SECTION 3

cDNA cloning and characterization of rice Fe-superoxide dismutase

Materials and Methods

Isolation and characterization of a cDNA for rice Fe-SOD -

Prior to the isolation of a cDNA for rice Fe-SOD, a nucleotide sequence of rice EST sequence (accession number, C26547), which is homologous to known plant Fe-SOD cDNAs (*A. thaliana* and *N. plumbaginifolia* (Van Camp et al., 1990), and *Glycine max* (Crowell and Amasino, 1991a)), was obtained by a search of DNA databases (Fig. III-1A). A partial fragment of the EST clone (Fig. III-1B) was obtained using PCR with a pair of primers, RFeSODF-1 (5'-AGAAAAGATGGCGGC TTTCGCCTC-3') and RFeSODR-1 (5'-TTGCTGATGCTTACCCCAGTG-3'), which were synthesized based on this rice EST sequence. Using the PCR fragment, a cDNA library, which was made from leaves of rice seedlings using *Eco*RI predigested λZIPLOX arms as described in section 1, was screened and a full-length cDNA clone, designated as RFeSOD4, was then obtained and used for further analysis. All recombinant DNA techniques and sequencing were performed according to the procedures in section 1.

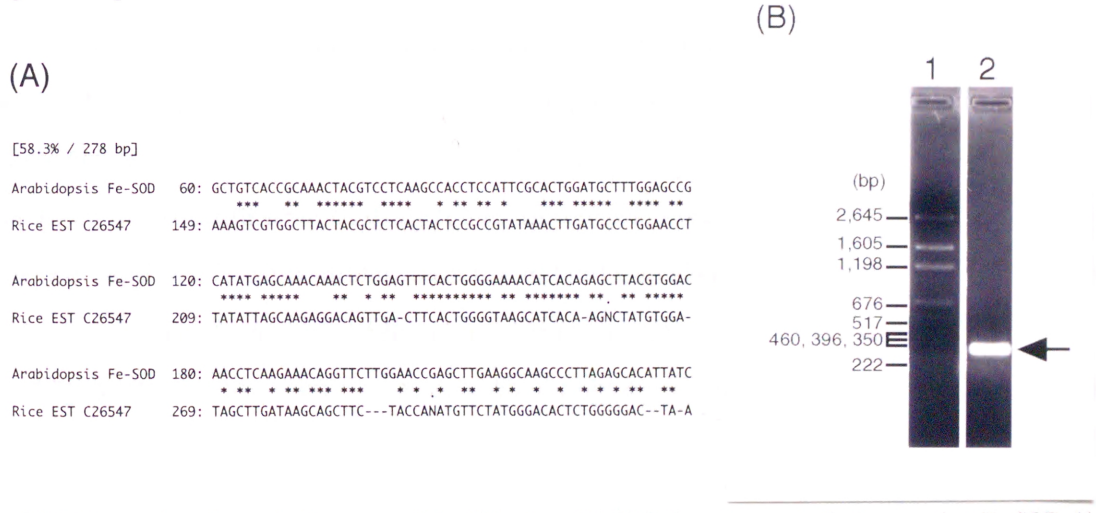


Fig. III-1. Preparation of a partial fragment of rice EST clone (C26547) homologous to the known plant Fe-SOD. (A) Comparison of the nucleotide sequence among rice EST clone (C26547) and A. thaliana Fe-SOD (FSD1) (Van Camp et al., 1990). The identical nucleotides were represented as asterisks. (B) Electrophoresis analysis stained by ethidium bromide; lane 1: pGEM marker (Promega Japan), lane 2: PCR products amplified with a pair of primer, RFESODF-1 and RFESODR-1, whose sequence was shown in Fig. III-2. Arrows indicated the recovered band by electroelution.

Purification of recombinant rice Fe-SOD protein expressed in E. coli -

For the production of histidine-tagged recombinant protein for rice Fe-SOD, a *Xho*I-*Hin*dIII (Fig. III-2, underlined) fragment from RFeSOD4 was inserted in frame into *Sal*I (compatible 5' overhang to *Xho*I) and *Hin*dIII sites of plasmid pQE32 (QIAGEN K.K., Tokyo, Japan; Fig. III-5A)

in *E. coli* strain JM109. This *E. coli.* was cultured at 25 °C for 20 h after induction with 0.2 mM IPTG. The expression of recombinant protein in *E. coli* was recognized by means of 20% SDS-PAGE and Coomassie brilliant blue (CBB) staining (Fig. III-5B). For purification from the native state, cytoplasmic fractions of *E. coli* were prepared as follows. The culture solution treated with IPTG was centrifuged at 4,000 x *g* for 10 min, and the pellet was resuspended in a sonication buffer (20 mM Na-phosphate (pH 7.6), 300 mM NaCl, 10 mM imidazole and 0.2% Triton X-100). Lysozyme was then added to 1 mg/ml, and the pellet was incubated on ice for 30 min. The resulting solution was sonicated and centrifuged at 10,000 x *g* for 20 min. These procedures were repeated twice. In order to concentrate SOD activity from the recombinant protein, the supernatant was passed through a His Trap chelating column (Amersham Pharmacia Biotech Japan) according to the manufacturer's instructions. Prior to metal analysis and SOD activity assays, the purified protein was dialyzed with 50 mM K-phosphate (pH 7.5) and 0.1 mM EDTA using Slide-A-Lyzer Dialysis Cassette (PIERCE, IL, USA) at 4 °C. The purity of the purified proteins was checked by densitometric scanning of the electrophoresed 20% SDS-PAGE gel using a Image Master 1D software (Amersham Pharmacia Biotech Japan).

Metal analysis -

Metals of the purified recombinant protein and *E. coli* SODs (Fe-SOD and Mn-SOD: purchased from Sigma (St Louis, MO)) were determined by inductively coupled plasma-atomic emission spectrophotometry (ICP-AES) using SPS 1500VR atomic spectrometer (Seiko Instruments Inc., Chiba, Japan). The concentration of dialyzed recombinant protein, *E. coli* SODs, and the reference metal ions (Fe^{2+} and Mn^{2+}) were diluted with 0.1 N HNO_3 to about 0.1 ppm and subjected to the metal analysis. The metal contents of the recombinant protein was revised with the purity determined by densitometric scanning as described above and indicated as μg-atom/mg protein.

SOD activity assays -

The solution assay of SOD activity was measured spectrometrically by the nitroblue tetrazolium (NBT) method (Beyer and Fridovich, 1987) as follows. In this assay, 1 unit of SOD is defined as the amount required to inhibit the photoreduction of NBT by 50% with the $O_2^{\cdot-}$ generated by the xanthine-xanthine oxidase system. Changes in absorbance (560 nm), which dued to the formation of formazan, were measured by a BioSpec-1600 spectrometer (Shimadzu).

The SOD activity was localized on the gels as described by Kanematsu and Asada (1989) as follows. The purified recombinant rice Fe-SOD protein (5 u) prepared as described above were

separated on a native 8% polyacrylamide gel at 4 °C for 2-3 h. The gel was treated with riboflavin solution (36 mM K-phosphate (pH 7.8), 28 mM N',N',N',N'-tetramethylethylenediamide (TEMED), and 28 μM riboflavin) and then shaking for 20 min. After washing in water twice, 2.5 mM NBT was added and shaking for 20 min. These procedures were performed in the dark. The treated gel was stained by irradiation with a fluorescent lamp until the bands would completely appear. Three types of SOD isoforms were distinguished by adding the inhibitors (3 mM H_2O_2 or 5 mM KCN) for 20 min prior to treatment with the riboflavin solution. Protein concentrations were determined colorimetrically with Coomassie brilliant blue (CBB) (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

Genomic Southern blot analysis of rice Fe-SOD gene (sodB gene) -

All procedures in Genomic Southern blot were carried out as described in section 1, without used probe. An *Sal*I-digested fragment of RFe-SOD-4, full length cDNA of rice Fe-SOD, was used for the hybridization probe.

Results

Characterization of a cDNA for rice Fe-SOD -

Before the isolation of Fe-SOD cDNA from rice, a nucleotide sequence of a rice EST clone (accession number C26547) was obtained by searching the DNA databases with the nucleotide sequence of known plant Fe-SOD cDNAs (*A. thaliana* and tobacco (*N. plumbaginifolia*) (Van Camp et al., 1990), and soybean (Crowell and Amasino, 1991a)). Using a partial fragment of this EST clone, which was obtained as described in Materials and Methods, a cDNA, designated RFeSOD4, was isolated from the cDNA library of rice leaves. Both strands of the nucleotide sequence were identified and used for further analysis. RFeSOD4 (accession number AB014056) is 1,352 bp in length and has an open reading frame encoding 255 amino acids with a molecular weight of 29,476 (Fig. III-2). The deduced amino acid sequence from RFeSOD4 has hydrophobic residues in the amino-terminal region (residues 1 to 40). This region is assumed to be a putative transit peptide for the transportation to chloroplasts because characterized Fe-SOD in higher plants is localized in chloroplasts (Bowler et al., 1994; Van Camp et al., 1990).

The deduced amino acid sequence was compared with those of the known plant Fe-SODs (Fig. III-3). The sequences of recently characterized plant Fe-SODs, two cDNAs from *A. thaliana* (these cDNAs were designated *A. thaliana* FSD2 and FSD3, while the previously characterized *A. thaliana* Fe-SOD cDNA was FSD1) (Kliebenstein et al., 1998) and maize cDNA (accession

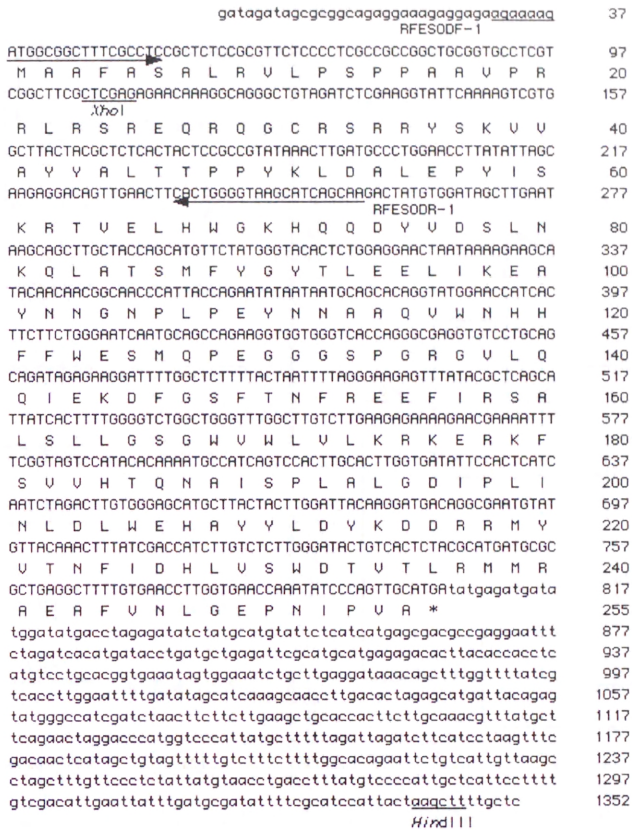


Fig. III-2. Nucleotide and deduced amino acid sequences of rice Fe-SOD cDNA, RFeSOD4. The nucleotide sequence of the putative open reading frame and non-coding regions are shown in uppercase and lowercase letters, respectively. The deduced amino acid sequence is presented in the single-letter code under the nucleotide sequence. An asterisk indicates a termination codon. PCR primers for amplification of a partial rice EST clone (C26547) are underlined with an arrowhead indicating polarity. Restriction sites (*Xho*I and *Hin*dIII) for obtaining the fragment to produce a recombinant protein are underlined with the name of each restriction endonuclease. The nucleotide sequence data in this figure (RFESOD-4) have been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AB014056.

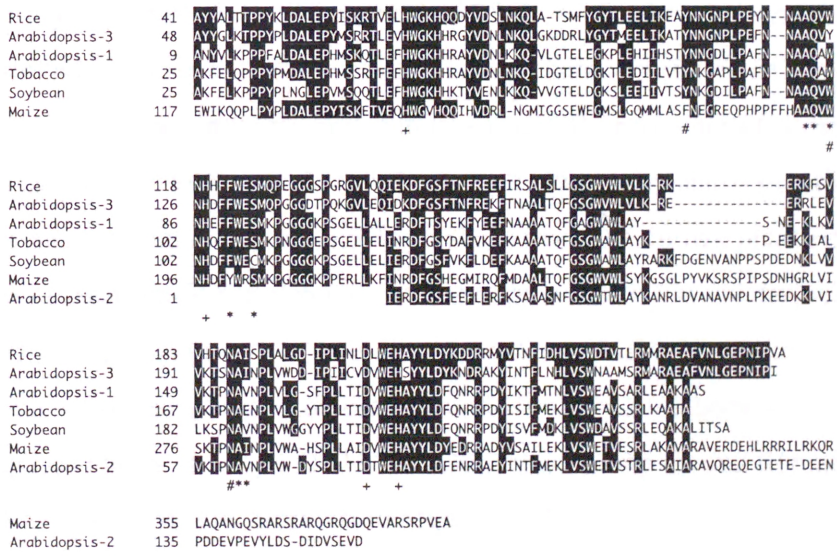


Fig. III-3. Comparison of the deduced amino acid sequence from rice Fe-SOD cDNA with those from plant Fe-SOD cDNAs, *A. thaliana*-1,2,3 (FSD1, 2, and 3) (Van Camp et al., 1990; Kliebenstein et al., 1998), *N. plumbaginifolia* (Van Camp et al., 1990), *G. max* (Crowell and Amasino, 1991a), and maize (accession number Y11914). Identical residues with rice Mn-SOD are enclosed in black boxes. Residues for catalytic activity and metal ligand binding are shown with sharp and plus marks, respectively. Residues that distinguish Fe-SOD from Mn-SOD are indicated as asterisks.

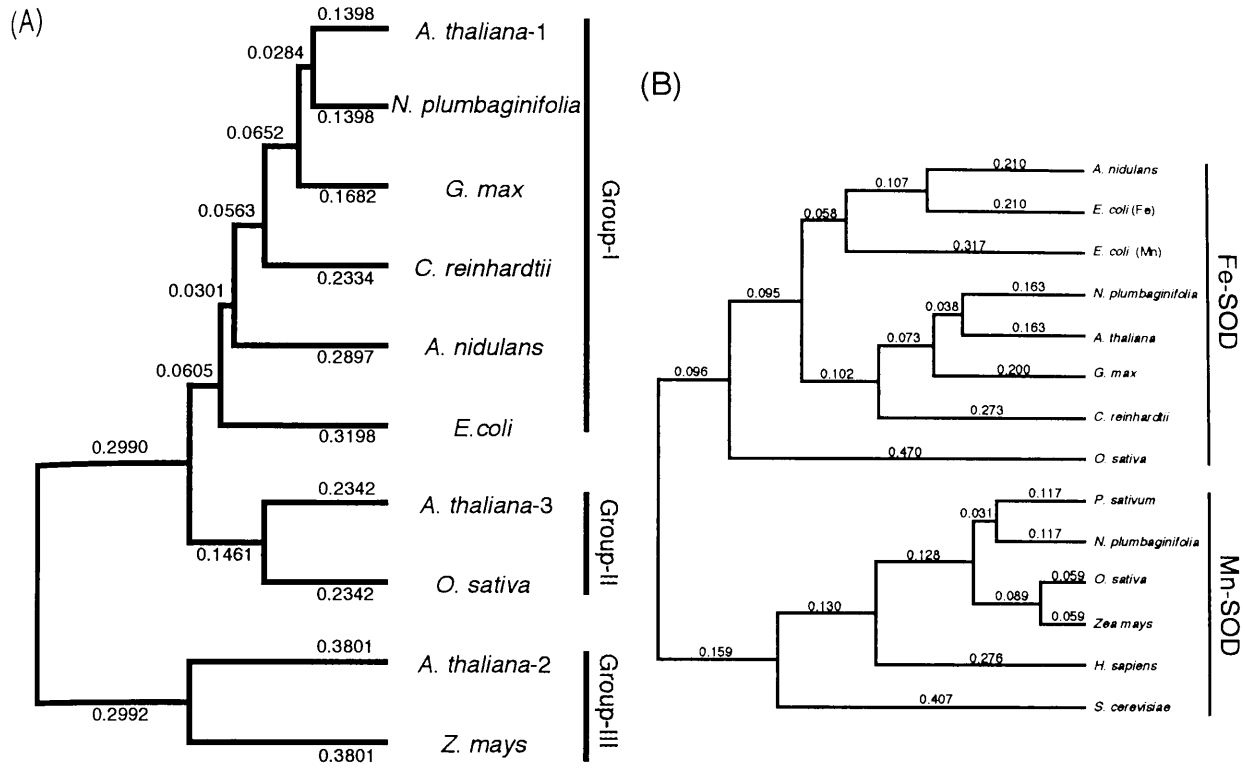


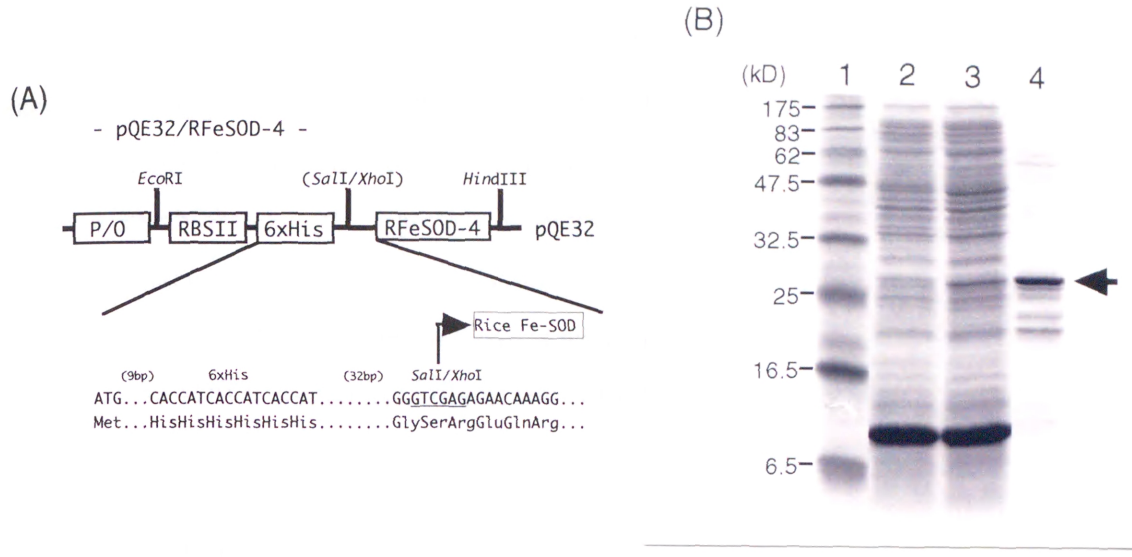
Fig. III-4. Molecular phylogenic tree of (A) plant Fe-SODs or (B) Fe-SOD and Mn-SOD. The phylogenic tree was constructed with GeneWorks software (IntelliGenetics). Numbers indicate the branch length as proportional genetic divergence. The sequences of these SODs are shown in Fig. III-3, and Fe-SODs from *C. reinhardtii* (accession number U22416), *A. nidulans* (accession number X17431) and *E. coli* (accession number J03511) , and Mn-SODs from *O. sativa* (Sakamoto et al., 1993a) *Z. mays* (accession number M33119), *N. plumbaginifolia* (accession number X14482), *P. sativum* (accession number X60170), *E. coli* (accession number X03951), *S. cerevisiae* (accession number X02156) and *H. sapiens* (accession number X14322).

number Y11914), were also used in this and later analyses. This newly characterized rice Fe-SOD shows a high similarity only with *A. thaliana* FSD3 (66.2%), while the comparison with the previously characterized plant Fe-SODs and other recently characterized Fe-SODs showed relative low similarity (41 to 52%). The similarity between rice Fe-SOD and each of the previously reported plant Fe-SODs is less than that observed among the dicotyledonous plant Fe-SODs (about 70%). These results suggested that rice Fe-SOD is distinct from the known plant Fe-SODs. The phylogenic analysis of plant Fe-SODs also indicated that plant Fe-SODs are divided into three groups (Fig. III-4A): first group (group I) including *A. thaliana* FSD1, *N. plumbaginifolia*, and *G. max* Fe-SODs, which were characterized before the author's study have been started; second group (group II) including rice and *A. thaliana* FSD3 Fe-SODs; third group (group III) including *Z. mays* and *A. thaliana* FSD2 Fe-SODs. The amino acid sequences deduced from rice Fe-SOD cDNA and the Fe-SOD cDNAs from first and second groups shows a high preservation of the residues that distinguish Fe-SOD from Mn-SOD, as well as those for catalytic activity and metal ligand binding (Fig. III-3), while those of Fe-SOD cDNAs in group III did not conserve the several necessary residues for Fe-SOD.

Fe-SOD and Mn-SOD are structurally very similar and are thought to be evolved from a common ancestor before eukaryotes and prokaryotes diverged. But rice Fe-SOD have only about a 30% similarity compared with rice Mn-SOD. The phylogenic relationships among rice Fe-SOD and the following known Fe-SODs have been also analyzed (Fig. III-4B); the plant Fe-SODs in group I (*A. thaliana*, *N. plumbaginifolia*, and *G. max*), *Chlamydomonas reinhardtii* (accession number U22416), *Anacystis nidulans* (accession number X17431) and *E. coli* (accession number J03511) and Mn-SODs from *O. sativa* (Sakamoto et al., 1993b), *Zea mays* (accession number M33119), *N. plumbaginifolia* (accession number X14482), *Pisum sativum* (accession number X60170), *E. coli* (accession number X03951), *Sacharomyces cerevisiae* (accession number X02156) and *Homo sapiens* (accession number X14322). The more striking feature of the tree is the existence of two main groups classified by their metal content, except for *E. coli* Mn-SOD. The isolated rice Fe-SOD clone could nest on the branch of Fe-SODs but not on the known plant Fe-SODs (group I).

Characterization of the recombinant rice Fe-SOD -

To clarify that RFeSOD4 actually encodes rice Fe-SOD, RFeSOD4 was fused in frame into pQE32 (QIAGEN K.K.) in the *E. coli* strain JM109 (Fig. III-5A). A specific band was induced by IPTG, and the molecular size estimated by SDS-PAGE is approximately 29 kD, an expected size from the deduced amino acid sequence of RFeSOD4 (Fig. III-5B, lanes 2,3). The His-tagged recombinant RFeSOD4 protein was partially purified as described in Materials and Methods. The partial purified protein (Fig.III-5B, lane 4) was estimated as the purity of approximately 50% by the densitometric scanning (data not shown). The metal contents of the partial purified protein and *E. coli* Fe-SOD and Mn-SOD were measured by ICP-AES as described in Materials and Methods (Table III-1). In the case of the recombinant protein, the metal contents were revised with the purity determined by densitometric scanning. The metal contents of recombinant RFeSOD4 protein were similar to those of *E. coli* Fe-SOD but apparently distinct from those of *E. coli* Mn-SOD. This showed that RFeSOD4 encodes an iron-containing SOD in rice. Furthermore, the purified recombinant RFeSOD4 protein (5 u) was used for the assay of SOD activity on the gels. Four bands were observed (Fig III-5C, lane 2), and three of these bands were derived from *E. coli* endogenous SOD activity (Fig. III-5C, lane 1). The SOD activity from the recombinant protein (shown with a tailed arrow) was not inhibited by 5 mM KCN (Fig. III-5C, lane 3) or by 3 mM H_2O_2 (Fig. III-5C, lane 4) treatments, although the known Fe-SODs were inhibited by H_2O_2. The concentrations of cyanide and H_2O_2 were enough to inhibit the known SOD activities (cyanide for Cu/Zn-SOD, H_2O_2 for Cu/Zn-SOD and Fe-SOD) in both tobacco leaves extracts on a gel (data not shown).



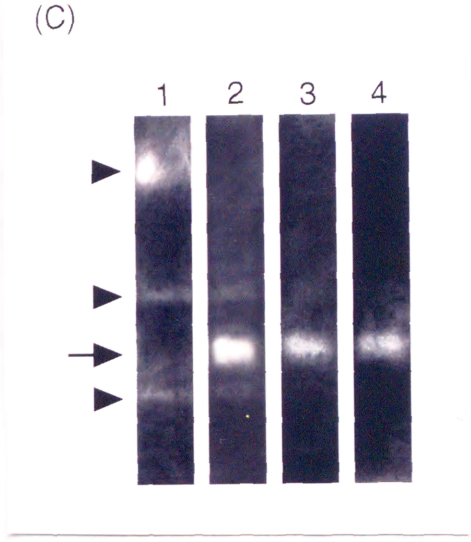


Fig. III-5. Expression in *E. coli* (B) and SOD activity (C) of Recombinant Rice Fe-SOD. (A) Fusion construction in frame of pQE32/RFeSOD-4. (B) SDS-PAGE gel (20%) was stained with CBB. Molecular marker (lane 1; Prestained protein marker, New England Biolabs); The cytoplasmic fractions of *E. coli* strain JM109 harboring pQE32/RFeSOD4 (not induced, lane 2; IPTG-induced, lane 3); 2.5 μg of purified recombinant rice Fe-SOD protein (lane 4). (C) A partially purified recombinant rice Fe-SOD protein (5 u) was fractionated by a native 8% polyacrylamide gel electrophoresis and stained for SOD activity as described in Materials and Methods. Inhibitors (3 mM KCN and 5 mM H_2O_2) were added to the gels before activity staining. SOD activity from recombinant rice Fe-SOD protein is indicated with a tailed arrow, and bands of SOD activity from *E. coli* are shown as arrowheads. Lane 1, SOD activity from the cytoplasmic fraction of JM109 harboring pQE32; lane 2, SOD activity from the purified recombinant RFeSOD4 protein; lane 3, KCN-resistant SOD activity of lane 2; lane 4, H_2O_2-resistant SOD activity of lane 2.

Table III-1. Metal Contents of Recombinant Rice Fe-SOD, *E. coli* Fe-SOD and Mn-SOD

Metal contents were determined by ICP-AES as described in Materials and Methods. Values are given as means ±SD of three determinations.

samples	metal content (μg-atom/mg of protein)	
	Fe	Mn
Recombinant rice Fe-SOD	0.354±0.011	0.053±0.002
E. coli Fe-SOD	0.692±0.038	0.040±0.009
E. coli Mn-SOD	0.049±0.038	1.271±0.011

Genomic Southern blot analysis of rice Fe-SOD gene -

To clarify the copy number of rice Fe-SOD gene (hereafter *sodB* gene) in rice genome, genomic Southern blot analysis was carried out as described in section 1, with ^{32}P-labelled full-length fragment of RFeSOD-4 (Fig. III-6A). At the high stringency washing (0.1 x SSC, 55 °C), one or two bands were observed by the digestion of restriction enzyme (*Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I). This result was also obtained by washing at the low stringency (2 x SSC, 42 °C). Without the case of the digestion by *Bam*HI, two digested bands were detected. The restriction map of RFeSOD-4 indicated that the cDNA contains the digest sites of *Eco*RI, *Hin*dIII, and *Pst*I (Fig. II-6B). Thus, *sodB* gene is supposed to be a single copy gene in rice genome.

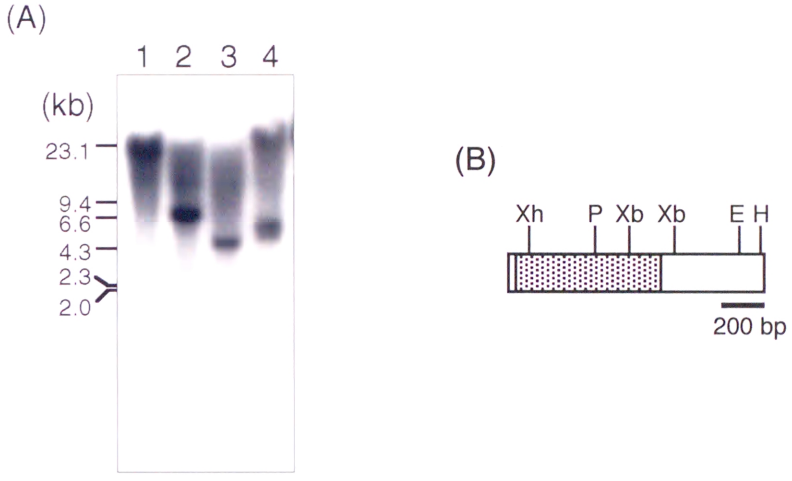


Fig. III-6. (A) Genomic Southern blot analysis of rice Fe-SOD. Total genomic DNA (10 μg) from rice was digested with *Bam*HI (1), *Eco*RI (2), *Hin*dIII (3), and *Pst*I (4), transferred onto nylon membrane, probed with a ^{32}P-labelled full-length cDNA (RFeSOD-4) and washed at the stringency (0.1 x SSC, 65 °C). Size markers (λ/*Hin*dIII; New England Biolabs.) are indicated on the left. (B) Restriction map of rice Fe-SOD cDNA (RFeSOD-4). Open reading frame is indicated as shaded box. E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Xb, *Xba*I; Xh, *Xho*I.

Discussion

In this section, the author reported the isolation of a cDNA for Fe-SOD using the information of one rice EST clone homologous to the known plant Fe-SOD cDNA. Furthermore, the isolated cDNA was identified to encode iron-containing SOD using the recombinant protein expressed in *E. coli*. In such analyses, it was particularly interesting that the sensitivities of the recombinant protein to inhibitors were different from those of the characterized plant Fe-SODs. This is the first report of the sequence and characterization of Fe-SOD from a monocotyledonous plant (Kaminaka et al., 1999a). Fe-SOD has been thought to be absent in rice and other monocotyledonous plants because Fe-SOD activity has not been found by SOD activity staining (Briges and Salin, 1981; Kanematsu and Asada, 1989). Recently, however, the presence of putative Fe-SOD activity, as cyanide-

insensitive and H_2O_2-sensitive SOD activity, has been reported in barley and rice (Casano et al., 1994; Ushimaru et al., 1995). The transcripts of Fe-SOD homologues in barley and rice, further, could be detected using the characterized plant Fe-SOD cDNAs as the heterologous probes (Casano et al., 1994; our unpublished data). On the other hand, the characterized cDNAs for plant Fe-SODs have been isolated only from several dicotyledonous plants (Van Camp et al., 1990; Crowell and Amasino, 1991a). Therefore the author assumed that monocotyledonous plants have also gene(s) and proteins for Fe-SOD(s), and then started the attemption of cDNA cloning for Fe-SOD in rice.

The metal analysis (Table III-1) and SOD assays (Fig. III-5C) of the recombinant protein apparently indicated that the isolated rice cDNA encodes an iron-containing SOD. However the newly characterized rice Fe-SOD is different in several respects from other known Fe-SODs. The SOD activity was not inhibited by H_2O_2 or cyanide in the recombinant rice Fe-SOD examined (Fig. III-5C, lane 3,4), unlike the activities of the exogenous *lac*Z fusion proteins of both *N. plumbaginifolia* and *G. max* Fe-SOD in *E. coli*, which were completely inhibited by H_2O_2 (Van Camp et al., 1990; Crowell and Amasino, 1991b). All known Fe-SOD activities are sensitive to H_2O_2. The tryptophan residue at 117 of rice Fe-SOD in Fig. III-3 has been believed to be responsible for this H_2O_2-sensitivity (Bowler et al., 1994; Yamakura et al, 1998). This, however, may not be the case, as the residue is completely conserved in rice Fe-SOD (Fig. III-3). No Fe-SOD activities in the rice tissues might, so far, have been identified by the SOD activity staining method misjudged as one of Mn-SOD activities because the characterized rice Fe-SOD is insensitive to H_2O_2. The location of rice Fe-SOD, which is encoded by RFeSOD4, has not been identified, but characterized Fe-SODs in higher plants are found in the chloroplasts (Bowler et al., 1994; Van Camp et al., 1990). The deduced amino acid from rice Fe-SOD have a putative transit peptide in the amino-terminal region. Mn-SOD activity has been identified as thylakoid-bound Mn-SOD in spinach, which has been reported as H_2O_2- or cyanide-insensitive SOD activity in chloroplasts (Hayakawa et al., 1985). However, no genes for chloroplastic Mn-SOD have been characterized. The Mn-SOD activity reported in chloroplasts may be attributed to the same type of Fe-SOD activity as that found in rice.

Recently, in addition to the characterized plant Fe-SOD cDNAs from *A. thaliana*, *N. plumbaginifolia*, and *G. max* before the start point of the author's study, large-scale analysis of *A. thaliana* SOD genes using the information of DNA databases have indicated *A. thaliana* has seven SOD genes, including newly characterized two Fe-SOD homologue cDNAs (*A. thaliana* FSD2 and FSD3) (Kliebenstein et al., 1998). Furthermore, a cDNA for Fe-SOD homologue have also been isolated from maize (accession number Y11914). The evolutionary analysis of the phylogenic tree among rice Fe-SOD and these plant Fe-SODs indicated that the plant Fe-SODs are divided into

three different groups (Fig. III-4A). The tryptophane residue, which has been thought to be associated with the sensitivity to H_2O_2 (Bowler et al., 1994; Yamakura et al., 1998), was completely conserved in the Fe-SOD sequences of group I and II but not of group III (Fig. III-3). The primary structures of group III Fe-SODs are quite different from those of group I and II Fe-SODs and not conserve several important residues for Fe-SODs. Therefore the cDNAs in group III might not encode Fe-SOD. Actually, Fe-SOD activity from these cDNAs, which are characterized only as Fe-SOD homologues, have not been examined.

Rice Fe-SOD is highly homologous only with *A. thaliana* FSD3, and both were classified in same category, group II (Fig. III-4A). This cDNA sequence was deduced from the *A. thaliana* genomic DNA, which was identified as a single gene on *A. thaliana* chromosome V, and the corresponding cDNA could not be found in the public domain EST databases (Kliebenstein et al., 1998). This result suggested the expression level of *A. thaliana FSD3* gene is very low. Likewise rice Fe-SOD gene is also a single copy gene revealed by genomic Southern blot analysis, and the expression level of rice Fe-SOD gene was seemed to be relatively lower than other SOD genes (data not shown). These results and speculations suggested that there are group II Fe-SODs, which have such characters as the author identified, in all plant species. On the other hand, the phylogenic analysis using Fe-SODs and Mn-SODs from various organisms indicated that rice Fe-SOD has a distinct evolutionary process from Mn-SODs but belongs to the different group from the other known Fe-SODs (Fig. III-4B). This result and the nature of sensitivity to inhibitors suggested that rice Fe-SOD encoding by RFeSOD4 is a novel Fe-SOD, which is distinct from the known Fe-SODs. The author wonder whether there are such three distinct Fe-SOD isoforms in rice. There are no evidences to explain for this question, but the author could detect the transcript using a hetelogous probe, *Nicotiana tabacum* Fe-SOD cDNA prepared as described in CHAPTER III (data not shown). Therefore the further analysis about rice Fe-SODs seemed to be necessary. The gene expression of the novel Fe-SOD gene in rice have been analyzed, and the results and discussion will be shown in the next section.