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Characterization of CuZn-superoxide dismutase gene from the green alga *Spirogyra* sp. (Streptophyta): Evolutionary implications for the origin of the chloroplastic and cytosolic isoforms

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When organisms appeared on the earth's terrestrial surface, the ancestors of land plants needed to develop the ability to avoid the harmful action of reactive oxygen species (ROS). To elucidate the adaptation of superoxide dismutase (SOD) to oxidative stress during evolution, we examined the protein and gene, by purification and cloning, of CuZn-SOD from the eukaryotic alga *Spirogyra*, a streptophyte alga that led to the evolution of land plants.

The purified CuZn-SODs resembled those of land plants in respect of physicochemical properties including N-terminal amino acid sequence. A cDNA of the enzyme encoded a protein of 196 amino acid residues containing a transit peptide of 42 residues, thus *Spirogyra* possesses a gene for the chloroplastic CuZn-SOD isoform. This is the first direct evidence of the occurrence of the chloroplastic type of CuZn-SOD isoform in algae. The genomic gene consisted of nine exons as compared to eight for the chloroplastic genes of higher plants. Except the first intron, the remaining exon-intron structure of the *Spirogyra* gene was identical with those of higher plants in terms of splicing points, although the average length of intron for the *Spirogyra* gene was shorter than those of land plants, indicating a closer evolutionary relationship in green plant lineage. The organization of *cis*-elements in the promoter region of the *Spirogyra* gene resembled that of rice chloroplastic CuZn-SOD. The responsiveness of CuZn-SOD to Cu was also observed. Phylogenetic analyses of *Spirogyra* chloroplastic CuZn-SOD with recently available genomes of prasinophyte green algae indicated that the chloroplastic CuZn-SOD gene was derived from an ancestral cytosolic CuZn-SOD gene at an early phase in the evolution of prasinophyte algae.

Key words: eukaryotic algae, reactive oxygen species, *Spirogyra*, streptophytes, superoxide dismutase.

INTRODUCTION

The appearance of organisms on the earth's terrestrial surface was one of the most dramatic turning points in the evolution of life. The first conqueror of land, which is believed to have been a moss, and its descendants enlarged their habitats by developing the ability to adapt to new environments, even those with more oxygen, finally leading to present-day ecosystems (Bhattacharya and Medlin 1998, Becker and Marin 2009). During the evolution of life from water to land, adaptation to a terrestrial environment was crucial for survival. Compared to the aquatic environment, the terrestrial environment would result in increased production of reactive oxygen species (ROS), because under stress conditions such as dehydration, ultraviolet irradiation and high intensity of light, ROS are inevitably generated (Asada 1999, 2006). The ROS including superoxide, H_2O_2, hydroxyl radical, and singlet oxygen are toxic to all organisms by non-specifically oxidizing proteins, DNA and membranes (Fridovich 1995). Thus, ancestors of land plants needed to reinforce their ability to deal with ROS.

Superoxide dismutase (SOD) catalyzes the dismutation reaction of superoxide to H_2O_2 and molecular oxygen, and plays a role in protecting cells from oxidative damage caused by ROS (Fridovich 1995). SOD is a metalloenzyme and consists of four isozymes, i.e. CuZn-SOD, Mn-SOD, Fe-SOD and Ni-SOD. It is distributed ubiquitously in aerobes, and is even present in some anaerobes. In plants, CuZn-SOD consists of chloroplastic and cytosolic isoforms that are immunologically distinguishable by characteristics in amino acid sequences. Mn-SOD is a mitochondrial enzyme, whereas Fe-SOD, a paralogous protein of Mn-SOD, is a chloroplast enzyme although its gene is not necessarily expressed (Kanematsu and Asada 1994). Ni-SOD (Youn et al. 1996) has not been reported from plants.

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The abbreviations used are: PPFD, photosynthetic photon flux density; ROS, reactive oxygen species; SOD, superoxide dismutase; SOD-I, CuZn-SOD isozyme I; SOD-III, CuZn-SOD isozyme III.

Green plants (Viridiplantae) form a monophyletic lineage and consist of all green algae and land plants (embryophytes) including bryophytes, pteridophytes and spermatophytes. Land plants are thought to have evolved from some green algae (charophytes) in the course of evolution 450 million years ago. Thus, green algae can be divided into two groups on the basis of cytological and molecular criteria such as the mode of cell division and the enzyme for glycolate oxidation: chlorophyte algae (e.g. *Chlamydomonas*) and charophyte algae (e.g. *Nitella* and *Spirogyra*). The former are characterized by phycoplast formation and glycolate dehydrogenase, and the latter by phragmoplast formation and glycolate oxidase. Since charophytes are close relatives of embryophytes in a green plant lineage, charophytes and embryophytes are subsumed under the term streptophytes (Becker and Marin 2009). Chlorophyte algae and streptophyte algae form a sister clade with each other in a green plant lineage.

Most eukaryotic algae such as red, brown and green (chlorophytes) algae, diatom and *Euglena* lack CuZn-SOD (Asada et al. 1977) but charophyte green algae including *Spirogyra* (Kanematsu and Asada 1989b), *Nitella* and *Chara* (Henry and Hall 1977) contain CuZn-SOD. The recent whole genome sequencing of the red alga *Cyanidioschyzon merolae* (Matsuzaki et al. 2004), the diatoms *Thalassiosira pseudonana* (Armbrust et al. 2004) and *Phaeodactylum tricornutum* (Bowler et al. 2008), and the green alga *Chlamydomonas reinhardtii* (Merchant et al. 2007) confirmed the absence of CuZn-SOD in most eukaryotic algae including chlorophytes. Although CuZn-SOD activity was detected in several charophyte algae, the detailed nature of algal CuZn-SOD including gene structure was not revealed.

To elucidate the adaptation of SOD isozymes and their isoforms to oxidative stress in the course of evolution, we examined CuZn-SOD and its gene from the green alga *Spirogyra*, a streptophyte alga that led to the evolution of land plants. In this paper, we report the purification and characterization of *Spirogyra* CuZn-SOD and the cloning of its cDNA and genomic gene. The results show that the *Spirogyra* CuZn-SOD gene resembles those of land plants in terms of amino acid sequence and exon-intron structure. Furthermore, we compare the *Spirogyra* CuZn-SOD gene with the recently available genomes of the prasinophyte algae (green algae), *Ostreococcus lucimarinus* (Derelle et al. 2006, Palenik et al. 2007) and *Micromonas* sp. (Worden et al. 2009) and discuss the origin of the algal CuZn-SOD isoform genes.

MATERIALS AND METHODS

Materials. DEAE Sephacel, Phenyl Sepharose CL-4B and Sephadex G-100 were purchased from Pharmacia Biotech (Uppsala, Sweden). *TaKaRa Taq*, *TaKaRa LA Taq*, Oligotex-dT30 Super and RNA PCR kit (AMV) ver 2.1 were obtained from Takara (Kyoto, Japan). Isogen is a product of Nippon Gene (Toyama, Japan). DNeasy Plant Maxi Kit, Geneclean II Kit and Quantum Prep Plasmid Miniprep Kit were from Qiagen (Valencia, CA, USA), BIO 101 (Vista, CA, USA) and Bio-Rad (Hercules, CA, USA), respectively. SuperScript First-Strand Synthesis System for RT-PCR was purchased from Life Technologies (Rockville, MD, USA). SMART RACE cDNA Amplification Kit and Universal GenomeWalker Kit were obtained from Clontech (Palo Alto, CA, USA). pGEM-T Easy Vector System I was from Promega (Madison, WI, USA). The other reagents were commercial products of the highest grade available. Antibodies raised against spinach chloroplastic and cytosolic CuZn-SODs were prepared as described previously (Kanematsu and Asada 1989a, Rubio et al. 2009). Bacterial strain, medium and growth conditions were described in a previous paper (Kanematsu and Fujita 2009).

Culture of *Spirogyra* cells. *Spirogyra* cells were collected at two sites: a dam in Aoyama, Himeji city, Hyogo prefecture, and a pond at Kougedani in Shibushi city, Kagoshima prefecture. The cells from Hyogo were transported to Miyazaki under cold, then frozen immediately at −20°C on arrival, and used for CuZn-SOD purification. The fresh cells obtained in Kagoshima were cultured in a slightly modified Reichardt's medium (Reichardt 1967, Fujii et al. 1978). Unless otherwise stated, culturing was conducted in 1/5-strength medium supplemented with soil extract at 20°C under 16h-8h light-dark cycle (PPFD of 50 μmol $m^{-2}s^{-1}$) with aeration. The cultured cells were used for cloning of cDNA and genomic gene of CuZn-SOD.

Isolation of poly(A)$^+$ mRNA and genomic DNA. Total RNA of *Spirogyra* was obtained from the intact cells using Isogen by a similar method to that described previously (Kanematsu and Fujita 2009). The cultured cells were washed five times with distilled water, then illuminated further in water for 2 h under fluorescent lamps (PPFD of 50 μmol $m^{-2}s^{-1}$). After washing with dH_2O twice, the cells were blotted on Kimwipes and squeezed to remove excessive water. The cells (1.0 g) were ground in liquid nitrogen with a mortar and pestle, and the resulting fine powder was added to 10 ml Isogen preheated at 50°C, mixed vigorously and further incubated for 10 min at 50°C. After adding 2 ml of chloroform and shaking vigorously, the mixture was centrifuged, and an aqueous phase (6 ml) containing RNA was obtained. RNA was precipitated with the same volume of isopropanol by centrifugation. The removal of jelly material from white RNA pellets, formed during isopropanol precipitation, effectively improved the yield and purity of RNA. The RNA pellets were washed with 75% ethanol, dissolved in dH_2O, collected by ethanol precipitation and used as total RNA. Poly(A)$^+$ mRNA was obtained from the total RNA (200 μg) using Oligotex-dT30 Super as described previously (Kanematsu and Fujita 2009). Genomic DNA of *Spirogyra* was isolated from the intact cells (3 g) using a DNeasy Plant Maxi Kit according to the manufacturer's manual after being disrupted in liquid nitrogen with a mortar and pestle.

Amplification of cDNA core fragments by RT-PCR. cDNA cloning of CuZn-SOD was conducted by combination of RT-PCR and 5'- and 3'-RACE. To amplify the core region of the gene by RT-PCR, single-strand cDNA was reverse transcribed from 400 ng mRNA using the RNA PCR kit (AMV) (Takara) with OligodT-Adaptor Primer and AMV Reverse Transcriptase according to the manufacturer's instructions. The core region was amplified from a pool of the single-strand cDNA using the GeneAmp PCR System 9700 (Applied Biosystems) with *Takara Taq* polymerase and the following degenerate primers: sense primer, CARGARGAYGAYGGNCCNAC (SPGY-SENSE); and antisense primers, CCNCCYTTNCCAARRTCRTC (SPGY-AS-A), CCNCCYT-

TNCCGARRTCRTC (SPGY-AS-G), CCNCCYTTNCC-TARRTCRTC (SPGY-AS-T), and CCNCCYTTNCC-CARRTCRTC (SPGY-AS-C). These degenerate sense and reverse primers were designed on the basis of the N-terminal amino acid sequence of the purified *Spirogyra* CuZn-SOD-I (QEDDGP) and a sequence of the highly conserved region (DDLGKG) between both chloroplastic and cytosolic CuZn-SODs from land plants, respectively. PCR conditions were as follows: preheating at 94°C for 2 min, then 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and propagation at 72°C for 1 min, with post-heating at 72°C for 3 min.

5'- and 3'-RACE. The upstream and downstream regions of the core cDNA were amplified by the RACE method using a SMART RACE cDNA Amplification Kit according to the manufacturer's instructions. First-strands cDNA for 5'- and 3'-RACE were synthesized using 750 ng each of poly(A)$^+$ mRNA and PowerScript reverse transcriptase. In PCR, the following gene-specific primers designed from the core cDNA were used: SP5GSP#13 (5'-GCTTCGGCGATTCCTTCCTCGTTC-3') for 5'-RACE and SP3GSP#3 (5'-CCACAGGACCGCATCT-CAACCCC-3') for 3'-RACE. 5'-RACE was conducted using the GeneAmp 9700 under the following conditions: 94°C for 1 min, 5 cycles of 94°C for 5 s and 72°C for 3 min, 5 cycles of 94°C for 5 s, 70°C for 10 s and 72°C for 3 min, 30 cycles of 94°C for 5 s, 68°C for 10 s and 72°C for 3 min, with post-heating for 72°C for 3 min. For 3'-RACE, the final step was reduced to 25 cycles. The PCR products were purified by the glass milk method, and then ligated into pGEM-T EZ, and transformed by *E. coli* XL1-Blue MRF'. For the products in 5'-RACE, colonies having the larger insert were selected by insert check with PCR and used for further analysis.

Cloning of the genomic gene by PCR. The *Spirogyra* CuZn-SOD genomic gene was obtained by a two-step PCR amplification method as previously described (Kanematsu and Fujita 2009), which consisted of PCR amplifications for a central portion of the gene with gene-specific primers based on the cDNA sequence, and for the 5' upstream and 3' downstream regions of the gene using the Universal GenomeWalker Kit. The central portion of the gene was amplified from 300 ng of RNase-treated genomic DNA using *LA Taq* with the sense primer SPF3-34 (5'-GGACGCTGTCCGAATTTCG-TACACTCGACAAG-3') and antisense primer SPB724-691 (5'-AAACCAGAGGTTGGATGCAGGATTGAAC-TCTTGG-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, then 72°C for 10 min. The amplified fragment of 1.5 kbp was cloned and sequenced as described before (Kanematsu and Fujita 2009).

GenomeWalker libraries that consisted of adaptor-ligated genomic DNA fragments were constructed from RNase-treated genomic DNA (2.2 mg) digested either with *Dra*I, *Eco*RV, *Puv*II or *Stu*I according to the manufacturer's instructions. Primary and nested PCR of each library were performed with *LA Taq* using the following primers, respectively: SPGW5#1-1 (antisense primer, 5'-GACTCGGACAAGTTTGCCGAAAACCTG-3') for 5' upstream amplification and SPGW3#1-1 (sense primer, 5'-CCGACTCCAATCCCCAAGAGTTCAATC-3') for 3' downstream amplification, and SPGW5#1-2 (antisense primer, 5'-AAGGCTTGCGAGTCTTGTCGAGTG-TACG-3') for 5' upstream and SPGW3#1-2 (sense primer, 5'-CACTGCATTTTCACAGGTGTCATTGGG-3') for 3' downstream amplification. PCR conditions were as follows: for primary PCR, preheated at 94°C for 3 min, 7 cycles of 94°C for 2 s and 72°C for 3 min, then 32 cycles of 94°C for 2 s and 67°C for 3 min, with post-heating at 67°C for 4 min, and for nested PCR, preheated at 94°C for 3 min, 5 cycles of 94°C for 2 s and 72°C for 3 min, then 20 cycles of 94°C for 2 s and 67°C for 3 min, with post-heating at 67°C for 4 min.

Other methods for DNA and RNA manipulations. Agarose gel electrophoresis, purification and vector ligation of amplified DNA fragments were conducted as previously described (Kanematsu and Sato 2008, Kanematsu and Fujita 2009). The glass powder method for DNA purification employed Genobind (Clontech). Plasmids were prepared using the alkaline lysis method (Sambrook et al. 1989) in the early phase of experiments, and later using the Quantum Prep Plasmid Miniprep Kit. DNA inserts in pGEM vectors were checked by PCR with *Takara Taq* and universal primers (-21M13 and M13RV) under the following conditions: preheated at 94°C for 2 min, then 25－30 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 30 s and propagation at 72°C for 1.5 min, with post-heating at 72°C for 5 min. Promoter analysis was conducted using the Plant cis-Acting Regulatory DNA Elements (PLACE) database (Higo el al. 1999). Cycle sequencing reaction and DNA sequencing analysis were performed as previously described (Kanematsu and Sato 2008).

SOD assay and protein characterization. SOD was assayed by the xanthine-xanthine oxidase-Cyt *c* system as described previously (Kanematsu and Asada 1990) and the activity was expressed in McCord and Fridovich units in 3 ml reaction volume (McCord and Fridovich, 1969). Since our assay system employing 0.5 ml volume gave 6-fold activity units as compared to that of McCord and Fridovich, the values were divided by 6. Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Native-PAGE, SDS-PAGE, SOD activity staining, protein staining and immunoblotting were performed as described previously (Kanematsu and Asada 1990, Ueno and Kanematsu 2007).

Nucleotide sequence accession numbers. The nucleotide sequences of the cDNA and the genomic gene of *Spirogyra* chloroplastic CuZn-SOD have been submitted to the DDBJ, EMBL and GenBank under Accession Numbers AB075698 (cDNA) and AB098508 (genomic gene). Part of the present results have been presented elsewhere (Kanematsu et al. 2002, Kanematsu and Asada 2003, Kanematsu et al. 2003).

RESULTS

***Spirogyra* SOD isozymes and their isoforms.** *Spirogyra* cells collected in Kagoshima and Hyogo showed different band patterns in SOD activity staining on native-PAGE. The cells from Kagoshima indicated three activity bands on a 7.5% gel at pH 8.3 (Fig. 1A). The major band at the anodic side on the gel was inhibited by both cyanide and H_2O_2, indicating CuZn-SOD. The bands at the middle and the cathodic side were assigned

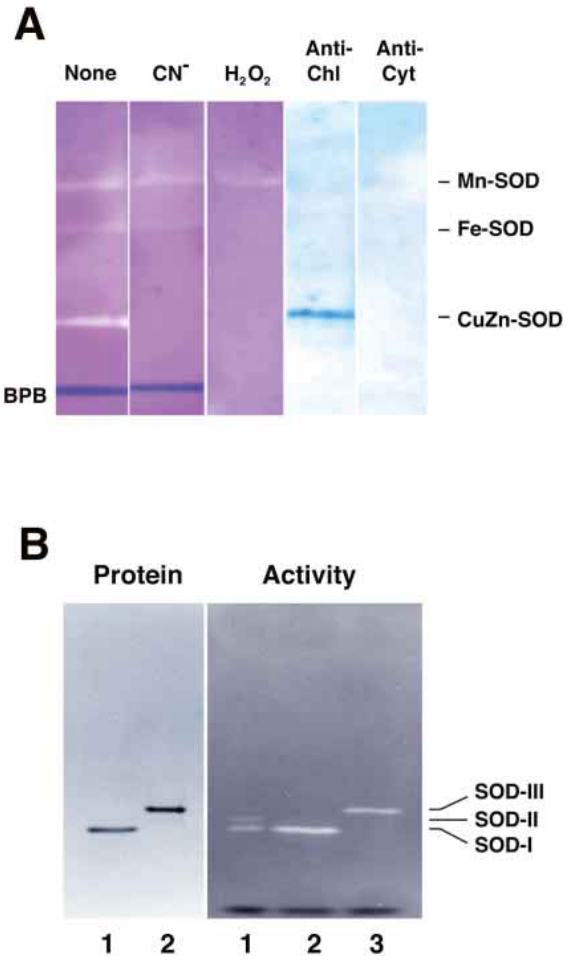

Fig. 1. *Spirogyra* SOD isozymes in native-PAGE. (A) SOD activity staining and Western blotting of the cell extract from *Spirogyra* sp. collected in Kagoshima. In each lane 100 μg of protein was applied. Activity staining (left three lanes). Left lane, no treatment; middle lane, 2 mM CN^- treatment during activity staining; right lane, pre-treated with 5 mM H_2O_2 for 30 min before staining. Western blotting (right two lanes). Left lane, anti-spinach chloroplastic CuZn-SOD serum; right lane, anti-spinach cytosolic CuZn-SOD serum. BPB, tracking dye. (B) Homogeneity of SOD-1 and SOD-III purified from *Spirogyra* cells obtained in Hyogo. Left panel, protein staining (each 10 μg protein). Lane 1, SOD-I; lane 2, SOD-III. Right panel, SOD activity staining (each 5 units). Lane 1, cell extract; lane 2, SOD-I; lane 3, SOD-III. Top, cathode; bottom, anode.

to Fe-SOD and Mn-SOD, respectively, according to their response to cyanide and hydrogen peroxide. In some cases, a faint cyanide-sensitive CuZn-SOD band was detected near to the major CuZn-SOD at the anodic side. The CuZn-SOD was shown to be a chloroplast-localizing isoform on the basis of the reactivity with anti-spinach chloroplastic CuZn-SOD serum and not with anti-spinach cytosolic CuZn-SOD (Fig. 1A). Thus, *Spirogyra* contains three types of SOD isozymes, i.e. CuZn-, Mn- and Fe-SODs.

The cells from Hyogo showed three cyanide-sensitive CuZn-SOD activity bands, which were termed SOD-I, -II and -III in anodic order, at the anodic side on a gel in 7.5% native-PAGE (Fig. 1B). Two bands (SOD-I and -III) were confirmed to be chloroplastic CuZn-SOD by purification (see below). A band corresponding to Mn-SOD or Fe-SOD did not appear on the gel, but this does not necessarily indicate the absence of both types of SOD isozyme in *Spirogyra* cells from Hyogo.

Purification of CuZn-SOD isoforms. CuZn-SODs were purified from *Spirogyra* cells collected in Hyogo. The cells (1.2 kg) were disrupted by Polytron for 20 min in 50 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA, and then by sonication for 5 min. After centrifugation, ammonium sulfate fractionation was conducted with 40－90% saturation. The precipitate was dissolved in 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA and dialyzed against the same buffer. The dialyzed enzyme was applied to a column of DEAE-Sephacel equilibrated with 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. SOD was eluted with 150 mM KCl in the equilibrating buffer. The active fraction was concentrated by ultrafiltration through an Amicon PM-10 membrane and the buffer was changed to 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA during the concentration.

The concentrated enzyme was subjected to a linear gradient elution of KCl (0 → 300 mM) in 10 mM Tris-HCl, pH 7.4, on a DEAE-Sephacel column. SOD activity was separated in three fractions. The first eluted active fraction was SOD-III and the last eluted fraction was SOD-I. Since the SOD-II fraction overlapped with SOD-I and -III, further purification of SOD-II was not conducted. SOD-I and -III fractions were separately pooled, concentrated and further purified by second linear gradient elution on DEAE-Sephacel column chromatographies. Each active fraction eluted was pooled and concentrated by ultrafiltration with PM-10, during which the buffer was changed to 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA and 35% ammonium sulfate.

Each enzyme solution was separately applied to a column of Phenyl-Sepharose equilibrated with the same starting solution. SOD was eluted by a simultaneous cross-linear gradient of ammonium sulfate (35 → 0%) and ethylene glycol (0 → 40%) in 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. Active fractions were pooled, concentrated and gel-filtrated through a column of Sephadex G-100 equilibrated with 10 mM potassium phosphate, pH 7.8, containing 150 mM KCl. The SOD fraction was pooled, concentrated and dialyzed against 10 mM potassium phosphate, pH 7.8 and used for characterization. Yield and specific activity of the enzymes during the purification are summarized in Table 1.

Characterization of SOD-I and -III, and their N-terminal amino acid sequences. In accordance with their negative charge, which was estimated by native-PAGE at pH 8.3 (Fig. 1B), the order of the elution of three CuZn-SOD isoforms on a linear gradient column chromatography of DEAE-Sephacel was SOD-III, SOD-II then SOD-I (Table 1). The purified SOD-I and SOD-III were indicated to be almost homogeneous by electrophoresis (Fig. 1B). Both enzymes were cross-reacted with anti-spinach chloroplastic CuZn-SOD serum in Western blotting after native- and SDS-PAGE, indicating that the purified SODs were the chloroplastic type of CuZn-SOD (data not shown).

The subunit molecular mass of SOD-I and -III were estimated by SDS-PAGE to be 22 and 20 kDa, respectively, in the presence of 2-mercaptoethanol, and 20 and

Table 1. Purification of CuZn-SODs from *Spirogyra* sp.

Purification step	Total protein (mg)	Total activity[c] (units)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
Crude extract	5,786	34,133	5.9	100	1
40-90% $(NH_4)_2SO_4$	373.80	18,333	49	54	8
1st DEAE-Sephacel	9.57	17,500	1,829	51	313
2nd DEAE-Sephacel					
Pooled Fr. #1	0.85	1,650	1,941	5	334
Pooled Fr. #3	1.65	9,984	6,051	29	1,037
SOD-I(Fr. #3)					
3rd DEAE-Sephacel	2.16[a]	7,676	3,554	22	609
Phenyl-Sepharose	0.33[a]	4,305	13,045	13	2,236
Sephadex G-100	0.20[b]	4,216	21,080	12	3,614
SOD-III(Fr. #1)					
3rd DEAE-Sephacel	0.64[a]	1,260	1,969	4	338
Phenyl-Sepharose	0.60[a]	1,240	2,067	4	354
Sephadex G-100	0.13[b]	1,344	10,338	4	1,772

[a] Protein was determined by the Lowry's method to 2nd DEAE-Sephacel step, then spectrophotometrically using $A^{1\%}_{1cm}$ at 280 nm = 10.
[b] $A^{1\%}_{1cm}$ at 258 nm = 4 was assumed.
[c] McCord-Fridovich units.

19 kDa, respectively, in the absence of the reductant (data not shown). The molecular masses of the enzymes were determined to be 32 kDa for both SOD-I and -III by the gel-filtration method (data not shown). N-terminal amino acid sequences with 56 residues of the purified SOD-I and -III revealed the characteristic sequences for the chloroplastic type of CuZn-SOD with four amino acid substitutions between both enzymes (Fig. 2). It should be noted that both sequences of SOD-I and -III were not identical to the deduced amino acid sequence from the cDNA for chloroplastic CuZn-SOD of *Spirogyra* cells obtained in Kagoshima (Fig. 2, and see below).

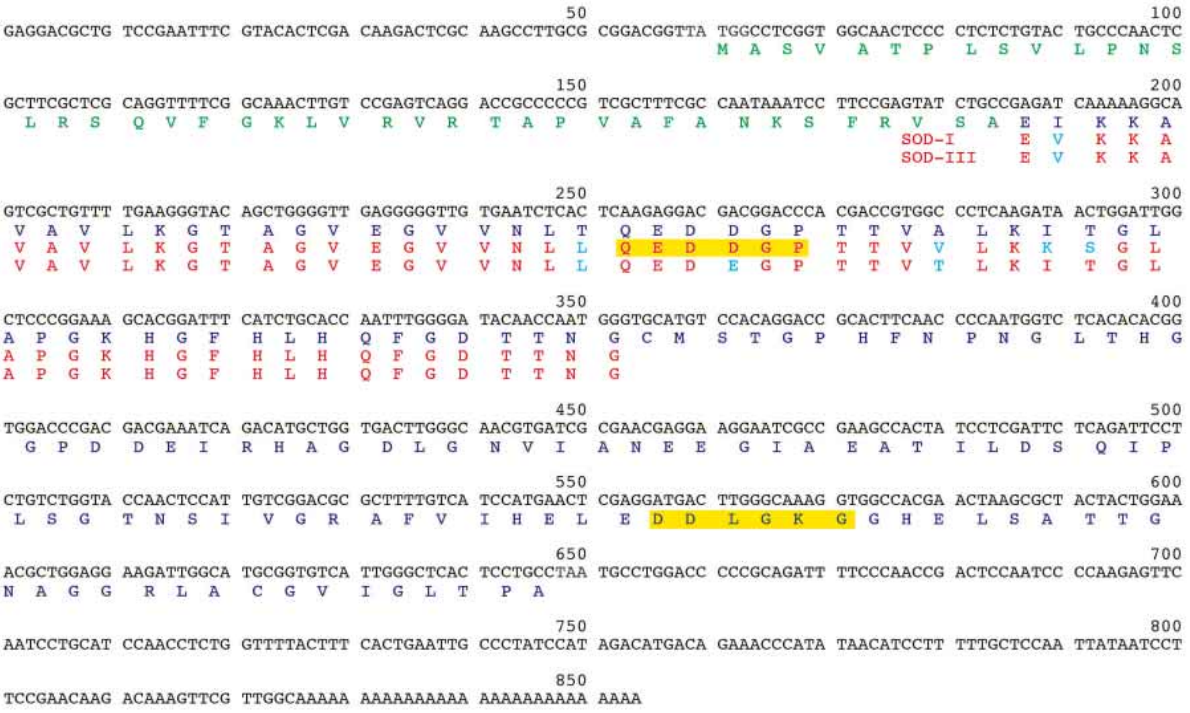

Fig. 2. Nucleotide sequence of *Spirogyra* CuZn-SOD cDNA and its deduced amino acid sequence. The nucleotide sequence of 854 bp was obtained from *Spirogyra* cells in Kagoshima. The nucleotide G at the first position is an artifact due to adapter ligation. The deduced amino acid sequence is indicated in green letters for a transit peptide and blue for mature protein, respectively. N-terminal amino acid sequences (56 residues) of SOD-I and -III in red letters are aligned with the deduced sequence. Mismatched residues are indicated in light blue. Amino acid sequences designed for degenerated primers used for isolation of the core portion are shaded in yellow.

Fig. 3. Nucleotide sequence of *Spirogyra* CuZn-SOD gene. The nucleotide sequence of the DNA fragment of 3,402 bp encoding chloroplastic CuZn-SOD is presented. Exons and UTRs are indicated in red and orange letters, respectively. Start and stop codons are underlined. The deduced amino acid sequence is shown in blue letters, in which the chloroplast transit peptide is in green. Two sets of long and short direct repeat sequences are indicated in light blue.

Cloning of *Spirogyra* CuZn-SOD cDNA. *Spirogyra* cells collected in Kagoshima were used for cDNA cloning of chloroplastic CuZn-SOD. The cloning was conducted by combination of RT-PCR for the core region using degenerate primers, and 5'- and 3'-RACE for the flanking region with gene-specific primers. DNA fragments of 0.32 kbp were obtained by RT-PCR and sequenced. The core fragments had a length of 283 bp (without primers) and encoded a partial sequence homologous to those of chloroplastic CuZn-SOD. The DNA fragments obtained by 5'- and 3'-RACE were 0.55 kbp and 0.5 kbp, respectively. The 5'-upstream fragment contained the ATG start codon, and sequences for the chloroplast transit peptide and for the N-terminal region of

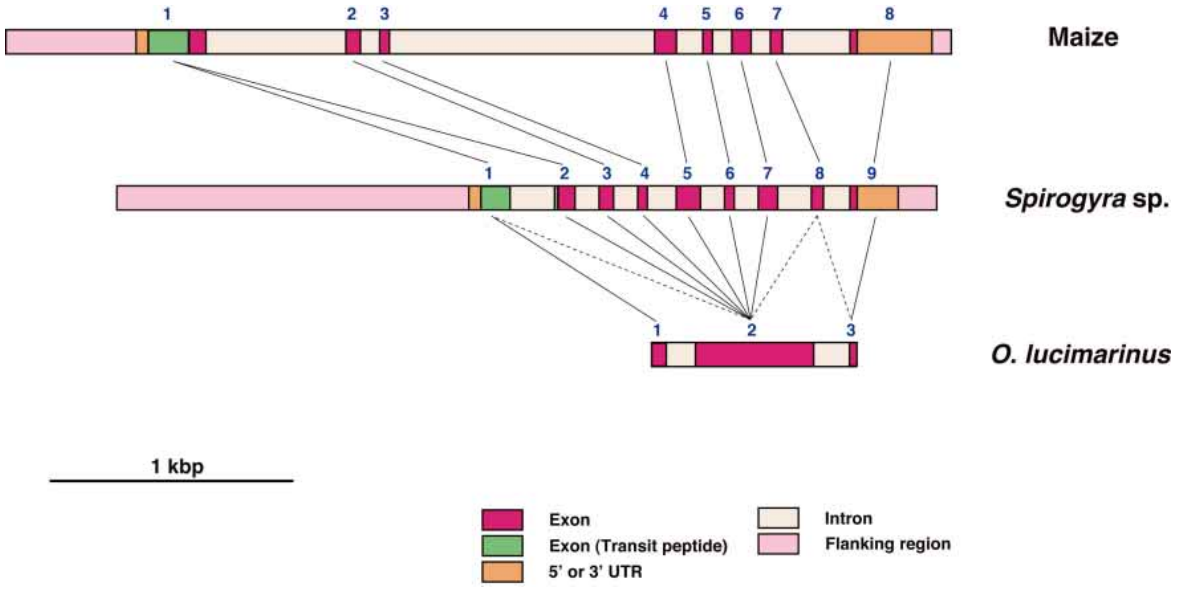

Fig. 4. Comparison of exon-intron structure of chloroplastic CuZn-SOD genes from *Spirogyra*, maize and *O. lucimarinus*. Maize, (*sod*-1) AB093580; *Ostreococcus lucimarinus* CCE9901, XP_001422430. The gene of *O. lucimarinus* is shown in coding sequence (CDS) without indication of the transit peptide region, because of no information for UTR. The exon number is indicated on each schematic drawing of the gene. The solid line shows the corresponding exon, and the dotted line indicates the dividing of exon or merging of partial exons.

mature chloroplastic CuZn-SOD. The 3'-downstream fragments also showed the complete 3'-downstream sequence. The complete cDNA nucleotide sequence of *Spirogyra* chloroplastic CuZn-SOD and its deduced amino acid sequence are shown in Fig. 2. The cDNA encoded a protein of 196 amino acid residues, of which 42 residues were for a transit peptide, revealed by the comparison with N-terminal sequences of the purified proteins.

We also analyzed chloroplastic CuZn-SOD genes in the *Spirogyra* cells from Hyogo by RT-PCR using the same degenerate primers. The amplified core fragments of 283 bp revealed the presence of at least two chloroplastic CuZn-SODs with four amino acid substitutions in 95 residues. This indicates the absence of protein modification during purification procedures since the cells from Hyogo contained at least two chloroplastic CuZn-SODs revealed by purification.

Cloning and structure of *Spirogyra* CuZn-SOD gene. The genomic gene was obtained by combination of PCR amplifications for the central region, and for upstream and downstream regions of the chloroplastic CuZn-SOD gene. First, the central portion of the gene was amplified using gene-specific primers based on the cDNA sequence. Amplified fragments of 1.5 kbp were cloned, and three clones were selected for whole sequencing. Then, using gene-specific primers based on both end sequences of a central region, upstream and downstream fragments of the gene were obtained by the DNA walking method. We obtained 5' upstream DNA fragments of 1.4, 5.5 and 3.5 kbp from the *Dra* I, *Eco* RV and *Pvu* II GenomeWalker libraries, respectively, and 3' downstream fragments of 1.4, 1.4 and 3.4 kbp from *Eco*RV, *Pvu*II and *Stu*I libraries, respectively. The candidate fragments containing the target sequence were analyzed by restriction enzyme digestion and sequencing. Finally, DNA fragments obtained from the 5' upstream *Eco*RV library (5.5 kbp) and from the 3' downstream *Eco*RV library (1.4 kbp) were fully sequenced. Thus, we obtained 3,402 bp of a genomic gene encoding chloroplastic CuZn-SOD by combination of 5'-side, central and 3'-side DNA fragments (Fig. 3).

In the 5' upstream region, two sets of long and short tandem repeated-sequences were observed (Fig. 3). The *Spirogyra* chloroplastic CuZn-SOD gene contained nine exons and eight introns. The comparison of exon-intron structure of the *Spirogyra* gene with those of other organisms is shown in Fig. 4. Analysis of *cis*-elements in the promoter region of the SOD gene using the PLACE database (Higo et al. 1999) revealed the resemblance in their arrangements with those of the rice chloroplastic CuZn-SOD gene (Kaminaka et al. 1997) as shown in Fig. 5.

Phylogenetic analyses of chloroplastic and cytosolic CuZn-SOD isoforms. Recently, whole genomes of the prasinophyte algae that belong to the division Chlorophyta have become available in public databases and exhibited the occurrence of CuZn-SOD genes (Derelle et al. 2006, Palenik et al. 2007, Worden et al. 2009). Alignment of amino acid sequences of CuZn-SOD from *Spirogyra* sp. with those of two prasinophytes, *Ostreococcus lucimarinus* and *Micromonas* sp., are shown in Fig. 6. The amino acid sequence homology in transit peptide and mature protein regions were 33% and 66%, respectively, between *Spirogyra* and *O. lucimarinus*, and 45% and 69%, respectively, between *Spirogyra* and *Micromonas* sp., indicating the close evolutionary relationship between prasinophytes and charophytes in a monophyletic lineage of green plants.

A phylogenetic tree of CuZn-SOD in green plants

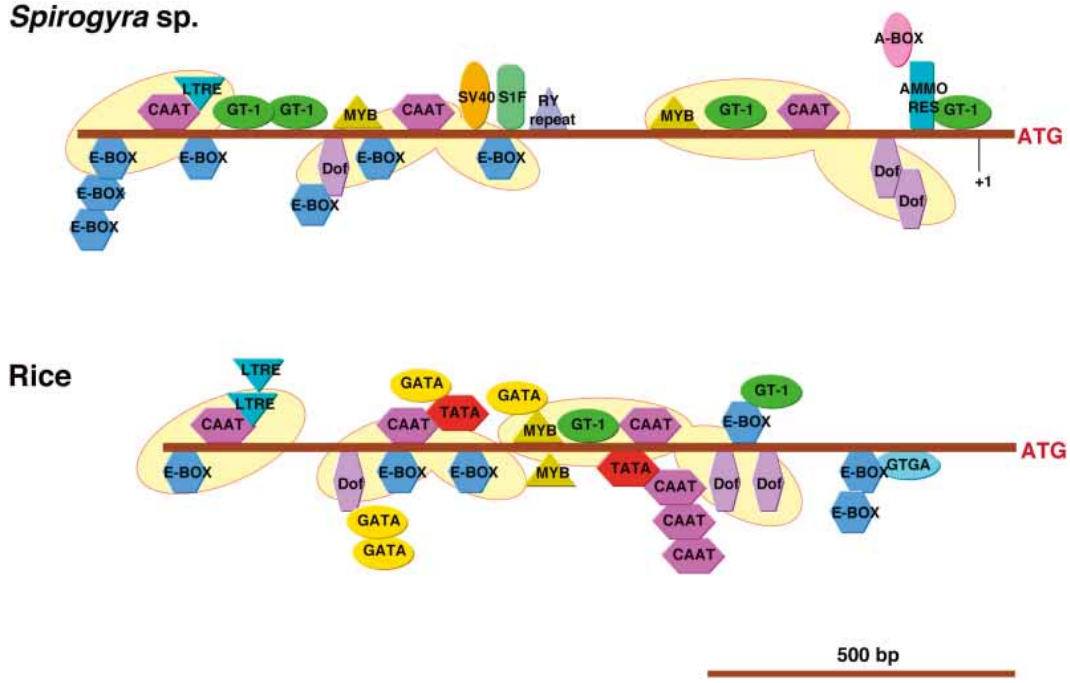

Fig. 5. Similarity in promoter regions between *Spirogyra* and rice chloroplastic CuZn-SOD genes. *Cis*-elements in the promoter region of the *Spirogyra* SOD gene were compared with those of rice chloroplastic CuZn-SOD gene (*sodCp*, AB026724) using the PLACE database. Selected putative *cis*-elements upstream of the ATG start codon are depicted in the figure. Where global similarity is observed in terms of organization, the *cis*-elements are shaded in yellow.

Abbreviations in the diagram, ID names of PLACE, motif sequences and some features of *cis*-elements are as follows: A-BOX, ACGTABOX, TACGTA, responsible for sugar repression; AMMO RES, AMMORESIVDCRNIA1, CGAACTT, ammonium response; RY repeat, RYREPEATVFLEB4, CATGCATG, quantitative seed expression; S1F, S1FBOXSORPS1L21, ATGGTA, negative element; SV40, SV40COREENHAN, GTGGWWHG, core enhancer, respectively. Other abbreviations of the elements in the diagram were described in our previous paper (Fig. 9 in Kanematsu and Fujita 2009).

(Viridiplantae) is shown in Fig. 7. In the phylogenetic analyses, we employed chloroplastic and cytosolic CuZn-SOD isoforms from the moss *Pogonatum inflexum* and the fern *Equisetum arvense* (Kanematsu unpublished), and maize as representative organisms in land plants in addition to chloroplastic CuZn-SODs from *Spirogyra* and the prasinophyte algae. The tree indicates a sister-group relationship between chloroplastic and cytosolic CuZn-SODs.

To date, no complete sequence data of cytosolic CuZn-SOD in algae have been reported except one partial nucleotide sequence, which was annotated as putative SOD (EST data), of the prasinophyte *Mesostigma viride*. We examined its isoform type by sequence alignment with authentic chloroplastic and cytosolic CuZn-SODs (Fig. 8). The results showed that *M. viride* contains cytosolic CuZn-SOD, which would make it the oldest cytosolic CuZn-SOD in algae.

Effect of Cu on CuZn-SOD activity in *Spirogyra*. To obtain insight into regulation mechanism of algal CuZn-SOD, we examined the responsiveness of CuZn-SOD in *Spirogyra* to Cu. SOD activity was increased by the addition of Cu to the culture medium and reached a maximum with doubled activity at 1 μM Cu, then decreased with the increase of Cu concentration (Fig. 9A). This activity was attributable to the chloroplastic CuZn-SOD as judged from SOD activity staining after native-PAGE (Fig. 9B). A Western blot clearly showed that the increased activity was due to the induction of SOD protein and not the activation of apo-CuZn-SOD (Fig. 9B). The SOD protein synthesis reached a plateau at 1 μM Cu, then was constant thereafter, whereas the activity gradually decreased as Cu concentration increased. Although the reason for the inactivation is not clear, it seems likely that ROS generated at or near the active site disturbs the microenvironment of Cu ligands. To examine the possible involvement of ROS in Cu treatment for SOD induction, we analyzed the effect of methylviologen on the SOD activity of *Spirogyra.* The treatment of methylviologen from 0.1 μM to 1 mM for 2 h under light resulted in a decrease of the activity, which was revealed by activity staining (data not shown), excluding the involvement of ROS in the CuZn-SOD induction by Cu.

DISCUSSION

Species of *Spirogyra*. In this experiment, we used *Spirogyra* cells collected from two different sites, Hyogo and Kagoshima, without further identifying their species because of the difficulty in species identification. *Spirogyra* is classified based on the conjugation process and zygospores, whereas they are mostly in the vegetative stage (Hainz et al. 2009). The two *Spirogyra* from Hyogo and Kagoshima showed morphological differ-

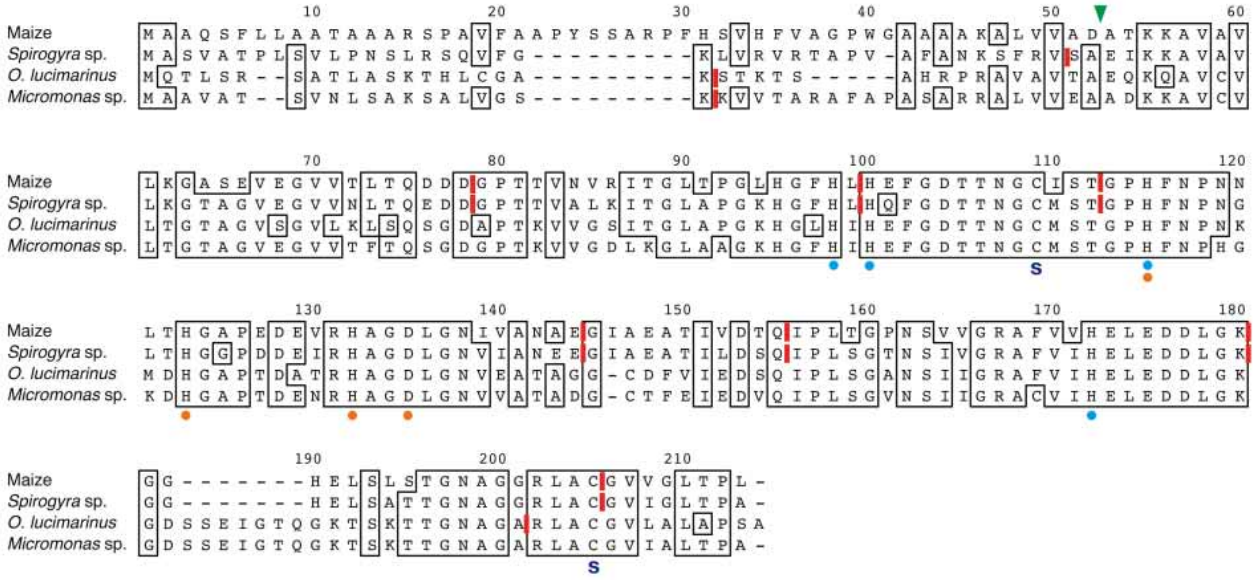

Fig. 6. Amino acid sequence comparison of CuZn-SODs from *Spirogyra*, maize and prasinophyte algae. The amino acid sequence of the *Spirogyra* CuZn-SOD was aligned with those of maize (Kanematsu and Fujita 2009) and the prasinophyte green algae *Ostreococcus lucimarinus* CCE9901 (XP_001422430) and *Micromonas* sp. RCC299 (XP_002508398) by the ClustalW program. Maize SOD is chloroplastic type (AB093580). Identical amino acid residues are boxed. The closed upside-down triangle shows the experimentally determined cleavage sites for the mature proteins of *Spirogyra* and maize CuZn-SOD. Ligands to copper and zinc are indicated with light blue and orange closed circles, respectively. Cysteine residues forming internal S-S bond are indicated by the letter S in blue. The red thick vertical lines show the positions of introns.

ences in cell length and diameter, indicating that they belong to different species.

SOD activity staining after separation of cell extracts in native-PAGE revealed a different mobility and pattern of activity bands for both cells (Fig. 1). The cells from Hyogo showed three CuZn-SOD bands on a gel while the cells from Kagoshima exhibited only one CuZn-SOD band. The occurrence of three CuZn-SOD isoforms in cells from Hyogo was in accordance with our previous results (Kanematsu and Asada 1989b). Comparison of the N-terminal amino acid sequences of CuZn-SODs from both cells, which were directly determined or deduced from cDNA, revealed homologous sequences with several amino acid substitutions (see below). Thus, it is obvious that the cells from both sites are different species of *Spirogyra*. However, this does not affect the conclusion obtained from the present results.

Properties and N-terminal amino acid sequence of purified algal CuZn-SODs. Previously, we partially purified CuZn-SOD from *Spirogyra* sp., but did not characterize it in detail due to a low amount of the enzyme (Kanematsu and Asada 1989b). Here, we purified two *Spirogyra* CuZn-SOD isoforms from the Hyogo cells (Table 1), characterized some of their properties and determined their N-terminal amino acid sequences. The two isoforms, SOD-I and -III, revealed a molecular mass of 32 kDa and a homodimeric subunit structure, which is characteristic of CuZn-SOD, confirming our previous results (Kanematsu and Asada 1989b). N-terminal amino acid sequences of the two purified enzymes clearly showed that they were the chloroplastic type of CuZn-SOD similar to those of land plants (Fig. 2), although this was suggested by the immunological reactivity of the previous enzyme preparation with anti-spinach chloroplastic CuZn-SOD (Kanematsu and Asada 1989b).

Structural characteristics of *Spirogyra* CuZn-SOD gene. *Spirogyra* contains a gene encoding the chloroplastic type of CuZn-SOD. This is the first direct evidence of the occurrence of chloroplastic CuZn-SOD in algae. The amino acid sequence of *Spirogyra* CuZn-SOD exhibited high homology with those of land plant CuZn-SODs, reflecting a close relationship in a monophyletic lineage of green plants. A sequence comparison of CuZn-SODs between *Spirogyra* and maize gave 21% and 80% homology in transit peptide and mature protein, respectively (Fig. 6).

The *Spirogyra* chloroplastic CuZn-SOD genomic gene contained nine exons while the chloroplastic SOD genes of higher plants contain eight (Fig. 3 and 4). Except the first intron, the remaining exon-intron structure of the *Spirogyra* gene was identical with those of higher plants in respect of splicing points (Fig. 6), although the average length of introns for the *Spirogyra* gene was shorter than those of land plants (Fig. 4), again reflecting an evolutionary link in a lineage of green plants.

The extra first 191 bp-intron of the *Spirogyra* gene was located at 9 bp upstream from the cleavage site in the chloroplast transit peptide coding region. The corresponding intron to the algal extra intron was also found in 5'-UTR of the moss *P. inflexum* chloroplastic CuZn-SOD gene (*sod*-2) (Kanematsu unpublished), but not in the fern *E. arvense* chloroplastic gene (*sod*-1) (Kanematsu unpublished) as well as higher plants (Fig. 4), indicating that the corresponding intron was lost in vascular plants. The recently available genome sequence of the moss *Physcomitrella patens* (Rensing et al. 2008) also con-

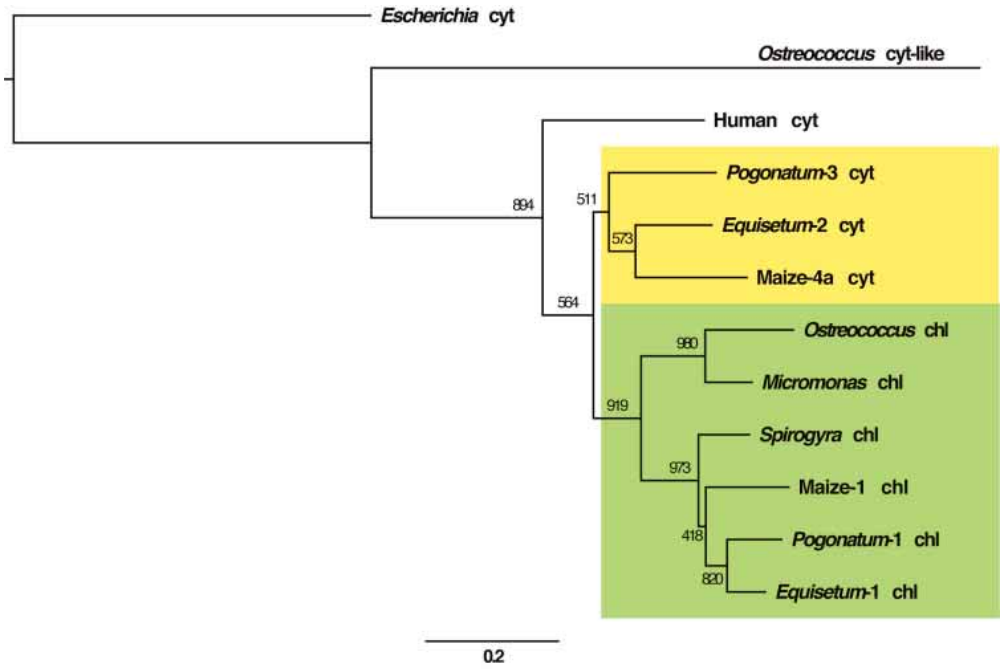

Fig. 7. Phylogenetic relationships among CuZn-SOD isoforms from green algae and land plants. CuZn-SODs were aligned by the ClustalW method with default settings (DDBJ). For chloroplastic SODs, sequences of mature proteins were used. The phylogenetic tree was constructed with the neighbor-joining method using a distance matrix of Kimura and bootstrapping of 1000 replications. The tree is rooted by using *Escherichia coli* CuZn-SOD as an outgroup. The bootstrap values are indicated on the left of each node. The clades of cytosolic and chloroplastic CuZn-SOD from streptophytes are shown in orange and green fields, respectively. *Ostreococcus*, *O. lucimarinus* CCE9901; *Micromonas, Micromonas* sp. RCC299; *Pogonatum*-1, SOD-1 *of Pogonatum inflexum* (moss); *Pogonatum*-3, SOD-3 of *P. inflexum; Equisetum*-1, SOD-1 of *Equisetum arvense* (fern); *Equisetum*-2, SOD-2 of *E. arvense.* Maize-1 and Maize-4a, chloroplastic and cytosolic SODs of maize, respectively. cyt, cytosolic; chl, chloroplastic.

firmed the presence of the corresponding intron in 5'-UTR in two chloroplastic CuZn-SOD genes. The promoter region of the *Spirogyra* SOD resembled that of rice chloroplastic CuZn-SOD in respect to the organization of *cis*-elements (Fig. 5). These results indicate that algal chloroplastic CuZn-SOD is an ancestor of land plant chloroplastic CuZn-SOD in an evolutionary sense.

Evolutionary relationships of *Spirogyra* chloroplastic CuZn-SOD gene with those of the prasinophyte algae. We reported that the most eukaryotic algae including chlorophytes are devoid of CuZn-SOD but charophyte algae contain CuZn-SOD, and suggested that ancestral CuZn-SOD diverged into the cytosolic and chloroplastic isoforms immediately after CuZn-SOD was acquired by photosynthetic organisms, and evolved independently thereafter (Kanematsu and Asada 1989b). Recently, the absence of the CuZn-SOD gene in the genome of the chlorophyte green alga *C. reinhardtii* (Merchant et al. 2007) was confirmed as well as in those of the red alga *C. merolae* (Matsuzaki et al. 2004), and the diatoms *T. pseudonana* (Armbrust et al. 2004) and *P. tricornutum* (Bowler et al. 2008). Thus, the exact location of the origin of chloroplastic CuZn-SOD in the green plant lineage is an intriguing issue.

After the accomplishment of *Spirogyra* CuZn-SOD gene sequencing (Kanematsu et al. 2003), the whole genome sequences of the prasinophyte algae *Ostreococcus lucimarinus* (Derlle et al. 2006, Palenik et al. 2007) and *Micromonas* sp. (Worden et al. 2009) have revealed the presence of the CuZn-SOD gene in these eukaryotic algae in addition to charophytes such as *Spirogyra*. Because the prasinophyte algae are considered to be an ancestor of green algae, the evolutionary position of *Spirogyra* is located between the prasinophytes and moss in the green plant lineage.

The amino acid sequence alignment of CuZn-SOD of *Spirogyra* with those of the prasinophyte algae clearly shows that the prasinophyte CuZn-SOD are chloroplastic SOD that contain the transit peptide to chloroplasts (Fig. 6). However, the exon-intron structures of the prasinophyte algal CuZn-SOD genes are completely different from that of the *Spirogyra* gene in terms of the number and position of introns. The prasinophyte algal gene contains only two introns, one in the transit peptide region and the other in the mature protein region, while the *Spirogyra* gene possesses eight introns, one of which is located in the transit peptide region (Fig. 4 and 6). Since the positions of introns differ between the prasinophyte and the *Spirogyra* genes, losses and acquisitions of the introns might have occurred during the early phase of evolution of green algae. On the contrary, no loss of the introns occurred in the course of evolution from charophyte algae to land plants, except for the first intron. It should be noted that the *Spirogyra* gene possesses almost the same exon-intron structure as those of embryophytes.

A phylogenetic tree of CuZn-SOD in green plants shows a sister-group relationship between chloroplastic

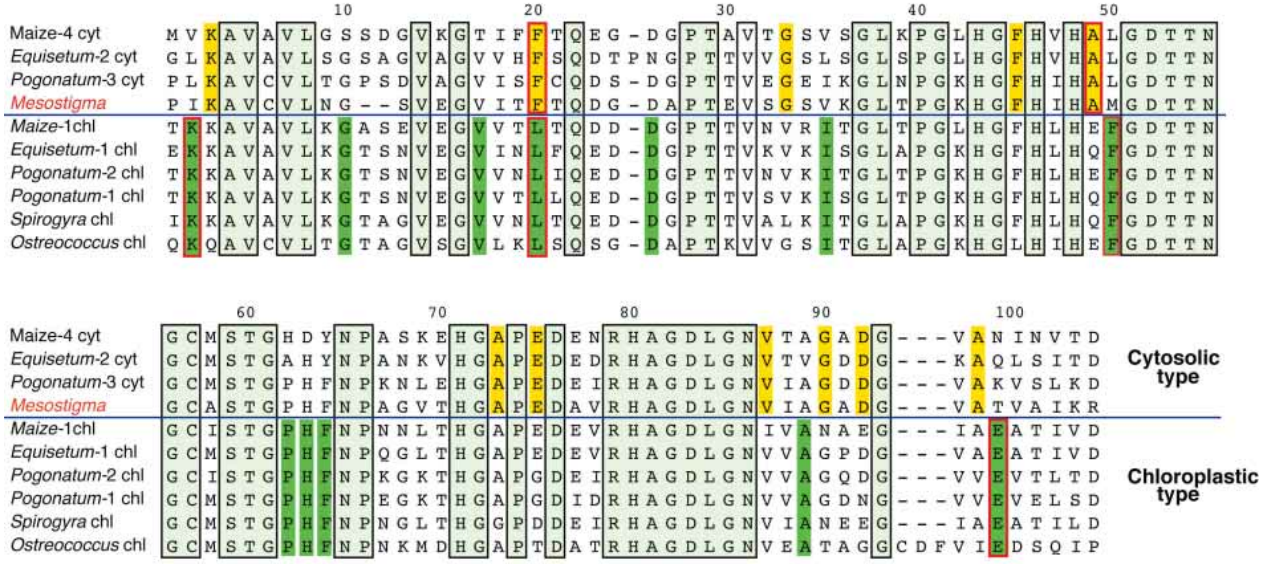

Fig. 8. Classification of a partial amino sequence of *Mesostigma* CuZn-SOD by sequence alignment with cytosolic and chloroplastic isoforms. A partial amino acid sequence of *Mesostigma* CuZn-SOD (EST sequence, 107 residues) was aligned with those of cytosolic and chloroplastic CuZn-SOD isoforms from green algae and land plants by the ClustalW method. To fit the sequence length of *Mesostigma* SOD, the sequences of other SODs are truncated. Conserved residues are boxed with a pale green background. Residues conserved in each cytosolic and chloroplastic isoform are highlighted in yellow and green, respectively. In addition, characteristic residues for either isoform are boxed in red. Names of SOD (organisms) are the same as those in Fig. 7.

and cytosolic CuZn-SODs, suggesting that the chloroplastic CuZn-SOD gene was derived from an ancestral cytosolic CuZn-SOD gene during early evolution in green plants, probably just before the divergence of charophytes (streptophyte algae) from chlorophytes (Fig. 7). It seems that chloroplastic CuZn-SOD of the prasinophyte algae may be the oldest chloroplastic CuZn-SOD in the monophyletic group of green plants.

Does the cytosolic CuZn-SOD gene occur in streptophyte algae? Genomes of *O. lucimarinus* and *Micromonas* sp. contain genes annotated as putative CuZn-SOD (referred to hereafter as CuZn-SOD-like protein) genes in addition to chloroplastic CuZn-SOD genes, but lack a typical cytosolic CuZn-SOD gene that resembles those of land plants. Both SOD-like proteins of these organisms exhibit 32% and 81% homology with each other, in the transit peptide and mature protein regions of amino acid sequences, respectively, but are devoid of three histidine residues that are essential for ligands of Cu. Thus, the SOD-like protein may have another function other than catalysis of the disproportionation reaction for superoxide. Interestingly, these proteins form a clade with bacterial CuZn-SODs in phylogenetic analyses (data not shown).

Although we immunologically detected the cytosolic CuZn-SOD isoform in *Spirogyra* cell extract in our previous paper (Kanematsu and Asada 1989b), its cDNA was not amplified from *Spirogyra* cells using the same degenerate primers that were used for the amplification of chloroplastic CuZn-SOD cDNA (data not shown). These primers could amplify both chloroplastic and cytosolic CuZn-SOD cDNAs from the moss *P. inflexum*, the fern *E. arvense* (Kanematsu 2005) and maize (Kanematsu and Fujita 2009). Although the reason for this discrepancy is uncertain, whole genome sequencing of *Spirogyra* will solve the problems.

At present, there are no complete sequence data for cytosolic CuZn-SOD in algae. Recently a partial EST sequence annotated as CuZn-SOD was obtained from the scaly green flagellate of streptophytes, *Mesostigma viride* (Simon et al. 2006), whose evolutionary position was recently shown to be at the bottom of divergence to the streptophytes and the chlorophytes (Nedelcu et al. 2006, Petersen et al. 2006, Rodríguez-Ezpeleta et al. 2007). We found that *Mesostigma* CuZn-SOD belongs to a group of land plant cytosolic CuZn-SOD by ClustalW analysis with *Mesostigma*'s 107 amino acid residues (Fig. 8). This is the first evidence of the presence of cytosolic CuZn-SOD in algae, which resembles those of land plants.

Phylogenetic analyses of cytosolic CuZn-SODs from land plants, bacteria, fungi, invertebrates and vertebrates show that the plant cytosolic enzyme forms a sister clade with chloroplastic CuZn-SOD within a monophyletic green plant lineage and the fungi and invertebrate cytosolic CuZn-SOD form a paraphyletic group with the plant cytosolic enzyme (data not shown). The results suggest that the chloroplastic CuZn-SOD gene diverged from ancestral plant cytosolic CuZn-SOD at an early phase of evolution of streptophytes.

Induction of CuZn-SOD by Cu. Copper ions are toxic to all cells due to their ability to produce ROS, therefore the concentration of free Cu in a cell is kept extremely low and most Cu exists in chelated form. In land plants, there is competition for Cu as a prosthetic metal mainly between plastocyanin, which is essential for photosynthesis, and CuZn-SOD. A mechanism for the priority usage of Cu by plastocyanin in the case of Cu

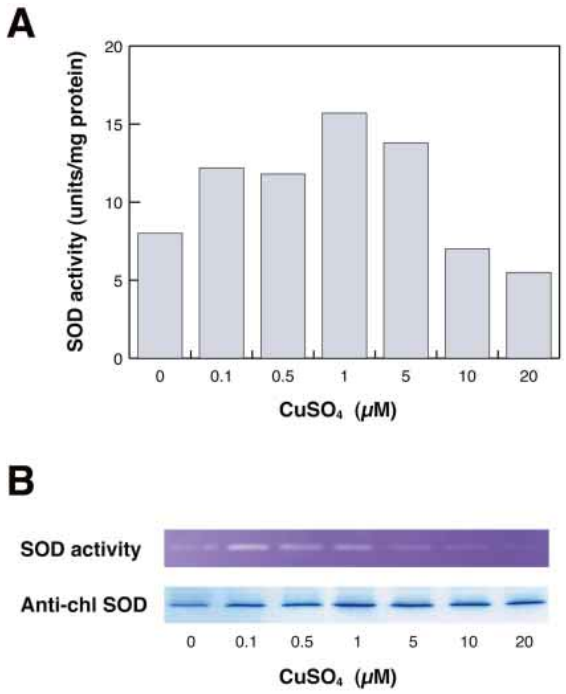

Fig. 9. Induction of CuZn-SOD by Cu. (A) Effect of $CuSO_4$ on SOD activity of *Spirogyra*. *Spirogyra* cells maintained by a culture in half strength Reichardt's medium were treated with the indicated concentration of $CuSO_4$ in the same medium for 24 h under light conditions (PPFD of 50 μmol $m^{-2}s^{-1}$). (B) SOD activity staining after native-PAGE and Western blotting with anti-spinach CuZn-SOD after SDS-PAGE. Each lane contains 100 μg of protein.

deficiency of *Arabidopsis thaliana* has been reported, where Cu deficiency induces a microRNA, miR398, which in turn down-regulates CuZn-SOD mRNA, resulting in a decrease of CuZn-SOD (Abdel-Ghany and Pilon 2008).

We observed the induction of CuZn-SOD activity and protein in *Spirogyra* by adding Cu to a culture medium (Fig. 9), confirming the previous observation that CuZn-SOD activity in the moss *Marchantia paleacea* var. *diptera* was increased by Cu (Tanaka et al. 1995). The medium we used, a modified Reichardt's medium, contained no Cu, therefore the *Spirogyra* cells were likely to be in a Cu-deficient state and to be responsive to Cu. *Spirogyra* freshly obtained from natural habitats revealed a similar CuZn-SOD induction profile. These results suggest that the down-regulation of CuZn-SOD in *Spirogyra* is operational, and if Cu is available in cells, then CuZn-SOD will be induced. Therefore, in addition to post-transcriptional regulation of CuZn-SOD by the microRNA, transcriptional regulation by Cu might be involved in the induction process of CuZn-SOD by Cu in *Spirogyra*.

Concluding remarks. Three types of SOD, namely Fe-, Mn- and CuZn-SOD, appeared on earth one by one in response to environmental changes in the course of evolution (Kanematsu and Asada 1994). Briefly, Fe-SOD was acquired by anaerobic bacteria, then Mn-SOD was derived from Fe-SOD in aerobic bacteria when the atmosphere was oxygenic, and lastly CuZn-SOD was added to plants, fungi and animals. In higher plants, CuZn-SOD consists of chloroplastic and cytosolic isoforms. We have been interested in the origin of both isoforms and have investigated CuZn-SOD and its gene from the streptophyte alga *Spirogyra*, which is an ancestor of land plants in an evolutionary sense. In the present study, we found that the *Spirogyra* CuZn-SOD is the chloroplastic isoform, which resembles those of land plants and the prasinophyte algae. Using available sequence data, we concluded that both CuZn-SOD isoforms diverged from a common ancestor, probably ancestral cytosolic CuZn-SOD, at a very early phase in the divergence of streptophyte and chlorophyte algae. CuZn-SOD of green plants might have appeared in an ancestor of prasinophyte algae, which are thought to be ancestral organisms in the green plant lineage.

The reason why Cu was adopted by SOD as a prosthetic metal is an intriguing question. One possible explanation is the availability of Cu ions in oxygenic environments due to the photosynthesis of cyanobacteria, since Cu exists in an insoluble form, copper sulfide, in anaerobic environments. Thus, it seems rational that CuZn-SOD biosynthesis could be induced by an increasing amount of Cu, because the binding of Cu by the protein has two functions: it can reduce the harmful action of free Cu ions and also catalyze the disproportionation reaction of superoxide which is produced by Cu. Further clarification of the gene regulation of CuZn-SOD will provide new insights into the role of CuZn-SOD isoforms in green plants.

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