PREFACE

Plants are invariably exposed to various forms of stress during normal condition, and their growth and productivity are strongly affected and limited by such stresses. Therefore, they have developed the abilities for the adaptation to the environmental changes in the evolution process. Much of the injury to plants caused by stress exposure is associated with oxidative damage by active oxygen species (AOS) at the cellular level. It is known that the production of AOS is promoted by the exposure to various environmental stresses such as drought, chilling, salinity, airpollutants, herbicides, and so on (Bowler et al., 1992). AOS such as superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) are generated as by-product of many biological reactions, and the highly toxic AOS, hydroxyl radical ($OH^{\cdot}$), generated from $O_2^{\cdot-}$ and H_2O_2 via metal-dependent conversion (Herber-Weiss reaction) is thought to be responsible for the majority of the biological damage associated with these molecules (Elstner, 1982). To minimize the damaging effect from AOS to organisms, they have evolved various enzymatic and non-enzymatic mechanisms.

In enzymatic mechanism, superoxide dismutase (SOD: EC 1.15.1.1) catalyzes the first step in the AOS-scavenging system by the disproportionation of $O_2^{\cdot-}$ to H_2O_2 and O_2 (Fridovich, 1986). SOD is classified by their metal co-factor: copper/zinc (Cu/Zn), manganese (Mn) and iron (Fe) forms. These isoforms are distributed in different subcellular locations (Bowler et al., 1994). In higher plants, Cu/Zn-SOD is localized mainly in chloroplasts or cytosol, and Mn-SOD localized predominantly in the mitochondrial matrix. Fe-SOD have been found only in several dicotyledonous plants, and is localized in chloroplasts.

In higher plants, the generated H_2O_2 by SOD or other cellular metabolic reactions is converted to H_2O by ascorbate peroxidase (APX), electron donor-nonspecific peroxidase (EC 1.11.1.7) like guaiacol peroxidase (GuPX), and catalase (CAT: EC 1.11.1.6). APX is thought to play a central role for the reaction in plants, and the physiological function is distinguished from CAT and GuPX because CAT and GuPX are localized exclusively in peroxisome, and cell wall and vacuole, respectively. APX (EC 1.11.1.11) occurs only in plants and needs ascorbic acid as a electron donor (Asada, 1992). Ascorbic acid (vitamin-C) is much abundant in plant cell and itself also functions as a non-enzymatic antioxidant in this reduced form (AsA) together with such as glutathione and α-tocopherol. APX have multiple isoforms and are classified four different isoforms by their locations: cytosolic (Chen and Asada, 1989), chloroplastic (stromal and thylakoid-bound types) (Chen and Asada, 1989; Miyake et al., 1993), and microbody isoforms (Yamaguchi et al., 1995). The primary oxidation product of the peroxidation is monodehydroascorbate (MDA) (Miyake and

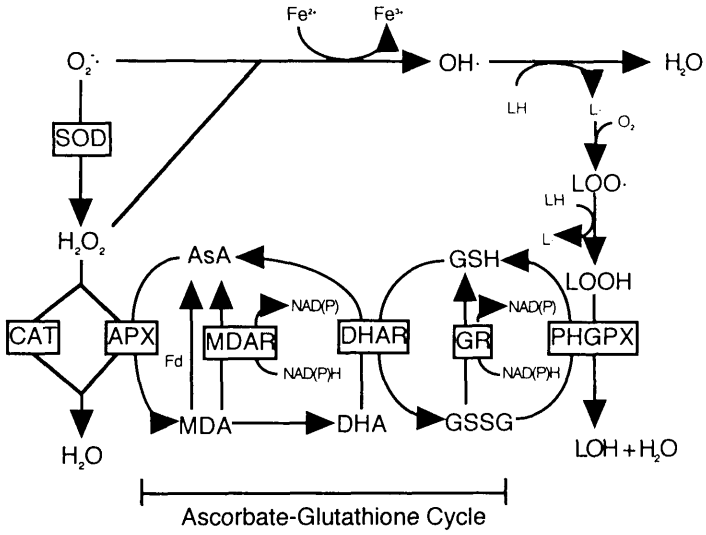


Fig. A. AOS-scavenging pathway in plants.

Asada, 1992), and then itself spontaneously disproponates to dehydroascorbate (DHA) and AsA (Asada, 1994). For the regeneration of oxidized ascorbic acids (MDA and DHA) to AsA, the substrate specific reductases, monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR), occur in plants. MDA is also reduced by ferredoxin in chloroplasts (Miyake et al., 1994). MDAR (EC 1.6.5.4) catalyzes MDA to AsA using NAD(P)H as a electron donor (Hossain et al., 1984). While DHAR (EC 1.8.5.1) catalyzes DHA to AsA using GSH as a electron donor (Hossain and Asada, 1984a). Furthermore, glutathione reductase (GR; EC 1.6.4.2) catalyzes the reduction of oxidized glutathione (GSSG), which is the product dued to DHAR or other glutathione-dependent reactions, to reduced glutathione (GSH) using NAD(P)H as an electron donor (Meister and Anderson 1983). MDAR, DHAR, and GR are found not only in chloroplast and cytosol but also in mitochondria, peroxisomes (Jimenz et al., 1997; del Rio et al., 1998) and apoplasts (Castillo and Greppin, 1988). Therefore the reduced state of ascorbic acid supposed to be maintained by coordinate working of these enzymes. These enzymes are involved in the so-called ascorbate-glutathione cycle (Foyer and Harbinson, 1994).

On the other hand, ascorbate-independent AOS-scavenging pathway, recently, have been characterized. This pathway consists of glutathione-dependent peroxidase (GPX) and GR. GPX, which is well-known in mammalians, catalyzes the reduction of organic hydroperoxides and lipid peroxides (LOOH) as well as H_2O_2 using GSH as the electron donor. In plants, GPX activity has been found only in cell-free extracts from suspension cultures, and cDNAs homologous to mammalian GPX have recently been isolated from several plants (Criqui et al., 1992; Holland et al., 1993; Mullineaux et al., 1998). Plant GPX is closely associated with the reduction of phospolipid hydroperoxide produced by OH· (Beeor-Tzahar et al., 1995), and therefore be called as

phospholipid hydroperoxide-glutathione peroxidase (PHGPX; EC 1.1.1.9). Thus, PHGPX is also significant for the scavenging of AOS in plants because this enzyme is associated with the detoxification of end product of AOS. In this thesis, these enzymes, including SOD, APX, GR, MDAR, DHAR, and PHGPX, mainly associated with the scavenging of AOS are designated AOS-scavenging enzymes (Fig. A).

Heretofore, the activity and gene expression of the AOS-scavenging enzymes under the stress conditions have been examined so far because the AOS-scavenging enzymes are strongly supposed to be associated with the AOS-scavenging produced under such conditions. Actually, the elevated activity or expression of AOS-scavenging enzymes in response to the stress-associated various stimuli, such as environmental stresses, phytohormone and chemical treatments, have been previously shown by many reports. To date, however, the overall speculation in the gene regulation of plant AOS-scavenging enzymes could not be obtained from these reports because the previous analyses were carried out only by focusing on one or several of AOS-scavenging enzymes. Furthermore to clarify the correlation between the AOS-scavenging enzymes and stress tolerance, the importance of AOS-scavenging enzymes for the protection against environmental stresses have been explored by transgenic approaches (Allen, 1995; Foyer et al., 1994). Most of transgenic plants, which have the elevated activities of SOD, GR, or CAT in chloroplasts or cytosol, have been reported to be more tolerant against environmental stresses, such as air-pollutants, low temperature, and drought, compared with the non-transgenic plants. However several transgenic plants overexpressed SOD or APX could not confer the stress tolerance to plants. To date, therefore, the molecular mechanisms or necessary factors to confer the stress tolerance to plant cells is not clarified only because these studies can indicate the correlation between the elevated activity of AOS-scavenging enzymes and stress tolerance.

In the present thesis, the author studied on the followings using rice, which is a main crop in Japan, to clarify the molecular mechanisms in the gene regulation of AOS-scavenging enzymes, and using tobacco, which is useful plant for the gene transformation, to clarify the important factor for the enhancement of stress tolerance in plants:

1) Cloning and characterization of all SOD genes in rice, and the analyses of gene expressions of rice SOD isoforms under various stress conditions.
2) Cloning, characterization of two of rice AOS-scavenging enzymes, GR and MDAR, and the analyses of gene expressions of rice AOS-scavenging enzymes, focusing on the cytosolic isoforms.
3) Evaluation of stress tolerance in SOD- or APX-overexpressing transgenic tobaccos.

CHAPTER I

SUPEROXIDE DISMUTASES IN RICE

Introduction

Superoxide dismutase (SOD, EC 1.15.1.1.), originally discovered from bovine erythrocytes (McCord and Fridovich, 1969), catalyzes the dismutation of $O_2^{\cdot-}$ to H_2O_2 and O_2. The importance of SOD has been demonstrated by the isolation of SOD-deficient mutants in *Escherichia coli* (*E. coli*) (Carlioz and Touati, 1986), which exhibits increased sensitivity to methyl viologen (paraquat), a redox-active compound that promotes the production of $O_2^{\cdot-}$. Furthermore, a null mutation of Cu/Zn-SOD in *Drosophila* caused to be infertility and reduce the life span (Phillips et al., 1989), while mutations in human cytosolic Cu/Zn-SOD are associated with the familial amyrotrophic lateral sclerosis known as a hereditary disease (Rosen et al., 1993).

SOD has multiple isoforms, which are classified by the active site metal co-factors (Cu/Zn, Mn, and Fe). In addition, a novel nickel-containing SOD was found in *Streptomyces* (Youn et al., 1996). Among these SODs, Mn-SOD and Fe-SOD have very similar three-dimentional structure (Stallings et al., 1984) and utilize the same amino acid ligands to bind the metals (Parker and Blake, 1988), while Cu/Zn-SOD is not related. Therefore Mn-SOD and Fe-SOD are assumed to have a common ancestral origin. These isoforms are distinguishable by their differential sensitivities to cyanide and H_2O_2; Cu/Zn-SOD to cyanide and H_2O_2, Fe-SOD to H_2O_2 (Bowler et al., 1994). SODs are distributed in different subcellular locations, presumably because $O_2^{\cdot-}$ cannot cross membranes and must therefore be dealt with at their sites of production (Takahashi and Asada, 1983). In higher plants, there are two types of Cu/Zn-SODs for the difference of their subcellular locations, in cytosol and plastids. The plastidic Cu/Zn-SOD is localized in the stroma (Asada et al., 1973), and the analysis of immunocytochemistry suggested that the SOD is attached to thylakoid membranes at the $O_2^{\cdot-}$ generation site, primary electron acceptor in PS I (Asada et al., 1974), in spinach chloroplasts (Ogawa et al., 1995). Beyond these isoforms, peroxisomal Cu/Zn-SOD have been characterized in watermelon cotyledons (Bueno et al., 1995). Mn-SOD is characterized mainly in the mitochondrial matrix. In addition to mitochondrial Mn-SODs, this activity was found in spinach chloroplast (Hayakawa et al., 1985) or in microbodies (peroxisomes and glyoxysomes) of several plants (Jimenz et al., 1997). Cu/Zn-SODs and Mn-SODs are distributed among all plant species, while Fe-SOD has been characterized only in several dicotyledonous plant species (Salin and Bridges, 1980; Bridges and Salin, 1981; Kwiatowski et al., 1985; Almansa et al., 1991). The

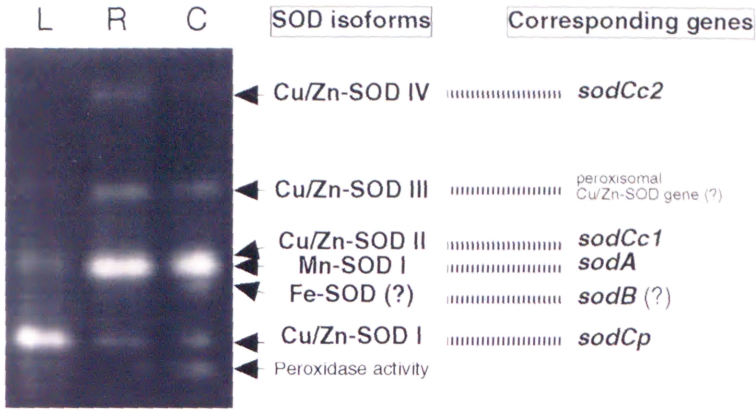


Fig. B. SOD isoforms and corresponding genes in rice. SOD activity staining of rice various tissues, leaves (L) and roots (R) of young seedlimgs and suspension culture of embryogenic calli (C), was carried out as described in Fig. III-5C.

characterized Fe-SOD are found in the chloroplasts (Bowler et al., 1994; Van Camp et al., 1990), and three cDNAs for Fe-SOD have been characterized (Van Camp et al., 1990; Crowell and Amasino, 1991a). Recently large-scale analysis of SOD genes, recently, have been carried out in *Arabidopsis thaliana* (Kliebenstein et al., 1998), which is most useful plant for various molecular analyses because of the availability of a large number of cDNA and genomic DNA sequences, and mutants. This report indicated that there are seven SOD genes, including newly characterized two Fe-SOD homologue cDNAs and putative peroxisomal Cu/Zn-SOD cDNA, in *A. thaliana.*

In rice, four Cu/Zn-SODs and two Mn-SODs have been isolated from rice leaves, as well as from non-photosynthetic tissues, and characterized (Kanematsu and Asada, 1989) (Fig. B). The amino-terminal amino acid residues of four Cu/Zn-SOD isozymes have been sequenced (Kanematsu and Asada, 1990). The Cu/Zn-SOD isozymes of rice are classified into two types. Cu/Zn-SOD I is assumed to be plastidic because it is the major isozyme in leaves. Cu/Zn-SODs (II, III and IV) are found in seed embryos and etiolated seedlings and at extremely low levels in leaves. Therefore, these latter isozymes are assumed to be cytosolic. One of the Mn-SODs isoforms (Mn-SOD I) have been characterized only by SOD activity staining on a gel with the inhibitor treatment (H_2O_2). In this analysis, the other Mn-SOD (Mn-SOD II), with a pI of about 9, and any Fe-SOD activity could not be detected. cDNAs and genes corresponding to two cytosolic Cu/Zn-SODs, and cDNA to Mn-SOD have previously been isolated from rice in the author's laboratory (Sakamoto et al. 1992a; Sakamoto, 1992; Sakamoto et al., 1993a; 1995a; 1995b). Furthermore, it have been shown that two of the cytosolic Cu/Zn-SOD genes are differentially regulated in response to abscisic acid by promoter analysis in rice protoplasts (Sakamoto et al., 1995b).

Plant SOD genes were known to respond to various stimuli, such as environmental stresses, phytohormone and chemical treatments (Bowler et al., 1992). To date, however, the mechanisms by

which the gene expression of SOD isozymes is regulated under stress conditions are not clarified. At the starting point of this study, the informations for all rice SOD genes were not enough to speculate the gene regulation of rice SOD genes. Therefore, prior to the analysis of the gene regulation, the author have isolated and characterized cDNA (Kaminaka et al., 1997) and gene for plastidic Cu/Zn-SOD, gene DNA for Mn-SOD, and cDNA for Fe-SOD (Kaminaka et al., 1999a) (Fig. B). Furthermore, to elucidate the regulatory mechanisms of rice SOD genes under various stress conditions, the author examined the transcripts of SOD isoforms in rice seedlings with the various stress treatments using the gene-specific probes to all rice SOD isoforms, and demonstrated that phytohormone and AOSs play as signals for the regulation of rice SOD genes (Kaminaka et al., 1999b).

SECTION 1

cDNA and genomic cloning of rice plastidic Cu/Zn-superoxide dismutase

Materials and Methods

Plant material and growth condition -

Rice (*Oryza sativa* L. cv Nipponbare) was used in all experiments. Unless otherwise mentioned, rice seedlings were grown hydroponically in a growth cabinet maintained at 28 °C and a 16 h photoperiod at 170 μmol quanta m^{-2} s^{-1} for 10-14 d after germination. Etiolated seedlings were grown in darkness for the same period. The experimental samples were immediately frozen in liquid nitrogen and stored at -80 °C until use.

Preparation of total RNA and construction of cDNA library -

The 10-14 d dark-grown etiolated seedlings were exposed to the continuous white-light for 2 h or 10 h prior to the isolation of total RNA for the construction of cDNA libraries. Total RNA was isolated using the guanidium-isothiocyanate (GIT) extraction and precipitation by cesium chloride (CsCl) ultracentrifugation according to Sakamoto (1992) with slight modification as follows. Less than 2 g of frozen tissues were powdered in liquid nitrogen and ground in GIT solution (4 M guanidium isothiocyanate, 25 mM sodium citrate, 0.5% *N*-lauroylsarcosinate, and 0.1 M 2-mercaptoethanol) with a prechilled mortar and pestle. The homogenate was centrifuged using swing rotor for 20 min at 3,500 rpm to remove the debris. 1.2 g CsCl was added into 3 ml of the supernatant and layered over the bottom solution (5.7 M CsCl in 0.1 M EDTA (pH 7.5)) and centrifuged for 3.5 h at 100,000 rpm (400,000 x *g*) by Optima-TLX or TL-100 (Beckman, CA, USA). The prepiciate was dissolved in resuspension buffer (10 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 0.1% SDS). The resulting solution was extracted with equal volume of phenol/chloroform/isoamylalchol (25:24:1), centrifuged at 15,000 rpm for 5 min, recovered the aqueous phase, and precipitated with ice-cold ethanol.

Poly(A)$^+$ RNA was purified from the total RNA using Oligotex-dT30 (TAKARA Shuzo, Kyoto, Japan). Unidirectional double-stranded cDNA was synthesized using SUPERSCRIPT Lambda System (GIBCO BRL, MD, USA), ligated with λZIPLOX *Not*I-*Sal*I arms (GIBCO BRL), and then packaged into phage particles using MaxPlax Packaging Extract (EPICENTRE TECH. Co., WI, USA). All procedures were carried out according to the manufacturers' instructions. Consequently, a cDNA library of approximately 1.8 x 10^5 independent recombinants was

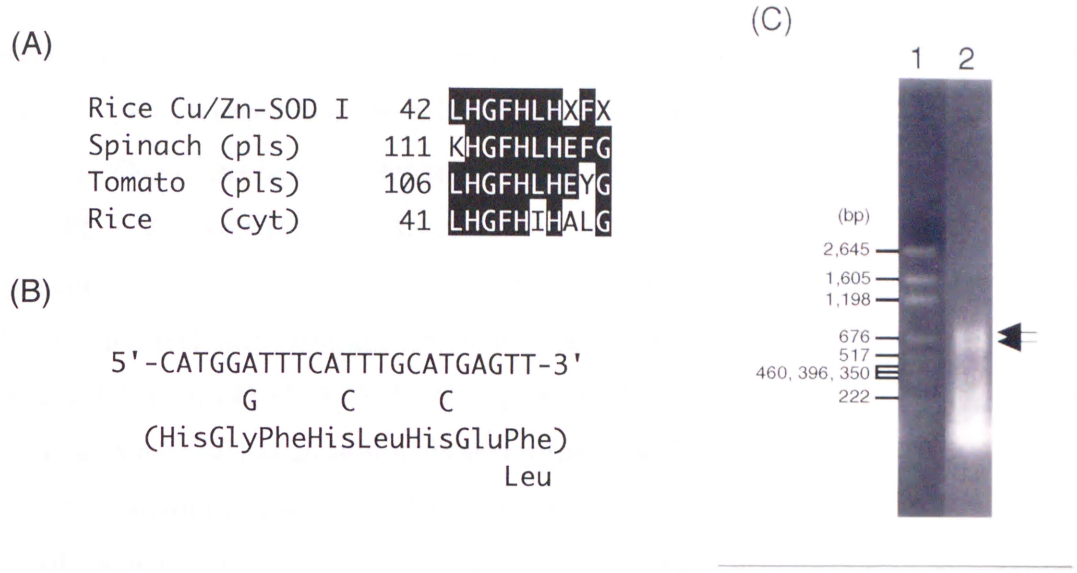


Fig. I-1. Preparation of a partial fragment of rice plastidic Cu/Zn-SOD cDNA. (A) Alignment of the amino acid sequence from a part of the known plant plastidic Cu/Zn-SOD, Rice Cu/Zn-SOD I (Kanematsu and Asada, 1990), Spinach (Sakamoto et al., 1993a), Tomato (Perl-Treves et al. 1988), and of cytosolic Cu/Zn-SOD from rice (Sakamoto et al., 1992a) (B) Sequence of synthesized primer, FM-II. (C) Electrophoresis analysis stained by ethidium bromide; lane 1: pGEM marker (Promega Japan, Tokyo, Japan), lane 2: PCR products amplified with FM-II and NotI-dT_{18} (Amersham Pharmacia Biotech Japan). Arrows indicated the recovered bands by electroelution for subcloning.

constructed.

Isolation and characterization of a cDNA for rice plastidic Cu/Zn-SOD -

Prior to the isolation of the cDNA for plastidic Cu/Zn-SOD from the rice leaf cDNA library, a probe for screening was prepared by PCR using the pair of primers (Fig. I-1), NotI-$d(T)_{18}$ (Amersham Pharmacia Biotech Japan, Tokyo, Japan) and FM-II (5'-CATGG[A/G]TTTCA[T/C]TT GCA[T/C]GAGTT-3') (Fig. I-1B), which was synthesized by reference to other plant plastidic Cu/Zn-SODs (Sakamoto et al., 1993a; Perl-Treves et al., 1988) (Fig. I-1A,B). The amplified DNA (Fig. I-1C) was electroeluted from the electrophoresis gel, and then subcloned into LITMUS 38 (New England Biolabs, MA, USA), which is modified as a T-vector for the direct cloning of products of PCR as described by Marchuk et al. (1991). The screening of the cDNA library was performed by plaque hybridization using this fragment, which was radiolabelled with [α-^{32}P]dCTP (ICN radiochemicals, CA, USA) using a *Bca*Best labeling kit (Takara Shuzo, Kyoto, Japan) and then purified with ProbeQuant G-50 Micro Columns (Amersham Pharmacia Biotech Japan) according to the manufacturers' instructions. The phages were grown on a lawn of *E. coli* Y1090(ZL) at a density of 5 x 10^4 plaques/150 mm plate. Plaques from a cDNA library were separately lifted to the nylon membranes (Hybond-C^+; Amersham Pharmacia Japan, Tokyo, Japan).

The blotted membranes was hybridized with the labelled-probe in the hybridization buffer (50% formamide, 6 x SSPE, 5 x Denhart solution, and 0.1% SDS) containing 0.1 mg/ml denatured salmon sperm DNA, washed twice with 2 x SSC containing 0.1% SDS at 42 °C, and then exposed to X-ray films. One of the isolated clones from about 3.0 x 10^5 plaques, a putative full-length clone designated PL-1, was used for the further analysis.

To determine the nucleotide sequence of both strands of this clone, PL-1 was digested with several restriction endonucleases and subcloned the fragments into pBluescript SK(+) (Stratagene, La Jolla, CA, USA) or LITMUS 38 (New England Biolabs). The recombinant plasmid DNAs were purified through the CsCl-equilibrium ultracentrifugation. These clones were sequenced with a THERMO Sequenase cycle sequence kit (Amersham Pharmacia Biotech Japan, Tokyo, Japan) by a DSQ-1000L sequencer (Shimadzu, Kyoto, Japan) according to manufacturers' instructions. The nucleotide sequence was analyzed using GENETYX software (Software Development Co. Ltd., Tokyo, Japan).

Preparation of genomic DNA and genomic Southern blot analysis -

Total genomic DNA was isolated from rice seedlings using the standard CTAB method (Sambrook et al., 1989) as follows. About 10 g of etiolated or green seedlings were powdered in liquid nitrogen with a mortar and pestle, added into 20 ml of prewarmed (60-70 °C) 2% CTAB solution (2% CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 1.4 M NaCl), and incubated at 65 °C for 30 min with continuous mixing. The consequent solution was extracted with equal volume of chloroform/isoamyalcohol (24:1) solution by centrifugation (3,000 rpm, 20 min), and then the top (aqueous) phase was recovered. This procedure was repeated twice. To precipitate the nucleic acids, 1-1.5 volumes of 1% CTAB solution (1% CTAB, 50 mM Tris-HCl (pH 8.0), and 10 mM EDTA) was added into the aqueous phase, mixed well until precipitate was visible (for 1 h), and centrifuged for 20 min at 3,000 rpm. The precipitate was dissolved with 1 ml of 1 M NaCl and 5 μl of 10 mg/ml RNAse A, and then incubated at 65 °C for 30 min with continuous mixing until completely dissolved. The genomic DNA prepiciated by adding ice-cold 10 ml of 100% ethanol was recovered with Pasteur pipet, washed twice with 100% ethanol, and dissolved in TE (pH 8.0).

The rice genomic DNA (10 μg) was digested with 50 units of each restriction enzyme (*Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Sac*II), electrophoresed in a 0.7% agarose gel, and then blotted into the nylon membrane (Hybond-N^+: Amersham Pharmacia Biotech Japan). The *Eco*RI and *Bam*HI digested fragment of PL-1 (full-length cDNA) was used as the hybridization probe. The hybridization and labeling of the probe DNA were performed as described in the screening of a

cDNA. The hybridized membrane was washed twice at 65 °C for 30-45 min in 0.1 x SSC containing 0.1% SDS and then subjected to the autoradiogram.

Isolation of a genomic clone for rice plastidic Cu/Zn-SOD -

The library constructed from rice germ genomic DNA with λEMBL3 arms (Sakamoto, 1992) was used to isolate the genomic clones for rice plastidic Cu/Zn-SOD gene. Screening of this library (2 x 10^5 plaques) was performed by the same method in the screening of cDNA clone. As the hybridization probe, a full-length fragment of PL-1 was prepared as described in genomic Southern blot analysis. The λphage DNA was prepared by polyethyleneglycol (PEG) method according to Sambrook et al. (1989) as follows. *E. coli* LE392 infected with the phage of positive clone (10^5 or 10^6 pfu/culture) was cultured in NZY (1% NZ amine, 0.5% NaCl, 0.5% Yeast extract, and 0.2% $MgSO_4$) liquid medium at 37 °C for overnight. 0.2% (v/v) of chloroform was added into the culture, continuously mixed for 15 min, and then centrifuged at 3,000 rpm for 10 min. 5 μg/ml RNAse A and DNAse I were added into the supernatant and incubated at 37 °C for 30 min. An equal volume of 20% PEG 6,000 in 2 M NaCl was added, incubated on ice for more than an hour, and then centrifuged at 3,000 rpm at 4 °C for 20 min. The precipitate was dissolved in SM buffer (50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 10 mM $MgSO_4$, and 0.01% (w/v) gelatin) and centrifuged at 8,000 rpm for 2 min. The concentration of EDTA and SDS was adjusted to 10 mM and 0.1%, respectively, and incubated at 60-65 °C for 15 min. This resulting solution was extracted with an equal volume of phenol/chloroform/isoamylalchol (25:24:1), centrifuged at 15,000 rpm for 3 min, and recovered the aqueous phase. This step was repeated twice and precipitated the nucleic acids with an equal volume of ice-cold iso-propanol. All recombinant DNA techniques and sequencing were performed according to the procedures in characterization of cDNA.

Results

Characterization of a cDNA for rice plastidic Cu/Zn-SOD -

Prior to the isolation of cDNA for plastidic Cu/Zn-SOD in rice, the oligonucleotide, designated FM-II (Fig. I-1B), which is based on the amino acid sequences of the known plant plastidic Cu/Zn-SOD and previously determined rice Cu/Zn-SOD-I (Kanematsu and Asada, 1989), was synthesized (Fig. I-1A). The fragment amplified with FM-II and NotI-$d(T)_{18}$ (Amersham Pharmacia Biotech Japan) (Fig. I-1C) was sequenced to confirm that it was part of the cDNA for rice plastidic Cu/Zn-SOD (data not shown). Using this DNA fragment as a probe, full-length cDNA clone of rice

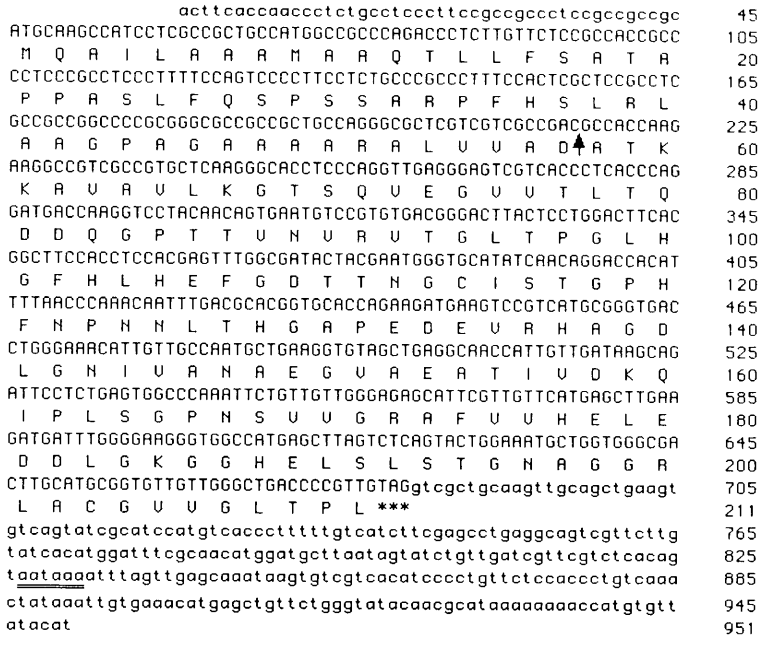


Fig. I-2. Nucleotide and deduced amino acid sequences of a cDNA for rice plastidic Cu/Zn-SOD, PL-1. The nucleotide (upper line) and amino acid residues (lower line) are numbered on the right. Arrow indicates the site of cleavage of a transit peptide, determined from a comparison with the amino-terminal amino acid sequence of rice Cu/Zn-SOD I (Kanematsu and Asada, 1990). Nucleotide sequence corresponding to the primer, FM-II, is underlined and the putative polyadenylation signal is double-underlined. Asterisks indicate a termination codon. The nucleotide sequence data in this figure (PL-1) have been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number D85239.

plastidic Cu/Zn-SOD, designated PL-1, was isolated and characterized as described in Materials and Methods. The sequence of 951 nucleotides of PL-1 (accession number D85239) included a region corresponded to the primer, FM-II, from position 343 to 365 (Fig. I-2, underlined), and a putative polyadenylation signal (-AATAAA-) was located from position 827 to 832 (Fig. I-2, double-underlined). The first ATG codon, from position 46 to 48, was predicted to be the initiation codon. Thus, PL-1 encoded a precursor protein of 211 amino acid with a molecular weight of 21,300. The sequence of the 57 amino-terminal amino acids from the putative processing site (Fig. I-2, shown as arrowhead) contained many hydrophobic and basic residues and a few residues that were negatively charged. This feature is typical of a plastid-specific transit peptide (Keegstra and Olsen 1989).

The sequence of amino-terminal amino acids deduced from PL-1 was identical as far as the 39th residue (Fig. I-3A) to that of rice plastidic Cu/Zn-SOD that was determined by Edman degradation (Kanematsu and Asada, 1989). However, several mismatches were found beyond the 40th residue. The primary structure of the mature polypeptide from rice plastidic Cu/Zn-SOD was compared with the amino acid sequences deduced for plastidic Cu/Zn-SOD from wheat (Wu et al., 1996), pea (Scioli and Zilinskas, 1988), spinach (Kitagawa et al., 1986; Sakamoto et al., 1993b) and tomato

(Perl-Treves et al., 1988) and for cytosolic Cu/Zn-SOD from rice (Sakamoto et al., 1992a) (Fig. I-3B). The sequence of rice plastidic Cu/Zn-SOD was very similar to those of the enzymes from other plant species. The identities compared with plastidic Cu/Zn-SODs from wheat, pea, spinach and tomato were 93.5, 91.6, 87.7, and 87.0%, respectively, while that with rice cytosolic Cu/Zn-SOD was only 64.2%. However, seven amino acid residues that coordinate with the copper and zinc atoms and two cysteine residues that form a disulfide bridge were conserved (Fig. I-3B, indicated by asterisks and plus signs, respectively).

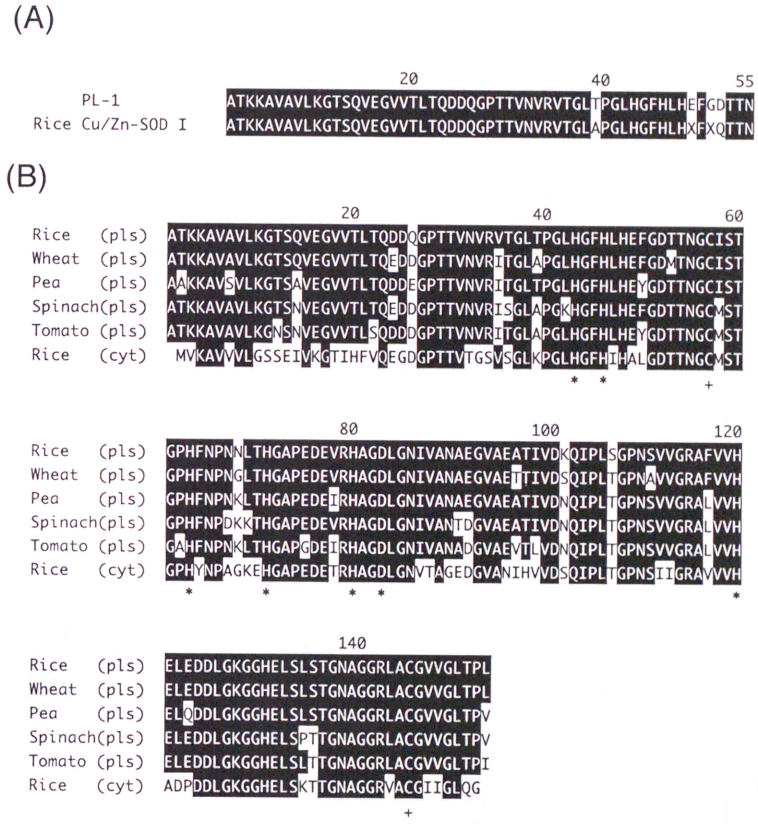


Fig. I-3. Comparison of the deduced amino acid sequence of the mature rice plastidic Cu/Zn-SOD with (A) the amino terminal sequence of rice Cu/Zn-SOD I and (B) those from wheat (Wu et al., 1996), pea (Scioli and Zilinskas, 1988), spinach (Kitagawa et al., 1986; Sakamoto et al., 1993b), and tomato (Perl-Treves et al., 1988) and with that of cytosolic Cu/Zn-SOD from rice (Sakamoto et al, 1992a). Identical residues with rice plastidic Cu/Zn-SOD are enclosed in black boxes. The residues that coordinate copper and zinc atoms and that form the singl e disulfide bridge are indicated with asterisks and plus signs, respectively. pls, plastidic; cyt, cytosolic.

Structure of rice plastidic Cu/Zn-SOD gene (sodCp) -

Prior to the isolation of the genomic clone for plastidic Cu/Zn-SOD, the copy number of rice plastidic Cu/Zn-SOD gene (hereafter *sodCp* gene) in the rice genome was evaluated by Southern blot analysis with a full-length fragment of PL-1 (Fig. I-4A). At the high stringency washing (0.1 x SSC, 65 °C), two or three bands were observed by the digestion of restriction enzyme (*Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Sac*II). This result was also obtained by washing at the low stringency (2 x SSC, 42 °C). Only two digested bands were detected by the digestion of *Sac*II, which can digest

one site of PL-1. Restriction map of isolated genomic DNA (Fig. I-4B) is also completely corresponding to the result of genomic Southern blot analysis. Thus, *sodCp* gene is a single copy gene in rice genome.

The genomic clone for *sodCp* gene was isolated using full-length fragment of PL-1 as a probe from a rice germ genomic library (5 x 10^5 plaques), and the five positive clones were obtained. From the result of Southern blot analysis of these clones, only one clone, designated gsodCp-1, was supposed to contain the full-length coding region and 5'-flanking region of *sodCp* gene (data not shown), and mapped. Three of *Sph*I fragments were subcloned into plasmid vector, and then re-subcloned for the sequencing. Physical map of *sodCp* gene region in gsodCp-1 is described in Fig. I-4B. 4297 bp of the nucleotide sequence from gsodCp-1 was completely determined (Fig. I-5) and its structure analyzed (Fig. I-4B). This sequence contains about 1.2 kb of promoter region and coding region spanning about 3 kb of genomic DNA. Structural alignment provided not only perfect agreement between the sequences analyzed for the cDNA and the putative exons in this gene, but also the existence of 7 introns in the coding region. All introns showed GT-AG intron border sequences. In summary, this gene (*sodCp*) is composed of 8 exons interrupted by 7 introns.

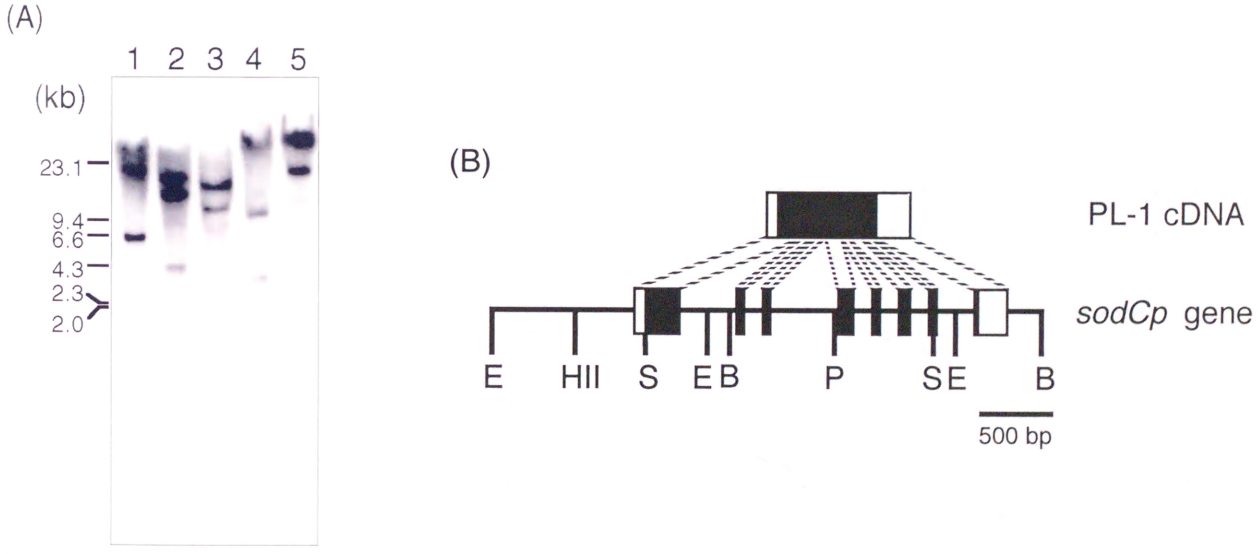


Fig. I-4. Analysis of rice plastidic Cu/Zn-SOD (*sodCp*) gene. (A) Southern blot analysis of rice genomic DNA. Rice genomic DNA was digested with *Bam*HI (1), *Eco*RI (2), *Hin*dIII (3), *Pst*I (4), and *Sac*II (5), transferred onto nylon membrane, probed with a ^{32}P-labelled full-length cDNA (PL-1) and washed at the stringency (0.1 x SSC, 65 °C). The molecular size of λ/*Hin*dIII marker is indicated at left. (B) Structure and restriction maps of *sodCp* gene. The boxes correspond to the exons; translational and untranslational regions are indicated as dark boxes and light boxes, respectively. B, *Bam*HI; E, *Eco*RI; HII, *Hin*cII; P, *Pst*I; S, *Sph*I.

Characterization of 5'-flanking region of sodCp gene -

To search for any known motifs or regulatory elements of other plant genes in the flanking region of *sodCp* gene (Fig. I-5), the homology search of the nucleotide sequence was performed by

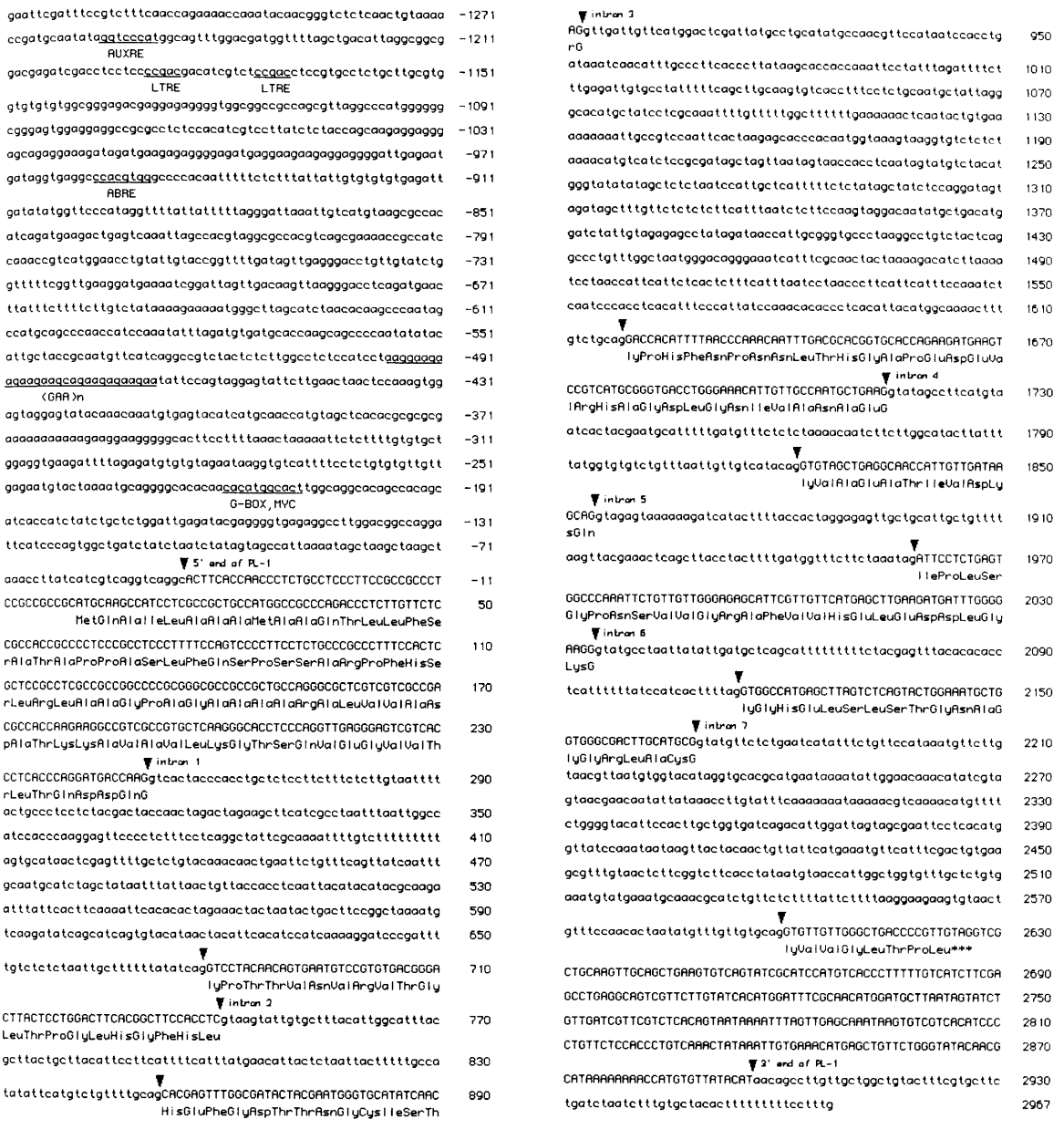


Fig. I-5. Nucleotide sequence of rice *sodCp* gene. The translational start site is shown as +1, and the deduced amino acids in the exon are represented using the triple-letter amino acid code. The 5'-flanking region and introns are in lower case, while the exons are represented in upper case. The known *cis*-elements, auxin-responsive element (AUXRE), ABRE, G-BOX, and MYC are labeled below the corresponding nucleotide sequences in 5'-flanking region. The repeat sequence of GAA is indicated as (GAA)n.

Fasta 1.7 software (Pearson and Lipman, 1988) in a database of plant *cis*-elements (PLACE) (Higo et al., 1998). In the sequence of promoter region in *sodCp* gene, the consensus sequence of a TATA box (5'-TATAA-3') was not found but a homologous sequence (5'-ATTAAAAT-3') found at position -92. Ten repeat sequence of GAA was found at positions -495. The phytohormone-responsive elements, a core sequence of auxin-responsive element (AUXRE: 5'-KGTCCCAT-3') (Ballas et al., 1993) and ABA-responsive element (ABRE; 5'-CCACGTGG-3') (Busk and Pages,

1998), were found at position -1257, and -958, respectively. Two core sequences of low temperature-responsive element (LTRE: 5'-CCGAC-3'; Baker et al., 1994) were found at positions -1191 and -117. G-BOX consensus sequence (5'-CACATGGCACT-3'; Gilmartin et al., 1990) was found at position -220. Further, through a search of DNA databases the long homologous region among the promoter regions of *sodCp* gene and other following rice genes was found from position -1036 to -658 (Fig. I-5,6): 26 kD globulin gene (accession number D50643), waxy genes (waxy A and B) (accession number X86325, AB008794, and AB008795), serine carboxylpeptidase gene (*cpd3*) (accession number D10985), and heat shock protein 70 gene (accession number X67711). Especially, the nucleotide sequence from the position -779 to -658 in *sodCp* gene was homologous to those of that rice genes. The nucleotide sequence of this core region also shows a high homology to those of two rice EST clones (accession number D23755 and AU033306). However any significant homologous sequences to the known *cis*-elements were not observed in DNA database.

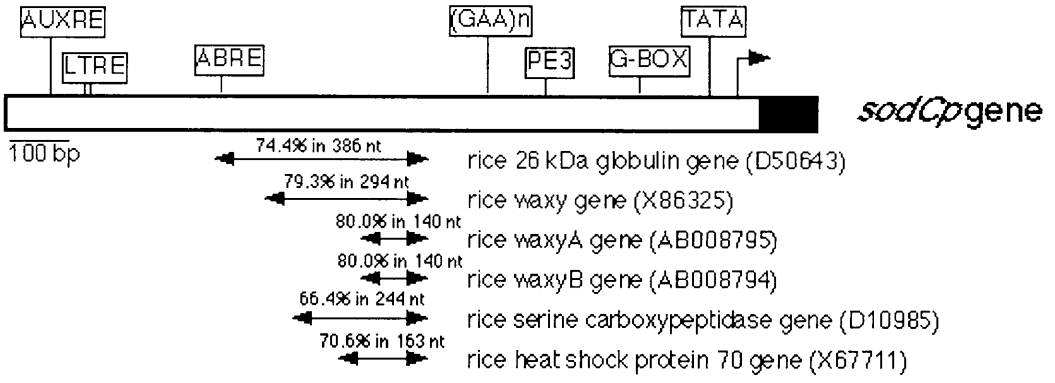


Fig. I-6. Highly homologous region on the 5' flanking sequence of *sodCp* gene with various rice genes, 26 kD globulin gene (accession number D50643), waxy genes (waxy A and B) (accession number X86325, AB008794, and AB008795), serine carboxylpeptidase gene (*cpd3*) (accession number D10985), and heat shock protein 70 gene (accession number X67711). The promoter region and a part of translational region are indicated as light and dark, respectively.

Discussion

In this section, the author reported that the isolation and characterization of a cDNA, PL-1, for plastidic Cu/Zn-SOD from rice (Kaminaka et al., 1997). Using this cDNA, the nucleotide sequence and structure of the corresponding genomic DNA, furthermore, was characterized. In particular, the characterization of 5'-flanking region in *sodCp* gene is thought to be available to analyze the gene regulation of rice SOD, which was carried out in latter section of this chapter.

Before this study, the cloning of this cDNA have already been attempted using spinach plastidic Cu/Zn-SOD cDNA as the heterologous probe but failed (Sakamoto, 1992). First, the author also attempted using cDNAs for spinach plastidic Cu/Zn-SOD or rice cytosolic Cu/Zn-SOD as such probe, or the antibody against spinach Cu/Zn-SOD. However the author could not isolate the

cDNA, but only several pseudo-positive clones. One of these clones, which was isolated by immunoscreening, have been identified as a cDNA for rice glyoxysomal malate dehydrogenase (Kaminaka et al., 1998a). The author have accomplished the cloning using a partial rice plastidic Cu/Zn-SOD cDNA, which was prepared by PCR using the pair of primers, NotI-d$(T)_{18}$ (Amersham Pharmacia Biotech Japan) and FM-II (Fig. I-1B), which was synthesized by reference to other known plant plastidic Cu/Zn-SODs. The difficulty for the isolation of the rice plastidic Cu/Zn-SOD cDNA might due to the highly GC-content than that of cytosolic Cu/Zn-SOD.

The several mismatches were found beyond the 40th residue between the amino acid sequences deduced from PL-1 and of rice Cu/Zn-SOD I (Kanematsu and Asada, 1989) (Fig. I-2A). There was no other transcript for a rice plastidic Cu/Zn-SOD because genomic Southern blot analysis indicated the existence of only a single copy of the gene (Fig. I-4A). The discrepancies between the present and previously reported primary structures are probably the result of polymorphism between the two cultivars (Sasanishiki and Nipponbare) or of errors in sequencing at the later stages of analysis by Edman degradation because such mismatches were also observed in the comparison among rice cytosolic Cu/Zn-SODs (Sakamoto, 1992).

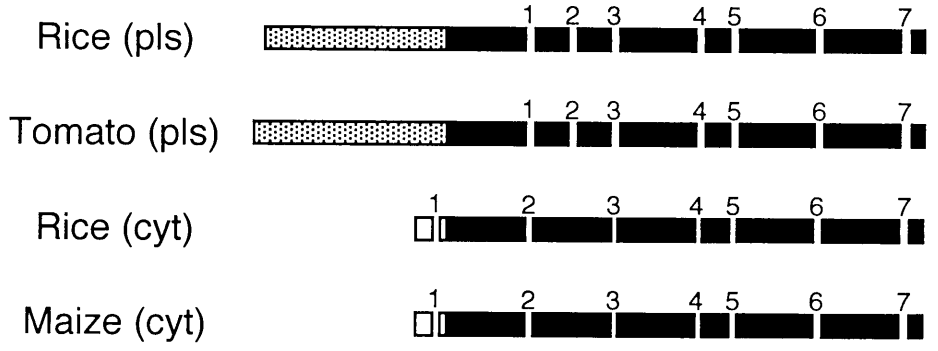


Fig. I-7. Comparison of exon/intron location in the amino acid sequences of plant Cu/Zn-SODs. Genomic sequences of plastidic Cu/Zn-SOD gene from tomato (Kardish et al., 1994), and cytosolic Cu/Zn-SOD from rice (Sakamoto et al., 1992b), and maize (*sod4A*, accession number U34727) were used for comparison. Each intron number is indicated above the amino acid sequences.

The nucleotide sequence of *sodCp* gene was completely determined (Fig. I-6) and its structure analyzed (Fig. I-4B). To the authors knowledge, this is the first report of the information of gene for plastidic Cu/Zn-SOD in monocotyledonous plants. The intron number and the inserted positions in *sodCp* gene was compared with those in the known plant Cu/Zn-SOD genes; plastidic Cu/Zn-SOD gene from tomato (Kardish et al., 1994), and cytosolic Cu/Zn-SOD from rice (Sakamoto et al., 1992b), and maize (*sod4A*, accession number U34727). The intron/exon location in the amino acid sequences (Fig. I-7) indicated that the intron number of these genes are same each other, and

the inserted position of introns are very similar, without second introns of plastidic Cu/Zn-SOD genes, and first introns of cytosolic Cu/Zn-SOD genes. Only one of mismatch intron positions among these genes suggested that the insertion event of such introns and division of plant Cu/Zn-SODs into two or more types occurred at the same time.

By analysis of the promoter region in *sodCp* gene, several known regulatory elements were characterized (Fig. I-5). Two phytohormones-associated regulatory elements, a core sequence of auxin-responsive element (AUXRE) and ABA-responsive element (ABRE) were found. This AUXRE was characterized in the primary indoleacetic acid (IAA)-inducible gene, PS-IAA4/5, in pea (Ballas et al., 1993). This ABRE was found in rice *RGA1* gene (accession number L35844), which encode a G protein alpha subunit, and conserved ABRE core sequence (5'-ACGTSSSC-3'; S=C/G) characterized in rice *rab21* and wheat *Em* genes (see review by Busk and Pages, 1998). Two core sequences of low temperature-responsive element (LTRE: 5'-CCGAC-3') were also found. LTRE was characterized in *A. thaliana cor15a* (cold-regulated) gene and other cold temperature-inducible genes (Baker et al., 1994). The expression of *Nicotiana plumbaginifolia* plastidic Cu/Zn-SOD gene was repressed by IAA- and ABA-treatment (Kurepa et al., 1997). Therefore these elements may not be associated with the gene regulation of *sodCp* gene. The G-BOX sequence, which was found in pea ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit (*rbcS*) gene, was also found at near the start site of transcription, which is similar to the case of pea *rbcS* gene. This G-BOX sequence was characterized as a light-responsive element and as the binding site of trans-acting factor protein, GBF (CG-1) (Gilmartin et al., 1990). The expression of rice *sodCp* gene may be regulated by light signals because this gene completely conserved the G-BOX sequence and most of genes for chloroplast-localized proteins are regulated by such signals. The response of rice *sodCp* gene to light, ABA, or other stress treatments will show and discuss in detail in later section of this chapter. On the other hand, the unknown repeat sequence (GAA-repeat) and consensus sequences were also observed (Fig. I-5,6). In particular, the long (more than 140 bp) homologous region among several rice genes, which are not associated each other, was observed (Fig. I-6). Yet the function of this homologous region is unknown, but *E. coli* harbored the plasmid DNA containing this region was inhibited to increase the copy number in the cells (data not shown). This result suggested that this homologous region might be associated with the replication in cells or contain the unknown regulatory elements.

SECTION 2

Genomic cloning of rice Mn-superoxide dismutase

Materials and Methods

Genomic Southern blot analysis and characterization of rice Mn-SOD gene(s) -

Genomic Southern blot was performed as described in the previous section, without used probe. An *Eco*RI-digested fragment of M14 (Sakamoto et al., 1993b), full length of rice Mn-SOD cDNA, was used for the hybridization probe.

The genomic clone, designated gMN9, was used for the further analysis. This clone have already been isolated and subcloned into plasmid vectors in the author's laboratory (data not shown). All recombinant DNA techniques and sequencing were performed according to the procedures in the previous section.

Results

Comparison of the amino acid sequences of plant Mn-SODs -

In the previous study, a cDNA for rice Mn-SOD was isolated in the author's laboratory. The information for the characterization of this cDNA was described in Sakamoto et al. (1993b). The deduced amino acid sequence from the cDNA, which was designated M14, was compared with those from the known plant Mn-SODs, maize (White and Scandalios, 1988), pea (accession number X60170), and tobacco (*N. plumbaginifolia*) (Bowler et al., 1989) (Fig. II-1). The identities compared with the Mn-SODs from maize, pea, and tobacco were 93.5, 91.6, 87.7, and 87.0%, respectively. The putative processing site (shown as arrowhead) was presumed same as that of maize Mn-SOD (SOD-3), which was characterized by the N-terminal sequence of the purified SOD-3 and the importation of precursor SOD-3, which was synthesized *in vitro*, into mitochondria (White and Scandalios, 1987).

Gene structure of rice Mn-SOD (sodA gene) -

Prior to the isolation of the genomic clone for Mn-SOD, the copy number of rice Mn-SOD gene (hereafter *sodA* gene) in the rice genome was evaluated by Southern blot analysis with a full-length fragment of M14 (Fig. II-2A). At the high stringency washing (0.5 x SSC, 55 °C), two or three bands were observed by the digestion of the following restriction enzyme: *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Sac*II, while only one band was detected using *Sma*I. This result was also obtained by

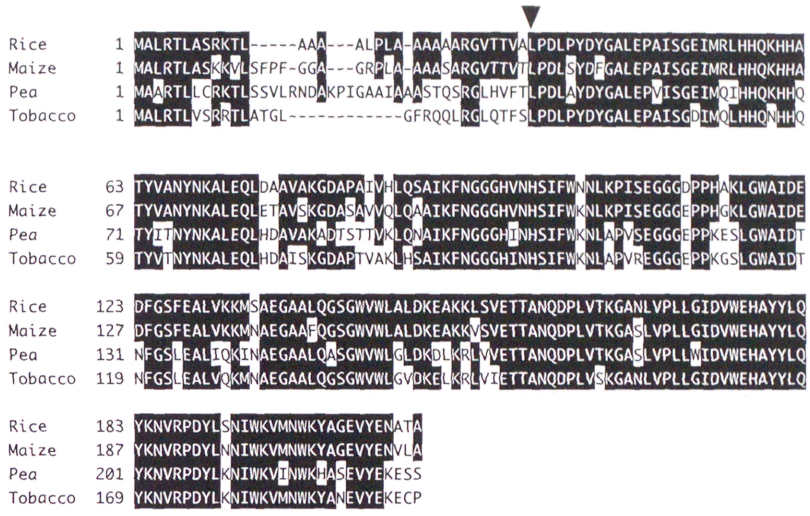
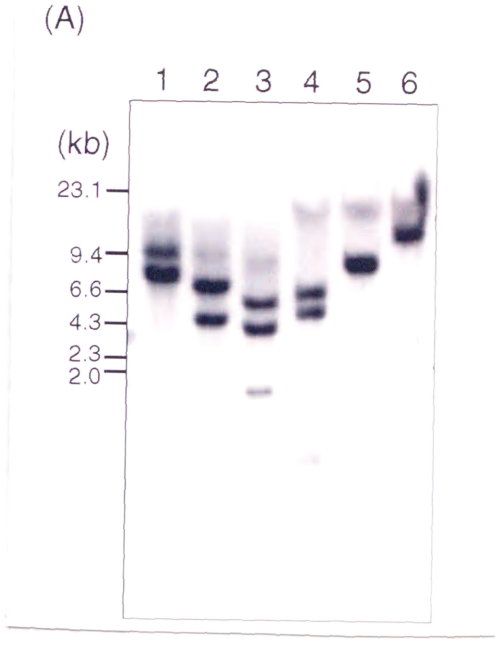


Fig. II-1 Comparison of the deduced amino acid sequence of rice Mn-SOD with those from maize (White and Scandalios, 1988), pea (accession number X60170), and tobacco (Bowler et al., 1989). Identical residues with rice Mn-SOD are enclosed in black boxes. The putative processing site, which was assumed by the characterization of the N-terminal sequence of the purified maize Mn-SOD (White and Scandalios, 1987), was indicated as an arrowhead.



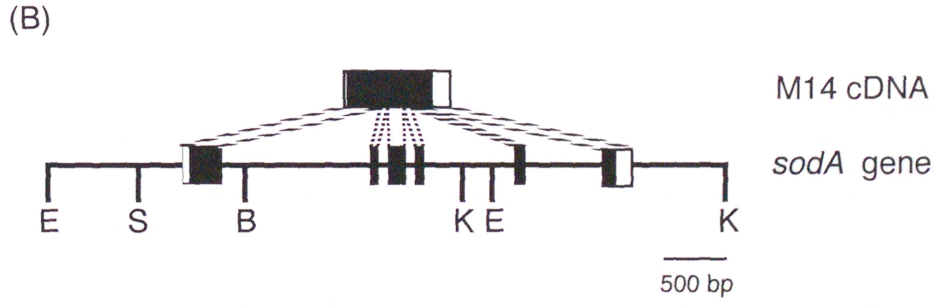


Fig. II-2. Analysis of rice Mn-SOD (*sodA*) gene. (A) Southern blot analysis of rice genomic DNA. Rice genomic DNA was digested with BamHI (1), *Eco*RV (2), *Hin*cII (3), *Hin*dIII (4), *Pst*I (5), and *Sma*I (6), transferred onto nylon membrane, probed with a ^{32}P-labelled full-length cDNA (M14) and washed at the stringency (0.1 x SSC, 65 °C). The molecular size of λ/*Hin*dIII marker is indicated at left. (B) Structure and restriction maps of *sodA* gene. The boxes correspond to the exons; translational and untranslational regions are indicated as dark boxes and light boxes, respectively. B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; S, *Sma*I.

washing at the low stringency (4 x SSC, 37 °C). Only two digested bands were detected by the digestion of *Sac*II, which can digest one site of M14. Furthermore, the restriction map of the isolated genomic DNA (Fig. II-2B) is also completely corresponding to the result of genomic Southern blot analysis. Thus, *sodA* gene is a single copy gene in rice genome.

Fig. II-3. Nucleotide sequence of rice *sodA* gene. The translational start site is shown as +1, and the deduced amino acids in the exon are represented using the triple-letter amino acid code. The 5'-flanking region and introns are in lower case, while the exons are represented in upper case. A putative TATA box, the core sequences of IBOX, MYB, GT-I, and BOX I, and *A. thaliana* MYB and MYC binding sites are labeled below the corresponding nucleotide sequence in 5'-flanking region.

Two genomic clone for rice Mn-SOD have already been isolated and subcloned into plasmid vectors (Sakamoto, data not shown). One of these clones, gMN-9, was used for the further analysis because putative exon sequence in this genomic clone is completely corresponded to the nucleotide sequence of M14, and the other clone (gMN-1) was supposed not to contain the full-length of

coding region for Mn-SOD (data not shown). Physical map of *sodA* gene region in gMN-9 is described in Fig. II-3. 4937 bp of the nucleotide sequence from gMN-9 was completely determined (Fig. II-3) and its structure analyzed (Fig. II-2B). This sequence contains about 1.1 kb of promoter region and coding region spanning about 3.8 kb of genomic DNA. Structural alignment provided not only perfect agreement between the sequences analyzed for the cDNA and the putative exons in this gene, but also the existence of 5 introns in the coding region. All introns showed GT-AG intron border sequences. In summary, *sodA* gene is composed of 6 exons interrupted by 5 introns.

Characterization of the 5'-flanking region of sodA gene -

To search for any known motifs or regulatory elements of other plant genes in the flanking region of *sodA* gene (Fig. II-3), the homology search of the nucleotide sequence was carried out as described in the previous section. In the sequence of promoter region in *sodA* gene, the putative TATA box sequence (5'-TATATA-3') was found at position -93. Several light-responsive elements (Terzaghi and Cashmore, 1995) were found as follows; the core sequence of I-BOX (5'-GATAA-3') and the complementary sequence (5'-TTATC-3') were found at positions -1027 and -1086, respectively. The consensus sequences of GT-1 binding site (5'-GGGTAAT-3') and Box I (5'-GTCCMTCMAACCTAMC-3'; M=A/C) were found at positions -380 and -175, respectively. Four consensus sequences of MYB binding site (5'-CNGTTR-3'; N=A/C/G/T, R=A/G) were found in this promoter region. Further the other binding site for one of the plant MYB protein (ATMYB2) (Urao et al., 1993) was also found at position -159.

Discussion

In this section, the author reported that characterization of gene (*sodA*) for Mn-SOD from rice using rice Mn-SOD cDNA (M14), which was previously isolated (Sakamoto et al., 1993b). The characterization of 5'-flanking region in *sodA* gene is, particularly, thought to be available for the latter analysis as the case in the former section.

The putative processing site of rice Mn-SOD was determined by the alignment with the amino acid sequence of maize Mn-SOD (SOD-3). The amino-terminal sequence of the purified SOD-3 was determined, and the precursor of SOD-3 synthesized *in vitro* was imported into mitochondria (White and Scandalios, 1987). In the author's laboratory, such experiment using the *in vitro* synthesized chimeric protein, which was including non-specific protein (*E. coli* ß-glucuronidase) fused with the amino-terminal region of rice Mn-SOD, was carried out and corresponded to the result of maize Mn-SOD (data not shown).

The nucleotide sequence of *sodA* gene was completely determined (Fig. II-2A) and its structure analyzed (Fig. II-2B). This is the first report of the information of gene for Mn-SOD in monocotyledonous plants. Even if there are two Mn-SOD proteins (Kanematsu and Asada, 1989), this gene is single copy gene in rice genome by genomic Southern blot analysis. The Mn-SOD encoded by M14 is seemed to be corresponded to Mn-SOD I because the pI of the mature Mn-SOD encoded by this cDNA is calculated at 6.14. Whereas any corresponding gene or informations for Mn-SOD 2, the pI of 9, have not been reported. Maize Mn-SOD genes constitute a multiple gene family, including four distinct Mn-SOD genes (Zhu and Scandalios, 1993), but the SOD activity staining on a gel was indicated there was only one Mn-SOD activity (SOD-3). The copy number of rice cytosolic Cu/Zn-SOD genes (Sakamoto, 1992; Sakamoto et al., 1995b) are corresponded to that of maize genes (Guan and Scandalios, 1998). The inconsistence of the copy number for Mn-SOD gene(s) in monocot could not be clarified but may due to the difference from the genome size of rice and maize.

The intron number and the inserted positions in *sodA* gene was compared with those in the known Mn-SOD genes from pea (accession number U30841), rubber tree (Miao and Gaynor, 1993), mouse (accession number L35525-L35528), *Caenorhabditis elegans* (accession number D12984), yeast (accession number X02156), and *E. coli* (accession number M94879). The intron/exon location in the amino acid sequences (Fig. II-4) indicated that the intron number and inserted positions among plant Mn-SOD genes are completely conserved regardless of monocot or dicot, and have no correlation for those of Mn-SODs in other organisms. This result suggested that the insertion event of introns occurred with the evolution process.

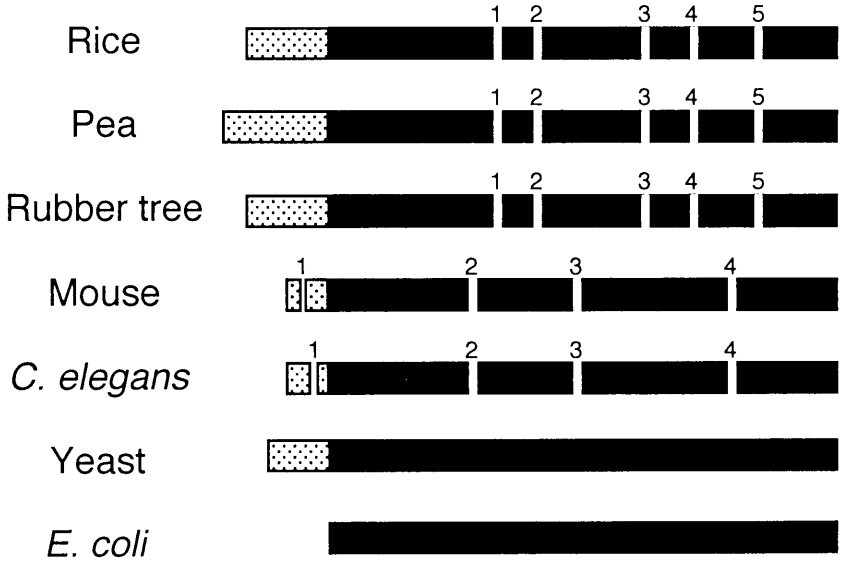


Fig. II-4. Comparison of exon/intron location in the amino acid sequences of Mn-SODs. Genomic sequences of Mn-SOD gene from pea (accession number U30841), rubber tree (Miao and Gaynor, 1993), mouse (accession number L35525-L35528), *C. elegans* (accession number D12984), yeast (accession number X02156), and *E. coli* (accession number M94879) were used for comparison. Each intron number is indicated above the amino acid sequences.

By analysis of the promoter region in *sodA* gene, several known regulatory elements were characterized (Fig. II-3). This sequence is including several light-responsive elements, I-BOX, GT-1 and BOX I (Terzaghi and Cashmore, 1995). The core sequences of I-BOX (5'-GATAA-3') and GT-1 binding site (5'-GGGTAAT-3') are the conserved sequence upstream of light-regulated genes in both monocots and dicots . Box I consensus sequence (5'-GTCCMTCMAACCTAMC-3') was found in mustard and parsley chalcone synthase genes and characterized as an essential element for light regulation. The existences of the light-responsive elements suggested that the expression of rice *sodA* gene is responded to light signals. Four consensus sequences of MYB binding site (5'-CNGTTR-3'), which is conserved in the binding sites of all animal MYBs and at least two plant MYB proteins, and the plant MYB protein (ATMYB2) were also found in this promoter region. The binding site of ATMYB2 was characterized in *A. thaliana* RD22 gene (Urao et al, 1993). This MYB protein together with MYC are strongly associated with ABA-mediated gene regulation (Abe et al., 1997). Actually, two similar sequences to the MYC binding site (5'-CACATG-3') were identified near the MYB binding site (Fig. II-3). These results strongly suggested that *sodA* gene is regulated by one of the ABA-mediated signal transduction pathway. The response of rice *sodA* gene to light, ABA, or other stress treatments will also show and discuss in detail in later section of this chapter.