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Molecular Detection of a Drought Stress-Inducible D-Amino Acid Oxidase Gene from *Zea mays* L.

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Abstract: In this research, the induction of maize DAAO gene was investigated under drought stress conditions. RT-PCR end product analysis of the mRNA samples purified from stressed and non-stressed leaves showed that DAAO gene is only expressed in stressed plants. The results indicated that DAAO transcript is consistently detected overtime as stress conditions continued, but it fell below the limit of detection when plants are completely dried/died. This result may be consistent with the earlier reports that points the accumulation of D-amino acids and expression of DAAO activity during mammalians aging or tissue developments in which PCD is involved. Cloning and sequencing of the expressed product revealed that the induced cDNA nucleotide and deduced amino acid sequences are 100% identical to DAAO gene that is expressed when maize plants utilize D-alanine as nitrogen source. This finding may provide new insights into the active role of D-amino acid oxidase gene and lead to the ways for the new studies on plant DAAO in the future.

Key words: D-amino acids, *Zea mays*, stress, expression, cDNA cloning

INTRODUCTION

D-Amino Acid Oxidase (DAAO) is a flavoenzyme that catalyzes stereospecifically the oxidative deamination of D-amino acids to the corresponding ketoacids, ammonia and hydrogen peroxide (Tishkov and Khoronenkova, 2005). DAAO have been characterized in a wide variety of organisms including animals and micro-organisms (Momoi *et al*., 1988; Tada *et al*., 1990; Pollegioni *et al*., 1993; Sarower *et al*., 2003, 2005). Newly, it was first identified and cloned from plant system (Gholizadeh and Kohnehrouz, 2009).

Because of its stereospecificity, it has important biotechnological and industrial applications. The main interest for DAAO in industrial biotechnology is due to its use in production of 7 ACA (7 Amino Cephalosporanic Acid) and several cephem antibiotics from the natural antibiotic cephalosporin C (Riethorst and Reichert, 1999) and in analytical biotechnology is because of its application as a biosensor in medical diagnostics (Kulys and Schmid, 1991). Generation of cytotoxic hydrogen peroxide with D-amino acid oxidase has been used to make it as anticancer prodrug in cancer medication (Stegman *et al*., 1998).

Although, it was discovered more than 60 years ago, but the physiological and biological roles of this enzyme remained obscure and not fully been clarified. In micro-organisms, DAAO allows D-amino acids to be used as growth substrates providing carbon and nitrogen (Fischer *et al*., 1996). In mammalians, the main role of D-amino acid oxidase in kidney and liver cells is detoxification of endogenous D-amino acids accumulated due to racemization process by Tishkov and Khoronenkova (2005). The important role of DAAO in maintaining the necessary levels of D-serine in different brain tissues has been reported in recent years by (Tishkov and Khoronenkova, 2005). Despite the presence of DAAO structures in plant system, no information is available about the activity of these enzymes or their biological roles in plants, thus far (Erickson *et al*., 2004; Gholizadeh and Kohnehrouz, 2009).

Induction of D-amino acid metabolizing enzyme activity following exougenous application of D-amino acids has already been reported in various organisms including mammalians and micro-organisms. Injection of D-alanine has stimulated DAAO activity in the kidneys of germ-free mice (Lyle, 1968). Characterization of an induced DAAO from common carp *Cyprinus carpio* with exogenous free D-alanine has been reported recently by Golam *et al*. (2003). In micro-organisms, the induction of D-amino acid oxidase with different D-amino acids and their derivatives has been detected in *Trigonopsis*

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variabilis, *Rhodotorula gracilis* and *Neurospora crassa* (Sikora and Marzluf, 1982; Simonetta *et al*., 1989; Hörner *et al*., 1996). In plants, following finding two Expressed Sequence Tags (ESTs) from *Arabidopsis thaliana* (GenBank accession No. NM_126129) and *Oriza sativa* (GenBank accession No. BAD31345.1) expression libraries, a D-alanine inducible DAAO gene was identified and cloned from maize plant (Gholizadeh and Kohnehrouz, 2009).

Some recent reports have shown that D-amino acids/D-amino acid oxidases are expressed during natural biological processes such as aging and tissue/organ developments in mammalians. Accumulation of D-amino acids in mammalian cells has been found to be one of the characteristics of organism aging (Fischer, 1998; Mothel *et al*., 2006). In addition, the induction of DAAO in these cells suggests the possible *in vivo* biological role of DAAO that is to act as detoxifying agents to metabolize D-amino acids accumulated during aging (D'Aniello *et al*., 1993; Sato *et al*., 1996). Spatiotemporal expression of zebrafish D-amino acid oxidase activity during early embryogenesis revealed that DAAO might have a role in developmental processes (Chen *et al*., 2007). In rice plant the D-amino acid content has been seen to be increased with age (Manabe *et al*., 1981; Gamburg and Rekoslavskaya, 1991).

It has been well understood that all of these processes are attributed to the Programmed Cell Death (PCD) that is triggered by the accumulation of Reactive Oxygen Species (ROS). Hydrogen peroxide (H_2O_2) is one of these species that is generated by oxidative enzymes (Greenberg, 1997). D-amino acid oxidases are a group of these enzymes that catalyzes deamination process of D-amino acids and produce H_2O_2 that recently been found to be involved in cell death progamme in mammalian cells (Ande *et al*., 2006).

Drought stress in plants as well as in other organisms leads to programmed cell death that has been found to be due to the production of reactive oxygen species (Dat *et al*., 2000; Mittler, 2002). Considering this information, present experiments were conducted to analyze the expression of a ROS producing enzyme, D-amino acid oxidase, during drought stress in plant system. Detection of expression was carried out by RT-PCR technique.

MATERIALS AND METHODS

This study was conducted in 2007 at Research Institute for Fundamental Sciences, Tabriz University, Tabriz City, Iran.

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; RNX™; CinnaGen) was used for total RNA isolation. mRNA purification kit was provided by QIAGEN, USA (Cat. No. 70022). AcessQuick™ 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 were of molecular biology grades.

Plant material and drought stress treatments: The seeds of *Zea mays* L. were provided by Dr. B. Baghban Kohnehrouz, University of Tabriz, Tabriz, Iran. Test plants were grown in glasshouse conditions. Plants were well watered until sixth leaf stage and then after the water was withheld till the plants were visibly wilted. Drought stress conditions were continued for 4 weeks. The leaf materials for the experiments were collected from the youngest leaves of control (non-stressed) and test (stressed) plants at four time intervals after treatments. A control sample was also taken from the test plant 1 day before stress treatment.

Total RNA isolation and mRNA purification: Total cellular RNA was isolated from the leaf samples using Trizol reagent. About 0.2 g of leaf material was fine powdered using liquid N_2 and 2 mL of Trizol reagent was added to homogenize it at Room Temperature (RT). 200 μL of chloroform was added to the mixture, mixed for 15 sec, incubated on ice for 5 min and centrifuged at 13000 g 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 1 mL of 75% ethanol, dried at RT and dissolved in 30 μL 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 kit protocol. The integrity of the purified mRNA was also analyzed by electrophoresis using 1% non-denaturing agarose gel.

Primer designing and amplification of maize DAAO: Specific primers for maize DAAO transcript were designed using Primer 3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The same primes had been previously designed based on the 5'and 3'ends of the overlapping maize ESTs (expressed s e quence tags) in GenBank databases (Gholizadeh and Kohnehrouz, 2009). The nucleotide

sequences of the primer pair were as follows: Fw: 5'CTGCACGGCCTACTTCCTC3', Rv: 5'CAACGCCTGCTCCTTCTC3'. In order to analyze the expression of the maize D-amino acid oxidase transcript, the RT-PCR reactions were performed using one-step AcessQuick™RT-PCR System (Cat. No. A1701; Promega). About 0.5 μg of mRNA was mixed with 25 μL Master Mix (2x) and 1 μL of each primer. The mixture was adjusted to a final volume of 50 μL using nuclease-free water. The reaction mixture was incubated at 45°C for 45 min and proceeded with PCR cycling. PCR was carried out after a pre-denaturation stage at 95°C for 3 min in 25 cycles of 1 min denaturation at 95°C, 1 min annealing at 60°C and 1.5 min of extension at 72°C. The reaction was finally extended at 72°C for 10 min. Amplified product was extracted from the agarose gel using Fermentas DNA extraction kit (Cat. No. K0513; CinnaGen), cloned in pGEM-T easy cloning vector and proceeded for the sequencing in Microsynth DNA sequencing center at Switzerland.

Sequence analysis of the isolated fragments: The nucleotide and deduced amino acid sequences of the isolated cDNA were analyzed for primary structure similarity by computing at BLAST (Basic Local Alignment Search Tool) and Multalin servers developed by NCBI (National Center for Biotechnology Information, USA) and Expasy proteomic tools.

RESULTS AND DISCUSSION

In this study, we investigated the induction of maize DAAO transcript under drought stress conditions. The specific primers for priming of the expressed gene were designed based on the maize DAAO cDNA sequence that has already been reported by Gholizadeh and Kohnehrouz (2009).

Expression of DAAO transcript was detected by RT-PCR using the same amount of the starting mRNA material for all test samples. Experiments were conducted at five time points for stressed and non-stressed plants with two replicates as described in methods. Analysis of RT-PCR end products (5 μL) on 1% agarose gel showed that the expression of DAAO transcript is only detectable in those samples collected from stressed plants. The samples from non-stressed plants as well as the one collected before stress treatment, failed to show evidence of DAAO gene expression (Fig. 1; the gel photograph for non-stressed plant not presented). The results showed that the expression of DAAO gene in stressed plants is detected overtime as stress continued, except for the time point fifth, when the plants dried/died completely.

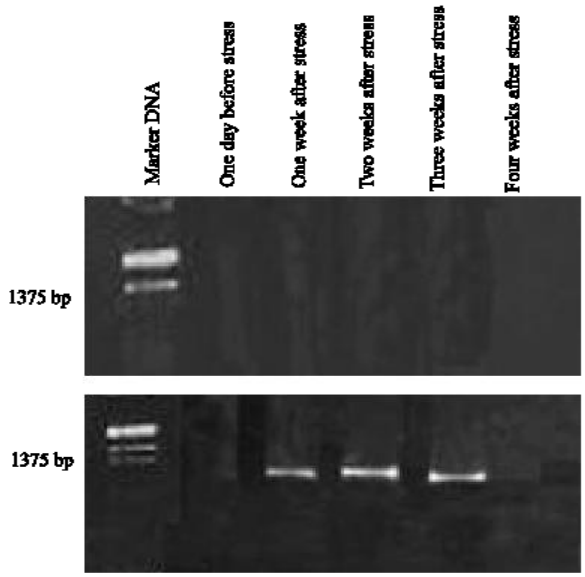

Fig. 1: Time-course analysis of DAAO gene expression. Expression of DAAO transcript was detected by RT-PCR at five time points including before (upper photograph) and after (lower photograph) stress treatments. As the results shows the expression of DAAO gene is detectable only after stress treatment and is the RT-PCR end product is observed on the gel overtime as stress continued, except for the time point fifth when the plants dried/died completely

Cloning and sequence analysis of the expressed product revealed that it is 100% identical to the DAAO cDNA isolated from maize plant grown in D-alanine containing solution medium. The nucleotide and deduced amino acid sequences of the expressed cDNA has been elucidated in Fig. 2. The sequence comparison of this cDNA with D-alanine induced DAAO gene from maize plant has been shown in Fig. 3. Sequence alignment of drought stress-induced maize DAAO with those of different origins and analysis of their phylogenetic tree revealed that the maize DAAO is much similar to *Oriza sativa* DAAO as well as others (including animals and micro-organisms) having different evolutionary pathways (Fig. 4; alignment data not shown).

This study for the first time reports the expression of DAAO transcript under an environmental stress condition in plant system. Previously, the accumulation of D-amino acids and expression of their metabolizing enzyme, DAAO, have been reported from mammalians and micro-organisms under exogenous application of different D-amino acids or their derivatives (Lyle, 1968; Sikora and Marzluf, 1982; Simonetta *et al*., 1989; Hörner *et al*., 1996;

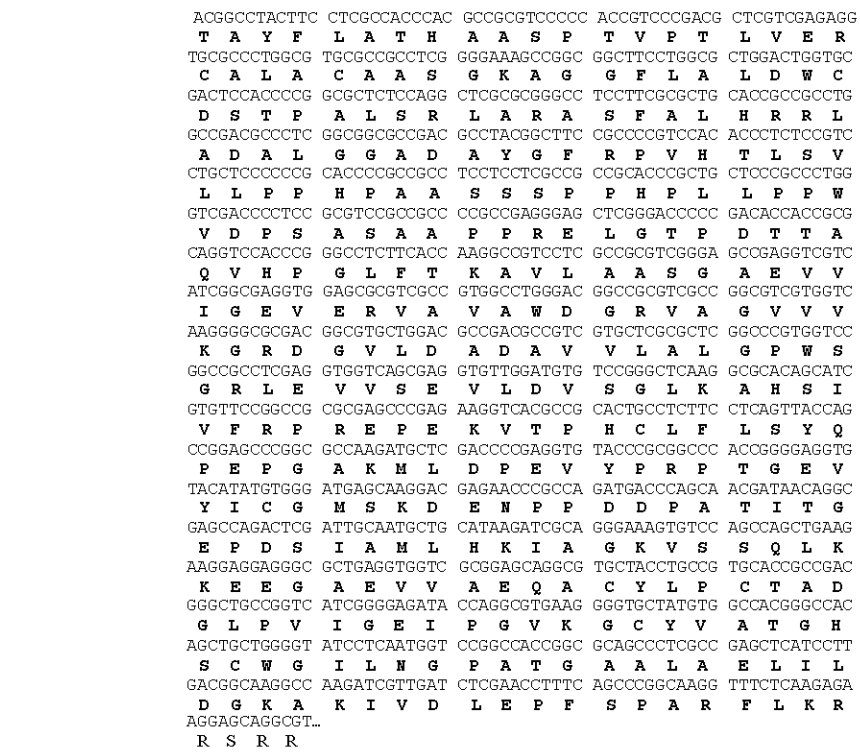

Fig. 2: Sequence characterization of the isolated cDNA. The induced cDNA was cloned in pGEM-T easy vector and sequenced. Deduced amino acid sequence of the clone was identified by using translation tool of expassy proteomic server (http://www.expasy.ch/tools)

Golam *et al*., 2003). Newly, the first inducible plant D-amino acid oxidase gene was identified using exogenous D-amino acid as an inducer and nitrogen source (Gholizadeh and Kohnehrouz, 2009). The reported cDNA sequence for maize DAAO has been submitted to EMBL databases under accession number AM407717.

Although, D-amino acids or their different derivatives have been found to be the common exogenous inducing compounds for the expression of DAAO genes in all organisms investigated, but the recent reports suggest the accumulation of D-amino acids and the expression of D-amino acid oxidase activity during different cellular/organism processes such as aging, tissue developments and cell death phenomenon (Chen *et al*., 2007; Mothel *et al*., 2006; Fisher, 1998; Sato *et al*., 1996; D'Aniello *et al*., 1993). In rice plant, D-amino acid content has been seen to be increased with age (Manabe *et al*., 1981). All of these indicating the *in vivo* biological roles for DAAO that need to be investigated in details.

It has been well known that both aging and every pattern of developmental processes are shared similar genetical, biochemical and morphological evidences. In particular, Programmed Cell Death (PCD) is a conserved phenomenon that has been widely observed to function in organism senescence and in all the predictable developmental processes (Greenberg, 1997). It has also been well understood that PCD is involved in response to different biotic/abiotic environmental stresses in different organisms (Greenberg, 1997).

Accumulation of Reactive Oxygen Species (ROS) has been found to be a general trigger for PCD process.Hydrogen peroxide (H_2O_2) is one of these species that is generated by oxidative enzymes such as D-amino acid oxidase that catalyzes the stereoselective deamination of D-amino acids and produces hydrogen peroxide (Greenberg, 1997). Induction of programmed cell death/apoptosis has been found to be attributed to H_2O_2 produced by DAAO in Jurkat cells (Ande *et al*., 2006). The induction of cytotoxic oxidative stress with DAAO enzyme in brain tumer cells has provided a useful tool in human cancer gene therapy (Stegman *et al*., 1998).

Drought stress in plants leads to programmed cell death that has been dmonstrated to be due to the production of reactive oxygen species (Dat *et al*., 2000; Mittler, 2002). Present experiment result showed that the expression of DAAO is induced during the period of

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daao1/ MSAAPPRRVVICGGGVVGACTAYFLATHAASPTVPTLVERCAPACAASGKAGGFLALDWCDSTPALSRLARASFALHRRLAD
daao2/ -------------------CTAYFLATHAASPTVPTLVERCAPACAASGKAGGFLALDWCDSTPALSRLARASFALHRRLAD
       -------------------**********************************************************
daao1/ ALGGADAYGFRPVHTLSVLLPPHPAASSSPPHPLLPPWVDPSASAAPPRELGTPDTTAQVHPGLFTKAVLAASGAEVVIGEV
daao2/ ALGGADAYGFRPVHTLSVLLPPHPAASSSPPHPLLPPWVDPSASAAPPRELGTPDTTAQVHPGLFTKAVLAASGAEVVIGEV
       **************************************************************************************
daao1/ ERVAVAWDGRVAGVVVKGRDGVLDADAVVLALGPWSGRLEVVSEVFDVSGLKAHSIVLRPREPEKVTPHCLFLSYKPEPGAK
daao2/ ERVAVAWDGRVAGVVVKGRDGVLDADAVVLALGPWSGRLEVVSEVFDVSGLKAHSIVLRPREPEKVTPHCLFLSYKPEPGAK
       **************************************************************************************
daao1/ MLDPEGYPRPTGEVYICGMSKDENPPDDPATITGEPDSIAMLHKIAGKVSSQLKKEEGAEVVAEQACYLPCTADGLPVIGEI
daao2/ MLDPEGYPRPTGEVYICGMSKDENPPDDPATITGEPDSIAMLHKIAGKVSSQLKKEEGAEVVAEQACYLPCTADGLPVIGEI
       **************************************************************************************
daao1/ PGVKGCYVATGHSCWGILNGPATGAALAELILDGKAKIVDLEPFSPARFLKRRSRR
daao2/ PGVKGCYVATGHSCWGILNGPATGAALAELILDGKAKIVDLEPFSPARFLKRRSRR
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Fig. 3: Sequence alignment between deduced amino acids of isolated cDNA and D-alanine induced gene in maize plant. The isolated cDNA clone under drought stress condition is 100% identical to the DAAO transcript induced in plants grown in D- alanine containing medium. daao1: The clone isolated under D-alanine containing medium; daao2: The clone isolated under drought stress conditions. The alignment has been carried out using CLASTALW at http://www.genome.ad.jp

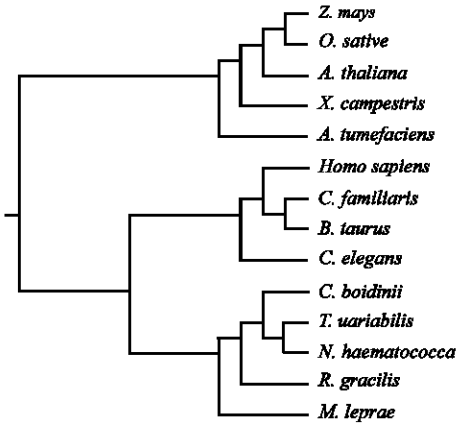

Fig. 4: Phylogenetic tree of DAAOs from different origins. *Zea mays* L. (accession No. AM407717); *Oriza sativa* (accession No. BAD31345.1); *Arabidopsis thaliana* (accession No. NM126129); *Xanthomonas campestris* (accession No. XCC2414); *Agrobacterium tumefaciens* (accession No. AGRC4235); *Homo sapiens* (accession No. BC029057); *Canis familiaris* (accession No. 486317); *Bos taurus* (accession No. 615334); *Caenorhabditis elegans* (accession No. C47A10.5); *C. andida boidinii* (accession No. AB042032); *Trigonopsis variabilis* (accession No. AY514426); *Nectria haematococca* (accession No. P24552); *Rhdotorula gracilis* (accession No. RGU60066); *Mycobacterium leprae* (accession No. MLCOSL672)

drought stress in maize plant, but the expression level is fell below the assay limit of detection, when plants leaves are completely dried/died. This may be consistent with the earlier reports that points the accumulation of D-amino acids and expression of DAAO activity during mammalians aging or tissue developments in which PCD is involved.

Since, the expression of DAAO transcript is detected over the time course of experiment, except for the death time, it is suggested that oxidative D-amino acid oxidase is biologically active enzyme in plants and might have a possible role in the induction of cell death in maize plant grown under environmental stress condition.

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