

Wang Feng · Wang Hongbin · Liu Bing · Wang Jinfa

Cloning and characterization of a novel splicing isoform of the iron-superoxide dismutase gene in rice (*Oryza sativa* L.)

Received: 29 April 2005 / Accepted: 14 June 2005 / Published online: 12 October 2005
© Springer-Verlag 2005

Abstract Superoxide dismutases (SODs) are ubiquitous metalloenzymes in aerobic organisms that play a crucial role in protecting organisms against ROS. Here, we report the molecular cloning and functional characterization of a novel alternatively spliced variant of the iron-superoxide dismutase gene, *OsFe-SODb*, from a rice panicle cDNA library. The alternative splicing event occurred in the fourth exon of the *OsFe-SOD* gene, and led to the translation of two isoforms of different sizes. The 5' flanking region of the *OsFe-SOD* was cloned and many cis-acting regulatory elements were found that are involved in light responsiveness, including a G-box and an I-box. RT-PCR analysis showed that the two alternative forms of *OsFe-SOD* were expressed in both the vegetative and reproductive tissues of Cps17. Moreover, accumulation of both isoforms was upregulated by light induction. In addition, the alternative splicing of *OsFe-SOD* mRNA was sensitive to low temperature. High yield production of the two recombinant OsFe-SOD isoforms was achieved in *Escherichia coli*. SOD assays showed that C-terminal truncation in OsFe-SODb did not result in a loss of SOD enzyme activity.

Key words Rice (*Oryza sativa* L.) · Iron-superoxide dismutase · cDNA · Alternative splicing · RT-PCR · SOD assay

Abbreviations SOD: Superoxide dismutase · ROS: Reactive oxygen species · RT-PCR: Reverse transcription-polymerase chain reaction · ORF: Open reading frame · bp: Base pair · kb: Kilobase pair · EST: Expressed sequence tag

Communicated by R. Reski

W. Feng · W. Hongbin · L. Bing · W. Jinfa (✉)
The State Key Laboratory for Biocontrol and The Key Laboratory of Gene Engineering of Ministry of Education, School of Life Sciences, Sun Yat-sen University, Guangzhou, 510275 People's Republic of China
e-mail: Ls19@zsu.edu.cn
Tel.: +86-20-84039179
Fax: +86-20-84039179

Introduction

Oxygen is essential for the existence of aerobic life, but toxic oxygen species, including oxygen-centered superoxide ($\cdot\text{O}_2^-$) and hydroxyl ($\cdot\text{OH}^-$) free radicals, as well as hydrogen peroxide, are generated in all aerobic cells. Oxygen toxicity had been reported in various species, and the damage resulting from these oxygen derivatives is known as oxidative stress (Cadenas, 1989; Halliwell, 1987). The superoxide radical ($\cdot\text{O}_2^-$) is generated as a by-product in oxygen-metabolizing organisms from a number of physiological reactions, such as the electron flow in chloroplasts and mitochondria and some redox reactions in cells. Exposure to chemicals such as plant hormones, fungal toxins/herbicides, and environmental stressors such as air pollutants, cold, drought and flooding can increase $\cdot\text{O}_2^-$ levels in plants. The superoxide radical then can react with hydrogen peroxide (H_2O_2) to produce the hydroxyl radical ($\cdot\text{OH}^-$), which is one of the most reactive molecules in living cells. Hydroxyl radicals cause the peroxidation of membrane lipids, breakage of DNA strands, and enzyme inactivation in cells (Bowler et al. 1992). Superoxide dismutase (SOD; EC 1.15.1.1) is a ubiquitous metalloenzyme that catalyzes the dismutation of superoxide anion radical to hydrogen peroxide and molecular oxygen (Fridovich, 1978). This reaction is the first step in the scavenging of reactive oxygen species (ROS). SOD isoforms are classified into four types based on the metal co-factor present at the catalytic center: copper/zinc (Cu/Zn-SOD), nickel (Ni-SOD), manganese (Mn-SOD) and iron (Fe-SOD). These isoforms are distributed in different subcellular locations. Ni-dependent enzymes have only been described from *Streptomyces* species (Youn et al. 1996). Cu/Zn-SODs are widely found in the cytosol, plastids and periplasm of prokaryotes (Steinman et al. 1990) as well as in eukaryotes (Bordo et al. 1994). Mn-SOD is localized predominantly to the mitochondrial matrix and the peroxisome, while Fe-SOD is observed mainly in prokaryotes and chloroplasts of limited plant species (Bowler et al. 1994; Bridges and Salin, 1981; Van Camp et al. 1990). Fe-SOD and Mn-SOD are structurally

very similar, and are thought to have evolved from a common ancestor before the divergence of prokaryotes and eukaryotes (Bowler et al. 1994; Stallings et al. 1984).

Two cDNAs for cytosolic Cu/Zn-SODs (Sakamoto et al. 1992a), one for plastidic Cu/Zn-SOD (Kaminaka et al. 1997), one for Mn-SOD (Sakamoto et al. 1993) and one for Fe-SOD (Kaminaka et al. 1999) were isolated previously from rice, an important crop and model monocot species. Fe-SOD was thought to be absent in rice and other monocotyledonous plants. However, Kaminaka et al. (1999) reported for the first time the sequence of *OsFe-SODa* cDNA from rice. Metal analysis and SOD assay of the recombinant protein overexpressed in *E. coli* showed that this cDNA encodes an iron-containing SOD. Furthermore, promoters of two rice cytosolic *Cu/Zn-SOD* genes were found and they differentially responded to externally supplied phytohormone ABA, although both responded to the antioxidant sulfhydryl reagent dithiothreitol DTT (Sakamoto et al. 1992b; Sakamoto et al. 1995). As one approach to define the molecular mechanisms responsible for plant *Fe-SOD* regulation and to identify the actual inducers of gene expression, we investigated the structures of the 5'-flanking sequences of rice *OsFe-SOD*. In addition, we acquired detailed information on exon/intron organization and examined the expression pattern of this gene.

Alternative splicing of heterogeneous nuclear RNA is an important cellular mechanism that increases the diversity of gene products and, therefore, plays a key role in the regulation of gene expression. It has been estimated that about 40% of all human genes have alternatively spliced variants. In contrast, far fewer alternatively spliced genes have been reported as of yet in plants. Intron removal efficiency and cryptic splice site utilization can affect the amount of mRNA produced as well as alter mRNA stability, translational efficiency and the sequence of the resulting protein product. Alternatively spliced forms have been associated with diverse physiologies and phenotypes. The most represented functional categories of alternatively spliced genes are enzymatic activity and cell growth. Four genes are known to be alternatively spliced in more than one plant species. They are rubisco activase (*Rca*), *MADS box*, granule-bound starch synthase (*GBSS/Waxy*) and starch branching enzyme (*SBE*). The categories and number of alternatively spliced genes in rice thus far are very limited, but will probably begin to increase at a greater pace as a result of the rapidly growing numbers of genomic and EST sequences (Zhou et al. 2003). To date, there have been no reports of an alternatively spliced member of the *SOD* gene family in any species. In this study, we identified a novel alternative splicing isoform, *OsFe-SODb*, of the rice iron-superoxide dismutase gene. This novel splicing event fuses exon 4, exon 5 and intron 4 of *OsFe-SODa*, thereby generating a new transcript encoding a C-terminal truncated protein. Therefore, it was interesting to study the differential regulation and tissue distribution of alternatively spliced variants of *OsFe-SOD*, and intriguing to determine if this new Fe-SOD isoform retains enzymatic activity.

Materials and methods

Plant materials

Rice (*Oryza sativa* L. cv. Cpslo17) panicles shorter than 3 cm were collected 15–20 day after panicle initiation. Seedlings from seven cultivars of two main subspecies of Asian cultivated rice (two *japonica* cultivars, Zhonghua 11 and Fengjin; five *indica* cultivars, Yuehang, Zhenjinzhan, Guangchaosimiao, Fongsizhan and Cpslo17) used in the experiments were grown in a growth cabinet maintained at 28 °C with a 16 h photoperiod for 14 day after germination. Embryogenic calli were maintained by weekly subculture in N6 medium containing 2 mg/L of 2,4-dichlorophenoxyacetic acid. For light induction experiment, dark-grown etiolated seedlings aged 14 day were exposed to light (60 $\mu\text{Em}^{-2}\text{s}^{-1}$) during the treatment. For cold treatment, 2-week-old seedlings grown under greenhouse conditions were transferred to 4 °C for 24 h and then returned to the 28 °C growth room. All samples were harvested at the indicated times, quickly frozen with liquid nitrogen and stored at –70 °C until used.

Construction of the rice cDNA library

Total RNA was isolated from rice panicles shorter than 3 cm in length using Concert™ Plant RNA Reagent (Invitrogen, USA) according to the manufacturer's instructions. Panicle cDNA was amplified using the SMART cDNA library construction kit (Clontech, USA). About 2 μg of total RNA was used to carry out ds cDNA synthesis following the manufacturer's protocols. After fractionation by CHROMA SPIN400, the ds cDNA was ligated into λ Triplex2 and packaged using the MaxPLax™ Lambda Packaging Extract Kit (EPICENTRE, USA).

Isolation of a cDNA clone and its 5' upstream sequence

Randomly selected clones from the panicle cDNA library were PCR amplified using lambda TriplEx2 insert screening primers (P1, 5' TCCGAGATCTGGAC-GAGC3', P2, 5' TAATACGACTCACTATAGGG 3'). The pTriplEx2 plasmids containing cDNA inserts of more than 1.0 kb in length were partially sequenced from the 5' ends. DNA sequence analysis and database comparisons were performed against GenBank entries. Primers (P3, 5' GGGTGCATCGATCGTGTCCAGGA3', P4 5'TTCTTCTCCTCTTTCTCCTGCGG3') based on the corresponding sequence of a rice BAC clone and primers (P5, 5' ACAGGTATGGAACCATCAC3', P6, 5'TCATGCAACTGGGATATTTGGT3') based on the cDNA sequence of *OsFe-SODb* were designed for cloning the 5' flanking region and partial genomic fragment of the *OsFe-SOD* gene. Cpslo17 DNA was used as the template for PCR amplification with LA Taq Polymerase

(TaKaRa, Japan). Purified PCR products were cloned into the pMD-18 T-Vector (TaKaRa, Japan) for sequencing.

Phylogenetic analysis

The amino acid sequences of OsFe-SODs were compared to those of various Fe-SODs. The sequences were obtained from the GenBank database and aligned with default parameters using CLUSTALX1.81. A phylogenetic tree was constructed using the neighbor-joining method of Saitou and Nei (1987) in the PHYLIP program package. The tree was developed with TreeView. To estimate the reliability of the branches of the tree, we used the bootstrap procedure (1,000 replicates).

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

To study the expression pattern of *OsFe-SOD* forms, total RNA was extracted from the leaves of different cultivars and various tissues of rice plants grown in soil. Semi-quantitative RT-PCR was performed with the primer pair of P7 (5' TCACTGGGGCAAGCATCAGCAAGAC3') and P8 (5' AAGCAGATTTCCACTATTTCCACCGT3'), which are located upstream and downstream of the additional 149 bp unspliced intron sequence of *OsFe-SODb*. The RT-PCR reaction for the house-keeping *Actin* gene, was performed using specific primers P9 (5' AGAGCTACGAGCTTCCTGATGGAC 3') and P10 (5' GAGAGATGCCAAGATGGATCCTCC 3') under the same conditions as those for the *OsFe-SODa* and *OsFe-SODb* reaction. The expression levels of *OsFe-SODa* and *OsFe-SODb* were calculated using a gray value ratio of amplified target gene cDNA fragment/*Actin* cDNA fragment in each lane. Reverse transcription was carried out in a 25 μ l reaction mixture containing 2 μ g of total RNA as template, 1 μ l of 10 μ M Oligo (dT)15 primer, 5 μ l of 5x reverse transcription buffer, 2.5 μ l of 10 mM dNTPs, 30 U of RNase inhibitor and 30 U of AMV reverse transcriptase (Promega, USA). In the control reactions, reverse transcriptase was omitted. Reverse transcription was carried out at 42 °C for 1 h. Two μ l of this reaction was then amplified by PCR to monitor the presence or absence of a transcript with one denaturation cycle of 5 min at 94 °C and 27 cycles of 30 s at 94 °C, 30 s at 58 °C, and 80 s at 72 °C.

Construction of expression vector and protein expression in *E. coli*

Primers were synthesized for subcloning the ORFs encoding rice Fe-SOD isoforms into the pET-11a expression vector (Novagen, USA) as follows:

P11, 5' TATCATATGCGCGCTTTCGCCTCCGCT3' (*Nde* I site underlined, start codon in bold),

P12, 5' ATGGATCCTCAGACTTTAAAACTAAATTTAG3' (*Bam*H I site underlined, stop codon in bold),

P13, 5' ATGGATCCTCATGCAACTGGGATATTTGGT3' (*Bam*H I site underlined, stop codon in bold).

P14, 5' TTGGCTTGTCTTGAAGAGAAAAGAACGA A3',

P15, 5' TCTCTTCAAGACAAGCCAAACCCAGCC3'.

Pfu DNA polymerase was used for PCR and a modified overlap extension PCR method (Wurch et al. 1998) was carried out to produce *OsSODa* fragment. Both the pET-11a vector and the PCR products were digested with *Nde* I and *Bam*H I and ligated to form the expression plasmids pET11a-*OsSODa* and pET11a-*OsSODb*. All constructs were confirmed by direct sequencing (Bioasia, Shanghai, China).

For production of recombinant rice Fe-SOD proteins, the final plasmid constructs were transformed into the *E. coli* bacterial host strain BL21(DE3). Five ml of starter culture was inoculated into 500 ml of Luria-Bertani media containing 50 μ g/ml carbenicillin. The cultures were vigorously shaken at 37 °C until the OD₆₀₀ approached 0.4–0.6, and were immediately shifted to 28 °C prior to induction. The recombinant enzymes were overexpressed in *E. coli* cells by using the strong inducible T7 promoter. After 10 h of induction with 1 mM IPTG, cells were harvested by centrifugation and the cell pellet was washed with an equal volume of PBS. Cells were again harvested by centrifugation, the supernatant was decanted, and the pellet was resuspended in PBS. The solution was sonicated and centrifuged at 10,000 g for 20 min. The expression of recombinant protein was analyzed by means of 12% SDS-PAGE and Coomassie Brilliant Blue staining.

Superoxide dismutase assay on SDS-PAGE gels

Sonicates were mixed with an equal volume of 2x SDS sample buffer, namely 25 mM Tris-HCl buffer (pH 6.8) containing 4% SDS, 20% glycerol and 0.05% bromophenol blue without β -mercaptoethanol at 4 °C. After electrophoresis at 4 °C, the gel was cut into two parts. One was stained with Coomassie Brilliant Blue G-250. The other was washed twice with PBS for 20 min, after which it was soaked in isopropanol: acetic acid : H₂O (5:2:13) twice for 30 min at 4 °C to remove the SDS (Guo 1999). The gel was then washed (2x) in PBS for 20 min before activity staining. A simple, rapid and convenient modified in-situ activity staining protocol, based on the photochemical method of Beauchamp and Fridovich (1971), was employed to locate SOD activity on the gel. SOD activity was identified by first incubating the gel in 25 mL of distilled water containing 2.45 mM nitroblue tetrazolium solution for 25 min in the dark at 4 °C. It was then soaked in a 50 mL solution containing 28 mM TEMED, 2.8x10⁻² mM riboflavin and 100 mM potassium phosphate (pH 7.8) for another 25 min in the dark at 25 °C. The SOD activity was developed by photoreaction. The soaking steps were performed with shaking at 75 rpm.

Results and discussion

Isolation of a cDNA clone encoding rice Fe-SODb and sequence analysis

In an attempt to acquire a cDNA clone from the panicle cDNA library, we selected insert fragments of more than 1.0 kb in length for partial sequencing from the 5' ends. Many EST sequences were generated by random sequencing. Based on the BLAST alignment results of these EST sequences, we finally obtained a cDNA clone of 1546 bp in length from the library that contains a 630 bp open reading frame (ORF) encoding a 209 amino acid protein with an estimated molecular mass of 24.2 kDa and a calculated pI of 9.03. After careful comparison with similar sequences, we found that it was a novel isoform of rice *OsFe-SOD* resulting from alternative splicing. We named this cDNA clone *OsFe-SODb*. It consists of a 49 bp 5' untranslated region (UTR) and an 866 bp 3'UTR that contains a poly(A+) tail. The sequence surrounding the initiation codon is in agreement with Kozak's rule (Kozak 1992), and a polyadenylation signal (gataaa) is found downstream of the stop codon. Subcellular localization predictions were carried out using TargetP V1.0 (Emanuelsson et al. 2000; Nielsen et al. 1997) and ChloroP (Emanuelsson et al. 1999). The corresponding results indicated that the first 32 residues in the N-terminal region code for a putative transit peptide for trans-

port to chloroplasts. Through residue composition analysis of these 32 residues, we found that it is enriched for Arginine (21.9%) and Alanine (18.8%), and that most arginines occur at the carboxy-terminal domain. Additional analysis by ProtScale at <http://us.expasy.org/tools/protscale.html> showed a highly hydrophobic central region (VLPSP-PAAV), followed by regions containing polar amino acids.

Although Fe-SOD and Mn-SOD are very similar in structure, they can be distinguished by their overall homologies and specific residues. This newly characterized rice Fe-SODb shows a high similarity with the known plant Fe-SODs (33.9% to 45.4%), and only about a 28.7% similarity with rice Mn-SOD. The alignment of amino acid sequences (Fig. 1) revealed that the deduced amino acids of *OsFe-SODb* have 45.4%, 40.5%, 40.2%, 39.1%, 37.7%, 33.9% similarity to Fe-SOD from tomato, soybean, *Arabidopsis thaliana*, tobacco, *Raphanus sativus* and pea. The two chief residues for distinguishing Fe-SOD and Mn-SOD, Alanine-188 and Glutamine-115, are found in both *OsFe-SOD* isoforms. Residues 115 and 188 are closely positioned in the tertiary structures of both Fe-SOD and Mn-SOD. However, the Mn-SOD has a small residue (Glycine) at position 115 adjacent to Glutamine-188, whereas Fe-SOD has a small residue (Alanine) at position 188 (Van Camp et al. 1990; Parker and Blake 1988). In addition, Tryptophan-117 reportedly confers H₂O₂ sensitivity and, therefore, can be considered specific for

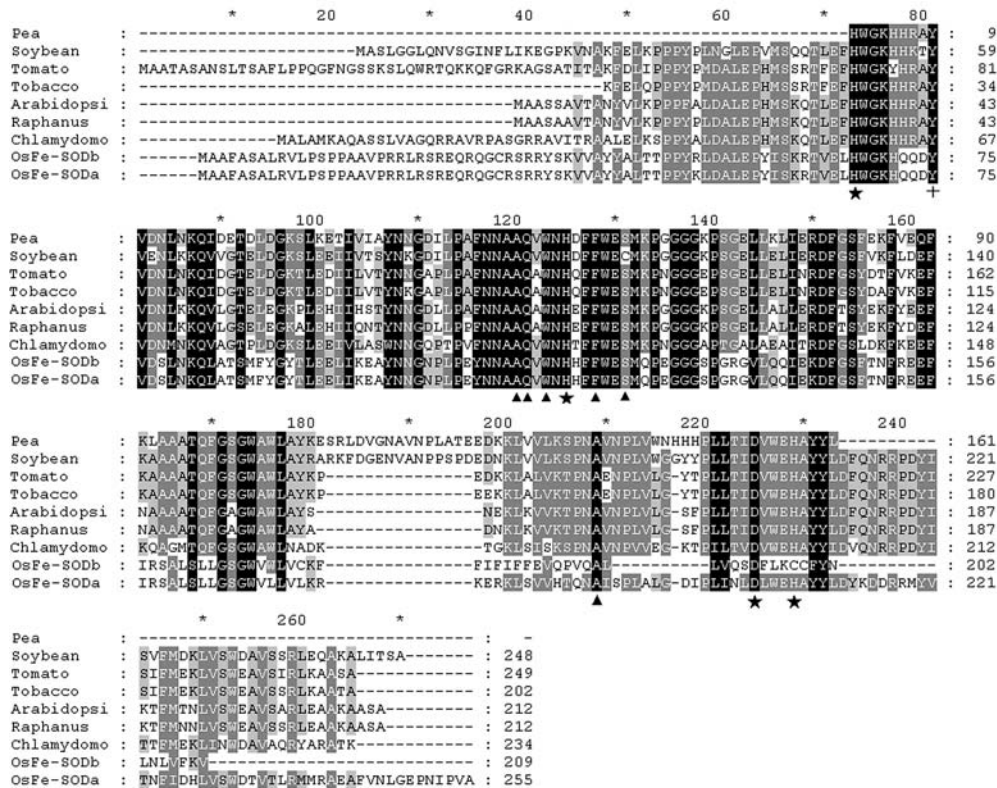


Fig. 1. Sequence alignment of the deduced amino acid sequence of rice Fe-SODs with those of *Chlamydomonas reinhardtii*, tomato, tobacco, *Arabidopsis thaliana*, *Raphanus sativus*, pea and soybean. The amino acid sequences were analyzed with the program

CLUSTALX1.81. Residues important for distinguishing Fe-SOD from MnSOD are indicated with arrowheads. Residues essential for catalytic activity and putative conserved metal ligand binding sites are shown with plus and asterisk marks, respectively

Table 1 Nucleotide sequence analysis of exon-intron junctions of rice iron-superoxide dismutase gene

Exon	GC content(%)	Length	Splice donor site	Intron	GC content(%)	T content(%)	Length	Splice acceptor site	Potential branch site sequence
A1B1	65.28	121	GCTTCGCTCGgtgagctcct	A1B1	40.23	36.16	860	tttgttcagAGAGAACAAA	cttgactatgatgttttagtg
A2B2	46.41	84	GTATAAACTTgtatgttctc	A2B2	37.10	36.08	97	tggtgtccagGATGCCCTGG	atnttaactatagctgtgc
A3B3	42.85	189	TGCAGCACAGgtaatgttgt	A3B3	32.21	40	180	tgaatacagGTATGGAACC	tatgttctaattttcaatc
A4	47.67	172	TGGCTTGCTgtagttttt	A4	32.20	46.30	149	ctgtttcagTGAAGAGAAA	ttaaagtctgatttataac
B4	39.99	395	TGGTGATATTgtatgttttc						
A5	37.82	74	TGGTGATATTgtatgttttc	A5B4	35.47	39.78	93	tctcttcagCCACTCATCA	cccaagtataaacctttgtg
A6B5	48.14	27	CTTGTGGGAGgtaaggaaat	A6B5	29.99	38.57	70	tggtttcagCATGCTTACT	cattatgctaataattgaca
A7B6	37.49	24	GGATTACAAAGgtattttcta	A7B6	32.67	41.58	101	gatgcttcagGATGACAGGC	ccaagtactaagctatcata
A8B7	41.75	182	TCATCATGAGgtaaaatcca	A8B7	36.60	33.12	1117	caattttcagCGACGCCGAG	gactatgctaataattttt
A9B8	40.60	490	AAGCTTTTGC						

Intron sequence is shown in lowercase and exon sequence in uppercase. A indicates the *OsFe-SODa* isoform and B indicates the *OsFe-SODb* isoform. Putative branch sites are boxed

Fe-SOD (Yamakura, 1984), is well conserved in both *OsFe-SOD* isoforms. Furthermore, as shown in Fig. 1, *OsFe-SODb* contains three of four putative Fe-binding sites (Histidine-67, Histidine-119, Asparagines-194), while *OsFe-SODa* contains all four (Histidine-67, Histidine-119, Asparagines-203, Histidine-207).

Genomic organization and exon-intron junction structure

Both the *OsFe-SODb* cDNA and the partial genomic sequences were compared to *japonica* and *indica* rice genomes using the BLAST program, and were then optimized manually. There was very little difference between the *japonica* and *indica* rice genomic sequences for the *OsFe-SOD* gene, only a few nucleotide substitutions that did not lead to a modification of key residues needed for enzyme function. The *OsFe-SOD* gene was located on the *japonica* rice BAC clone P0535G04 (Accession number AP000399) that was mapped to 10.4 cM on the short arm of chromosome 6, and spans approximately 4.0 kb along the chromosome. Detailed comparisons suggested that *OsFe-SODb* had 8 exons and 7 introns. All sequences at the exon-intron junctions in both isoforms of *OsFe-SOD* mRNA are consistent with the GT-AG rule (Table 1). The average length of introns and exons of *OsFe-SODb* are 359.7 bp and 184.1 bp, and the average GC content of introns and exons of *OsFe-SODb* are 37.2% and 43.4%, respectively. In agreement with properties essential for the splicing of plant introns, *OsFe-SOD* introns are strongly enriched throughout for U residues (Ko et al. 1998). AU-rich elements that play an important role in the definition of intronic borders (Merritt et al. 1997) are also found in *OsFe-SOD* introns. The potential branch points of all *OsFe-SOD* introns were revealed by NetPlantGene, a splice site prediction program (Hebsgaard et al. 1996).

Alternatively spliced *OsFe-SODb*

An alternative splicing event results in a fusion of exon 4, exon 5 and intron 4 of *OsFe-SODa*. As seen in Fig. 2, intron 4 (149 bp in length) of *OsFe-SODa* is unspliced in

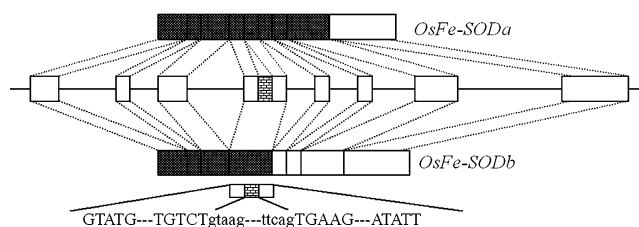


Fig. 2. Genomic organization of the rice *Fe-SOD* gene. Exons are shown as boxes. Solid boxes denote coding regions and open boxes represent non-coding regions. The genomic sequences of the exon/intron boundaries of the *Fe-SODa* (exon 4 to 5) are shown at the bottom

OsFe-SODb. The average GC content of this intron 4 is 32.2% and it has a splice donor and a splice acceptor site at its 5' and 3' end, respectively. The branch point sequence ctgat of intron 4 of *OsFe-SODa* fits the consensus sequence CT(A/G)A(A/T) (Tolstrup et al. 1997) and the underlined A is located 37 bases upstream of the acceptor site. The presence of this extra intron 4 in alternatively spliced *OsFe-SODb* leads to a frameshift mutation, which results in a complete change in the amino acid sequence downstream of the Valine-169 residue. To the best of our knowledge, although many different *SOD* genes have been isolated in plants and animals, this is the first report describing alternatively spliced forms in the *SOD* gene family. Thus, we believe that characterization of this gene augments the group of alternatively spliced genes.

Phylogeny

A phylogenetic tree based on Fe-SOD sequences from higher plants, chlorophytes, cyanobacteria and *E. coli* was plotted. Phylogenetic analysis showed the existence of three distinct clusters supported by high bootstrap values: the prokaryotic *E. coli* cluster, the prokaryotic cyanobacteria cluster and the Eukaryotic algae and plants cluster. The relationships between these clusters were consistent with their phylogenetic distributions. As shown in Fig. 3, the green algae *Chlamydomonas reinhardtii* nests on a branch of the known plant Fe-SODs. A feature of the eukaryotic plant cluster is that *OsFe-SOD* did not

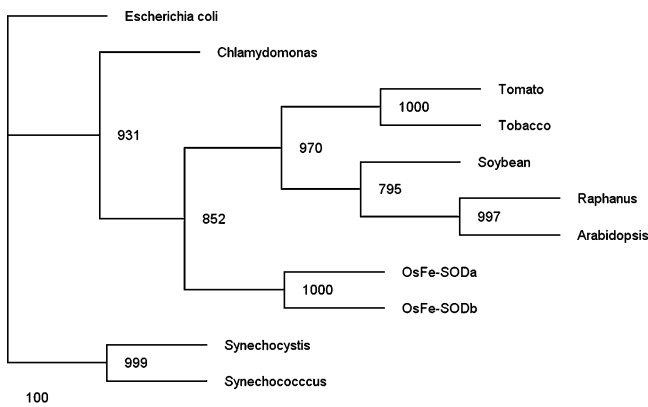


Fig. 3. Phylogenetic tree of Fe-SODs. A phylogenetic tree derived from the amino acid sequences of Fe-SODs from different species was constructed using the Neighbor-Joining method. The numbers shown at the branches are bootstrap values. GenBank numbers are: *Chlamydomonas reinhardtii*, U22416; *Synechococcus elongatus*, X17431; *Escherichia coli*, J03511; tomato (*Lycopersicon esculentum*), AY262025; tobacco (*Nicotiana plumbaginifolia*), P22302; *Arabidopsis thaliana*, P21276; *Raphanus sativus*, AAC15842; soybean (*Glycine max*), P28759; OsFe-SODa (*Oryza sativa*), JG0179; *Synechocystis sp.* P77968

group with that from the dicotyledonous plants, suggesting that monocotyledonous OsFe-SOD had a distinct evolutionary process separate from the Fe-SODs of the dicots. Fe-SOD was absent in animals, but was found in the cytosol of cyanobacteria. Meanwhile, most eukaryotic algae and plants contained Fe-SODs in the chloroplast stroma. It has been proposed that the Fe-SOD genes were not transferred independently from prokaryotes, but were originally encoded by chloroplasts and moved to the nuclear genome during a later stage of evolution (Van Camp et al. 1990; Grace 1990). All together, phylogenetic analysis of Fe-SOD was consistent with the hypothesis that chloroplasts are evolutionary derivatives of an endosymbiotic cyanobacterium.

Expression pattern of *OsFe-SOD* isoforms

Expression of two *OsFe-SOD* forms in the leaves of seven cultivars and different organs of Cpslo17 was examined by RT-PCR. Results showed that alternative splicing of *OsFe-SOD* stably occurred in two subspecies of rice *indica* and *japonica* (Fig. 4A). Expression levels of the two *OsFe-SOD* forms were different in the seven genotypes. The level of *OsFe-SODb* in leaves was higher than in *OsFe-SODa*, while a single *OsFe-SODa* mRNA of approximately 1,500 nt was detected in the vegetative tissues of rice (*Oryza sativa* L. cv Nipponbare) by Kaminaka et al. (1999). However, both *OsFe-SOD* transcripts were detected also in reproductive organs, such as the panicles and seeds, of Cpslo17 (Fig. 4B). Expression levels of two *OsFe-SOD* isoforms were also relatively high in young and meristem tissues. *OsFe-SODb* was highly expressed in leaves and young panicle, but *OsFe-SODa* was more abundant in panicles at the

flowering stage. Taken together, these data show that expression of *OsFe-SOD* forms was neither tissue nor cultivar specific.

Accumulation of *OsFe-SOD* isoforms by light inductor

Previously, Kaminaka (1999) examined the effect of light induction on the level of *OsFe-SODa* mRNA in rice etiolated seedlings. In our study, observation of the expression of the two alternative *OsFe-SOD* forms in etiolated seedlings after light induction (Fig. 4C) suggested that both *OsFe-SOD* isoforms were not expressed in etiolated seedlings and the mRNA levels of both forms increased steadily after light induction. Furthermore, the extent of *OsFe-SODa* transcript induction was higher than that of *OsFe-SODb*. A careful look at the 1.3 kb 5' upstream sequence of the *OsFe-SOD* ORF revealed the presence of various putative cis-acting regulatory motifs/elements which are listed in Table 2. Consistent with our observation, analysis of the cloned potential promoter sequence using the PlantCARE program (Lescot et al. 2002) indicated that a number of regulatory elements, such as an I-box and a G-box, that are functionally important in many light-regulated plant promoters, were frequently found in the *OsFe-SOD* promoter region. However, in contrast to these highly abundant light regulation elements, ABA-responsive elements (ABREs) were found in the 5'-flanking regions of the rice *SODCc2* (*Cu/Zn-SOD*) gene (Sakamoto et al. 1995). Further studies need to be conducted in order to clarify the function of these elements that may play an important role in expression of *OsFe-SOD* by light stimulation.

Alternative splicing in response to low temperature

Following cold treatment, a significant accumulation of *OsFe-SODa* was observed, with the transcript reaching maximum levels at 2 h, followed by a gradual decrease thereafter (Fig. 4D). However, expression of *OsFe-SODb* decreased dramatically when transferred to 4 °C. It has been presumed that the retention of introns by alternative splicing in plants probably originated from poor recognition of the intron rather than by active processes inhibiting the splicing reaction (Brown and Simpson 1998). However, generation of the *OsFe-SODb* form was strongly inhibited after 12 h of cold treatment, while levels of *OsFe-SODa* remained somewhat constant, indicating a good recognition of the intron at low temperature. Recovery of the expression of both *OsFe-SOD* transcript isoforms was observed after transfer of the seedling back to 28 °C. The results described above clearly revealed that splicing of the *OsFe-SOD* transcripts was sensitive to low temperature. Subsequent experiments should be performed to understand the mechanism of the regulation of alternative splicing by temperature.

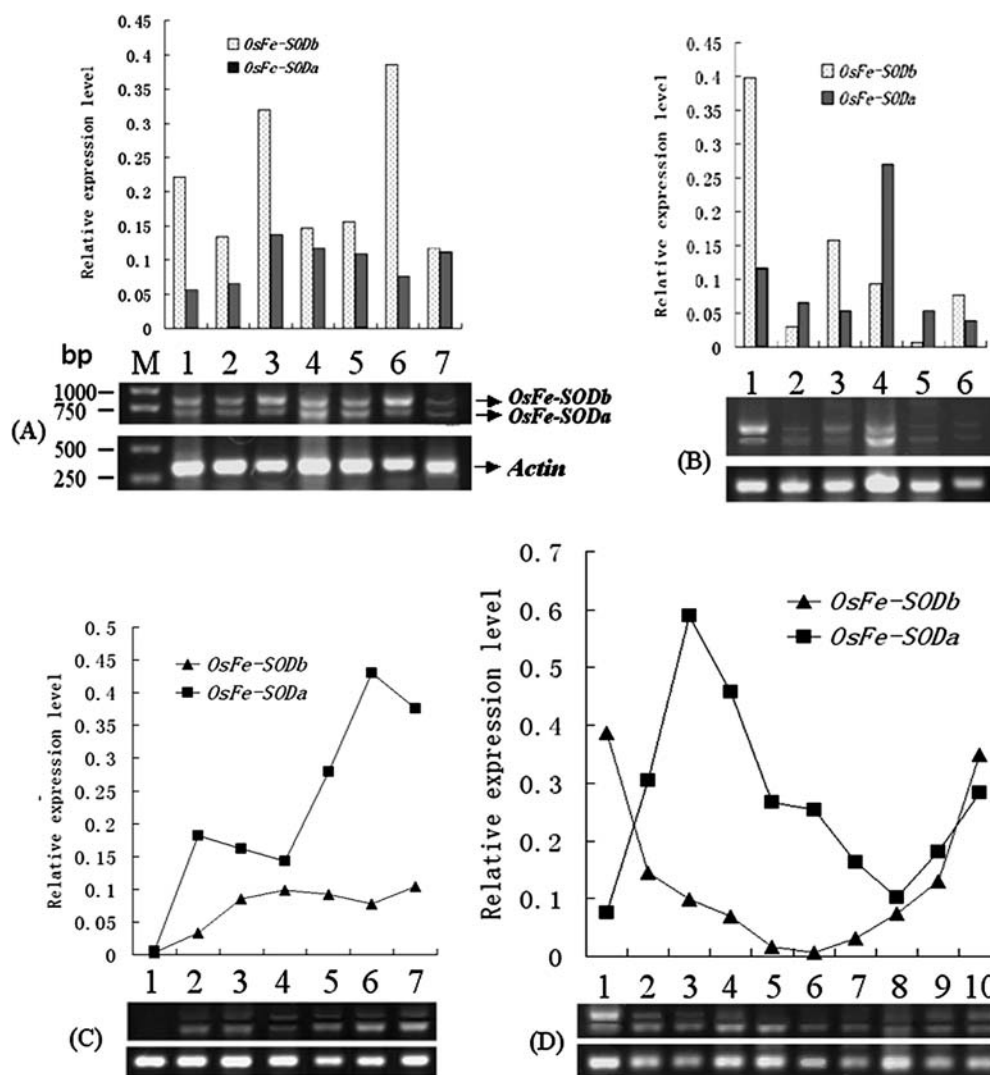


Fig. 4. RT-PCR expression analysis of alternatively spliced *OsFe-SOD* transcripts. A 738 bp *OsFe-SODa* fragment and a 887 bp *OsFe-SODb* fragment were amplified by 27 PCR cycles. As control, a 335 bp *Actin* fragment was simultaneously amplified. (A) Leaves of different rice cultivars, lane 1, Yuehang; lane 2, Fengjin; lane 3, Zhenjinzhan; lane 4, Guangchaosimiao; lane 5, Zhonghua 11; lane 6, Cpslo17; lane 7, Fengsizhan; M DL2000 DNA marker. (B) Different tissues of Cpslo17, lane 1, young leaf; lane 2, stem; lane 3, young

panicle; lane 4, panicle at flowering stage; lane 5, immature seed; lane 6, callus. (C) Light induction, lane 1 etiolated seedlings; lane 2, 1 h; lane 3, 3 h; lane 4, 9 h; lane 5, 24 h; lane 6, 36 h; lane 7, 72 h. (D) Cold treatment, lane 1, before treatment (control); lane 2, 4 °C for 1 h; lane 3, 4 °C for 2 h; lane 4, 4 °C for 3 h; lane 5, 4 °C for 6 h; lane 6, 4 °C for 12 h; lane 7, 4 °C for 24 h; lane 8, returned to 28 °C for 6 h; lane 9, returned to 28 °C for 9 h; lane 10, returned to 28 °C for 12 h

Both recombinant OsFe-SOD isoforms retain SOD activity

To obtain a large quantity of soluble and active enzyme, a series of temperature trials were performed. When expressed at 37 °C, the bulk of the recombinant proteins were expressed as mixtures in inclusion bodies. We ultimately ascertained that induction is optimal at 28 °C, and that the proteins are expressed as a soluble form at this temperature. In comparison with the control culture (BL21 containing plasmid pET-11a), the two proteins were expressed at a tremendously high-level within 10 hours after induction with IPTG at 28 °C. As was estimated from gel scanning, the synthesized *OsFe-SODa* and *OsFe-SODb* proteins constituted up to 21.6% and 27.2% of the total soluble protein

of *E. coli* cells, respectively. NBT staining was conducted after removal of SDS from the PAGE gel. As seen in (Fig. 5), the superoxide dismutase activity band, a clear zone against a brown background, showed that both of the recombinant isoforms retained specific enzymatic activity. Compared with *OsFe-SODa*, *OsFe-SODb* has a truncated C-terminal that still contains some conserved residues such as Alanine-188 and Asp-194. However, Tyrosine-75, which is believed to interact with Glutamine-115 in Fe-SOD and is thought to be important for catalytic activity (Van Camp et al. 1990), and Glutamine-115 are present in both *OsFe-SOD* isoforms. It appears that the domain that contributes to SOD activities is not located at the C-terminal of Fe-SOD. There is a fifteen residue insertion in the C-terminal of pea and soybean Fe-SOD. All of these findings denote that the

Table 2 Putative cis-acting regulatory elements found in 5' flanking region of *OsFe-SOD*

Elements/motifs	Organism	Function
I-box	<i>Flaveria trinervia</i> <i>Pisum sativum</i> etc	Part of a light responsive element
G-Box	<i>Hordeum vulgare</i> <i>Zea mays</i> , etc	Cis-acting regulatory element involved in light responsiveness
GAG-motif	<i>Arabidopsis thaliana</i> <i>Hordeum vulgare</i> etc	Part of a light responsive element
ATC-motif	<i>Arabidopsis thaliana</i>	Part of a conserved dna module involved in light responsiveness
TCCC-motif	<i>Spinacia oleracea</i>	Part of a light responsive element
GA-motif	<i>Arabidopsis thaliana</i> <i>Glycine max</i> , etc	Part of a light responsive element
GATA-motif	<i>Pisum sativum</i>	Part of a light responsive element
HSE	<i>Brassica oleracea</i>	Cis-acting element involved in heat stress responsiveness
WUN-motif	<i>Brassica oleracea</i>	Wound-responsive element
CATT-motif	<i>Zea mays</i>	Part of a light responsive element

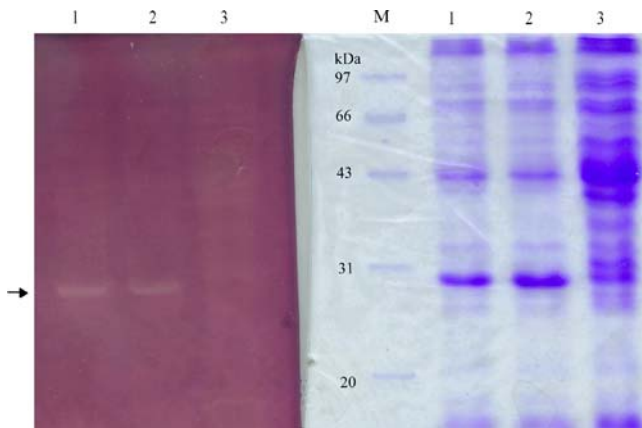


Fig. 5. Expression in *E. coli* and SOD activity of recombinant *OsFe-SOD* isoforms. Left, gel stained for SOD activity as described in the section on "Materials and Methods." Right, gel stained with Coomassie brilliant blue. Lanes 1, 2, 3 contain sonicates of *E. coli* strain BL21 harboring pET11a-*OsSODa*, pET11a-*OsSODb* and pET11a, respectively. M, Protein marker. Cultures were induced by incubation with 1mM IPTG for 10 h at 25 °C

C-terminal end of plant Fe-SOD is not essential for SOD activity. In plants, some genes have been shown to encode two or more transcripts that may have different functions. The intact rice *OsMAPK5a* isoform was shown to have kinase activity, but neither autophosphorylation nor MBP kinase activity was detected for the truncated *OsMAPK5b* isoform (Xiong and Yang 2003). Conversely, the alternatively spliced transcripts of a tomato diacylglycerol kinase gene were reported to encode two isoforms, both of which were catalytically active in vitro (Snedden and Blumwald 2000).

In this report, we defined a novel spliced isoform of *OsFe-SOD* from rice, the first member of the *SOD* gene family that has been shown to undergo alternative splicing. We also carried out SOD assays that demonstrated that both *OsFe-SOD* isoforms exhibit specific SOD activity. In conclusion, the cloning and functional expression of Fe-SOD from rice provided an opportunity to study the

biochemical characteristics of this enzyme and to explore the possibilities of its functional role in plants.

Supplementary material

The nucleotide sequences reported in this paper have been submitted to GenBank under accession numbers **AY770495** (*OsFe-SOD* mRNA, complete cds), **AY770496** (5' UTR sequence and 5' flanking region of *OsFe-SOD*) and **AY871310** (partial genomic sequence of *OsFe-SOD* gene).

Acknowledgements This research was supported by the Foundation of National Plant Transgenic Program (J00-A-009), the National Natural Science Foundation of China (30370809) and the Natural Science Foundation of Guangdong Province (990258), China.

References

- Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* 44:276–287
- Bordo D, Djnovic K, Bolognesi M (1994) Conserved patterns in the Cu,Zn superoxide dismutase family. *J Mol Biol* 238:366–386
- Bowler C, Van Camp W, Van Montagu M, Inze D (1994) Superoxide dismutase in plants. *Crit Rev Plant Sci* 13:199–218
- Bowler C, Van Montagu M, Inze D (1992) Superoxide dismutase and stress tolerance. *Annu Rev Plant Physiol Plant Mol Biol* 43:83–116
- Bridges SM, Salin ML (1981) Distribution of iron-containing superoxide dismutase in vascular plants. *Plant Physiol* 68:275–278
- Brown JWS, Simpson CG (1998) Splice site selection in plant pre-mRNA splicing. *Annu Rev Plant Physiol Plant Mol Biol* 49:77–95
- Cadenas E (1989) Biochemistry of oxygen toxicity. *Annu Rev Biochem* 58:79–110
- Emanuelsson O, Nielsen H, Brunak S, Heijne GV (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J Mol Biol* 300:1005–1016

- Emanuelsson O, Nielsen H, Heijne GV (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Sci* 8:978–984
- Fridovich I (1978) The biology of oxygen radicals. *Science* 201:875–880
- Grace SC (1990) Phylogenetic distribution of superoxide dismutase supports an endosymbiotic origin for chloroplasts and mitochondria. *Life Sci* 47:1875–1886
- Guo YJ (1999) Technique of electrophoresis of protein. Science Press, Beijing, pp. 154–156.
- Halliwell B (1987) Oxidative damage, lipid peroxidation and antioxidant protection in chloroplasts. *Chem Phys Lipids* 44:327–340
- Hebsgaard SM, Korning PG, Tolstrup N, Engelbrecht J, Rouze P, Brunak S (1996) Splice site prediction in *Arabidopsis thaliana* DNA by combining local and global sequence information. *Nucleic Acids Res* 24:3439–3452
- Kaminaka H, Morita S, Tokumoto M, Yokoyama H, Masumura T, Tanaka K (1999) Molecular cloning and characterization of a cDNA for an iron-superoxide dismutase in rice (*Oryza sativa* L.). *Biosci Biotech Biochem* 63:302–308
- Kaminaka H, Morita S, Yokoi H, Masumura T, 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
- Ko CH, Brendel V, Taylor RD, Walbot V (1998) U-richness is a defining feature of plant introns and may function as an intron recognition signal in maize. *Plant Mol Biol* 36:573–83.
- Kozak M (1992) Regulation of translation in eukaryotic systems. *Annu Rev Cell Biol* 8:197–225
- Lescot M, Dehais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouz P, Rombauts S (2002) PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Res* 30:325–327
- Merritt H, McCullough AJ, Schuler MA (1997) Internal AU-rich elements modulate activity of two competing 3' splice sites in plant nuclei. *Plant J* 12:937–43
- Nielsen H, Engelbrecht J, Brunak S, Heijne GV (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng* 10:1–6
- Parker MW, Blake CC (1988) Iron and manganese containing superoxide dismutases can be distinguished by analysis of their primary structures. *FEBS Lett* 229:377–382
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Sakamoto A, Nosaka Y, Tanaka K (1993) Cloning and sequencing analysis of a complementary DNA for manganese-superoxide dismutase from rice (*Oryza sativa* L.). *Plant Physiol* 103:1477–1478
- Sakamoto A, Ohsuga H, Tanaka K (1992a) Nucleotide sequences of two cDNA clones encoding different Cu/Zn-superoxide dismutases expressed in developing rice seed (*Oryza sativa* L.). *Plant Mol Biol* 19:323–327
- Sakamoto A, Okumura T, Kaminaka H, Sumi K, Tanaka K (1995) Structure and differential response to abscisic acid of two promoters for the cytosolic copper/zinc-superoxide dismutase genes, *SodCc1* and *SodCc2*, in rice protoplasts. *FEBS Lett* 358:62–66
- Sakamoto A, Okumura T, Ohsuga H, Tanaka K (1992b) Genomic structure of the gene for copper/zinc-superoxide dismutase in rice. *FEBS Lett* 301:185–189
- Snedden WA, Blumwald E (2000) A calmodulin-binding diacylglycerol kinase from tomato is produced by alternative splicing at its calmodulin-binding domain. *Plant J* 24:317–326
- Stallings WC, Pattridge KA, Strong RK, Ludwig ML (1984) Manganese and iron superoxide dismutases are structural homologs. *J Biol Chem* 259:10695–10699
- Steinman HM, Ely B (1990) Copper-zinc superoxide dismutase of *Caulobacter crescentus*: cloning, sequencing, and mapping of the gene and periplasmic location of the enzyme. *J Bacteriol* 172:2901–2910
- Tolstrup N, Rouze P, Brunak S (1997) A branch point consensus from *Arabidopsis* found by non-circular analysis allows for better prediction of acceptor sites. *Nucleic Acids Res* 25:3159–3163
- Van Camp W, Bowler C, Villarroel R, Tsang EWT, Van Montagu M, Inzé D (1990) Characterization of iron superoxide dismutase cDNAs from plants obtained by genetic complementation in *Escherichia coli*. *Proc Natl Acad Sci USA* 87:9903–9907
- Wurch T, Lestienne F, Pauwels PJ (1998) A modified overlap extension PCR method to create chimeric genes in the absence of restriction enzymes. *Biotechnol Techniques* 12:653–657
- Xiong LZ, Yang YN (2003) Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. *Plant Cell* 15:745–759
- Yamakura F (1984) Destruction of tryptophan residues by hydrogen peroxide in iron-superoxide dismutase. *Biochem Biophys Res Commun* 122:635–641
- Youn HD, Kim EJ, Roe JH, Hah YC, Kang SO (1996) A novel nickel-containing superoxide dismutase from *Streptomyces* spp. *Biochem J* 318:889–896
- Zhou Y, Zhou CL, Ye L, Dong JH, Xu HY, Cai L, Zhang L, Wei LP (2003) Database and analyses of known alternatively spliced genes in plants. *Genomics* 82:584–595