Atypical cytosol-localized Fe-superoxide dismutase in the moss *Pogonatum inflexum*

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cDNA clones encoding iron superoxide dismutase (Fe-SOD) of the moss *Pogonatum inflexum* were classified into two groups on the basis of nucleotide sequence. The major group showed an open reading frame (ORF) of 753 bp, encoding a typical chloroplastic Fe-SOD subunit of 28,768 Da. The minor group consisted of four splicing variants that differ in the sequence of the 5′-UTR 30 bp upstream from the start codon. All variants gave the same ORF of 636 bp encoding a protein of 23,785 Da. Alignment of the amino acid sequence of the minor Fe-SOD with those of other plant Fe-SODs indicated the absence of a chloroplast transit peptide, suggesting its atypical cytosolic localization. Recombinant cytosolic Fe-SOD was enzymatically active. Inactivation with H_2O_2 showed the active site metal to be Fe. The nucleotide sequences of both chloroplastic (4.5 kbp) and cytosolic (3.2 kbp) Fe-SOD genes revealed virtually the same exon–intron structure in terms of splicing positions. The cytosolic Fe-SOD gene lacks one intron in the coding region, and contains three consecutive splicing donor sites and one common acceptor site in the 5′-UTR, by which three of the four splicing variants are generated. The existence of the cytosolic Fe-SOD gene in the moss suggests the wide distribution of the enzyme among land plants, although it has been observed so far only in cowpea, radish, and *Arabidopsis thaliana*.

Key words: iron-containing superoxide dismutase, splicing variants, recombinant Fe-SOD, exon–intron structure, *Pogonatum inflexum*.

INTRODUCTION

Since mosses are devoid of vascular bundles and absorb water directly from the environment, they are always faced with environmental stresses, including desiccation (Proctor et al. 2007). As these stresses favor the production of reactive oxygen species (ROS), which have deleterious effects on all cells, such as superoxide, H_2O_2, hydroxyl radical, and singlet oxygen (Asada 1999, 2006), mosses might possess a sophisticated defense system against ROS.

Superoxide dismutase (SOD), a key enzyme in ROS detoxification, occurs in four isozyme forms: CuZn-, Mn-, Fe-, and Ni-SOD (Kanematsu and Asada 1994, Fridovich 1995, Dupont 2008, Kanematsu et al. 2012). In higher plants, Fe-SOD has been detected only in chloroplasts of limited species and has an apparently inconsistent distribution (Kanematsu and Asada 1994). However, recent genome analyses of higher plants (genome papers in http://genomevolution.org/wiki/index.php/Sequenced_plant_genomes) indicate the wide distribution of chloroplastic Fe-SOD genes among higher plants, suggesting an unknown underlying mechanism by which its expression is regulated. To elucidate such a control mechanism, we investigated genes encoding Fe-SOD and its homolog, Mn-SOD, in the moss *Pogonatum inflexum*, and cloned its Mn-SOD cDNAs (Kanematsu et al. 2012).

Here, we describe the occurrence of an atypical cytosolic Fe-SOD gene in addition to a typical chloroplastic Fe-SOD gene in *P. inflexum*. The presence of the cytosolic Fe-SOD gene in the moss suggests its wide distribution among land plants, although only a few examples have been reported.

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Abbreviations: *PiFe-sod-c*, *P. inflexum* cytosolic Fe-SOD gene; *PiFe-sod-p*, chloroplastic Fe-SOD gene; PiFe-SOD, gene product of *PiFe-sod*; ROS, reactive oxygen species; SOD, superoxide dismutase.

Accession numbers: *PiFe-sod-p* cDNA (chloroplastic, major, *sodB-1*), AB201828; *PiFe-sod-c* cDNA (cytosolic, minor, *sodB-2*), AB201829, AB811692-AB811695; *EaFe-sod* cDNA (chloroplastic, *sodB*), AB201831; *ZmFe-sod* cDNA (chloroplastic), AB201543; *PiFe-sod-p*, AB811691; *PiFe-sod-c*, AB811690. Some of the results have been presented elsewhere (Okayasu et al. 2005, Kanematsu 2005 and 2007, Kanematsu et al. 2005).

MATERIALS AND METHODS

Materials. The SMART RACE cDNA Amplification Kit and Universal GenomeWalker Kit were purchased from Clontech (Palo Alto, CA, USA). The pGEX-6P-2 expression vector, GSH-Sepharose 4B beads, and PreScission Protease were obtained from Amersham Bioscience (Buckinghamshire, UK).

The moss *Pogonatum inflexum* and the fern *Equisetum arvense* were collected on the Takanabe campus of Minami-Kyushu University, Miyazaki, Japan, as described previously (Kanematsu et al. 2011). Maize was grown as previously (Kanematsu and Fujita 2009).

Assay and protein analysis. SOD activity measurement, protein determination, native polyacrylamide gel electrophoresis (PAGE), SDS-PAGE, and SOD activity staining were conducted as previously described (Kanematsu and Asada 1990, Ueno and Kanematsu 2007). Classification of three types of SOD was done as previously described (Kanematsu and Asada 1990).

Extraction of mRNA and genomic DNA. mRNA and genomic DNA were isolated as described before (Kanematsu et al. 2010, 2011). Genomic DNA of *P. inflexum, E. arvense*, and 10-day-old maize seedlings were isolated by using an Isoplant kit (Nippon Gene, Toyama, Japan).

cDNA cloning. cDNA cloning was conducted in two steps: PCR amplification of the central portion of cDNA with degenerate primers, and then 5′- and 3′-RACE with gene-specific primers. cDNA pools for the partial cDNA amplification and for the RACE were prepared by the SMART RACE cDNA Amplification Kit described previously (Kanematsu et al. 2012).

Partial cDNA fragments obtained by PCR from the pools were ligated into the pGEM-T EZ vector and sequenced. Amplification by 5′- and 3′-RACE using the SMART RACE cDNA Amplification Kit was conducted as described (Kanematsu et al. 2010, 2011). LA-*Taq* was used for PCR-amplification. The gene-specific primers were as follows: for *P. inflexum PiFe-sod-p*, POFmaj-5GSP (5′ antisense primer) 5′-TGTCTTCACGATTGTGAGCTTGGCG-3′ and POFmaj3GSP (3′ sense) 5′-TGGCACTGATTGAGAGAGACTTCGGTTC-3′; for *P. inflexum PiFe-sod-c*, POFmin5GSP (5′ antisense) 5′-TCTGGACTGTCAATTTGCCATCCTGG-3′ and POFmin3GSP (3′ sense) 5′-CGTGATTTCGGCTCTTACGACCAGTTTG-3′; for *E. arvense* cDNA, EQF5GSP (5′ antisense) 5′-CACTACAAGCCATACCCAGCCAGACCC-3′ and EQF3GSP (3′-downstream sense) 5′-GGGGTAAAAAGCCAAGTGGAGAGGTCC-3′; for maize cDNA, MZF5GSP (5′ antisense) 5′-CAAGACAAGCCAAACCCAACCAGACC-3′ and MZF3GSP (3′ sense primer) 5′-TGGCTTACCTGAGGGAGGTGTGCTG-3′. Full-length cDNAs were constructed with central sequences and 5′- and 3′-flanking sequences.

PCR amplification of the central portion of Fe-SOD genomic genes. The two *P. inflexum* Fe-SOD genomic genes (*PiFe-sod-p* and *PiFe-sod-c*) were obtained as fusions of the central portion and the adjacent 5′-upstream and 3′-downstream portions of the gene as described (Kanematsu and Fujita 2009). Each central portion was amplified by PCR using LA-*Taq* polymerase (Takara, Kyoto, Japan) with gene-specific primers based on the cDNA sequences, and each flanking portion was obtained by using the Universal GenomeWalker Kit (Clontech) with gene-specific primers designed from the sequence of the central portion.

The following primers were used for amplification of the central portion of the *P. inflexum* genes: sense and antisense primers for *PiFe-sod-p*, POFmajGC F30 (sense) 5′-ACAGCGAGCTTGGGCTCCTCGCGTACATTC-3′ and POFmajGC B903 (antisense) 5′-AGCACCACACATTGATGGCGTGATTCTCATG-3′; for *PiFe-sod-c*, POFminGC F1321 (sense) 5′-TCCGCTCCCTTTGACAACAGTGGATCTGAGAC-3′ and POFminGC B1917 (antisense) 5′-CGTTTGGCAACCGCATCCCAAGAAACC-3′.

Amplified fragments measuring 2.4 kbp (*PiFe-sod-p*) and 1.6 kbp (*PiFe-sod-c*) were cloned and sequenced as described (Kanematsu and Fujita 2009). At least four clones for each portion of the gene were used for sequencing.

Amplification of the 5′ and 3′ portions of Fe-SOD genomic genes. The Universal GenomeWalker Kit was used to construct pools of adaptor-ligated genomic DNA fragments (referred to as the GenomeWalker *Dra*I, *Eco*RV, *Pvu*II, and *Stu*I libraries) from which DNA fragments adjacent to the central sequence of the gene were amplified, and to conduct PCR of each library using the gene-specific primers, according to the manufacturer's instructions. The following primers were used for amplification of the *P. inflexum* genes. For 5′-upstream amplification: for *PiFe-sod-p*, POFmajGW 5-1 (antisense primer) 5′-CTGCATACGAAAACGCCAGGTCCTTAG-3′ and POFmajGW 5-2 (nested antisense primer) 5′-AAACGCCAGGTCCTTAGAAGTCTTCCG-3′; for first walk of *PiFe-sod-c*, POFminGW 5-1 (antisense) 5′-CCCAGTGATACTCCAGCGTCTGTTTGC-3′ and POFminGW 5-2 (nested) 5′-GCATCCAAAGCATAAGGGGGAGGTCTC-3′, and second walk, POFminGW 5-2 (antisense) and POFminGW 5-3 (nested) 5′-TACAACTCTCTTCCACATTCACCTGGG-3′. For 3′-downstream amplification: for *PiFe-sod-p*, POFmajGW 3-1 (sense primer) 5′-GCCATCATCATGGAGAATCTTATCAACTG-3′ and POFmajGW 3-2 (nested sense primer) 5′-CTACGGCTTGAAAGAGCAAAAGCATTC-3′; for *PiFe-sod-c*, POFminGW 3-1 (sense) 5′-ACCTCGATTACCAGGTCAGTTAATGGC-3′ and POFminGW 3-2 (nested) 5′-ACTCGCATCTTGGTTGAAAGAATCTGG-3′. The adaptor primers AP1 and AP2 (Clontech) were used as outer and inner (nested) primers, respectively. The amplified fragments were cloned and sequenced as described (Kanematsu and Fujita 2009). At least four clones for each portion of each gene were sequenced.

Overexpression and purification of recombinant cytosolic Fe-SOD. The coding sequence of *PiFe-sod-c* including two adaptors was obtained by PCR with LA-*Taq* from the *P. inflexum* cDNA pools (libraries), which were prepared for 5′-RACE by using the SMART RACE cDNA Amplification Kit, with the primers POFminExF (sense) 5′-AAGAATTCTGTTAAGTATGGCAACAGTTCCGC-3′ and POFminExB (antisense) 5′-TTGTCGACTCTCAAATTGAAAGGAGCTTCCG-3′. The sense primer adds three additional amino acid residues, LLS, between the glutathione S-transferase (GST) and SOD. The antisense primer is located from the 3rd to the 33rd position after the stop codon in the 3′-UTR. The primers included the *Eco*RI site at the 5′ end and the *Sal*I site at the 3′ end. The cDNA fragment was ligated between the *Eco*RI and *Sal*I sites of the pGEX-6P-2 GST expression vector (Amersham Bioscience) and expressed in *E. coli* BL21 cells to yield a GST-fused protein. The protein was induced with 0.075 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 25 °C for 9 h.

The protein was purified as described (Kanematsu et al. 2012). In brief, after the cells were disrupted by sonication, the cell extract was passed through a GSH-Sepha-

rose column to capture the fused protein. Then the Fe-SOD was cleaved from GST with PreScission protease. The Fe-SOD was purified to an apparently homogeneous state by gel filtration through a Superdex 75 column and ion-exchange chromatography through Mono Q medium.

Modeling of protein three-dimensional structure. The 3D structure of PiFe-SODs and PiMn-SOD were predicted from their amino acid sequences by using the CPHmodels-2.0 protein homology modeling server (http://www.cbs.dtu.dk/services/CPHmodels-2.0/). Cowpea Fe-SOD (1UNF) and human Mn-SOD (1NOJ) were used as modeling templates.

RESULTS

Fe-SOD cDNAs of *Pogonatum inflexum*. Although Fe- and Mn-SOD are homologous proteins, PCR amplified cDNA fragments encoding Fe-SOD only, not Mn-SOD, from moss, fern, and maize using the degenerate primers for Fe-SOD (Kanematsu et al. 2012). PCR amplified two types of cDNA fragments from *P. inflexum*, each of 254 bp (without primers): *PiFe-sod-p*, designated major (65% of transcripts), and *PiFe-sod-c*, designated minor (35%).

We cloned full-length cDNAs encoding the Fe-SODs of *P. inflexum* by 5′- and 3′-RACE. RACE amplified *PiFe-sod-p* (major) cDNA fragments of 557 bp (5′-RACE) and 599 bp (3′-RACE), with an overlap of 134 bases. Assembly of the two sequences gave the full-length *PiFe-sod-p* cDNA of 1,022 bp (Fig. 1). On the other hand, 5′-RACE of *PiFe-sod-c* (minor) was complicated. The 5′-RACE products revealed four splicing variants that differ in the sequence of the 5′-UTR 30 bp upstream from the start codon (Fig. 2C). The genomic sequence obtained here (see below) shows the presence of three donor sites and one acceptor site in the 5′-UTR region that generate the variants (Fig. 2A, B). Among 23 clones, 65% of the transcripts were alternatively spliced in comparison with the genomic sequence. To construct the full-length cDNA

***PiFe-sod-p* cDNA (chloroplastic)**

```
                                                                      50                                                                  100
CAGCAGATTG AGAAGCTATG GCGACCTGCA CAGCGAGCTT GGGCTCCTCG CGTACATTCC TCCGCCATAG CTTCGGTGTT TCAAAGAAGT ATTCCACACC
                  M   A   T   C   T   A   S   L   G   S   S   R   T   F   L   R   H   S   F   G   V   S   K   K   Y   S   T   P
                                                                     150                                                                 200
GAGGATGCGG TCTGTTGGCA TTCCTAGGGC ACAATTTTTC CTGAAGCCTC TTCCATATGA GCTTGATGCT TTGGAACCCC ACATGAGCAA GAAGACTTTA
  R   M   R   S   V   G   I   P   R   A   Q   F   F   L   K   P   L   P   Y   E   L   D   A   L   E   P   H   M   S   K   K   T   L
                                                                     250                                                                 300
GAGTTCCATT ATGGCAAACA TCACCGGGCA TATGTTGATA ACCTCAATAA GCAAATAGAG AATACAGATT TTGAGAGGAA CACTCTTGAA GAAATTGTCA
  E   F   H   Y   G   K   H   H   R   A   Y   V   D   N   L   N   K   Q   I   E   N   T   D   F   E   R   N   T   L   E   E   I   V
                                                                     350                                                                 400
AAATTTCTTA CAATAACGGA AACCCCTTAG CTCCTTTCAA CAATGCTGGT CAGGCATGGA ACCATGATTT CTTTTGGTCT TCCTTATGTC CTGGTGGAGG
K   I   S   Y   N   N   G   N   P   L   A   P   F   N   N   A   G   Q   A   W   N   H   D   F   F   W   S   S   L   C   P   G   G   G
                                                                     450                                                                 500
TGGCACACCT GAAGGCGAGC TACTGGCACT GATTGAGAGA GACTTCGGTT CTTATGAAGA TTTTGAAAAA GAATTCAAGC AGGCTGGTGC AACTCAATTT
  G   T   P   E   G   E   L   L   A   L   I   E   R   D   F   G   S   Y   E   D   F   E   K   E   F   K   Q   A   G   A   T   Q   F
                                                                     550                                                                 600
GGAGCCGGTT GGGTCTGGCT AGTTTTGAAG GACGCCAAGC TCACAATCGT GAAGACACCA AATGCTCTCA CTCCCATTAT TTGGAATTAC TTTCCTTTGC
  G   A   G   W   V   W   L   V   L   K   D   A   K   L   T   I   V   K   T   P   N   A   L   T   P   I   I   W   N   Y   F   P   L
                                                                     650                                                                 700
TGGTGGTCGA CATCTGGGAG CATGCTTACT ACCTTGACTA TCAGAATCGA AGACCGGACT ATATGGCCAT CATCATGGAG AATCTTATCA ACTGGAAGAC
L   V   V   D   I   W   E   H   A   Y   Y   L   D   Y   Q   N   R   R   P   D   Y   M   A   I   I   M   E   N   L   I   N   W   K   T
                                                                     750                                                                 800
AGTAGAACTA CGGCTTGAAA GAGCAAAAGC ATTCATAAAT CTTGGGAAGC CGACCATCCC TGAACTTTAG AACTTCATCT TACACATACT TGCCTGTAAT
  V   E   L   R   L   E   R   A   K   A   F   I   N   L   G   K   P   T   I   P   E   L
                                                                     850                                                                 900
TTTGACATAC TACTTTCAAG AGTTTTTTAC GTCTCCTGGA GATGAGAGTT TCCAGCGATC GGAAAAGGTT GTCATGAGAA TCACGCCATC AATGTGTGGT
                                                                     950                                                                1000
GCTAGATACA GTAAAGTGAC ACATTCTGCA GATCACAATG TATTTTTATG ATTCATAATT CTGCCAGAAG CTATGTTAAT GCGTTTCGTA TTATTGCGAA

AAAAAAAAAA AAAAAAAAAA AA
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***PiFe-sod-c* cDNA (cytosolic)**

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                                                                      50                                                                  100
ATCATTACAT CTCTGAGTAG CTGTTAAGTA TGGCAACAGT TCCGCTCCCT TTGACAACAG TGGATCTGAG ACCTCCCCCT TATGCTTTGG ATGCATTGGA
                              M   A   T   V   P   L   P   L   T   T   V   D   L   R   P   P   P   Y   A   L   D   A   L   E
                                                                     150                                                                 200
GCCTCACATG AGCAAACAGA CGCTGGAGTA TCACTGGGGC AAGCACCACC GCTCCTACGT AGACAACCTG AAGAAGCAGA TAGAAGGTAC TGAGTTGGCC
  P   H   M   S   K   Q   T   L   E   Y   H   W   G   K   H   H   R   S   Y   V   D   N   L   K   K   Q   I   E   G   T   E   L   A
                                                                     250                                                                 300
ACTAAAAGCT TGGAGGAGAT TGTGAAAGTT TCCTACAACA ACGGACAGCC AACAGCTGCT TTCAATAACG CGGGTCAGGC GTGGAACCAT GAGTTCTTCT
  T   K   S   L   E   E   I   V   K   V   S   Y   N   N   G   Q   P   T   A   A   F   N   N   A   G   Q   A   W   N   H   E   F   F
                                                                     350                                                                 400
GGCTGTCGAT GTCACCAGAC GGTGGTAAGG CCCCCGAGGG AGAGCTGTTG GGGCTTCTCA AACGTGATTT CGGCTCTTAC GACCAGTTTG TAACGCAGTT
W   L   S   M   S   P   D   G   G   K   A   P   E   G   E   L   L   G   L   L   K   R   D   F   G   S   Y   D   Q   F   V   T   Q   F
                                                                     450                                                                 500
CAAGCAAGCA GGTGCCACTC AGTTCGGATC AGGGTGGGCT TGGTTGACCA TCCAGGATGG CAAATTGACA GTCCAGAATA GCCCCAACGC TATCAACCCG
  K   Q   A   G   A   T   Q   F   G   S   G   W   A   W   L   T   I   Q   D   G   K   L   T   V   Q   N   S   P   N   A   I   N   P
                                                                     550                                                                 600
CTTGTGTTTG GCCACACTCC ACTGCTCGTC GCAGACGTCT GGGAGCACGC ATATTACCTC GATTACCAGA ACCGACGGCC TGATTACTTG ACCACATTCA
  L   V   F   G   H   T   P   L   L   V   A   D   V   W   E   H   A   Y   Y   L   D   Y   Q   N   R   R   P   D   Y   L   T   T   F
                                                                     650                                                                 700
TGAATGAGCT GGTTTCTTGG GATGCGGTTG CCAAACGCCT ACAGGCCGCC CTGTCCGAGA GCTGATCCGG AAGCTCCTTT CAATTTGAGA ATTATGACCC
M   N   E   L   V   S   W   D   A   V   A   K   R   L   Q   A   A   L   S   E   S
                                                                     750                                                                 800
CCAAGGCGCA GTCCGGTTAA TATGTAATAA ACCCATTATG TGAATTTGTA TATGTAAGTG AAATGTATGG ATCAATAAAT ACGTTTTTAC CAAAAAAAAA

AAAAAAAAAA AAAAAAA
```

Fig. 1. Nucleotide and deduced amino acid sequences of *PiFe-sod-p* (1,022 bp) and *PiFe-sod-c* (817 bp) cDNAs. A transit peptide of PiFe-SOD-p is shown in red letters. Stop codons at 5' ends are underlined.

sequence of *PiFe-sod-c*, we used a sequence up to the 5′ end (29 bp upstream from ATG) of the exon containing the ATG start codon as the 5′-end sequence (477 bp), and a 3′-end sequence of 455 bp obtained by 3′-RACE. With an overlap of 115 bp, the two sequences gave a *PiFe-sod-c* cDNA of 817 bp (Fig. 1). Thus, *P. inflexum* contains two

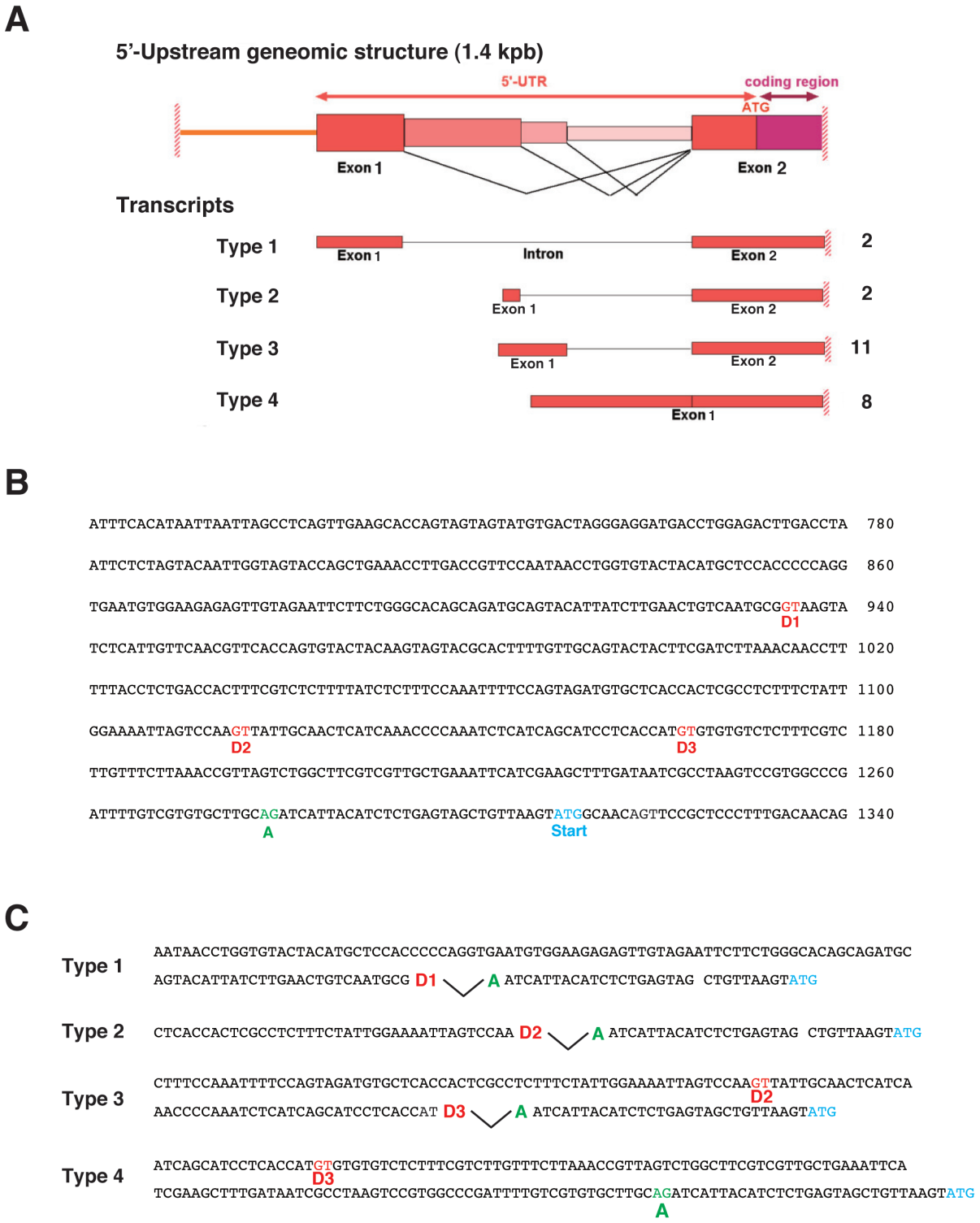

Fig. 2. Structure of splicing-variant-generated *PiFe-sod-c* transcripts. (A) Schematic drawings of 5'-upstream genomic structure of *PiFe-sod-c* and exon–intron structure of four splicing variants (Type 1–4). Numbers of clones in each type are indicated on the right. Each variant was compared with the genomic sequence. The 5′-upstream regions including the start codon and the 5′ end of the coding region are shown. Among splicing variants, bars indicate exons and lines indicate introns. (B) 5′-Upstream genomic sequence of *PiFe-sod-c*. The region from the base position at 701 to 1340 is presented. D1–D3, splicing donor sites; A, acceptor site. Start codon is blue. (C) Nucleotide sequences of the four variants. Deletions of introns are indicated by splicing donor and acceptor sites. Type 4 is a intron-retained transcript.

genes for Fe-SOD isoforms.

PiFe-sod-p cDNA had a complete open reading frame (ORF) of 753 bp and a sequence for a chloroplast transit peptide. Thus, *PiFe-sod-p* encodes a chloroplastic Fe-SOD of 28,768 Da consisting of 250 amino acid residues (Fig. 1). Alignment of the amino acid sequences of other Fe-SODs suggested that the mature enzyme has 215 residues (Fig. 3). *PiFe-sod-c* cDNA also had a complete ORF of 636 bp that encoded a protein of 23,785 Da consisting of 211 amino acid residues. *PiFe-sod-c* had no chloroplast transit peptide sequence, and thus encodes a cytosolic Fe-SOD (Figs. 1, 3).

Fe-SOD cDNAs of *Equisetum arvense* and maize. To survey the occurrence of the cytosolic form of Fe-SOD in other land plants, we cloned cDNAs from *E. arvense* and maize (*Z. mays*). Full-length cDNAs were obtained by extending the 5′ and 3′ ends of previously amplified cDNA fragments (254 bp without primers for *E. arvense* and 260 bp for maize) by 5′- and 3′-RACE (Kanematsu et al. 2012). An *E. arvense* Fe-SOD (EaFe-SOD) cDNA of 1,168 bp was obtained by combining the 5′-RACE product of 642 bp with the 3′-RACE product of 657 bp with 131-bp overlap (data not shown). A maize Fe-SOD (ZmFe-SOD) cDNA of 1,370 bp was obtained by combining the 5′-RACE product of 554 bp and the 3′-RACE product of 943 bp with127-bp overlap (data not shown).

Both cDNAs had a complete ORF. The deduced amino acid sequences consisted of 284 residues for *E. arvense* and 258 for maize. Alignment of the amino acid sequences with those of PiFe-SOD-p and PiFe-SOD-c indicated that both SODs are chloroplastic and have a transit peptide (Fig.

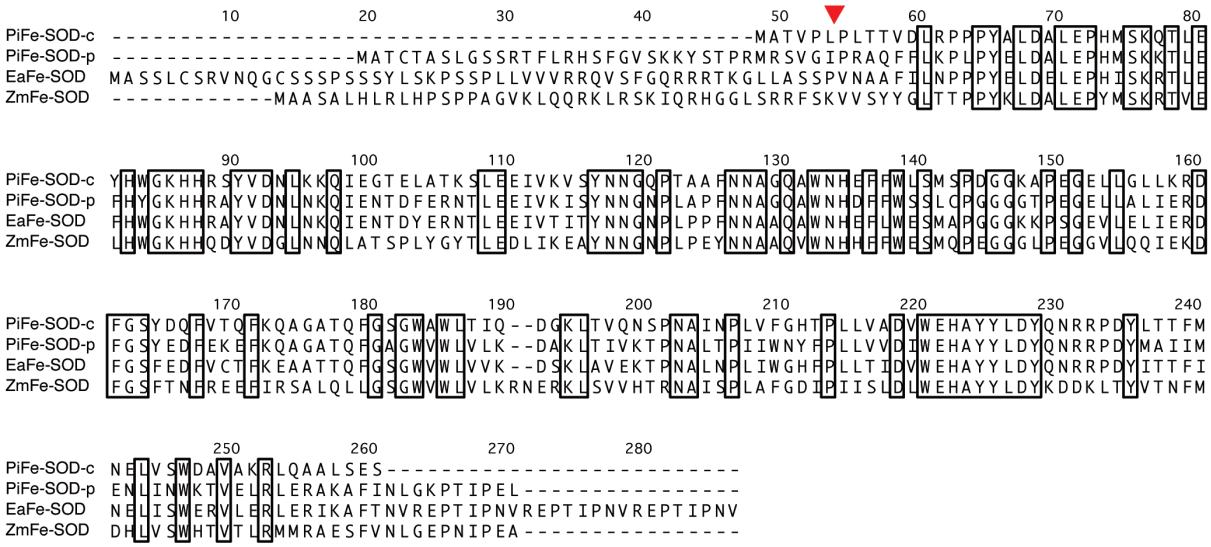

Fig. 3. Alignment of the amino acid sequences of PiFe-SOD-p, PiFe-SOD-c, EaFe-SOD and ZmFe-SOD. Identical amino acid residues are boxed. ▼, Estimated cleavage sites for the mature proteins of PiFe-SOD-p, EaFe-SOD and ZmFe-SOD.

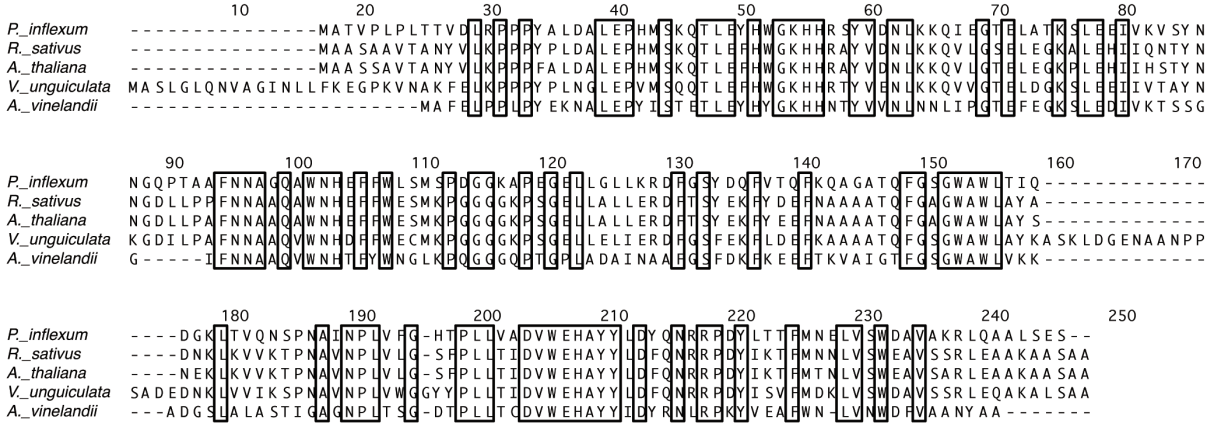

Fig. 4. Amino acid sequence comparison of PiFe-SOD-c and other cytosolic Fe-SODs. *P. inflexum*, PiFe-SOD-c; *R. sativus* (*Raphanus sativus*), AF061583; *A. thaliana* (*Arabidopsis thaliana*, FSD1), NP_194240; *A. vinelandii* (*Azotobacter vinelandii*), AB025798.

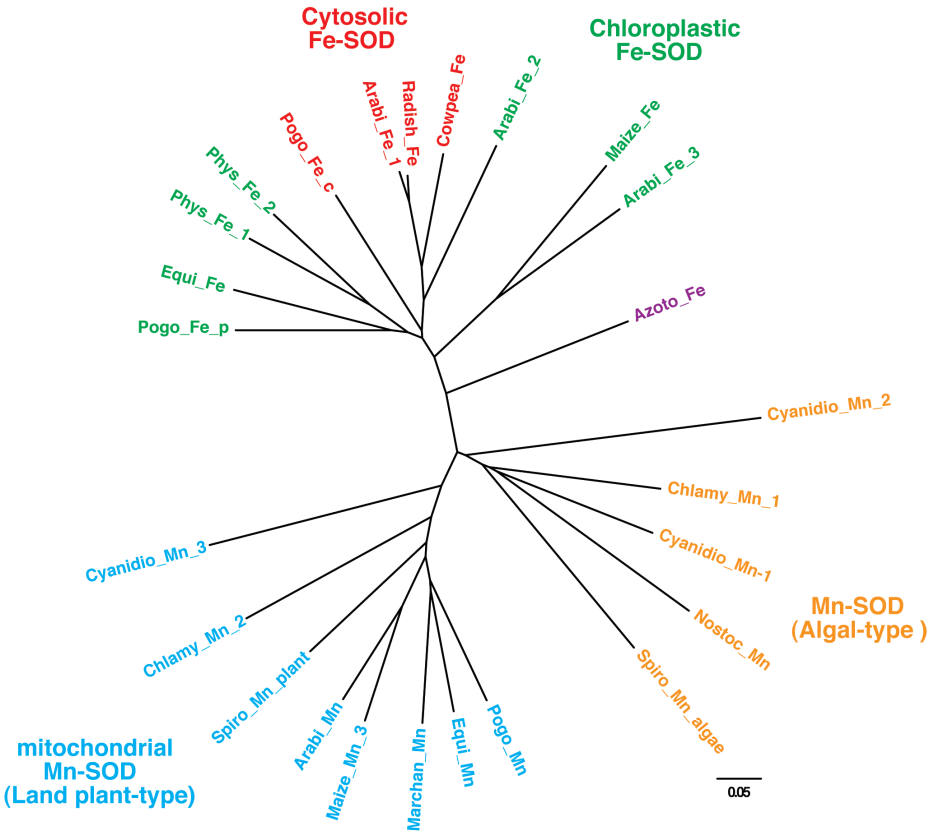

Fig. 5. Phylogenetic relationships among Fe- and Mn-SODs from cyanobacteria, red and green algae, mosses, ferns, and higher plants. Amino acid sequences were aligned by ClustalW (ver 2.1) at DNA Data Bank of Japan (DDBJ) with default settings. The phylogenetic tree was constructed by the neighbor-joining method. Clades indicate chloroplastic and cytosolic Fe-SODs, algal-type Mn-SODs, and land-plant-type mitochondrial Mn-SODs. Cytosolic Fe-SODs are indicated in red. The horizontal bar represents a distance of 0.05 amino acid substitutions per site.

Fe-SODs are as follows: Equi_Fe (*E. alvense*), maize_Fe (*Z. mays*), Pogo_Fe_p and Fe_c (*P. inflexum* Fe-SOD-p and -c), this study; Arabi_Fe_1, 2, 3 (*A.thaliana* FSD1, FSD2, and FSD3), NP_194240, NP_199923, and NP_197722, respectively; Azoto_Fe (*Azotobacter vinelandii*), AB025798; Cowpea_Fe (*V. unguiculata*), AAA80639; Phys_Fe_1, 2 (*P. patens*), XP_001756362 and XP_001751975, respectively; Radish_Fe (*R. sativus*), AF061583. Mn-SODs are as follows: Arabi_Mn (*A. thaliana*), NP_187703; Chlamy_Mn_1, 2 (*Chlamydomonas reinhardtii*), XP_001700058 and XP_001699077, respectively; Cyanidio_Mn_1, 2, 3 (*Cyanidioschyzon merolae*), BAM83058, BAM81167, and BAM82408, respectively; Equi_Mn (*E. arvense*), AB201830; Maize_Mn_3 (*Z. mays*), AAA33512; Marchan_Mn (*Marchantia paleacea*), BAD13494; Nostoc_Mn (Nostoc sp. PCC7120), NP_484114; Pogo_Mn (*P. inflexum*), AB201827; Spiro_Mn_algal and plant (*Spirogyra* sp.), AB201833 and AB201832, respectively.

3). No cytosolic Fe-SOD was found in either plant. The EaFe-SOD had extra 16 residues in the C-terminus compared with the PiFe-SOD-p and ZmFe-SOD (Fig. 3).

Comparison of amino acid sequences among cytosolic Fe-SODs. Amino acid sequence alignment of PiFe-SOD-c and other cytosolic Fe-SODs from radish (Kwon and An 2006), cowpea (Moran et al. 2003), *A. thaliana* (Arabidopsis Genome Initiative 2000) and *Azotobacter vinelandii* (Kanematsu and Sato 2008) are shown in Fig. 4. The position of the N-terminus of PiFe-SOD-c is identical to those in radish and *A. thaliana*, but not in cowpea, in which a short peptide is removed from the N-terminus (Moran et al. 2003). The sequence homology between PiFe-SOD-c and *A. thaliana* Fe-SOD, and between PiFe-SOD-c and radish Fe-SOD were 65 %.

Phylogenetic analysis of PiFe-SOD-p and PiFe-SOD-c. A phylogenetic tree was constructed with the amino acid sequences of PiFe-SOD-p and PiFe-SOD-c, and of other Fe-SOD and Mn-SOD from cyanobacteria, red and green algae, mosses, ferns, and higher plants (Fig. 5). The tree shows three discrete clades as shown previously: chloroplastic Fe-SOD, algal-type Mn-SOD and mitochondrial Mn-SOD (Kanematsu et al. 2012). PiFe-SOD-c forms a sister group with cytosolic Fe-SODs of radish and *A. thaliana* among Fe-SODs. Chloroplastic Fe-SOD (FSD2) of *A. thaliana* is also placed in this group although its chloroplast localization was demonstrated (Myouga et al. 2008). PiFe-SOD-p shows more close relation to Ea-SOD than to PiFe-SOD-c.

Genomic structure of *PiFe-sod-p* and *PiFe-sod-c*. PCR amplification of the central region of *PiFe-sod-p* and *PiFe-sod-c* gave fragments of 2.4 and 1.6 kbp, respectively. The fragments were fully sequenced. Using gene-specific primers based on the central sequences, we amplified the upstream and downstream fragments ad-

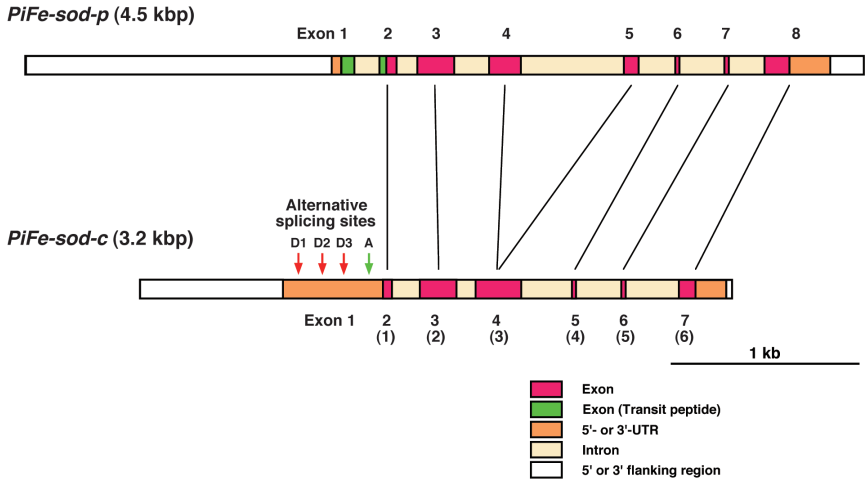

Fig. 6. Exon–intron structure of *PiFe-sod-p* and *PiFe-sod-c*. The vertical solid lines show the corresponding exons. D1–D3, splicing donor sites; A, acceptor site. The numbers in parentheses are for the exon numbers of transcript Type 4.

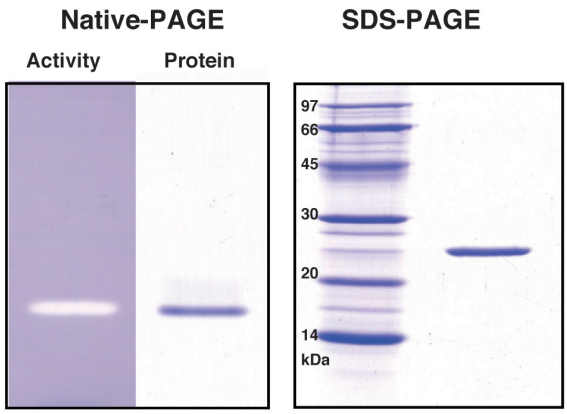

Fig. 7. Native- and SDS-PAGE of recombinant PiFe-SOD-c. Both analyses used 2 µg of the purified recombinant protein. Bands are stained with Coomassie Brilliant Blue R-250. For SOD activity staining, 10 units of the enzyme was applied.

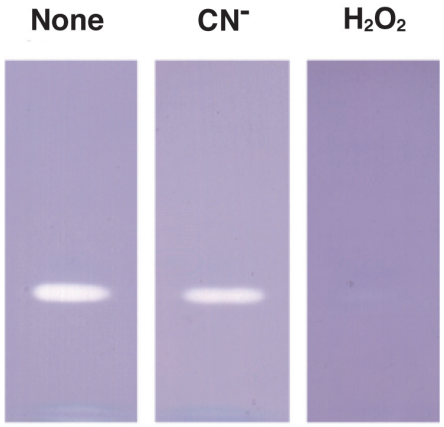

Fig. 8. Determination of the active-site metal in the recombinant PiFe-SOD-c by cyanide and H_2O_2 treatment after Native-PAGE. Ten units of purified recombinant PiFe-SOD-c was used. "CN^-" indicates 2 mM CN^- during activity staining. "H_2O_2" indicates incubation of gel for 20 min in 10 mM H_2O_2 before staining.

jacent to the central region. The *PiFe-sod-p* 5′-upstream fragment of 5 kbp was amplified from the EcoRV library and was sequenced up to 1.9 kbp from the 3′ end. The 3′-downstream fragment of 0.9 kbp was amplified from the *Stu*I library and was sequenced up to 0.5 kbp from the 5′ end. The first PCR of *PiFe-sod-c* gave a 0.6 kb-fragment for the 5′-upstream sequence from the *Pvu*II library. To obtain a longer fragment, we conducted the second PCR and obtained a 5.5-kbp fragment from the *Dra*I library. The fragment was sequenced up to 0.8 kbp from the 3′ end. A 5′-end sequence of 0.4 kbp was determined for a 3′-downstream fragment (3 kbp) obtained from the *Pvu*II library. Finally, by assembling the sequences of the three portions, we revealed nucleotide sequences of 4,541 bp for *PiFe-sod-p* and 3,185 bp for *PiFe-sod-c*.

PiFe-sod-p has eight exons and seven introns (Fig. 6). An intron is located in the transit-peptide-coding region in *PiFe-sod-p*. *PiFe-sod-c* has seven (type 1, 2, 3 in Fig. 2) or six (type 4) exons, depending on an alternative splicing at the 5′-UTR of the gene (Figs. 2, 6). Except for this and the deletion of one intron in *PiFe-sod-c*, which corresponds to the intron between exon 4 and exon 5 of *PiFe-sod-p*, the exon–intron structures are the same: the splicing positions of both amino acid sequences are identical. *PiFe-sod-p* showed the same exon–intron structure in coding sequence as those of *A. thaliana* cytosolic and chloroplastic Fe-SOD genes (*FSD1, FSD2, FSD3*) (TAIR and RIKEN Arabidopsis Full-Length Clone Database).

Overexpression and characterization of recombinant PiFe-SOD-c. To examine whether the *P. inflexum* cytosolic Fe-SOD is functional, we produced a recombinant protein and characterized it. The purified recombinant PiFe-SOD-c was homogeneous as judged from the single

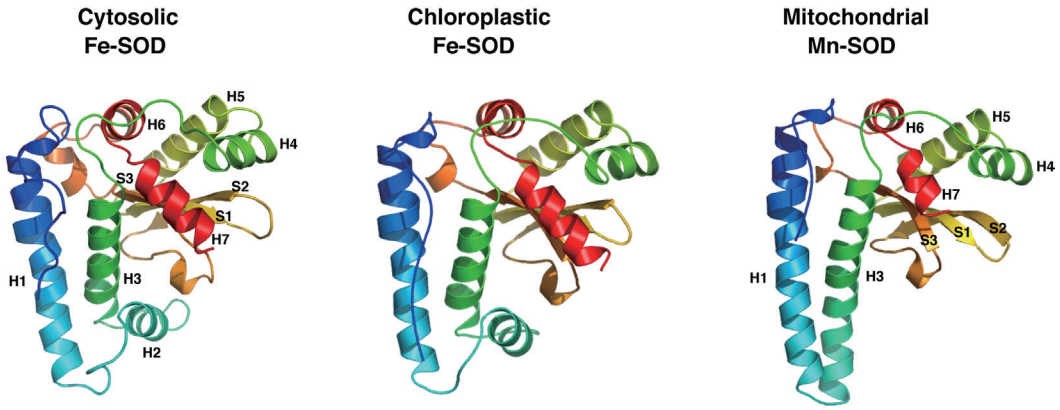

Fig. 9. Comparison of 3D structures of *P. inflexum* Fe- and Mn-SOD monomers. Cytosolic Fe-SOD, PiFe-SOD-c; chloroplastic Fe-SOD, PiFe-SOD-p; mitochondrial Mn-SOD, AB201827. H1–H7, α helices; S1–S3, β strands.

band on both native- and SDS-PAGE gels (Fig. 7). The coincidence of the single SOD activity band with a protein band on the native-PAGE gel indicates no modification during the purification procedure (Fig. 7). The presence of Fe but not Mn at the catalytic site of the enzyme was confirmed by the effect of inhibitors on activity: the recombinant enzyme was inactivated by 10 mM H_2O_2 but was not inhibited by 2 mM cyanide (Fig. 8), suggesting that PiFe-SOD-c is an Fe-containing SOD. The molecular mass and the subunit molecular mass of the recombinant PiFe-SOD-c were determined to be 50 kDa by gel filtration (data no shown) and 24 kDa by SDS-PAGE (Fig. 7), respectively. This result suggests that PiFe-SOD-c is a homodimer.

Three-dimensional structural comparison of PiFe-SOD-p, PiFe-SOD-c and PiMn-SOD. Since the PiFe-SOD-p and PiFe-SOD-c amino acid sequences share 62% homology (Fig. 3), and phylogenetic analysis indicated that PiFe-SOD-p is more closely related to EaFe-SOD than to PiFe-SOD-c (Fig. 5), we determined their 3D structures and examined the structural differences. The 3D monomer structures of mature PiFe-SOD-p and PiFe-SOD-c, which both consist of seven α-helixes and three β-strands, showed no prominent differences (Fig. 9). Likewise, there were no apparent structural differences between PiFe-SOD-p and EaFe-SOD (data not shown).

We previously showed that one of two Mn-SOD isoforms of *Spirogyra* sp. (SpMn-SOD-L) is similar to Mn-SOD form land plants, but the other (SpMn-SOD-A) resembles Fe-SOD in 3D structure, where the region consisting of the H1–H3 helixes in Fe-SOD is shorter than in Mn-SOD (Kanematsu et al. 2012). H1 and H3 of Fe-SOD are connected with a short alpha helix (H2), while the corresponding helixes (H1 and H3) of Mn-SOD are connected with a short turn. PiFe-SOD-p and PiMn-SOD showed similar structural differences in H1–H3 (Fig. 9).

DISCUSSION

Occurrence of a gene encoding cytosolic Fe-SOD in *P. inflexum*. Our discovery of two Fe-SOD genes (*PiFe-sod-p* and *PiFe-sod-c*) in *P. inflexum* suggests the wide distribution of Fe-SOD genes among land plants. Unexpectedly, however, *PiFe-sod-c* is an atypical cytosolic Fe-SOD gene that lacks a chloroplast transit sequence, whereas *PiFe-sod-p* encodes a chloroplastic Fe-SOD, which is similar to other plant Fe-SODs. Although most Fe-SODs in land plants are chloroplast-localized enzymes, a few cytosolic Fe-SODs has been reported from cowpea (Moran et al. 2003) and radish (Kwon and An 2006). Furthermore, FSD1, previously annotated as a chloroplastic Fe-SOD in *Arabidopsis thaliana*, has been shown to be localized in the cytosol (Myouga et al. 2008). Thus, in addition to chloroplastic Fe-SOD, cytosolic Fe-SOD seems to occur widely among land plants.

Alternative splicing of *PiFe-sod-c* transcripts. 5′-RACE of *PiFe-sod-c* gave four splicing variants. Comparison with the genomic sequence of *PiFe-sod-c* revealed three splicing donor sites located consecutively upstream of one common acceptor site in the 5′-UTR (Fig. 2). The nucleotide sequences of variants indicate that the three donor sites are not obligatory. Thus, the splicing variants may be generated by alternative donor site–type splicing and intron retention–type splicing.

In general, some patterns of alternative splicing result in the generation of isoforms that differ in their subcellular localization by inserting or deleting the sequence for a transit peptide or signal peptide (Stamm el al. 2005). However, this is not the case for *PiFe-sod-c*. Since stop codons exist in all three frames between the common acceptor site in the 5′-UTR and the start codon, no splicing variants affect the N-terminal sequence of the protein. Thus, it can be concluded that a cytosolic Fe-SOD is not produced from a chloroplastic Fe-SOD gene by alternative splicing, but is a product of a distinct Fe-SOD gene that is devoid of a transit peptide sequence.

Exon–intron structure comparison among moss Fe-SODs. Since the exon–intron structures of chloroplastic and cytosolic Fe-SODs from *P. inflexum* and *A. thaliana* are the same or similar in terms of splicing points (Fig. 6), we investigated Fe-SOD genes in the database of *Physcomitrella patens*, the first moss to have its whole genome decoded (Rensing et al. 2008). We compared genomic clones (DOE JGI) with cDNA clones from the *P. patens* full-length cDNA clone database (RIKEN BioResource Center). The results indicated that the two *P. patens* Fe-

SOD genes present are chloroplastic genes that contain a transit peptide sequence. To our surprise, both are devoid of introns. In contrast, the exon–intron structures of the CuZn-SOD genes of *P. inflexum* (Kanematsu et al. 2011) resemble those of *P. patens*. The absence of introns may be a special case of deletion from an ancestral gene after the divergence of *P. patens* in the evolution of mosses.

Characterization of recombinant PiFe-SOD-c. The recombinant Fe-SOD had similar specific activity to those of other Fe-SODs (Kanematsu and Asada 1994), indicating that *PiFe-sod-c* and its product are functional. Thus, the possibility of its being a pseudogene is excluded. The recombinant PiFe-SOD-c was determined to be a homodimer consisting of two equal subunits of 24 kDa, consistent with the deduced subunit molecular mass of 23,785 Da for PiFe-SOD-c, with 211 amino acid residues. The catalytic metal was confirmed to be Fe (Fig. 8). These results indicate that PiFe-SOD-c is similar to other Fe-SODs from prokaryotes and eukaryotes in terms of enzymatic and protein properties.

Concluding remarks. We found a cytosolic Fe-SOD gene in the moss *P. inflexum* that has a similar exon–intron structure to those of higher plants (Fig. 6) and has the identical N-terminus position in amino acid sequence to those of cytosolic Fe-SODs in radish and *A. thaliana* (Fig.4). Phylogenetic analysis confirmed that cytosolic PiFe-SOD-c is more related to cytosolic Fe-SODs of higher plants than to PiFe-SOD-p (Fig. 5). These findings suggest that cytosolic Fe-SOD genes originated from chloroplastic Fe-SOD genes in the evolution of land plants, and are widely present among land plants.

PiFe-sod-c is alternatively spliced to generate at least four transcript variants, which encode the same product. We do not know whether the alternative splicing in the 5′-UTR has a physiological role, but it may affect gene expression, by which the inconsistent distribution of Fe-SOD activity among land plants could partly be explained.

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