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Full Length Research Paper

Estimation of D-amino acid oxidase gene expression in different organs of drought-challenged maize

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To enhance our exploitations in plant system of D-amino acids oxidizing enzymes (D-amino acid oxidases; DAAO), our efforts were made to unravel the relative expression patterns of maize DAAO transcript using basic and quantitative reverse transcription and polymerase chain reaction (RT-PCR) with template materials collected separately from three different organs of test plants grown under drought stress conditions. Relative expression analysis using $2^{-\Delta\Delta Ct}$ values indicated that maize DAAO transcript is detectable in all samples, showing about 2.9 to 3.1 folds increase in stress-challenged leaf and root tissues. The results also revealed that the expression of maize DAAO in leaf tissues is prior to root tissues, reflecting the quick impression and response of leaf tissues to drought stress in comparison to roots. Our results may provide a basis and a new research gate to study the novel biological roles of DAAO gene product in plant system.

Key words: D-amino acid oxidase, drought, expression, maize, stress.

INTRODUCTION

D-amino acid oxidases (DAAO) are flavoproteins that metabolize D-amino acids by oxidative deamination manner (Momoi et al., 1988; Tishkov and Khoronenkova, 2005). DAAO have been discovered and well characterized in mammalian system and micro-organisms (Momoi et al., 1988; Tada et al., 1990; Pollegioni et al., 1993; Sarawer et al., 2003, 2005). They have been first cloned and identified from plants grown in the media containing D-amino acids as nitrogen source and partially characterized from plants grown under stress conditions (Gholizadeh and Baghbankohnehrouz, 2009a, 2009b). The physio-biological roles of these enzymes have not been fully understood till date. They have been generally known to provide carbon and nitrogen source for microbial growth and developments (Fischer et al.,1996). In mammalian cells, DAAO detoxify endogenous D-amino acids accumulated in kidney and liver cells (Tishkov and Khoronenkova, 2005). In brain tissues, DAAO maintain the functional level of D-serine (Tishkov and Khoronenkova, 2005). However, there are no reports regarding the activity or the biological roles of these enzymes in plant system (Gholizadeh and Baghbankohnehrouz, 2009a, 2009b).

Usually, studies on a gene expression profiles enhance our knowledge about its putative physio-bilogical roles. With respect to DAAO gene expression, its stimulation in the kidneys of germ-free mice (Lyle 1968) as well as its induction in *Cyprinus carpio, Trigonopsis variabilis*, *Rhodotorula gracilis* and *Neurospora crassa* under D-alanine containing media have been studied (Golam et al., 2003; Sikora and Marzluf, 1982; Simonetta et al., 1989; Horner et al., 1996). Accumulation of D-amino acids and activation of its detoxifying enzyme DAAO during aging in mammalian system (Sato et al., 1996; Mothel et al., 2006), expression of Zebrafish DAAO during its early embryogenesis (Chen et al., 2007) and an increase in the concentration of D-amino acids in rice plant during aging (Manabe et al., 1981; Gamburg and Rekoslavskaya, 1991), all reveal that DAAO might have a key role in developmental processes in different organisms. However, its specific gene expression profiles remained obscure in plants and were not reported (Gholizadeh and Baghbankohnehrouz, 2009a, 2009b). For the first time, we reported a drought stress-inducible D-amino acid oxidase gene from the leaves of maize plant suggesting the possible role of DAAO in drought stress resistance (Gholizadeh et al., 2009b).

Due to the commercial importance of maize plant, there is significant interest in understanding its expressed sequences and identification of the biological functions of

Forward primer: 5′ CTGCACGGCCTACTTCCTC 3′	
Reverse primer: 5′ CAACGCCTGCTCCTTCTC 3′	
Denaturation	93°C / 3 min
Annealing	60°C / 1 min
Extension	72°C / 1.5 min
Final extension	72°C / 10 min
Amplification was performed in Techneh (Germany) type thermal cycler	

Figure 1. Primer set and PCR amplification steps.

the encoded proteins. Thus far, there is a wealth of information available from developmental / phenotypic descriptions, stress effects and resistance for this plant.

This information can be synergistically integrated with well-defined genes at transcriptional and functional levels. To date, the undergoing maize complete genome sequencing project along with large scale EST (expressed sequence tags) and full length cDNA sequencing projects offers a huge advantage in this regards (Alexandrov, 2008).

Therefore, to increase our knowledge regarding the possible roles of DAAO in plant system, an attempt was made to investigate the expression profile of DAAO gene expression in three different organs of drought-challenged maize plants.

MATERIALS AND METHODS

Bacterial strains and chemicals

E. coli strain DH5α was used for bacterial transformation. Plasmid vector pGEM-T easy (Cat. no. A1360; Promega) was used for PCR product cloning. Trizol reagent (Cat. no. RN7713C; RNXTM; CinnaGen) was used for total RNA isolation. mRNA purification kit was provided by QIAGEN, USA (Cat. No.70022). AcessQuickTM RT-PCR System was purchased from Promega (Cat. no. A1701). Fermentas DNA extraction kit (Cat. no. K0513) was used for the purification of the PCR product from the agarose gel. All of the other chemicals used in this research work were of molecular biology grades.

Plant materials

The seeds of *Zea mays L.* were provided by Dr B. Baghban Kohnehrouz, (Genetic Engineering Lab, Dept. Plant Breeding and Biotechnology, University of Tabriz, Tabriz, Iran). Test plants were allowed to grow completely in greenhouse conditions (day to night period of 12h: 12h and humidity of about 70%). In order to collect the leaf and root materials, plants were well watered until sixth leaf stage and then after the water were withheld till the plants were visibly welted. Drought stress conditions were continued for 4 weeks. Experimental materials were collected from the root and leaf tissues before and after stress treatment at four time intervals. To collect the seed materials, test plants were subjected to stress after seed production.

Total RNA isolation and mRNA purification

Total cellular RNA was separately isolated from test materials using Trizol reagent, separately. About 0.2 g of each material was fine powdered using liquid N2 and 2 ml of Trizol reagent was added to homogenize it at room temperature (RT). 200µl of chloroform was added to the mixture, mixed for 15 second, incubated on ice for 5 min and centrifuged at 13000g for 15 min. The upper phase was transferred to another tube and RNA was precipitated with an equal volume of isopropanol. The pellet was washed in 1ml of 75% ethanol, dried at RT and dissolved in 30µl RNase-free water. The integrity of the RNA was tested on 1% non-denaturing agarose gel using TBE running buffer. Poly (A^+) RNA was purified from total RNA using oligo dT-columns according to the provided kit protocol. The integrity of the purified mRNA was also analyzed by electrophoresis using 1% non-denaturing agarose gel. The quantity of the RNA in the starting materials for the next experiments was measured spectrophotometrically (Ausubel et al., 1991).

Primer designing and RT-PCR amplification

Specific primers used for the amplification were designed based on already reported DAAO transcript sequence (Gholizadeh et al., 2009b). The primer sequences were designed by Primer3 software (http:/www.primer3plus.com/web_0.4.0/input.htm). In order to analyze the expression pattern of DAAO gene, the RT-PCR reactions were separately performed using one-step AcessQuickTM RT-PCR System (Cat. no. A1701; Promega). About 0.5µg of each mRNA sample was mixed with 25µl Master Mix (2x) and 1µl of correspondent primer set. The mixtures were adjusted to a final volume of 50µl using nuclease-free water. The reaction mixtures were incubated at 45°C for 45 min and preceded with PCR cycling. PCR was carried out after a pre-denaturation stage at 95°C for 3 minutes in 25 cycles. The nucleotide sequence and the details of PCR steps were presented in Figure 1.

In the next step, the amplified products were extracted from the agarose gel, cloned in pGEM-T easy cloning vector (Ausubel,

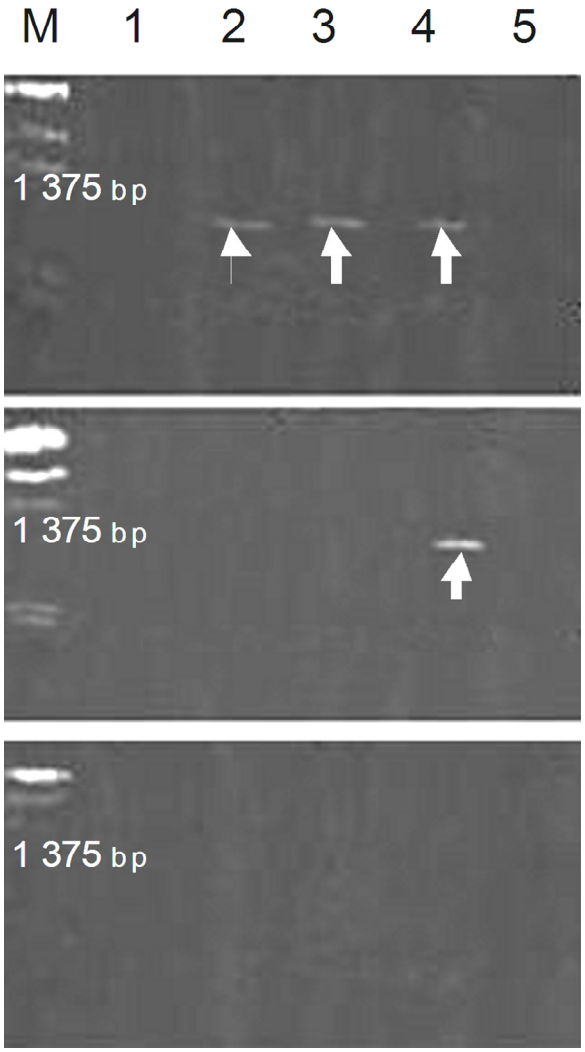

Figure 2. Analysis of DAAO gene expression.

1991). The cloned fragments proceeded for the sequencing in Microsynth DNA sequencing center at Switzerland.

Expression analysis by real-time reverse transcription PCR

The expression level of the DAAO transcripts in test samples were analyzed by real-time RT-PCR using iQ SYBR Green supermix (Bio Rad, USA) on Miniopticon Real Time PCR Detection System (Bio Rad, USA). Experiments were performed in three replicates and the mean values of the data were presented. All the expression data were normalized by adjusting the expression level of actin gene in test samples and the relative fold expression was calculated using the $2^{-\Delta\Delta Ct}$ value (Livak and Schmittgen, 2011).

Sequence analysis of the isolated fragments

The nucleotide sequences of the isolated cDNA were analyzed by computing at BLAST (Basic Local Alignment Search Tool) at http://www.ncbi.nlm.blast.com/, and CLASTALW sequence alignment software at http://www.genome.jp/ and Expasy proteomic tools at http://www.expasy.org/tools/.

RESULTS

To prime the expressed DAAO gene in different organs, previously reported primer pair for the leaf DAAO was used (Gholizadeh et al., 2009b). The expression of DAAO transcript was analyzed by basic and quantitative RT-PCR methods using the same amount of template mRNA / cDNA for all test samples collected from leaf, root and root tissues at five time points for stressed and non-stressed plants.

Analysis of basic RT-PCR end products on 1% agarose gel revealed that the expression of DAAO transcript is only dateable in the leaf and root tissues of plants under stressed. The samples collected from seed tissues failed to show evidence of DAAO gene expression (Figure 2). No RT-PCR positive signals were observed for non-stressed plant tissues. The results showed that the expression of DAAO gene in leaf tissues is prior to root tissues (Figure 2). As it is shown on the photograph, the expression signal is detected in leaf tissues one week after stress treatment and continued over time as stress condition continued, except for the time point fifth when the plants dried completely. The results show that the expression of DAAO is far below the detection limit in root tissues till third week of stress treatment.

The presence of DAAO transcript was detected by RT-PCR at five time points including before and after stress treatment. 5µl of RT-PCR end products were separated on 1% agarose gel and analyzed. M: *Eco*RI and *Hin*dIII Lambda DNA marker; 1: one day before stress treatment; 2: one week after stress; 3: two weeks after stress; 4: three weeks after stress and 5: four weeks after stress. Upper photograph: samples collected from leaf tissues; middle photograph: samples from root tissues. Lower photograph: samples collected from seed tissues.

The presence and the level of the expression of DAAO transcript were also analyzed by real-time RT-PCR using iQ SYBR Green dye. Figure 3 shows the expression and quantification of the relative fold change in the expression level of DAAO gene in different samples. All data were normalized by actin and related to L0 (non-stressed leaf sample considered as control) using $2^{\Delta\Delta Ct}$ value (Livak and Schmittgen, 2011).Despite the results obtained by agarose gel, analysis of the $2^{-\Delta\Delta Ct}$ values reliably revealed that maize DAAO is expressed in all test samples. However, its expression level varies in different samples. Data showed that the expression level of DAAO gene is considerably higher in stress-challenged tissues (Figure 3). In comparison with non-stressed tissues, there is about 2.9 to 3.1 folds increase in stressed leaf and root samples. The expression level of DAAO is found to be very low in seed tissues so that it was not detectable by agarose gel.

Time course experiment analysis demonstrated that the expression of DAAO in leaf tissues is detectable one week after stress treatment, while the expression signal of root tissues is observed after three weeks of stress treatment. Transcription level of DAAO gene was

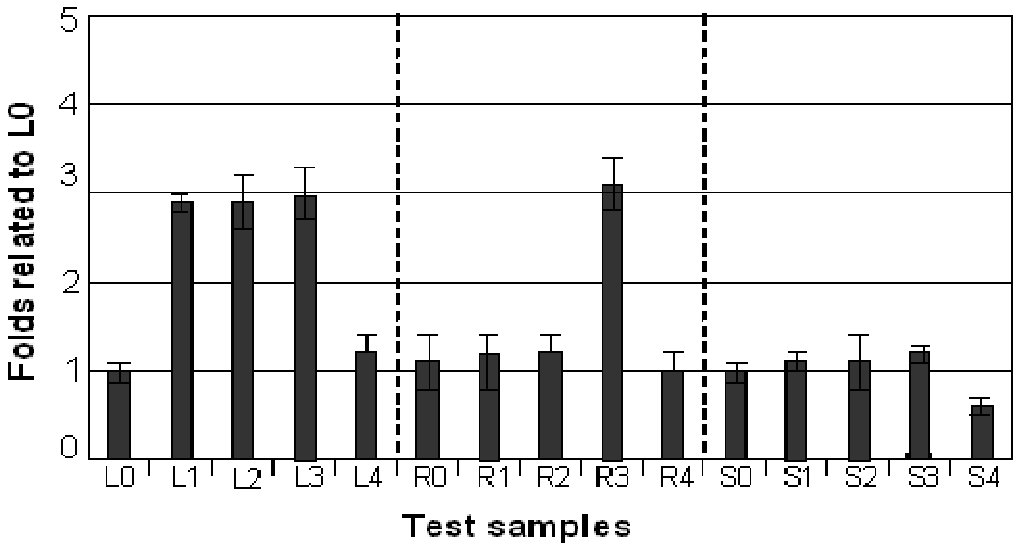

Figure 3. Real-time based quantitative RT-PCR analysis of DAAO transcript.

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Leaf DAAO/ CTAYFLATHAASPTVPTLVERCALACAASGKAGGFLALDWCDS
Root DAAO/ CTAYFLATHATSPTVPTLVERCALACAASGKAGGFLALDWCDS
                     ACG
Leaf DAAO/ TPALSRLARASFALHRRLADALGGADAYGFRPVHTLSVLLPPH
Root DAAO/ TPALSRLARASFALHRRLADALGGADAYGFRPVHTLSVLLPPH

Leaf DAAO/ PAASSSPPHPLLPPWVDPSASAAPPRELGTPDTTAQVHPGLFT
Root DAAO/ PAASSSPPHPLLPPWVDPSASAAPPRELGTPDTTAQVHPGLFT

Leaf DAAO/ KAVLAASGAEVVIGEVERVAVAWDGRVAGVVVKGRDGVLDADA
Root DAAO/ KAVLAASGAEVVIGEVERVAVAWDGRVAGVVVKGRDGVLDADA

Leaf DAAO/ VVLALGPWSGRLEVVSEVLDVSGLKAHSIVFRPREPEKVTPHC
Root DAAO/ VVLALGPWSGRLEVVSEVLDVSGLKAHSIVFRPREPEKVTPHC

Leaf DAAO/ LFLSYQPEPGAKMLDPEVYPRPTGEVYICGMSKDENPPDDPAT
Root DAAO/ LFLSYQPEPGAKMLDPEVYPRPTGEVYICGMSKDENPPDDPAT

Leaf DAAO/ ITGEPDSIAMLHKIAGKVSSQLKKEEGAEVVAEQACYLPCTADI
Root DAAO/ ITGEPDSIAMLHKIAGKVSSQLKKEEGAEVVAEQACYLPCTAD

Leaf DAAO/ GLPVIGEIPGVKGCYVATGHSCWGILNGPATGAALAELILDGK
Root DAAO/ GLPVIGEIPGVKGCYVATGHSCWGILNGPATGAALAELILDGK

Leaf DAAO/ AKIVDLEPFSPARFLKRRSRR
Root DAAO/ AKIVDLEPFSPARFLKRRSRR
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Figure 4. Comparison of deduced amino acid sequences.

analyzed in three different tissues of maize using iQ SYBR Green dye (provided by Bio Rad, USA) on Miniopticon Real Time PCR Detection system (Bio Rad, USA). L: leaf sample; R: root sample; S: seed sample; 0: one day before stress treatment; 1: one week after stress; 2: two weeks after stress; 3: three weeks after stress and 4: four weeks after stress. Data presented as the mean values of three replicates ± SD.

For sequence characterization, we cloned and sequenced the RT-PCR end products of leaf and root tissues. The sequence analysis results showed that the root DAAO sequence is identical to the leaf DAAO except for the position 11 in which Thr is substituted with Ala (Figure 4).

The isolated cDNA clones from maize drought stressed leaf and root tissues were sequenced and their deduced

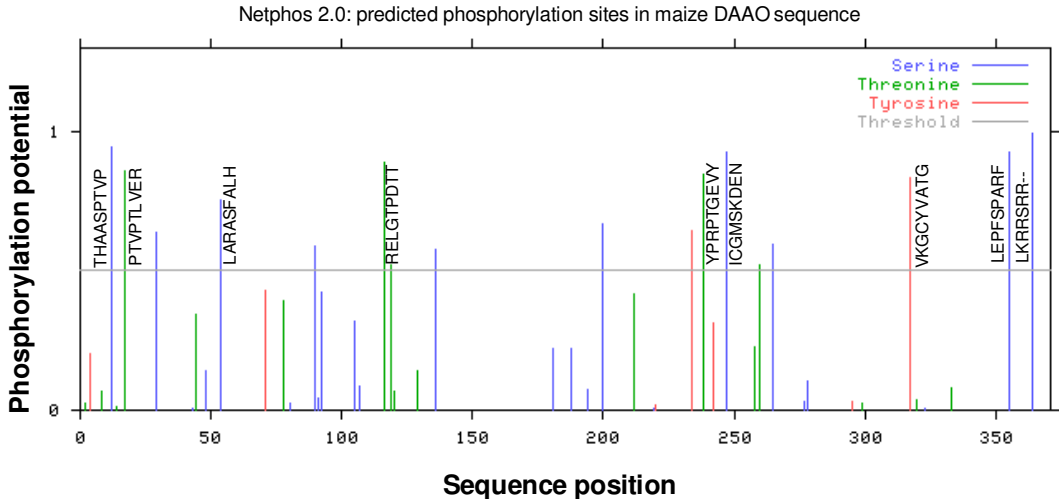

Figure 5. Prediction of high potential phosphorylation sites.

amino acid sequences were aligned by CLASTALW sequence alignment software at http//www.genome.ad.jp. Computed analysis of the potential phosphorylation sites revealed that maize DAAO sequence has got five Serin phosphorylation sites including THAASPTVP, LARASFALH, ICGMSKDEN, LEPFSPARF, LKRRSRR--, three Theronine phosphorylation sites including PTVPTLVER, RELGTPDTT, YPRPTGEVY and one Tyrosin phosphorylation site VKGCYVATG. These sites were predicted to have high potential for phosphorylation (Figure 4). The number, position and the sequence of the highly conserved phosphorylation peptides were predicted on the amplified sequence by proteomic server at http//www.expasy.ch.

DISCUSSION

In the previous work for the first ever time, we identified a drought stress inducible D-amino acid oxidase gene from the leaves of maize plants (Gholizadeh et al., 2009b). Herein, we extended our expression studies on three different organs of test plants including leaf, root and seeds. Analysis of basic RT-PCR end products on 1% agarose gel revealed that the expression of DAAO transcript is only dateable in the leaf and root tissues of plants under stressed. The samples collected from seed tissues failed to show evidence of DAAO gene expression on agarose gel (Figure 2). The results also showed that the expression of DAAO gene in leaf tissues is prior to root tissues (Figure 2).

Analysis of the presence and the level of the expression of DAAO transcript in different test samples (by real time RT-PCR) reliably revealed that maize DAAO is expressed in all test samples, but the expression level is considerably higher in stress challenged tissues showing about 2.9 to 3.1 folds increase in leaf and root samples (Figure 3). The overall results not only indicate that the expression level of DAAO gene is increased in stressed leaf and root tissues, but also reflect the quick impression and response of leaf tissues to drought stress in compare to roots. The expression of DAAO gene has been previously reported from mammalians and microorganisms under various conditions such as aging, tissue developments and cell death phenomenon (D'Aniello et al. 1993; Sato et al., 1996; Fischer 1998; Mothel et al., 2006; Chen et al., 2007). In rice plant, D-amino acid content was reported to be increased with age (Manabe et al., 1981; Camburg and Rekoslavskaya, 1991). However, there is no knowledge regarding the presence and activity of D-amino acids metabolizing enzyme in plants, thus far. N-malonylation of D-amino acids is known in higher plants and is considered to be a detoxification mechanism for D- amino acids (Liu et al., 1983). However, there are no evidences for the detoxifying activity of plants DAAO. Despite mammalians and microorganisms, there were no reports to our knowledge regarding the DAAO inducing factors in plant system. For the first time in our laboratory, an inducible DAAO gene was identified from the leaves of maize plants grown under drought stress conditions as well as from the plants grown in D-alanine containing media (Gholizadeh et al., 2009a, 2009b).

In a previous large scale comparative phosphoproteomics study, it has been generally suggested that almost all the known key proteins in plants contain highly conserved phsphorylation sites (Nakagami et al., 2010). In this study, we predicted high potential sites in maize DAAO sequence that was isolated and characterized in our laboratory (Figure 5) (Gholizadeh et al., 2009a, b). On the basis of the presence of high phosphorylation potential, it can be suggested that maize DAAO like other key proteins may contributes common and important roles in response to

different endogenous and exogenous stimuli such as aging, tissue developments and various environmental cues and stresses in plant system, as it has been reported for mammalians. Since drought stress is like as aging, developmental processes and other environmental stimuli are shared similarly with genetical, biochemical and morphological evidences in plant system (Dat et al., 2000; Mittler, 2002), therefore, it is predicted that plant DAAO might be triggering all developmental processes, senescence and stress responses as like as for mammalians. However, more experimental evidences are recommended in the future investigations.

As the first ever time report, we hope our results will provide a basis and a new research gate to study the detailed biological roles of DAAO gene product in plants.

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