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MOLECULAR CHARACTERIZATION OF A SALT-INDUCIBLE MONODEHYDROASCORBATE REDUCTASE FROM THE HALOPHYTE *AVICENNIA MARINA*

Kumaresan Kavitha,[1,*] Balasundaram Usha,† Suja George,† Gayatri Venkataraman,† and Ajay Parida[2,†]

*Department of Plant Biotechnology, School of Bioscience and Technology, VIT University, Vellore, India 632014; and †M. S. Swaminathan Research Foundation, Third Cross Street, Taramani Institutional Area, Chennai, India 600113

Salinity poses a major threat to crop productivity. Our earlier work has used the halophytic plant *Avicennia marina* as a model organism for mining genes that function in salinity stress tolerance. Here we report the isolation and characterization of a monodehydroascorbate reductase (MDAR) from this plant. MDAR plays a key role in regeneration of ascorbate from monodehydroascorbate for reactive oxygen species scavenging. A cDNA clone encoding MDAR was isolated from a cDNA library created from a salt-stressed leaf of *A. marina*. A transit peptide at the N-terminal region of Am-MDAR suggested chloroplastic localization. Transcript profiling for *Am-MDAR* revealed that the gene is expressed in response to salinity and oxidative stress (high-intensity light, H_2O_2, and iron overload). The genomic clone of *Am-MDAR* contained 16 exons. The presence of two identical MDAR transcripts, with and without exon 3, indicated possible exon skipping. A 1167-bp fragment corresponding to the *5′* upstream region of *Am-MDAR* was isolated, and transient reporter gene expression studies revealed it to be a functional promoter. In-silico analysis of this sequence revealed the presence of putative light- and abiotic-stress regulatory elements. The possible reasons for changes in gene expression during stress in relation to the host plant's stress tolerance mechanisms are discussed.

Keywords: ascorbate, *Avicennia marina*, monodehydroascorbate reductase, salt stress.

Introduction

Abiotic stress is the principal cause of crop failure worldwide, causing a dip of more than 50% in average yields for most major crops (Boyer 1982; Bray et al. 2000). A large portion of land available for cultivation is already under threat of drought, and more than 50% of all arable land is predicted to be affected by salinity by the year 2050 (Flowers and Yeo 1995). This condition is alarming and necessitates increasing agricultural productivity per unit of land and water. The development of crop plants able to grow in these adverse conditions assumes importance in this emerging global context.

Despite the wealth of information on abiotic stress and stress tolerance in plants, many aspects still remain unclear. One effective way of analyzing a stress response is to use model organisms chosen for their relative amenability for study and/or their tolerance to the stress in question. The use of stress-tolerant model organisms helps to identify the pathways/genes that make them tolerant. *Avicennia* is a monotypic, pantropical mangrove genus with eight species, of which *Avicennia marina* is widely distributed both latitudinally and longitudinally. The high salt tolerance of *A. marina* is a consequence of water use efficiency, which balances the relations between carbon gain, water loss, and ion uptake with the transpiration stream on a low but constant level. *Avicennia marina* grows in coastal regions where the salt concentration can be as high as 9%. The regulation of inorganic ions occurs partially by exclusion at the roots and also by excretion via salt glands, the excretion rate for sodium and chloride ions being 0.4 and 0.046 μmol m^{-2} s^{-1}, respectively (Mehta et al. 2005). It is thus an ideal candidate plant for mining genes for salt tolerance.

In this article, we report the isolation and characterization of a monodehydroascorbate reductase (MDAR) gene from *A. marina*. MDAR (EC 1.6.5.4) is a flavin-adenine dinucleotide (FAD) enzyme that uses NAD(P)H as an electron donor to reduce MDA to ascorbate (AsA; Hossain et al. 1984). It is the only enzyme known to use an organic radical as an enzyme substrate. In the chloroplast, MDA is reduced to AsA by photoreduced ferredoxin at a high rate, and this is likely to constitute the main pathway in the vicinity of the thylakoid membrane (Miyake and Asada 1994). Away from the thylakoid membrane, reduction of MDA can occur via two enzymes in the ascorbate-glutathione pathway, dehydroascorbate reductase (DHAR) and MDAR. The specific activity of MDAR was found to be 10 times that of DHAR in both mitochondria and chloroplasts of some plants (Mittova et al. 2000). Computer simulations based on known enzyme concentrations and properties in the chloroplast led to the conclusion that the majority of MDA is reduced by MDAR (Polle 2001).

With its ability to directly regenerate AsA, MDAR plays an important role in maintaining a reduced pool of AsA. Activity of MDAR has been described in several plant cell compartments, such as chloroplasts (Hossain et al. 1984), mitochondria (Jiménez et al. 1997), cytosol (Dalton et al. 1993), glyoxysomes (Bowditch and Donaldson 1990), and leaf peroxisomes

[1] Current address: Oil Seed Research Station, Tamil Nadu Agriculture University, Tindivanam, India 604002.

[2] Author for correspondence; e-mail: ajay@mssrf.res.in.

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(Jiménez et al. 1997). Upregulation of different MDAR isoforms in response to various environmental stresses have been reported (Grantz et al. 1995; Ye and Gressel 2000; Yoon et al. 2004).

In this investigation, we isolated an *A. marina* MDAR (*Am-MDAR*) cDNA clone from a cDNA library created from a salt-stressed *A. marina* leaf. The expression profile of *Am-MDAR* under different conditions and in different tissues was analyzed. The gene organization of *Am-MDAR* and the presence of various *cis*-acting elements in its promoter were examined. Our results point to the suitability of overexpressing *Am-MDAR* in transgenic systems for protection against salt stress.

Material and Methods

Isolation of Am-MDAR *cDNA Clone and In-Silico Analysis*

A cDNA library was created from the leaf tissue of salt-stressed month-old *Avicennia marina* seedlings (Mehta et al. 2005). Random expressed sequence tag (EST) sequencing of this library led to the identification of an EST that showed maximum homology to MDAR (CD777105). This EST was sequenced completely with the BigDye terminator method (ABI 310, Applied Biosystems). Similarity searches were done with the BLASTX program in the National Center for Biotechnology Information (NCBI) database. Open reading frames (ORFs) were identified in this cDNA with the NCBI's ORF finder (http://www.ncbi.nlm.nih.gov), and the longest ORF with predicted start and stop codons was identified as the putative coding region. The conserved domains present in the ORFs were identified through the NCBI Conserved Domain Database (NCBI-CDD; Marchler-Bauer et al. 2007). Protein-targeting predictions were done via PSORT. Molecular-mass predictions were made with Prot Param (Gasteiger et al. 2005). Multiple sequence alignments were performed with the ClustalW program at NPS (Network Protein Sequence analysis). Phylogenetic analyses were performed with MEGA, version 4.0, via the neighbor-joining method. Genomic sequences were aligned with the Plant GDB database. The *Arabidopsis* genome database was searched to determine the number and location of MDAR genes in the *Arabidopsis* genome.

RNA Isolation and Transcript Profiling of Am-MDAR

Avicennia marina seedlings were grown according to Mehta et al. (2005). One-month-old seedlings (four-leaf stage) were used in this study. Stress conditions imposed on *A. marina* seedlings (light [500 microequivalent m^{-2} s^{-1}], salinity [500 mM NaCl], H_2O_2 [90 mM], and iron overload [1 mM Fe(III) citrate]) are as described by Jithesh et al. (2006). To study tissue-specific expression, RNA was isolated from *A. marina* leaves, shoots, and roots. Total RNA from frozen leaf tissue was isolated according to Chomczynski and Sacchi (1987). Total RNA (20 μg $lane^{-1}$) was fractionated on a 1.5% denaturing formaldehyde-agarose gel and transferred to nylon membrane (Hybond-N, Amersham). RNA blot hybridizations were carried out at 65°C with α-^{32}P-labeled *Am-MDAR* cDNA as probe, as per Mehta et al. (2005).

Assay for MDAR activity in A. marina

Avicennia marina leaf tissue subjected to salinity, excess-light, dark, H_2O_2, iron overload, or iron-limiting conditions for 6 h each were ground in liquid nitrogen in homogenization buffer (1 : 4 w/v; 25 mM Tris-Cl [pH 7.8], 0.1 mM EDTA, 5% [w/v] glycerol, and 250 μM PMSF). The homogenate was centrifuged at 12,000 *g* for 20 min, and the supernatant was used for the MDAR assay, as described by Hossain et al. (1984). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 0.2 mm NADH, 2.5 mm ascorbic acid (ASC), and 1 unit of ASC oxidase. The reaction was initiated by adding ASC oxidase (EC 1.10.3.3 from *Cucurbita*; Sigma-Aldrich, St. Louis, MO) to the mixture, thus

Table 1

Oligonucleotides Used in This Study

Primer	Oligonucleotides (5′ to 3′)
MDAR-UTR-F	GCACACGC GTTCAATGTGCATG
MDAR2R	CCCTTTCTCCTTGTACCAGTCA
MD AR2F	GCGTTGGCACTGGTGGTGAGA
MDAR1R	GCCACCACCAACC ACCACAA C
MDAR1F	CCTGGAGTTCATTATATTCGTGAT
MDAR1R	GCCACCACCAACCACCACAAC
MDAR3R	GCTGTGCAGACCGACGAGCAT
MDAR 3F	GCTCGGTTGAACATGTTGATCA
MDAR-UTR-R	GCACACGCGTTCAATG TGCATG
MDAR5F	GGAGGTGGAAATGCAG CTGGT
MDAR-GSP1	GGCCGAGGAATTTGATT AACCG
MDAR-GSP2	CCCGTGCTTCAGTGAAGGGG
MDAR-GSP3	CGCCATTATTGCTCTACAAACTG
AD1	NGTCGASWGANAWGAA
AD2	TGWGNAGS ANCASAGA
AD3	WGTGNAGWANCANAGA
AD4	STTGNT ASTNCTNTGC
MDAR PROM 1F	CGCGGATCCCTCTCGATCTTATCATGAAAATACT
MDAR PROM 1R	CCGGAATTCTATTGCTCTACAAACTGTAAGCTT

generating the substrate MDA. Activity was measured as the ASC oxidase–induced oxidation of NADH. The reaction was monitored at 340 nm (extinction coefficient for NADH = 6.2 mM^{-1} cm^{-1}). Assays were carried out in triplicate.

Isolation of the Am-MDAR *Genomic Clone*

Genomic DNA of *A. marina* leaves was isolated by the method of Dellaporta et al. (1983). Primers corresponding to different regions of *Am-MDAR* cDNA were used to generate overlapping genomic fragments. Fragments of 1.285 kb, 1.507 kb, 246 bp, 1.75 kb, and 1.65 kb were amplified with primer pairs MDAR-UTR-F/ MDAR2R, MDAR2F/MDAR1R, MDAR1F/ MDAR1R, MDAR1F/MDAR3R, and MDAR 3F/MDAR–UTR-R, respectively (table 1) and cloned in T/A vector (MBI, Fermentas). The overlapping clones were sequenced completely and compared with the *Am-MDAR* cDNA sequence.

Genomic DNA Blot Analysis

Avicennia marina genomic DNA (15 μg) was digested with restriction enzymes—namely, *Eco*RI, *Bam*HI, and *Hind*III—resolved on 1.0% agarose gel, and then blotted onto positively charged nylon membrane (Hybond-N+, Amersham) and hybridized with an MDAR full-length cDNA probe. The probe was radiolabeled with (α-^{32}P) dCTP, using random primer labeling beads (Amersham).

Reverse Transcription PCR to Confirm Exon Skipping

One microgram of total RNA from *A. marina* root was used as a template for the reverse transcriptase (RT) reaction using the primers MDAR5F and MDAR2R. The amplified PCR products were gel eluted, cloned, and sequenced. Correspondingly, the PCR product obtained in an RT reaction was loaded on 2% agarose, transferred to positively charged nylon membrane (Hybond-N+, Amersham), and hybridized with an MDAR probe specific to exons 2 and 3 of the cDNA of MDAR (152 bp).

Isolation of the 5′ Upstream Region of Am-MDAR

The sequence corresponding to the 5′ upstream region of *Am-MDAR* was amplified from genomic DNA via thermal asymmetric interlaced PCR (TAIL-PCR), with modifications according to Sessions et al. (2002). Three nested gene-specific reverse primers (MDAR-GSP1–MDAR-GSP3) and one of four arbitrary degenerate primers (AD1–AD4) were used in successive rounds of TAIL-PCR cycling. The primary PCR product was diluted 40-fold and used in the secondary reaction, while the secondary product was diluted 10-fold for the tertiary reaction. The products of the primary, secondary, and tertiary reactions were analyzed on a 0.8% agarose gel. Fragments exhibiting a difference in size consistent with nested gene-specific primer positions were cloned with an InsTA Clone PCR cloning kit according to the manufacturer's instructions (MBI Fermentas) and sequenced completely. The AD primers, as well as the gene-specific reverse primers, are listed in table 1. The specific products obtained were gel eluted and sequenced. The putative *cis*-acting DNA elements in the isolated 5′ regions were identified using the PLACE database (Higo et al. 1999).

Promoter Functional Analysis by Transient Reporter Gene Expression

The *Am-MDAR* putative promoter was reamplified from *A. marina* genomic DNA with the primers MDAR PROM 1F and MDAR PROM 1R (table 1) and cloned in T/A cloning vector (InsTA Clone PCR cloning kit; MBI Fermentas) according to the manufacturer's instructions. After the sequence was confirmed, *Am-MDAR* putative promoter was released from the T/A cloning vector by restriction digestion with *Eco*RI and *Bam*HI and cloned in the promoter fusion vector pCAMBIA 1391z in the *Eco*RI and *Bam*HI sites, with *UidA* (GUS) as the reporter gene (pPRM-1391z). The gene pPRM-1391z was transformed into *Agrobacterium* strain LBA4404 by the freeze-thaw method. Recombinant *Agrobacterium* colonies were selected on YEP medium supplemented with rifampicin (rif; 10 mg mL^{-1}) and kanamycin (kan; 50 mg mL^{-1}); colony PCR was then performed with the primers MDAR PROM 1F and MDAR PROM 1R. A single positive *Agrobacterium* colony was resuspended in 100 μL of sterile distilled water and then spread-plated on rif$^+$, kan$^+$ YEP. After 2 d of incubation in the dark at 28°C, the lawn of culture was scraped off carefully and resuspended in half-strength Murashige-Skoog (MS) medium to obtain an optical density of 1–2 at 595 nm. Acetosyringone (100 μM) was added to the suspension and gently mixed for ~10 min. Greenhouse-grown tobacco cv. Petit Havana leaves were infiltrated in the epidermal region with this culture by means of a syringe and needle. After 2 d, GUS staining was carried out in infiltrated leaf regions according to Jefferson (1987). The leaf pieces were mounted in water and examined under the Nikon Optiphot-2 phase contrast microscope using ×10, ×20, and ×40 objectives and photographed with a Nikon CoolPix 4300.

Results

Isolation of Am-MDAR *cDNA and In-Silico Analysis*

An EST (GenBank ID CD777105) showing maximum homology to chloroplast MDAR was identified from the *Avicennia marina* cDNA library and was sequenced completely. The full-length cDNA (*Am-MDAR*, 1916 bp) has a 114-bp-long 5′ untranslated region (UTR) and a 407-bp-long 3′ UTR with an ORF of 1395 bp. This is the first MDAR gene reported from this halophyte (GenBank ID FJ887789). The deduced protein has a theoretical molecular mass of 50.47 kDa and an isoelectric point (pI) of 6.73. The protein has a transit peptide cleavage site between amino acids 46 and 47. This protein showed maximum similarity (80%) to the chloroplast MDAR of *Lycopersicon esculentum* (GenBank ID ABG57052), followed by chloroplastic MDARs of *Brassica oleracea* (GenBank ID BAD14933) and *Arabidopsis thaliana* (GenBank ID BAA12349).

Am-MDAR possesses motifs conserved in other plant MDARs. The amino acid residues between Glu-55 and Val-73 and between Ile-84 and Gly-89 are reported to be involved in the binding of FAD, while the amino acid residues between Lys-210 and Val-228 and between Ise-237 and Asp-242 are involved in the binding of NADH. An additional domain between Thr-333 and Asp-343 is important in the binding of the flavin moiety of FAD (fig. 1). The signal peptide analysis of Am-MDAR indicated a possible localization

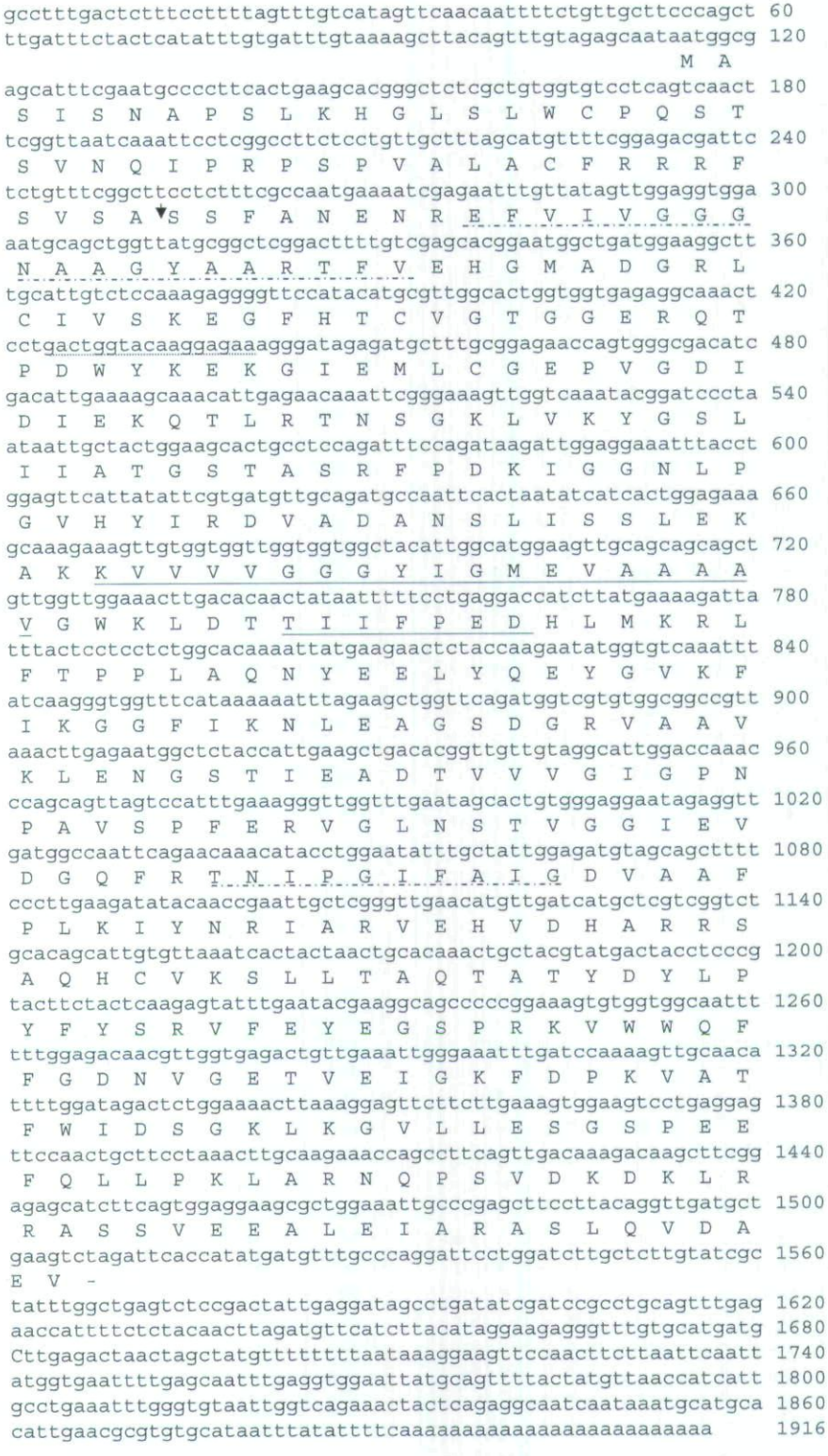

Fig. 1 cDNA and deduced amino acid sequence of chloroplastic monodehydroascorbate reductase from *Avicennia marina* (*Am-MDAR*). The transit peptide cleavage site is indicated by an arrow. Flavin-adenine dinucleotide (FAD)– and NADH-binding domains are indicated by dot-dashed and solid underscores, respectively.

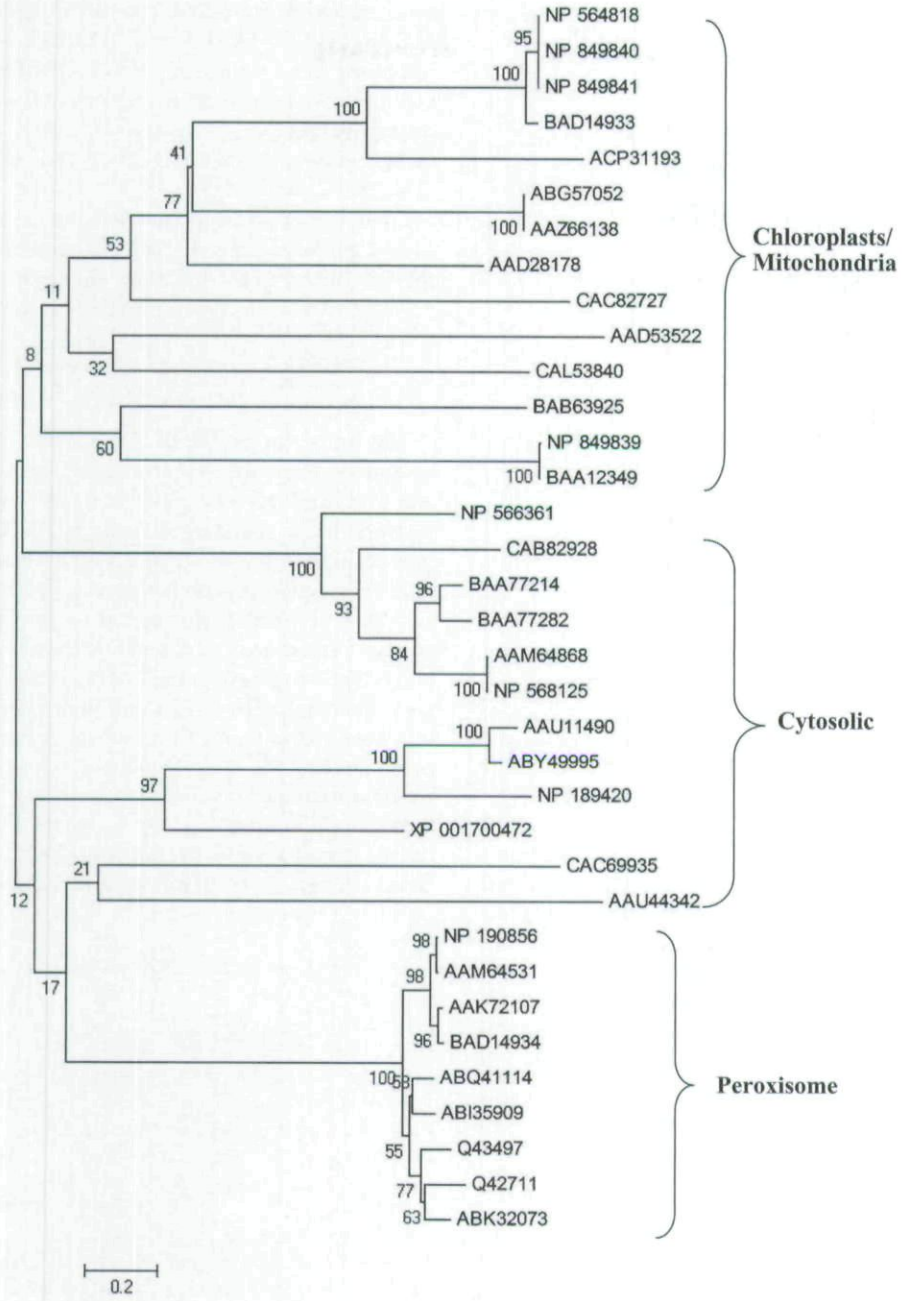

Fig. 2 Phylogenetic analysis of *Avicennia marina* monodehydroascorbate reductase (Am-MDAR) with other plant MDARs. The evolutionary history was inferred through the neighbor-joining method. The optimal tree, with the sum of branch lengths of 19.86292232, is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in units of amino acid substitutions per site. Phylogenetic analyses were conducted in MEGA4. The source plant and the protein identifications (GenBank) are shown. The analysis shows proteins clustering according to their cellular localization, with Am-MDAR clustering with the other chloroplastic MDARs.

in chloroplast or mitochondria. Am-MDAR, upon phylogenetic analysis with MDARs from higher plants, clustered with putative chloroplasts/mitochondrial isoforms, well separated from those that are localized in compartments, such as peroxisomes and cytosol, further confirming the possibility of a chloroplast/mitochondrial localization (fig. 2).

Gene Structure of Am-MDAR

The full-length genomic clone of *Am-MDAR* (GenBank ID FJ903500; 6058 bp) obtained by genome-walking methods, consists of 16 exons and 15 introns. All sequences bordering the introns/exons conform to the GT/AG rule (Mount 1996) for splice junctions. The gene organization of *Am-MDAR*

Fig. 3 PCR Southern blot analysis confirmation of exon skipping in monodehydroascorbate reductase (*Am-MDAR*). *PC* indicates cDNA of *Am-MDAR* as positive control. *Root* indicates products of reverse transcriptase PCR on total RNA from unstressed *Avicennia marina* root. Band at 227 bp has retained exon 3, while exon 3 is skipped in the 152-bp band.

was compared with that of four chloroplastic MDAR genes from *Arabidopsis* in chromosome 1. *Am-MDAR* was found to be most similar to NM_179510 and NM_105067 in exon number, sequence, and intron position in the protein sequence, even though the intron size varied.

The comparison of the genomic sequence of *Am-MDAR* with the cDNA revealed that exon 3 was absent in the cDNA. An RT-PCR analysis using total RNA from *A. marina* revealed the presence of an additional MDAR isoform that had exon 3. Further confirmation with PCR Southern blot analysis using *Am-MDAR* as probe detected two bands, confirming the presence of two isoforms of MDAR, one with and the other without exon 3 (fig. 3).

Genome Organization of MDAR *in* A. marina

Southern blot analysis of *A. marina* genomic DNA showed multiple bands that revealed the presence of multiple gene copies of *Am-MDAR* in the *A. marina* genome (fig. 4). The band intensity and the size of the hybridized fragments indicated that at least three copies of chloroplastic MDAR isoforms were present in *A. marina*.

In-Silico Characterization of Am-MDAR *Promoter*

To identify the *cis*-acting elements that are responsible for regulation of the gene expression under various environmental factors, an 1167-bp fragment in the 5′ upstream region of *Am-MDAR* was isolated with TAIL-PCR (GenBank ID FJ903500). Putative *cis*-acting elements were identified with the program PLACE. A putative TATA box is located 161 bp upstream of the translation start site of *Am-MDAR*. Many *cis*-acting elements previously reported to be involved in stress-responsive gene regulation were found in the 5′ upstream region of *Am-MDAR*: ACGTATERD1 and MYB1AT, involved in drought-responsive gene regulation; MYCCONSENSUSAT, involved in cold/freeze-responsive gene regulation; GARE, involved in GA-responsive gene expression; and several light-responsive elements, such as ASF1MOTIFCAMV, CIACADIANLELHC, GATABOX, and I BOX.

Intense GUS staining was detected in tobacco leaves transiently expressing the *Am-MDAR* promoter, a *UidA* construct (pPRM-1391z). This indicated that the isolated *Am-MDAR* promoter was a functional promoter in tobacco.

MDAR Transcript Profile and Activity under Various Abiotic Stress Conditions

The transcript profile of *Am-MDAR* under different stress conditions—namely, salinity, excess light, H_2O_2 application, and iron overload—were analyzed (fig. 5*A*–5*D*). Salt stress application led to a sudden increase in RNA expression from 10 min until 30 min after application, with a slight decline thereafter followed by upregulation at 2 h, after which the expression level declined. Light seemed to have a profound influence in the expression of *Am-MDAR*. When dark-acclimatized plants were exposed to high light, increased expression levels were observed as early as a half-hour later, and high induction was observed at 6 and 12 h, but the transcript level was found to decline at 24 h. Gene expression in response to H_2O_2 application also showed a similar pattern, with high induction observed at 6 h, followed by a sharp decline. Iron overload caused the induction of *Am-MDAR* from 1/2 to 6 h later. Basal expression level was regained after withdrawal of the

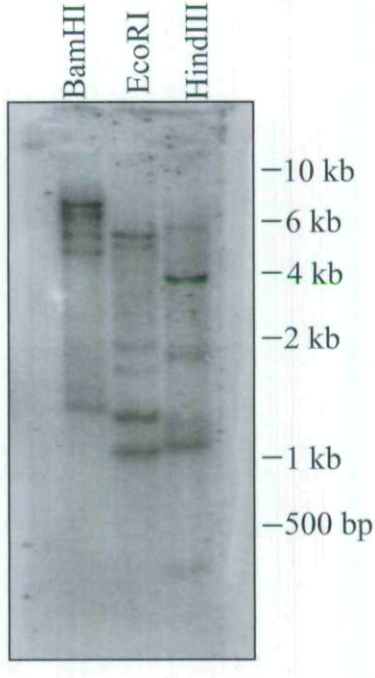

Fig. 4 Genomic Southern blot analysis of *Am-MDAR*, revealing copy number in *Avicennia marina*. Genomic DNA was digested with *Bam*HI, *Eco*RI, and *Hind*III and hybridized with *Am-MDAR* cDNA-specific probe. The molecular sizes of the standards are indicated on the right.

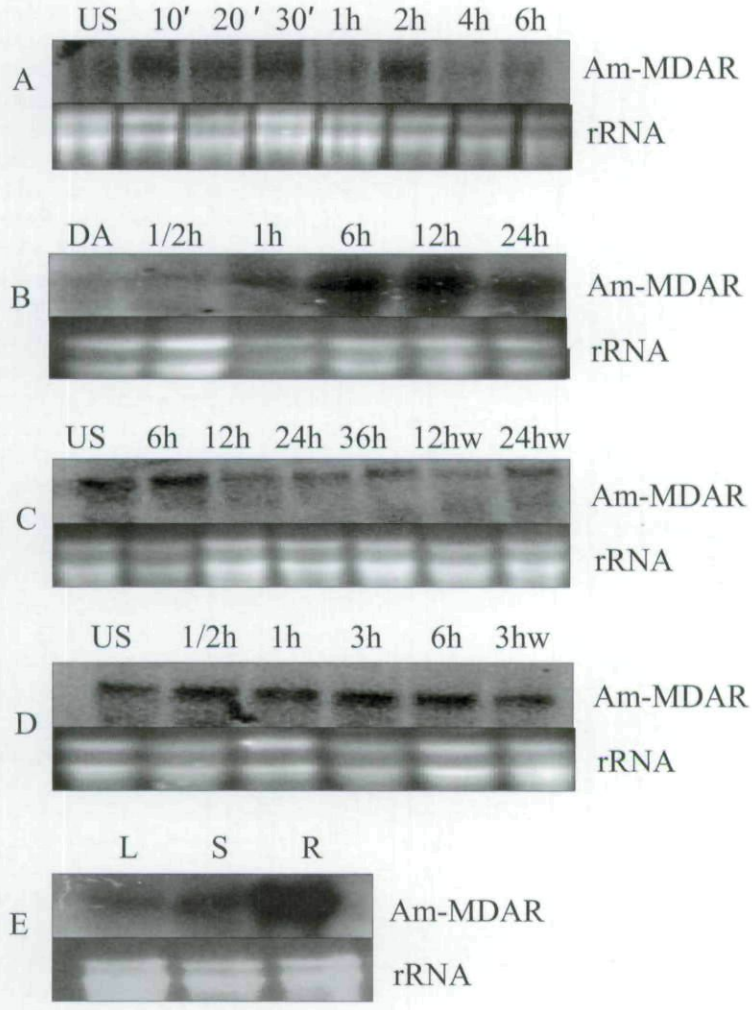

Fig. 5 Expression analysis of *Avicennia marina* monodehydroascorbate reductase (*Am-MDAR*) under several abiotic stresses and in different tissues. In each panel, the upper half indicates the Northern blot while the lower half represents the ethidium bromide–stained gel before transfer. Time points of RNA isolation are indicated at the top of the panel in minutes or hours. *A*, NaCl (500 mM); *B*, light stress (500 microequivalent m^{-2} s^{-1}); *C*, H_2O_2 stress (90 mM); *D*, Fe(III) citrate (1000 μM). *US* = unstressed: plants were acclimatized with half-strength Murashige-Skoog medium, and leaf tissue was frozen for RNA isolation before stress (0 h). *DA* = dark-adapted plants; *hw* = hours after withdrawal. *E*, Tissue-specific expression of *Am-MDAR*. RNA was isolated from unstressed leaf (*L*), shoot (*S*), and root (*R*) of *A. marina* seedlings.

stress. The induction of *Am-MDAR* by different abiotic stresses suggests a common signaling pathway that is activated after the perception of all stress types.

The tissue-specific expression pattern (leaf, shoot, and root) of *Am-MDAR* in *A. marina* under normal, unstressed conditions was analyzed by Northern blot analysis (fig. 5*E*). The *Am-MDAR* transcription was found to be highest in root and lowest in leaf tissue.

The MDAR enzyme activities were also analyzed in *A. marina* leaf tissue under conditions such as dark acclimation, salinity stress, excess light, H_2O_2 application, and iron deficit and overload (at 6 h; fig. 6). After the *A. marina* seedlings were shifted to the dark, a marked decrease in MDAR activity was observed, relative to that in the unstressed controls maintained at normal light, whereas activity increased when seedlings were exposed to high light. Stress treatments with NaCl and H_2O_2 resulted in increases in MDAR activity relative to unstressed controls. Iron deficiency did not alter MDAR activity much in comparison with the controls, while iron overload led to an increase in activity.

Discussion

Despite the wealth of information on abiotic stress and stress tolerance in plants, many aspects still remain unclear. Our previous study used *Avicennia marina*, a highly salt-tolerant species, as a model plant system for identifying and isolating genes functioning in abiotic-stress tolerance (Mehta et al. 2005). In this article, we report the isolation and molecular characterization of a putative chloroplastic MDAR from *A. marina*.

MDAR has been reported to be distributed widely across kingdoms, pointing toward a universal role. In plants, it has been found to localize in chloroplasts, glyoxysomes, mitochondria, and cytosol, which is consistent with the fact that the MDA radical seems to be ubiquitous in the cell. Recent work has shown that MDAR is one of the major antioxidant

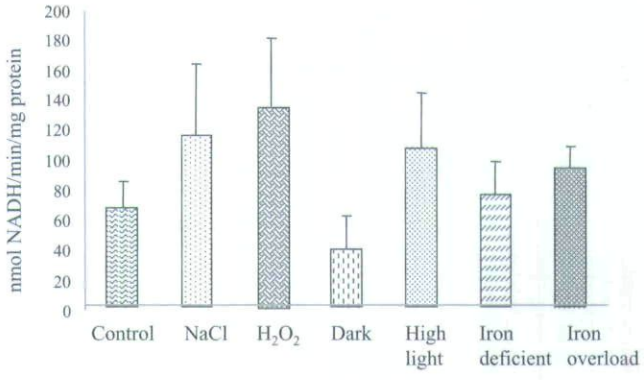

Fig. 6 Monodehydroascorbate reductase activity in leaf extracts of *Avicennia marina* seedlings exposed to different abiotic stresses. *Avicennia marina* seedlings were exposed to dark, light (500 microequivalent m^{-2} s^{-1}), NaCl (500 mM), H_2O_2 (90 mM), iron deficiency, and iron (III) citrate (1000 μM) overload for 6 h. The data are means from a minimum of three independent measurements, and error bars indicate standard error of the mean.

systems in plant cells for protection against the damage produced by reactive oxygen species (ROSs; Noctor and Foyer 1998). In-silico analysis of *Am-MDAR* cDNA revealed the presence of an N-terminal signal peptide for chloroplastic localization. In a previous report, two *Arabidopsis* MDARs, identical except for an extended stretch of amino acids at the N-terminal, were found to localize in different cellular organelles (Obara et al. 2002). The MDAR with the longer N-terminal region (D84417) was imported into mitochondria, whereas the one with the shorter N-terminal region (NP_564818) was imported into the chloroplast. Obara et al. (2002) suggested that first 50 or 43 amino acids are sufficient to target this protein to the mitochondria or the chloroplast, respectively. The Am-MDAR is identical to NP_564818 at the N-terminal region that is targeted toward the chloroplast.

The cDNA and genomic DNA sequences of *Am-MDAR* and its copy number in *A. marina* were examined. A comparative analysis of the genomic regions of the *Arabidopsis* putative chloroplastic MDAR (At1g63950) and *Am-MDAR* revealed a strong conservation of exon sequence and length, while the length and sequence of the introns varied significantly. Exon 3 (75 bp) in the genomic DNA of *Am-MDAR* was found to be missing in the cDNA. An RT-PCR using total RNA isolated from *A. marina* root under unstressed conditions led to the identification of two cDNAs, one with and one without exon 3. It is possible that two chloroplastic MDAR isoforms exist in *A. marina* that differ in the presence of exon 3. Genomic Southern blot analysis also confirmed the presence of more than one highly homologous chloroplastic MDAR isoform. Exon skipping has been observed in several *Arabidopsis* mutants (Simpson et al. 1998). Bischoff et al. (2001) reported skipping of a potential exon as a prerequisite for the production of a functional protein in tomato. All the functional domains important in protein function (FAD-binding domains, NADH-binding domains, and the domain important in the binding of the flavin moiety of FAD) are present in the *Am-MDAR* cDNA. *Am-MDAR* cDNA was obtained from a cDNA library created from an *A. marina* salt-stressed leaf. RT-PCR analysis on the root RNA of *A. marina* resulted in the obtaining of two isoforms of *Am-MDAR* (one with and one without exon 3). The search of the salt-stressed-leaf cDNA library revealed the presence of only one cDNA for MDAR. It is possible that the cDNA without exon 3 is preferentially expressed during salt stress in *A. marina* leaf. Further experimental evidence may be needed to confirm this. Our results are the first report on exon skipping in chloroplastic MDAR genes.

It is widely accepted that diverse environmental conditions can induce oxidative stress, which in turn increases the enzyme activity of the ascorbate-glutathione cycle (Foyer et al. 1994). Considering that MDAR is an enzyme of the ascorbate-gluthatione cycle, we examined its activity and mRNA levels under several abiotic-stress conditions (NaCl, H_2O_2, high light intensity, iron deficit, and iron overload). Early induction of *Am-MDAR* gene was observed under salt stress, H_2O_2, high light intensity, and iron overload (within 6 h). In *Brassica campestris*, cytosolic MDAR is upregulated by H_2O_2, salicylic acid, paraquat, and ozone treatments 4 h after application (Yoon et al. 2004). MDAR mRNA accumulation was higher in tomato after wounding or mechanical stimulation (Ben Rejeb et al. 2004), higher in pea after low-temperature treatment, wounding, and 2,4-D application (Leterrier et al. 2005), and higher in *Conyza bonariensis* after paraquat treatment (Ye and Gressel 2000). In general, under abiotic stresses such as salt, drought, cold/freeze, and high light intensity, an ROS is generated. The upregulation of *Am-MDAR* under these stress conditions and its localization in the chloroplast plays a pivotal role in *A. marina*'s stress tolerance.

Regulatory elements that are involved in drought, light, cold/freeze, and gibberellic acid response were found in the promoter of *Am-MDAR*. The induction of the *Am-MDAR* gene under NaCl stress, H_2O_2, high light intensity, and iron and copper overload can be accounted for by the presence of these *cis*-acting elements in the promoter. A Northern blot analysis also indicated higher gene expression in root tissues of *A. marina* than in leaf and shoot tissues. In peas, fruits, stem, and flowers recorded higher peroxisomal MDAR expression, whereas the expression was lower in leaves (Leterrier et al. 2005). But our result is not similar to the expression pattern of *B. campestris* MDAR, where expression of *Bc-MDAR* was higher in leaves than in roots and stems (Yoon et al. 2004). The results for the enzyme activity assay for MDAR in *A. marina* leaves under different stress conditions were in accordance with the Northern blot analysis. Under our experimental conditions, activity was upregulated under NaCl, H_2O_2, high light intensity, and iron overload but reduced on exposure to dark. There was no significant difference in MDAR activity between control plants and those grown in iron-deficit conditions. The increase in MDAR activity has been described in several stress conditions, for instance, in tomato under salinity (Mittova et al. 2003) and high light intensity (Gechev et al. 2003). Leterrier et al. (2005) reported that activity of MDAR was significantly higher under high light intensity and cadmium treatment and was reduced by the herbicide 2,4-D in peas. Under dark conditions, Am-MDAR activity is reduced when compared to control plants. This might be due to reduced photosynthesis under dark conditions. Similar results are reported in wheat seedlings, wherein the activities of MDAR, ascorbate peroxidase, superoxide dismutase, DHAR, glutathione reductase, and catalase were much lower in seedlings grown under low-light

conditions than in those grown under high-light conditions (Mishra et al. 1995).

Our results confirm the vital role played by Am-MDAR in *A. marina*'s stress tolerance. Since generation of an ROS is a common element in many abiotic stresses, such as drought, heat, and cold/freeze, it is possible that *Am-MDAR* overexpression might improve survival under these stresses in transgenic systems.

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