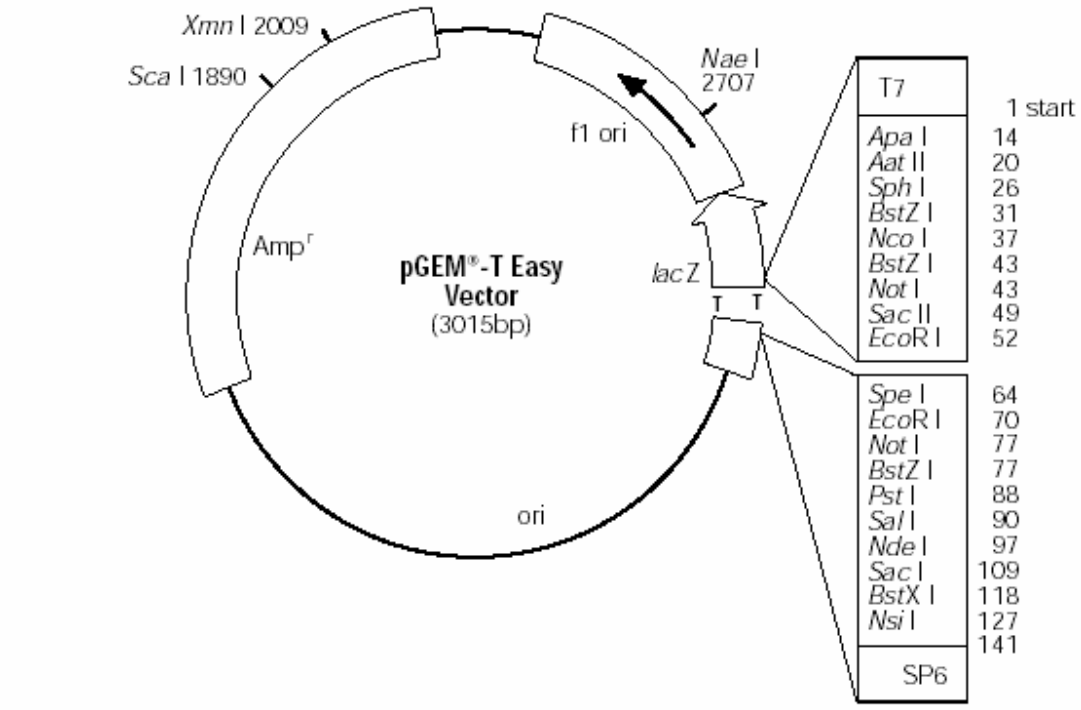
APPENDIX A

PGEM®-T Easy vector circle map and sequence reference points

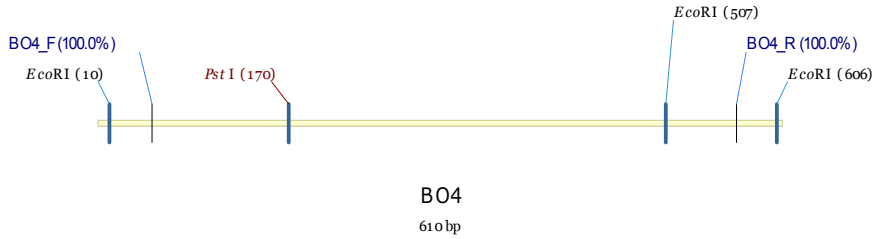


pGEM®-T Easy Vector sequence reference points:

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

APPENDIX B

Primer binding and restriction sites of BO4



EcoRI BO4_F 100.0%

1 GGCCGCGGGAATTCGATTCA TTATGCTGAG TGATATCTT T TT TT TTAGCC GATCTTAGTA TTGGTGTTTT TTATGGATTG CCATTTAAAG TATAGCTCCT
CCGGCGCCCT TAAGCTAAGT AATACGACTC ACTATAGAAA AAAAAATCGG CTAGAATCAT AACCACAAAA AATACCTAAC GGTAAATTTC ATATCGAGGA

PstI

101 ATTGGTC TTC TTATGGCGGG ATATAGCTCA AATAAAAAAT ATTCT TT TTC AGGCGGTCTA CGAGCTGCAG CTCAATCCAT TAGTTATGAA ATACCATTAA
TAACCAGAAG AATACCGCCC TATATCGAGT TTATT TT TTA TAAGAAAAAG TCCGCCAGAT GCTCGACGTC GAGTTAGGTA ATCAATAC TT TATGGTAATT

201 CTT T TTGTG T GCTAGCAATA TC TCTACGTG TGATTCG TTA AAATGGATCT TT TTCC TATA AAATCCATTA ACTATTTATA TTCCTT TT CT TATTTAGTAT
GAAAAACACA CGATCG TTAT AGAGATGCAC ACTAAGCAAT TTTACCTAGA AAAAGGATAT TTTAGGTAAT TGATAAATAT AAGGAAAAGA ATAAATCATA

301 TTGGGT TGGT AAGTTAAACT AGATAGCTAT ATGAGTGAAA CAAAACAGCT TATAAATTTG TAGTAAAAAG AAAAAATCTC ATT T CCTACG TACAAGAAAA
AACCCAACCA TTCAA TTTGA TC TATCGATA TACTCACTTT GT T TTGTCGA ATATTTAAAC ATCATT TT TC TT TT TTAGAG TAAAGGATGC ATGTTC TT TT

401 AAGTGGAAGT AAACATAAGC AGTGTAAACT CT TT ATCCCA AGGTTGATAT TT TT TAATTA GTCATCATAT CTTGAAGCGG CCAAGAA TAA AGGATTCACG
TT CACCTTCA TTT GTA TTCG TCACA TTTGA GAAATAGGGT TCCAAC TATA AAAAATTAAT CAGTAGTATA GAAC TTCGCC GGTTC TT ATT TCC TAAGTGC

EcoRI

501 ATATGGAATT CCA TTACTAG AATATT CCTA GTTA TTATAT TACTATAACT TAATAATCCA TAAGAAGAAT CTACCAGCAT TTAGTGAGGGTTAAAATCAC
TATA CCTTAA GGTAATGATC TT ATAAGGAT CAATAATATA ATGATATTGA ATTAT TAGGT ATTCTT C TTA GATGGTCGTAAATCAC TCCCAATT TTAGTG

EcoRI BO4_R 100.0%

601 TAGTGAATTC
ATCACTTAAG

Primer binding and restriction sites of BO10


```
            EcoRI             BO10_for 100.0%
           ~~~~~~~          ~~~~~~~~~~~~~~~~~~~~~~~~
  1  GGCCGCGGGA ATTCGATTAT TAACCCTCAC TAAATCGGTT CATAGACAAG TGAAAGTGGA TACTCTAAAA TACGCAAGAT AAGCGTGAGT GCTATGGATG
     CCGGCGCCCT TAAGCTAATA ATTGGGAGTG ATTTAGCCAA GTATCTGTTC ACTTTCACCT ATGAGATTTT ATGCGTTCTA TTCGCACTCA CGATACCTAC

                           BamHI
                           ~~~~~~~
                                                              ~~~~~~~~~~~~~~~~~~~~
                                                               BO10_rev 100.0%
101  GCGTTCTCGT AGGGAGACGG GAGCGGATCC ATAGTGGTGT ATTGATATGG TGAATATGTG GACTCGTGTG CGCCACCTAA AAAAAAAGAT ATCACTCAGC
     CGCAAGAGCA TCCCTCTGCC CTCGCCTAGG TATCACCACA TAACTATACC ACTTATACAC CTGAGCACAC GCGGTGGATT TTTTTTTCTA TAGTGAGTCG
                         EcoRI
                      ~~~~~~~
201  ATAATGAATC ACTAGTGAAT TC
     TATTACTTAG TGATCACTTA AG
```

APPENDIX C

Autoclave:	Hirayama, Hiclave HV-110, JAPAN
Balance:	Sartorius, BP 221 S,
	Schimadzu, Libror EB-3200 HU, JAPAN
Centrifuge:	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
Cassette:	Kodak Biomax MS casette with intensifying screen, USA
Deep-freeze:	-80ºC, Thermo Electron Corporation, USA
	-20ºC, Bosch, TURKEY
Deionized water:	Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Biogen Inc., USA
	Biorad Inc., USA
	SCIE-PLAS, TURKEY
Gel documentatiton:	UVITEC, UVIdoc Gel Documentation System,UK
	BIO-RAD, UV-Transilluminator 2000, USA
Gel dryer:	E-C corp., EC 355 gel dryer, USA

Heating block: Bioblock Scientific, FRANCE

Hybridization oven: Shel Lab, Model 1012 hyb. oven, USA

Ice machine: Scotsman Inc., AF20, USA

Incubator: Memmert, Modell 300, GERMANY

Memmert, Modell 600, GERMANY

Laminar flow: Kendro Lab. Prod., Heraeus, Herasafe HS12, GERMANY

Magnetic stirrer: VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY

VELP Scientifica, Microstirrer, ITALY

Micropipette: Gilson, Pipetman, FRANCE

Eppendorf, GERMANY

Microwave Oven: Bosch, TURKEY

pH meter: WTW, pH540 GLP Multical®, GERMANY

HANNA, pH213 microprocessor pH meter, GERMANY

Power Supply: Wealtec, Elite 300, USA

Biogen, AELEX, USA

Real-Time detection system: BIO-RAD, iCyclerİQ™ Multicolor Real-Time Detection System,

Refrigerator:	+4º, Bosch, TURKEY
Shaker:	GFL, Shaker 3011, USA
	New Brunswick Sci., Innova™ 4330, USA
Spectrophotometer:	VARIAN, Cary 300 Bio Uvi-visible spec., AUSTRALIA
Speed vacuum:	Savant, Refrigerated Vapor Trap RVT 400, USA
Thermocycler:	PE Applied biosystems, GeneAmp PCR System 9700, USA
	MJ Research, PTC-100, USA
Water bath:	TECHNE, Refrigerated Bath RB-5A, UK
	JULABO, TW 20, USA