SECTION 4

Gene expression of rice superoxide dismutase isoforms

- expression in vegetative tissues and

gene regulation under environmental stresses -

Materials and Methods

Preparation of Experimental samples from rice vegetative tissues -

The leaves, stems and roots were prepared by cutting out the rice seedlings, which were grown hydroponically as described in section 1. Suspension cells derived from rice embryogenic calli were maintained by weekly subculture in Murashige and Skoog (MS) liquid medium (Murashige and Skoog, 1962) containing 1 mg/l of 2,4-dichlorophenoxyacetic acid. The senescence samples were prepared from rice leaves at the flowering stage; leaf 1 is the first apical leaf and leaf 6 is most senescence leaf. 14 DAF of rice developing seeds were divided into pericarp, endosperm and embryo in grinding buffer for RNA extraction with tweezers, and then immediately subjected to the RNA extraction. For the experiment of light induction, dark-grown 10 d etiolated seedlings were exposed to the light (170 μmol quanta m^{-2} s^{-1}) during the treatment. The samples without seeds immediately frozen in liquid nitrogen were stored at -80 °C until use.

Stress treatments -

Rice (*Oryza sativa* L. cv Nipponbare) seedlings were grown hydroponically as described in section 1, and used for the following stress treatments. For the treatments of ABA (Sigma), salinity stress, H_2O_2, and metal ions (0.1 mM), the seedling roots were placed in ABA (1 mM), NaCl (250 mM), H_2O_2 (1 mM), and each metal sulfate (0.1 mM; $CuSO_4$, $FeSO_4$, $MnSO_4$, and $ZnSO_4$, 50 μM; $Al_2(SO_4)_3$) solutions. In the case of ABA, rice seedlings treated with the different concentration of ABA (10^{-3},10^{-4},10^{-5} M) for 16 h were also used. In the metal treatments, both stress-treated seedlings and control seedlings were kept for 16 h. For chilling stress, the seedlings were exposed to 10 °C. Drought stress was induced by withholding water. The treatment of paraquat was performed by splaying plants with 100 ml of 10^{-3} M methyl viologen (paraquat). These stress treatments were performed under continuous light (170 μmol quanta m^{-2} s^{-1}) unless otherwise mentioned. The salinity stress in the dark was performed in the darkness by the same treatment in the light. Samples were collected at 0, 3, 6, 12, 24 and 48 h (drought, salinity, chilling and ABA treatments) or 0, 2, 4, 8, 16 and 24 h (paraquat and H_2O_2 treatments) after the start of each treatment, immediately frozen

in liquid nitrogen, and stored at -80 °C until use.

Preparation of rice SOD gene-specific probes -

To prepare the gene-specific probe to each rice SOD gene, 3'-untranslational region (UTR) of each gene was used in the case of *sodA*, *sodCc1* and *sodCc2*, and full-length cDNAs used in the case of *sodB* (Fe-SOD gene) and *sodCp*. The 3'-UTR fragments of *sodA*, *sodCc1* and *sodCc2* were obtained by PCR with a pair of primer, which were synthesized by the based on the nucleotide sequence of each genomic DNA (Sakamoto et al., 1992b; 1995a; 1995b; Sakamoto, 1992; section 2 in this thesis). To obtain the fragment of full-length cDNAs, the plasmids containing cDNAs (RFeSOD-4 and PL-1) were digested by the restriction enzymes (*Sal*I for *sodB*, *Eco*RI and *Bam*HI for *sodCp*). All probes were recovered by the electroelution after the agarose electrophoresis. The used probes and primers were summarized in Table IV-1.

Table IV-1. Rice SOD gene specific probes used in Northen blot analysis.

Isozyme	Gene	Gene-specific probe	Primer
Mn-SOD	*sodA*	*sodA* 3'-UTR	sodA-3UF: 5'-TGAAGGCAACGCTCATGGTT-3' sodA-3UR: 5'-AGAACGGGAATTGGCACAGA-3'
Fe-SOD	*sodB*	RFeSOD4	
Cu/Zn-SOD			
Plastidic	*sodCp*	PL-1	
Cytosolic	*sodCc1*	*sodCc1* 3'-UTR	sodCc1-3UF: 5'-AGGCTGAAACCTGGAGGTGTGAAC-3' sodCc1-3UR: 5'-TGATTGCCAATCTTACTTGCGTTCCG-3'
	sodCc2	*sodCc2* 3'-UTR	sodCc2-3UF: 5'-ACGTCTCAACTTTCCAACATACAGAA-3' sodCc2-3UR: 5'-GTACTCAAGTCCACCAATGATACT-3'

Northern blot analysis -

Total RNA of each sample was prepared using GIT-CsCl method as described in CHAPTER I, without seeds, which was used the SDS-phenol method (Sakamoto, 1992) with minor changes as follows. Seeds were powdered in liquid nitrogen with a mortar and pestle, and then homogenated in grinding buffer (0.1 M Tris-HCl (pH 9.0), 0.1 M NaCl, 1% SDS, and 2 M 2-mercaptoethanol). The homogenate was extracted with an equal volume of phenol/chloroform/isoamylalcohl (25:24:1), centrifuged at 15,000 rpm for 5 min, and the aqueous phase was recovered. This step was repeated at least three times until no interface of denatured protein was obtained. Following extraction, twentieth volume of 5 M NaCl and two volume of ethanol added into the aqueous solution to precipitate nucleic acids. The resultant precipitate chilled at -80 °C for at least 30 min was recovered by centrifugation and resolved with DEPC-treated distilled water. Third volume of 10 M

lithium chloride was added, and the resultant prepiciate chilled at 4 °C for at least 2 h (recommended for overnight) was recovered by centrifugation and used for Northern blot analysis.

Northern blot analysis was performed as follows. Total RNA (15-20 μg) was resolved in loading buffer (50% (v/v) formamide, 2.2 M formaldehyde, 20 mM MOPS, 4% (v/v) glycerol, 0.8% (w/v) bromophenol blue) and denatured at 65 °C for 10 min. The resultant solution added with ethidium bromide was separated by 5% (v/v) formaldehyde-containing 1.2% agarose gel and then blotted to nitrocellulose membrane (Hybond-C extra; Amersham Pharmacia Biotech Japan) in 20 x SSC for overnight. The membrane was baked at 80 °C for 2 h and subjected to the hybridization. The hybridization with ^{32}P-labelled SOD gene specific probes was performed as described in the previous sections but the washing was carried out at the high stringency (0.5 x SSC, at 55 °C).

Results

Preparation of SOD gene specific probes -

Prior to Northern blotting, the specific probes to rice SOD genes were prepared as described in Materials and Methods, and summarized in Table IV-1. For the detections of *sodB* and *sodCp* genes, full-length cDNAs were used as the hybridization probes because these genes exist as the single copy gene in rice genomes (see in the section 1,3). In the case of cytosolic Cu/Zn-SOD genes and Mn-SOD gene, 3'-UTR prepared by PCR were used because cytosolic Cu/Zn-SOD genes consist of two genes, *sodCc1* and *sodCc2* genes (Sakamoto, 1992), and only *sodA1* gene have been characterized in rice Mn-SOD gene (see in the section 2). The cross-hybridization among these probes have not been occurred in Southern blot analysis washed at a high stringency (data not shown). With these probes, Northern blot analysis of rice SOD genes was carried out using rice vegetative tissues, or rice seedlings treated with chemical treatments (ABA, methyl viologen, H_2O_2, metal ions) and environmental stresses, such as drought, salinity and chilling.

Expression of rice SOD genes in vegetative tissues -

In order to clarify the tissue-specific expression of each rice SOD isoform, the transcripts of rice SOD isozymes in various rice vegetative tissues were analyzed. Northern blot analysis in green leaves, stems, and roots of young seedlings, and calli were performed (Fig. IV-1A). *sodA* gene is strongly expressed in non-photosynthesis tissues, especially in calli. Whereas both transcripts of cytosolic Cu/Zn-SOD genes (*sodCc1* and *sodCc2*) were observed strongly in roots but a little in calli. The expression pattern between both genes for plastid-localized SOD isoforms (*sodB* and

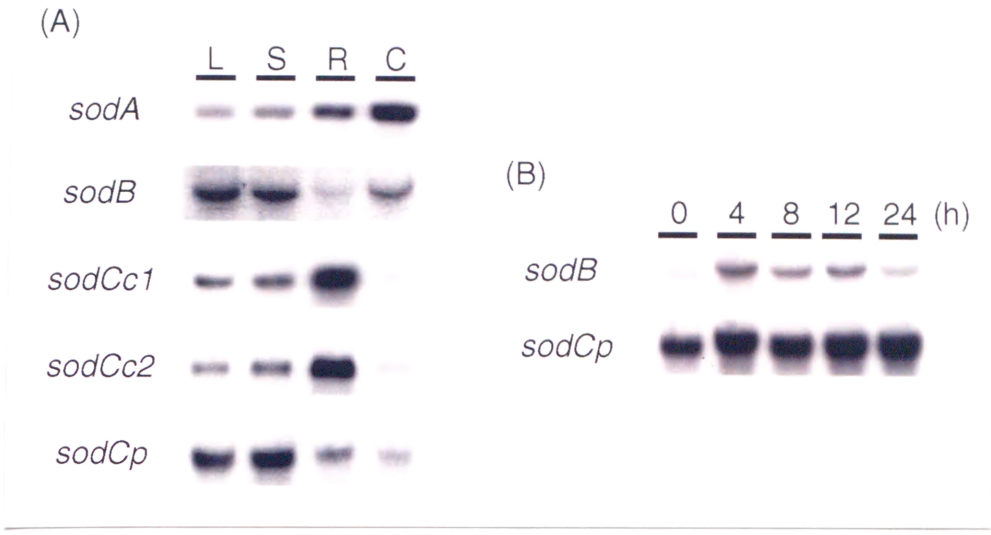

Fig. IV-1. mRNA abundance of rice SOD isoforms in vegetative tissues (A) and effect of light signal on the gene expressions of rice chloroplast-localized SOD, Fe-SOD and plastidic Cu/Zn-SOD (B). Total RNA (20 μg) was isolated from leaves (L), stems (S), roots of young seedlings and suspension culture of embryogenic-calli (C) or from greening leaves of etiolated seedlings treated by continuous illumination (170 μmol quanta m^{-2} s^{-1}), and subjected to Northern blot as described in Materials and Methods. ^{32}P-labeled gene-specific probes for SOD isozymes (Table IV-1) were used for the hybridization.

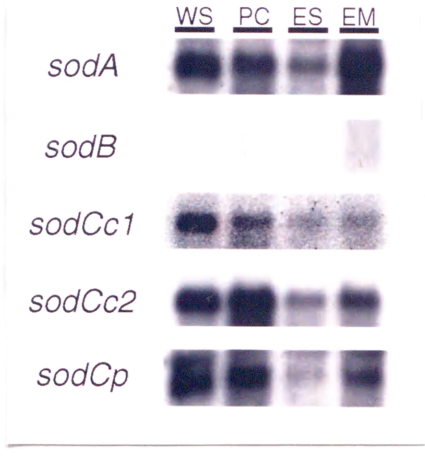

Fig. IV-2. mRNA abundance of rice SOD isoforms in various tissues of developing seed. Total RNA (20 μg) was isolated from whole seed (WS), pericarp (PC), endosperm (ES), and embryo (EM) of rice developing seeds (14 DAF), and subjected to Northern blot as described in Fig. IV-1.

Fig. IV-3. Effect of senescence on rice SOD mRNA abundance. Total RNA (20 μg) was isolated from developing leaves at the flowering stage (leaf 1 is the first apical leaf and leaf 6 is most senescence leaf), and subjected to Northern blot as described in Fig. IV-1.

sodCp) is similar, strongly expressed in photosynthesis tissues, leaves and stems. In the light induction experiment (Fig. IV-1B), these genes were strongly and similarly induced by light signal at 4 h after on set of the treatment. However the mRNA level of *sodB* gene in calli was more lower than that of *sodCp* gene and decreased at 24 h after the strating of light induction experiment.

Furthermore, the abundance of rice SOD mRNA in different tissues of developing seeds (14 DAF) was analyzed by Northern blot analysis (Fig. IV-2). The transcript of *sodB* gene was undetectable in all seed tissues, even if that of *sodCp* gene was detectable. The low levels of other

transcripts in endosperm may due to large amount of mRNAs of genes for storage proteins and amylose synthesis in this tissues. These transcripts could be detected in all seed tissues. The strong expression of *sodA* gene was observed in embryo. The expressions of *sodCc1* and *sodCp* genes is supposed to be constitutive in the tissues of developing seeds, while the mRNA of *sodCc2* gene is relative high amount especially in pericarp.

To clarify effects of senescence on rice SOD genes, Northern blot analysis in leaves of different age was carried out (Fig. IV-3). The transcripts of *sodA* and *sodCp* genes in old leaves were more abundant than those in young leaves, while two cytosolic Cu/Zn-SOD mRNAs is decreased as developing of senescence. The significant changes of *sodB* mRNA was not observed in developing of senescence.

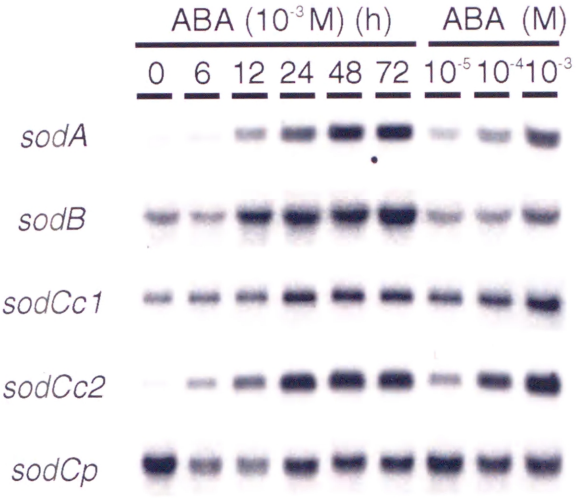

Fig. IV-4. Effect of ABA on rice SOD mRNA abundance. Total RNA (20 μg) was isolated from rice seedlings treated with 10^{-3}M ABA (0-48 h) or different concentration of ABA (10^{-3}, $10^{-4}$and 10^{-5}M) for 16 h under continuous illumination (170 μmol quanta m^{-2} s^{-1}), and subjected to Northern blot as described in Fig. IV-1.

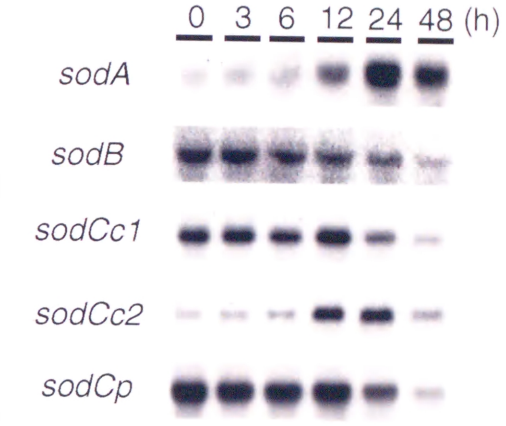

Fig. IV-5. Effect of drought on rice SOD mRNA abundance. Northern blot analysis of rice seedlings, treated by withholding water (0-48 h) under continuous illumination (170 μmol quanta m^{-2} s^{-1}), was performed as described in Fig. IV-5.

Responses of abscisic acid (ABA)-inducible genes for rice SOD isoforms to environmental stresses -

Under stress conditions such as drought, high concentration of salts, and low temperature, the ABA content increases in plant tissues (Skriver and Mundy, 1990). Therefore we first examined the responses of rice SOD genes to ABA treatments (Fig. IV-4). The expressions of *sodA* and *sodCc2* genes were strongly induced by 10^{-3} M ABA treatment and the induction was dose-dependent on ABA concentrations (10^{-3}-10^{-5} M) at 16 h after the start of the treatment. mRNAs of these genes were also increased by drought (Fig. IV-5) and salinity (250 mM NaCl; Fig. IV-6A) treatments.

Similar inductions of these genes were observed by the salinity treatment in the dark (Fig. VI-6B). In both cases of ABA and drought treatment, however, the accumulation of *sodCc2* mRNA at maximum level was early induced than that of *sodA* gene. On the other hand, these genes were differentially responded to chilling treatment at 10 °C for 48 h (Fig IV-7). *sodA* gene was rapidly responded to chilling (at 3 h after the treatment), while the significant change of *sodCc2* gene was not observed.

While the expressions of *sodCc1* and *sodB* genes were also induced by ABA treatment but not dose-dependent induction (Fig. VI-4). *sodCc1* gene was induced gradually by chilling but not by drought and salinity treatments (Fig. IV-5,6,7). Whereas the mRNA level of *sod*B gene was increased by salinity treatments both in the light and in the dark (Fig. IV-6), and rapidly by chilling (Fig. IV-7) but decreased by drought treatment (Fig. IV-5).

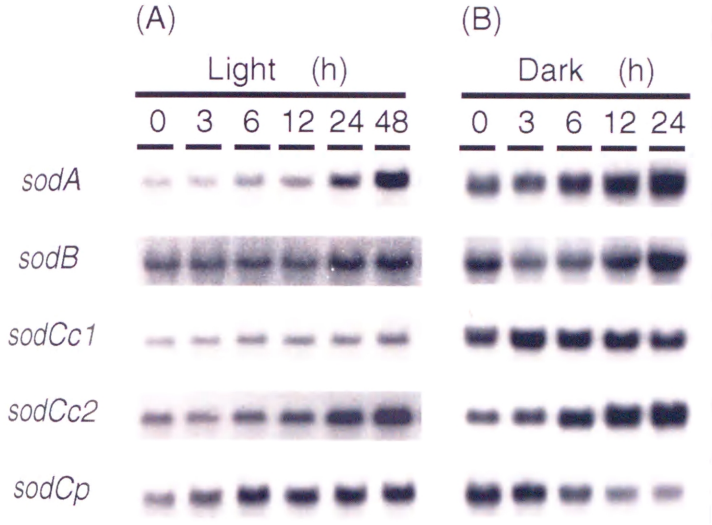

Fig. IV-6. Effect of salinity treatment on rice SOD mRNA abundance in the light (A) and dark (B). Northern blot analyses, of which (A) rice seedlings treated with 250 mM NaCl under continuous illumination (170 μmol quanta m^{-2} s^{-1}, 0-48 h) and (B) the 16 h-dark-adapted rice seedlings was treated with 250 mM NaCl in the dark (0-24 h), were performed as described in Fig. IV-5.

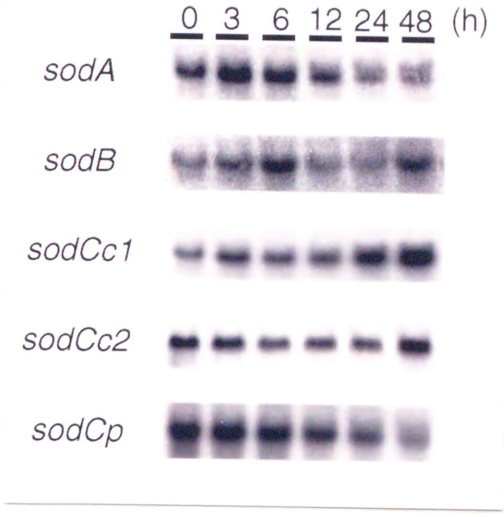

Fig. IV-7. Effect of chilling treatment on rice SOD mRNA abundance. Northern blot analysis of rice seedlings, subjected to chilling condition (10 °C, 0-48 h) under continuous illumination (170 μmol quanta m^{-2} s^{-1}), was performed as described in Fig. IV-5.

Regulation of sodCp gene under salinity stress may be associated with light-dependent AOS generation -

sodCp gene was quickly responded to salinity (Fig. IV-6A) in the light although the expression of *sodCp* was suppressed by ABA, drought and chilling treatments (Fig. IV-4,5,7). However decrease of *sodCp* mRNA by salinity treatment in dark-adapted seedlings was observed under the darkness (Fig. IV-6B). These results indicated that the expression of *sodCp* gene is regulated by the light-dependent signal transduction pathway under salinity condition. To clarify whether this light-dependent signal transduction pathway is mediated by AOS derived from chloroplasts, we have

examined the effects of methyl viologen (10^{-5} M), which generate $O_2^{\cdot-}$, and H_2O_2 (10^{-3} M) under illumination on rice SOD mRNAs. All SOD genes were differentially induced by methyl viologen (Fig. IV-8), and the degree of each gene induction was different. In the H_2O_2 treatment (Fig. IV-9), no response of cytosolic Cu/Zn-SOD genes was observed. *sodA* and *sodCp* genes were rapidly induced by H_2O_2 treatment, while *sodB* mRNA was gradually increased. Such rapid induction of *sodCp* gene was similar to the case of salinity treatment (Fig. IV-6A).

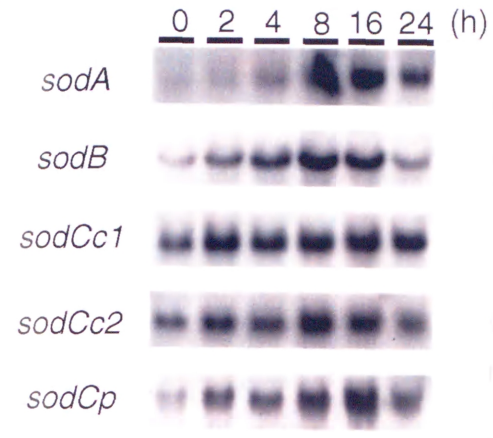

Fig. IV-8. Effect of methyl viologen (paraquat) on rice SOD mRNA abundance. Northern blot analysis of rice seedlings, treated with 10^{-5} M methyl viologen (0-24 h) under the illumination (170 μmol quanta m^{-2} s^{-1}), was performed as described in Fig. IV-5.

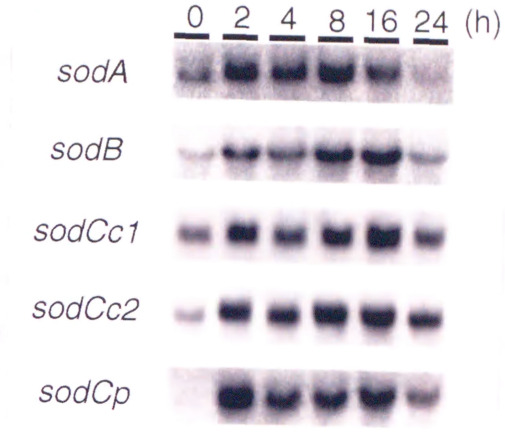

Fig. IV-9. Effect of H_2O_2 on rice SOD mRNA abundance. Northern blot analysis of rice seedlings, treated with 10^{-3} M H_2O_2 (0-24 h) under the illumination (170 μmol quanta m^{-2} s^{-1}), was performed as described in Fig. IV-5.

Effects of heavy metal ions on the gene expression of rice SOD isoforms -

It is known that the high amount of heavy metals in cells induce AOS generation and caused to oxidative stress. Now, however, the molecular mechanisms for induction of oxidative stress by heavy metals are not clear. Therefore effects of various heavy metal ions (Al^{3+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+}) on rice SOD genes by Northern blot analysis (Fig. IV-10). The expression of *sodA* mRNA was induced by Cu^{2+} and Zn^{2+}, and slightly by Fe^{2+}. The significant changes in mRNA level of *sodCc1* and *sodCc2* genes were not observed by all heavy metal treatments. The expression of both *sodB* and *sodCp* genes were induced by the treatments of Al^{3+}, Fe^{2+}, Mn^{2+}, and Zn^{2+}.

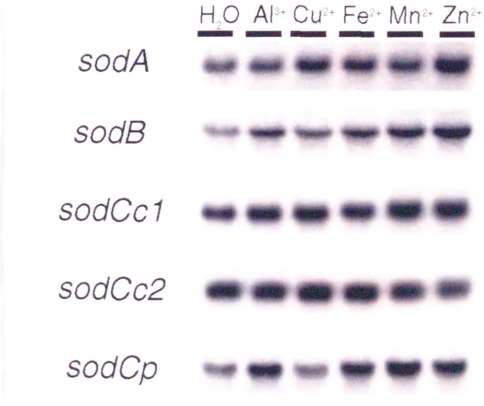

Fig. IV-10. Effect of heavy metal ions on rice SOD mRNA abundance. Northern blot analysis of rice seedlings, treated with 100 mM heavy metals (Al^{3+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+}) for 16 h under the illumination (170 μmol quanta m^{-2} s^{-1}), was performed as described in Fig. IV-5.

Discussion

In this section, the author reports the expression of each rice SOD gene in vegetative tissues and the responses of SOD genes to various stimuli, such as chemical and environmental stresses. These experiments with gene specific probes to all SOD isoforms will be much available to understand the role of each SOD isoform and the regulation of plant SOD genes. The major part of these results will be published in Kaminaka et al. (1999b).

The result of Fig. IV-1 suggested that the expression levels of rice SOD genes in each vegetative tissue (Fig. IV-1A), leaves, stems, roots and calli, may be different according to the location of encoding SODs. mRNA of *sodA* gene for mitochondrial Mn-SOD was observed mainly in embryogenic-calli rather than roots, which is similar to rubber tree Mn-SOD gene (Miao and Gaynor, 1993), while the high level expressions of two cytosolic genes (*sodCc1* and *sodCc2*) were detected only in roots and almost undetectable in calli. However SOD activity staining analysis indicated that rice Mn-SOD activity (Mn-SOD I) occupied the predominant SOD activity in roots and calli (Fig. B). Therefore *sodA* transcripts in these tissues may be much higher amount than those of cytosolic genes' transcripts. Both *sodB* and *sodCp* genes were strongly expressed in photosynthesis tissues. These genes were also dramatically induced by light induction (Fig. IV-1B), and which is corresponding result of *N. plumbaginifolia* Fe-SOD gene (Tsang et al., 1991) and tomato plastidic Cu/Zn-SOD gene (Perl-Treves and Galun, 1991). However relative high level of *sodB* transcript was also detected in calli. The suspension culture of embryogenic-calli is thought to be always subjected to high osmotic condition from the culture medium. Such differential expression levels of chloroplast-localized SOD isoforms suggested that *sodB* gene is strongly induced by various stress-related stimuli.

In the analysis of rice SOD transcripts in various seed tissues (Fig. IV-2), the high levels of *sodA* and *sodCc2* mRNAs were observed in embryo and pericarp, respectively. Similar results were reported as follows; maize Mn-SOD gene (SOD-3) was also strongly expressed in scutella (embryo) (White and Scandalios, 1988), and the large amount of tomato cytosolic Cu/Zn-SOD mRNA was detected in pericarp of the ripening fruit at all stage of developing (Perl-Treves and Galun, 1991). The clear necessity of AOS-scavenging at the developing stage of rice seed is unknown, but the level of ABA synthesis is elevated at late embryogenesis stage and induced many ABA-responsive genes (Skriver and Mundy, 1990). *sodA* and *sodCc2* are also ABA-responsive genes (Fig. IV-4) and so may be up-regulated at developmental stage of seed. On the other hand, the transcript of *sodCp* gene was detected in all seed tissues although this gene encode plastidic isoform. However because the SOD activity from rice Cu/Zn-SOD I, which is encoded by *sodCp*

gene, was detected in seed extracts (Kanematsu and Asada, 1989). In promoter region of *sodCp* gene, an unknown homologous region with several rice genes including 26 kD globulin and waxy genes, which are strongly associated with seed developing, was observed (section 1). The existence of this homologous region and plastidic Cu/Zn-SOD activity in rice seed suggested that rice plastidic Cu/Zn-SOD may have important roles, such as AOS-scavenging in amyloplast developing, in rice seeds.

In rice developing leaves at flowering stage (Fig. IV-3), differential responses of rice SOD genes were observed and indicated that rice SOD genes are differentially regulated during developing. These results was inconsistent with that about *N. plumbaginifolia* chloroplast-localized SOD genes (Kurepa et al., 1997), and which indicated that the transcript of plastidic Cu/Zn-SOD gene was detected only in young leaves of mature plants, while Fe-SOD mRNA was abundant in young and old leaves of mature plants. Only the increases of *sodA* and *sodCp* mRNA abundance during developing were similar to the response of these genes to oxidative stress treatments, methyl viologen and H_2O_2 (Fig. IV-8,9). Oxidative stress has a important role and accelerates in the development of senescence because the damages by senescence, such as lipid peroxidation or decrease in photosynthesis capacity, may due to oxidative stress. Therefore the regulation of these gene at developing stages may be directly associated with AOS-generation and, in that case, AOS itself may play a role as a signalling factor.

Responses of *sodA* and *sodCc2* genes, which were strongly induced by ABA (Fig. IV-4), were observed to drought and salinity stress (Fig. IV-5,6), while only *sodA* gene responded to chilling (Fig. IV-7). It is well-known that the gene regulation under these stress is associated with ABA-mediated signal transduction pathways. The promoter analysis of rice *sodCc1* and *sodCc2* genes in rice protoplasts indicated that these genes were differentially expressed in response to ABA, and the sequence analysis suggested that the differential responses were associated with the numbers of ABRE, which was the consensus sequence found in many ABA-responsive genes, in each promoter region (Sakamoto et al., 1995b). Responses of these genes are corresponded to the result in this section, ABA treatment directly induced the expression of *sodCc2* but not *sodCc1* because the induction of *sodCc1* gene was not dose-dependent of ABA concentration (Fig. IV-4). In maize, similar results to the results of this section were observed (Guan and Scandalios, 1998). On the other hand, Mn-SOD genes are also induced by ABA and high osmoticum, which was induced in cells by drought, salinity and abnormal temperature (Skriver and Mundy, 1990), in developing maize embryo (Zhu and Scandalios, 1994), and by ABA, salinity and high osmoticum in *N. tabacum* Bright Yellow 2 (BY-2) cell cultures (Bueno et al., 1998). In addition, the expression level of Mn-SOD genes in *N. plumbaginifolia* (Bowler et al., 1989) and rubber tree (Miao and Gaynor,

1993) were also increased by high osmoticum treatment. These consensus results suggested that Mn-SOD gene(s) in all plants are regulated by ABA-mediated signal transduction pathway(s) under osmotic stress-realted environmental stresses, such as drought, salinity and high or low temperatures. In the promoter sequence of rice *sodA* gene (section 2), there were the binding sequence of MYB and MYC, which are associated with ABA-mediated signal transduction pathway distinct from ABRE-mediated pathway (Abe et al., 1997). Therefore *sodA* and *sodCc2* genes may be regulated by different signal transduction pathways via ABA under these stress conditions. The induction of *sodCc2* gene by ABA, drought, and salinity occurred apparently early than that of *sodA* gene (Fig. IV-4,5,6), which may due to the difference of regulational mechanisms for these genes. Actually, differential responses of *sodA* and *sodCc2* genes to chilling was observed (Fig. IV-7). Plant MYB- and MYC-mediated signal transduction pathway is not associated with chilling stress (Urao et al., 1993). On the other hand, H_2O_2 concentration was remarkably increased under chilling at 4 °C but the increase was starting at 4 d afte the treatment (Fadzillah et al., 1996). *sodCp*, which is also H_2O_2 inducible gene, mRNA abundance was decreased during chilling treatment (Fig. IV-7). Therefore responses of rice SOD genes might not be directly associated with AOS-mediated siganl transduction pathway(s).

While responses of *sodCc1* and *sodB* genes, which was also induced by ABA (Fig. IV-4), to such environmental stresses were not similar to those of *sodA* and *sodCc2* (Fig. VI-5,6). We suggest that the regulation of *sodB and sodCc1* genes under stress conditions is not directly associated with ABA such as *sodA* and *sodCc2* genes because the induction of these genes by ABA was not dose-dependent. *N. plumbaginifolia* Fe-SOD gene was induced by the treatments of chilling (Tsang et al., 1991) and phytohormones (giberellin and kinetin) but not by ABA (Kurepa et al., 1997), and no response of Fe-SOD gene by ABA and salinity was also observed in *N. tabacum* BY-2 cells (Bueno et al., 1998). These results about responses to ABA and salinity are not consistent with our results. In the previous section, rice Fe-SOD was characterized as a distinct type of Fe-SOD from *N. plumbaginifolia* Fe-SOD. Therefore the inconsistent results may due to the difference of examined Fe-SOD genes.

Similar and specific responses of *sodCp* gene to salinity and H_2O_2 (Fig. IV-6,9) strongly suggest that the regulation of *sodCp* gene under salinity needs a light signal, and AOS may play as a signaling factor under such stress. The presence of salt in assay inhibit the PS II activity of tobacco thylakoid membrane but not PS I activity (Murota et al., 1994). Photosynthetic electron transport is inhibited by the inactivation of PS II, and then AOS is supposed to be generated. Currently, the author considers that H_2O_2 rather than $O_2^{\cdot-}$ might play as an important signaling factor for the regulation of *sodCp* gene, because H_2O_2 is well-known as a signal molecule in the environmental

stresses such as chilling, ozone exposure, drought and infection (Foyer et al., 1997) In *E. coli*, there is H_2O_2-activated transcriptional regulator, OxyR, which activates the expression of defense genes under stress conditions(Demple and Amabile-Cuevas, 1991). The existence of such H_2O_2-mediated signal transduction pathway was recently suggested because of the isolation of OxyR homologue, H_2O_2-regulated annexin-like protein, from *A. thaliana* by complementation of Δ*OxyR* strain in *E. coli* (Gidrol et al., 1996). On the other hand, because the activities of key enzyme for CO_2 fixation, such as fructose 1,6-bisphosphatase, was inhibited by H_2O_2 (Charles and Halliwell, 1980), the genes for AOS-scavenging and other defense enzymes in chloroplasts might be regulated by sensing the elevation of H_2O_2 concentration under various stress conditions. In order to demonstrate this hypothesis, it seemed to be necessary to evaluate the concentration of H_2O_2 in rice seedlings under salinity condition.

Excess supplement of heavy metals inhibits plant growth and metabolisms although these are essential micronutrients. Now, however, the physiological investigations about the toxicity of heavy metals are poor for understanding the mechanisms to cause the damages, but is thought to be strongly associated with Haber-Weiss reaction, which is metal-mediated AOS-generating reaction. The increased SOD activity by excess Cu^{2+}, Mn^{2+}, and Zn^{2+} with the induction of lipid peroxidation was reported so far. The author's experiments in this section (Fig IV-10) is first report of large-scale analysis for responses of plant SOD genes to heavy metals. Cytosolic Cu/Zn-SOD activity was increased by excess Mn^{2+} in *Phaseolus vulgaris* (Gonzalez et al., 1998), and *A. thaliana* cytosolic Cu/Zn-SOD gene was strongly induced by Al^{3+} at 8 h after the treatment (Richards et al., 1998), while responses of rice cytosolic Cu/Zn-SOD genes were not observed. Pea Mn-SOD activity was induced by excess Mn^{2+}, and Zn^{2+} (del Rio et al., 1985), which is partial corresponded to the response of rice *sodA* gene. The similar responses of chloroplast-localized SOD genes, *sodB* and *sodCp*, to heavy metals suggested that there is necessity to be regulated in coordinated fashion under such stresses and this regulation may be associated at least with AOS-generation and with Haber-Weiss reaction in chloroplasts because these genes were induced by almost all heavy metals.