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Isolation, molecular cloning and expression analysis of *Aeluropus littoralis* Monodehydroascorbate reductase (*MDHAR*) gene under salt stress

Azadeh Mohseni[1], Ghorban Ali Nematzadeh[2], Ali Dehestani[2], Behzad Shahin[2] & Elham Soleimani[2]

1. Department of Plant Breeding, Sari Agricultural Sciences and Natural Resources University, Sari, Iran.
2. Genetics and Agricultural Biotechnology Institute of Tabarestan, Sari Agricultural Sciences and Natural Resources University, Sari, Iran.

*Corresponding Author, Email: azadehmohseni34@yahoo.com

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Abstract

Plants are naturally exposed to frequent changes in environmental conditions such as salt stress affecting their growth and development, and consequently a considerable reduction in total productivity. Monodehydroascorbate reductase (MDHAR), a key enzyme in ascorbate-glutathione cycle, serves as an important antioxidative enzyme in scavenging of reactive oxygen spices (ROS). In this study, *MDHAR* gene from *Aeluropus littoralis* was isolated using specific primers by RT-PCR and its expression variation was investigated at 0, 150, 300, 450 and 600 mM of NaCl through Real-time PCR. The results indicated that *A. littoralis* MDHAR gene compromised 1436 bp without any introns, showing a high similarity with *Sorghum bicolor*. The only alteration of *MDHAR* expression was observed at 300 mM NaCl, i.e. cytosolic MDHAR in both shoot and root was increased 1.3 and 1.4 fold compared to control samples. It could be concluded that accumulation of MDHAR reduces at Nacl concentrations higher than 300 mM.

***Key words*:** *Aeluropus littoralis*, Ascorbate-glutathione cycle, MDHAR, Real-Time PCR, ROS.

Introduction

Salt stress is a major environmental challenge leading to significant decreases in plant growth and productivity (20). Normally, upon an abiotic stress like salinity, the disrupted cellular homeostasis leads to the production of reactive oxygen species (ROS) e.g. superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) which interact with different cellular molecules and metabolites (1, 20). The overproduction of ROS damages membranes, DNA and

proteins and even leads to cell death (16). To protect themselves from ROS-induced oxidative damage, crop plants have evolved a wide range of defense systems that could be enhanced to resist unfavorable abiotic stress conditions, including antioxidant molecules and antioxidative enzymes (18). Enzymes involved in restoring the redox state of the glutathione and ascorbate pools such as monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) could play a key role in maintenance of ascorbate (AsA) pool and protection against abiotic stresses (10, 17). In plant cells, ascorbate peroxidase is a major scavenger of H_2O_2 using ascorbate as a reducing substrate to convert it to H_2O (6). In this reaction, ascorbate is oxidized to a short half-life as monodehydroascorbate (MDHA) which is then reduced to AsA by monodehydroascorbate reductase (MDHAR), or spontaneously disproportionated to AsA and dehydroascorbate (DHA). DHA is reduced to ascorbate through a GSH-dependent reaction by dehydroascorbate reductase (5). Glutathione disulfide (GSSG) is reduced by glutathione reductase using NADPH as the electron donor. These series of reactions scavenging ROS as described above are known as the ascorbate–glutathione cycle (6).

The Poaceae are a large family of monocotyledonous plants which are known as stress-tolerant plants (8). *Aeluropus littoralis*, a halophyte with C_4 photosynthesis, belongs to Poaceae family that can endure salt (NaCl) up to 600 mM (14, 9). *A. littoralis* grows in marshes, salty and drought lands. This plant includes diploid genome (2n=2X=14) with about 342Mb length (12). C_4 photosynthesis system in *A. littoralis* is the one of the reasons for being more flexible against salt and drought stresses. Due to these characteristics, *A. littoralis* may serve as a rich genetic resource for the identification of new genes related to salt, drought and heat tolerance (23). It also can be used in productivity breeding in cultivated crops (8).

Materials and methods

Plant materials and growth conditions

A. littoralis seeds were collected from natural habitats in Esfahan and cultured in acid washed and nutrient free sand in plastic pots in green-house (16 hours light, 8 hours darkness and 25º C). Pots were irrigated with half Hoagland's solution for 15 days. For the analysis of gene expression, plants were transferred to hydroponic media in plastic chambers with half-strength Hoagland's solution (pH 5.6) for another 15 days at 25 ˚C, 16 h photoperiod and relative humidity of 60-75%. The Hoagland's solution was changed every 3 days and pH was adjusted to 5.6. After adaption of seedlings with hydroponic media, they were exposed to salt stress under absence and presence of varying concentrations of salinity which increased 150 mM NaCl daily (0, 150, 300, 450 and 600 mM NaCl). Following treatment, the samples of shoots and roots were collected from plants and immediately frozen in liquid nitrogen

and stored at -80 ℃ for RNA isolation and DNA synthesis.

MDHAR gene isolation

Specific primers were designed using Oligo software based on the conserved sequences of *MDHAR* genes in Poaceae family. Total RNA was extracted from the shoots with the TRIZOL reagent (Invitrogen, USA) according to the manufacturer's protocol. RNA quantify was evaluated by spectrophotometry. The single-strands cDNA was synthesized using the M-MULV reverse transcriptase kit (Fermentas) and oligo (dT) primers according to manufacturer's instructions. RT-PCR was performed using 1µl cDNA, 10 Pico moles of specific primers, 200µM dNTPs, 1x PCR-buffer and 2.5 unit *Taq* DNA polymerase. PCR products were separated on 1% agarose gel, isolated using High Pure PCR product purification kit (Roche, USA). The isolated fragment was sequenced and specific primers were designed according to the exact gene sequence.

Real-Time PCR analysis

The expression profiles of *MDHAR* gene after different treatments were detected through Real-time PCR analysis. Gene-specific primers (forward: 5- GAAAGCCAGAAA CA-GATACCT -3; reverse: 5- CAATA-ACTAACCGCAGCAAT-3) were designed using oligo (ver. 5) and the gene of actin (forward primer, 5-GGA-TCTTTACGGCAATGTC-3, reverse primer, 5-GGCGCAACTACCTTCA-CCT-3) was chosen as control. Isolation of total RNA from control and treated samples was performed with Trizol as described above. The RNA samples (3 µg) were treated with RNase-free DNaseI (2 units) in 37℃ for 30 minutes to eliminate traces of DNA, followed by the quantification using the spectrophotometer. Synthesis of single strand cDNA was done using by MMULV reverse transcriptase as above description. To ensure gene-specific amplification, the mentioned primers were used to amplify the MDHAR fragment through regular PCR. The resulting 115-bp PCR fragment was amplified, and the expected MDHAR fragment was then confirmed through sequence analyses. Aliquots of the first-stand cDNA mixtures served as the templates for real-time PCR analysis by using the SYBRGreen Real-time PCR, Master Mix Reagent Kit (Thermo scientific) in ABI Prism 7700 and Sequence (Applied Biosystems, Foster City, CA, USA). Real time RT-PCR was carried out under the following program: 95℃ for 15 min, 15 S of denaturation, 30 S of annealing and extension at 57 ℃ for 40 cycles. Melt curve analysis was performed to check the specificity of the amplified product. To minimize sample variation, the samples were normalized using actin expression mRNA expression. The relative transcript levels were calculated using the actin as the housekeeping gene.

Results

MDHAR gene isolation

According to the sequences from Poaceae family which applied in primer design for MDHAR gene, 1436

nucleotides was predicted for full-length cDNA of MDHAR which was obtained using RT-PCR. After cloning MDHAR gene in PTZ57R/T vector, the cDNA insert was completely sequenced by M13 forward and M13 reverse universal primers separately (Bioneer, Korea). The sequenced fragments were analyzed in Bio-Edit software to earn full length MDHAR gene. Bioinformatics analysis indicated that MDHAR gene includes 1436 nucleotides without intron which encodes protein whit 479 amino acid in theory with 51757.4 molecular weight. The theoretical pI was calculated as 8.46.

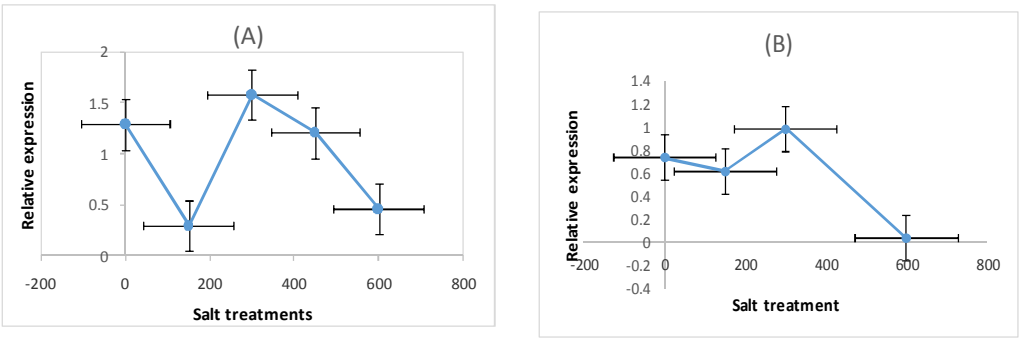
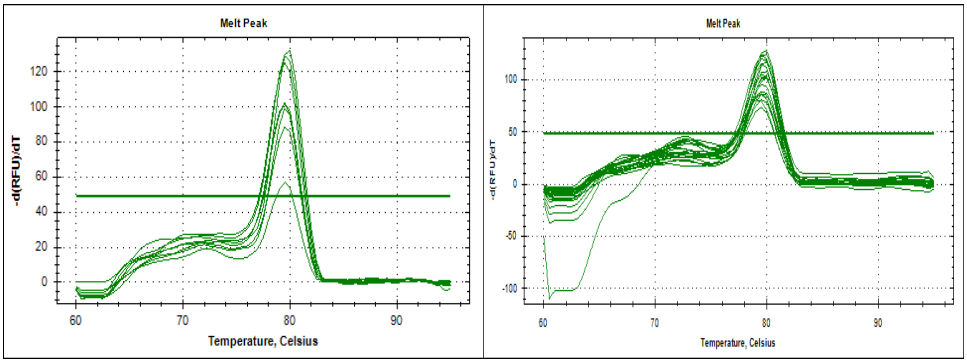


Figure 1. relative expression of MDHAR under 0, 15, 300, 450 and 600 mM NaCl in Aeluropus littoralis. (A) The relative abundance of MDHAR shoot. (B) The relative abundance of MDHAR in shoot. X axis is salinity treatments and Y axis indicates relative expression which calculated with 2- ΔΔct formula based on actin gene as reference.



(A) (B)

Figure 2. Melt curve resulted from Real-time PCR in MDHAR amplification in various salinity in shoot (A) and root (B). All of the curves includes the same temperature peak.

Expression pattern of MDHAR gene under salt stress

The MDHAR transcript was measurable in both stressed and non-stressed control seedlings.To assess the salt stress expression of MDHAR from *A. littoralis* seedlings, total RNA was isolated from shoots and roots in both NaCl treatment and control plants. The relative expression levels of MDHAR in stressed samples compared with its corresponding control one were detected using by Real-time PCR. The data were normalized using actin gene

as reference for internal control. In an attempt to capture salinity changes in MDHAR gene expression, the *A. littoralis* seedlings we sampled at 0. 150, 300, 450, 600 mM salinity stress. The transcription of the MDHAR gene in shoots was somewhat similar to root's in that they both were decreased under 150mM NaCl and both of them were peaked in 300mM concentration of salinity compared with their corresponding control samples. In shoots, MDHAR transcript was decreased significantly 4.37 fold less than control samples under 150 mM salinity, while in roots this decreasing was observed 1.19 fold. The most transcription under 300 mM NaCl was almost 1.3 and 1.4 fold higher compared with control sample in shoots and roots respectively (Figure 1A) and B). In roots, the expression of MDHAR was remarkably suppressed in following NaCl concentration; however we lost our data under 400mM NaCl because of the nonsense result. The least expression under 600 mM NaCl was reduced 0.7 fold rather than control sample in roots (Figure 1B).

The melt curve indicated all of the curves have the same temperature peak, it has proved that non-specific amplification did not occurred in Real-time PCR production (Figure 2A) and B).

Discussion

The main adverse effects of salt stress on plant growth and its productivity are due to ion cytotoxicity ($Na^{+,}$ Cl^{-} and SO_4^{-}) and osmotic stress which also lead to oxidative stress (24). In this paper, we have cloned and identified a cDNA encoding MDHAR which were located in cytosol and also we studied relative expression of MDHAR gene under various salinities in *A. littoralis*. Alignment of the deduced protein sequence with MDHAR from other plants and NCBI protein Blast (http://www.ncbi.nlm.-nih.gov/BLAST.cgi) suggested the cDNA indeed code the MDHAR isozymes with the same physiology function, locating in cytoplasm. Previous studies supported the cytosolic forms of antioxidants involved in the AsA-GSH cycle have physiological importance (21, 22). Lunde *et al*. (15) suggested that cytosolic MDHAR is likely to play important roles in protecting the whole plant during abiotic stresses. The phylogenic tree demonstrates that MDHAR has the most similarity with MDHAR from *Sorghum bicolor* (89%). Protein analysis sequences were indicated the total number of negatively and positively charged residues was 48 and 52 respectively; and its half-life was estimated 30 hours (mammalian reticulocytes, in vitro), less than 20 hours (yeast, in vivo) and less than 10 hours (Escherichia coli, in vivo).

The functions of MDHAR have been determined in a wide range of plants because of its important role in AsA-GSH cycle and ROS scavenge. In our study, reduction of the gene transcript under first treatment (150mM NaCl) in shoots was 3.67 fold less than roots. The most increased MDHAR transcription was observed under 300 mM NaCl in both roots and shoots

which in roots was 1.091 fold higher compared with shoots. In following treatments the gene expression was down-regulated. Up-regulation in mRNA level of MDHAR under 300mM NaCl suggests this gene may plays role in salinity tolerance in *A. littoralis*; however, the response of *A. littoralis* to salt stress may depend on the concentration of NaCl and duration of exposure of the plants to salt, leading to varying degrees of adaptation. It was reported the cellular localization and the ability to confer salt stress tolerance in transgenic tobacco of this salt inducible Am-MDAR. Transgenic tobacco plants overexpressing Am-MDAR survived better under conditions of salt stress compared to untransformed control plants (11). A cDNA encoding MDHAR from the mangrove plant *Acanthus ebracteatus* was introduced into rice to examine its role in salt tolerance. The transgenic rice lines overexpressing AeMDHAR showed a significant increase in MDHAR enzyme activity compared to untransformed plants under both NaCl and control conditions (19). DHAR and MDHAR over expressions in cytosol with increasing ASA to DHA ratio enhance salt stress tolerance (22, 21, 2, 3). Lower lipid peroxidation and ability to maintain high chlorophyll content under salt stress conditions resulting from higher MDHAR activity was also reported (11, 7, 4, 13).

Generally it could be concluded that the ratio of MDHAR reduces under more than 300 mM concentration of NaCl. It also was suggested the transcription reduction under first treatment (150 mM NaCl) can result from this fact that plants needed a time to adapt to stress conditions.

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جداسازی، همسانه‌سازی مولکولی و بررسی بیان ژن مونودهیدروآسکوربات ردوکتاز (MDHAR) از گیاه آلوروپوس لیتورالیس تحت تنش شوری

آزاده محسنی[1*]، قربانعلی نعمت‌زاده[2]، علی دهستانی[2]، بهزاد شاهین[1]، الهام سلیمانی[2]

۱. گروه زراعت و اصلاح نباتات، دانشگاه علوم کشاورزی و منابع طبیعی ساری، ساری، ایران

۲. پژوهشکده ژنتیک و زیست‌فناوری کشاورزی طبرستان، ساری، ایران

*نویسنده مسوول: azadehmohseni34@yahoo.com

چکیده

گیاهان به طور طبیعی در معرض تغییرات محیطی بسیاری از جمله شوری هستند که رشد و نمو آنها را تحت تاثیر قرار داده و سبب کاهش قابل توجهی در میزان محصولات خواهد شد. مونودهیدرو آسکوربات ردوکتاز (MDHAR)، یکی از آنزیم‌های کلیدی در چرخه‌ی گلوتاتیون-آسکوربات است که نقش مهمی را به عنوان آنزیم‌های آنتی‌اکسیدان در پاکسازی گونه‌های فعال اکسیژن (ROS) بر عهده دارد. در این مطالعه، ژن MDHAR از گیاه آلوروپوس لیتورالیس با پرایمرهای اختصاصی با روش RT-PCR جدا شده و تغییرات بیان آن در شرایط شوری با غلظت‌های ۰، ۱۵۰، ۳۰۰، ۴۵۰ و ۶۰۰ میلی‌مولار NaCl از طریق Real-Time مورد بررسی قرار گرفت. نتایج نشان داد که MDHAR از ۱۴۳۶ جفت باز بدون اینترون تشکیل شده است و بیشترین شباهت را با گیاه سورگم نشان داده است. مطالعات بیانی ژن MDHAR نیز حاکی از آن بود که بیان این ژن هم در شاخساره و هم در ریشه تنها در غلظت ۳۰۰ میلی‌مولار به ترتیب به میزان ۱/۳ و ۱/۴ برابر نسبت به گیاه کنترل افزایش یافته است و در غلظت‌های بالاتر افزایش بیانی مشاهده نشد. از نتایج به دست آمده می‌توان نتیجه گرفت که بیان ژن مذکور در غلظت‌های بالاتر از ۳۰۰ میلی‌مولار کاهش می‌یابد.

کلمات کلیدی: آلوروپوس لیتورالیس، چرخه‌ی آسکوربات-گلوتاتیون، مونودهیدروآسکوربات رداکتاز، Real-Time PCR، گونه‌های اکسیژن فعال.