IDENTIFICATION OF GENES UP-REGULATED IN RESPONSE TO LEAD EXPOSURE IN SUNFLOWER (*Helianthus annuus* L.)

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Heavy metals are elements that have atomic number over 20 (Nieboer and Richardson, 1980) and a density higher than 5.0 g/cm^3 (Sanità di Toppi and Gabbrielli, 1999). The most common heavy metals are cadmium (Cd), chromium (Cr), mercury (Hg), lead (Pb), silver (Ag), tin (Sn) etc. (Kabata-Pendias and Pendias, 1992). Heavy metals are important environmental pollutants, and many of them are toxic even at very low concentrations. Pollution of the biosphere with heavy metals has accelerated dramatically since the beginning of the Industrial Revolution (Padmavathiamma and Li, 2007). The primary sources of this pollution are the burning of fossil fuels, mining and smelting of metalliferous ores, municipal wastes, fertilizers, pesticides, and sewage (Passariello *et al*., 2002; Yadav, 2010). Heavy metal poisoning may occur from industrial exposure, polluted soil, air, water, feeds or medicine or improperly coated food containers (National Organization of Rare Disorder, USA, report, 1998).

Lead (Pb) is considered one of the major contaminants, it cause brain, liver and kidney damage in children and nerve damage in adults (Spiro and Stiglani, 2002).

It is assumed that plants can be used for decontaminating waters and soils without any problems. The use of plants to accumulate toxic metals from polluted soils and waters especially heavy metals is known as phytoremediation (Raskin *et al*., 1997; Yadav *et al*., 2009). Phytoremediation offers a low cost and an environmentally friendly approach for decontaminating soils and waters of heavy metals. In addition some extracted metals may be recycled for useful value (Chaney *et al*., 1997; Kotrba *et al*., 2009; Chehregani *et al*., 2009).

Despite more than 10 years of intensive research focused on the phytoremediation, very few commercial phytoextraction operations have been realized (Eisa and Eid, 2011). Therefore, selection of plant material is an important factor for successful field phytoremediation. Sunflower (*Helianthus annuus* L.) is a fast growing high biomass crop with extensively growing deep root system and it is the fifth largest among the oilseed crops. Furthermore, sunflower can accumulate

various toxic metals from contaminated soils (Lombi *et al*., 1998). Therefore, sunflower could be a potential candidate for phytoremediation of soils contaminated with excessive Pb.

Cloning and characterization of the heavy metals stress-induced genes offer a chance for understanding the physiological responses of the plant cell to the heavy metal stress, Moreover, it produce a source of genes for producing transgenic plant tolerant to heavy metal stress. Differential display-polymerase chain reaction (DD-PCR) (Liang and Pardee, 1992) is a simple, powerful and sensitive method for the isolation of differentially expressed genes. It has been useful in characterizing and cloning of expressed sequence tags (EST) preferentially expressed in different tissues and/or under different conditions (Cushman and Bohnert, 2000; Martin-Laurent *et al*., 1997; Roux and Perrot-Rechenmann, 1997; Visioli *et al*., 1997; Deleu *et al*., 1999; Wei *et al*., 2000; Zhang *et al*., 2005; Yong *et al*., 2007; Yu *et al*., 2008; Venkatachalam *et al*., 2009). Here we employed the DD-based approach to understand the molecular mechanism of Pb-accumulation and tolerance in sunflower grown in Pb-contaminated soil.

MATERIALS AND METHODS

Plant material and Lead-stress experiment

In order to assess relative responses to lead stress; *H. annus* seeds were germinated in greenhouse conditions under 22-24°C and relative humidity 80%, seeds were grown in three pots (30 cm in diameter). Five seeds were planted in each pot. Seeds were irrigated with two different concentrations of lead nitrate Pb $(NO_3)_2$ (100 and 1000 ppm). Control plants were simultaneously irrigated with tap water. After 30 days of Pb- treatment, plants were harvested and used for RNA extraction.

RNA extraction and cDNA synthesis

Total RNAs were extracted from 700 mg of the harvested control and lead treated whole plant tissues according to the procedure of Chomczynski (1993), using the TriPure isolation reagent (Roche Molecular Biochemicals, Germany). First and second cDNA strands were synthesized using Improm^{TM} Reverse Transcription System (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions.

Differential display analysis and Re-PCR amplification

Differential display was carried out according to Liang and Pardee (1992) with some modifications, where PCR amplification of cDNA was carried out by GenHunterRNAimage kit according to the manufacturer's instructions with the anchor primers (T11G- 5'- TTT TTT TTT TTG -3', T11A- 5'- TTT TTT TTT TTA-3) in combination with arbitrary primers (AP1- 5'- AAG CTT GAT TGC C -3'), (AP2- 5'- AAG CTT CGA CTG T -3'), (AP3- 5'- AAG CTT TGG TCA G -3'), (AP4- 5'- AAG CTT CTC AAC G -3'), (AP6- 5'- AAG CTT GCA CCA T -3'),

(AP7- 5'- AAG CTT AAC GAG G -3') and (AP8- 5'- AAG CTT TTA CCG C -3'). Supertherm gold DNA polymerase Taq (Hoffmann-La-Roche) was used for amplification. Separation of amplified fragments was carried out on 6% denaturing polyacrylamide gels using Sequi-Gen® Sequencing Cell (Bio-Rad Laboratories, Hercules, California, USA).The gels were silver stained using the silver sequence kit (Promega, Madison, Wisconsin, USA), following the manufacturer's instructions.

Isolation and reamplification of cDNA fragments

Sterile scalpel blades were used to cut the desired bands from the gel. Gel slices were incubated in 50 µl dd H_2O at 65°C for 30 min, and then left at room temperature for elution. Three µl of the aliquot were used for re-amplification in a total volume of 25 ml, using the same set of corresponding primers. The reactions of PCR and the re-PCR involving selected DD fragments were carried out in a GeneAmp® PCR System 9700 instrument, programmed for 94°C for 1 min (1 cycle); 94°C for 30 sec, 38°C for 2 min, 72°C for 30 sec (40 cycles); 72°C for 5 min (1 cycle), then held at 4°C.

Cloning of cDNA fragments

The reamplified DD fragments were inserted into pGEM-T Easy Vector System (Promega, Madison, Ws, USA) using *E. coli* DH5α competent cells strain according to Sambrook *et al*. (2001). After transformation, all cells were spread on LB plates containing ampicilin with IPTG and X-Gal and incubated overnight at 37°C. Competent cells transformed with pGEM-T easy vector were detected by blue/white colony screening.

DNA Sequencing and computer analysis

The DNA sequence was determined by Applied Biosystems 3730XL sequencer. Sequences were analyzed using BLAST programs of the National Center for Biotechlogy (NCBI), USA [www.ncbi.nlm.nih.gov/Blast].

RESULTS AND DISCUSSION

Expression pattern of DD-cDNA transcripts

Differential display technique was carried out to isolate novel genes associated with lead tolerance and accumulation in *Helianthus annuus* plants.

Differential display-PCR was performed according to Gao *et al*. (2008). The amplified products were analyzed on 6% urea polyacrylamide sequencing gels (Fig. 1). We have successfully identified twenty fragments differentially expressed due to Pb treatment and/or concentration of treatment. The isolated ESTs were named HaP1 to HaP20. The ESTs were submitted to GenBank under accession number from AB823195 to AB823214.

Sequence analysis of DD- fragments

The isolated expressed sequence tags were classified according to their

levels of expression (Table 1). In response to the Pb exposure, seven cDNA fragments (HaP2, HaP3, HaP5, HaP12, HaP16, HaP17 and HaP19), were induced only at concentration of 100 ppm of Pb $(NO_3)_2$. Ten cDNA fragments (HaP1, HaP4, HaP6, HaP7, HaP8, HaP9, HaP11, HaP14, HaP15 and HaP18), were induced only at concentration of 1000 ppm of Pb $(NO_3)_2$, while three cDNA fragments (HaP10, HaP13 and HaP20) were induced at both concentrations.

The isolated fragments were analyzed using BLAST programs of the National Center for Biotechlogy (NCBI) (Table 1). Scanning of HaP1 and HaP3 cDNA fragments in the GeneBank showed no significant homology in BlastN.

Blasting of HaP10 and HaP20 cDNA fragments in the GeneBank showed similarity to unclassified and unknown genes.

The HaP2 fragment when analyzed by BlastN alignment, it showed significant homology with Expressed Sequence Tags of *Glycine max* similar to serine/threonine-protein kinase gene.

Protein kinases such as serine/threonine- protein kinase plays an important role in the signaling cascade pathway, by which the plant can regulate the cellular processes in response to different stresses includes heavy metal stress.

The mitogen-activated protein kinases (MAPK) are widely expressed serine/threonine kinases that mediate signals for the regulation of important cellular functions cascades. They are composed of three functionally linked protein kinases. MAP kinase activation is regulated by upstream dual-specificity kinases, which are known as MAPK kinases (MAPKKs; also known as MKKs). The activation of MAPKKs is regulated by other upstream kinases, known as MAPKK kinases (MAPKKKs; also known as MKKKs) and requires the phosphorylation of conserved threonine and tyrosine residues in the so-called TEY (Thr, Glu, Tyr) activation loop by a specific MAPK kinase (MAPKK), (Triesmann, 1996).

In *Arabidopsis*, AtMEKK1 (a MAPKinase-kinase-kinase) and AtMPK3 (a MAPKinase) are activated by dehydration, touch and cold (Mizoguchi *et al*., 1996). Jonak *et al*. (1996) reported the role of AtMPK3 during dehydration, which is transcriptionally upregulated upon drought stress. In *Arabidopsis*, AtMPK4 and AtMPK6 are post-translationally activated by cold, osmotic stress, and wounding (Ichimura *et al.*, 2000). Rentel *et al.* (2004) confirmed the role of OXI1, a serine/threonine kinase homologue identified as a downstream component to the AtMPK3 and AtMPK6.

After analysis by BlastN alignment, we found that there were two cDNA fragments showed homology with salinity responsive genes (HaP8 and HaP15), three cDNA fragments showed homology with drought responsive genes (HaP5, HaP7 and HaP11), one cDNA fragment showed homology with cold responsive gene (HaP

6), one cDNA fragment showed homology with oxidative responsive genes (HaP14) and one cDNA fragment showed homology with biotic stress resistance gene (HaP16).

Responses to heavy metal stress depend on a complicated signal transduction pathway within the cell that begins with the sensing of heavy metal (or heavy metal associated symptoms) and converges in transcription regulation of metal-responsive genes (Singh *et al*., 2002). Still much remains unknown about the molecular components of the metal-induced signal transduction, and only recently through differential-expression analyses, it has been possible to identify transcription factors (TFs) putatively responsive to heavy metal stress (Fusco *et al.*, 2005). As commonly found for other stress-related TFs, heavy metal responsive TFs also share the same signal transduction pathway and are therefore activated by abiotic stresses such as cold, dehydration, Salicylic Acid (SA) and H_2O_2 (Singh *et al*., 2002). In addition, cross-talk also exists between Cd tolerance mechanisms and pathogen defense signaling (Suzuki *et al.*, 2001).

Heavy metals affect the expression of ERF proteins that belong to the APETALA2 (AP2)/ethylene-responsive-element-binding protein (EREBP) family. Members of these TFs can bind to several pathogenesis-related promoters and dehydration-responsive elements (DRE motif) (Singh *et al.*, 2002). It has been shown that *ERF1* and *ERF2* genes are induced after 2 h of Cd-treatment in *A. thaliana* roots (Weber *et al*., 2006). Moreover, it has been reported that DREB2A is induced by Cd: DREB2A specifically interacts with the DRE motif in the promoter region of the rd29A and activates its transcription in Cd-exposed plants. Rd29A is already known to be induced by cold, salt and dehydration stresses (Suzuki *et al.*, 2001). OBF5, a bZIP-type DNA binding protein, was shown to be Cd-induced: it binds to the promoter region of the gene coding for the glutathione S-transferase, an enzyme involved in ROS scavenging and xenobiotic detoxification. Furthermore, it has recently been demonstrated that the expression of BjCdR15, a bZIP protein identified in *B. juncea*, is induced after short Cd treatment. This TF controls the expression of several metal transporters, is involved in long distance root-to-shoot heavy metals transportation and its overexpression in *A. thaliana* and tobacco plants enhances heavy metals tolerance and accumulation in the shoot (Suzuki *et al.*, 2001).

WRKY53, a TF belonging to the WRKY family, was isolated as being differentially expressed in Cd-treated *Thlaspi caerulescens* plants. This gene is also modulated by other environmental stresses such as salinity, drought, cold and salicylic acid and seems to participate in the stress-related signal transduction pathway regulating the activity of other TFs rather than directly activating gene expression (Wei *et al.*, 2008).

The HaP4 fragment when analyzed by BlastN alignment, it showed significant homology with EST of *Arabidopsis thaliana* putative anion transporter 2 (ANTR2) mRNA.

Numerous families of metal ion transporters have been identified in *Arabidopsis*. Some members of these gene families have been identified through the complementation of yeast mutants, while many others have been identified through sequence homology with well characterized animal and yeast genes. The biochemical function and specificity of some transporters have been well-characterized using expression systems in *E. coli*, yeast and *Xenopus* oocytes. Thus far, however, there are relatively few reports of *Arabidopsis* mutant or transgenic plants being affected by metal transport. Plants exhibiting such effects include lead and nickel tolerance in transgenic plants with a modified calmodulin-binding channel; Cd-excluding mutants and Mn-tolerant transgenic plants expressing AtCAX2. The role that these transporters might play in phytoremediation processes has yet to be determined. In some cases the identification and characterization of these genes in *Arabidopsis* has led to the study of the apparent homologues of these genes in metal-tolerant and/or hyper-accumulating species, particularly other members of the *Brassicacea* (Persans *et al*., 2001).

The HaP9 fragment when analyzed by BlastN alignment, it showed significant homology with Expressed Sequence Tags of *Helianthus annuus* delta-12 oleate desaturase (FAD2-2) gene.

Priya and Shivendra (2011) prepared a suppression subtractive hybridization (SSH) library using cDNA generated from plants treated with high Phosphorus (p) as the 'tester'. Based on the results of dot blot analysis, 360 positive cDNA clones were selected from the SSH library for sequencing. A total of 89 expressed sequence tags (ESTs) were identified as high P-responsive genes; the delta-12 oleate desaturase (FAD2-2) gene is represented as one of these ESTs.

The HaP12 fragment when analyzed by BlastN alignment, it showed significant homology with EST of *Dianthus caryophyllus* putative MtN3-like protein mRNA. Betania (1999) demonstrated that the MtN3-like protein has a role in pathogen resistance. The HaP13 and HaP19 fragments when analyzed by BlastN alignment, they showed significant homology with Expressed Sequence Tags for NADH dehydrogenase chain 3 (*nad3*) genes. Mitochondrial NADH dehydrogenase is one of the enzymes comprising the respiratory chain and consists of approximately 30 different subunits (Kanrar, 2005). The HaP17 fragment when analyzed by BlastN alignment, it showed significant homology with EST from *Volvox sp.* similar to cytochrome oxidase subunit 1(CoxI).

Bourdineaud (2006) studied CoxI gene expression by real-time quantitative PCR in *Crassostrea gigas* which was exposed for 10 or 14 days to 0.13 lM Cd^{2+} in

controlled laboratory conditions, he demonstrated that in this species CoxI gene was up-regulated by Cd. The HaP18 fragment when analyzed by BlastN alignment, it showed significant homology with Expressed Sequence Tags of *Medicago truncatula* MLP-like protein.

Marjo and Naoise (2006) used proteomic profiling to identify differences in protein intensities among three *Thlaspi caerulescens* accessions with pronounced differences in tolerance, uptake and root to shoot translocation of Zn and Cd. Proteins were separated using two-dimensional electrophoresis. Major latex protein-related/MLP-like protein was induced in *Thlaspi caerulescens* due to these stresses.

The twenty isolated and characterized cDNA fragments were deposited in the Genbank as a result of screening in response to Pb exposure in sunflower. Success in isolation of these fragments opens the door to several future aspects, like: isolation of full-length genes which have important roles to help plants survive under severe stress conditions, cDNA fragments with no significant similarities or cDNA fragments with unknown function can be used to discover new genes related to the stress response mechanisms and transformation of isolated genes to important crops will increase the tolerance of these plants to stress.

SUMMARY

In this study, Differential display reverse transcription (DDRT) technique was used to analyze differentially up-regulated genes in *Helianthus annuus* in response to Pb exposure. Seeds of sunflower were germinated in lead contaminated soils and untreated plants were used as control. Twenty differentially expressed fragments were identified and characterized. The fragments were classified according to their expression levels. The significance of the function of the identified differentially expressed genes was discussed in relation to their possible roles as stress genes. Two fragments showed no significant homology with any database sequences in the GenBank. Results of the database sequence alignment identified two fragments showed homology to unclassified and unknown genes; two cDNA fragments show homology with salinity responsive genes, three fragments showed homology with drought responsive genes, one fragment showed homology with cold responsive gene, one fragment showed homology with oxidative responsive gene and one fragment showed homology with biotic stress resistance gene.

More importantly, a fragment had significant homology with EST of *Glycine max* similar to serine/threonine-protein kinase gene, a fragment showed significant homology with EST of *Arabidopsis thaliana* putative anion transporter 2 (ANTR2) mRNA, a fragment had significant homology with EST of *Helianthus annuus* delta-12 oleate desaturase (FAD2-2) gene, a fragment showed significant homology with EST of *Dianthus caryophyllus* putative MtN3-like protein mRNA, two fragments showed significant

homology with EST for NADH dehydrogenase chain 3 (nad3) genes, a fragment had significant homology with EST from *Volvox sp.* similar to cytochrome oxidase subunit 1(CoxI) and a fragment showed significant homology with EST23 of *Medicago truncatula* MLP-like protein.

These results implicate that several pathways are involved in the plant's response to Pb exposure which still needs to be elucidated further.

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Table (1): Description of DD-fragment sequences as compared to database sequences & expression patterns of differentially expressed fragments.

Fragment No.	Length (bp)	Accession No.	Differential Expression/time			Homology	E-Value	Max. ident.
			C	100 ppm	1000 ppm			
HaP1	89	AB823195	-	-	+	no significant homology .	-	-
HaP2	199	AB823196	-	+	-	mRNA sequence from *Glycine max* similar to serine/threonine-protein kinase, XM_003523069.1.	5	96%
HaP3	130	AB823197	-	+	-	no significant homology .	-	-
HaP4	203	AB823198	-	-	+	mRNA sequence of Arabidopsis thaliana putative anion transporter 2 (ANTR2), NM_116261.1.	3	88%
HaP5	209	AB823199	-	+	-	mRNA sequence from *Cicer arietinum* under drought stress, FE671109.1.	3e-14	88%
HaP6	400	AB823200	-	-	+	mRNA sequence from *Pyramimonas gelidicola* under cold stress, FS594725.1.	1e-85	85%
HaP7	500	AB823201	-	-	+	mRNA sequence from *Sorghum bicolor* under drought stress, CF758797.1.	2e-14	76%
HaP8	493	AB823202	-	-	+	mRNA sequence from *Dunaliella salina* under salt stress, HO847679.1.	0	96%
HaP9	360	AB823203	-	-	+	mRNA sequence of *Helianthus annuus* delta-12 oleate desaturase (FAD2-2) gene, AY802997.1.	2e-64	100%
HaP10	342	AB823204	-	+	+	mRNA sequence of *Vitis vinifera* cDNA clone WIN0829, EC968791.1.	0.1	91%
HaP11	340	AB823205	-	-	+	mRNA sequence from *Vigna unguiculata* under drought stress, FF538704.1.	1.6	86%
HaP12	199	AB823206	-	+	-	mRNA sequence of *Dianthus caryophyllus* putative MtN3-like protein, AF151726.1.	2e-8	96%
HaP13	239	AB823207	-	+	+	mRNA sequence of *Helianthus annuus* mitochondrial gene for NADH dehydrogenase subunit 3, Z49774.1.	3e-27	81%
HaP14	200	AB823208	-	-	+	mRNA sequence from *Citrus clementine* under oxidative stress, DY301041.1.	3	80%
HaP15	206	AB823209	-	-	+	EST from *Puccinellia tenuiflora* under salt stress, JZ104269.1.	0.002	85%
HaP16	200	AB823210	-	+	-	mRNA sequence of *Triticum aestivum* Powdery Mildew Resistant, BE401393.1.	3	83%
HaP17	207	AB823211	-	+	-	mRNA sequence from *Volvox sp.* similar to cytochrome oxidase subunit 1, BAA09814.1.	0.008	52%
HaP18	217	AB823212	-	-	+	mRNA sequence of *Medicago truncatula* MLP- protein, XM_003628152.1	1e-2	100%
HaP19	294	AB823213	-	+	-	mRNA sequence from *Nicotiana tabacum* similar to NADH dehydrogenase chain 3 (nad3) genes, HO663928.1.	2e-62	83%
HaP20	300	AB823214	-	+	+	mRNA sequence of *Arabidopsis lyrata* expressed protein, XM_002886273.	1e-8	95%

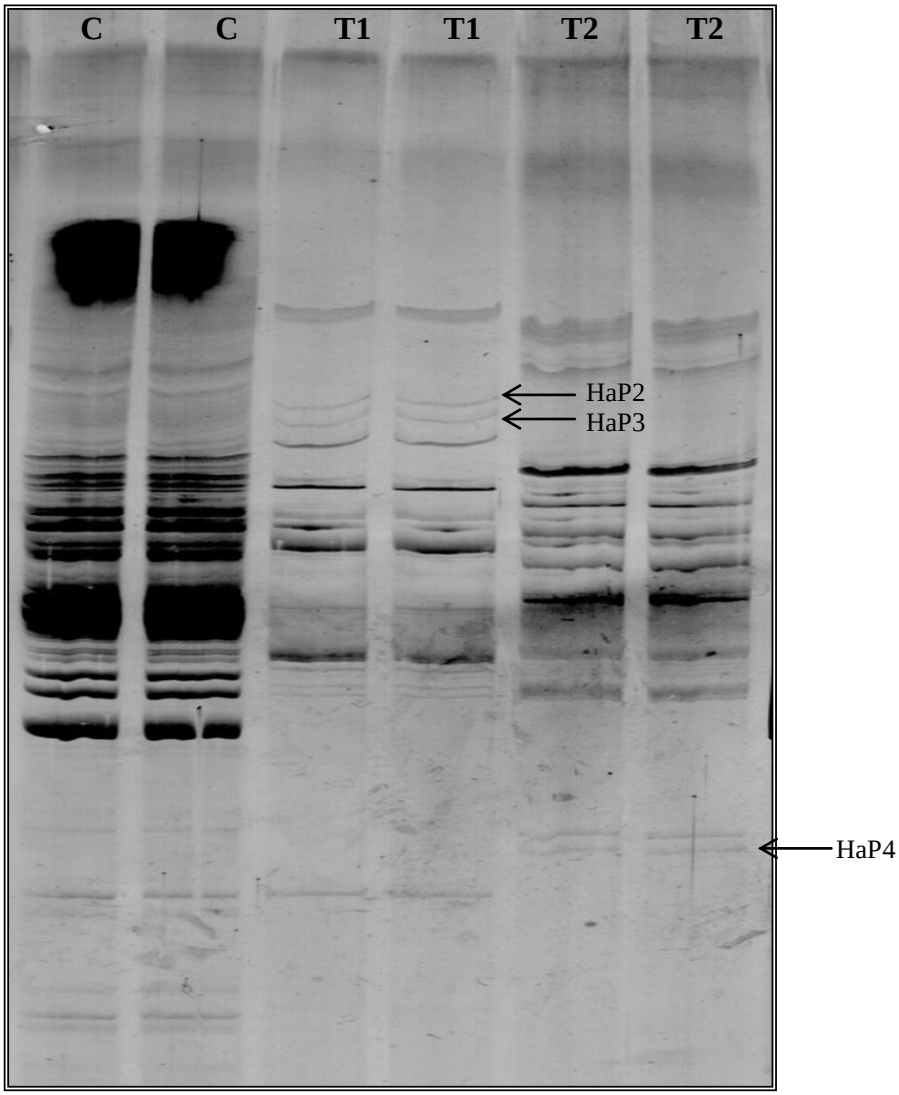

Fig. (1): DD-polyacrylamide gel showing differentially expressed bands. C; control, T1 and T2 treatment refers to plants grown in Pb polluted soil of 100 ppm and 1000 ppm, respectively. Arrows indicate a number of differentially expressed bands. The figure represents a part of DD gels.