Assignment of new cDNA for maize chloroplastic CuZn-superoxide dismutase (SOD-1) and structural characterization of *sod-1* genes

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A cDNA encoding chloroplastic CuZn-SOD (SOD-1) from maize revealed an unusual deduced amino acid sequence (Kernodle and Scandalios 2001) compared to those in other plants. To elucidate whether a typical type of chloroplastic CuZn-SOD is also present in addition to this unique SOD, we purified and characterized SOD-1, and cloned its cDNA and genomic genes.

The purified SOD-1 from maize leaves was a homodimer of 32 kDa, and exhibited an N-terminal amino acid sequence characteristic of the chloroplastic type of CuZn-SOD. A SOD-1 cDNA of 890 bp was obtained by combination of RT-PCR and RACE. Although the cDNA showed incomplete ORF, the deduced amino acid sequence contained a partial chloroplast transit peptide and a complete sequence of mature protein (154 residues) that was identical to the N-terminal sequence of the purified protein up to the 35th residue. The amino acid sequence of mature SOD-1 was 95% identical to that of rice chloroplastic CuZn-SOD. Thus, it is clear that the previously reported "SOD-1 cDNA" from maize is not the cDNA responsible for SOD-1.

Genomic SOD-1 gene (*sod-1*) was obtained by long PCR for the central region and gene-walking method for the 5'-upstream and 3'-downstream regions, and was sequenced for 3.9kbp. The *sod-1* gene was allelic, termed *sod-1a* and *sod-1b*, and contained 8 exons and 7 introns, which is the same as the chloroplastic CuZn-SOD gene in rice. The Cu-responsive element (TNNNGCTG) and a sequence similar to the target sequence of *Arabidopsis thaliana* microRNA 398 that regulates CuZn-SOD gene expression in response to Cu availability were observed upstream near the TATA box in the promoter region and in the 4th exon of maize *sod-1*, respectively.

Key words: *Zea mays,* reactive oxygen species, *sod-1* gene, superoxide dismutase, SOD-1 cDNA.

INTRODUCTION

Photosynthesis is the process by which solar energy is converted to chemical energy. Although many factors including environmental stress affect the efficiency of photosynthesis, plants have evolved and adapted to fluctuating environment by means of sophisticated systems to ensure optimal photosynthesis. At normal conditions, electrons derived from water are used for reduction of $NADP^+$ to NADPH for CO_2 fixation, but under unfavorable conditions resulting in a shortage of electron acceptor $NADP^+$, oxygen acts as an alternative electron acceptor for water-derived electrons, protecting the photosynthetic machinery against over-reduction that blocks the electron flow in photosystems (Asada 1999, 2006). In this process, called the water-water cycle, the superoxide derived from oxygen by one-electron reduction at PSI is further reduced to water by several enzyme systems. Although superoxide production in chloroplast is inevitable for the protection of photosynthesis, superoxide itself constitutes a reactive oxygen species (ROS) that nonspecifically oxidizes biomolecules such as DNA, proteins and membranes, and brings a deleterious effect to organisms.

Superoxide dismutase (SOD) is a metalloenzyme that catalyzes the dismutation of superoxide to hydrogen peroxide and molecular oxygen, and protects cells against harmful superoxide radicals and other ROS (Fridovich 1995). SOD is ubiquitous in aerobes to ensure their existence in oxygenic environments, and is present even in some anaerobes for the occasional encounter with oxygen in their habitats. With respect to metal cofactors at active sites, four SOD isozymes have been found: CuZn-, Mn-, Fe-, and Ni-containing SOD (Kanematsu and Asada 1994).

Plants contain chloroplastic and cytosolic CuZn-SOD that can be distinguished by amino acid sequence. Chloroplastic CuZn-SOD binds weakly to thylakoid membranes and plays an important role in scavenging ROS in the water-water cycle (Ogawa et al. 1995, Asada 1999). The chloroplastic enzyme shows less variation in amino acid sequence compared to the cytosolic enzyme, indicating the importance of its binding to thylakoids. Mn-SOD is found in all plants and is localized in mito-

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The abbreviations used are: ME, malic enzyme; ORF, open reading frame; PSI, photosystem I; PSII, photosystem II; ROS, reactive oxygen species; SOD, superoxide dismutase; SOD-1, maize chloroplastic CuZn-SOD; *sod-1*, maize SOD-1 gene; SODCp, rice chloroplastic CuZn-SOD; *sodCp*, rice SODCp gene.

chondria, whereas Fe-SOD, a protein homologous to Mn-SOD, is associated with chloroplasts in only some plants. Ni-SOD (Youn et al. 1996) has not yet been detected in plants.

The complete genomes of *Arabidopsis thaliana* and rice have revealed their number and type of SOD. *A. thaliana* contains at least 7 SOD genes: three CuZn-SOD genes (*CSD1*, *CSD2*, *CSD3*), three Fe-SOD genes (*FSD1*, *FSD2*, *FSD3*) and one Mn-SOD gene (*MSD1*) (Kliebenstin et al. 1998, TAIR GBrowse http://www.arabidopsis.org/cgi-bin /gbrowse/arabidopsis/). Rice contains three CuZn-SOD genes (*sodCp*, *sodCc1*, *sodCc2*), one Fe-SOD gene (*sodB*) and one Mn-SOD gene (*sodA*) (NBRP Oryzabase http://www.shigen.nig.ac.jp/rice/oryzabase/). Although complete genome information is not yet available for maize, which belongs to the same Poaceae family as rice, maize contains chloroplastic CuZn-SOD (SOD-1), which is a major SOD in the leaf, as well as four cytosolic CuZn-SODs (SOD-2, SOD-4a, SOD-4b and SOD-5). In addition, four Mn-SODs (SOD-3) constituting a gene family are present in mitochondria (Scandalios 1997, Zhu and Scandalios 1993), but Fe-SOD activity has not been detected. We have cloned a full-length cDNA encoding chloroplastic Fe-SOD, indicating the presence of this type of isozyme in maize (Kanematsu, unpublished; Accession Number AB201543).

Maize achieves NADP-ME type of C_4 photosynthesis with the cooperation of two types of cells: mesophyll cells for accumulation of CO_2 and bundle-sheath cells for CO_2 fixation. The two types differ in oxygen concentration because the bundle-sheath cells show a higher rate of cyclic electron flow around PSI compared to that for linear electron flow from PSII to PSI, indicating little oxygen evolution at PSII (Takabayashi et al. 2005). Thus, it seems that SOD regulation differs between the two types of cells. Furthermore, since the cyclic electron flow around PSI in bundle-sheath chloroplasts competes for electron with the water-water cycle, in which SOD plays a role in rapid conversion of superoxide to hydrogen peroxide and molecular oxygen, SOD might determine the direction of electron flow. Therefore, it is very important to investigate the gene regulation of chloroplastic CuZn-SOD in maize.

Although the availability of genes or cDNA for chloroplastic CuZn-SOD from maize is necessary for such studies, a cDNA reported to encode chloroplastic CuZn-SOD (SOD-1) showed unusually low homology in the deduced amino acid sequence (Kernodle and Scandalios 2001) to that of chloroplastic CuZn-SOD from other plants. To elucidate whether this unusual type of SOD is the sole chloroplastic CuZn-SOD in maize or whether a typical type of chloroplastic CuZn-SOD is also present, we conducted the purification of maize SOD-1 and the cloning of cDNA responsible for the purified SOD-1. Furthermore, we sequenced the entire SOD-1 gene (*sod-1*) covering 3.9 kbp for comparison. The results indicate that the previously reported "maize SOD-1 cDNA" does not encode the chloroplastic CuZn-SOD, and that maize contains typical chloroplastic CuZn-SOD genes.

MATERIALS AND METHODS

Materials. *TaKaRa Taq*, *TaKaRa LA Taq* and Oligotex-dT30 Super were purchased from Takara (Kyoto, Japan). Isogen was obtained from Nippon Gene (Toyama, Japan). Nucleon Phytopure for plant DNA extraction kit was from Amersham Life Science (Buckinghamshire, UK). Geneclean II Kit was from BIO 101 (Vista, CA, USA). Quantum Prep Plasmid Miniprep Kit was from Bio-Rad (Hercules, CA, USA). SuperScript First-Strand Synthesis System for RT-PCR was from Life Technologies (Rockville, MD, USA). SMART RACE cDNA Amplification Kit and Universal GenomeWalker Kit are products of Clontech (Palo Alto, CA, USA). pGEM-T Easy Vector System I was purchased from Promega (Madison, WI, USA). The DNA sequencing kit (BigDye Terminator Cycle Sequencing Ready Reaction) was from Applied Biosystems (Foster City, CA, USA). All other reagents were commercial products of the highest grade. Antibody raised against spinach chloroplastic CuZn-SOD was prepared as described previously (Kanematsu and Asada 1989).

Plant material and growth conditions. Hybrid seeds (F1) of maize (*Zea mays* L. cv. Honey Bantam) were obtained from Atariya Farm (http://www.atariya.net). Seeds were imbibed for 24 h in distilled water and grown on paper towels moistened with distilled waster in trays in growth chambers at 25°C with a 16-h photoperiod and PPFD of 100 μmol $m^{-2}s^{-1}$. The plants were fertilized occasionally with 1000-fold diluted Hyponex. For enzyme extraction, maize was field grown for two months under natural conditions.

Isolation of chloroplasts and preparation of stromal fraction. Maize chloroplasts were obtained by differential centrifugation method. Leaves (50 g) from two-week-old plants were cut into small pieces and homogenized in 100 ml of 50 mM MES-KOH medium using a Polytron at maximum speed for a few seconds. The MES-KOH medium contained 0.33 M sorbitol, 1 mM $MgCl_2$, 2 mM Na_2-EDTA, 10 mM NaCl, 0.5 mM KH_2PO_4, 1 mM ascorbic acid and 50 mM MES adjusted to pH 6.2 using KOH. The homogenate was filtered through four layers of cheesecloth, and the filtrate was centrifuged at 700×g for 30 s to remove cell debris. The supernatant was centrifuged at 1,000×g for 30 s and the resulting precipitate was resuspended in 4 ml of 50 mM HEPES-KOH medium. The HEPES-KOH medium was the same as MES-KOH medium except that 50 mM HEPES-KOH, pH 7.6, was used instead of MES-KOH, pH 6.2. The suspension was again centrifuged at 1,000×g for 30 s and the precipitated chloroplasts were collected and lysed osmotically in a small amount of 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. After centrifugation at 18,000×g for 10 min, the supernatant was used as the stromal fraction.

Plasmid, bacterial strain, medium and growth conditions. pGEM-T Easy and *Escherichia coli* XL1-Blue MRF' (Stratagene, La Jolla, CA, USA) were employed as cloning plasmid vector and host strain, respectively. *E. coli* harboring plasmids was cultured in Luria-Bertani medium (LB) containing 100 μg/ml ampicillin (amp) at 37°C for 16−17 h. If necessary, isopropyl-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) were added at 200 μM and 40 μg/ml, respectively.

N-terminal amino acid sequencing. The purified SOD (0.6 μg) was fixed on a PVDF membrane using ProSorb (PerkinElmer) according to the manufacturer's instructions. The PVDF membrane was subjected to N-

terminal Edman sequencing using a Procise 494/HT Protein Sequencer (Applied Biosystems, Foster City, CA, USA). Analysis was conducted up to the 45th amino acid residue.

Isolation of total RNA and purification of Poly(A)$^+$ mRNA. Total RNA was isolated from maize leaves using Isogen according to the manufacturer's instructions with some modification. Leaves (0.5 g) from plants grown for 10 days were pulverized in liquid nitrogen with a mortar and pestle, and the resulting fine powder was added to 10 ml Isogen preheated at 50°C, spun for 30 s and further incubated for 10 min at 50°C. After adding 2 ml chloroform and shaking vigorously, the mixture was centrifuged at 4,500×g for 10 min, and aqueous phase (6 ml) was obtained. After adding an equal volume of isopropanol and allowing it to stand for 10 min at room temperature, the mixture was centrifuged at 4,500×g for 10 min. To the pellet thus obtained, 1 ml of 75% EtOH was added and the solution was spun vigorously, and centrifuged at 18,000×g for 5 min. The precipitate (300 μg RNA) was dissolved in 200μl distilled water. Poly(A)$^+$ mRNA was obtained from the total RNA using Oligotex-dT30 Super according to the manufacturer's instructions. Poly(A)$^+$ mRNA was recovered in distilled water and stored with 2.5 vol 100% EtOH and 0.1 vol sodium acetate, pH 5.2, at −80°C until use.

Amplification of cDNA fragments by RT-PCR. cDNA fragments encoding CuZn-SOD were obtained by two-step RT-PCR. First-strand cDNA was prepared from 500 ng mRNA using the SuperScript First-Strand Synthesis System for RT-PCR (Life Technologies) with Oligo $(dT)_{12\text{-}18}$ Primer and SuperScript II Reverse Transcriptase according to the manufacturer's instruction manual. Amplification of CuZn-SOD cDNA fragments was conducted using *Taq* polymerase and gene-specific degenerate primers as follows: sense primer CARGARGAYGAYGGNCCNAC (SPGY-SENSE) and antisense primers CCNCCYTTNCC**A**ARRTCRTC (SPGY-AS-A); CCNCCYTTNCC**G**ARRTCRTC (SPGY-AS-G); CCNCCYTTNCC**T**ARRTCRTC (SPGY-AS-T); CCNCCYTTNCC**C**ARRTCRTC (SPGY-AS-C). PCR was conducted using the GeneAmp PCR System 9700 (Applied Biosystems) under the following conditions: preheated at 94°C for 2 min, 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 30 s.

Purification and vector ligation of amplified DNA fragments. PCR products were separated on an agarose gel, and a major band on the gel corresponding to the amplified DNA fragments was excised and purified by glass powder method using the Geneclean II Kit. The purified DNA fragments were ligated into the pGEM-T Easy Vector and transformed by competent cells of *E. coli* XL1-Blue MRF'. Positive colonies were selected from LB/amp plates supplemented with X-gal and IPTG. Plasmids were prepared using the Quantum Prep Plasmid Miniprep Kit.

First-strand cDNA synthesis and 5'- and 3'-RACE. The upstream and downstream regions of the cDNA encoding chloroplastic CuZn-SOD were amplified by RACE method using a SMART RACE cDNA Amplification Kit according to the manufacturer's instructions. In first-strand cDNA synthesis for 5'- and 3'-RACE, each 150 ng poly(A)$^+$ mRNA was used. The cDNA for 5'-RACE (5'-RACE-Ready cDNA) was synthesized with PowerScript reverse transcriptase using a modified oligo(dT) primer (5'-CDS) and the SMART II oligo (Clontech). The cDNA for 3'-RACE (3'-RACE-Ready cDNA) was synthesized with the reverse transcriptase using a special oligo(dT) primer (3'-CDS) (Clontech).

RACE PCR reactions were performed with Advantage 2 Polymerase Mix (Clontech) using Universal Primer Mix (UPM, Clontech) and the following gene-specific primers designed from the cDNA core nucleotide sequence of chloroplastic CuZn-SOD: MZ5GSP13 (5'-TGGGTATCAACAATGGTTGCCTCGG-3') for 5'-RACE and MZ3GSP9 (5'-CTTCATGGCTTCCACCTC-CACGAG-3') for 3'-RACE. PCR for 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, and 72°C for 3 min. For 3'-RACE, the final step was reduced to 25 cycles.

The PCR products were gel-purified, ligated into pGEM-T EZ, and transformed by *E. coli* XL1-Blue MRF'. Positive colonies were selected from LB/amp plates containing IPTG/X-gal and the inserts were checked by PCR. For the products in 5'-RACE, colonies having the larger insert were used for further analysis. Plasmids were purified using the Quantum Prep Plasmid Miniprep Kit and subjected to sequencing.

Isolation of genomic DNA. Genomic DNA was isolated from 12-day-old maize leaves using a Nucleon Phytopure Plant DNA Extraction Kit according to the manufacturer's instructions with slight modification. Green leaves (0.1 g) in a 1.5-ml microtube were pulverized in liquid nitrogen with a small pestle driven by electric motor, and DNA was extracted from the resulting fine powder. The DNA was finally dissolved in 20μl of 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA.

Amplification of genomic gene by PCR. Chloroplastic CuZn-SOD genomic gene was obtained by two-step PCR amplification. First, a central portion of the gene was amplified using gene-specific primers based on the obtained cDNA sequence. Then, 5'-upstream and 3'-downstream of the central portion of the gene were obtained by DNA walking using the Universal GenomeWalker Kit.

The central portion of the gene was amplified from 300 ng of genomic DNA using *LA Taq* with the sense primer MZF2-21 (5'-TCCCTATTCCTCCGCACGCC-CTTTCCACTC-3') and antisense primer MZB717-685 (5'-CCTGCAAAGGCTATTACGCACCATACCACAT-GC-3'). PCR was conducted in the 7600 mode of the GeneAmp PCR System 9700. PCR conditions were: 94°C for 1 min, 35 cycles of 98°C for 10 s and 68°C for 10 min, 72°C for 10 min. About 3 kbp of the major PCR product was gel-separated, purified by glass-milk method, ligated into pGEM-T Easy, and transformed by *E. coli* XL1-Blue MRF'. Positive colonies were selected from LB/amp plates containing IPTG/X-gal.

Pools of adaptor-ligated genomic DNA fragments (GenomeWalker libraries) were constructed according to the manufacturer's manual. Genomic DNA (2.5 μg) was digested either with *Dra*I, *Eco*RV, *Puv*II or *Stu*I, and the DNA fragments were purified as recommended in the manual and dissolved in 20 μl of 10 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA. Each fragment derived

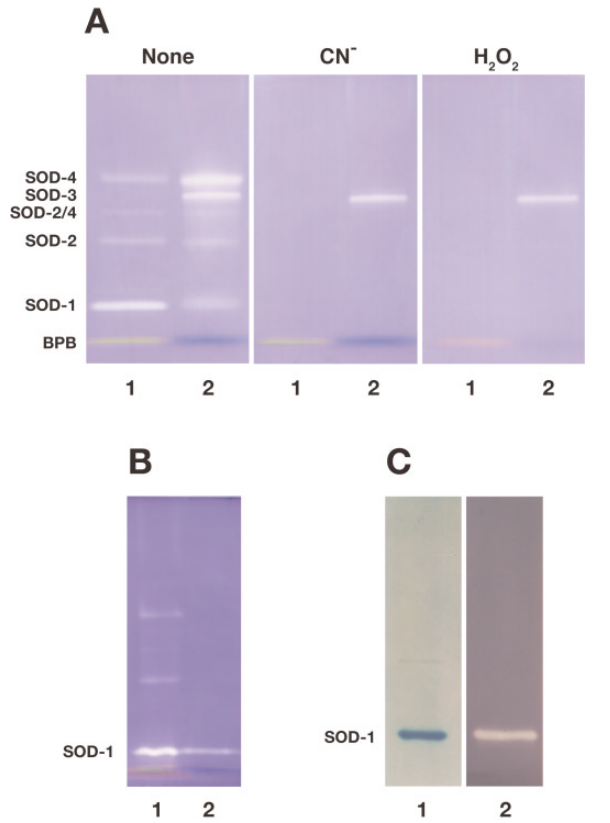

Fig. 1. Maize SOD isozymes in native-PAGE. (A) SOD activity staining of the cell extract from maize seeds and leaves. Left panel, no treatment; middle panel, 2 mM CN^- treatment during activity staining; right panel, pre-treated with 5 mM H_2O_2 for 30 min before staining. Lane 1, leaf extract (100 μg protein); Lane 2, seed extract (100 μg protein). BPB, tracking dye. SOD-2/4, a homodimer composed of each subunit of SOD-2 and SOD-4. Seeds imbibed in distilled water for 12 h were used. The leaves were obtained from the 14-dpi plants. (B) Chloroplast localization of SOD-1. Lane 1, leaf extract (14 dpi); Lane 2, stromal fraction. (C) Homogeneity of purified SOD-1. Lane 1, protein staining (Coomassie Brilliant Blue R-250) of the purified SOD-1 (10 μg); Lane 2, activity staining of the purified enzyme (5 units). Top, cathode; bottom, anode.

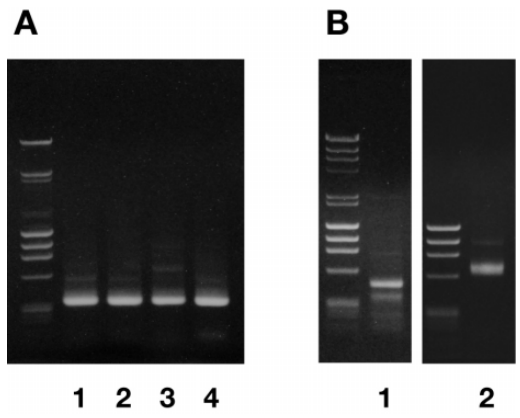

Fig. 2. Cloning of SOD-1 cDNA. (A) Amplification of the central portion of SOD-1 cDNA (323 bp) by PCR with degenerate primers. The sense primer SPGY-SENSE was used. Lanes 1 to 4, degenerate antisense primer SPGY-AS-A, SPGY-AS-G, SPGY-AS-T and SPGY-AS-C, respectively. (B) Amplification of the 5'- and 3'-flanking regions of the cDNA central portion by RACE. Lane 1, 5'-RACE with the gene-specific primer MZ5GSP13; Lane 2, 3'-RACE with the primer MZ3GSP9. The leftmost lane of each panel, size marker.

from restriction enzyme digestion was ligated to GenomeWalker Adaptor (Clontech), and the reaction mixture was aliquoted and used for amplification.

Primary PCR of each GenomeWalker library was performed with *LA Taq* using Adapter Primer 1 (AP1, Clontech) and antisense primer MZ2GW5#1 (5'-AGAGAGGAATGCACCCACCCGATTAAC-3') for 5'-upstream amplification, or sense primer MZ2GW3#1 (5'-TGCGTTGCTGAATCATTTCCTCTTGTG-3') for 3'-downstream amplification under the following PCR conditions: 94°C for 3 min, 7 cycles of 94°C for 2 s and 72°C for 3 min, 32 cycles of 94°C for 2 s and 67°C for 3 min, and 67°C for 4 min.

Nested secondary PCR of each library was conducted with *LA Taq* using AP2 (Clontech) and antisense primer MZ2GGW5#2 (5'-AGGTGAGAAGATGGGGGGACAGGATAG-3') for 5'-upstream, or sense primer MZ2GW3#2 (5'-GGGTTGCAGTTGCAGCTACGCTGTTAC-3') for 3'-downstream amplification under the conditions of preheating at 94°C for 3 min, 5 cycles of 94°C for 2 s and 72°C for 3 min, 20 cycles of 94°C for 2 s and 67°C for 3 min, with post-heating at 67°C for 4 min.

Promoter analysis. Promoter analysis was conducted using the Plant cis-Acting Regulatory DNA Elements (PLACE) database (Higo el al. 1999).

Analytical methods. Cycle sequencing reaction and DNA sequencing analysis were performed as described previously (Kanematsu and Sato 2008). SOD was assayed as described previously (Kanematsu and Asada 1990) and the unit of activity was based on McCord and Fridovich (1969). Protein content was measured following the method of Lowry et al. (1951) with bovine serum albumin as the standard. Native-PAGE, SDS-PAGE, SOD activity staining, and immunoblotting were performed as before (Kanematsu and Asada 1990, Ueno and Kanematsu 2007). Agarose electrophoresis was performed according to the procedures described by Sambrook et al. (1989).

Nucleotide sequence accession numbers. The nucleotide sequences of the cDNA and the genomic genes (*sod-1a* and *sod-1b*) of maize chloroplastic CuZn-SOD (SOD-1) have been submitted to the DDBJ, EMBL and GenBank under Accession Numbers AB078737 (SOD-1 cDNA), AB093580 (*sod-1a*) and AB093581 (*sod-1b*). Part of the present results have been presented elsewhere (Kanematsu and Fujita 2002, Kanematsu et al. 2003).

RESULTS

Activity staining of maize SOD isozymes and isoforms in native-PAGE. In early investigations, maize SOD isozymes and isoforms separated on a starch gel and stained for SOD activity were designated as SOD-1, SOD-2, SOD-3 and SOD-4 in order of mobility (Baum

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                                                                                                    100
CTCCCTATTC CTCCGCACGC CCTTTCCACT CGGTCCACTT CGTCGCCGGC CCGTGGGGCG CCGCCGCCGC CAAGGCGCTC GTCGTCGCGG ACGCCACTAA
   P  Y  S   S  A  R   P  F  H   S  V  H  F   V  A  G   P  W  G   A  A  A  A   K  A  L   V  V  A   D  A  T  K
                                                                                                   A  T  K

                                                                                                    200
GAAGGCCGTA GCCGTCCTCA AGGGCGCGTC TGAGGTCGAG GGCGTGGTCA CGCTCACGCA GGACGACGAT GGACCTACAA CTGTGAATGT CCGTATCACT
  K  A  V   A  V  L   K  G  A  S   E  V  E   G  V  V   T  L  T  Q   D  D  D   G  P  T   T  V  N  V   R  I  T
  K  A  V   A  V  L   K  G  A  S   E  V  E   G  V  V   T  L  T  Q   D  D  D   G  P  P   T  V  N  V   R  I

                                                                                                    300
GGACTTACCC CTGGACTTCA TGGCTTCCAC CTCCACGAGT TTGGTGATAC TACCAATGGG TGCATATCGA CAGGACCACA TTTTAATCCA AACAATCTGA
 G  L  T   P  G  L  H   G  F  H   L  H  E   F  G  D  T   T  N  G   C  I  S   T  G  P  H   F  N  P   N  N  L

                                                                                                    400
CACACGGTGC ACCAGAAGAC GAAGTCCGTC ATGCGGGTGA CCTGGGAAAC ATTGTTGCAA ATGCTGAGGG CATAGCCGAG GCAACCATTG TTGATACCCA
T  H  G  A   P  E  D   E  V  R   H  A  G  D   L  G  N   I  V  A   N  A  E  G   I  A  E   A  T  I   V  D  T  Q

                                                                                                    500
GATTCCTTTG ACTGGCCCAA ATTCAGTTGT TGGGAGAGCA TTTGTGGTTC ATGAGCTTGA AGATGATTTG GGGAAAGGGG GCCATGAGCT CAGCCTCTCT
  I  P  L   T  G  P   N  S  V  V   G  R  A   F  V  V   H  E  L  E   D  D  L   G  K  G   G  H  E  L   S  L  S

                                                                                                    600
ACTGGAAATG CTGGTGGAAG ACTGGCATGT GGTGTTGTTG GCCTGACTCC ATTGTAGGTT GCTCGCCGGG TGCCCGGGTT GCAGTTGCAG CTACGCTGTT
 T  G  N   A  G  G  R   L  A  C   G  V  V   G  L  T  P   L

                                                                                                    700
ACATCCTTGC CTTGGTGGAC TTTTGTCTTC AATCGTGTTA TTGGGACAGT GACCTTTTTT TTGTATTACT TGGATTCATT CCATGCATGT GGTATGGTGC

                                                                                                    800
GTAATAGCCT TTGCAGGTCG ACTGTATAAA AAGCAATATT TAGTTGAGGA AATAAGTGTT GCCACATATC CAAATCGATC TATGAAGCAC TATGAACATC

                                                                                                    890
AAATGTTATT TTGATTTTGT TTTAACCTTT TTTAAAGCTA TTTCTAAAAA GTAATATTCG AGAAAAAAAA AAAAAAAAAA AAAAAAAAAA
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Fig. 3. Nucleotide sequence of maize SOD-1 cDNA and its deduced amino acid sequence. The nucleotide sequence of 890 bp was obtained from the combination of PCR amplification for central, and 5'-and 3'-flanking regions. The deduced amino acid sequences of transit peptide and mature SOD-1 are shown in green and blue letters, respectively. N-terminal amino acid sequence up to the 35th residue of SOD-1 is indicated in red letters with mismatch at the 29th residue, which is probably due to poor resolution in the analysis.

and Scandalios 1982, Baum et al. 1983). SOD-1 was a dominant activity band in leaves and was assigned to chloroplastic CuZn-SOD, whereas SOD-2 and SOD-4, and SOD-3 were determined to be cytosolic CuZn-SOD and Mn-SOD, respectively.

In 7.5% native-PAGE at pH 8.3, cell extracts from maize leaves revealed a major SOD activity band at the furthermost anodic side on a gel, whereas seed extracts showed a major band at the cathodic side (Fig. 1A). Seeds exhibited considerable activity of Mn-SOD (SOD-3), which was assigned according to the response against cyanide and hydrogen peroxide. The chloroplast stromal fraction revealed a single activity band corresponding to the major band of leaf extract (Fig. 1B). Thus, this band was assigned as chloroplastic CuZn-SOD (SOD-1), which was confirmed by the reactivity with anti-spinach chloroplastic CuZn-SOD serum (data not shown). This antibody could distinguish chloroplastic and cytosolic CuZn-SODs from land plants (Kanematsu and Asada 1989).

To the best of our knowledge, N-terminal amino acid sequence analysis of maize chloroplastic CuZn-SOD (SOD-1) has not previously been done; therefore, we purified SOD-1 to determine the N-terminal sequence of the protein.

Purification and N-terminal amino acid sequence of SOD-1. Mature leaves (1 kg) of maize were homogenized by Polytron in 50 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA with polyvinylpyrrolidone. After centrifugation, the cell extract was fractionated with ammonium sulfate and the fraction corresponding to 40－80% saturation was collected. The precipitate was dissolved in and dialyzed against 10 mM potassium

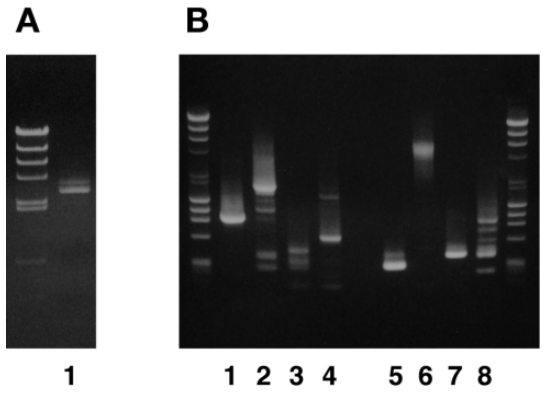


Fig. 4. Cloning of SOD-1 genomic gene. (A) Amplification of the central portion of the genomic gene (Lane 1) by PCR. The DNA fragment (3 kbp) was amplified with the sense primer MZF2-2 and the antisense primer MZB717-685 using *LA Taq*. Left lane, size marker. (B) Amplification of the 5'-upsteam and 3'-downstream regions of the genomic gene fragment by GenomeWalker method. PCR was conducted with each GenomeWalker library that was made by treating genomic DNA with the restriction enzyme *Dra*I, *Eco*RV, *Puv*II or *Stu*I. Nested secondary PCR with the antisense primer MZ2GGW5#2 for 5'-upstream amplification (Lanes 1 to 4) and with the sense primer MZ2GW3#2 for 3'-downstream amplification (Lanes 5 to 8). Lanes 1 and 5, *Dra*I; Lanes 2 and 6 *Eco*RV; Lanes 3 and 7, *Puv*II; Lanes 4 and 8, *Stu*I-treated GenomeWalker library. Both sides of lanes, size marker.

Fig. 5. Nucleotide sequences of two alleles (*sod-1a* and *sod-1b*) of maize SOD-1 genomic gene. Two allelic genes are shown in consensus sequence (black letters) with different nucleotides at alleles (light blue letters), which are placed above the consensus sequence. *Sod-1a* and *sod-1b* are indicated with green and blue letters, respectively. A nucleotide deletion is shown with a dot. Deduced amino acid sequence is shown in blue letters, in which chloroplast transit peptide is in green letters. Exons 1 to 8 are indicated in red letters, but 5'-UTR in exon 1 is not shown due to uncertainty of transcription start site. Start and stop codons are underlined in red. TATA box as well as Cu-responsive sequence and a possible target sequence of miRNA are shaded in yellow. A putative polyadenylation signal is underlined in blue.

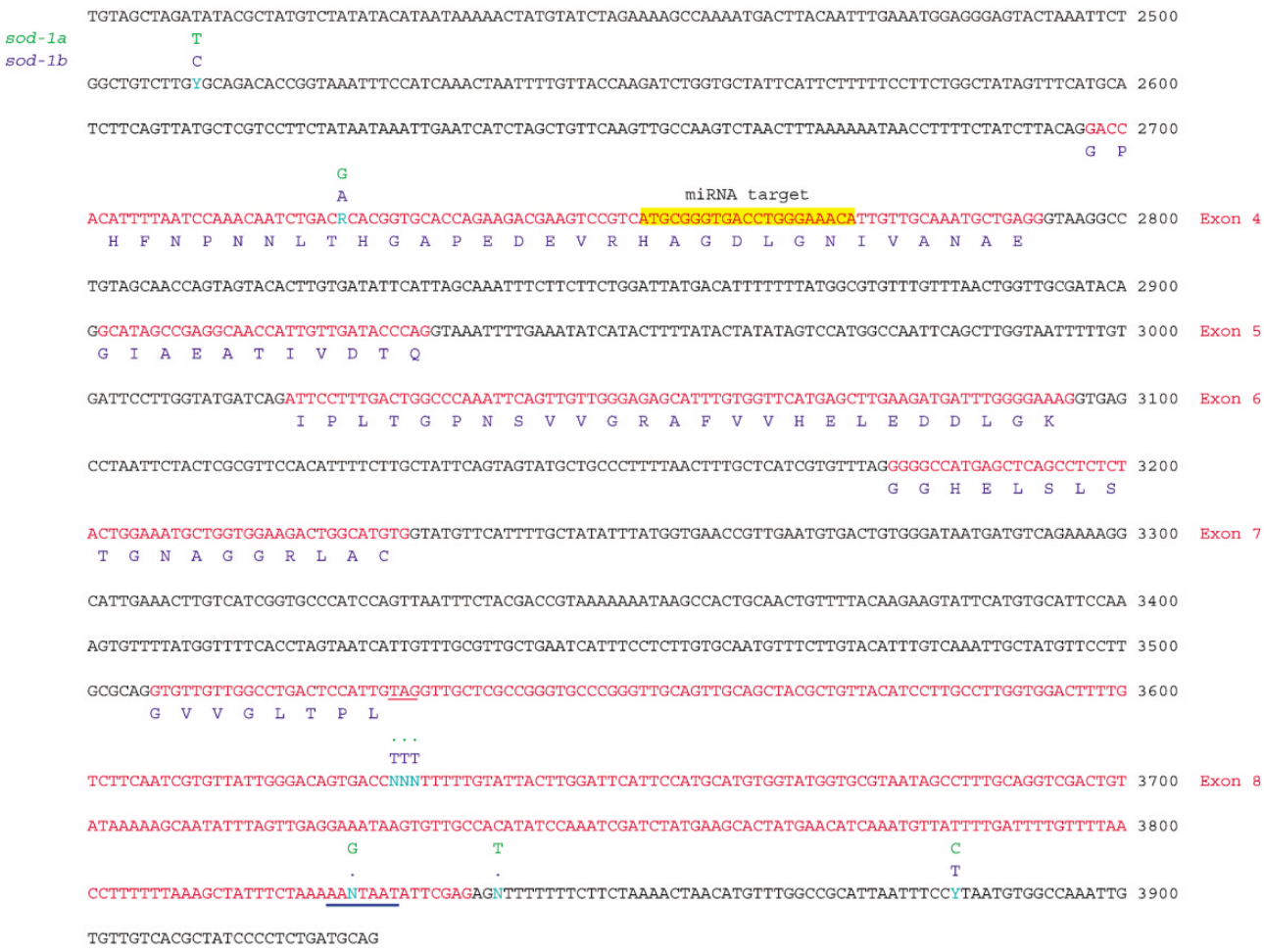


Fig. 5. ***Continued.***

phosphate, pH 7.8, containing 0.1 mM EDTA. The dialyzed enzyme was applied to a column of DEAE Sephadex A-50 equilibrated beforehand with 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. After washing the column with 50 mM KCl in the equilibrating buffer, SOD was eluted with 150 mM KCl in the buffer.

The 150 mM KCl fraction was concentrated by ultrafiltration using Amicon PM-10 and the buffer was changed to 10 mM Tris-HCl, pH 7.4, during the concentration. The concentrated enzyme was subjected to linear gradient elution using two phases (0→30 mM KCl steep gradient and 30→35 mM KCl gentle gradient in 10 mM Tris-HCl, pH 7.4) on a Mono Q HR5/5 column fitted to FPLC (Pharmacia Biotech). Active fractions eluted at 30→35 mM KCl gradient were pooled and concentrated, during which the buffer was changed to 0.1 M potassium phosphate, pH 7.8, containing 2 M ammonium sulfate. The enzyme solution was adsorbed on Phenyl Superose HR5/5 equilibrated with the same buffer. SOD was eluted using a simultaneous cross-linear gradient of 2→0 M ammonium sulfate and 0→40% ethylene glycol in 0.1 M potassium phosphate, pH 7.8. Active fractions were pooled, concentrated and passed through a column of Superdex 75 HR10/30 equilibrated with 10 mM Tris-HCl, pH 7.8, containing 150 mM KCl. The SOD fraction was pooled, concentrated and dialyzed against 10 mM potassium phosphate, pH 7.8.

Electrophoresis revealed that the purified enzyme was almost homogeneous (Fig. 1C). The cross reactivity with anti-spinach chloroplastic CuZn-SOD serum in Western blotting after native-PAGE clearly indicated that the purified CuZn-SOD is a chloroplastic type of the enzyme (data not shown), and accordingly, corresponds to SOD-1, as designated by Baum et al. (1983). Gel-filtration method estimated a molecular mass of 32 kDa and SDS-PAGE indicated a homodimer subunit structure (data not shown).

N-terminal amino acid sequence (35 residues) of the purified CuZn-SOD showed typical characteristics of a chloroplastic type of the enzyme. The amino acid sequence was identical to the deduced amino acid sequence from the cDNA for chloroplastic CuZn-SOD (see below).

Cloning of SOD-1 cDNA. cDNA encoding chloroplastic CuZn-SOD was obtained by combination of RT-PCR using degenerate primers based on amino acid sequences, and 5'- and 3'-RACE with gene-specific primers. In the primer design for RT-PCR, the forward degenerate primer was based on the N-terminal amino acid sequence of the chloroplastic CuZn-SOD of the green alga *Spirogyra* sp. (Kanematsu, unpublished) and the reverse primer was based on the highly conserved region of amino acid sequences between both chloroplastic and cytosolic CuZn-SOD isozymes from land plants (Fig. 10.3 in Kanematsu and Asada 1994). Using these

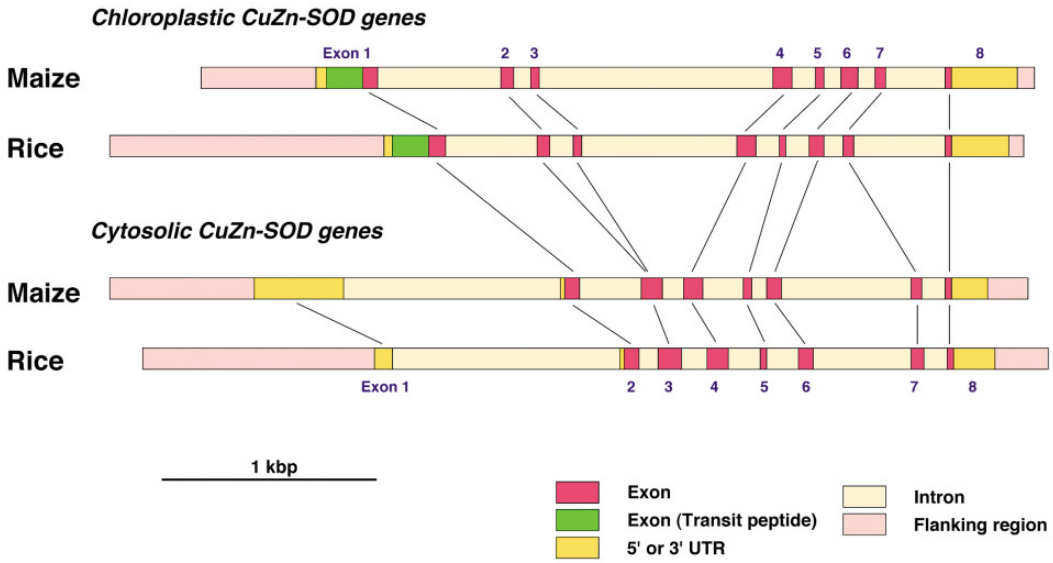


Fig. 6. Comparison of exon-intron structure of chloroplastic and cytosolic CuZn-SOD genomic genes. Chloroplastic CuZn-SOD genes, maize (*sod-1*) and rice (*sodCp*, Accession No. AB26724); cytosolic CuZn-SOD genes, maize (*sod-4A*, U34727) (Kernodle and Scandalios 1996) and rice (*sodCc1*, L19435). The transcription start site of maize *sod-1* was rationally estimated to be localized between the TATA box and ATG start codon so that the 5'-UTR in exon 1 could be compared with that of rice.

primer sets, we successfully amplified cDNA fragments for chloroplastic CuZn-SOD of the green alga *Spirogyra* sp. and for both chloroplastic and cytosolic CuZn-SOD of the moss *Pogonatum inflexum* and the fern *Equisetum arvense* (Kanematsu, unpublished).

A DNA fragment of 323 bp with two extra A was amplified in RT-PCR using the degenerate sense primer SPGY-SENSE, and antisense primer SPGY-AS-A, SPGY-AS-G, SPGY-AS-T or SPGY-AS-C, as shown in Fig. 2A. Each antisense primer that differed at one base position was used to increase its concentration in the PCR reaction. After cloning the amplified fragments into the vector pGEM-T EZ followed by transformation with *E. coli* XL1-Blue MRF', seven positive colonies were collected and plasmids were sequenced. The results indicated that out of seven clones, five clones contained a partial nucleotide sequence for chloroplastic CuZn-SOD, whereas two clones possessed a sequence belonging to cytosolic CuZn-SOD based on its amino acid sequence alignment in many plants.

Upstream and downstream sequences of chloroplastic CuZn-SOD cDNA were obtained by 5'- and 3'-RACE using gene-specific primers MZ5GSP13 and MZ3GSP9, respectively (Fig. 2B). The PCR products in 5'-RACE revealed their truncated 5'-upstream sequence, being devoid of ATG start codon, although they contained the sequence covering the N-terminal region of mature chloroplastic CuZn-SOD gene. The DNA fragments amplified in 3'-RACE showed a complete 3'-downstream sequence. The combined nucleotide sequence is shown in Fig. 3. To obtain the complete ORF sequence, we compensated for the lacking sequence by isolating the chloroplastic CuZn-SOD genomic gene, instead of finding the longer PCR products in 5'-RACE.

Cloning of SOD-1 genomic gene. The central region of the chloroplastic CuZn-SOD gene was amplified by long PCR using *LA Taq* and gene-specific primers MZF2-21 and MZB717-685 (Fig. 4A). Amplified fragments of 3 kbp were cloned, and six clones were selected for whole sequencing. We found two nucleotide sequences derived from both parents of the hybrid maize Honey Bantam, differing at several alleles (see below).

Using gene-specific primers designed from the sequence of the central region of the gene, upstream and downstream regions of the gene were obtained by DNA walking from both sides of the central portion. PCR products from the *Dra*1 library contained a 5'-side of the target sequence of 870 bp and the products from *Dra*I and *Pvu*II libraries contained a 3'-side of the target sequence of 310 bp and 400 bp, respectively (Fig. 4B). The fragments from *Eco*RV and *Stu*I were false positive. Thus, the 5'-upstream and 3'-downstream DNA fragments obtained from *Dra*I and *Pvu*II libraries, respectively, were fully sequenced. Both sides of the DNA fragments revealed two allelic sequences derived from both parents, the same as with the central fragment.

Using allele positions, we could correctly connect 5'-side, central and 3'-side fragments, and finally we assembled 3.9 kbp of DNA for maize chloroplastic CuZn-SOD genomic gene (Fig. 5). Hereafter, the two allelic genes are referred to as *sod-1a* and *sod-1b*.

Exon-intron structure of *sod-1a* and *sod-1b*. Maize SOD-1 genomic genes, *sod-1a* and *sod-1b*, contained 8 exons and 7 introns as revealed by comparison with SOD-1 cDNA (Fig. 5). The exon-intron structure of *sod-1* (*sod-1a* and *sod-1b*) was compared with that of maize cytosolic (*sod-4a*) and rice chloroplastic (*sodCp*) and cytosolic (*sodCc1*) CuZn-SOD genes (Fig. 6). The comparison revealed that the structure of the maize *sod-1* highly resembled that of rice *sodCp*, and exon 2 and 3 of maize *sod-1* had merged into exon 3 of maize cytosolic *sod-4A*, as those of the rice counterparts.

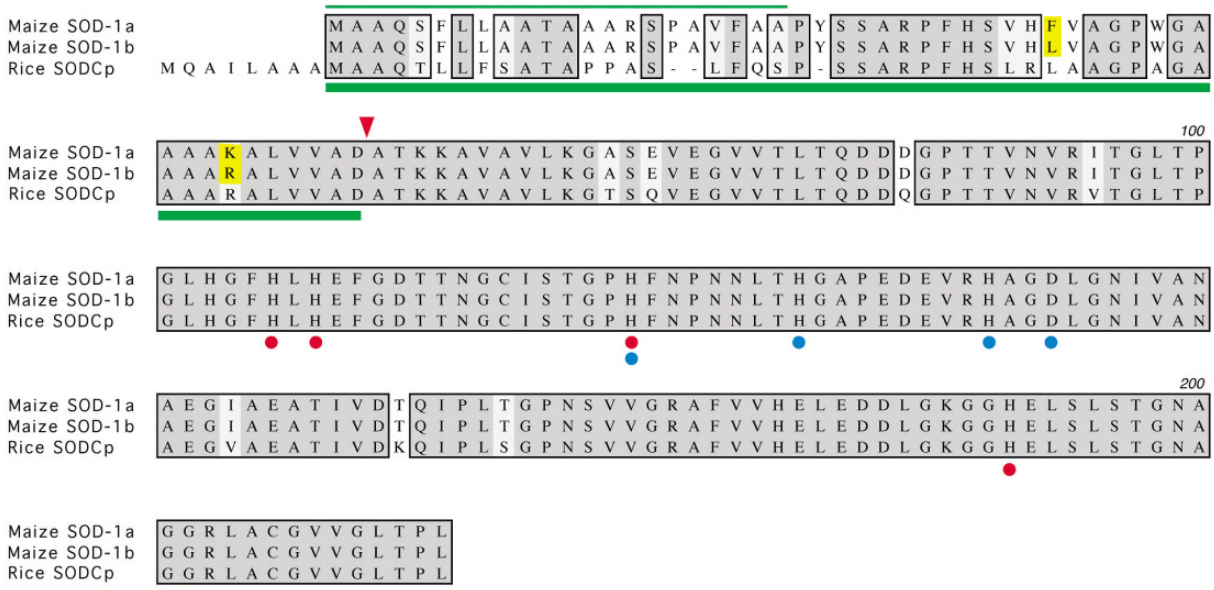


Fig. 7. Comparison of amino acid sequences of chloroplastic CuZn-SODs from maize and rice. The amino acid sequences of maize SOD-1a and SOD-1b were compared with that of rice chloroplastic SODCp (Kaminaka et al. 1997). Alignment was conducted using the ClustalW program. Incomplete ORF of SOD-1 cDNA was complemented by maize SOD-1 genomic sequence (thin green line). The thick green line indicates transit peptide of maize SOD-1. Red closed upside-down triangle shows a cleavage site for mature proteins. Identical and similar amino acid residues are boxed with dark and light shades, respectively. Ligands to copper and zinc are indicated with red and blue closed circles, respectively.

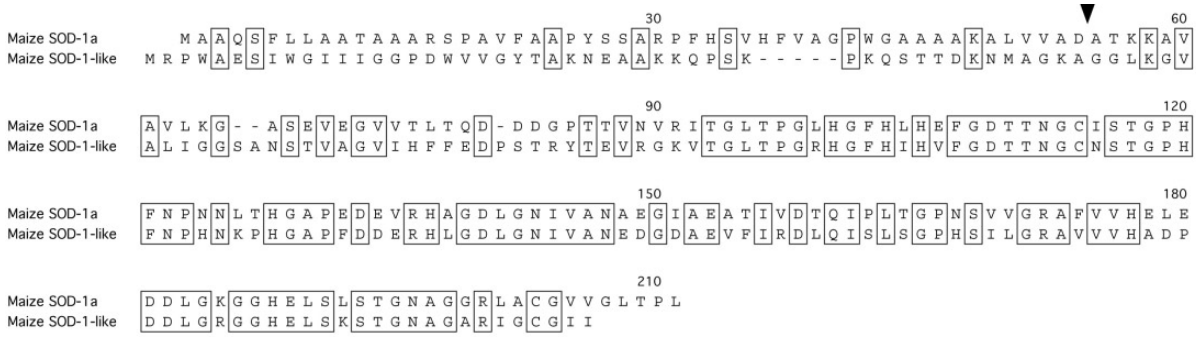


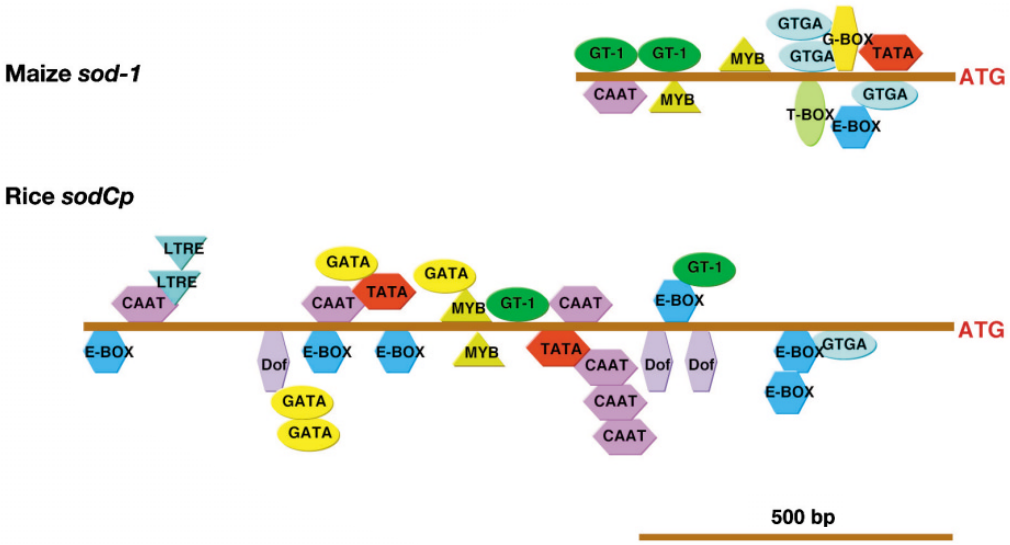
Fig. 8. Amino acid sequence alignment of chloroplastic CuZn-SOD (SOD-1a) and the previously assigned "SOD-1" (SOD-1-like). Maize SOD-1a and SOD-1-like, which was previously assigned to SOD-1, were aligned using ClustalW. Identical amino acid residues are boxed. Closed upside-down triangle above SOD-1a sequence shows a cleavage site for mature SOD-1 protein.

Amino acid sequence comparison of SOD-1. The complete amino acid sequence of SOD-1 was deduced from the cDNA and compensation for 22 residues of the N-terminal by the genomic sequence, or deduced from the allelic genomic sequences (*sod-1a* and *sod-1b*) for the entire region. We obtained two amino acid sequences with two different amino acid residues in the transit peptide (Fig. 7). No difference in coding region for mature protein was observed. The SOD-1 cDNA that we cloned was shown to be derived from *sod-1a*.

Amino acid sequences of maize SOD-1 were compared with that of rice chloroplastic CuZn-SOD (SODCp) (Fig. 7). The alignment indicated a high resemblance of 95% in the coding region and 63−67% in the transit peptide. All amino acid residues for metal ligands were conserved. Thus, SOD-1 is a typical chloroplastic CuZn-SOD. Comparison of SOD-1 amino acid sequence with the deduced sequence previously reported as "SOD-1" (Kernodle and Scandalios 2001) (SOD-1-like) clearly showed that the present SOD-1 is a product of the *sod-1* gene (Fig. 8).

Regulatory motifs in promoter region of *sod-1*. Regulatory elements in the promoter region of *sod-1a* and *sod-1b* were analyzed using the PLACE database (Fig. 9). The putative TATA box was found 90 bp upstream from the ATG start codon, and several stress-related elements such as G-box, E-box and MYB were indicated around the TATA box. Comparison of these arrangements of *sod-1* promoter region with those of rice

A



B

Factor or site name	Motif	Description
CAATBOX1(CAAT)[1)]	CAAT	promoter consensus sequence
EBOXBNNAPA(E-BOX)	CANNTG	ABA response
GT1CONSENSUS(GT-1)	GRWAAW	light responsive element
GBOXLERBCS(G-BOX)	MCACGTGGC	light responsive element
GTGANTG10(GTGA)	GTGA	GTGA motif in promoter
MYBCORE(MYB)	CNGTTR	dehydration responsive element
TATABOX2	TATAAAT[2)]	promoter consensus sequence
MARTBOX(T-BOX)	TTWTWTTWTT	motif found in matrix attachment region
DOFCOREZM(Dof)	AAAG	auxin response, phytochrome signaling
GATABOX(GATA)	GATA	light responsive promoter
LTRECOREATCOR15(LTRE)	CCGAC	low temperature responsive element

1) The names in parentheses are used in schematic diagrams (A).
2) Maize *sod-1* contains putative TATA sequence, TATAATAAA.

Fig. 9. Comparison of *cis*-elements in promoter regions between maize *sod-1* and rice chloroplastic *sod* gene. (A) Schematic diagrams of *cis*-elements in promoter regions of maize chloroplastic *sod-1* and rice chloroplastic *sodCp* (AB026724). *Cis*-elements were searched using the PLACE database and selected putative *cis*-elements upstream ATG start codon are depicted in the figure. (B) Names of the selected *cis*-elements, motif sequences and descriptions for their responses.

sodCp revealed low similarity, indicating the occurrence of species-specific regulation.

DISCUSSION

Identification of chloroplastic CuZn-SOD termed SOD-1 in native-PAGE. SOD isozymes in maize have been well characterized genetically and biochemically, and used as a model system in analyzing gene control under oxidative stress (Baum and Scandalios 1982, Scandalios 1997). To our surprise, however, a cDNA reported to be encoding SOD-1, assigned to chloroplastic CuZn-SOD in a starch gel, revealed an unusual deduced amino acid sequence compared to that of chloroplastic CuZn-SOD from other plants (Kernodle and Scandalios 2001). The fact that the differences in amino acid sequence among chloroplastic CuZn-SOD are smaller than those observed in cytosolic CuZn-SOD (Kanematsu and Asada 1990) prompted us to investigate the gene responsible for chloroplastic CuZn-SOD.

First, we assigned the activity band of maize SOD-1 on a polyacrylamide gel by correlating a SOD zymogram on the starch gel (Baum and Scandalios 1982) to that on a 7.5% native-PAGE. The extracts from maize leaves and seeds gave the same number of SOD activity bands with different band intensity on the gel. The most anodic band in leaf extract, which corresponds to a minor one in seed extract, was a major SOD band among leaf SODs, indicating that the major band is SOD-1 (Fig. 1A). The position of this SOD band in leaf coincided with that of SOD in chloroplast stroma (Fig. 1B). Thus, it is clear that the major activity band (SOD-1) from leaf extract on the polyacrylamide gel is chloroplastic CuZn-SOD.

Characterization of SOD-1 and corresponding

cDNA. The purified SOD-1 revealed molecular mass of 32 kDa and homodimer structure, which is characteristic of plant CuZn-SOD, confirming the previous results (Baum et al. 1983). SOD-1 N-terminal amino acid sequence of 35 residues showed higher homology with that of mature chloroplastic CuZn-SOD from other plants compared to that of cytosolic CuZn-SOD, indicating that the purified SOD-1 was chloroplastic CuZn-SOD.

The cloned cDNA for SOD-1 consisted of 890 bp nucleotides, lacking a 5'-end region including the ATG start codon. However, the deduced amino acid sequence showed the presence of transit peptide for chloroplast location in SOD-1 pre-protein. The lacking coding sequence for transit peptide was complemented by the genomic sequence of *sod-1* (see below). The deduced amino acid sequence for the mature SOD-1 (154 residues) coincided perfectly with the N-terminal sequence of the purified protein up to the 35th residue, excluding the 29th residue, where P was called instead of T. The mismatch may be due to overlapping of the precedent residue P next to T in the Edman degradation reaction. The rest of the sequence after the 35th residue also had high homology with that of chloroplastic CuZn-SOD. Thus, these results definitely show that maize contains normal chloroplastic CuZn-SOD, which is SOD-1, as a major SOD in leaves. Accordingly, it is clear that the previously reported "maize SOD-1 cDNA" (Kernodle and Scandalios 2001) is not the cDNA responsible for SOD-1.

Comparison of amino acid sequence of maize SOD-1 with that of other plant chloroplastic CuZn-SOD. Although the maize cDNA was truncated at the 5'-end region, it revealed to be encoding SOD-1 chloroplast-localizing pre-protein The lacking 22 amino acid residues in transit peptide were deduced from the *sod-1* gene obtained in this experiment, and joined to the truncated N-terminal sequence, PYSS-, to form a complete ORF. The absence of introns in the DNA sequence from which the lacking residues were deduced, was estimated by alignment of the deduced amino acid sequence of 22 residues with the sequence of transit peptide of rice chloroplastic CuZn-SOD (SODCp), as shown in Fig. 7. High homology in the sequence indicated the absence of introns in this region.

The transit peptide of maize SOD-1 contained 52 amino acid residues. The N-terminal sequence of mature SOD-1, ATKKAVA-, was characteristic of chloroplastic CuZn-SOD, based on protein and cDNA sequences. The maize SOD-1 highly resembled rice SODCp in amino acid sequence, where the sequence for transient peptide and mature protein were 63−67% and 95% identical, respectively, reflecting their close phylogenetic relationship.

Recently, four chloroplastic CuZn-SOD isoforms resolved by two-dimensional PAGE were reported in maize (Mauro et al. 2005). Three of the four isoforms exhibited the same N-terminal amino acid sequence (10 residues) and the remaining one had a blocked N-terminus. However, it is not known whether these are products of different genes or products derived through post-translational modification. We did not observe the separation of any CuZn-SOD activity from SOD-1 fraction on ion-exchange and hydrophobic chromatography during purification by monitoring with native-PAGE.

SOD-1 allelic genes. In the present experiment, we used an F1 hybrid of maize, Honey Bantam. As expected, we detected two allelic genes for SOD-1 genomic gene (*sod-1*) that were derived from maternal and paternal lines (Fig. 5).

PCR amplification for the central region of *sod-1* from maize genomic DNA using gene-specific primers resulted in two groups of *sod-1* fragments of 3 kbp with several alleles (Fig. 5). Five clones of each group were determined for nucleotide sequence. The alleles were composed of SNPs and small indels (maximum 7 base), but devoid of long indels. Gene walking using PCR to the upstream and downstream regions from each end of the *sod-1* central fragment also gave two groups of *sod-1* allelic fragments. Fortunately, upstream and downstream fragments were successfully connected to each group of central fragment using alleles in overlapping regions. We arbitrarily termed each *sod-1* gene as *sod-1a* and *sod-1b* (Fig. 5). The alleles found in *sod-1* gene resulted in two amino acid substitutions in the transit peptide but not in the mature protein. It is not clear whether or not this mutation in transit peptide affects the efficiency of transport of SOD-1 to chloroplasts. It should be noted that SOD-1b possessed the same amino acid residues at two substitution positions of transit peptide as those of rice SODCp (Fig. 7).

Exon-intron structure of *sod-1*. Comparison of maize *sod-1* nucleotide sequence with SOD-1 cDNA sequence revealed its exon-intron structure. The *sod-1* of 3.9 kbp contained 8 exons and 7 introns, which is characteristic of chloroplastic CuZn-SOD gene from higher plants. Since the cDNA was truncated and devoid of the ATG start codon, the transcription start site and 5'-UTR of exon 1 was not certain in *sod-1*. However, it is possible to assume the absence of an intron between the ATG translation start site and the position of the 5'-end of the truncated cDNA, because of the high similarity between the deduced amino acid sequence from this region and that of rice SODCp pre-protein. Furthermore, the possibility of another intron in the upstream region from the ATG start site to promoter (TATA box) might be excluded, judging from the length (90 bp) between them and the case of rice *sodCp*. If any, an intron should be very short.

We compared the exon-intron structure of *sod-1* with those of chloroplastic and cytosolic CuZn-SOD genes from other plants (Kaminaka et al. 1997, Kernodle and Scandalios 1996). Figure 6 shows one of such results. In accordance with the high similarity in amino acid sequence, *sod-1* possessed the identical exon-intron structure to that of rice *sodCp*. Exon 2 and 3 of *sod-1* was found to fuse in maize cytosolic *sod-4A*, as shown between rice chloroplastic and cytosolic CuZn-SOD genes, *sodCp* and *sodCc1*.

Promoter analysis of *sod-1* and its expression control. Biotic and abiotic stresses regulate SOD gene expression through the production of ROS inside cells (Alscher et al. 2002, Mylona et al. 2007). Cold and drought induce several antioxidant enzymes including CuZn-SOD in maize (Iannelli et al. 1999, Pastori et al. 2000, Stepien and Klobus 2005). Thus, overexpressed SOD in transgenic plants increases their resistance against environmental stress (Breusegem et al. 1999).

We observed several regulatory *cis*-elements in a 500-bp-length upstream region from the ATG start codon of *sod-1* using the PLACE database (Higo et al. 1999). Such motifs were TATA, GTGA, G-box, E-box, T-box, MYB, GT-1 and CAAT, some of which were found in the promoter region of photosynthesis-related genes.

The type of elements and their arrangement in the promoter region of *sod-1* did not resemble that of rice *sodCp*. Rather, the arrangement of motifs in rice *sodCp* was correlated to that of chloroplastic CuZn-SOD gene from the green alga *Spirogyra* sp. (Kanematsu, unpublished), indicating a difference in the regulatory control of gene expression between maize *sod-1* and rice *sodCp*. It should be noted that C_4 plants such as maize were derived from C_3 plants such as rice to obtain more efficient photosynthetic performance.

The Cu-responsive element TNNNGCTG was located 47 bp upstream from the TATA box in the *sod-1* promoter region (Fig. 5), indicating transcriptional regulation of *sod-1* by copper, as shown in maize *sod4/4A* and the previous maize "*sod-1* gene" (Ruzsa and Scandalios 2003). On the other hand, Nagae et al. (2008) reported a *cis*-acting element that responded negatively to the copper level in the chloroplastic Fe-SOD gene of the moss *Barbula unguiculata*. Thus, CuZn-SOD and Fe-SOD seem to be reciprocally regulated by copper in chloroplasts. However, there are contradictory reports on the presence/absence of Fe-SOD in maize chloroplasts (Iannelli et al. 1999, Mauro et al. 2005, Pastori et al. 2000). We cloned chloroplastic Fe-SOD cDNA from maize (Accession Number AB201543), indicating the presence of the Fe-SOD gene, but did not observe any Fe-SOD band in the activity staining after native-PAGE using sensitivity to H_2O_2 as criteria. Thus, the expression level of maize Fe-SOD seems to be vey low if not absent.

Recent studies suggest that a microRNA, miR398, of *Arabidopsis thaliana* targets its chloroplastic and cytosolic CuZn-SOD genes (Bonnet et al. 2004, Jones-Rhoades and Bartel 2004). It has been shown that the post-transcriptional induction of both types of CuZn-SOD in *A. thaliana* was mediated by down-regulation of miR398 in response to oxidative stress (Sunkar et al. 2006). Furthermore, low copper availability downregulated CuZn-SOD gene expression through the induction of miR398 (Abdel-Ghany and Pilon 2008). A similar nucleotide sequence of target sequence of *A. thaliana* miR398 was also found in exon 4 of maize *sod-1* with 19/21 base matches, for which the amino acid sequence (HAGDLGN) was conserved among higher plants. Thus, maize *sod-1* might also be regulated by microRNA in a similar manner to *A. thaliana*. However, miR398-like microRNA is not observed in the microRNA database of maize (miRBase, http://microrna.sanger.ac.uk/) so far accumulated.

Localization of SOD-1 in bundle-sheath chloroplasts. Maize leaves contain two types of cells: mesophyll and bundle sheath. Bundle-sheath chloroplasts show little PSII activity, indicating no oxygen evolution and an absence of oxygenase activity of RuBP carboxylase/oxygenase (Takabayashi et al. 2005). Interestingly, it has been shown that mesophyll chloroplasts are devoid of SOD activity, whereas bundle-sheath chloroplasts do contain SOD (Doulis et al. 1997, Pastori et al. 2000). Thus, the chloroplasts obtained by differential centrifugation in this experiment might be derived from bundle-sheath cells. The mechanical method would selectively disrupt mesophyll cells and their chloroplasts but not bundle-sheath cells, because the latter is more resistant against shearing stress. We confirmed the absence of CuZn-SOD in mesophyll chloroplasts obtained from maize protoplasts (Kanematsu, unpublished). The preferential distribution of CuZn-SOD between two types of chloroplasts indicates an unknown function of CuZn-SOD in bundle-sheath chloroplasts.

Since CuZn-SOD in bundle-sheath chloroplasts of C_4 plants might determine the direction of electron flow from PSI to the water-water cycle or to the cyclic electron flow around PSI under the various environmental conditions by regulating the rate in the conversion of superoxide to hydrogen peroxide and molecular oxygen, chloroplastic CuZn-SOD genes of C_3 and C_4 plants appear to be controlled in a different fashion. The present nucleotide sequence of *sod-1* might be a clue to solve this problem.

Concluding remarks. In this study, we demonstrated that the previously reported "maize SOD-1 cDNA" (Kernodle and Scandalios 2001) was not the cDNA encoding chloroplast CuZn-SOD (SOD-1), and we assigned a new cDNA responsible for maize SOD-1. The reason why an unrelated cDNA was screened is obviously due to cross-hybridization of the 27-mer oligo DNA probe derived from a conserved region of the tomato chloroplastic CuZn-SOD cDNA. In addition, the unavailability of information on the N-terminal amino acid sequence of SOD-1 did not allow identification of the correct cDNA. On the other hand, we amplified SOD-1 cDNA by PCR using the degenerate primer set and classified the PCR products into two groups of cDNAs for chloroplastic and cytosolic CuZn-SODs. With adequate N-terminal sequence data we could correctly obtain SOD-1 cDNA, followed by SOD-1 genomic genes using the cDNA sequence.

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REFERENCES

Abdel-Ghany, S.E. and Pilon, M. (2008) MicroRNA-mediated systemic down-regulation of copper protein expression in response to low copper availability in *Arabidopsis. J. Biol. Chem*. **283**: 15932-15945.

Asada, K. (1999) The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **50**: 601-639.

Asada, K. (2006) Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiol*. **141**: 391-396.

Alscher, R.G., Erturk, N. and Heath, L.S. (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J. Exp. Bot*. **53**: 1331-1341.

Baum, J.A., Chandlee, J.M. and Scandalios, J.G. (1983) Purification and partial characterization of a genetically-defined superoxide dismutase (SOD-1) associated with maize chloroplasts. *Plant Physiol*. **73**: 31-35.

Baum, J.A. and Scandalios, J.G. (1982) Multiple genes controlling superoxide dismutase expression in maize. *J. Hered*. **73**: 95-100.

Bonnet, E., Wuyts, J., Rouzé, P. and Van de Peer, Y. (2004) Detection of 91 potential conserved plant microRNAs in *Arabidopsis thaliana* and *Oryza sativa* identifies important target genes. *Proc. Natl. Acad. Sci. USA* **101**: 1511-11516.

Breusegem, F.V., Slooten, L, Stassart, J.-M., Moens, T., Botterman, J., Van Montagu, M. and Inzé, D. (1999)

Overproduction of *Arabidopsis thaliana* FeSOD confers oxidative stress tolerance to transgenic maize. *Plant Cell Physiol*. **40**: 515-523.

Doulis, A.G., Debian, N., Kingston-Smith, A.H. and Foyer, C.H. (1997) Differential localization of antioxidants in maize leaves. *Plant Physiol*. **114**: 1031-1037.

Fridovich, I. (1995) Superoxide radical and superoxide dismutases. *Annu. Rev. Biochem*. **64**: 97-112.

Higo, K., Ugawa, Y., Iwamoto, M. and Korenaga, T. (1999) Plant cis-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Res*. **27**: 297-300.

Jones-Rhoades, M.W. and Bartel, D.P. (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Molecular Cell* **14**: 787-799.

Kaminaka, H., Morita, S., Yokoi, H., Masumura, T. and Tanaka, K. (1997) Molecular cloning and characterization of a cDNA for plastidic copper/zinc-superoxide dismutase in rice (*Oryza sativa* L.). *Plant Cell Physiol*. **38**: 65-69.

Kanematsu, S. and Asada, K. (1989) CuZn-superoxide dismutases in rice: occurrence of an active, monomeric enzyme and two types of isozyme in leaf and non-photosynthetic tissues. *Plant Cell Physiol*. **30**: 381-391.

Kanematsu, S. and Asada, K. (1990) Characteristic amino acid sequences of chloroplast and cytosol isozymes of CuZn-superoxide dismutase in spinach, rice and horsetail. *Plant Cell Physiol*. **31**: 99-112.

Kanematsu, S. and Asada, K. (1994) Superoxide dismutase. *In* Molecular Aspects of Enzyme Catalysis. Edited by Fukui, T. and Soda, K. pp. 191-210. Kodansha/VHC, Tokyo.

Kanematsu, S. and Fujita, K. (2002) Cloning and characterization of cDNA and gene for maize chloroplastic CuZn-superoxide dismutase (SOD-1). *In* Abstracts of the 75th Annual Meeting of the Japanese Biochemical Society. The Japanese Biochemical Society, Tokyo, Japan. Abstr. p. 736.

Kanematsu, S., Fujita, K., Shinpei, U. and Asada, K. (2003) Exon-intron structure of the chloroplastic CuZn-SOD genes in eukaryotic alga and land plants: its implication in the molecular evolution. *In* Abstracts of Plant Biology 2003. American Society of Plant Biologist, USA. Oxidative stress, Abstr. # 114.

Kanematsu, S. and Sato, S. (2008) Cloning of Fe-superoxide dismutase gene from the diazotroph *Azotobacter vinelandii* and the stimulation of its expression under anaerobic conditions in *Escherichia coli. Bull. Minamikyushu Univ*. **38A**: 7-18.

Kernodle, S.P. and Scandalios, J.G. (1996) A comparison of the structure and function of the highly homologous maize antioxidant Cu/Zn superoxide dismutase genes, *Sod4* and *Sod4A. Genetics* **144**: 317-328.

Kernodle, S.P. and Scandalios, J.G. (2001) Structural organization, regulation, and expression of the chloroplastic superoxide dismutase *Sod1* gene in maize. *Arch. Biochem. Biophys*. **391**: 137-147.

Kliebenstein, D.J., Monde, R.-A. and Last, R.L. (1998) Superoxide dismutase in Arabidopsis: an eclectic enzyme family with disparate regulation and protein localization. *Plant Physiol.* **118**: 637-650.

Iannelli, M.A., Van Breusegem, F., Van Montagu, M., Inzé, D. and Massacci, A. (1999) Tolerance to low temperature and paraquat-mediated oxidative stress in two maize genotypes. *J. Exp. Bot*. **50**: 523-532.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. **193**: 265-275.

Mauro, S., Van Eycken, F., Challou, N., Lucas, P. and L'Oiseau, M. (2005) Characterization of new maize chloroplastic copper/zinc superoxide dismutase isoforms by high resolution native two-dimensional polyacrylamide gel electrophoresis. Identification of chilling responsive chloroplastic superoxide dismutase isoforms. *Physiol. Plant*. **124**: 323-335.

McCord, J.M. and Fridovich, I. (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **244**: 6049-6055.

Mylona, P.V., Polidoros, A.N. and Scandalios, J.G. (2007) Antioxidant gene responses to ROS-generating xenobiotics in developing and germinated scutella of maize. *J. Exp. Bot.* **58**: 1301-1312.

Nagae, M., Nakata, M. and Takahashi, Y. (2008) Identification of negative cis-acting elements in response to copper in the chloroplastic iron superoxide dismutase gene of the moss *Barbula unguiculata. Plant Physiol.* **146**:1687-1696.

Ogawa, K., Kanematsu, S., Takabe, K. and Asada, K. (1995) Attachment of CuZn-superoxide dismutase to thylakoid membranes at the site of superoxide generation (PSI) in spinach chloroplasts: detection by immuno-gold labeling after rapid freezing and substitution method. *Plant Cell Physiol*. **36**: 565-573.

Pastori, G., Foyer, C.H. and Mullineaux, P. (2000) Low temperature-induced changes in the distribution of H_2O_2 and antioxidants between the bundle sheath and mesophyll cells of maize leaves. *J. Exp. Bot.* **51**: 107-113.

Ruzsa, S.M. and Scandalios, J.G. (2003) Altered Cu metabolism and differential transcription of Cu/Zn*Sod* genes in a Cu/ZnSOD-deficient mutant of maize: evidence for a Cu-responsive transcription factor. *Biochemistry* **42**: 1508-1516.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Scandalios, J.G. (1997) Molecular genetics of superoxide dismutases in plants. *In* Oxidative Stress and the Molecular Biology of Antioxidant Defenses. Edited by Scandalios, J.G. pp. 527-568. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Stepien, P. and Klobus, G. (2005) Antioxidant defense in the leaves of C3 and C4 plants under salinity stress. *Physiol. Plant*. **125**: 31-40.

Sunkar, R., Kapoor, A. and Zhu, J.-K. (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by downregulation of miR398 and important for oxidative stress tolerance. *Plant Cell* **18**: 2051-2065.

Takabayashi, A., Kishine, M., Asada, K., Endo, T. and Sato, F. (2005) Differential use of two cyclic electron flows around photosystem I for driving CO_2-concentration mechanism in C_4 photosynthesis. *Proc. Natl. Acad. Sci. USA* **102**: 16898-16903.

Ueno, S. and Kanematsu, S. (2007) Immunological and electrophoretic characterization of proteins exhibiting superoxide dismutase activity in the moss *Pogonatum inflexum. Bull. Minamikyushu Univ.* **37A**: 1-9.

Youn, H.-D., Kim, E.-J., Roe, J.-H., Hah, Y.C. and Kang, S.-O. (1996) A novel nickel-containing superoxide dismutase from *Streptomyces* spp. *Biochem. J.* **318**: 889-896.

Zhu, D. and Scandalios, J.G. (1993) Maize mitochondrial manganese superoxide dismutases are encoded by a differentially expressed multigene family. *Proc. Natl. Acad. Sci. USA* **90**: 9310-9314.