

GENE AND ENZYMATIC ACTIVITIES OF ARABIDOPSIS D-AMINO ACID OXIDASE IN SALTY MEDIA

Gholizadeh A.

Research Institute of Fundamental Sciences, University of Tabriz, Tabriz, Iran

Received: 30 January 2014 Accepted: 29 May 2014

Summary: The relative gene expression and the enzymatic activity of D-amino acid oxidase (DAAO) were assessed in the leaves of *Arabidopsis* plants grown under salt stress and a subsequent recovery period. Analysis of the distribution patterns of the gene expression by using semi-quantitative and real-time reverse transcription and polymerase chain reactions (RT-PCR) revealed that *daao* gene was inducible in the leaves of the test plants under salty growth medium. The distribution patterns of DAAO enzyme activity towards D-aspartate, D-alanine and D-serine showed that D-aspartate oxidase was more likely an inducible DAAO enzyme in salt-challenged test plants. Parallel experiments related to antioxidant abilities provided further assessment criteria for clarification of a potent resistance mechanism. It was indicated for the first time that D-aspartate oxidase might be a DAAO of pivotal importance for salt-challenged *Arabidopsis* plants, proposing a DAAO-dependent strategy as a new candidate and promising target for salt resistance engineering in plant systems.

Citation: Gholizadeh A., 2013. Gene and enzymatic activities of Arabidopsis D-amino acid oxidase in salty media. *Genetics and Plant Physiology*, 3(3–4): 133–145.

Keywords: Arabidopsis; salt stress; D-amino acid oxidase activity; gene expression.

INTRODUCTION

As one of the major environmental abiotic stresses, soil salinity has an adverse affect on plant growth in irrigated arid regions of the world (Schleiff, 2008). Current estimations show that about 10-35% of the agricultural lands are now affected all around the world. Each year, large significant areas are becoming unusable (<http://www.liv.ac.uk/>).

Salinity affects many biological processes, so that the resistance or tolerance mechanisms mostly involve a complex interplay of physiological

and biochemical characters in the plant system. Salt causes injury in plants usually through osmotic stress, ionic effect, nutrient deficiency and reactive oxygen species (ROS), thereby affecting the overall plant growth and developmental processes (Munns, 2002). Facilitating ion exclusion, accumulation of compatible solutes, metabolic switching to C4 type of photosynthesis and detoxification of free radicals by antioxidant systems are the well known plant strategies to withstand salt stress.

*Corresponding author: aghz_bioch@yahoo.co.in

In plants, salt stress responses and tolerance mechanisms are genetically complex, often modulated by multiple metabolic and signaling pathways. To date, a large-scale gene expression profiles under salt stress are available for many plant species, including *Arabidopsis*, rice, barley, and ice plant (reviewed by Jamil et al., 2011). The identification of such gene expression profiles helped us to understand more about the complex regulatory pathways affecting plant salt tolerance and potentially functional characterization of unknown genes, which may be good candidates for developing salt-resistant plants.

D-amino acid oxidase (EC.1.4.3.3; DAAO) is a FAD-dependent oxidoreductase that metabolizes the enantioselective oxidative deamination of D-amino acids to yield the corresponding α -imino acids and their hydrolyzed products α -oxoacids and ammonia. This catabolic reaction is accompanied with the reoxidation of the reduced FAD by molecular oxygen and the release of hydrogen peroxide (Curti et al., 1992). The structures and functions of DAAO have been discovered and well characterized in mammalian system and microorganisms (reviewed by Tishkov and Khoronenkova, 2005). For the first time, they have been cloned from plants grown in the media containing D-amino acids as a nitrogen source and partially characterized from plants grown under drought stress conditions by our laboratory research group (Gholizadeh and Baghbankohnerouz, 2009; Gholizadeh et al., 2009). To date, it has been revealed that DAAO is a ubiquitous enzyme in all kinds of organisms, but their physiological and biological roles have not been fully understood yet.

They have been generally known to play catabolic and developmental roles in microorganisms, regulatory functions on the neuromodulator D-serine in the human brain, detoxifying roles towards all accumulated D-amino acids in kidney and liver cells (reviewed by Tishkov and Khoronenkova, 2005). However, the overall knowledge of D-amino acids in plant system is very poor and obscure.

Most of the information on the DAAO putative physiological and biological functions has been achieved from studies with respect to its inducible gene expression as well as its enzyme activity patterns. Its stimulation in the kidneys of germ-free mice (Lyle, 1968), its induction in *Cyprinus carpio*, *Trigonopsis variabilis*, *Rhodotorula gracilis* and *Neurospora crassa* in D-alanine-containing media are the well studied cases (Golam et al., 2003; Horner et al., 1996; Simonetta et al., 1989; Sikora and Marzluf, 1982). Besides, accumulation of D-amino acids and activation of related DAAO during aging in mammals (Fischer, 1998; Mothel et al., 2006), expression of *Zebrafish daao* during the early embryogenesis step (Chen et al., 2007) and the increase in the concentration of D-amino acids in aging rice plants (Gamburg and Rekoslavskaya, 1991), elevated expression of *daao* gene in drought-challenged maize plants (Gholizadeh et al., 2009), all reveal that DAAO might play key roles in growth and developmental processes as well as in response to stressful stimuli.

The initial reports of DAAO expression in plant systems have shown that the metabolism of D-amino acids might be stress-related (Gholizadeh, 2009). Later on, studies on *daao* gene expression profiles

in different organs of maize plants under drought stress conditions have provided a basis to study the potent biological roles of D-amino acid oxidases in plant systems (Gholizadeh, 2011).

To our knowledge, so far, no attention has been given to *daao* gene expression and enzymatic activity profiles in salt-challenged plant species. Therefore, an attempt was made: 1) to investigate the relative expression profiles of *Arabidopsis daao* (accession number: NP_201530) gene in leaf tissues under salt stress conditions and the subsequent recovery period, and 2) to elucidate the relative distribution patterns of the DAO activity in the leaf enzyme extracts of salt-challenged and recovered *Arabidopsis* plants. To characterize further the possible DAO-dependent resistance mechanism in salt-challenged plants, the antioxidant capacity of the test leaves was examined and discussed.

MATERIALS AND METHODS

Materials

Seeds of *Arabidopsis thaliana* were provided by Dr B. Baghbankohneirouz (Laboratory of Plant Genetic Engineering, Dept. Plant Breeding and Biotechnology, University of Tabriz, Iran). Trizol reagent used for total RNA extraction was purchased from Gibco BRL, USA (Cat. No. 15596-013). The mRNA purification kit was from QIAGEN, USA (Cat. No.70022). Chemicals used for the cDNA synthesis were provided from Promega (USA) cDNA synthesis kit (Cat. No. C4360). All other chemicals were of analytical and molecular biology grades and purchased from Merck AG (Darmstadt, Germany) or Sigma (St. Louis, MO, USA).

Plant growth and treatments

Seeds of *Arabidopsis thaliana* were incubated at 4°C for 3 days and then germinated in autoclaved soil at 25°C and a 12h/12h day/night photoperiod and light intensity of 150 mE m⁻² s⁻¹. Seedlings with the same size and vigor were collected and then transferred to Hoagland solution containing NaCl at concentrations of 50, 100, 150, 200, 250 and 300 mM. Six NaCl treatments were considered for a period of one week and for each treatment three replications were included. After one week, plants were transferred to NaCl-free Hoagland solution for one week recovery period. Both gene expression and enzyme activity assessments were performed on leaf materials collected from non-stressed, stressed and recovered plants. Two terminal leaves of the test plants with the same height, age and vigour were selected for the experiments. All data are represented as the means ± SD of three replicates per each treatment.

Total RNA isolation

For the isolation of total cellular RNA from the leaf tissues, about 0.2 g leaf material (collected separately from non-stressed control, salt-stressed and recovered leaves) was fine powdered using liquid N₂ and 2ml 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 13,000 x 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 RNA was tested on 1% non-denaturing agarose gel using TBE running buffer.

mRNA purification and cDNA synthesis

Poly (A⁺) RNA was purified from the total cellular RNA using oligo-dT columns according to the Qiagen mRNA mini preparation and purification kit manufacturer. Single and double-stranded cDNAs were then synthesized according to the protocol of Promega cDNA synthesis kit. The integrity of the purified mRNA and synthesized double strand cDNA were analyzed by electrophoresis using 1% non-denaturing agarose gel. The quantities of the cDNA in the starting test materials were measured spectrophotometrically for the next experiments (Ausubel et al. 1991).

Semi-quantitative RT-PCR assay

The coding region of *Arabidopsis* DAAO cDNA was amplified using polymerase chain reaction. PCR was carried out using test leaves cDNA populations as templates and a specific primer set (forward: 5'-CCCGACGAACAATCACGATC-3' and reverse: 5'-GCTCGAGACTGTTTT CGCAA-3'). Each reaction was carried out by mixing the following components in a 0.5 ml PCR tube containing 100 pmol of each primer, 50 ng of cDNA, 2.5 µl of 10x PCR buffer, 10 mM dNTP, and 20 units *Taq* polymerase. The reaction mixtures were processed in thermocyclers (Techne, Germany) under the following cycling program: denaturation at 93°C for 1 min, annealing at 58°C for 1.5 min and extension at 72°C for 2 min. The reactions were finally extended at 72°C for 10 min. The PCR end products were separately analyzed on a 1.2 % agarose

gel. The amplified fragment was cloned in pGEM-T Easy vector system I and transformed to *E. coli* strain DH5α. Transformants were grown on isopropyl β-D-thiogalactopyranoside (IPTG)/X-gal media and a single recombinant colony was selected and processed for plasmid extraction using the alkaline lysis method (Birnboim and Dolly 1979). The isolated plasmid was digested with *Eco*RI restriction enzyme and separated on 0.8 % agarose gel. The partial sequencing of the amplified fragment was done at Microsynth DNA sequencing center, Switzerland.

Real-time quantitative RT-PCR assay

The relative expression levels of DAAO gene in the leaf materials of *Arabidopsis* test plants were analyzed by real-time RT-PCR using iQSYBR Green supermix (Bio Rad, USA) on Bio Rad MiniOpticon Real Time PCR Detection System. All expression data were normalized by adjusting the expression level of actin gene in test samples and the relative expressions were calculated using the $2^{-\Delta\Delta C_t}$ value (Livak and Schmittgen 2001). Experiments were performed in triplicates and the mean values are presented on the graph.

Enzyme extraction

200 mg of leaf material (collected separately from non-stressed control, salt-stressed and recovered leaves) were homogenized in 1ml of 0.1 M phosphate buffer (pH 7.0) containing 0.5 µl of β-mercaptoethanol and a pinch of polyvinyl polypyrrolidone (PVP). The homogenates were then centrifuged at 12,000 x g for 10 min and the supernatants were used for total D-amino acid oxidase activity assay.

Relative DAAO activity assay

DAAO activity of the test leaf samples was estimated using a peroxidase-coupled system by measuring the increase in A_{435} resulting from the oxidation of D-amino acids. To carry out this assay, 3 ml of enzyme extract solution containing 0.05 M of test D-amino acid, 0.0065 % o-dianisidine, and 0.1 ml of 1 % peroxidase enzyme were incubated at 37°C for 10 min. The absorbance of the samples was measured spectrophotometrically (UV-1800 spectrophotometer, RAY Leigh, China) at 435 nm. Compared to non-stressed control sample, the percentage of increase or decrease in the absorbance was considered as assessment criteria for the relative DAAO activity.

Total antioxidant assay

Total antioxidant activity of the leaf materials was determined using ferric reducing antioxidant power (FRAP) assay (Benzie and Strain, 1999). To 1 ml of leaf extract in 0.1 M phosphate buffer (pH 7.0), 3 ml of FRAP reagent (10 mM TPTZ: tripyridyl triazine, 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 300 mM sodium acetate buffer (pH 3.6) in the ratio of 1:1:10) was added and the reaction mixture was incubated at 37°C for 4 min. Measurements were carried out spectrophotometrically at 593 nm. Antioxidant potential of the samples was determined against a standard curve of ferrous sulphate (Fe^{II} , 100-1000 μM). Ascorbic acid (100 μM) served as a positive standard and BSA considered as a negative standard. FRAP values were calculated as follows: FRAP value ($\mu\text{mol Fe}^{II}$ 100mg⁻¹) = A_{593} of sample / A_{593} of standard) \times FRAP value of standard. FRAP values of all samples were presented as ($\mu\text{mol Fe}^{II}$ 100 mg FW^{-1}).

Computational analysis

Arabidopsis putative DAAO sequence (accession number: NP_201530) was extracted from publicly available database using NCBI site at: <http://www.ncbi.nlm.com/>. The specific primer set was designed by Primer3 v.4.0 software at http://www.primer3plus.com/web_0.4/input.htm. Nucleotide sequence analysis was performed by BLAST at NCBI site. Two sequences identities were detected by CLASTAW software. The molecular interaction between DAAO 3D structure and FMN/FAD was detected by using molecular docking software at AutoDock VINA.

RESULTS AND DISCUSSION

Since the discovery of DAAO and its complex biochemical and genetic characterization in different mammals and microorganisms, the active presence of its gene has not been well investigated in plant system. The first studies on DAAO ability in plants have been carried out on maize after finding putative *daao* sequences thought the genome projects outcomes (Gholizadeh and Baghbankohnerouz, 2009; Gholizadeh et al., 2009; Gholizadeh, 2011).

To continue the studies on plant DAAO, we conducted our experiments towards further identifications of DAAO ability at transcriptional and bio-functional levels in other plants. We planned to search this capability in *Arabidopsis thaliana* as a genetically well characterized plant. The sequence data survey for *Arabidopsis daao* using bioinformatic tools revealed us the presence of a newly annotated and released putative *daao* sequence (GenBank accession number

NP_201530). To our knowledge, there was no any investigation with respect to this predicted *daao* sequence at transcriptional and bio-functional levels, as yet. Therefore, we supposed that this putative *daao* gene like that of maize plant might be induced in response to inducing compounds or environmental cues.

To prime the *Arabidopsis* DAAO transcript, a specific primer pair was designed and synthesized based on its already reported sequence (accession number: NP_201530). The expression analysis was performed by semi-quantitative and real-time quantitative methods using the prepared cDNA materials of different leaf tissues collected from non-stressed, salt-stressed and recovered plants after the stress release. Separation of RT-PCR end products on 1% agarose gel revealed a detectable band with a molecular size of about 900 bp that was consistent to the calculated size of *daao* amplicon (Fig. 1). The results showed that the expression of *daao* gene is only detectable in salt-stressed *Arabidopsis* plants including those exposed to 150 and 200 mM of NaCl concentrations. The leaf tissues of non-stressed and recovered plants failed to show evidence of *daao* gene expression (gel photograph not presented). The highest level of *daao* expression was found to be related to the plants exposed

to 150 mM of NaCl concentration. The expression of DAAO transcript was shown to be apparently decreased in 200 mM NaCl-treated plants and ended in those exposed to higher concentrations of NaCl.

For further confirmation, the amplified RT-PCR product was partially sequenced and presented (Fig. 1). Analysis of the obtained nucleotide sequence was carried out by using BLAST server and its complete sequence identity with the already reported *Arabidopsis daao* sequence (accession number: NP_201530) was confirmed by CLASTAW software (the results not presented).

The presence and the level of *daao* gene expression were also analyzed by real time RT-PCR using iQ SYBR Green dye. All data were normalized by actin expression and related to sample 150 (sample treated with 150 mM NaCl considered as control) using $2^{-\Delta\Delta Ct}$ values (Fig. 2). The results indicated that the expression patterns of *daao* gene were consistent with the distribution profiles of *daao* expression obtained by semi-quantitative analysis. The expression of *daao* gene was not considerably detected except for the samples exposed to the salt concentrations of 150 and 200 mM. It was revealed that the level of DAAO transcripts in the plants treated with 200 mM NaCl was reduced by about 10 % compared to the plants treated

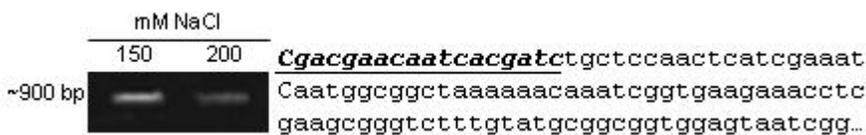


Figure 1. RT-PCR analysis of *daao* expression. The RT-PCR experiments were carried out for non-stressed, salt-stressed and recovered *Arabidopsis* plants and the end products were analyzed on 1% agarose gel. The detectable bands related to plants treated with 150 and 200 mM NaCl and the partial nucleotide sequence of the amplified fragment are presented.

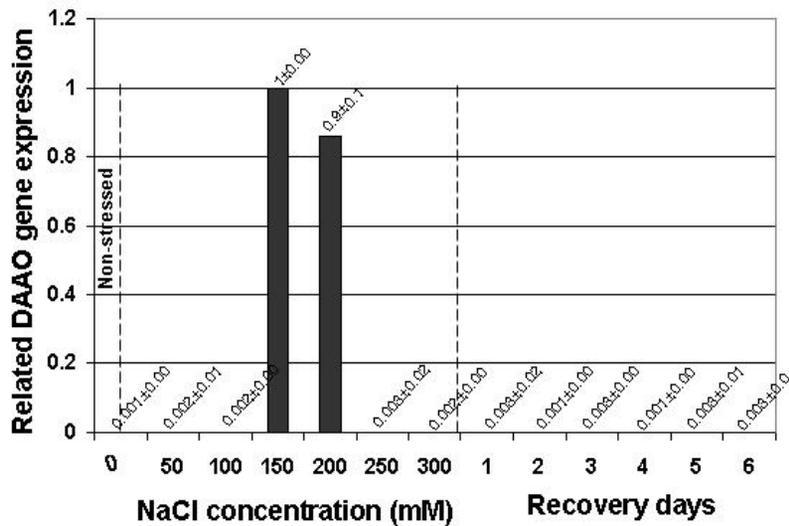


Figure 2. Real-time based expression analysis. The expression of *Arabidopsis daao* transcript was analyzed by real-time RT-PCR method using iQ SYBR Green dye. The experiments were carried out for non-stressed, salt-stressed and recovered *Arabidopsis* plants. Using $2^{-\Delta\Delta Ct}$ values, the relative expressions of *daao* were compared. Data are presented as the means of triplicates \pm SD.

with 150 mM NaCl.

As a general rule, *daao* is known as a controllable gene in different mammals and microorganisms. It is usually induced by D-enantiomers of amino acids in mammals and microorganisms (Horner et al., 1996; Molla et al., 2003). Numerous reports have shown that *daao* genes of mammals are expressed during natural biological processes such as aging and tissue/organ developments (Fischer, 1998; Mothel et al., 2006). Despite microbial and animal systems, the inductive expression of plant *daao* has only been studied and reported in maize plants exposed to D-alanine as a nitrogen source and drought stress (Gholizadeh and Baghbankohnehouz, 2009; Gholizadeh et al., 2009; Gholizadeh, 2011).

This study for the first time reports that *Arabidopsis daao* is an inducible gene that can be activated and expressed in leaf tissues under salt stress conditions. But

how different salt concentrations effort the inductive or suppressive effects on *daao* gene expression remain to be elucidated. In overall, the present results indicate that DAO gene might have an important role in salt resistance in *Arabidopsis* plant. Based on the activity of *daao* gene in salt-atressed *Arabidopsis* plants as well as drought-stressed maize plants, it may be suggested that *daao* is a common stress-related gene in plants. Therefore, this gene can be nominated and utilized as a potential biotechnological tool in plant genetic engineering.

In order to predict the functionality and the substrate specificity of the expressed *daao* gene product in salt-stressed plants, we randomly assessed DAO enzymatic activity of the leaf extracts towards D-aspartate, D-alanine and D-serine. The percentage of increase or decrease in the absorbance of the test samples at 435 nm was considered as a

criterion for the relative DAAO activity assessment. The results showed that the activity of DAAO towards D-aspartate was sharply increased in the leaf tissues of test plants exposed to 150 mM NaCl

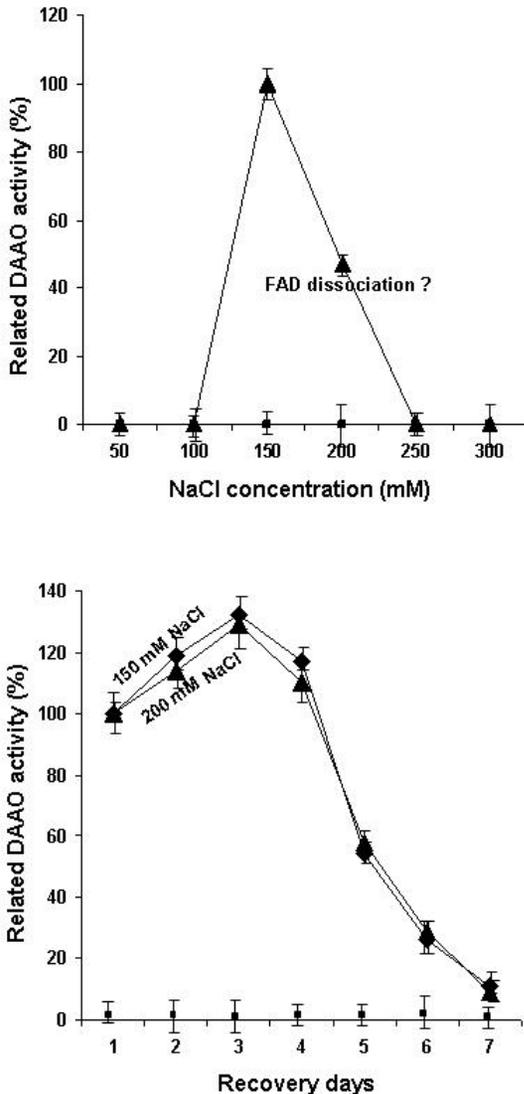


Figure 3. Assessment of D-aspartate oxidase activity. DAAO activity towards D-aspartate in the leaf extracts of non-stressed, salt-stressed (upper) and recovered (lower) *Arabidopsis* plants were estimated spectrophotometrically using a peroxidase-coupled system by measuring the increase in the absorbance of the samples at 435 nm. Data are presented as the means of triplicates \pm SD.

(Fig. 3). It was decreased by about 65% in plants treated with 200 mM NaCl. Data revealed that D-aspartate oxidase activity was sharply decreased close to the non-stressed level at salt concentrations higher than 200 mM. In non-stressed, low-stressed and very high stressed plants no DAAO activity was detected. Despite D-aspartate, D-alanine and D-serine failed to show evidence of DAAO activity in all test samples.

The activity of DAAO in test samples was also analyzed during 7 days of recovery period (Fig. 3). The results showed that the activity of D-aspartate oxidase was slightly increased during the first 3 days and then reverted back to the level verified before the stress or to the level of low stressed plants. The activity of DAAO towards D-alanine and D-serine was not detected for the recovered plants as for salt-stressed plants. This result may be partially consistent with the reports of Erickson's group that introduced D-alanine oxidase gene into *Arabidopsis* and developed a new selectable marker system based on the absence of D-alanine metabolism in this plant (Erickson et al., 2004).

Comparison of the distribution patterns in the activity of D-aspartate oxidase with the *daao* gene expression profiles showed similar induction/modulation patterns. This similarity most likely revealed the substrate specificity of the expressed DAAO gene in *Arabidopsis* plants under salt stress conditions. Despite this, a sharp decrease in the enzyme activity in comparison to the gene expression level under high salty media is a point of interest in our experiment. Most likely, this controversy is related to the direct inhibitory effects of high salt concentrations on the activity of DAAO enzyme.

Previously, the inhibition of flavoenzymes activity in the presence of different salts has been studied *in vitro* (Neujahr, 1983; Pollegioni et al., 2003; Hefti et al., 2003). It has been generally suggested that flavoenzyme inhibition by high salt concentrations is due to the abundance of negatively charged ions, which leads to the conformational perturbations preventing holoprotein formation. Recently, the significant inhibition of recombinant *Tv*DAAO activity has been studied in the presence of increasing concentrations of NaCl (Kopf et al., 2011). In this report, the inhibitory effect of NaCl was predicted to be due to the deflavination of the holoenzyme and induction of FAD dissociation by chloride negative ions as already been reported for cyanate or cyanide (Neujahr, 1983). For the confirmation of the previous reports, we predicted the three-dimensional structure of *Arabidopsis* DAAO and studied its molecular interaction with FMN/FAD by using bioinformatic tools. Our results indicated that FMN and FAD tend to interact with the inner part of the test DAAO molecule and be placed away from the sides exposed to water (Fig. 4). Therefore, considering this type of interaction, the effects of NaCl on the holoenzyme (flavoDAAO) might have two explanations: 1) the influence of NaCl on holoenzyme may be due to the competitive inhibitory effect of chloride ions on flavin and its subsequent dissociation, and 2) the inhibitory effect of NaCl on holo DAAO activity may be related to the chaotropic destructive effect of sodium positive ions on overall three-dimensional structure of DAAO and the consequent dissociation of flavin molecule from the perturbed enzyme structure. As the chloride ions have been reported to be less chaotropic, they

will be less effective in the removal of flavin.

To our idea, considering the interior location of flavin molecule on holoDAAO

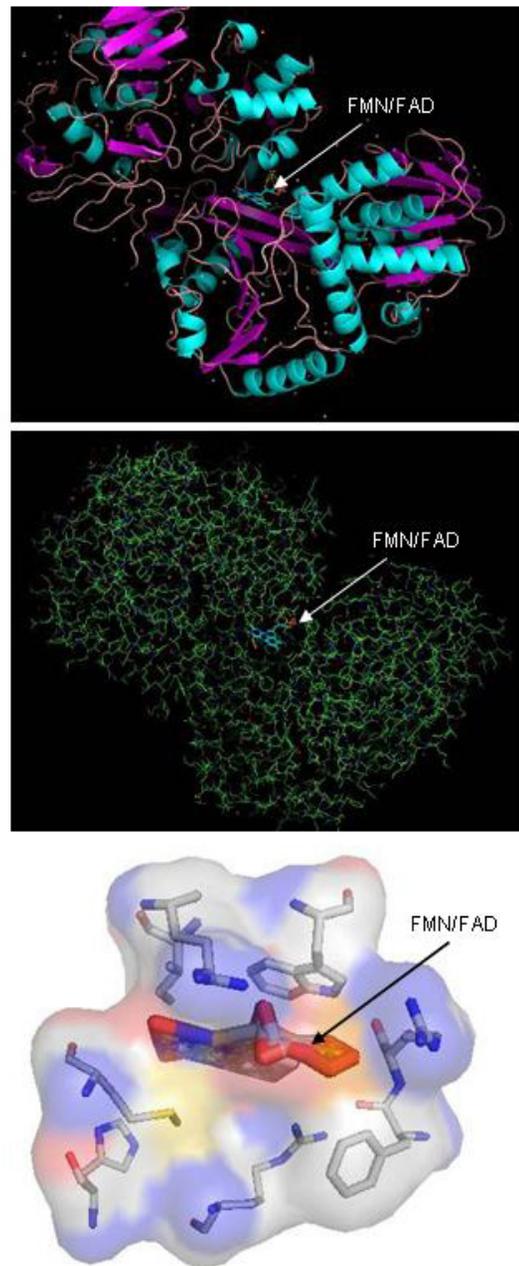


Figure 4. Computational analysis of FMN/FAD interaction with DAAO. *Arabidopsis* DAAO molecular interaction with FMN/FAD was predicted as three different presentations by using AutoDock VINA software.

and its less availability to chloride ions, the probability of chloride effects on deflavination process of DAAO may not be significant. On the other hand, the strong chaotropic effect of sodium ions and the

higher electrostatic force between sodium ions and flavin molecules may signify the influence of sodium ions (rather than chlorides) on the deflavination process of DAAO.

Our experiment results suggest that D-aspartate oxidase could be a component of pivotal importance in salt-induced resistance phenomenon in the test plant. In order to provide further assessment criteria for characterization of a possible resistance mechanism, we planned a parallel experiment to assay the total antioxidant potential of the plants. To test this capacity, FRAP assay was performed. The results showed that the antioxidative capacity was sharply increased up to 1.35 units in the leaves of plants exposed to 150 mM NaCl (Fig. 5). The capacity was found to decrease to 0.94 units in 200 mM NaCl-treated plants and it reached close to the normal level during high salty and recovery periods. The experimental results clarified the parallel increase/decrease modulation patterns in the antioxidant ability and DAAO activity of salt-treated *Arabidopsis* plants.

This is an interesting result and a probable explanation could be the altered antioxidant ability as a consequence of the H₂O₂ release by DAAO activity. Therefore, the decrease in antioxidant ability may be correlated with the decrease in the DAAO activity in plants treated with 200 mM NaCl (Fig. 5).

Earlier studies have shown that salt stress induces activation of the antioxidant system in plants (Gossett et al., 1994; Sreenivasula et al., 2000; Stepien and Klobus, 2005; Hichem et al., 2009; Kholova et al., 2009; Gholizadeh and Baghbankohnerouz, 2010). However, so far, no attention has been given to the

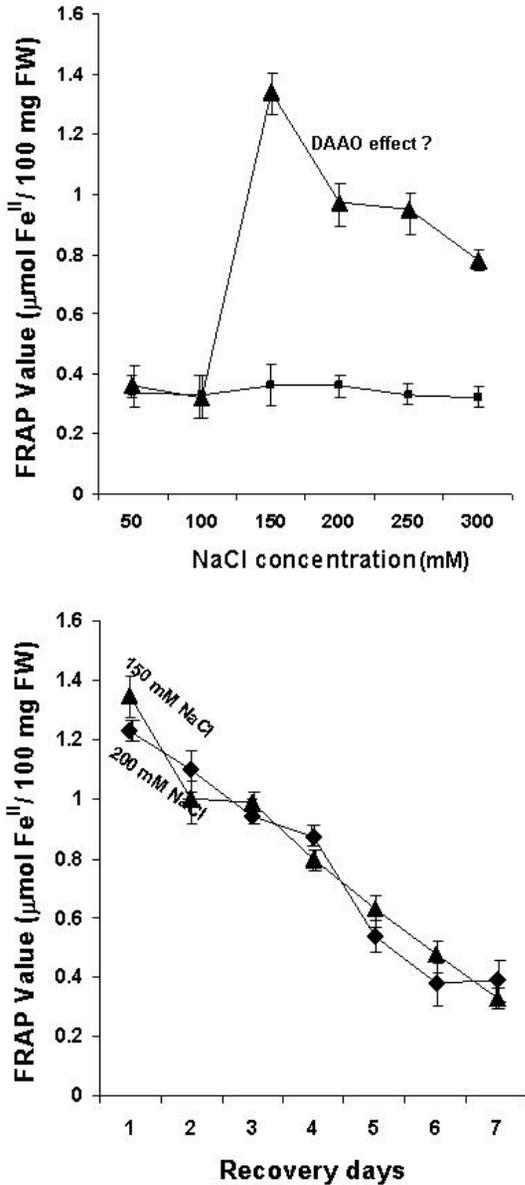


Figure 5. Assessment of antioxidant ability. The total antioxidant capacities of the leaf extracts were assessed in non-stressed, salt-stressed (upper) and recovered (lower) *Arabidopsis* plants using ferric reducing antioxidant power (FRAP) assay. Data are presented as the means of triplicates ± SD.

possible correlation between antioxidant and DAAO abilities. Therefore, our results may help to understand and characterize a potent resistance mechanism of salt-stressed plants by a putative DAAO-dependent strategy. But, how and to what extent DAAO exerts its effects on salt resistance in plants remain to be elucidated by further experiments. Also, the question whether DAAO plays a direct role in salt-challenged plants or exerts its effect indirectly by elevating the antioxidant system needs to be answered. Nevertheless, the overall results indicate that DAAO can be considered as a new candidate for plant genetic engineering for salt resistance.

ACKNOWLEDGMENTS

The author of this paper is thankful to the Research Institute for Fundamental Sciences (RIFS), University of Tabriz, Iran for the financial support of this work.

REFERENCES

- Ausubel F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seiolman, J.A. Smith, R. Struhi, 1991. *Current Protocols in Molecular Biology*, New York, John Wiley & Sons.
- Benzie F.F., J.J. Strain, 1999. The ferric reducing ability of plasma as leisure of antioxidant power, The FRAP assay. *Analytical Biochemistry*, 239:70–76.
- Birnboim H.C., J.A. Dolly, 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research*, 7:1513–1515.
- Chen Y.H., W.L. Chen Y.H., Wang, M.Y. Huang, M.K. Chern, 2007. Spatiotemporal expression of zebrafish D-amino acid oxidase during early embryogenesis. *Journal of Fish Physiology and Biochemistry*, 33: 73–80.
- Curti B., S. Ronchi, S.M. Pilone, 1992. D- and L-amino acid oxidases. In: *Chemistry and Biochemistry of Flavoenzymes* (edited by Muller F.), CRC Press, 69–94.
- Erickson O., M. Hertzberg, T. Nasholm, 2004. A conditional marker gene allowing both positive and negative selection in plants. *Nature Biotechnology*, 22: 455–458.
- Fischer L., 1998. D-amino acid oxidases in biotechnology. *Recent Research Developments in Microbiology*, 2: 295–317.
- Gamburg K.Z., N.L. Rekoslavskaya, 1991. Formation and functions of D-amino acids in plants. *Plant cell physiology (Fiziologiya Rastenii)*, 38: 1236–1246.
- Gholizadeh A., 2011. Estimation of D-amino acid oxidase gene expression in different organs of drought-challenged maize. *Scientific Research and Essays*, 6: 5290–5295.
- Gholizadeh A., B. Baghbankohnerouz, 2009. Molecular cloning and expression in *Escherichia coli* of an active fused *Zea mays* L. D-amino acid oxidase. *Biochemistry (MOSCOW)*, 74: 137–144.
- Gholizadeh A., B. Baghbankohnerouz, 2010. Activation of phenylalanine ammonia lyase as a key component of the antioxidative system of salt-challenged maize leaves. *Brazilian Journal of Plant Physiology*, 22: 217–223.
- Gholizadeh A., M.H. Faizi, B.

- Baghbankohnerouz, 2009. Molecular detection of drought stress-inducible D-amino acid oxidase gene from *Zea mays* L. Asian Journal of Plant Sciences, 8: 224–229.
- Golam S.M., S. Okada, H. Abe, 2003. Molecular characterization of D-amino acid oxidase from common carp *Cyprinus carpio* and its induction with exogenous free D-alanine. Archives in Biochemistry and Biophysics, 420: 121–129.
- Gossett D.R., E.P. Millhollon, M.C. Lucas, 1994. Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivars of cotton. Crop Science, 34: 706–714.
- Hefti M.H., J. Vervoort, W.J.H. Van Berkel, 2003. Deflavination and reconstitution of flavoproteins: tackling fold and function. European Journal of Biochemistry, 270: 4227–4242.
- Hichem H., D. Mounir, A. Naceur, 2009. Differential responses of two maize varieties to salt stress: changes on polyphenols composition of foliage and oxidative damages. Industrial Crop production, 30: 144–151.
- Horner R., F. Wagner, L. Fischer, 1996. Induction of the D-amino acid oxidase from *Trigonopsis variabilis*. Applied Environmental Microbiology, 62: 2106–2110.
- Jamil A., S. Riaz, M. Ashraf, M.R. Foolad, 2011. Gene Expression Profiling of Plants under Salt Stress. Critical Reviews in Plant Sciences, 30: 435–458.
- Kholova J., R.K. Sairam, R.C. Meena, G.C. Srivastava, 2009. Responses of maize genotypes to salinity stress in relation to osmolytes and metal-ions contents, oxidative stress and antioxidant enzymes activity. Biologia Plantarum, 53: 249–256.
- Kopf J., D. Hormigo, J.L. Garc'ia, C. Acebal, I. de la Mata, M. Arroyo, 2011. Inhibition of Recombinant D-Amino Acid Oxidase from *Trigonopsis variabilis* by Salts. Enzyme Research, 1–5.
- Livak K.J., T.D. Schmittgen, 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. Methods, 25: 402–408.
- Lyle L.R.D., 1968. D-amino acid oxidase induction in the kidneys of germ-free mice. Journal of Bacteriology, 96: 606–608.
- Molla G., L. Motteran, L. Piubelli, M.S. Pilone, L. Pollegioni, 2003. Regulation of D-amino acid oxidase expression in the yeast *Rhodotorula gracilis*. Yeast, 20: 1061–1069.
- Mothel J.P., E. Rouaud, P.M. Sinet, B. Potier, A. Jouvenceau, P. Dutar, C. Videau, J. Epelbaum, J.M. Billard, 2006. Critical role for the glial-derived neuromodulator D-serine in the age-related deficits of cellular mechanisms of learning and memory. Aging Cell, 5: 267–271.
- Munns R., 2002. Comparative physiology of salt and water stress. Plant Cell and Environment, 25: 239–250.
- Neujahr H.Y., 1983. Effect of anions, chaotropes, and phenol on the attachment of flavin adenine dinucleotide to phenol hydroxylase. Biochemistry, 22: 580–584.
- Pollegioni L., S. Iametti, D. Fessas, et al., 2003. Contribution of the dimeric state to the thermal stability of the flavoprotein D-amino acid oxidase. Protein Science, 12: 1018–1029.

- Schleiff U., 2008. Analysis of water supply of plants under saline soil conditions and conclusions for research on crop salt tolerance. *Journal of Agronomy and Crop Science*, 194: 1–8.
- Sikora L., G.A. Marzluf, 1982. Regulation of L-amino acid oxidase and of D-amino acid oxidase in *Neurospora crassa*. *Molecular and General Genetics*, 186: 33–39.
- Simonetta M.P., R. Verga, A. Fretta, G.M. Hanozet, 1989. Induction of D-amino acid oxidase by D-alanine in *Rhodotorula gracilis* grown in defined medium. *Journal of General Microbiology*, 135: 593–600.
- Sreenivasulu N., B. Grimm, U. Wobus, W. Weschke, 2000. Differential response of antioxidant compounds to salinity stress in salt-tolerant and salt-sensitive seedlings of foxtail millet (*Setaria italica*). *Physiologia Plantarum*, 109: 435–442.
- Stepien P., G. Klobus, 2005. Antioxidant defense in the leaves of C₃ and C₄ plants under salinity stress. *Physiologia Plantarum*, 125: 31–40.
- Tishkov V.L., S.V. Khoronenkova, 2005. D-amino acid oxidase: structure, catalytic mechanism and practical application. *Biochemistry (Moscow)*, 70: 51–56.