Differential gene expression profiling in two cultivars of colza under aluminum stress (*Brassica napus* L.)

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**ABSTRACT**

Aluminum toxicity is one of the major limiting factors for crop production in acid soils. Exposure to this metal can cause changes in gene expression and lead to various signs of toxicity in plants. Gene expression of two cultivars of colza plant was analyzed using differential display - PCR technique under aluminum stress. Through the analysis and screening of experimental conditions and primers combination, a number of different bands of Opera cultivar were obtained. By submitting to GeneBank and BLAST comparison, identified chloroplast-related gene fragment.

**Key words:** Differential display, Stress response, Zarfam cultivar, Opera cultivar, Chloroplast

**Introduction**

Canola (*Brassica napus* L.) is an important rape seed, grown predominately under semi-arid condition. These plants are exposed to varied type of environmental conditions such as drought and salinity which could adversely affect plant growth and production [4].

Heavy metal ions play essential roles in many physiological processes. In trace amounts, several of these ions are required for metabolism, growth, and development. However, problems arise when cells are confronted with an excess of these vital ions or with non-nutritional ions that are potentially highly toxic to all organisms including animals and plants [8]. Numerous studies on the physiological responses to excess amounts of heavy metal ions indicate that plants have developed various mechanisms to cope with this environmental threat. Until now, however, the cellular mechanisms of heavy metal stress-induced signaling remained unclear. Metal ions can affect all the different classes of biomolecules [1]. It is widely recognized that metal compounds may have a profound effect on gene expression patterns, as demonstrated by the growing number of metal responsive genes that have been identified in different organisms [15,16].

Aluminum is the most abundant element of the earth crust, representing about 7% of its mass. It can be one of the most growth-limiting factors [13], possibly affecting approx. 40% of the worlds arable land that is potentially usable for food and biomass production [20]. Toxicity of Al concerns, however, only some of its soluble forms, where the most toxic monomer species Al$^{3+}$ prevails in acidic conditions [12,17,10]. Inhibition of root growth is well known effect of Al toxicity and root tips has been suggested as a primary site for Al-induced injury in plants [29]. Aluminum affects many aspects of physiology, biochemistry and molecular biology of the cell by disrupting a numerous components [26].

Transcriptome profiling of plants to environmental stresses can be studied using different techniques, which include DD-PCR, serial analysis of gene expression (SAGE), subtractive hybridization, DNA-chip, and cDNA microarray. mRNA differential display has been has been widely used to identify genes whose expression levels have been altered under different environmental conditions because of its technical simplicity and lack of requirement for previous genomic information of the species of interest [18,5]. In this research, the differential display technique applied to explore how exposure to aluminum might affect gene expression in two cultivars of *Brassica napus* L.

**Materials and Methods**

Two varieties of canola Zarfam and Opera were provided by Seed and Plant Institute of Karaj, equal number of seeds from each cultivar were selected and disinfected with 1% sodium hypochlorite for 10 minutes. Seeds in petri dishes with moist filter paper were placed until germination. Seeds germinated in plastic pots containing washed sand were transfered and placed in a greenhouse with appropriate
conditions. Seedlings were laid in Hoagland nutrient solution and treated at two-leaf stage by different Al concentrations. Four replications for each treatment were considered, in such way that three plants were in each pot. After 2 weeks, plant samples were collected and leaves of each seedling were separated for further work, snap frozen in liquid nitrogen and stored at -80°C, in plastic bags.

RNA extraction and cDNA synthesis:

Total RNA was isolated from leaf tissues of frozen plant samples using the plant RNeasy system (Qiagen, Canada), following the manufacturer’s instructions. The total RNA was quantified on a GeneQuant Pro (Amersham Biosciences) spectrophotometer and the quality of RNA was analyzed by electrophoresis on 1% agarose gel [30]. To remove DNA from RNA samples the volume of the solution is adjusted with 1µg extracted RNA and 1µl buffer DNase to a final volume of 10µl with DEPC and incubated for 30 min at 32°C. Then 1µl EDTA was added and again heated 60 min at 65°C.

DD-PCR [18] was done using the Fermentase Kit (Clontech Laboratories, Inc.) according to manufacturer’s instructions. DNA-free total RNA (4 Ag) was extracted from plants of control and Al-treated brassica was reverse-transcribed in a 20 µl final reaction volume containing 1µl oligo-dT, 1µl random primer, 4 µl reactant buffer, 1 µl dNTP, 0/5 µl RNAase inhibitor, 1 µl reverse transcriptase. DEPC was added to a final solution of 20 µl and incubated at 30°C for 5 min, 50°C for 50 min, the reaction mixture was heated in 95°C for 5 min and the cDNA was stored at -20°C for subsequent PCR reactions.

PCR amplification:

The PCR reaction was set by adding to a 1 mL microcentrifuge tube, 5.0 µL PCR buffer, 1µL dNTP (10mM), 2.5µLTaq DNA polymerase buffer (10X), 1.5µL MgCl2 (50 mM), 5µL anchored oligo-dT primer (10µM), 5µL arbitrary primer (10µM), 4.0 µL cDNA, 0.3 µL Taq DNA polymerase, 0.3 µL was added at the end and the reaction mixture was brought to 50µl final volume with sterile water (Fermentase Inc). Random primers used were sal1 (5'- AAG CTT GAT TGC-3'), sal2 (5'- AAG CTT TGG TCA-3'), sal3 (5'-AAGCTT TTA CGC-3') and anchored primer used was (5'-AAGCTTTTTTTTTTA-3'). All the contents were mixed gently. PCR reaction was run as follows: 94°C for 30 sec, 36°C for 2 min, 72°C for 1 min, 30 cycles, followed by a 10 min final extension at 72°C.

The PCR products were separated by 8% polyacrylamid gel at 90 V for 1h.

Sequencing and Comparison:

The acrylamide gels were stained by means of the silver staining protocol described by Creste et al., [9] PCR bands up-regulated by aluminum stress were chosen and sent for sequencing. Nucleotide sequences or the deduced amino acid sequences were compared with DNA sequences from NCBI database using the BLASTn.

Results:

The original differential display method relies on the use of an oligo-dT anchord primer and a random primer to amplify the cDNA pools obtained from the control and treated samples in order to allow easy identification and recovery of cDNAs that exhibit differential expression [18]. To ensure the quality and quantity of the cDNA pools derived from the control and treated cells of both cultivars, one housekeeping gene, ubiquitin was first amplified (Fig1). The cDNA synthesis of both cultivars were then compared using a pair of anchored and random primer. Fig 2 shows the results by the pairs of anchored primer with sal2 and sal3. In the case of sal1 clear difference was not observed in both cultivars but whenever sal2 and sal3 applied, clear difference was observed in the cDNA display from Opera cultivar. The bands exhibited a clear up-regulation in treated sample of Opera cultivar rather than of Zarfam cultivar. By submitting to NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrps.cgi) and comparing fragments, identified expression of chloroplast-related DNA sequences in Opera cultivar for sal3, while the fragments for sal2 did not show any similarity to the known genes from the GenBank database. The sequence homology of the up-regulated cDNA fragments was determined with other plant sequences using the program BLASTN.

The aluminum stress up-regulated cDNA fragment showed sequence similarity to two of the members of Brassicaceae family; 79% homology to the Olimarabidopsis pumila chloroplast DNA and 77% homology to Nasturtium officinale chloroplast DNA (Table1).
Fig. 1: Arrows indicates aluminum did not affect the expression housekeeping gene ubiquitin.

Fig. 2: Representative differential display band patterns of mRNA from control and aluminum-treated samples. Total RNA extracted from control and aluminum-treated *B. napus* plants was reverse-transcribed and amplified with the primer combination indicated on top of figure. PCR reactions were performed on two different dilutions of each cDNA sample to minimize artefacts. PCR products were separated on denaturing polyacrylamide gel. Arrows indicate differentially expressed cDNA fragments that were recovered from gel and analyzed further.

Table 1: The cDNA sequence producing significant alignment with other family members

<table>
<thead>
<tr>
<th>Description</th>
<th>Total score</th>
<th>Query Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Brassica napus</em> strain ZY036 chloroplast, complete genome</td>
<td>263</td>
<td>79%</td>
</tr>
<tr>
<td><em>B. rapa</em> chloroplast petA gene for cytochrome f</td>
<td>263</td>
<td>79%</td>
</tr>
<tr>
<td><em>Nasturtium officinale</em> chloroplast DNA, complete sequence</td>
<td>257</td>
<td>79%</td>
</tr>
<tr>
<td><em>Olimarabidopsis pumila</em> chloroplast DNA, complete sequence</td>
<td>252</td>
<td>77%</td>
</tr>
</tbody>
</table>

Discussion:

The presence of toxic levels of heavy metals triggers a wide range of cellular responses including changes in gene expression and synthesis of metal-detoxifying peptides. In this context, searched for genes whose expression may be affected by aluminum. For the identification and isolation of differentially expressed genes, several PCR based techniques are available.

The DD-PCR technique, developed by Liang and Pardee [18], has been widely used in plants to isolate genes that are differentially expressed in response to various stresses [21,35,19,36]. This technique is a suitable, low-cost technique, it is fast and requires small amounts of RNA to identify differentially displayed genes. It does not require cDNA cloning and blotting and can even be applied in laboratories where radioactive labelling is not available by using the silver staining method that
enables the detection and recovery of the PCR products from acrylamide gels.

The differential display method was used to perform a comparative gene analysis on cultured *B. napus* plants that were grown in the presence of aluminum. From this analysis, cDNA bands were identified corresponding to genes that were apparently modulated by the aluminum treatment. These bands were successively visualized by staining of acrylamid gel and, after sequencing, identified by comparison with sequences available in the data banks.

The sequence homology has indicated the effects of aluminum on the chloroplast region of plant cell affecting the most important process of cell’s function, the photosynthesis. Along with the plant cell growth retardation, photosynthesis is the prime process affected by drought [7] and salinity [25,2].

In recent research heavy-metal induced expression of several genes, such as *Pet* (genes encoding proteins of the cytochrome b6/f complex), *Ndh* (genes encoding NADH dehydrogenase protein), *Psa* (genes encoding PSI proteins) and *Psa* (genes encoding PSI proteins).

Multi-subunit complex of cyt b6/f is a crucial component for the photosynthetic electron transport chain of higher plants, green algae and cyanobacteria. This complex is catalyzing oxidation of quinols and the reduction of plastocyanin. This reaction allows to establish the proton force required for the ATP synthesis. In the vast majority of the chloroplast genomes that have been sequenced, the genes encoding the three major subunits of the b6/f complex are invariably found to be present: *petA* encoding cytochrome f, *petB* encoding cytochrome b6, and *petD* encoding subunit IV. In contrast, the *petC* gene, encoding the Rieske-type iron–sulfur protein, is always nucleus-localized. Four additional genes, *petG* (or *petE*), *petL*, *petM* and *petN* encoding small molecular mass subunits in the range of 3.0–4.0 kDa may be present or absent from chloroplast DNA (cpDNA) [33,3]. Expression of the chloroplast *petA* gene-encoding cytochrome f, a major subunit of the cytochrome b6/f complex, depends on two specific nucleusencoded factors: MCA1, required for stable accumulation of the *petA* transcript, and TCA1, required for its translation. MCA1 is a short-lived protein. Its abundance varies rapidly with physiological conditions that deeply affect expression of the *petA* gene in vivo, for instance in aging cultures or upon changes in nitrogen availability [27].

In *Chlamydomonas reinhardtii* removal of major nutrients (nitrogen, phosphate and sulfate), as well as temperature stress (L and H) and UV light exposure, accounted for the majority of the observed changes, although each condition could be correlated with at least some variation. With respect to nutrient stress, under nitrogen limitation, nearly all of the chloroplast mRNAs encoding photosynthetic proteins exhibited lower accumulation. For the cytochrome b6/f complex, nearly uniform increases were seen under three conditions: middle of the light period in synchronized cells (ML), low temperature (L), and exposure to UV light. PSI transcripts were particularly concerted, showing decreases under bright light (BL), in the dark (D), in -S, and under heat stress (H). PSI transcripts increased in-P. PSII transcripts also increased uniformly in -P and decreased nearly uniformly in the dark (D), in-N and -S, in minimal medium (M), and in heat stress (H). This result is consistent with regulatory elements or factors that recognize classes of genes or RNAs and has an evident functional logic.

Photosystem II (PSII) of the photosynthetic apparatus is a multi subunit pigment-protein complex in the thylakoid membrane, which performs light-induced oxidation of water and reduction of plastocyanine. Most of the cofactors needed for this process are associated with the D1 core protein of PSII. In case of the thermophilic cyanobacterium (*Thermosynechococcus elongates*), there are three gene copies each coding for a distinct protein. As shown for other cyanobacteria, the exchange of these D1 proteins seems to be a protective mechanism under high light stress conditions. Quantitative real time PCR analysis revealed an exchange of the *psbA* transcription within 30 minutes of high light conditions (500 µE). While the transcription of *psbA1* decreases from 90 % to 1.5 %, the *psbA3* transcription increases from 9 % to 98 %. The transcription of *psbA2* seems to be unaffected (1%) [31].

The plastid *ndh* genes encode components of the thylakoid *Ndh* complex, which is analogous to the NADH dehydrogenase or complex I of the mitochondrial respiratory chain and catalyzes the transfer of electrons from NADH to plastocyanine [32,6,28]. In concerted action with electron-draining reactions, the *Ndh* complex protects against photooxidative-related stresses [22,11], probably by contributing to poised the redox level of the cyclic photosynthetic electron transporters [6,23]. The higher sensitivity of *ndh* gene defective plants to stress and the consistent presence of the plastid *ndh* genes in most photosynthetic plants in the line leading from certain charophycean green algae to land plants suggest that the *Ndh* complex is necessary or provides advantages for photosynthesis in the highly fluctuating terrestrial environment [23]. Accordingly, the *Ndh* complex could be involved in the photosynthetic adaptation of leaves to the rapid and extreme light and temperature variations to which many perennial plants are exposed.

Thus, high expression of these genes in Opera cultivar under aluminum stress were involved in tolerance to this metal.
Reference


