Occurrence of two types of Mn-superoxide dismutase in the green alga *Spirogyra*: cDNA cloning and characterization of genomic genes and recombinant proteins

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Two types of cDNA encoding Mn-SOD were cloned from the green alga *Spirogyra*, which is located at an important position bridging from green algae to land plants in plant lineage. One, referred to as the land plant-type, encoded a protein whose sequence closely resembled those of mitochondrial Mn-SODs from land plants. The other, referred to as the algal-type, encoded a Mn-SOD similar to that of *Chlamydomonas reinhardtii* in amino acid sequence. The alignment of both SOD sequences revealed their resemblance in the transit signal region, indicating that both are mitochondria-localizing Mn-SODs. Phylogenetic analyses showed that the algal-type and land plant-type of Mn-SOD belong to separate subgroups and that the algal-type is contained in algae but not in land plants, while the land plant-type is involved in algae and land plants. Although the genomic genes of both types consisted of six exons, their exon-intron structures were completely different from each other. The structure of the land plant-type gene was identical with those of two Mn-SOD genes of *Arabidopsis thaliana*. Recombinant Mn-SODs of the algal- and land plant-type were determined to be homodimer and homotetramer, respectively. The three-dimensional structure of both types, predicted by homology modeling, showed a prominent difference at the N-terminal helices H1–H3 which may affect the quaternary structure. Thus, the present results clearly show that *Spirogyra* contains two types of Mn-SOD, algal- and land plant-type, differing in the quaternary structure. This suggests an earlier divergence of Mn-SOD genes before the emergence of plant lineage during the course of evolution. In addition, we found a first intron-retained splicing variant of the algal-type and discuss its function in gene regulation.

Key words: manganese-containing superoxide dismutase, overexpression, recombinant SOD, *Spirogyra*.

INTRODUCTION

Reactive oxygen species (ROS), including superoxide (O_2^-), H_2O_2, hydroxyl radical and singlet oxygen, are inevitably produced in biological processes such as respiration and photosynthesis, although they are highly toxic to cells (Asada 2006). Because of the high reactivity of ROS, aerobic organisms have developed defense systems against ROS with the simultaneous acquisition of systems that produce and/or use oxygen during the course of evolution. Since the superoxide is located at the uppermost site of the cascade reaction in ROS production, where H_2O_2 is formed from O_2^-, and HO· radical is produced from O_2^- and H_2O_2 in the presence of transition metal ions, the scavenging of superoxide finally determines the concentration of ROS in cells.

Superoxide dismutase (SOD) is a metalloenzyme that catalyzes the disproportionation of superoxide to form H_2O_2 and molecular oxygen and protect cells from the deleterious effect of ROS (Fridovich 1995). The enzyme is classified into four types depending on its prosthetic metals: Cu and Zn, Mn, Fe or Ni-containing SODs (Kanematsu and Asada 1994). A characteristic distribution of Fe-, Mn- and CuZn-SOD among organisms indicates the hierarchical appearance of each isozyme reflecting the phylogenetic evolution, i.e. Fe-SOD appeared first, then Mn-SOD, a homologous protein with Fe-SOD, was derived from Fe-SOD, and CuZn-SOD was acquired more recently. Ni-SOD, which is evolutionarily unrelated to other SODs, is found in some bacteria but not in ani-

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Abbreviations: *SpMnsod-L, Spirogyra* land plant-type Mn-SOD gene; *SpMnsod-A*, *Spirogyra* algal-type Mn-SOD gene; SpMnSOD-L, gene product of *SpMnsod-L*; SpMnSOD-A, gene product of *SpMnsod-A*; ROS, reactive oxygen species; SOD, superoxide dismutase.

Accession numbers of cDNAs and genomic genes: *SpMnsod-L* cDNA (major, *sodA-1*), AB201832; *SpMnsod-A* cDNA (minor, sodA-2), AB201833; *SpMnsod-A* fragment (5' upstream, genomic), AB201834; *Pogonatum inflexum* Mn-SOD cDNA (*sodA*), AB201827; *Equisetum arvense* Mn-SOD cDNA (*sodA*), AB201830. *SpMnsod-L* genomic gene, AB244736; *SpMnsod-A* genomic gene, AB244737. Part of the present results have been presented elsewhere (Okayasu el al. 2005, Kanematsu 2005, Kurogi et al. 2005, Kanematsu et al. 2006).

mals and plants, showing no phylogenetic distribution.

Superoxide is produced mainly in electron transport chains of mitochondria and chloroplasts. Since superoxide is not able to penetrate membranes due to its negative charge, that produced inside organelles must be removed by organelle-localizing SOD. In land plants, chloroplasts contain chloroplastic CuZn-SOD isoform and/or Fe-SOD while mitochondria possess Mn-SOD. The cytoplasm of cells also contains cytosolic CuZn-SOD isoform but neither Fe-SOD nor Mn-SOD. Furthermore, the short lifetime of superoxide does not allow it to diffuse to a significant extent, so SOD must be localized where superoxide is produced. Proximal localization of chloroplastic CuZn-SOD to the generation sites on thylakoid membranes has been reported (Ogawa et al. 1995).

We have previously investigated the divergence of chloroplastic and cytosolic CuZn-SOD isoforms during the course of evolution, and cloned the cDNA and genomic genes of chloroplastic CuZn-SOD from the green alga *Spirogyra* (Kanematsu et al. 2010), which is located at an important evolutional position bridging from green algae to land plants in plant lineage (Becker and Marin 2009, Bhattacharya and Medlin 1998). In the cDNA cloning of *Spirogyra*, the degenerate primers we used, which were able to amplify both chloroplastic and cytosolic CuZn-SOD cDNAs from land plants including moss and fern, resulted in amplification of chloroplastic but no cytosolic CuZn-SOD cDNA. These results suggest that *Spirogyra* may be devoid of the cytosolic gene, or if any, it might be little expressed. Thus, it would be interesting to know what type of SOD is present in the cytosolic fraction of *Spirogyra* cells.

In the present work, we extended the survey of SOD isozymes in *Spirogyra*, cloned two types of cDNA and genomic gene of mitochondrial Mn-SOD isoforms and characterized the genes and their recombinant proteins. The results show that both Mn-SOD genes and their products differ in exon-intron structure and in quaternary structure, respectively. Furthermore, both types of Mn-SOD seem to be contained in eukaryotic algae while one type is not found in land plants; the implication of this is discussed with respect to the molecular evolution of Mn-SOD in plant lineage. Finally, we discuss the function of the intron-retained splicing variant of algal-type Mn-SOD.

MATERIALS AND METHODS

Materials. Isogen, DNeasy Plant Maxi Kit, Quantum Prep Plasmid Miniprep Kit and pGEM-T Easy Vector System I were purchased from Nippon Gene (Toyama, Japan), QIAGEN (Valencia, CA, USA), Bio-Rad (Hercules, CA, USA) and Promega (Madison, WI, USA), respectively. The SMART RACE cDNA Amplification Kit and Universal GenomeWalker Kit are products of Clontech (Palo Alto, CA, USA). LA-*Taq* was from Takara (Kyoto, Japan). pGEX-6P-2 expression vector, GSH-Sepharose 4B and PreScission Protease were products of Amersham Bioscience (Buckinghamshire, UK).

Spirogyra sp. was collected at Kougedani pond, Shibushi city, Kagoshima prefecture, Japan and cultured as described previously (Kanematsu et al. 2010). The moss *Pogonatum inflexum* and the fern *Equisetum arvense* were collected in the Takanabe campus of Minami-Kyushu University, Miyazaki, Japan as described previously (Kanematsu et al. 2011).

Isolation of mRNA and genomic DNA. Isolation of mRNA and genomic DNA was conducted as described before (Kanematsu et al. 2010, 2011). Genomic DNA of *Spirogyra* was obtained using the DNeasy Plant Maxi Kit (QIAGEN) and those of *P. inflexum* and *E. arvense* were isolated using Isoplant (Nippon Gene).

cDNA cloning: Amplification of partial cDNA fragments by PCR. cDNA cloning was conducted by two successive steps of PCR, i.e. PCR amplification for the central portion of cDNA with degenerate primers designed from portions of amino acid sequences, and then 5'- and 3'-RACE with non-degenerate primers designed from nucleotide sequences of the central portion of the cDNA. Partial cDNA fragments were amplified by PCR using degenerate primers from a cDNA pool that was prepared by the SMART RACE cDNA Amplification Kit (Clontech). The following degenerate primers were used for partial cDNA amplification: Mn/Fe-SOD antisense primer, 5'-ARRTARTANGCRTGYTCCCA-3' (Mn-Fe AS); Mn-SOD sense primers, 5'-GGNGGNCAYATHAAYCAY-3' (M-Ile), 5'-GGNGGNCAYGTNAAYCAY-3' (M-Val), 5'-GGNGGNCAYTAYAAYCAY-3' (M-Tyr); Fe-SOD sense primers, 5'-AAYGCNGCNCARGTNTGGA-3' (F-Val), 5'-AAYGCNGCNCARGCNTGGA-3' (F-Ala). The antisense primer was designed on the basis of the highly conserved region (DVWEHAYYL) among plant Mn- and Fe-SODs. The sense primers were taken from the regions distinguishable for Mn-SOD (NGGGH(I/V/T)NHS) and Fe-SOD (NNAAQ(V/A)WNH). Amplification conditions were the same as before (Kanematsu et al. 2010). The amplified fragments were ligated into pGEM-T EZ and sequenced.

cDNA cloning: 5'- and 3'-RACE. Amplification by 5'- and 3'-RACE using the SMART RACE cDNA Amplification Kit was conducted as previously described (Kanematsu et al. 2010, 2011). The following gene-specific primers were used for 5'- and 3'-RACE of *Spirogyra* Mn-SOD cDNAs: for *SpMn-2* (renamed as *SpMnsod-A*) cDNA, SPM318 5GSP-1 (5'-upstream antisense) 5'-GGATTGTCCTGGTTCATTGTGGTCTCG-3' and SPM318 3GSP-1 (3'-downstream sense) 5'-CGAAGACGAGTTCCAATCCGTTGATG-3'; for *SpMn-1* (renamed as *SpMnsod-L*) cDNA, SPM264MJ 5GSP-1 (5'-upstream antisense) 5'-GCAGTTGTCTGAACGGCAAGGCG-3' and SPM264MJ 3GSP-1 (3'-downstream sense) 5'-CCGATTAGCCAAGGGGGAGGAGAAC-3'.

The gene-specific primers used for 5'- and 3'-RACE of *P. inflexum* and *E. arvense* Mn-SOD cDNAs were as follows: for the *P. inflexum* cDNA, POM5GSP-1 (5'-upstream antisense) 5'-CGTCCTGGTTAGCAGTTGTCTCCACCG-3' and POM3GSP-1 (3'-downstream sense) 5'-TGCCAAAATGAACGCAGCAGGTG-3'; for the *E. arvense* cDNA, EQM5GSP-1 (5'-upstream antisense) 5'-TGGCAGTCGGCTCCACAACAAGC-3' and EQM3GSP-1 (3'-downstream sense) 5'-TGCTGTTGGTACTGCTGTTCAAGGCTC-3'. For amplified DNA fragments in 5'-RACE, the clones having the largest insert were selected by insert check with PCR after cloning into vectors and used for further analysis. Full-length cDNAs were constructed with central sequences and 5'- and 3'-franking sequences.

Cloning of genomic genes: Amplification of central portion. Two *Spirogyra* Mn-SOD genomic genes were obtained by each combination of three sequences, i.e.

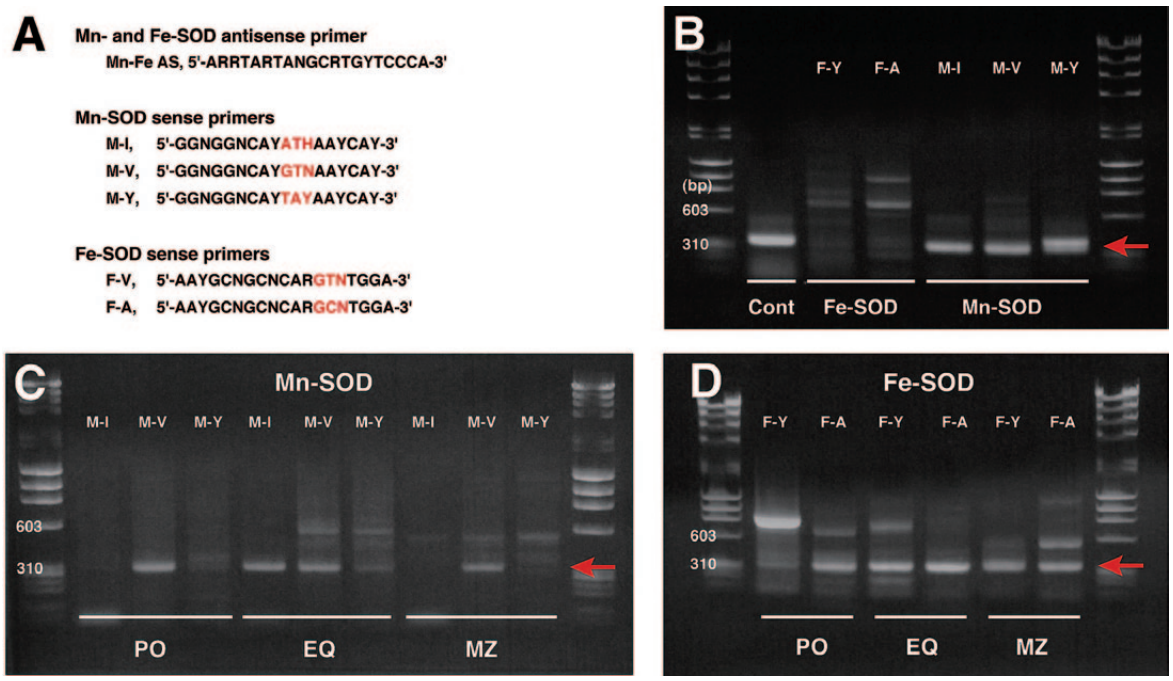

Fig. 1. Discriminating amplification of Mn- and Fe-SOD cDNA fragments from *Spirogyra* sp., *P. inflexum*, *E. arvense* and maize with degenerate primers. The cDNA fragments of Mn- and Fe-SOD were amplified by PCR using Mn-SOD specific sense primers (M-I, M-V and M-Y) and Fe-SOD specific sense primers (F-V and F-A), respectively, with the common antisense primer (Mn-Fe As) for both isozymes. (A) Degenerate primer sets for Mn- and Fe-SOD cDNAs. (B) Mn-SOD cDNA fragment amplification from *Spirogyra* sp. Cont, CuZn-SOD cDNA fragment (310 bp) with the degenerate primers for CuZn-SOD cDNA (Kanematsu et al. 2010); Fe-SOD and Mn-SOD, Fe- and Mn-SOD cDNAs, respectively. (C) and (D) Amplification for Mn- and Fe-SOD cDNA fragments, respectively. PO, *P. inflexum*; EQ, *E. arvense*; MZ, maize. Arrows indicate the expected fragments.

central portion and two adjacent 5'-upstream and 3'-downstream portions of the gene as previously described (Kanematsu and Fujita 2009). Each central portion was amplified by PCR using LA-*Taq* (Takara) with gene-specific primers based on the cDNA sequences, and each 5'-upstream and 3'-downstream portion was obtained using the Universal GenomeWalker Kit (Clontech) with gene-specific primers designed from the sequence of the central portion. LA-*Taq* was used for PCR-amplification in RACE.

The following primers were used for the amplification of the central portion of the *Spirogyra* genes: sense and antisense primers for *SpMnsod-A*, SPMcmdGC F26 (sense) 5'-CGGCATTGCCTTACGATTACAACGCTCTTGAGC-3' and SPMcmdGC B652 (antisense) 5'-ACTCCACGACTTTGTCCCAGTTCACCACGTTC-3'; for *SpMnsod-L*, SPMmarGC F315 (sense) 5'-ACTCCGCTCTGGAACCCGTTATCAGTGGGG-3' and SPMmarGC B879 (antisense) 5'-TTGCTGGATTCATACCTTTCGCCAACGTCTTTC-3'. PCR was conducted in the 7600 mode of the GeneAmp 9700 under the following conditions: 94°C for 1 min, 35 cycles of 98°C for 10 s and 68°C for 10 min, and 72°C for 10 min. The amplified fragments of 1 kbp (*SpMnsod-A*) and 1.5 kbp (*SpMnsod-L*) were cloned and sequenced as described before (Kanematsu and Fujita 2009). At least four clones for each portion of the gene were used for sequencing.

Cloning of genomic genes: Amplification of the 5'-upstream and 3'-downstream portions. Construction of pools of adaptor-ligated genomic DNA fragments (referred to as GenomeWalker *Dra* I, *Eco* RV, *Pvu* II and *Stu* I libraries) from which DNA fragments adjacent to the central sequence of the gene were amplified, and PCR of each library using the gene-specific primers were performed as before (Kanematsu et al. 2010). The following primers were used for 5'-upstream and 3'-downstream GenomeWalker amplification of the *Spirogyra* genes. For 5'-upstream amplification: for *SpMnsod-A*, SPM5GW-1 (outer antisense) 5'-AGTTCGTGTATCGGCTTCTGGATGTCG-3' and SPM5GW-2 (inner antisense) 5'-AAGAGCGTTGTAATCGTAAGGCAATGC-3';for *SpMnsod-L*, SPMmar5GW-1 (outer antisense) 5'-AAGGCGGCATTGAAATTCGTCACATAC-3' and SPMmar5GW-2 (inner antisense) 5'-TCTCCATAATTTCCCCACTGATAACGG-3'. For 3'-downstream amplification: for *SpMnsod-A*, SPMcmd3GW-1 (outer sense) 5'-AGAACTCCGCATCAAAATCCCAAGTTG-3' and SPMcmd3GW-2 (inner sense) 5'-ACCTATCTTACGAGAACCGCAAGGTCG-3'; for *SpMnsod-L*, SPMmar3GW-1 (outer sense) 5'-ACCCTTTGGTAACCACTGGCTTGGTTC-3' and SPMmar3GW-2 (inner sense) 5'-GGAATTGACGTTTGGGAACATGCTTAC-3'. The adaptor primers, AP1 and AP2 (Clontech), were used as outer and inner (nested) primers, respectively.

Constructs, overexpression and purification of two recombinant Mn-SODs. To construct expression vectors for two types of Mn-SOD, we used each coding sequence for mature protein, excluding each transit peptide sequence. For recombinant SpMnSOD-A, a coding sequence was obtained by PCR with LA-*Taq* from the *Spirogyra* cDNA library for 5'-RACE using the following primers: SPMcmdEXF#1 (sense) 5'-AAGAATTCAGCTCGGAGCACTTCAGAAGATG-3' and SPMcmdEXB#1 (antisense) 5'-CAAGGCATGGTCGACGACGCTCG-3'. The sense primer was designed to amplify the exon 1-spliced cDNA (see below) and to make eight amino acid residues, IQLGALQK, derived from transit peptide as a linker between glutathione S-transferase (GST) in vector and the second ATG codon of the mature protein. The *Eco* RI site was included at the 5' end of the sense primer. The antisense primer was designed to be located from the 10th to 32nd nucleotide position after the stop codon in 3' UTR and to include the *Sal* I site by changing one nucleotide at the middle of the primer. The coding sequence of SpMnSOD-L was also obtained as above using the following primers: SPMmarEXF#1 (sense) 5'-AAGAATTCGGCTTGCCTCCACTGCGGTTTTGC-3' and SPMmarEXB#1 (antisense) 5'-TTGTCGACAGATGTCGGGGTTGGTAGAGAGC-3'. The sense primer adds two amino acid residues, IA, adjacent to the N-terminus Leu at a possible cleavage site.

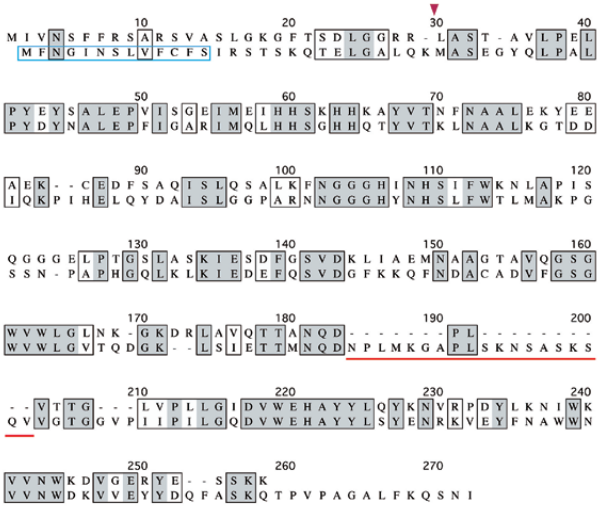

Fig. 2. Deduced amino acid sequences of two types of Mn-SOD from *Spirogyra*. The amino acid sequences of both algal-type (lower sequence) and land plant-type (upper sequence) Mn-SODs were compared by alignment. Identical (in dark gray background) and homologous (pale gray) amino acid residues are boxed. The compensated 13 residues of the algal-type SOD from the genomic gene are boxed by the blue line. An extra insertion in the algal-type SOD is underlined in red. The closed upside-down triangle shows the estimated cleavage sites for the mature proteins of both SODs.

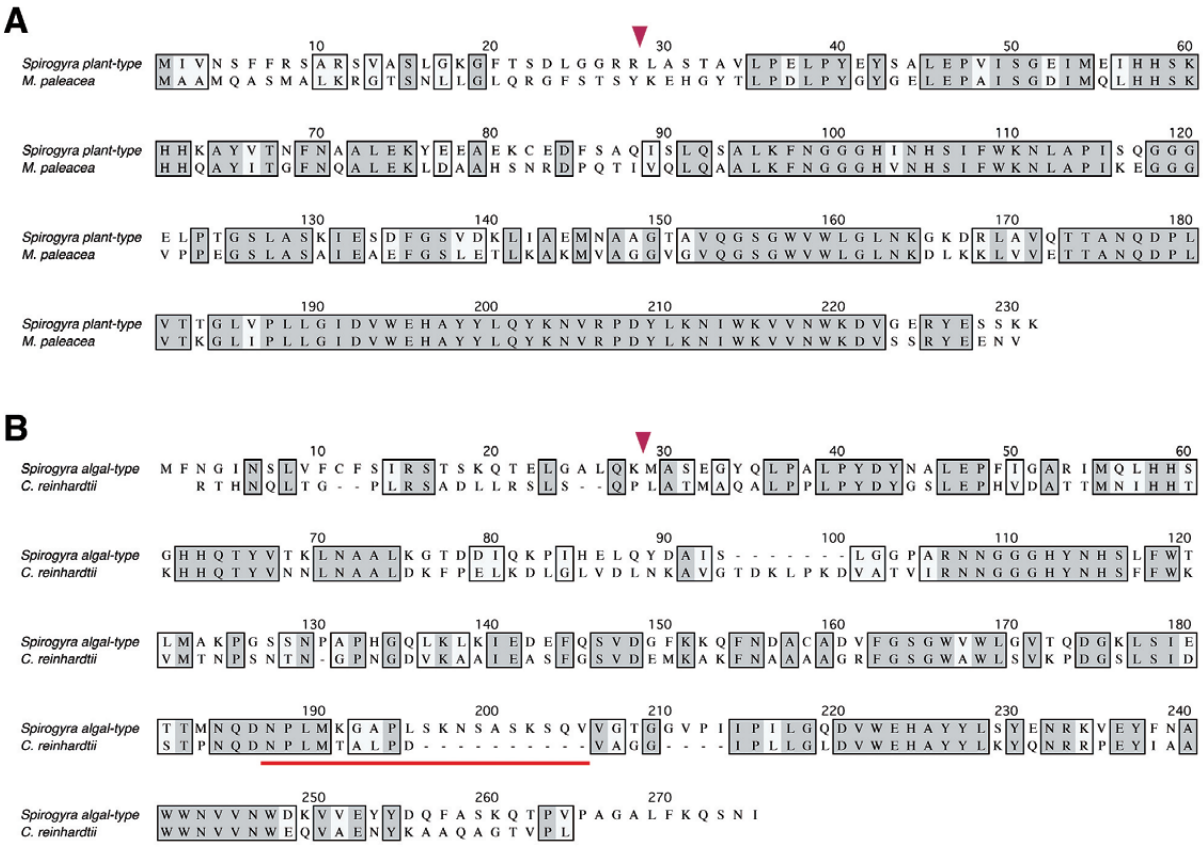

Fig. 3. Amino acid sequence comparisons between (A) *Spirogyra* land plant-type and *M. paleacea* Mn-SODs and between (B) *Spirogyra* algal-type and *C. reinhardtii* Mn-SODs. The amino acid sequences of land plant-type and algal-type *Spirogyra* Mn-SODs were aligned with those of *Marchantia paleacea* (BAD 13494) and the green alga *Chlamydomonas reinhardtii* (AAA80639) by the ClustalW program. The closed upside-down triangles show the estimated cleavage sites for the mature SODs. The extra insertion in the *Spirogyra* algal-type SOD is shown by a red bar.

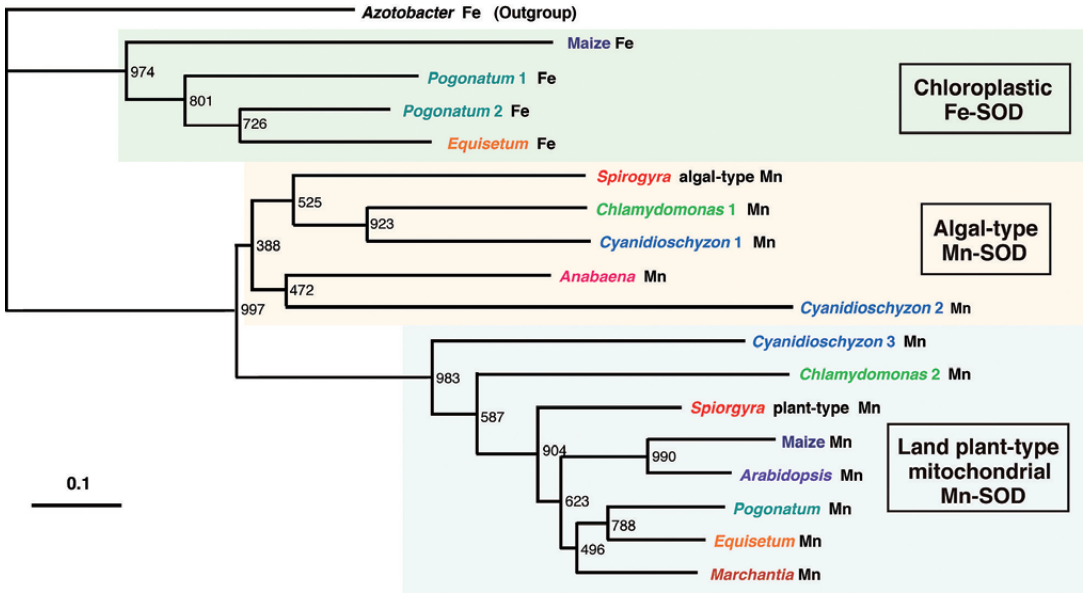

Fig. 4. Phylogenetic relationships among Mn-SODs from algae and land plants. The amino acid sequences from Mn- and Fe-SOD cDNAs of cyanobacteria, red and green algae, mosses, ferns and higher plants were aligned by ClustalW. Transit peptides for mitochondria and chloroplasts were excluded for ClustalW. The phylogenetic tree was constructed with the neighbor-joining method using the distance matrix of Kimura and bootstrapping of 1,000 replications. The bootstrap values are indicated in the right of each node. The tree is rooted by using *Azotobacter vinelandii* Fe-SOD as an outgroup. The clades of chloroplastic Fe-SODs, algal Mn-SODs and land plant mitochondrial Mn-SODs are shown in pale green, brown and blue backgrounds, respectively. Fe-SODs are as follows: maize, AB201543; *Equisetum* and *Pogonatum* 1 and 2, unpublished sequences (Kanematsu). Mn-SODs are as follows: *Anabaena*, 1GV3; *Arabidopsis*, At3g10920; *Chlamydomonas* 1, U24500; *Chlamydomonas* 2, Q9XS41; *Cyanidioschyzon* 1, CMN023C; *Cyanidioschyzon* 2, CMT028C; *Cyanidioschyzon* 3, CMR158C; *Equisetum*, AB201830; maize, M33119; *Marchantia*, BAD13494; *Pogonatum*, AB201827; *Spirogyra* algal-type, AB201833; *Spirogyra* land plant-type, AB201832.

The antisense primer is located from the 12th position after the stop codon in 3'-UTR. The sense and antisense primers included the *Eco* RI site at the 5' end and the *Sal* I site at the 3' end, respectively.

The amplified fragments were once ligated into pGEM-T EZ vector and transformed with *E. coli* XL1-Blue MRF'. Clones having the correct sequence were selected after sequencing, digested with *Eco* RI and *Sal* I and ligated into the *Eco* RI and *Sal* I sites of pGEX-6P-2 expression vector (Amersham Bioscience). The GST-fused Mn-SOD recombinant vectors were expressed in *E. coli* BL21.

The cells harboring the recombinant vectors were precultured in LB/amp media overnight at 37°C with vigorous shaking, and 4 ml of cells were inoculated into 200 ml of fresh LB/amp media and grown at 37°C for another one hour to $OD_{600\,nm}$ of 0.8. After cooling down the cultures to 25°C, each of the GST-fused *Spirogyra* Mn-SOD was induced with 0.04 mM IPTG at 25°C for 6 hours. The cells were collected by centrifugation at 4,000 × *g* for 5 min and stored at −20°C until use.

The frozen cells were thawed in extraction buffer consisting of 50 mM potassium phosphate, pH 7.8, 0.5 mM EDTA and 150 mM NaCl and disrupted by sonication for 5 min on ice. The cell extract was obtained by centrifugation at 30,000 × *g* for 20 min at 4°C. The cell extract was passed through a column of GSH-Sepharose pre-equilibrated with the extraction buffer and the adsorbed GST-fused Mn-SOD was eluted with 10 mM GSH in 50 mM Tris HCl, pH 8.0. After removal of GSH by dialysis against a buffer consisting of 50 mM Tris HCl, pH 7.0, 1 mM EDTA, 150 mM NaCl and 1 mM DTT, the fused Mn-SOD was cleaved from GST with PreScission protease (Amersham Bioscience), and then the protease was removed by passing through a column of GSH-Sepharose. Each SOD was further purified to near homogeneity by gel-filtration of Superdex 75 and ion-exchange chromatography of Mono Q.

Modeling of three-dimensional structure of Mn-SODs. The three-dimensional structures of *Spirogyra* Mn-SODs were predicted from their deduced amino acid sequences by a homology modeling method using the CPHmodels-2.0 Server (http://www.cbs.dtu.dk/services/CPHmodels-2.0/) with *Deinococcus radiodurans* Mn-SOD (1Y67) and Human Mn-SOD (1NOJ) as modeling templates for algal- and land plant-type Mn-SODs, respectively. Structures were rendered in MacPyMOL.

Other methods. DNA sequencing and other DNA and RNA manipulations were conducted as described previously (Kanematsu and Sato 2008, Kanematsu and Fujita 2009, Kanematsu et al. 2010 and 2011). SOD activity assay, protein determination, native-polyacrylamide gel electrophoresis (PAGE), SDS-PAGE, and SOD activity staining on gel were performed as before (Kanematsu and Asada 1990, Ueno and Kanematsu 2007).

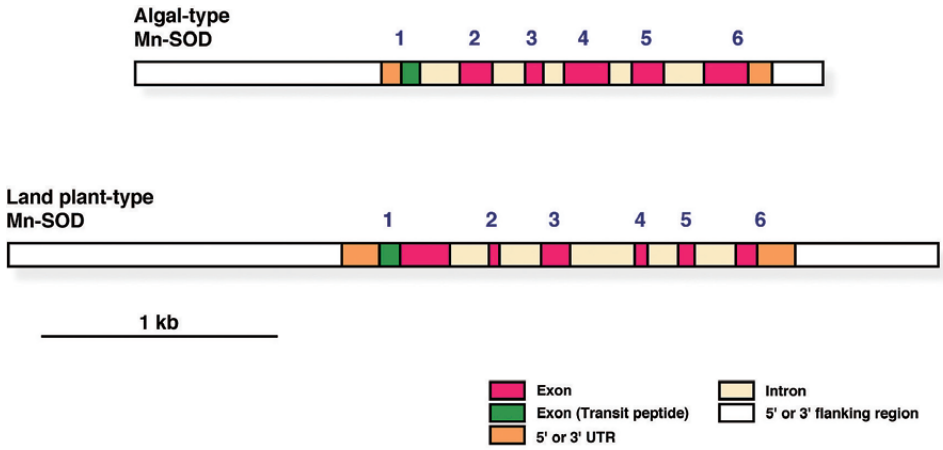

Fig. 5. Comparison of exon-intron structure of algal- and land plant types of Mn-SOD genes from *Spirogyra*. The exon-intron structure of the algal-type gene of 2,944 bp and the land plant-type gene of 3,980 bp are schematically drawn. The exon number is indicated on each gene.

RESULTS

Degenerate primers designed to amplify either Mn- or Fe-SOD cDNA fragments exclusively from algae, moss, fern and higher plants. To clone cDNAs encoding possible Mn- and/or Fe-SODs of *Spirogyra* by PCR, we employed the strategy that discriminates both isozymes from the initial step of the cloning procedure. Degenerate primer sets were designed based on highly conserved and diverse regions of amino acid sequence among plant Mn- and Fe-SODs (Fig. 1A). PCR using the primer sets for Mn-SOD resulted in amplification of Mn-SOD cDNA fragments from cDNA libraries of the green alga *Spirogyra* as well as from those of the moss *Pogonatum inflexum*, the fern *Equisetum arvense* and maize, but no Fe-SOD cDNA fragment (Fig. 1B and C). On the other hand, the primer sets for Fe-SOD amplified Fe-SOD cDNAs from *P. inflexum*, *E. arvense* and maize but not Mn-SOD cDNAs (Fig. 1D). Thus, the degenerate primers we used here have high specificity to discriminate both types of isozyme gene from plants including algae and lower plants.

Algal- and land plant-types of Mn-SOD cDNAs from *Spirogyra*. Using the degenerate primers for Mn-SOD, two cDNA fragments of 264 bp (without primers, *SpMn-1* fragment) and 318 bp (*SpMn-2* fragment) were obtained from *Spirogyra*, but no fragment of Fe-SOD cDNA was amplified with the degenerate Fe-SOD primers (Fig. 1B), suggesting the absence of the Fe-SOD gene or no expression of the enzyme in *Spirogyra*. In RACE reactions, we obtained *SpMn-1* cDNA fragments of 681 bp by 5'-RACE and 520 bp by 3'-RACE with overlapping regions of 127 bases, and *SpMn-2* cDNA fragments of 476 bp (5'-RACE) and 532 bp (3'-RACE) with overlapping regions of 91 bp. By assembling the sequences, finally we obtained *SpMn-1* cDNA of 1,074 bp and *SpMn-2* cDNA of 917 bp. Thus, it is evident that *Spirogyra* contains two genes of Mn-SOD isoforms.

SpMn-1 cDNA showed a complete ORF and contained a sequence for a mitochondrial transit peptide. Thus, *SpMn-1* encoded mitochondrial Mn-SOD. The deduced amino acid sequences consisted of 231 residues. The mature enzyme contained 203 residues, assumed by comparison with N-terminal sequences of other Mn-SOD (Fig. 2). *SpMn-2* cDNA contained an incomplete ORF consisting of 255 amino acid residues. The incomplete ORF was compensated by the genomic sequence as described below and the missing sequence of 13 residues was deduced. Thus, *SpMn-2* encodes a protein consisting of 263 amino acid residues. The absence of introns in the compensated sequence was confirmed by sequencing of the genomic gene (see below). *SpMn-2* also contained a sequence for mitochondrial transit peptide, and mature protein was estimated to contain 235 residues (Fig. 2).

By homology search, we found that *SpMn-1* cDNA most resembled that of the moss *Marchantia paleacea* (Sakaguchi et al. 2004) while *SpMn-2* cDNA resembled that of the green alga *Chlamydomonas reinhardtii* (Kitayama et al. 1995) (Fig. 3). Therefore, we refer hereafter to *SpMn-1* and *SpMn-2* as land plant-type gene (*SpMnsod-L*) and algal-type gene (*SpMnsod-A*), respectively.

Mn-SOD cDNAs from moss and fern. To compare *Spirogyra* Mn-SOD cDNAs with those of lower plants, the cloning of Mn-SOD cDNAs from *P. inflexum* and *E. arvense* was completed by 5'- and 3'-RACE for the flanking region of the core fragments (both 264 bp without primers) obtained above (Fig. 1C). Finally, we obtained *P. inflexum* Mn-SOD cDNA of 1,097 bp by assembling the upstream sequence (656 bp) by 5'-RACE and the downstream sequence (551 bp) by 3'-RACE with an overlapping region of 110 bp, and *E. arvense* Mn-SOD cDNA of 1,031 bp by combining the 5'-RACE product of 692 bp and the 3'-RACE product of 428 bp with 89-base overlap. Both cDNAs contained complete ORF and the deduced amino acid sequences for *P. inflexum* and *E. arvense* Mn-SODs consisted of 229 and 232 residues, respectively. Alignment of amino acid sequences of both Mn-SODs with those of other plant Mn-SODs clearly showed the presence of mitochondrial transit peptide (data not

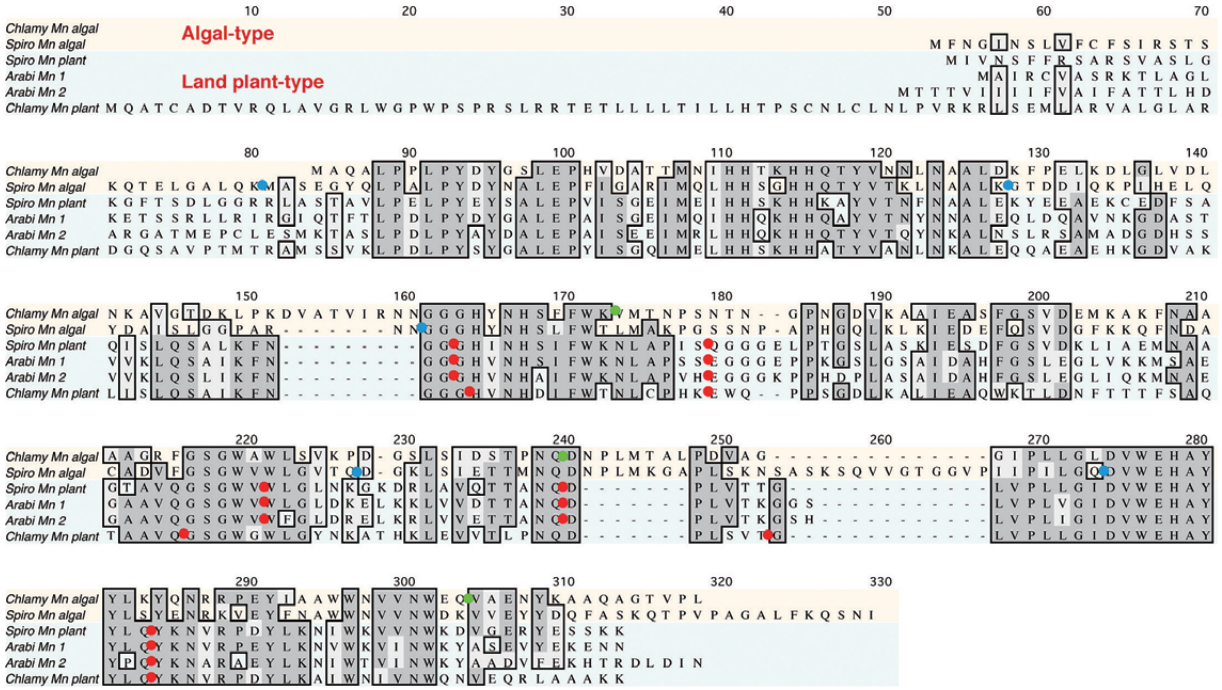

Fig. 6. Comparison of intron positions on amino acid sequences of Mn-SODs from *Spirogyra*, *C. reinhardtii* and *A. thaliana*. The amino acid sequences of the two *Spirogyra* Mn-SODs were aligned with those of *C. reinhardtii* and *A. thaliana* by ClustalW. Blue and green dots indicate intron positions of the algal-type Mn-SODs of *Spirogyra* and *C. reinhardtii* (U24500), respectively. Red dots show the intron sites of land plant-type Mn-SODs of *Spirogyra*, *C. reinhardtii* (Q9XS41) and *A. thaliana*. *Arabi Mn 1*, *A. thaliana* Mn-SOD (At3g10920); *Arabi Mn 2*, *A. thaliana* Mn-SOD (At3g56350).

shown). Thus, the *P. inflexum* and *E. arvense* Mn-SODs are mitochondrial SODs.

Phylogenetic analysis of two types of Mn-SOD from *Spirogyra*. A phylogenetic tree was constructed with the deduced amino acid sequences of Mn-SOD and Fe-SOD cDNAs from the algae and land plants along with the sequences from the whole genomes of the red alga *Cyanidioschyzon merolae* (Matsuzaki et al. 2004) and the green alga *Chlamydomonas reinhardtii* (Merchant et al. 2007) (Fig. 4). The tree clearly shows the occurrence of two types of Mn-SOD: algal-type Mn-SOD and land plant-type mitochondrial Mn-SOD. The land plant-type Mn-SOD is contained in eukaryotic algae as well as land plants while the algal-type is contained in prokaryotic and eukaryotic algae but not land plants. Fe-SOD forms another clade other than the two types of Mn-SOD.

Structure of *Spirogyra* Mn-SOD genomic genes. The genomic genes, *SpMnsod-A* and *SpMnsod-L*, were obtained by combining PCR-amplified DNA fragments for the central, upstream and downstream regions of the genes. PCR amplification for the central region of the *SpMnsod-A* and *SpMnsod-L* using gene specific primers based on the cDNA sequences gave fragments of 1,122 bp (with primers) and 1,484 bp, respectively. The upstream and downstream fragments adjacent to the central region were obtained by PCR from the GenomeWalker libraries, which are uncloned, adaptor-ligated genomic DNA fragment pools treated by restriction enzyme, using gene-specific primers based on the sequence of the central portions.

The 5'-upstream fragment of 3 kbp and 3'-downstream fragment of 2.0 kbp for the *SpMnsod-A* were amplified from the *Dra* I and *Eco*RV GenomeWalker libraries, respectively, and for the former, 1,442 bp (accession number, AB4231) at the 3'-side was sequenced and for the latter, 487 bp at the 5'-side. Finally, we determined a nucleotide sequence of 2,944 bp for the *SpMnsod-A*. For the *SpMnsod-L*, we amplified 5'-upstream (1.8 k) and 3'-downstream (2.5 k) fragments from the *Stu* I GenomeWalker library. The whole sequence of 1,756 bp for the 1.8-k fragment and the 5'-side sequence of 1,079 bp for the 2.5-k fragment were determined. By assembling the sequences of the three fragments, we revealed a nucleotide sequence of 3,980 bp for the *SpMnsod-L*.

Both the *SpMnsod-A* and SpMnsod-L are composed of six exons and five introns (Fig. 5). However, the positions of introns on amino acid sequences were completely different between those of the two types of Mn-SOD. Although the sequence of SpMnSOD-A closely resembled that of *C. reinhardtii* among other Mn-SODs, the intron positions differed from those of *C. reinhardtii*. On the other hand, the *SpMnsod-L* showed the same exon-intron structure as those of the two Mn-SODs of *Arabidopsis thaliana* (Fig. 6).

Intron-retained splicing variant of *Spirogyra* algal-type Mn-SOD transcript. In 5'-RACE of *SpMnsod-A* cDNA, we noted the presence of transcripts that contained an extra 164 bp sequence near the 5' end of cDNA, indicating the occurrence of alternative splicing. The 5'-upstream genomic sequence of *SpMnsod-A* clearly showed the occurrence of an intron of 164 bp adjacent to

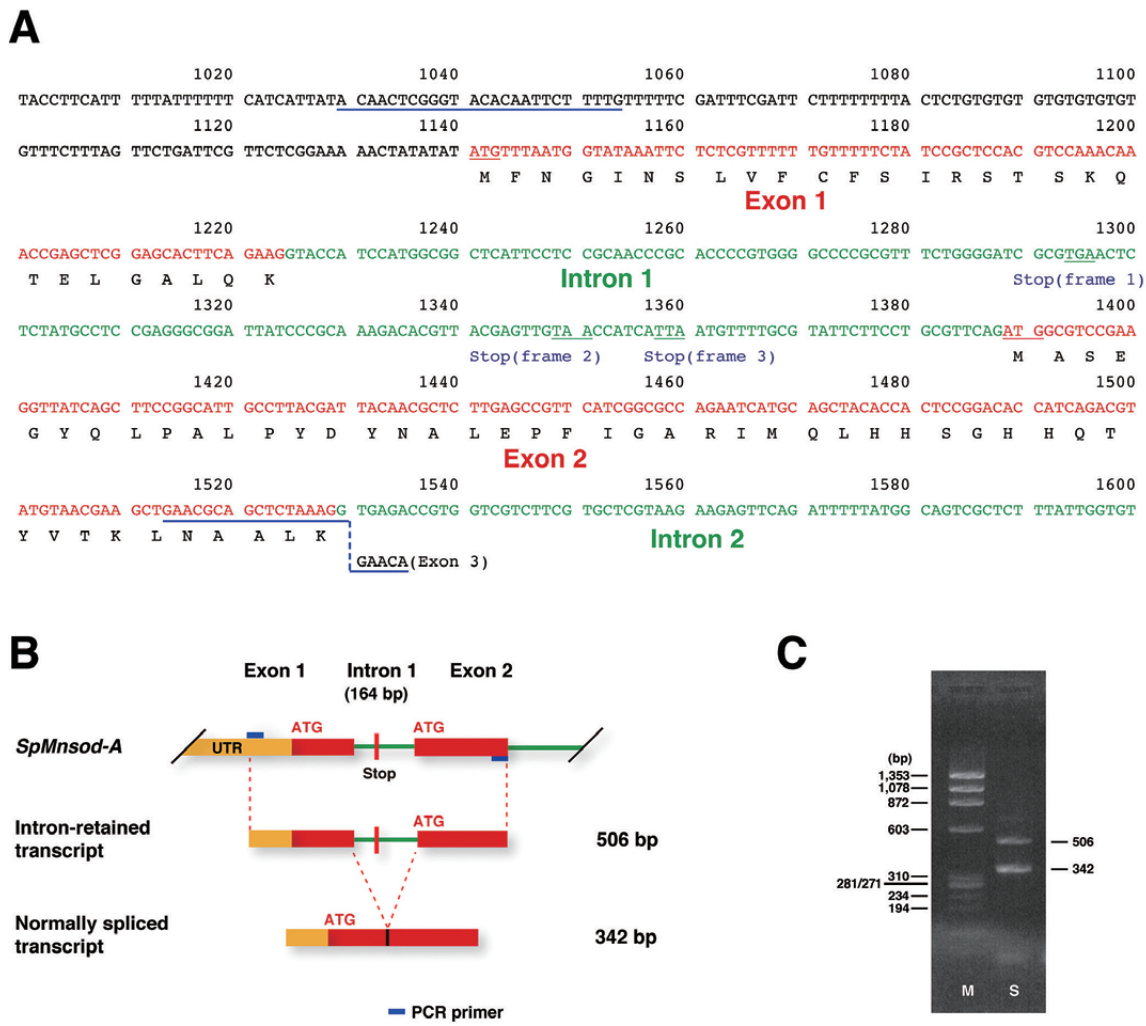

Fig. 7. Occurrence of two types of transcript from *SpMnsod-A* gene. (A) The 5'-upstream nucleotide sequence. The region from the base position at 1001 to 1600 is presented. Two possible ATG start codons, and three stop codons in forward frames within intron 1 are underlined. Primer sites (sense, SPMcmdF1 and antisense, SPMcmdB1) are indicated by blue lines. Note that five bases at 5' end of the antisense primer are derived from exon 3. (B) Schematic drawing of the splicing patterns of the 5'-upstream region. Primers are shown in blue bars. The transcript produced by splicing of the first intron results in a 342 bp fragment (with the primers). In case of intron retention, the transcript is 506 bp in length. (C) Abundance ratio of the two transcripts estimated by RT-PCR. Sense primer (SPMcmdF1), 5'-ACAACTCGGGTACACAATTCTTTTG-3'; antisense primer (SPMcmdB1), 5'-TGTTCCCTTTAGAGCTGCGTTC-3'.

the ATG codon (Fig. 7A) in exon 2. Exon 1 contains the first ATG and encodes a possible transit peptide for mitochondrial localization consisting of 26–28 amino acid residues. Thus, the transcript having the extra sequence was an intron-retained one while the transcript devoid of this sequence was a canonical one. Intron 1 contains stop codons in all three forward reading frames while the intron 1-spliced transcript encodes mitochondrial Mn-SOD with a signal sequence (Fig. 7B).

To determine the ratio of splicing variant derived from *SpMnsod-A*, RT-PCR using sense and antisense primers in 5'-UTR of exon 1 and in exon 2, respectively, was conducted. Figure 7C shows that the amount of the longer transcript (506 bp) retaining intron 1 was one third of that of the intron 1-spliced transcript (342 bp).

Overexpression and purification of recombinant Mn-SODs. The cDNA fragments encoding mature SpMnSOD-A and -L were ligated into pGEX-6P-2 expression vector and transformed with *E. coli* BL21. The regions of transit peptide were excluded. When induced with 1 mM IPTG at 37°C, most of the two types of GST-fused Mn-SODs were recovered in insoluble fractions. Lowering the temperature and IPTG concentration and prolonging the induction time produced adequate amounts of the fused SODs in soluble fractions. Finally, the GST-fused *Spirogyra* SODs were induced at 25ºC by 0.04 mM IPTG for 6 hours (Fig. 8). A single pass of the cell-free extracts through a column of GSH-Sepharose was not sufficient to purify the fused SOD, because chaperone proteins were contaminated. These contaminated proteins were removed by successive operation of gel filtration and ion-exchange chromatography after cleavage of the fused SOD by protease. The recombinant Mn-SODs were purified to near homogeneity on the basis of native- and SDS-PAGE (Fig. 9 and 10).

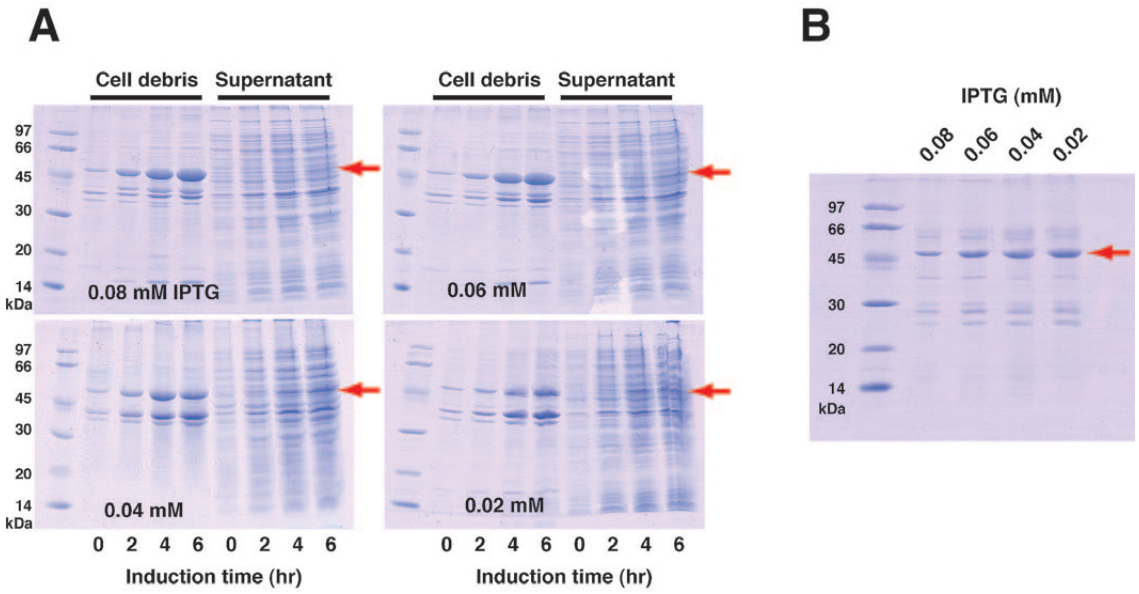

Fig. 8. Effect of different concentrations of IPTG on induction of recombinant SpMnSOD-A. *E. coli* cells harboring pGEX-6P-2-Mn that encodes algal-type Mn-SOD were pre-cultured for 18 h at 37°C, and then were induced by 0.02–0.08 mM IPTG at 25°C for up to 6 h with vigorous shaking. The cells were harvested at the indicated time, ruptured by sonication, and separated into supernatant and cell debris by centrifugation. Red arrows indicate the location of the recombinant SOD. (A) Recovery of the recombinant Mn-SOD in cell debris and supernatant. Aliquots of each fraction were subjected to SDS-PAGE, and proteins were stained by CBB. (B) GST-fused recombinant SOD obtained by GSH-Sepharose column chromatography. Aliquots of each supernatant obtained from cells induced by different concentrations of IPTG for 6 h at 25°C were applied to columns of GSH-Sepharose. Adsorbed GST-fused SOD was eluted by GSH and analyzed by SDS-PAGE.

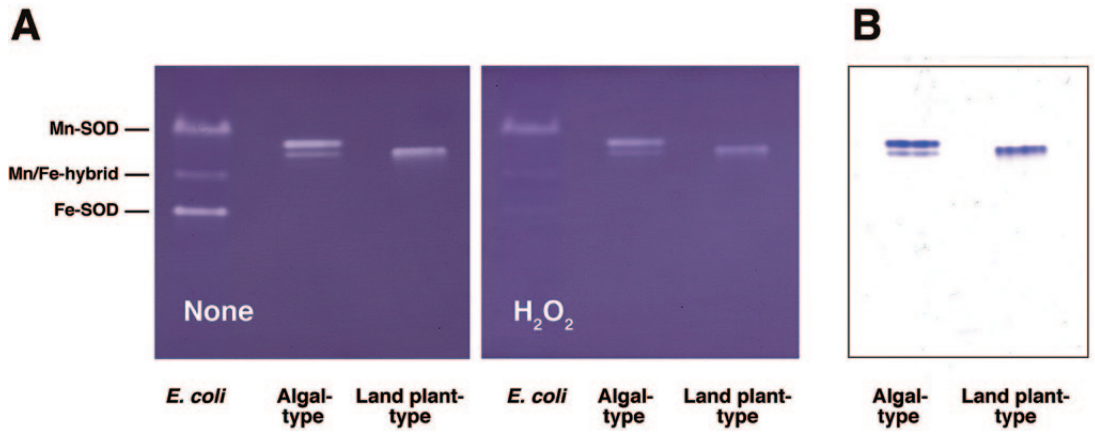

Fig. 9. Determination of metal type of the purified recombinant SODs by H_2O_2 treatment after native-PAGE. (A) Activity staining and H_2O_2 treatment. Left panel, none. Right panel, H_2O_2 treatment. 10 units of each of the purified recombinant SODs were used. H_2O_2 treatment was conducted by incubating a gel for 20 min in 10 mM H_2O_2 after electrophoresis. *E. coli* BL21 cell extract that contained Mn- and Fe-SODs and Mn/Fe-hybrid SOD was used as a control. Mn/Fe-hybrid, the hybrid SOD consisted of each subunit of Mn- and Fe-SODs. (B) Protein staining. 2 μg of each of the algal- and land plant-types of recombinant Mn-SODs were used.

Characterization of recombinant Mn-SODs. The purified recombinant Mn-SODs exhibited SOD activity, which was revealed by the cytochrome *c*-xanthine-xanthine oxidase assay system and activity staining after native-PAGE. The specific activities of both the enzymes were comparable judging from the gels for activity staining and protein staining in native-PAGE (Fig. 9).

The metal type at active sites of the recombinant Mn-SODs was confirmed by the response against inhibitor or inactivator on activity staining after native-PAGE. Both the recombinant SpMnSOD-A and -L were neither inhibited by 2 mM cyanide nor inactivated by 10 mM H_2O_2, suggesting that the SpMnSOD-A and -L are manganese-containing SODs (Fig. 9A).

The molecular masses of the recombinant SpMnSOD-A and -L were determined by gel filtration. The recombinant

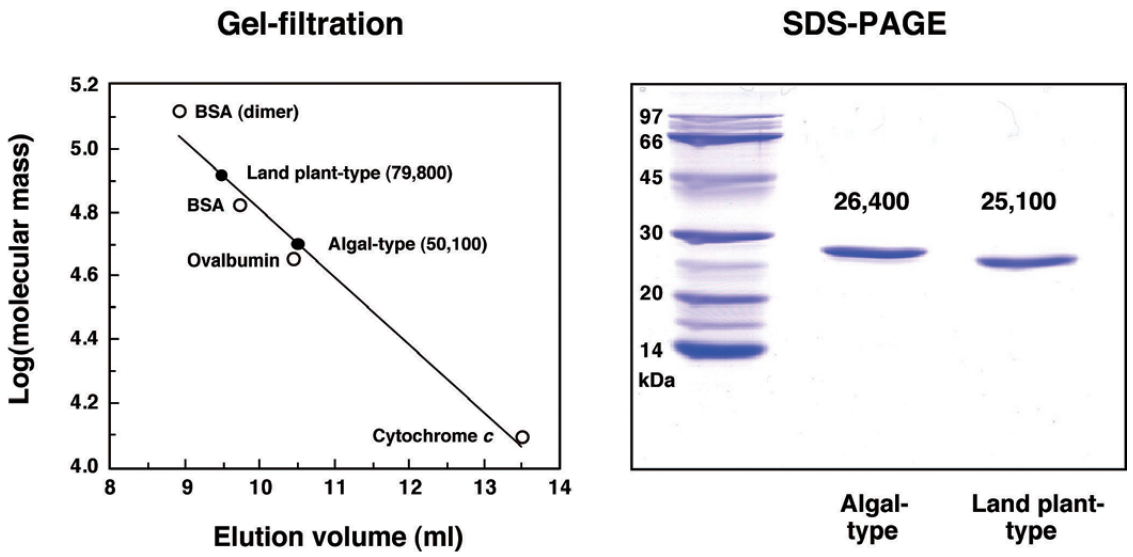

Fig. 10. Determination of molecular and subunit molecular masses of the two types of recombinant Mn-SODs. Left panel, molecular mass determination by gel-filtration. Gel-filtration was conducted using a Superdex G75 column fitted with FPLC. The column was calibrated with BSA, ovalbumin and cytochrome c. Right panel, subunit molecular mass determination by SDS-PAGE. 2 μg of each of the purified recombinant algal- and land plant types of Mn-SOD were electrophoresed. Proteins were stained with CBB.

SpMnSOD-A and -L gave molecular masses of 50,100 Da and 79,800 Da, respectively (Fig. 10). The subunit molecular masses of the recombinant SpMnSOD-A and -L were revealed to be 26,400 Da and 25,100 Da by SDS-PAGE (Fig. 10). Thus, it was suggested that the SpMnSOD-A was homodimer while the SpMnSOD-L was homotetramer.

Comparison of three-dimensional structure of two types of *Spirogyra* Mn-SOD. Since the amino acid sequences of SpMnSOD-A and SpMnSOD-L differed to some extent, the three-dimensional structures of their monomers were compared to examine structural differences. The modeling of the *Spirogyra* Mn-SODs was conducted by CPHmodels-2.0 Server using *Deinococcus radiodurans* Mn-SOD (1Y67) and human Mn-SOD (1N0J) as modeling templates, for the algal- and land-plant type, respectively (Fig. 11).

A prominent feature was noted in the region of the helices H1–H3: the H1–H3 of the SpMnSOD-A are shorter than those of the SpMnSOD-L (Fig. 11). In the SpMnSOD-A the H1 and H3 are connected with a short alpha helix (H2) while the corresponding helixes of the SpMnSOD-L are connected with a short turn.

DISCUSSION

Validity of the classification of Mn- and Fe-SODs by the degenerate PCR primers. The degenerate PCR primers used in the present study can discriminate between Mn- and Fe-SOD cDNAs in PCR amplification from total cDNA pools of land plants. With the primers targeted for Mn-SOD, Mn-SOD cDNAs were obtained from the moss *Pogonatum inflexum*, the fern *Equisetum arvense* and maize as well as *Spirogyra*. On the other hand, the primers for Fe-SOD amplified only Fe-SOD cDNAs from the same organisms except *Spirogyra* (Fig. 1). Thus, both degenerate PCR primers for Mn- and Fe-SODs are SOD metal-type specific.

The phylogenetic tree clearly shows that plant Mn- and Fe-SODs are widely separated even in lower plants such as mosses and ferns in an evolutionary sense (Fig. 4), suggesting that both the SOD can be distinguished on the basis of amino acid sequences. The "cambialistic" Mn/Fe-SOD that can use both Mn and Fe as a catalytic metal (Sugio et al. 2000) has not been detected in plants so far. Therefore, without direct analysis of metal at the active site of the enzymes, it may be safe to say that the cDNAs obtained with the primers for Mn-SOD are Mn-SOD cDNAs and with the primer for Fe-SOD are Fe-SOD cDNAs. The validity of the present classification of the SOD using the metal-type specific degenerate primers was confirmed by the response against H_2O_2 treatment with the recombinant *Spirogyra* Mn-SODs (Fig. 9) and *Pogonatum* Fe-SOD (Kanematsu, unpublished data).

Occurrence of two types of mitochondrial Mn-SOD gene in *Spirogyra* and its evolutional implication. A phylogenetic tree based on the amino acid sequences of Mn- and Fe-SODs showed three clusters: the land plant-type of mitochondrial Mn-SODs, algal-type of Mn-SODs and chloroplastic Fe-SODs (Fig. 4). The *Spirogyra* mitochondrial Mn-SOD of the land plant-type forms a clade with Mn-SODs of land plants including mosses, ferns and higher plants while the *Spirogyra* mitochondrial Mn-SOD of the algal-type belongs to a different clade which consists of Mn-SODs from prokaryotic and eukaryotic algae. It should be noted that the primitive red alga *Cyanidioschyzon merolae* (Matsuzaki et al. 2004), and the green alga *C. reinhardtii* (Merchant al. 2007), of which whole genomes were revealed, are found to contain the two types of Mn-SOD like *Spirogyra*. Considering the phylogenetic position of red algae in plant lineage, it

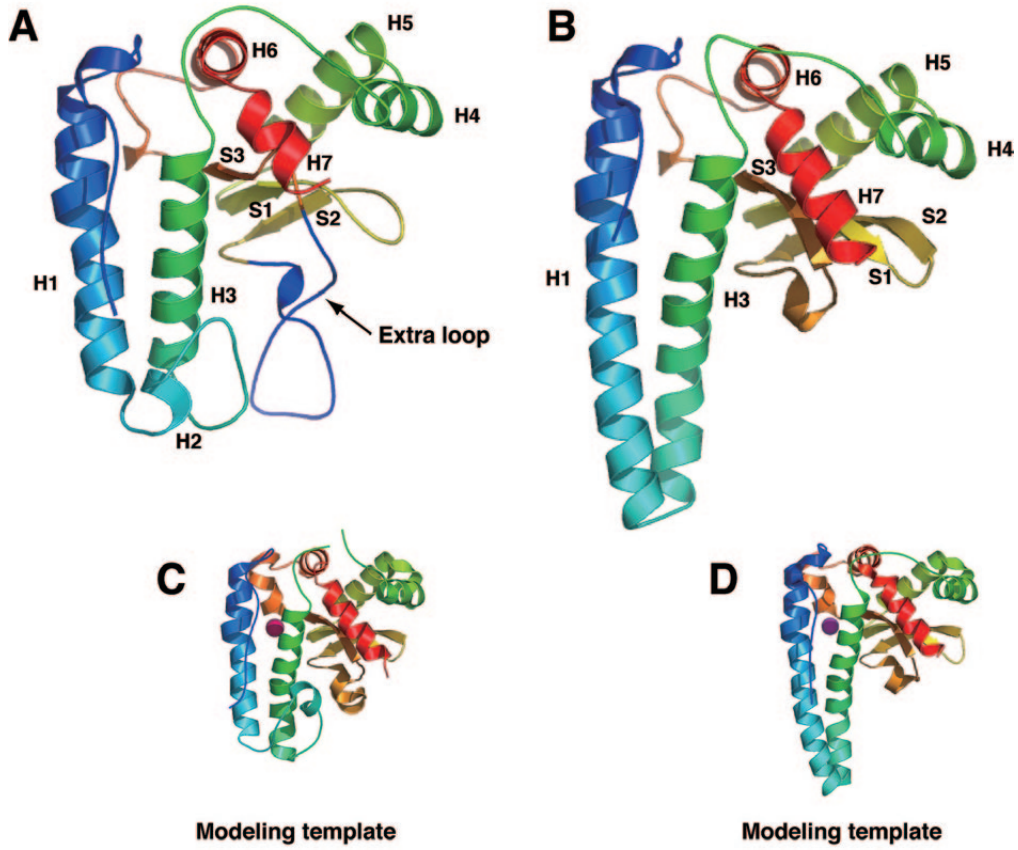

Fig. 11. Comparison of three-dimensional structures of two types of *Spirogyra* Mn-SOD. Each monomeric structure is presented. (A) SpMnSOD-A. H1-H7, α-helices; S1-S3, β-strands. The extra loop is indicated in blue. (B) SpMnSOD-L. The corresponding H2 is divided into two parts and involved in H1 and H3, respectively. (C) *Deinococcus radiodurans* Mn-SOD (1Y67) and (D) human Mn-SOD (1NOJ) as modeling templates for algal-type and land plant-type Mn-SODs, respectively.

seems that eukaryotic algae may contain the two types of Mn-SOD. The occurrence of the land plant-type in red algae and green algae suggests the early divergence of the land plant-type from ancestral Mn-SOD, probably before the divergence of prasinophyte algae (green algae). To the contrary, no algal-type of Mn-SOD is detected in land plants including mosses and ferns. The reason why only the land plant-type Mn-SOD was passed down to land plants but not the algal-type Mn-SOD in the course of evolution in plant lineage is obscure. Elucidation of the physiological function of both Mn-SODs may solve this problem.

Structural feature of the two types of *Spirogyra* Mn-SOD. The alignment of amino acid sequences of the two types shows the presence of a long insert in the algal-type (Fig. 2), which forms an extra loop between the β strands S1 and S2 in three-dimensional structure (Fig. 11). The extra loop is located at an open space in the L-shaped monomer of the algal-type, making the monomer more globular in shape. Since the recombinant algal-type possesses almost the same specific activity as that of the recombinant land-plant type, this insertion of a long loop might not affect the catalytic activity.

A prominent feature of the secondary structure between the algal- and land plant-type is noted in the first helix region: in the algal-type the H1 and H3 are connected with a short α helix, H2, while the helixes of the land plant-type are connected with a short turn (Fig. 11). Similar structural differences between the *Spirogyra* algal- and land plant-type Mn-SODs are also observed between Mn- and Fe-SODs from ferns, mosses and higher plants (data not shown). Fink and Scandalios (2002) reported that the H1 to H3 of four isoforms of maize Mn-SOD, which were the same in higher structure, differ from those of bacterial Mn-SODs.

Mitochondrial Mn-SODs in higher plants, i.e. the land plant-type, are tetramers while chloroplastic Fe-SODs are dimer (Kanematsu and Asada 1994). It is interesting to note that the land-plant type of *Spirogyra* Mn-SOD is tetramer while the algal-type is dimer (Fig. 10). Thus, it seems that the structure of the helices H1–H3 may determine the quaternary structure of Mn- and Fe-SOD in land plants.

Localization of SOD isoforms in *Spirogyra*. Several exceptions to the general rule of localization of SOD isozymes and isoforms in land plants as mentioned above have been reported. CuZn-SOD having an amino acid sequence of the chloroplast type was devoid of a transit peptide for chloroplasts and appeared in the cytosolic fraction of the moss *Marchantia paleacea* (Tanaka et al.

1996, 1998). Cytoplasm-localizing Fe-SODs of eukaryotes were found in radish (AF061583), cowpea root nodules (Moran et al. 2003) and *P. arvense* (Kanematsu, unpublished data). All these examples are products of genes without transit sequences.

Despite our efforts to determine which type of SOD participates in removing superoxide in the cytosolic fraction of *Spirogyra*, we could not detect any cytosolic Mn- or Fe-SOD. Cytosolic CuZn-SOD was not found in this organism, either. (Kanematsu et al. 2010). Since the superoxide produced in each compartment should be removed by SOD localized in each compartment due to the low membrane impermeability of superoxide, obviously, further work is needed. In addition to this, the chloroplastic Fe-SOD gene was not detected in *Spirogyra* by PCR. Thus, it seems that the Fe-SOD gene is also absent from this organism. Even so, chloroplast-localizing CuZn-SOD may play a role in scavenging superoxide in chloroplasts instead of Fe-SOD.

Function of intron-retained transcript from *SpMnsod-A*. We found an intron-retained splicing variant of the algal-type Mn-SOD transcript. The retention of intron 1 between exon 1 and exon 2 might result in the interruption of protein synthesis due to the occurrence of an in-frame stop codon. If the second ATG that is located at the 5' end of exon 2 acts as a start codon, the intron 1-retained transcript would produce Mn-SOD devoid of transit peptide, i.e. a cytosolic SOD (Fig. 7), but this is hardly the case. Dual targeting of proteins produced from a single gene to mitochondria and cytosol needs each capped transcript derived from the gene, because the capping of transcripts is essential for eukaryotic protein synthesis. In the present case in which the retained intron excluded a signal sequence encoded by the upstream exon by introduction of a stop codon, the mechanism by which the resulting intron-retained transcript obtains a capped sequence at a region downstream of the stop codon cannot be explained. Alternative trans-splicing which creates a long and a short spliced variant from a single gene and is responsible for dual targeting of products has been reported in Trypanosomes (Retting et al. 2011). Mn-SOD of the protozoa *Toxoplasma gondii* is targeted to both mitochondria and apicoplasts via a bipartite N-terminal targeting sequence (Pino et al. 2007).

The ratio of the level of the intron-retained transcript to that of the normally spliced transcript of *SpMnsod-A* was estimated to be one third (Fig. 7), indicating the high frequency of this type of alternative splicing in *SpMnsod-A*. Although intron-retained splicing is one of the rarest cases in humans (Ladd and Cooper 2002), 2-3% of *A. thaliana* genes conduct intron-retained splicing (Ner-Gaon et al. 2004). It is well known that splicing variants with stop codons introduced are subject to degradation by nonsense-mediated decay (NMD) (Stamm et al. 2005). Thus, the intron-retained *SpMnsod-A* transcript also would be degraded by the NMD mechanism. However, considering the high abundance of the intron 1-retained variant compared with the canonical one and the fact that no splicing variant was found other than the intron 1-retained one, it is suggested that *SpMnsod-A* conducts preferential splicing for unknown reasons. Thus, we postulate its regulatory function modulating the level of SpMnSOD-A. Further studies are currently underway in this regard.

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