Molecular Cloning and Characterization of a cDNA for an Iron-Superoxide Dismutase in Rice (Oryza sativa L.)


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We have isolated a cDNA encoding Fe-SOD from rice (Oryza sativa L.). The deduced amino acid sequence consists of a polypeptide with 255 amino acids, including a putative transit peptide (40 a.a.) in amino-terminal residues. This sequence is similar to the known plant Fe-SODs but not classified in the group of known Fe-SODs. The metal analysis and SOD assays of the partial purified recombinant protein expressed in E. coli showed that this cDNA encodes an iron-containing SOD. However this SOD activity was not inhibited by the treatment with hydrogen peroxide, which was expected to inhibit known Fe-SOD activity. mRNA of rice Fe-SOD was detected in all vegetative tissues examined, being especially abundant in calli, and strongly increased by light induction. These results suggested that this cDNA encodes rice Fe-SOD, which is apparently distinct from known plant Fe-SODs.

Key words: active oxygen; iron-superoxide dismutase (EC 1.15.1.1); cDNA cloning; gene expression; rice (Oryza sativa L. cv Nipponbare)

Superoxide dismutase (SOD, EC 1.15.1.1) catalyzes the first step in the scavenging system of active oxygens by the disproportionation of superoxide anion radicals to hydrogen peroxide and molecular oxygen.1) SOD has multiple isoforms, which are classified by their metal cofactor: copper/zinc (Cu/Zn), manganese (Mn), and iron (Fe) forms.2) Mn-SOD and Fe-SOD are structurally very similar, while Cu/Zn-SOD is not related.3) These isoforms are distinguishable by their differential sensitivities to cyanide and hydrogen peroxide, and are found in different subcellular locations. In higher plants, Cu/Zn-SOD is mainly in plastids and cytosol. Mn-SOD is predominantly in the mitochondrial matrix. Cu/Zn-SOD and Mn-SOD are found among all plant species, but Fe-SOD, which is located in chloroplasts,4) has been characterized only in several dicotyledonous plant species.5-8)

In rice, four Cu/Zn-SODs and two Mn-SODs were characterized.9) We have previously isolated cDNAs corresponding to these SOD isoforms, two cytosolic Cu/Zn-SODs, plastidic Cu/Zn-SOD and Mn-SOD.10-12) Plant SOD genes are regulated in response to various stimuli, such as environmental stress, phytohormones, and chemical treatments. Increases of SOD activity in plants confer tolerance against oxidative and environmental stresses.13,14) We have previously shown that two of the cytosolic Cu/Zn-SOD genes are differentially regulated in response to abscisic acid by promoter analysis.15) Fe-SOD has been thought to be absent in rice and other monocotyledonous plants16) because Fe-SOD activity has not been found by SOD activity staining.17,18) Recently, however, the presence of putative Fe-SOD activity, as cyanide-insensitive and hydrogen peroxide-sensitive SOD activity, has been reported in rice and barley.19,20) Therefore we can assume the presence of gene(s) for rice Fe-SOD in monocotyledonous plants. cDNAs for plant Fe-SODs have been isolated only from several dicotyledonous plants, Arabidopsis thaliana, Nicotiana plumbaginifolia,21) and Glycine max.22) We report here for the first time the sequence of Fe-SOD cDNA from a monocotyledonous plant, rice (Oryza sativa L.). We consider that this cDNA encodes a novel type of Fe-SOD, which is apparently distinct from the known plant Fe-SODs, from the analyses of primary structure, SOD activity assays, metal contents, and the gene expression in the vegetative tissues and by light signals.

Materials and Methods

Plant Materials. Rice (Oryza sativa L. cv Nipponbare) was used in all experiments. Seedlings were grown in a growth cabinet maintained at 28°C and a 16-h photoperiod at 170 µE m−2 s−1 for 10 d after germination. Etiolated seedlings were grown in darkness for the same period. Embryogenic calli were maintained by weekly subculture in MS liquid medium containing 1 mg/L of 2,4-dichlorophenoxyacetic acid. Samples for the extraction of total RNA were frozen in liquid N2 and stored at −80°C until use. For the experiment of light induction, dark-grown 10 d etiolated seedlings were exposed to the light (170 µE m−2 s−1) during the treatment. Escherichia coli (E. coli) Fe-SOD19) and Mn-SOD20) were purchased from Sigma.

Isolation and sequencing of a cDNA for rice Fe-SOD. Before the isolation of a cDNA for rice Fe-SOD, we ob-
tained a nucleotide sequence of rice EST sequence (accession number C26547), which is similar to known plant Fe-SOD cDNAs (A. thaliana, N. plumbaginifolia, and G. max)\textsuperscript{18}, with a search of DNA databases. A partial fragment of the EST clone was obtained using PCR with a pair of primers, RFeSOD1-1 (5'-AGAAAA-\textsuperscript{5}GATGGCGGCTTTCGCCCT-3') and RFeSOD1-2 (5'-TTTGTAGTGTCTACCCAGTG-3'), which were synthesized based on this rice EST sequence. Using the PCR fragment, we screened a cDNA library, which was made from leaves of rice seedlings using lambda-ZIPLOX arms as described,\textsuperscript{11} and a full-length cDNA clone, designated RFeSOD4, was then obtained and used for further analysis. The methods of screening and sequencing were followed as described previously.\textsuperscript{11} The sequence data from RFeSOD4 was analyzed using Gene Works software (IntelliGenetics, Mountain View, CA). The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number AB014056.

Purification of recombinant rice Fe-SOD protein expressed in E. coli. For the production of His-tagged recombinant protein for rice Fe-SOD, a XhoI-HindIII (Fig.1, underlined) fragment from RFeSOD4 was inserted in frame into SalI (compatible 5' overhang to XhoI) and HindIII sites of plasmid pQE32 (Qiagen K.K., Tokyo, Japan) in E. coli strain JM109. This E. coli was cultured at 25°C for 20 h after induction with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The expression of recombinant protein in E. coli was recognized by means of 20% SDS-PAGE and Coomassie brilliant blue staining. 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 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 g for 20 min. These procedures were repeated twice. To concentrate SOD activity from the recombinant protein, the supernatant was passed through a His Trap chelating column (Amersham Pharmacia Biotech Japan, Tokyo, Japan) according to the manufacturer’s instructions. Before 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, Rockhold, IL) at 4°C. The purity of the purified proteins was checked by densitometric scanning of the electrophoresed 20% SDS-PAGE gel using an Image Master 1D software (Amersham Pharmacia Biotech Japan).

Metal analysis. Metals of the purified recombinant protein and E. coli SODs (Fe-SOD\textsuperscript{19} and Mn-SOD\textsuperscript{20}) were measured by inductively coupled plasma-atomic emission spectrophotometry (ICP-AES) using an SPS 1550VR atomic spectrometer (Seiko Instruments Inc., Chiba, Japan). The dialyzed recombinant protein and E. coli SODs were diluted with 0.1 M HNO\textsubscript{3} and their metals were analyzed. The metal contents of the recombinant protein were revised with the purity measured by densitometric scanning.

Northern blot analysis and SOD activity assays. Extraction of total RNA and Northern blotting were done as described previously,\textsuperscript{10} except for the washing conditions and the probes used. The hybridization probe specific to rice Fe-SOD was prepared from a SalI fragment of RFeSOD4, containing full-length cDNA. The hybridized membrane was washed at a high stringency (0.5× SSC, 55°C).

SOD activity was assayed by the nitroblue tetrazolium (NBT) method.\textsuperscript{22} The unit of SOD activity was the amount of enzyme that gave 50% inhibition of NBT reduction. The purified recombinant rice Fe-SOD protein (5 µM) prepared as described above was separated on a native 8% polyacrylamide gel at 4°C. The SOD activity was localized on the gels, and three types of SOD isoforms were distinguished by adding the inhibitors (3 mM hydrogen peroxide or 5 mM KCN) before activity staining as described by Kanematsu and Asada.\textsuperscript{9} Protein concentrations were determined colorimetrically.\textsuperscript{22}

Results

Cloning and characterization of rice Fe-SOD cDNA. Before the isolation of Fe-SOD cDNA from rice, we obtained a nucleotide sequence of a rice EST clone (accession number C26547) by searching the DNA databases with the nucleotide sequence of known plant Fe-SOD cDNAs (A. thaliana, N. plumbaginifolia\textsuperscript{4} and G. max\textsuperscript{9}). 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 is 1,352 bp in length and has an open reading frame encoding 255 amino acids with a molecular weight of 29,476 (Fig. 1). 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.\textsuperscript{2,4} The deduced amino acid sequence was compared with those of the known plant Fe-SODs. This amino acid sequence 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. 2).\textsuperscript{2,4,23} This newly characterized rice Fe-SOD shows a high similarity with the known plant Fe-SODs (46 to 52%), while only about 30% similarity with rice Mn-SOD. 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%). Fe-SOD and Mn-SOD are structurally very similar and are thought to be evolved from a common ancestor before eukaryotes and prokaryotes diverged.\textsuperscript{2,3} We have analyzed the phylogenetic relationships among rice Fe-SOD.
and the following known Fe-SODs and Mn-SODs (Fig. 3); Fe-SODs from A. thaliana, N. plumbaginifolia, 8 G. max. 18 Chlamydomonas reinhardtii (accession number U22416), Anacyclus nidulans (accession number X17431) and E. coli (accession number J03511), and Mn-SODs from O. sativa. 12 Zea mays (accession number M33119), N. plumbaginifolia (accession number X14482), Pisum sativum (accession number X06170), E. coli (accession number X03951), Saccharomyces cerevisiae (accession number X02156) and Homo sapiens (accession number X14322). The more striking feature of the tree is the existence of at least two main groups classified by their metal content, except for E. coli Mn-SOD. The isolated rice Fe-SOD clone could not nest on the branch of the known plant Fe-SODs. Rice Fe-SOD is assumed to be a different type from the known plant Fe-SODs.

Characterization of the recombinant rice Fe-SOD

To clarify that RFeSOD4 actually encodes rice Fe-SOD, RFeSOD4 was fused into pQE32 (QIA-GEN K.K.) in the E. coli strain JM109. A specific band was induced by IPTG, and the molecular size estimated by SDS-PAGE is approximately 29,000 daltons, an expected size from the deduced amino acid sequence of RFeSOD4 (Fig. 4A, lanes 2,3). The His-tagged recombinant RFeSOD4 protein was partially purified as described in Materials and Methods. The partial purified protein (Fig. 4, 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 19 and Mn-SOD 20 were measured by inductively coupled plasma-atomic emission spectrophotometry (ICP-AES) as described in Materials and Methods (Table 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 Mn-SOD but apparently distinct from those of E. coli Mn-SOD. This showed that RFeSOD4 encodes an iron-containing protein rather than Mn-SOD. Furthermore, the purified recombinant RFeSOD4 protein (5 u) was used for the assay of sod activity on the gels. Four bands were observed (Fig. 4, lane 2), and three of these bands were derived from E. coli endogenous sod activity (Fig. 4, lane 1). The sod activity from the recombinant protein (shown with a tagged arrow) was not inhibited by 5 mM KCN (Fig. 4B, lane 3) or by 3 mM hydrogen peroxide (Fig. 4B, lane 4) treatments, although the known Fe-SODs were inhibited by hydrogen peroxide. 1,2 The concentrations of cyanide and hydrogen peroxide were enough to inhibit the known sod activities (cyanide for Cu/Zn-SOD, hydrogen peroxide for Cu/Zn-SOD and Fe-SOD) in both tobacco leaves and rice embryogenic calli extracts on a gel (data not shown).
Fig. 2. Comparison of the Deduced Amino Acid Sequence from Rice Fe-SOD cDNA with Those from *A. thaliana*, *N. plumbaginifolia* and *G. max*.

Residues conserved in all four species 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 with asterisks.

Fig. 3. Molecular Phylogenetic Tree of Fe-SOD and Mn-SOD.

The phylogenetic tree was constructed with GeneWorks software (IntelliGenetics, Mountain View, CA). Numbers indicate the branch length as proportional genetic divergence. The sequences of these SODs are shown in Fig. 2. and Fe-SODs from *C. reinhardtii* (accession number U22416), *A. nidulans* (accession number X17431) and *E. coli* (accession number 103511), and Mn-SODs from *O. sativa*, *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 X14222).

dicated that the characterized rice cDNA encodes an iron-containing SOD. However, rice Fe-SOD is different in several respects from other known Fe-SODs. The SOD activity was not inhibited by hydrogen peroxide or cyanide in the recombinant rice Fe-SOD examined (Fig. 4B, lane 3,4), unlike the activities of the exogenous lacZ fusion proteins of both *N. plumbaginifolia* and *G. max* Fe-SOD in *E. coli*, which were completely inhibited by hydrogen peroxide. The known Fe-SOD activities are sensitive to hydrogen peroxide. The tryptophan residue at 117 in Fig. 2 has been believed to be responsible for this hydrogen peroxide sensitivity. This, however, may not be the case, as the residue is completely conserved in all four plant Fe-SODs including rice Fe-SOD (Fig. 2). Because rice Fe-SOD activity is insensitive to hydrogen peroxide, Fe-SOD activity in the rice tissues has not so far been identified by the SOD activity staining method misjudged as one of Mn-SOD activities. Furthermore, an evolutionary analysis of the phylogenetic tree indicates that rice Fe-SOD has a distinct evolutionary process from the known plant Fe-SODs (Fig. 3). We therefore believe that RFeSOD4 cDNA encodes a novel type of iron-superoxide dismutase. 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. Mn-SOD activity, which has been reported as hydrogen peroxide- or cyanide-insensitive SOD activity in chloroplasts, has
Fig. 4.  Expression in E. coli (A) and SOD activity (B) of Recombinant Rice Fe-SOD.

(A) SDS-PAGE gel (20%) was stained with Coomassie brilliant blue. Molecular marker (lane 1); Prestained protein marker, New England Biolabs, MA; 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). (B) A partially purified recombinant rice Fe-SOD protein (5 μl) 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 hydrogen peroxide) 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, hydrogen peroxide-resistant SOD activity of lane 2.

Fig. 5.  Northern Blot Analysis of Rice Fe-SOD Gene, in Rice Vegetative Tissues (A) and the Effects of Light Induction (B).

Transcript was hybridized with 32P-labelled SauI fragment of RFeSOD4. Each lane was loaded with 20 μg of total RNA. rRNA indicates the result of blotted membrane stained with ethidium bromide. (A) Expression of rice Fe-SOD gene in rice vegetative tissues. Lane 1, shoots of etiolated seedlings; lane 2, green leaves; lane 3, stems; lane 4, roots; lane 5, embryogenic calli. (B) Changes of rice Fe-SOD mRNA in etiolated seedlings by the light induction. Lane S, rice seedlings.

been identified as thylakoid-bound Mn-SOD in spinach. 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. This rice Fe-SOD gene was expressed in all tissues ex-
Table 1. Metal Contents of Recombinant Rice Fe-SOD, E. coli Fe-SOD and Mn-SOD

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fe (μg-atm/mg of protein)</th>
<th>Mn (μg-atm/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant rice</td>
<td>0.356 ± 0.011</td>
<td>0.053 ± 0.002</td>
</tr>
<tr>
<td>E. coli Fe-SOD</td>
<td>0.692 ± 0.038</td>
<td>0.040 ± 0.009</td>
</tr>
<tr>
<td>E. coli Mn-SOD</td>
<td>0.049 ± 0.038</td>
<td>1.271 ± 0.011</td>
</tr>
</tbody>
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Metal contents were measured by ICP-AES as described in Materials and Methods. Values are given as means ± SD of three measurements.

...aminated, and the expression pattern was quite different from that of rice SOD isozymes. We are particularly interested in the lower expression level of this Fe-SOD gene in the leaves of rice seedlings than those in etiolated seedlings and stems (Fig. 5A) although plastidic Cu/Zn-SOD gene is expressed strongly only in the leaves, and this Fe-SOD is supposed to be localized in chloroplasts as described above. The expressions of two chloroplast-localized SOD isozymes, Fe-SOD and plastidic Cu/Zn-SOD, in N. plumbaginifolia have been reported to be differentially regulated during development and under various stimuli. The strong expression of rice Fe-SOD gene was also observed in embryogenic calli that were maintained as a suspension culture (Fig. 5A). In the case of the known plant Fe-SODs, mRNA of G. max Fe-SOD was also observed in cultured soybean cells but little was found in the vegetative tissues. Furthermore the effects of light induction on the level of Fe-SOD mRNA in rice seedlings showed that this gene was strongly induced by light (Fig. 5B). A similar result was observed in N. plumbaginifolia. These results suggested that rice Fe-SOD gene is complementarily expressed with plastidic Cu/Zn-SOD gene in vegetative tissues, and the expression is induced by special signals.

Fe-SOD activity and the mRNA of the Fe-SOD homologue gene have been observed in barley. In our attempt, A. thaliana Fe-SOD cDNA was not cross-hybridized with RFeSOD4 as a probe (data not shown). Therefore there may be two types of Fe-SODs in plants. The N. plumbaginifolia Fe-SOD gene was expressed in response to various stimuli. Furthermore, the increase of oxidative stress tolerance has been observed in transgenic tobacco plants overproducing Fe-SOD in chloroplasts. We are currently investigating the subcellular localization and gene structure of this rice Fe-SOD, and are producing transgenic plants overproducing this novel type of Fe-SOD in chloroplasts. Further study of this novel Fe-SOD will provide a new aspect on the function of Fe-SOD isozymes in plants.

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References


