SECTION 2

Cloning and characterization of two cDNAs for rice cytosolic monodehydroascorbate reductase

Introduction

MDAR (EC 1.6.5.4) catalyzes MDA to AsA using NAD(P)H as an electron donor (Hossain et al., 1984) and widely distributed in mammalians and plants (Arrigoni et al. 1981; Goldenberg et al., 1983; Sun et al., 1984). MDAR is a FAD- and monomeric enzyme (Hossain and Asada, 1985), and in plants is found not only in chloroplast stroma (Hossain et al., 1984) but also in cytosol, mitochondria (Arrigoni et al., 1981; Dalton et al., 1993) and glyoxysome membrane (Bowditch and Donaldson, 1990). The analysis of immunocytochemistry using soybean MDAR antibody indicated that cytosolic MDAR is located primarily in cell wall of soybean nodules (Dalton et al., 1993). The recent report indicated that the determined amino acid sequence of purified NADH-hexacyanoferrate oxidoreductase (NFORase), which was obtained from highly purified spinach plasma membranes and consisted of the majority of plasma membrane NFORase activity, was corresponded to that of the known plant MDARs, and the spinach MDAR is strongly associated with the cytoplasmic surface of plasma membranes (Berczi and Moller, 1998). MDAR is purified from cucumber fruits (Hossain and Asada, 1985), potato tubers (Borraccino et al., 1986; Leonardis et al., 1995) and soybean root nodules (Dalton et al., 1992). cDNAs encoding MDAR were also isolated from cucumber (Sano and Asada, 1994), pea (Murthy and Zilinskas, 1994) and tomato (Grantz et al., 1995). These cDNAs have been supposed to encode cytosolic isozymes because of lack of any signal sequence. Although many papers about MDAR have been published, there is little information of the expression or the regulation of MDAR gene. It have been reported only to be shown that the expression of tomato MDAR gene is induced by wounding (Grantz et al., 1995). In this section, the author reports the isolation and characterization of two cDNAs for rice MDAR.

Materials and Methods

Isolation and characterization of two cDNAs for rice MDARs -

Prior to the screening, any nucleotide sequences of rice EST clones that are highly homologous to the known plant MDAR cDNAs, from cucumber (Sano and Asada 1994) and pea (Murthy and Zilinskas, 1994), were searched using the program of Fasta 1.7 software (Pearson and Lipman, 1988). Subsequently the nucleotide sequences of four EST clones were obtained (Fig. VI-1A).

Based on two of these EST sequences (accession number D46186 and D39373), two oligonucleotides, RMDS-1 (5'-GGAGGCGTCGCCGCGGGATACGCTGC-3') and RMDA-1 (5'-CCAACGTTGTCGCCGTAGAATTGCCA-3') for use as forward and reverse primer, respectively, were synthesized. About 1.1 kb fragment (Fig. VI-1B) was amplified by PCR using rice double-strand cDNA of rice greening seedlings, which was prepared as described in CHAPTER I, as a template and subcloned into T-vector of LITMUS 38 (New England Biolabs), which was prepared as described in CHAPTER I. The partial nucleotide sequence of this fragment was determined to recognize as a partial fragment of MDAR cDNA by the comparison with other known plant MDAR cDNAs. Using this PCR fragment, the cDNA library, which was used in the cloning of rice plastidic Cu/Zn-SOD cDNA (CHAPTER I-1), was screened. Subsequently, 2 positive clones were obtained from 3.0 x 10^6 plaques. Further a rice EST clone (D39373), whose sequence was referred for the primer design, was purchased from the Rice Genome Research Program because both sequences of the isolated cDNAs are not corresponded to that of the EST clone. The isolated largest clone, designated RMDAR-1, and the purchased EST clone, designated RMDAR-2, were used for further analysis. The methods of screening and sequencing were followed as described in CHAPTER I.

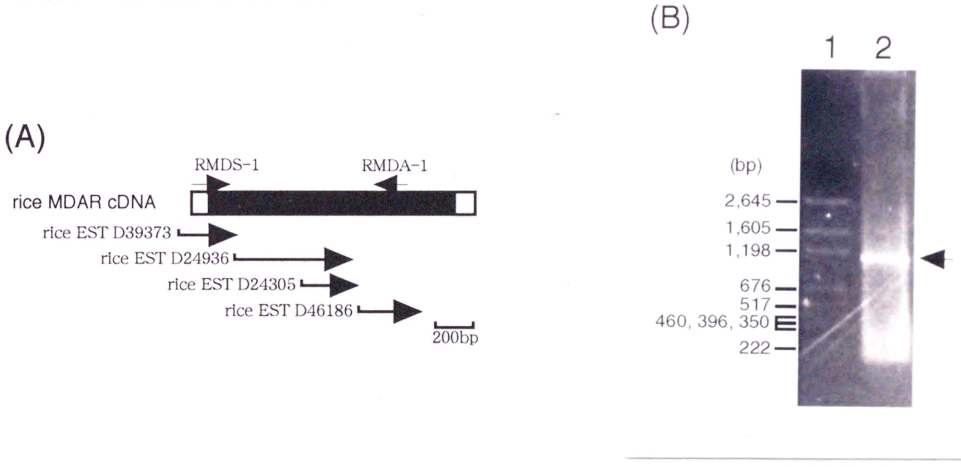

Fig. VI-1. Preparation of a partial fragment of rice MDAR cDNA. (A) Comparison among putative rice MDAR cDNA and rice EST clones. The positions of PCR primers for amplification of a partial rice MDAR are indicated by arrowheads as polarity. (B) Electrophoresis analysis stained by ethidium bromide; lane 1: pGEM marker (Promega Japan), lane 2: PCR products amplified with a pair of primer, RMDS-1 and RMDA-1. Arrows indicated the recovered band by electroelution for subcloning.

Genomic Southern blot, Northern blot, and immunoblot analyses -

Genomic Southern blot analysis was performed as described in CHAPTER I, with ^{32}P-labelled *Eco*RI and *Bam*HI-fragment of RMDAR-1 cDNA (full-length cDNA) and washing at high-stringency conditions (1 x SSC, 42 °C). The restriction endonucleases of *Hin*dIII, *Pst*I, and *Sph*I were used for the digestion of rice genomic DNA.

Northern blot analysis in vegetative tissues was carried out as described in the previous section,

with ^{32}P-labelled RMDAR-1 full-length cDNA, used in Southern blot analysis, and washing at the stringency (1 x SSC, 42 °C). The quantification of the probe hybridized to specific mRNAs were performed by densitometric scanning of the autographs using a Master Scan II IM-300 (Scanalytics, Billerica, MA, USA). The amount of each gene specific transcript was revised with the amount of the loading rRNA, scanning by same method.

Immunoblot analysis in vegetative tissues was carried out using polyclonal-antibody against cucumber cytosolic MDAR (kindly gift from Dr. Sano of Kyoto Prefectural University; Sano and Asada, 1994) as described in the previous section. 20 μg of the soluble fraction from each sample was separated by SDS-PAGE, electroblotted, and then the immunoreaction was carried out using the MDAR antibody (1:1000 dilution) as first antibody.

Results

Isolation and characterization of two cDNAs for rice cytosolic MDARs -

To isolate full-length cDNA(s) clone for MDAR, a cDNA library used in cloning of rice plastidic Cu/Zn-SOD (CHAPTER I-1) was screened with about 1.1 kb of rice MDAR partial clone (Fig. VI-1B) prepared as described in Materials and Methods. 2 positive clones were obtained from 3.0 x 10^6 plaques and then the 5' end of these clones was partially sequenced (data not shown). Both sequences are corresponded each other, but not corresponded to that of EST D39373. Therefore this EST clone was also purchased from the Rice Genome Research Program. A longest cDNA of two positive clones and the EST clone, which were designated RMDAR-1 and RMDAR-2, respectively, were used for further analysis. The nucleotide sequences of these cDNA were completely determined. The nucleotide and deduced amino acid sequences of RMDAR-1 and RMDAR-2 are presented in Figure VI-2. The RMDAR-1 (accession number D85764) contains a 1305 bp putative open reading frame and includes both 5'- and 3'-UTRs, while the RMDAR-2 contains 5'-UTR but is lack of about 2-300 bp at 3' end of cDNA, which is included 3' part of coding region and 3'-UTR. The 3' UTR in RMDAR-1 contains a putative polyadenylation signal (5'-AATAAA-3'), which was located 16-bp upstream from poly(A)$^+$ tails (Figure VI-2A, double-underlined). The deduced amino acid sequence from RMDAR-1 showed that this cDNA encode 435 amino acid residues with a molecular weight of 46,630, which corresponded to the size (47 kD) of protein synthesized from RMDAR-1 by *in vitro* transcription/translation system (data not shown). The comparison between the deduced amino acid sequences of RMDAR-1 and RMDAR-2 indicated 86.7% of identity. This result suggested that these cDNAs encode the same isoform of

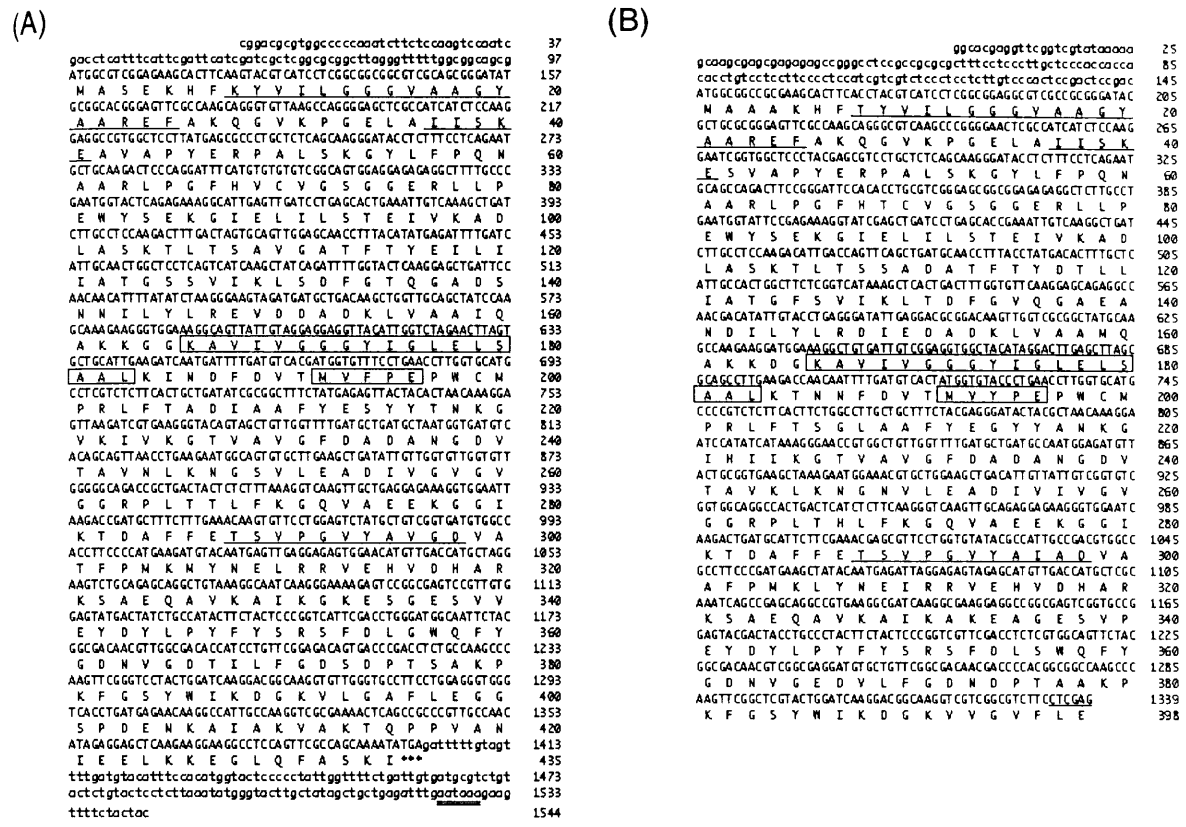

Fig. VI-2. Nucleotide and deduced amino acid sequences of two rice cytosolic MDAR cDNAs, RMDAR-1 (A) and RMDAR-2 (B). The nucleotide (upper line) and amino acid residues (lower line) are numbered on the right. The termination codon is indicated as asterisks. The putative polyadenylation signal (-AATAAA-) is double-underlined. The finger print of binding domain for FAD and NAD(P)H are underlined and boxed, respectively. The nucleotide sequence data of RMDAR-1 will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number D85764.

rice MDAR.

The primary structures deduced from rice MDAR cDNAs were compared with the deduced amino acid sequences of cytosolic MDAR from cucumber (Sano and Asada, 1994), pea (Murthy and Zilinskas, 1994) and tomato (Grantz et al., 1995), and putative chloroplastic MDAR from *A. thaliana* (accession number D84417) in Figure VI-3. The sequence of rice MDARs was highly conserved among those of the known plant cytosolic MDARs, and the identities compared with cytosolic MDARs from cucumber, pea and tomato were 76.7, 75.5, and 79.0%, respectively. While the identity compared with *A. thaliana* chloroplastic MDAR was 44.5%. Furthermore, the deduced amino acid sequences from rice MDARs were not contained any typical targeting signal in amino-terminal amino acid sequence. Therefore these cDNAs are seemed to encode cytosolic isoforms of rice MDAR similar to other characterized plant MDARs. The deduced amino acid sequence also contains putative flavin adenine dinucleotide (FAD) and NAD(P)H binding domains (Figure VI-2; underlined and boxed, respectively), which were conserved in other flavoprotein oxidoreductases (Eggink et al., 1990) and in other plant MDARs. In rice the sequences of these domain were

Fig. VI-3. Comparison of the deduced amino acid sequence of rice cytosolic MDAR with those of other MDAR cDNAs, cytosolic MDARs from cucumber (Sano et al., 1994), pea (Murthy and Zilinskas, 1994) and tomato (Grantz et al., 1995), and putative chloroplastic MDAR from *A. thaliana* (accession number D84417). Identical residues among more than three sequences are enclosed in black boxes.

strongly conserved only in the deduced amino acid sequences from cytosolic GR but not in those from other rice flavoproteins (data not shown). This results suggested that these domain within MDAR may be conserved only in cytosolic proteins of flavoprotein oxidoreductases.

Genomic Southern blot analysis of rice cytosolic MDAR genes -

To clarify the copy number of rice cytosolic MDAR gene(s), genomic Southern blot analysis was carried out as described with a full-length fragment of RMDAR-1 (Fig. VI-4A). One major band and minor band were detected by the digestion of *Hin*dIII, while two major bands and several

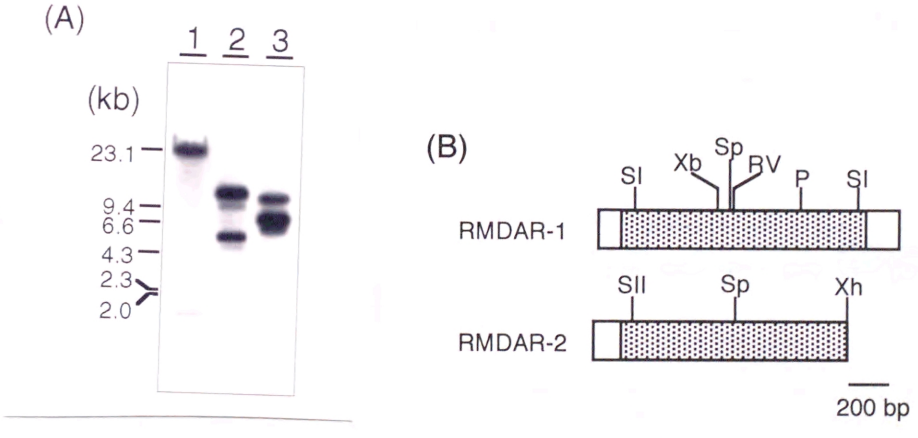

Fig. VI-4. (A) Genomic Southern blot analysis of rice cytosolic MDAR. Total genomic DNA (10 μg) from rice was digested with *Hin*dIII (1), *Pst*I (2), and *Sph*I (3), transferred to nylon membrane, and hybridized with a full-length fragment of RMDAR-1. Size markers (λ/*Hin*dIII; New England Biolabs.) are indicated on the left. (B) Restriction map of rice cytosolic MDAR cDNAs, RMDAR-1 and RMDAR-2. Open reading frame is indicated as shaded box. RV, *Eco*RV; P, *Pst*I; SI, *Sac*I; SII, *Sac*II; Sp, *Sph*I; Xb, *Xba*I; Xh, *Xho*I.

minor bands were detected with *Pst*I and *Sph*I, whose restriction sites were contained in the nucleotide sequence of both rice cytosolic MDAR cDNAs (Fig. VI-4B). This result showed that there is at least one more gene for cytosolic MDAR in rice nuclear genome, excepting the gene for RMDAR-1. These minor bands in Southern blot analysis were seemed to be corresponded to the gene for RMDAR-2. Therefore the genes of cytosolic MDAR may be consisted a small gene family in rice nuclear genome, containing two genes.

Expression of rice cytosolic MDAR genes in vegetative tissues -

To analyze the gene expression of rice cytosolic MDAR, Northern blot analysis of various vegetative tissues, such as etiolated seedlings, green leaves, stems, roots and calli, was carried out (Fig. VI-5A). Before this experiment, the author have attempted to detect the gene-specific transcripts for RMDAR-1 and RMDAR-2 using 3'- and 5'-UTR of each cDNA, respectively (data not shown). In the case of RMDAR-2, the specific transcript, however, was not able to be judged because 5'-UTR of RMDAR-2 was also hybridized to the non-specific transcripts. Therefore, we used a full-length rice MDAR cDNA (RMDAR-1) as a hybridization probe in this experiment. The steady level of about 1.6 kb mRNA, which mostly corresponded to the length of RMDAR-1 (1560 bp), was detected in all tissues examined. The amount of transcript for rice cytosolic MDAR was revised with the amount of the loading rRNA as described in Materials and Methods. This gene slightly strongly expressed in calli (2.4-fold, comparing to amount of mRNA in etiolated seedlings) and in green leaves, stem and roots the increase of the transcript was observed (1.4 to 1.7-fold, comparing to amount of mRNA in etiolated seedlings). Immunoblot analysis in the same samples was also done using anti-cucumber cytosolic MDAR antibody (Fig. VI-5B). Two major bands were detected (48 and 42 kD) in all tissues examined. The band corresponded to the isolated rice cytosolic MDAR cDNA (48 kD) was abundant in root and calli.

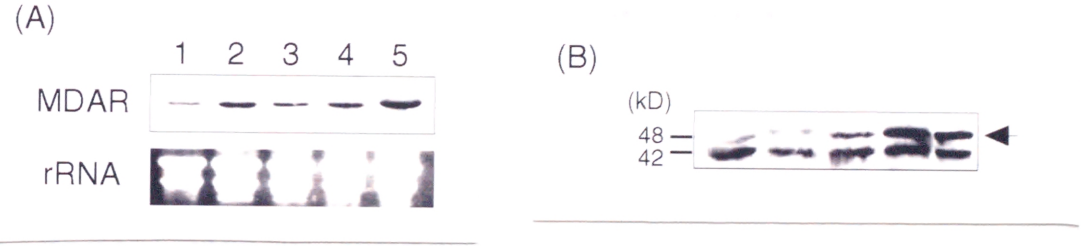

Fig. VI-5. Expression of rice cytosolic MDAR in vegetative tissues. (A) Northern blot analysis of rice cytosolic MDAR. Total RNA (15 mg) was fractionated by an electrophoresis on a 1.2% formamide-containing agarose gel, transferred to nylon membrane, and hybridized with a full-length fragment of RMDAR-1. Each rRNA band was used as size marker and to check the loading of equal amounts of total RNA with ethidium bromide. Lane 1, shoots of etiolated seedlings; lane 2, green leaves; lane 3, stems; lane 4, roots; lane 5, suspension culture of embryogenic-calli. (B) Immunoblot analysis of rice cytosolic MDAR. Each total protein (20 μg) of the same tissue as used in Northern blotting was separated by 10% SDS-PAGE, transferred to PVDF membrane and subjected to immunoblotting with the antibodies against cucumber cytosolic MDAR (Sano and Asada, 1994). An arrow indicates putative corresponding protein band to the isolated rice cytosolic MDAR cDNAs.

Discussion

In this section, two cDNAs for MDAR from rice were isolated, and the gene structure and the expression were characterized. The deduced amino acid sequences from rice MDAR cDNAs (Fig. VI-3) are highly homology to the amino-terminal amino acid sequence of purified MDAR II from soybean root nodules (Dalton et al., 1992) and the internal amino acid sequences of purified MDAR from cucumber fruit (Sano and Asada, 1994). This finding and the absence of any typical sequence of targeting signal to any organelles indicate that these rice MDAR cDNAs encode cytosolic isoform of MDAR as other plant MDARs. Moreover the molecular weight (46,630) of the deduced amino acid sequence from RMDAR-1 corresponds to those of cucumber MDAR and soybean root nodules (47 kD) but not to that of cytosolic MDAR from potato tubers (42 kD). Two distinct size (42 and 48 kD) of MDAR isoforms in rice were detected with anti-cucumber MDAR antibody (Ushimaru et al., 1997; Fig. VI-5B). These distinct molecular weight of MDARs are corresponded to two purified cytosolic MDARs as described above. The molecular weight of deduced amino acid from rice cytosolic MDAR cDNAs indicated that MDARs encoded by the isolated cDNAs are corresponded to the 47 kD of MDAR. The molecular weight of spinach MDAR, which is the purified plasma membrane-associated NFORase, is 45 kD (Berczi and Moller, 1998). The nucleotide or amino acid sequence informations about 45 or 47 kD of cytosolic MDARs are available, but that of 42 kD MDAR is not. Therefore 42 kD of cytosolic MDARs may be structurally similar to 47 kD of MDARs, but have distinct primary structures.

Genomic Southern blot analysis (Fig. VI-4B) and the existence of two cDNAs for rice cytosolic MDAR suggested that the gene of cytosolic MDAR consists a small gene family in rice nuclear genome, containing two genes. Likewise two isoforms, putative cytosolic isoform (Dalton et al., 1993), of MDAR have been isolated from soybean root nodules (Dalton et al., 1992) and the presence of different isoform(s) not corresponding to a cDNA isolated from cucumber seedlings has reported (Sano and Asada, 1994), while the existence of single copy gene for MDAR in tomato has reported (Grantz et al., 1995). In plants, the existence of multiple isoforms for MDAR has reported, in chloroplast stroma (Hossain et al., 1984), cytosol, mitochondria (Dalton et al., 1993; Leonardis et al., 1995) and on glyoxysome membranes (Bowditch and Donaldson, 1990). The difference between the amino acid sequences of these isoforms is not clear, but in DNA databases the sequence of putative chloroplastic isoform of MDAR from *A. thaliana* is observed (accession number D84417). The deduced amino acid sequence from this cDNA shows relatively low identities to other cytosolic MDARs (less than 50 %) and has a putative transit peptide sequence. This result suggested that the sequences of cytosolic MDAR is much difference with that of

chloroplastic MDAR. Therefore our analysis by genomic Southern blot suggested to characterize only genes for cytosolic isoforms of MDAR, which is presumably 47 kD of rice cytosolic MDAR.

The gene expression of cytosolic MDAR in rice is shown in Fig. VI-5. This gene is seemed to be expressed rather consitutively in all tissues, but relatively stronger in non-photosynthetic tissues. The expression of cytosolic MDAR in pea was detected in all tissues, which they examined, and slightly high level of the transcript was detected in flower tissues and etiolated seedlings relative to the light grown plants (Murthy and Zilinskas, 1994), while comparative high level of the transcript in tomato was detected in roots relative to other tissues (Grantz et al., 1995). Grantz and his colleagues illustrated that the abundance of the transcript of cytosolic MDAR has a correlation to MDAR activity and an inverse correlation to ascorbic acid in each tissue. On the other hand, the activity of MDAR increased by the treatment of L-galactono-1,4-lactone, which artificially increased the intracellular level of AsA (Hausladen and Kunert, 1990). In rice, the quantification of ascorbic acid in roots and shoots of etiolated seedlings and light grown plants was performed (data not shown). This result is mostly same result as in tomato; the amount of ascorbic acid in etiolated seedlings is slightly abundant relative to that of light grown plants. Our data also indicated an inverse correlation between the amount of ascorbic acid and transcript of cytosolic MDAR in these tissues.

SECTION 3

Gene regulation of rice cytosolic active oxygen scavenging enzymes under environmental stresses

Materials and Methods

Experimental materials: stress treatments and preparation of gene specific probes to rice cytosolic AOS-scavenging enzymes -

All materials and samples treated with various stresses were same as used in CHAPTER I-4 (study on the gene regulation and expression of rice SOD isoforms).

To prepare the gene-specific probe to each rice cytosolic AOS-scavenging enzymes, namely SOD, APX, GR, MDAR, and DHAR, 3'-UTR of each gene was used in the case of cytosolic Cu/Zn-SOD, APX and MDAR, and full-length cDNAs used in the case of cytosolic GR and DHAR. In the case of cytosolic Cu/Zn-SOD genes (*sodCc1* and *sodCc2*), cytosolic GR gene (*RGRC2*), and one of cytosolic MDAR gene (*RMDAR1*), gene specific probes were prepared as described in CHAPTER I-3, II-1, and II-2, respectively. The 3'-UTR fragments of cytosolic APX genes (*apxa and apxb*)(Morita et al., 1997) were obtained by PCR with the primer as follows; for apxa: APXA-3'F (5'-GAGGTTTCTAGTCTACTACTGC-3') and APXA-3'R (5'-GGATGCAGCA TTGCAGTTGAGC-3'), for apxb: APXB-3'F (5'-GAAGCCTTTAGAGAGCGGGATA-3') and APXB-3'R (5'-ATCTTGACAGCAAATAGCTTGG-3'). For the preparation of the gene specific probes to one of rice cytosolic MDAR gene (*RMDAR1*), 3'-UTR of RMDAR-1 was amplified by PCR with following primers; RMD1-3UF (5'-TGAGATTTTTGTAGTTTTGA-3') and RMD1-3UR (5'-TTGTAGTAGAAAACTTCTTT-3'). The coding region of rice DHAR cDNA was prepared by PCR, using rice DHAR cDNA as a template, with the pair of primers, EcoF (5'-GGCGTGGAGGT GTGCGTCAAGG-3') and EcoR (5'-TTACGCATTCACTTTTGGTGC-3') (kindly gift of cDNA and primers from Dr. Ushimaru, Shizuoka University; sequence data is not published). A partial cDNA of putative rice cytosolic PHGPX (Fig. VII-1B) was prepared by PCR using rice seedling double-strand cDNAs as a template with the pair of primers, RGPS1 (5'-CCATACACGAATTCA CCGTCAAGGA-3') and RGPA1 (5'-AATGACTTTGCCATTCTTATCAAT-3'). These primers were synthesized with the based on the nucleotide sequences of rice EST clones (accession number D22908, D48881, and D49202), which are highly homologous to *A. thaliana* putative cytosolic PHGPX cDNA (accession number AJ000470) (Fig. VII-1A). All probes were recovered by the electroelution after the agarose electrophoresis. The used probes for the hybridization were summarized in Table VII-1.

The extraction of total RNA and Northern blot analysis were performed as described in CHAPTER I-4, with ^{32}P-labelled each gene specific probes to rice cytosolic AOS-scavenging enzymes.

Table VII-1. Gene specific probes to rice cytosolic AOS-scavenging enzymes used in Northen blot analysis.

Enzyme	Gene	Gene-specific probe
SOD	*sodCc1*	3'UTR fragment of *sodCc1*
	sodCc2	3'UTR fragment of *sodCc2*
APX	*apxa*	3'UTR fragment of *apxa*
	apxb	3'UTR fragment of *apxb*
GR	*RGRC2*	full-length cDNA of RGRC2
MDAR	*RMDAR1*	3'UTR fragment of *RMDAR-1*
DHAR	*DHAR*	coding region of rice DHAR cDNA
PHGPX	*PHGPX*	partial PCR fragment of rice EST clone homologous to the known plant PHGPX

Results

Characterization of rice EST clones homologous to cytosolic PHGPX -

In order to detect the specific-transcript to cytosolic PHGPX, a nucleotide sequence of rice EST clones (accession number D22908, D48881, and D49202) were obtained by searching the DNA databases with the nucleotide sequence of putative cytosolic PHGPX cDNA (GPX2, accession number AJ000470) isolated from *A. thaliana* (Fig. VII-1A). A partial fragment of the concatenate sequence using these rice EST clones was amplified by PCR as described in Materials and Methods (Fig. VII-1B). Sequencing of this fragment revealed that this fragment included the sequences of rice EST clones (data not shown). Therefore this PCR-fragment was used as a partial DNA of rice cytosolic PHGPX cDNA for further analyses.

Preparation of gene-specific probes to cytosolic isoforms of AOS-scavenging enzymes -

Before Northern blot analysis, the gene-specific probes to rice AOS-scavenging enzyme were prepared as described in Materials and Methods and summarized in Table VII-1. For the detections of *RGRC2* gene, full-length cDNA was used as the hybridization probe because this gene exist as the single copy gene in rice genomes (see in the section 1). In the case of two cytosolic Cu/Zn-SOD genes (*sodCc1* and *sodCc2*), two cytosolic APX genes (*apxa* and *apxb*), and one of cytosolic MDAR genes (*RMDAR1*), 3'-UTR prepared by PCR were used because these have been characterized as two copy genes in rice genomes (Sakamoto et al., 1992b; Sakamoto, 1992; Morita

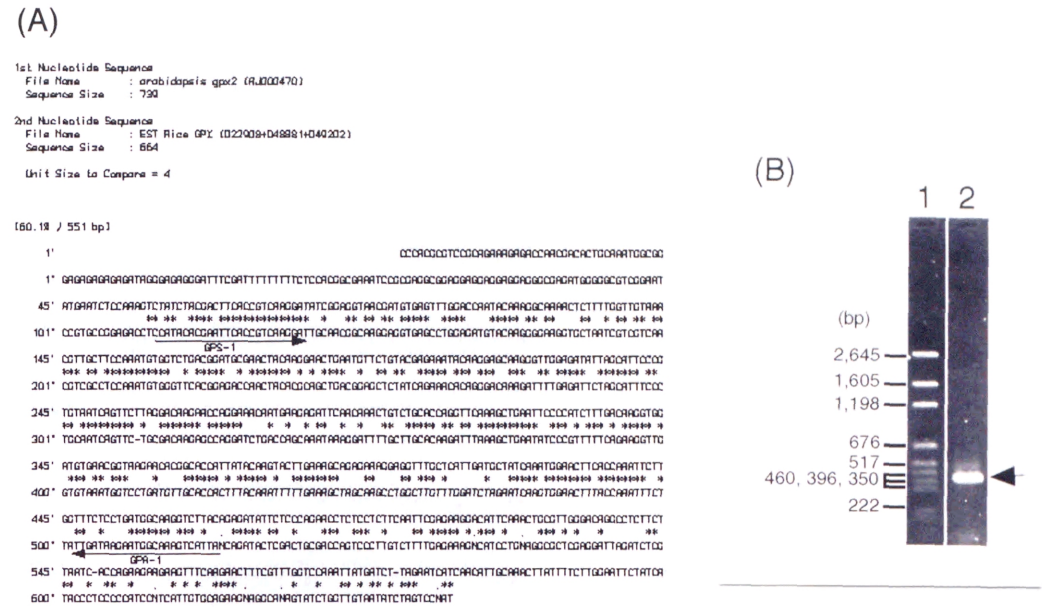

Fig. VII-1. Preparation of a partial fragment of rice cytosolic PHGPX cDNA. (A) Comparison between the nucleotide sequences of *A. thaliana* cytosolic PHGPX cDNA (GPX2, accession number AJ000470) and the concatenate sequence of rice EST clones (accession number D22908, D48881, and D49202). The positions of PCR primers, GPS-1 and GPA-1, for amplification of a partial rice cytosolic PHGPX are indicated by arrowheads as polarity. (B) Electrophoresis analysis stained by ethidium bromide; lane 1: pGEM marker (Promega Japan), lane 2: PCR products amplified with a pair of primer, GPS-1 and GPA-1. Arrows indicated the recovered band by electroelution for using as a hybridization probe.

et al., 1997; see in section 2). In the case of cytosolic DHAR and PHGPX genes, PCR-fragments were used for the hybridization probes. With these probes, Northern blot analysis was carried out using rice seedlings treated with ABA and environmental stresses, such as drought, salinity and chilling.

Coordinate regulation of the genes for cytosolic AOS-scavenging enzymes under *ABA and ABA-associated environmental stresses* -

In order to clarify the regulational mechanisms of genes for cytosolic AOS-scavenging enzymes under environmental stresses, the transcripts of these genes in rice seedlings treated with ABA and ABA-related environmental stress treatments, as done in CHAPTER I-4, were examined by Northern blot analysis. In the ABA treatment (Fig. VII-2), the mRNA levels of *sodCc2*, *apxa*, and *RGRC2* genes were increased significantly at 12 h after the treatment, and the induction was dose-dependent on ABA concentration. While the induction of *sodCc1*, *RMDAR1*, *DHAR*, and *PHGPX* genes by ABA treatment was not dose-dependent. The response of *apxb* gene to ABA was not observed. In the drought treatment (Fig. VII-3), *sodCc2*, *apxa*, *DHAR*, *RGRC2*, and *PHGPX* genes were strongly induced and the high expression level of these genes were kept at 24 h after the treatment, while those of other genes was dramatically decreased at same time. The salinity stress

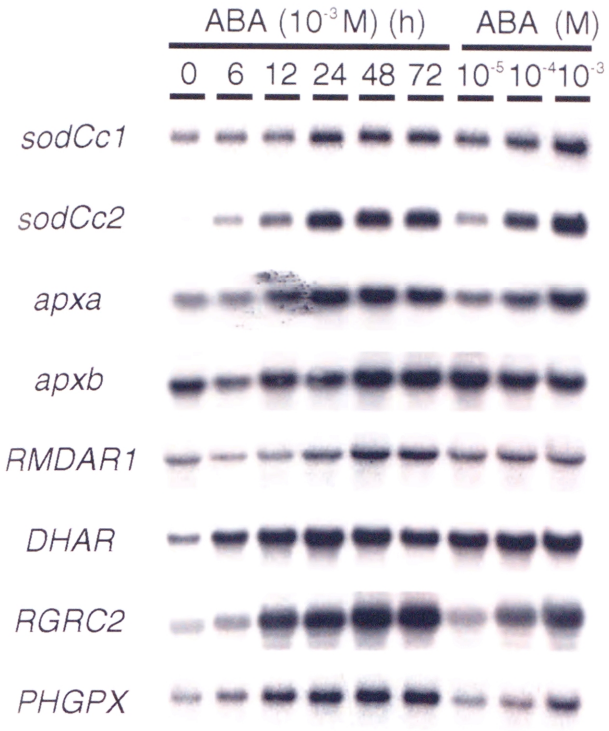

Fig. VII-2. Effect of ABA on the gene expressions of cytosolic AOS-scavenging enzymes. Total RNA (20 μg) was isolated from rice seedlings treated with 10^{-3} M ABA (0-48 h) or different concentration of ABA (10^{-3}, 10^{-4} and 10^{-5} M) for 16 h, and subjected to Northern blot as described in Materials and Methods. ^{32}P-labeled gene-specific probes for cytosolic AOS-scavenging enzymes (Table VII-1) were used for the hybridization.

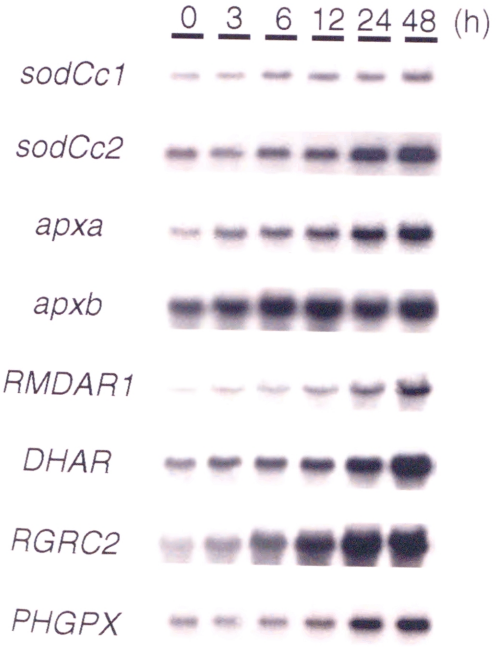

Fig. VII-4. Effect of salinity treatment on the gene expressions of cytosolic AOS-scavenging enzymes. Northern blot analysis of rice seedlings, treated with 250 mM NaCl under continuous illumination (170 μmol quanta m^{-2} s^{-1}), was performed as described in Fig. VII-2.

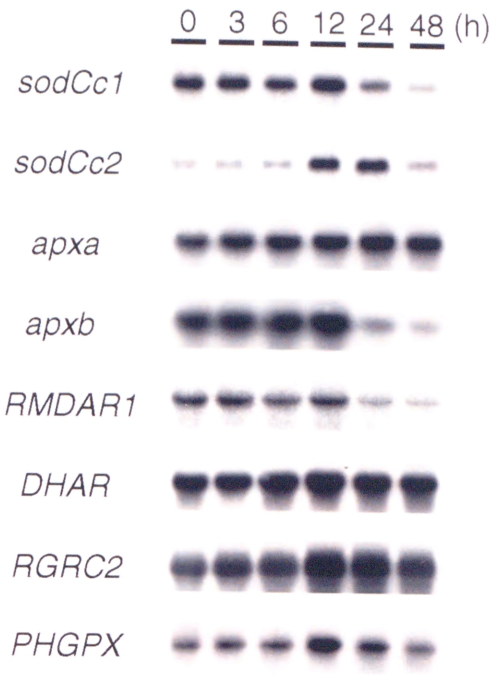

Fig. VII-3. Effect of drought on the gene expressions of cytosolic AOS-scavenging enzymes. Northern blot analysis of rice seedlings, treated by withholding water (0-48 h) under continuous illumination (170 μmol quanta m^{-2} s^{-1}), was performed as described in Fig. VII-2.

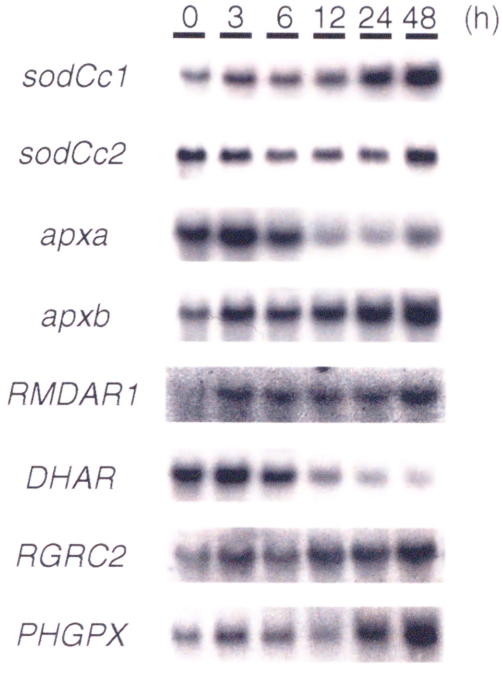

Fig. VII-5. Effect of chilling treatment on the gene expressions of cytosolic AOS-scavenging enzymes. Northern blot analysis of rice seedlings, subjected to chilling condition (10 °C, 0-48 h) under continuous illumination (170 μmol quanta m^{-2} s^{-1}), was performed as described in Fig. VII-2.

induced the expression of all genes without *sodCc1* gene, but there is difference in the induction patterns (Fig. VII-4). The mRNA level of *apxb* gene was increased from 3 h after the treatment and reached the maximum level at 6 h. Whereas the expressions levels of *sodCc2*, *apxa*, *RMDAR1*, *DHAR*, *RGRC2*, and *PHGPX* genes were gradually increased, and the maximum level of mRNAs were observed at 48 h after the treatment. In the chilling treatment at 10 °C (Fig. VII-5), mRNA levels of *sodCc1*, *apxb*, *RMDAR1*, *GR*, and *PHGPX* genes were gradually increased during 48 h of treatment, while rapid responses of *apxa* and *DHAR* genes was observed at 3 h after the starting of treatment. The significant change of *sodCc2* gene was not observed.

Discussion

In this section, the author reports the gene expression of cytosolic AOS-scavenging enzymes, namely SOD, APX, MDAR, DHAR, GR, and PHGPX, under ABA and ABA-associated stress treatments. Of course, this is first report of such large scale analysis of plant AOS-scavenging enzymes and carrying out the overall speculation about a part of regulational mechanisms.

Previous reports about response of each cytosolic SOD, MDAR, and GR gene to environmental stresses are referred to the former sections. Beside these results, several reports have indicated the responses of another cytosolic AOS-scavenging genes, APX and PHGPX, to various stimuli, including environmental stresses. The many informations about cytosolic APX gene have already been available, and so examined the responses of cytosolic APX genes to various stress. The amount of pea cytosolic APX mRNA was increased by the treatments of paraquat, ABA, high temperature and ethephone but the coordinate elevation of cytosolic APX protein was not observed (Mitller and Zilinskas, 1992). One of *A. thaliana* cytosolic APX gene (*APX1*) was induced strongly by methyl viologen and ethephone (Storozhenko et al., 1998) and slightly by ozone and sulfur dioxide (Kubo et al., 1995). PHGPX cDNA was, at first, isolated from *Nicotiana sylvestris* by differential screening, and the mRNA was abundant in germinating seed and increased by $HgCl_2$ treatment (Criqui et al., 1992). Further PHGPX cDNA was isolated using the antibody against one of salt-stress-associated protein from *Citrus sinensis* cultured cells (Holland et al., 1993). PHGPX cDNAs were categorized to two groups as their localized subcellular compartments, presumably cytosolic and chloroplastic isoforms (Mullineaux et al., 1998). In rice, there are no information about PHGPX. About DHAR in rice, the purified protein from rice brans was characterized (Kato et al., 1997) and corresponding cDNA was isolated (unpublished data by Dr. Ushimaru). The amount of rice DHAR was increased by air-adaptation, together with APX and MDAR (Ushimaru

et al., 1997).

Like this, because the previous analyses were carried out only by focusing on one or several of AOS-scavenging enzymes, the overall speculation in the gene regulation of plant AOS-scavenging enzymes has not been carried out so far.

In this section, similar responses of *sodCc2*, *apxa*, *RGRC2* genes to ABA and ABA-associated stress, salinity and drought, were observed (Fig. VII-2,3,4). While such responses of these genes were not observed to chilling at 10 °C (Fig. VII-5), but these responses were seemed to be not associated with ABA-mediated signal transduction pathway like the case of SOD genes (in CHAPTER I-4). *sodCc2* and *RGRC2* genes were seemed to be regulated by ABA-mediated signal transduction pathway because there are several ABREs in the promoter region of each gene and *sodCc2* gene responded to ABA by promoter analysis in rice protoplasts (Sakamoto et al., 1995b; in this thesis, CHAPTER II-1). Several results about such coordinate gene regulation of cytosolic AOS-scavenging enzymes were reported as follows. The parallel induction of pea cytosolic SOD and APX genes were observed under drought stress and the recovery period (Mitller and Zilinskas, 1994). Similar responses of cytosolic Cu/Zn-SOD and APX genes to ozone were observed in *A. thaliana* (Conklin and Last, 1995) and to ozone, sulfur dioxide and UV-B in *N. plumbaginifolia* (Willekens et al., 1994). *Citrus* cytosolic Cu/Zn-SOD, APX was increased by the treatment of salinity and methyl viologen, while PHGPX was increased only by salinity (Gueta-Dahan et al., 1997). The high levels of both SOD and GR activities were significant for the tolerance against drought and herbicide in maize (Malan et al., 1990), and against oxidative stress by transgenic analysis (Aono et al., 1995). All of these results suggested that the coordinate expressions of *sodCc2*, *apxa*, and *RGRC2* genes, presumably regulated by ABA-mediated signal transduction pathway(s), are important when plants are subjected to environmental stresses.

On the other hand, similar expression pattern was observed in *DHAR* and *PHGPX* genes. These genes were induced by ABA but not dose-dependent (Fig. VII-2), and induced by drought and salinity treatments (Fig. VII-3,4). *Citrus* PHGPX protein was induced by ABA and salinity but the salinity induction was independent to ABA (Gueta-Dahan et al., 1997). The mechanisms of the regulation for *sodCc1*, *apxb*, *RMDAR1*, *DHAR*, and *PHGPX* genes could not be clarified. Several cytosolic SOD and APX genes were regulated by cellular redox state; *N. plumbaginifolia* and rice cytosolic Cu/Zn-SOD gene is up-regulated by reduce reagents (Herouart et al., 1993; Sakamoto et al., 1995a), and mRNA levels only of *A. thaliana APX1* and *APX2*, which are cytosolic APX genes, were rapidly (within 15 min) increased by excess light treatment and the regulation was associated with the redox states of plastquinone (Karpinski et al., 1997). Therefore a part of cytosolic AOS-scavenging enzyme genes might be regulated by the cellular redox states under stress conditions.

One of rice cytosolic Cu/Zn-SOD gene, *sodCc1*, has 77 bp of homologous sequence with rice thioredoxin h (*Rtrxh*) gene (Ishiwatari et al., 1995; Sakamoto et al., 1995b). 28 bp core sequence in the homologous sequence is highly conserved in the promoter region of these genes and rice glutaredoxin gene (accession number D86744). Currently in the author's laboratory, this sequence has been identified as a *cis*-element for gene regulation by cellular redox state.