BIOLOGIA PLANTARUM 52 (4): 703-708, 2008

Differential expression of wheat transcriptomes in response to varying cadmium concentrations

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Abstract

This study aims to understand the changes in the transcriptome of durum wheat (*Tricitum durum* cv. Balcali-85) upon exposure to varying Cd concentrations using mRNA differential display (mRNA DD) technique. Sequence analyses of the two heavily induced genes upon exposure to Cd showed high homology to *NADH dehydrogenase subunit 1* (EC907725) and *PsaC* gene encoding a photosystem 1 (PS 1) 9 kDa subunit protein (EC907731). Additionally, three differentially expressed genes (EC907726, EC907729 and EC907730) were identified. Their sequence analyses revealed no significant homologies to known genes. The expressions of *NADH dehydrogenase subunit 1* and *PsaC* genes were confirmed by Northern blot analysis and quantified by real time PCR. This is the first report for the induction of *NADH dehydrogenase subunit 1* gene during Cd stress in wheat.

Additional key words: cadmium-responsive genes, functional genomics, mRNA differential display, *Triticum durum*.

Introduction

Cadmium is a highly toxic heavy metal and an important environmental pollutant (Liu *et al.* 2007b). Its exposure causes several physiological and metabolical changes in plants including decreased shoot and root growth, leaf chlorosis, changes in respiration and nitrogen metabolism and oxidative stress (Agrawal and Sharma 2006, Drajić *et al.* 2006, Scebba *et al.* 2006, Liu *et al.* 2007a). Cd has been reported to interfere with photosynthesis by damaging the photosynthetic apparatus, especially by competitively binding to essential calcium site of photosystem 2 (Krupa 1988, Hodoshima *et al.* 2006) and by altering the activities of photosystem 1 and 2 (Siedlecka and Baszynsky 1993). In *Brassica napus*, Cd treatment was proven to reduce total concentration of chlorophyll and carotenoid, and led to an increase in the non-photochemical quenching (Larsson *et al.* 1998). In addition, Cd was shown to induce expression of NADH dehydrogenase subunit 9 in a marine alga, *Nannochloropsis oculata* (Kim *et al.* 2005). The physiological functions of this enzyme are not clear; however it is known to be involved in several stress responses (Geisler *et al.* 2004). Furthermore, Cd has been reported to interact with water balance through inhibition of stomatal opening in leaves where it exerts this effect *via* interfering with movements of K^+, Ca^{2+}, and abscisic acid into guard cells (Barceló and Poschenrieder 1990).

Cd has clear damaging effects on vital cell constituents. 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 inhibits the activity of that protein (Hall 2002). Cd^{2+} is capable of replacing 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). 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 may interfere with the transcription and signal transduction mechanisms. Cd has been reported to alter RNA levels, to inhibit the activity of ribonuclease (Shah and Dubey 1995) and to stimulate the formation of free radicals and reactive oxygen species (ROS), resulting in oxidative stress (Tiryakioglu *et al.* 2005, Rodriguez-Serrano *et al.* 2006).

Plants have evolved a variety of mechanisms to tolerate the harmful effects of Cd inside the cells. These include synthesis of phytochelatins (PC), cysteine-rich peptides, which are able to chelate Cd heavy metal

Received 28 February 2007, *accepted* 7 August 2007.
Abbreviations: Ct - threshold cycle; GADPH - glyceraldehyde-3-phosphate dehydrogenase; GSH - glutathione; mRNA DD - mRNA differential display; PC - phytochelatin; PS 1 - photosystem 1; RT-PCR - reverse transcriptase polymerase chain reaction .
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and sequestration of the Cd-complexes in a safe compartment inside the cell, especially vacuoles, by means of Cd-PC complexes. PC synthase catalyzes PC biosynthesis and requires GSH or GSH-like peptides with blocked thiol groups for its activity (Vatamaniuk *et al.* 2000). Cd-PC complexes are transported into vacuole through a vacuolar ABC type ATP-binding cassette transporter (Ortiz *et al.* 1995). The expression of these mechanisms under Cd stress differs greatly among the plant species, and the genotypes of a given plant species (Grant *et al.* 1998, Hart *et al.* 1998, Ozturk *et al.* 2003). 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 tolerance to Cd toxicity to achieve a better human nutrition and health.

To our knowledge, there is no study in the literature reporting Cd-induced alterations in the transcriptome of wheat. In this study, a durum wheat cv. Balcali-85, generally more tolerant to Cd and known to accumulate more Cd than that of bread wheat (Liang and Pardee 1992, Grant *et al.* 1998, Hart *et al.* 1998), was used to screen for genes that alter their expression levels upon three different doses of Cd-treatments by using mRNA differential display (mRNA DD). This technique is a rapid and sensitive method to detect heavily expressed and rare mRNA transcripts that are differentially expressed between two cellular populations, or within a single cell type or in multiple cell populations using small amount of RNA (Liang and Pardee 1992, Ito *et al.* 1994, Lievens *et al.* 2001).

Materials and methods

Plant growth conditions and cadmium treatments: Wheat (*Tricitum durum* cv. Balcali-85) seeds were surface sterilized with 1 % NaOCl and germinated in *Perlite* moistened with saturated $CaSO_4$ for 5 d. Seedlings were then transferred to continuously aerated Hoagland's solution and grown under controlled conditions (16-h photoperiod, temperature 24/22 ºC, relative humidity 60/70 %, and photon flux density of 600 - 700 µmol m^{-2} s^{-1}) for 7 d. Plants were then treated with 0, 2, 10, or 30 µM Cd in the form of $CdSO_4$ for 7 d and grown with control plants under the same conditions. When the symptoms of Cd treatment became visible (like reduction in shoot elongation and development of necrotic patches on the older leaves), leaf tissues were sampled, immediately frozen in liquid nitrogen and stored at -80 ºC until analysis.

Total RNA isolation: Total RNA was isolated from 0.2 g leaf tissue using *Trizol* reagent (*Invitrogen*, Carlsbad, CA, USA) according to protocols outlined by Budak *et al.* (2006). Isolated RNA were then treated with DNaseI and ethanol precipitated before the determination of concentration and purity spectrophotometrically.

cDNA synthesis, mRNA differential display and subcloning of induced fragments: cDNA was synthesized from 3 µg total RNA using *Omniscript* Reverse Transcription kit (*Qiagen*, Valencia, CA, USA) according to manufacturer's instructions. Amplification of cDNA fragments to be used in mRNA differential display was performed using 72 different combinations of arbitrary forward (designed as "P") and reverse (designed as "T") primer pairs (Table 1) purchased from *Integrated DNA Technologies* (Coralville, IA, USA). The amplification was carried out in 0.02 cm^3 reaction mixture containing 500 ng cDNA, 1X PCR buffer, 2.5 mM $MgCl_2$, 50 µM dNTP mix, 74 kBq [α-^{33}P]dATP (*Institute of Isotopes Co.*, Budapest, Hungary), 1 µM "P" primer, 1 µM "T" primer and 1.25 units of Taq DNA polymerase (*Promega*, Madison, WI, USA). PCR was carried out in a DNA thermocycler GeneAmp PCR System 9700 (*PE Applied Biosystems*, Foster City, CA, USA) with the following conditions: one cycle at 94 ºC for 4 min, 40 ºC for 5 min, 72 ºC for 5 min; two cycles at 94 ºC for 1 min, 40 ºC for 1 min, 72 ºC for 5 min; 30 cycles at 94 ºC for 30 s, 58 ºC for 30 s, 72 ºC for 2 min, and a final extension step at 72 ºC for 7 min. The PCR products obtained from control and Cd treated plants were size-fractionated in parallel by electrophoresis in denaturing 6 % polyacrylamide/8 M urea gels as outlined by Ergen *et al.* (2007). After the completion of electrophoresis, gels were air-dried on *Whatmann 3MM* filter paper and exposed to X-ray films (*Kodak*) for visualization. The recovery of differentially expressed cDNAs from dried gels was performed by incubating gel

Table 1. Primers used in mRNA differential display.

Primer	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

fragments in 0.04 cm^3 10 mM tricine (pH 9.5) supplemented by 0.2 mM EDTA at 100 °C for 5 min. Eluted fragments were reamplified using the same set of primers and PCR conditions except that three initial rounds for nonspecific annealing were omitted and [α-^{33}P] was not added to the reaction mixture. Reamplified cDNAs were analyzed by agarose gel electrophoresis and recovered from gel using Qiaquick Gel Extraction kit (*Qiagen*). The fragments were subcloned in pGEM-T Easy Vector (*Promega*) for sequencing purposes.

Sequence analysis: The subcloned fragments were sequenced by *Iontek Company* (Istanbul, Turkey). The sequences were then analyzed by 'VecScreen' algorithm (http://www.ncbi.nlmn.nih.gov) to remove vector contamination, annotated on the basis of their homologies and deposited in EMBL/GenBank Data Libraries (EC907725, EC907731, EC907726, EC907729, and EC907730).

Northern blot analysis: The differentially expressed cDNA fragments were labeled using Gene Images Random Prime Labeling kit (*Amersham*, GE Healthcare, Buckinghamshire, England) and used as probes for Northern hybridization. Total RNA (25 µg) of the control and Cd treated plants were electrophoresed in denaturing 0.7 M formaldehyde/1.2 % (m/v) agarose gels. After completion of the run, the gels were blotted onto nitrocellulose membrane (*Schleicher&Schuell*, Dassel, Germany) and fixed by baking the filter at 80 °C in a vacuum oven for 2 h. The membrane was incubated in prehybridization solution for 30 min at 65 °C and hybridized overnight at the same temperature. The membrane was then washed twice with 1× SSC, 0.1 % (m/v) SDS at 65 ºC 15 min each, followed by low stringency wash in 0.5× SSC, 0.1 % (m/v) SDS at 65 ºC for 15 min. After stringency washes, the blot was incubated in anti-fluorescein-HRP conjugate (*GE Healthcare Bio-Sciences*, Piscataway, NJ, USA). With a final wash in 0.1 % (v/v) Tween-20 in buffer A (100 mM Tris-HCl, 600 mM NaCl, pH 7.5), unincorporated antibodies were removed. Finally, the blot was incubated in detection reagent and exposed to Hyperfilm ECL (*Kodak*). After 2 min incubation, the films were developed.

Quantitative real-time PCR analysis: First-strand cDNA was synthesized from total RNA isolated from control and Cd-treated leaf tissues, as described before. Diluted cDNA samples (400 ng) were used as templates in 0.025 cm^3 of Quantitect SYBR Green PCR Master mix (*Qiagen*) supplemented with 1 µM of gene specific primers (Table 2) designed using Vector NTI program (Vector NTI Advance 9, *Invitrogen*). PCR was performed with the iCyclerIQ (*Bio-Rad*, Hercules, CA, USA) with the following conditions: denaturation step at 94 ºC for 4 min, 36 cycles at 94 ºC for 30 s, 52 ºC for 1 min, 72 ºC for 30 s, and a final extension step at 72 ºC for 8 min. The threshold cycle (Ct) values of the duplicate PCRs were averaged and quantification was performed using relative standard curve method as described (Pfaffl 2001). As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used, since its expression was found to be constant upon Cd exposure in our previous experiments (data not shown). GADPH forward and reverse primers used in real-time PCR analysis were purchased from *Qiagen*.

Table 2. Primers (GeneBank number) designed for gene specific amplification of *NADH dehydrogenase subunit 1* (EC907725) and *PsaC* (EC907731) genes. F stands for forward primer and R for reverse primer.

Number	Sequence (5'-3')
EC907725_F	5'-GCCGATCTTAGTATTGGTGTTT-3'
EC907725_R	5'-AACCCTCACTAAATGCTGGTAGA-3'
EC907731_F	5'-TTCATTATGCTGAGTGATATCTTTTT-3'
EC907731_R	5'-ACCCTCACTAAATCGGTTCAT-3'

RT-PCR for *NADH deyhrogenase subunit 1* and *PsaC* genes: 1 µg total RNA isolated from control, 2 µM and 10 µM Cd-treated leaf samples were used for cDNA synthesis by Omniscript Reverse Transcription kit (*Qiagen*) according to manufacturer's instructions. The amplification of *NADH dehydrogenase subunit 1* and *PsaC* genes was performed using gene specific primers (Table 2) in 0.02 cm^3 reaction mix containing 100 ng cDNA, 1X PCR buffer, 2.5 mM $MgCl_2$, 50 µM dNTP mix, 1 µM of each primer and 1.25 units of Taq DNA polymerase (*Promega*) with following PCR cycle; denaturation step at 94 ºC for 4 min, 36 cycles at 94 ºC for 30 s, 52 ºC for 1 min, 72 ºC for 30 s, and a final extension step at 72 ºC for 8 min.

Results

Identification of induced genes by mRNA DD: The mRNA DD technique was used to identify induced genes in the leaf tissues of durum wheat that had been exposed to 0, 2, 10, and 30 µM concentrations of Cd. Nine 5'-arbitrary and eight 3'-oligo(dT) primers (Table 1) were used to amplify cDNAs synthesized from the control and Cd treated durum wheat leaf samples. A representative picture of mRNA DD gel is given in Fig. 1, through which five PCR products were identified as heavily induced upon exposure to different concentrations of Cd (Table 3).

Characterization of cDNA clones: The cDNA bands identified as differentially induced in leaf tissues of

durum wheat seedlings upon exposure to Cd were annotated based on their putative translation products (Table 4). The two of the genes showed significant homologies to the sequences available in databases. Accordingly, one of the genes (EC907725) were identified as *NADH dehydrogenase subunit 1* (*Tricitum aestivum*, E = 1e-20), and the other (EC907731) as a

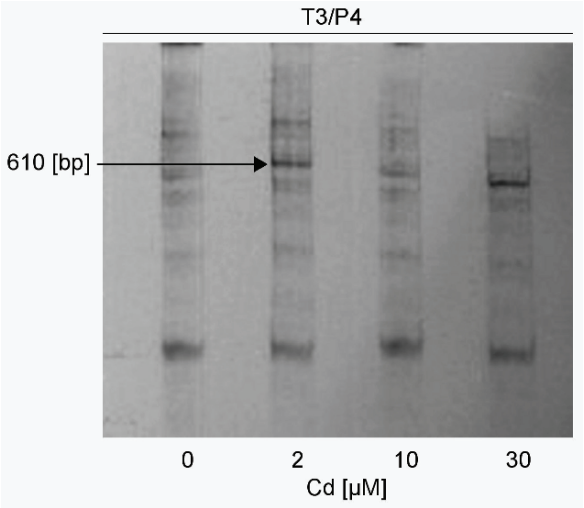

Fig. 1. A representative autoradiograph of an mRNA differential display gel after amplification with T3/P4 primer pair. The *arrow* indicates the position of *NADH dehydrogenase subunit 1* (EC907725) mRNA. The change in the band intensity indicates the differential expression upon exposure to different Cd concentrations.

Table 3. Sizes of the differentially expressed cDNA fragment bands and primer combinations used in mRNA DD.

GeneBank No.	PCR products [bp]	Primers
EC907725	610	T3/P4
EC907726	726	T9/P9
EC907729	233	T3/P3
EC907730	222	T3/P2
EC907731	585	T3/P2

homolog of *PsaC* gene encoding a PS 1 9 kDa protein from bread wheat (*Triticum aestivum*) with an E value of 2e-19. The results are in well agreement with the results found in a marine alga (Kim *et al.* 2005). The present study, for the first time, clearly demonstrated that these genes were also induced in durum wheat (*Triticum durum*) in response to Cd. One of the genes having differential induction upon Cd treatment showed a partial homology to several transcription factors and carries a DNA-binding motif similar signature (EC907726). The last two genes (EC907729 and EC907730), on the other hand, showed no significant homology to the known plant genes, which may be orphan genes (species specific genes). Further studies were focused on the cDNA clones showing homology to already known genes (EC907725; *NADH dehydrogenase subunit 1*, and EC907731; *PsaC* gene coding PS 1 9 kDa subunit) and the other three genes, which did not show any significant homology, were not our main focus.

Table 4. BLASTX search results of differentially expressed cDNAs isolated by mRNA DD.

Clone	Sequence homology	Effect of Cd	E-value	NCBI accession number
EC907725	NADH dehydrogenase subunit 1	+	1e-20	NP114313
EC907726	no hits	+		
EC907729	no hits	+		
EC907730	no hits	+		
EC907731	PS I 9 kDa subunit protein	+	2e-19	CAA31555

Northern blot analysis: Northern blot analysis was performed to confirm the induction of differentially expressed genes identified. Induction of NADH dehydrogenase subunit 1 and PS 1 9 kDa subunit mRNAs upon exposure to Cd were investigated using gene specific fragments as probes. A representative picture was depicted in Fig. 2 using *NADH dehydrogenase subunit 1* specific probes. The results were found to be matching the expectations from mRNA DD gel and confirmed the induction in the expression of genes identified.

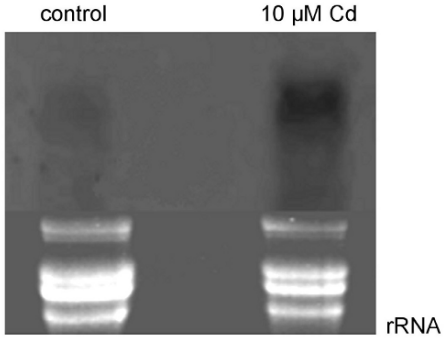

Fig. 2. A representative picture of Northern blot analysis of 25 µg total RNA from leaf tissues of control and 10 µM Cd treated seedlings after hybridization with fluorescently-labeled *NADH dehydrogenase subunit 1* gene (EC907725) specific probes. The ethidium bromide stained rRNA bands in the agarose gel is given as loading control.

Quantification of the induction in NADH dehydrogenase subunit 1 and PS 1 9 kDa subunit transcripts using real-time PCR: Real-time PCR was performed to quantify the expression levels of homologs of NADH dehydrogenase subunit 1 and PS I 9 kDa subunit. GADPH gene was selected as an internal control of the efficiency of the cDNA amplification, since the

gene expression of GADPH was shown to be not altered upon Cd exposure. The results indicated a 26.5 fold increase in the transcript amount of *NADH dehydrogenase subunit 1* gene (EC907725) and that of *PS I 9 kDa subunit* (EC907731) gene was found to be 8.5 fold when exposed to 2 µM and 10 µM Cd, respectively, compared to the expression in the control samples.

RT-PCR of *NADH dehydrogenase subunit 1* and *PsaC* genes: The visualization of changes in the gene expression of *NADH dehydrogenase subunit 1* and *PsaC* was performed through RT-PCR approach. The results were compared by agarose gel electrophoresis (Fig. 3). The expression of *NADH dehydrogenase subunit 1* gene under normal conditions was found to strongly induced after treatment with 2 µM Cd and returned to normal, even less, level after exposure to 10 µM Cd. On the other hand, the basal expression level of *PsaC* gene was found to be less than that of *NADH dehydrogenase* gene. Treatment with 2 µM Cd caused an induction in the expression of *PsaC* gene and exposure to 10 µM Cd resulted in a much higher transcript accumulation.

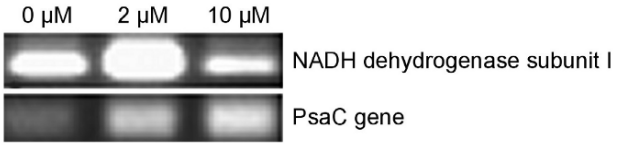

Fig. 3. RT-PCR analysis of transcript amounts of *NADH dehydrogenase subunit 1* and *PsaC* (Photosystem 1 9 kDa subunit) genes in control (0 µM), 2 µM and 10 µM Cd-treated durum wheat seedlings.

Discussion

In plants, Cd treatment has been reported to cause several metabolic adaptations in a species- and dose-dependent manner (Herbette *et al.* 2006, Rodriguez-Serrano *et al.* 2006, Semane *et al.* 2007). Although, changes in the antioxidative defense mechanism including glutathione metabolism has been excessively studied, there is not enough data available in literature about dosage-dependent changes in plant transcriptome upon exposure to Cd (Herbette *et al.* 2006, Rodriguez-Serrano *et al.* 2006, Semane *et al.* 2007). In this study, mRNA DD technique was used to identify heavily accumulated mRNAs upon exposure to Cd in a durum wheat cultivar. Differentially expressed genes were successfully identified from the leaf tissues of wheat seedlings treated with different concentrations of Cd. Two of the genes were identified as homologs of *NADH dehydrogenase subunit 1* and *PsaC* gene encoding photosystem 1 9 kDa subunit. This analysis revealed the potential use of mRNA DD technique for the detection of changes in the transcriptome of durum wheat in response to varying Cd concentrations. The induction in the *NADH dehydrogenase subunit 1* mRNA was confirmed with Northern blot analysis and quantified as about 26 fold change in transcript amount upon exposure to 2 µM Cd compared to untreated tissue. NADH dehydrogenase is known to play an important role in response to various plant stress mechanisms, and the present study indicated the significant induction of this gene in response to varying Cd exposure. Recently, an induction in the expression of *NADH dehydrogenase subunit 9* gene upon Cd exposure was reported in a marine alga *Nannochloropsis oculata* (Kim *et al.* 2005), which may suggest a Cd responsive role of this enzyme. The induction of the *NADH dehydrogenase subunit 1* transcript upon Cd treatment, however, was not reported before. Additionally, this is the first partial sequence of NADH deyhdrogenase subunit 1 gene obtained from durum wheat (*Triticum durum*).

The second gene that was shown to be induced upon Cd exposure was *PsaC* encoding PS 1 9 kDa subunit; the first report of partial sequence from durum wheat. Cd is shown to damage PS 1 and 2 in plants (Siedlecka and Baszynsky 1993). To alleviate the harmful effect of the Cd on photosynthetic apparatus, plants induce expression of *PsaC* gene which encodes for a subunit of PS 1 to compensate the decrease in the rate of photosynthesis. Real-time PCR data also validated that upon exposure to 10 µM Cd, this gene is up-regulated approximately 8.5-fold compared to the control plants.

The results obtained from Northern blot and real time PCR analyses were further confirmed by an RT-PCR approach. The transcript level of *NADH dehydrogenase subunit 1* was shown to be severely induced after treatment with 2 µM Cd, however, returned to a much lower amount after 10 µM Cd exposure. The changes in the transcript amount of *PsaC* gene was proven to be positively induced by the concentration of Cd used for stress treatment; the highest expression was detected upon 10 µM Cd treatment. The difference in the induction patterns of these two genes indirectly proves the gene specific behavior of plants upon Cd exposure.

Although mRNA DD technique is used for the detection of differentially expressed genes in response to several environmental conditions, the use of this technique for the identification of transcriptome of wheat upon heavy metal stress such as Cd has not yet been performed. In this study, the identification of the differentially expressed wheat cDNAs detected by mRNA DD was reported to alter their expression upon Cd stress, proving the potential use of mRNA differential display technique.

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