



## Research paper

Genome-wide analysis of superoxide dismutase genes in *Larix kaempferi*Xue-Min Han<sup>a,b,1</sup>, Qiang-Xin Chen<sup>b,1</sup>, Qi Yang<sup>b</sup>, Qing-Yin Zeng<sup>a,b</sup>, Ting Lan<sup>c,\*</sup>, Yan-Jing Liu<sup>a,\*\*</sup><sup>a</sup> State Key Laboratory of Tree Genetics and Breeding, Chinese Academy of Forestry, Beijing 100091, China<sup>b</sup> State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China<sup>c</sup> Guangdong Provincial Key Laboratory for Plant Epigenetics, College of Life Sciences and Oceanography, Shenzhen University, Shenzhen 518060, China

## ARTICLE INFO

## Keywords:

Gene expression  
Subcellular localization  
Biochemical properties  
Salt tolerance  
Functional divergence

## ABSTRACT

Superoxide dismutase is a key enzyme that scavenges superoxide anion and plays vital roles in plant antioxidant system. This study identified six SOD genes from the deciduous conifer *Larix kaempferi*, which is widely distributed across the cooler regions of the northern hemisphere. These six SOD genes were classified into three types: Cu/Zn-SOD (LkSOD1, 2, 3 and 4), Fe-SOD (LkSOD5) and Mn-SODs (LkSOD6). Three Cu/Zn-SOD proteins (LkSOD1, 3 and 4) were cytosolic-localized, while the other three proteins (LkSOD2, 5 and 6) were chloroplast-localized. *Larix* SOD proteins displayed catalytic activities toward superoxide anion, and retained > 55% of its maximum enzymatic activity between 10 °C and 40 °C. Over expressions of three *Larix* SOD genes (LkSOD2, 4 and 6) in *Arabidopsis thaliana*, respectively, showed increased germination rates and root lengths during salt stress. LkSOD5 gene could rescue pale green and dwarf phenotype of *Arabidopsis atfsd2-2* mutant. Taken together, this study provided comprehensive insight into the gymnosperm SOD gene family.

## 1. Introduction

Reactive oxygen species (ROS) are chemically reactive chemical species containing oxygen, mainly including peroxides, superoxide, hydroxyl radical, and singlet oxygen, etc. (Kliebenstein et al., 1998). ROS are metabolized and produced in several cell organelles, such as mitochondria, chloroplasts, plasma membranes and nucleolus. Chloroplasts and mitochondria are the main places for producing ROS (Karuppanapandian et al., 2011). ROS have dual functions. Low concentration of ROS is beneficial for plant growth. However, high concentration of SOD can induce oxidative stress, resulting in irreversible damage to cells (Brawn and Fridovich, 1981; Niki et al., 2005; Bose et al., 2014). Under normal circumstances, the active oxygen produced will be promptly removed to achieve a dynamic equilibrium state. Once exposed to UV, freezing, drought, high salt, heavy metals and other abiotic stresses, the dynamic balance will be broken, resulting in excess of ROS (Gill and Tuteja, 2010).

To reduce the damage caused by these oxidative stresses, plants have developed a complete set of active oxygen scavenging systems, including enzymatic and non-enzymatic antioxidative systems, respectively. Non-enzymatic antioxidative systems include ascorbic acid

(AsA), glutathione (GSH), vitamin E, carotenoids, flavonoids and other antioxidants (Mittler et al., 2004). Enzyme antioxidant system mainly includes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX), guaiacol peroxidase (GPOD), peroxidase (PRX), glutathione-S-transferase (GST) (Mittler et al., 2004; Gill and Tuteja, 2010; Hasanuzzaman et al., 2012). Among them, SOD (EC 1.15.1.1), which is a kind of metal enzyme, plays an important role as the first line of defense against oxidative damage. SOD catalyzes the convert of superoxide anion into hydrogen peroxide and oxygen (Ewing and Janero, 1995). And then, hydrogen peroxide is further decomposed into completely harmless water by CAT, GPX and APX, achieving the purpose of scavenging ROS. Based on the metal involved in the catalysis, SOD is divided into four main categories: Cu/Zn-SOD, Fe-SOD, Mn-SOD and Ni-SOD (Zelko et al., 2002; Miller, 2012). In plants, there are three major categories of SOD: Cu/Zn-SOD, Fe-SOD and Mn-SOD.

SOD plays a vital role in the growth and development of plants. In *Arabidopsis thaliana*, AtFSD2 and AtFSD3 played important roles in the early chloroplast growth and development (Myouga et al., 2008), while AtMn-SOD1 may be involved in root and ovule development (Morgan et al., 2008; Martin et al., 2013). Plant SOD genes not only showed

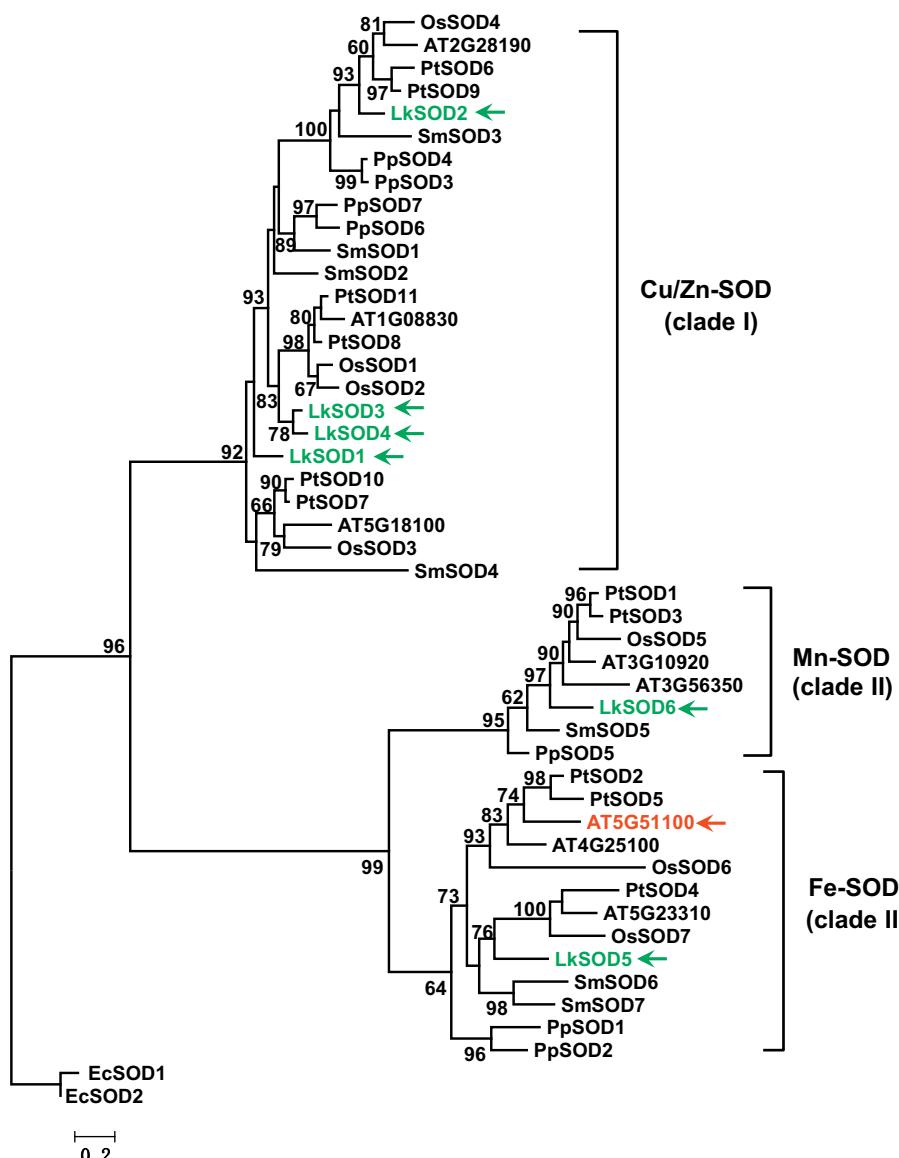
**Abbreviations:** SOD, Superoxide dismutase; ROS, Reactive oxygen species; AsA, ascorbic acid; GSH, glutathione; CAT, catalase; APX, ascorbate peroxidase; GR, glutathione reductase; DHAR, dehydroascorbate reductase; GPX, glutathione peroxidase; GPOD, guaiacol peroxidase; PRX, peroxidase; GST, glutathione-S-transferase

\* Corresponding author.

\*\* Correspondence to: Y.-J. Liu, State Key Laboratory of Tree Genetics and Breeding, Chinese Academy of Forestry, Beijing 100091, China.

E-mail addresses: [lantingchn@foxmail.com](mailto:lantingchn@foxmail.com) (T. Lan), [yan.jing.liu@163.com](mailto:yan.jing.liu@163.com) (Y.-J. Liu).

<sup>1</sup> These authors contributed equally to this work.



**Fig. 1.** Phylogenetic tree of 46 SOD genes from six land plant species. The abbreviations for the six species were as followed: Pp, *Physcomitrella patens*; Sm, *Selaginella moellendorffii*; Lk, *Larix kaempferi*; Os, *Oryza sativa*; At, *Arabidopsis thaliana*; Pt, *Populus trichocarpa*. Numbers at each node in the phylogenetic tree were bootstrap values, and only values higher than 50% were shown. The LkSOD genes were indicated by green arrows and AtFSD2 (AT5G51100) was indicated by red arrow. Two *Escherichia coli* SOD genes EcSOD1 (WP\_112836890.1) and EcSOD2 (WP\_086590367.1) were used as outgroup. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

different expression pattern under normal conditions, but also can be induced by the external environment. In *Arabidopsis*, *AtFSD1* was abundantly expressed in roots, stems, leaves and immature pods, weakly expressed in flowers and seeds. *AtFSD2* and *AtFSD3* showed highest expression in immature pods, no expression in roots, and moderate expression in other sites (Myouga et al., 2008). Under UV and high light stress, the expression levels of *AtCSD1* and *AtCSD2* increased (Huang et al., 2012; Xing et al., 2013). Under salt stress, the expression level of *AtCSD1* increased, while the expression level of *AtCSD2* and *AtCSD3* decreased (Attia et al., 2011). SOD also plays an important role in the response to abiotic stress. Overexpression of SOD gene in plants can enhance their ability to resist drought and salt stresses. Over expression of several different SOD genes in *Arabidopsis thaliana* showed that the salt tolerance of transgenic lines increased (Wang et al., 2004; Gill et al., 2010; Liu et al., 2015). Over expressing the Cu/Zn-SOD gene of pea into tobacco, the tolerance of transgenic plants toward low temperature and high temperature oxidative stress also increased (Gupta et al., 1993).

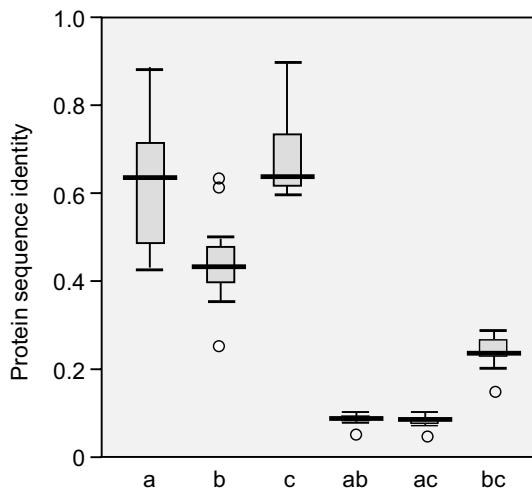
Gymnosperms represent a large group of plants with a long evolutionary history. But little is known about SOD in gymnosperms. In this study, we focused on functional characterizations of the SOD gene family in Japanese larch (*Larix kaempferi*). *L. kaempferi* is widely

distributed in China, with strong adaptability and valuable wood properties. As an important forest tree, *L. kaempferi* were also used as raw material for paper making. In this study we cloned six SOD genes from *L. kaempferi*. By integrating gene expression pattern, subcellular localization, biochemical characteristics and physiological function, this study provided comprehensive insight into the gymnosperm SOD gene family.

## 2. Material and methods

### 2.1. Identification of SOD genes from *L. kaempferi* genome

To identify SOD genes in *L. kaempferi*, primers used to amplify cDNA of *L. kaempferi* were designed based on putative *Pinus taeda* SOD gene sequences, which were identified from the GenBank™ EST database. Primers used to amplify *L. kaempferi* SOD genes were listed in Supplemental Table S1. Total RNA was isolated from five tissues (root, stem, leaf, bud and phloem) using an RNAPrep pure Plant Kit (TIANGEN, Beijing, China) and reverse transcribed into cDNA using an RNA PCR Kit (AMV) version 3.0 (Takara Bio Inc., Dalian, China). An actin gene amplified from *L. kaempferi* was used as an internal control. PCR conditions were optimized to consist of an initial denaturation of



**Fig. 2.** Pairwise sequence identity of 46 full-length SOD proteins from six land plant species. a, b, and c represent pairwise sequence identities of clade I, II, and III SOD proteins, respectively. ab, ac and bc represented pairwise sequence identities between clade I and II SOD proteins, clade I and III SOD proteins, and clade II and III SOD proteins, respectively. The box plot showed the median (black line), interquartile range (box), and maximum and minimum scores (whiskers) of each data set. Outliers were shown as circles outside of the whiskers.

3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 40 s at 65 °C, and 1 min at 72 °C, and a final extension of 3 min at 72 °C. The PCR products were separated on a 1% agarose gel. Fragments of expected lengths were purified and cloned into pEASY-T3 cloning vector (Transgen, Beijing, China) and sequenced to verify the gene sequences.

## 2.2. Conserved motifs and phylogenetic analyses

Conserved motif analysis of LkSOD genes was performed by the Multiple Em for Motif Elicitation (MEME Suite 4.12.0) server with the number of motifs was set to 4 (Bailey et al., 2009). The motifs found were determined through hmmsearch in HmmerWeb version 2.21.0 (<https://www.ebi.ac.uk/Tools/hmmer/search/hmmsearch>). The alignment of 56 full-length SOD protein sequences was carried out by MAFFT v.7 (<https://mafft.cbrc.jp/alignment/server/>). The phylogenetic trees were reconstructed using a maximum-likelihood procedure in PHYML software with the WAG amino acid substitution model (Guindon and Gascuel, 2003). One hundred bootstrap replicates were

performed in each analysis to obtain the confidence support.

## 2.3. Expression of SOD genes in *L. kaempferi* tissues

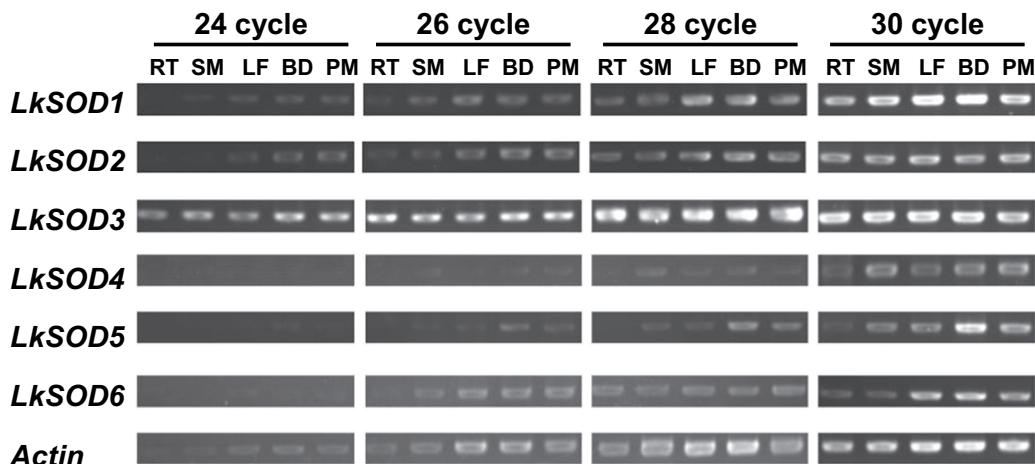
Samples were collected from three *L. kaempferi* trees in the Beijing Botanical Garden. To investigate the expression patterns of SOD gene family under normal conditions, total RNA were isolated from five different tissues, including root, stem, leaf, bud and phloem, and then reverse transcribed into cDNA as described above. For PCR analysis, specific primers were designed based on the multiple sequence alignment of *L. kaempferi* SOD genes (Supplementary Table S2). An actin gene amplified from *L. kaempferi* was used as an internal control. PCR conditions were optimized to consist of an initial denaturation of 3 min at 94 °C, followed by cycles of 30 s at 94 °C, 40 s at 65 °C, and 1 min at 72 °C, and a final extension of 3 min at 72 °C. To be in the linear range, the numbers of cycles used for amplification with each primer pair were 24, 26, 28, and 30, respectively.

## 2.4. Subcellular localization

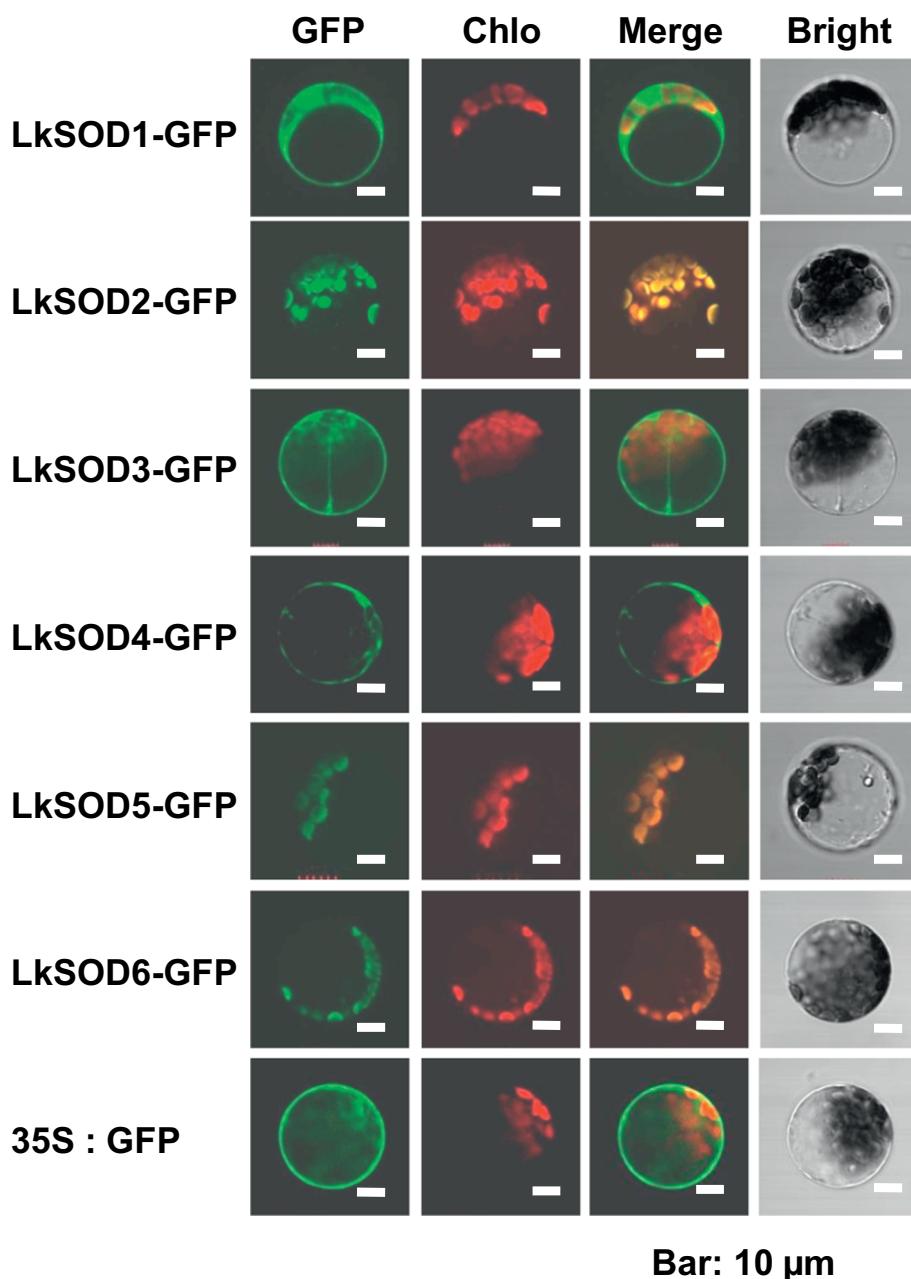
To investigate the subcellular divergence of *L. kaempferi* SOD genes, 6 *L. kaempferi* SOD proteins were subcloned into modified pGEM-T vectors (pPSN) to obtain C-terminal GFP tags (Supplementary Fig. S1). The primers used were listed in Supplemental Table S3. Colonies containing the target insert were confirmed by DNA sequencing. The resulting constructs were used for transient expression in *Arabidopsis* mesophyll protoplasts by PEG-calcium-mediated transfection method (Yoo et al., 2007). After culture for 24 h, the protoplasts were harvested and observed with a confocal laser microscope (Olympus FV1000 MPE). GFP fusion fluorescence was excited with a 488-nmlaser, while auto fluorescence of chlorophyll was imaged using a 543-nmlaser.

## 2.5. Expression and purification of recombinant *L. kaempferi* SOD proteins

To investigate the enzymatic characteristics of *L. kaempferi* SOD proteins, 6 *L. kaempferi* SOD genes were subcloned into pET-30a expression vectors (Novagen), and then sequenced to verify the insertion. The primers used to construct the SOD expression vectors were listed in Supplemental Table S4. *Escherichia coli* BL21 (DE3) cells harboring pET-30a/SOD plasmids were cultured and then induced by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside when the optical density (A600) reached 0.5. Then cells were harvested by centrifugation (7500 g, 3 min, 4 °C) after 10 h induction, re-suspended in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, and 20 mM imidazole, pH 7.4), and disrupted by cold sonication. The homogenate was then subjected



**Fig. 3.** Expression patterns of *Larix kaempferi* SODs. The expression of *Larix* SODs genes were examined using semiquantitative PCR under normal conditions. RT, SM, LF, BD and PM indicated root, stem, leaf, bud and phloem, respectively.



**Fig. 4.** Subcellular localization of *Larix kaempferi* SOD proteins. GFP signal (green) and chlorophyll autofluorescence (red) were observed using confocal laser-scanning microscopy, and an overlay was shown in yellow. Bars = 10  $\mu$ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to centrifugation (7500 g, 10 min, 4 °C). The supernatant was loaded onto a Nickel-Sepharose High Performance column (GE Healthcare Biosciences), which was pre-equilibrated with binding buffer. The SOD proteins that bound to the Nickel-Sepharose column were eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, and 500 mM imidazole, pH 7.4).

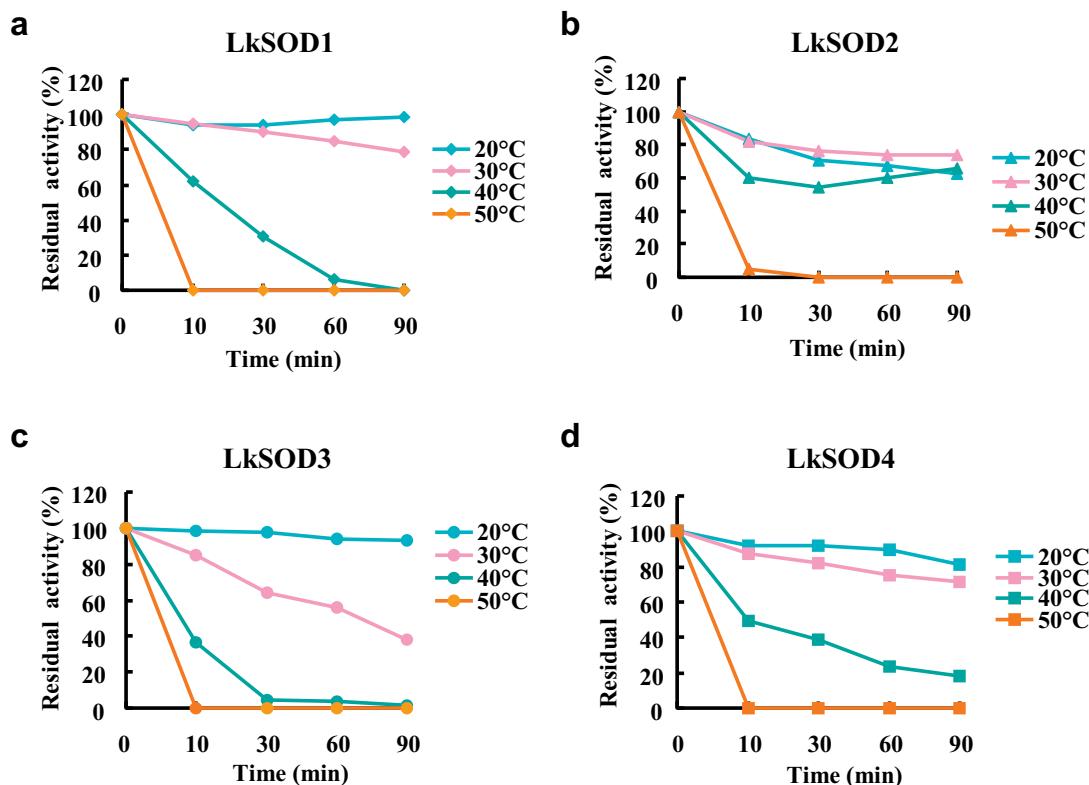
#### 2.6. Enzyme assays of *Larix* SOD

The activity of SOD was determined with hydroxylamine method by using Total Superoxide Dismutase (T-SOD) assay kit. The inhibiting rate of SOD to O<sub>2</sub><sup>−</sup> produced by the xanthine morpholine with xanthine oxidase was measured by a colorimetric method. And one unit of SOD activity (U) was defined as the quantity of SOD required to produce 50% inhibition of reduction of nitrite in 1 mL reaction solution by

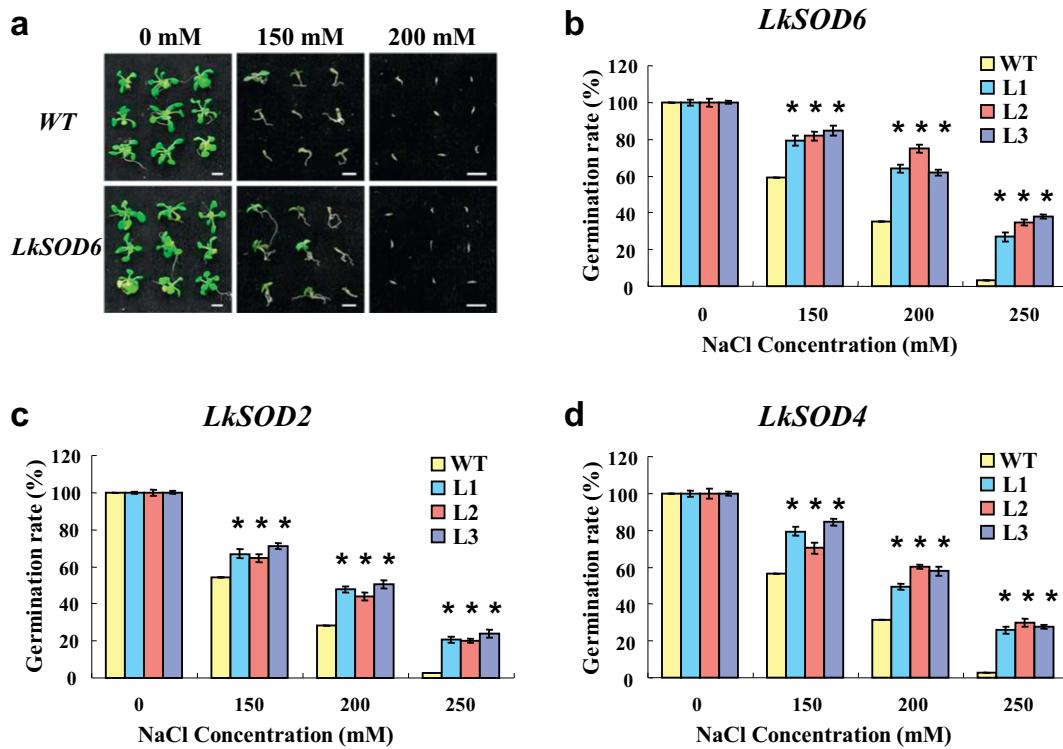
measuring the change of absorbance at 550 nm after 10 min of reaction at 37 °C. The assay was run in triplicate. The dependence of SOD activity on temperature was measured at various temperatures from 15 °C to 70 °C at 5 °C intervals. The thermal stability of SOD was determined by incubating the purified protein at 20 °C, 30 °C, 40 °C and 50 °C, respectively. And then the activity was measured after 0 min, 10 min, 30 min, 60 min and 90 min of reaction, respectively. Protein concentrations were determined by measuring A280 (Layne, 1957).

#### 2.7. Vector construction and obtainment of transgenic plants

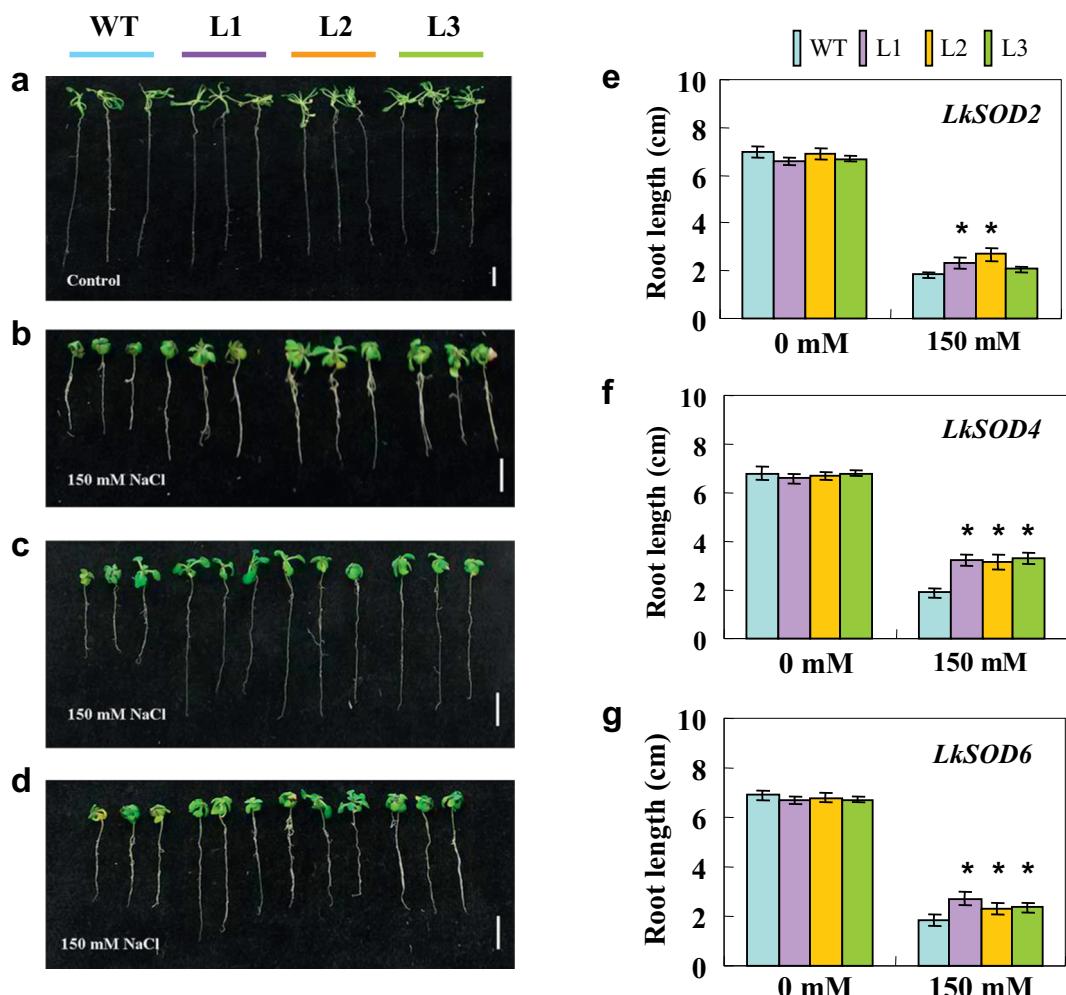
For transformation, the SOD sequences of *L. kaempferi* were cloned into modified pCAMBIA1302 vector (pCAMBIA1302OE) under cauliflower mosaic virus 35S (CaMV35S) promoter (Supplementary Fig. S2). The primers used were listed in Supplemental Table S5. The constructed



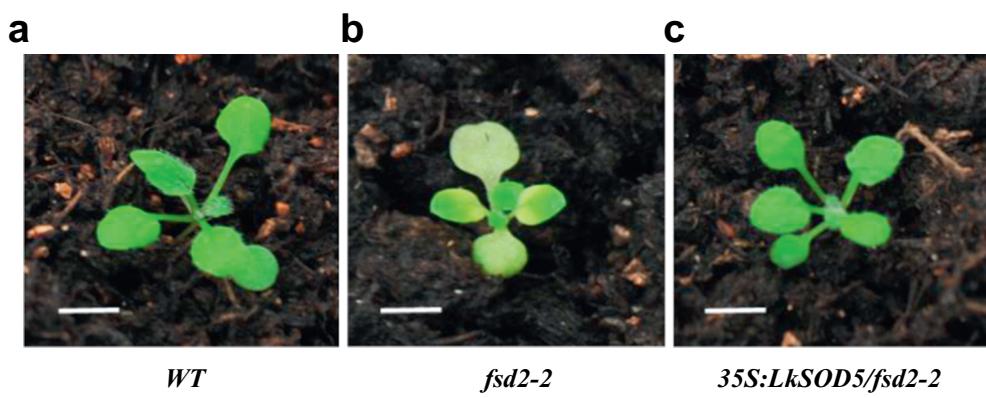
**Fig. 5.** Thermal inactivation of four *Larix kaempferi* SOD proteins. 20 °C, 30 °C, 40 °C and 50 °C was indicated by blue, pink, green and yellow separately. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 6.** Germination of WT and transgenic *Arabidopsis* lines expressing *Larix kaempferi* SOD genes under different concentrations of NaCl treatment. (a) Phenotype of WT and transgenic lines expressing LkSOD6 after growing on the plate with different concentrations of NaCl treatments for 25 days. (b, c and d), Statistical analysis of germination rates of WT and transgenic lines of LkSOD6, LkSOD2 and LkSOD4 before and after NaCl treatments. Asterisks indicated significant difference (independent-sample t-test,  $P < 0.05$ ). Bars = 0.5 cm.



**Fig. 7.** Root length of WT and different transgenic *Arabidopsis* lines expressing *Larix kaempferi* SOD genes under 0 mM and 150 mM NaCl treatment. (a), Roots of WT and transgenic line 1, 2 and 3 of LkSOD2 after growing on 1/2 MS plates for 25 days. (b, c and d), Roots of WT and transgenic line 1, 2 and 3 of LkSOD2, LkSOD4 and LkSOD6 after growing on 1/2 MS plates with 150 mM NaCl for 25 days. (e, f and g), Statistical analysis of roots length of WT and transgenic lines of LkSOD2, LkSOD4 and LkSOD6 before and after NaCl treatments. Asterisks indicated significant difference (independent-sample t-test,  $P < 0.05$ ). Bars = 1 cm.



**Fig. 8.** Complementation of *fsd2-2* mutant with 35S:LkSOD5. (a) WT, (b) *fsd2-2*, and (c) 35S:LkSOD5/*fsd2-2* plants were grown on soil for 8 days after germination. Bars = 0.5 cm.

pCAMBIA1302OE vectors containing SOD sequences were used for transformation of *Arabidopsis thaliana* Columbia-0 ecotype (Col-0) or *fsd2-2*, using *Agrobacterium tumefaciens* strain LBA4404 via floral dip method (Zhang et al., 2006). Primary transformants (T1) were selected on 1/2 Murashige-Skoog (MS) plate contain hygromycin and confirmed by polymerase chain reaction (PCR) using genomic DNA isolated from the leaves of transgenic plants, and by reverse transcriptase (RT)-PCR

using total RNA following the standard protocols.

#### 2.8. Salt tolerance analysis

For the germination test, seeds of transgenic lines (T3) over-expressing LkSOD and WT were grown in 1/2 MS plate supplemented with desired concentrations of NaCl (0, 50, 100 and 150 mM) for

20 days. Plate with 0 mM NaCl was taken as control. Finally, the germination rates were scored. For the plant growth assay, the 5-day-old seedlings grown on 1/2 MS plate were transferred onto plates containing 150 mM NaCl for 25 days. Then the root length was observed and calculated.

### 3. Results and discussion

#### 3.1. Sequence characteristics of *L. kaempferi* SOD genes

In this study, we cloned six full-length SOD genes from the *L. kaempferi* genome, which were named LkSOD1, 2, 3, 4, 5 and 6. Four motifs (motif 1 to motif 4) were identified in six LkSOD proteins by MEME (Supplementary Fig. S3). Among them, motif 1 and 2 was described as copper/zinc superoxide dismutase (SODC), motif 3 was related to iron/manganese superoxide dismutase, alpha-hairpin domain (IMA), and motif 4 was related to iron/manganese superoxide dismutase, C-terminal domain (IMC). As shown in Fig. 1, motif 1 and 2 were located within LkSOD1, 2, 3 and 4, which were thus classified into Cu/ZnSODs, and motif 3 and 4 were shared in LkSOD5 and 6, which were thus classified into Fe-Mn SODs.

In this study, we identified six and seven SOD genes from *Physcomitrella patens* and *Selaginella moellendorffii*, respectively, while the SOD genes in *Oryza sativa*, *Arabidopsis thaliana*, and *Populus trichocarpa* were identified in previous studies (Supplemental Table S6). We reconstructed the phylogenetic tree of 46 SOD genes from these six land plants using two *Escherichia coli* SOD genes EcSOD1 (WP\_112836890.1) and EcSOD2 (WP\_086590367.1) as outgroup. Phylogenetic tree showed plant SOD genes were divided into three clades (Fig. 1). Pairwise comparisons of the 46 full-length SOD protein sequences from six land plant species revealed that the protein sequence identities within each clade were higher than that between clades (independent-sample t-test,  $P < 0.0001$ ) (Fig. 2). The genes in clades I, II and III were belong to Cu/Zn-SOD, Mn-SOD and Fe-SOD, respectively. LkSOD1, 2, 3 and 4 were grouped with Cu/Zn-SOD genes, LkSOD5 with Fe-SOD, and LkSOD6 with Mn-SOD.

#### 3.2. Expression of *L. kaempferi* SOD genes under normal growth condition

To investigate the expression of SOD genes in *L. kaempferi*, RNA was extracted in root, stem, leaf, bud and phloem under normal growth conditions, respectively. The semi-quantitative RT-PCR showed the six *Larix* SOD genes were expressed under 30 amplification cycles (Fig. 3), suggesting that these six genes might be constitutively expressed in *L. kaempferi*. Among six *Larix* SOD genes, the expression of LkSOD3 was the highest under 24, 25 and 28 cycles, while LkSOD4, 5 and 6 were weakly expressed.

#### 3.3. Subcellular localization of *L. kaempferi* SOD proteins

The generation of ROS was detected in several organelles, such as chloroplast, mitochondria, apoplast, plasma membrane, and nucleolus (Gill et al., 2015). The divergence in subcellular localization could indicate divergence in function, which is an important mechanism for the retention of duplicated genes (Marques et al., 2008). To determinate the subcellular localization of *Larix* SOD proteins, we constructed six LkSOD-GFP fusion proteins. Confocal fluorescence microscopy was then used to monitor the localization of the fluorescent protein. Three Cu/Zn-SODs (LkSOD1, 3 and 4) showed cytosolic location (Fig. 4). The green fluorescent of one Cu/Zn-SOD (LkSOD2), one Fe-SOD (LkSOD5), and one Mn-SOD (LkSOD6) were co-localized with the red auto fluorescence of chlorophyll, indicating that these three proteins were localized to the chloroplast.

In eukaryotes, different cells have different functions. Proper protein localization is important for its function (Boruc et al., 2010). The differentiation of protein subcellular localization might be related to

the functional divergence of genes (Marques et al., 2008). Three *Larix* Cu/Zn-SODs had cytosolic location, while one *Larix* Cu/Zn-SOD had chloroplast location, indicating their functional divergence.

#### 3.4. Biochemical properties of *L. kaempferi* SOD proteins

To investigate the catalytic characteristics of *Larix* SOD proteins, which may be related to their biological functions, six *Larix* SOD proteins were expressed in *E. coli* BL21 (DE3). LkSOD5 and 6 proteins were expressed as inclusion bodies in *E. coli*. Four Cu/Zn-SOD proteins (LkSOD1, 2, 3, and 4) were expressed as soluble proteins. Therefore, the biochemical activities of these four Cu/Zn-SOD proteins were determined with substrate superoxide anion. All four SOD proteins showed catalytic activities toward superoxide anion. The activities of LkSOD1, 2, 3 and 4 were  $284.318 \pm 9.100$  U/mg,  $201.622 \pm 0.860$  U/mg,  $694.475 \pm 44.824$  U/mg and  $569.484 \pm 14.091$  U/mg, respectively.

The activity of LkSOD toward superoxide anion was temperature-dependent. The profiling of temperature dependent analysis among the four Cu/Zn-SOD proteins (LkSOD1, 2, 3 and 4) suggested a broad optimal temperature range for catalytic activity (Supplementary Fig. S4). All proteins showed  $> 55\%$  of their maximum activity over a temperature range of  $10^\circ\text{C}$ – $40^\circ\text{C}$ . However, there was a marked change in enzymatic activities among the four proteins over the temperature range of  $5^\circ\text{C}$ – $70^\circ\text{C}$ . For LkSOD3, a sharp drop in activity was observed when temperature rising to  $50^\circ\text{C}$ . For LkSOD1, 2, and 4, they retained 38%, 82% and 61% of their maximum activity at  $50^\circ\text{C}$ , separately. When the temperature continued to rise to  $60^\circ\text{C}$ , all LkSOD1, 3, and 4 lost their activity, but LkSOD2 still kept 25% of the maximum activity. LkSOD2 displayed  $> 82\%$  of the maximum activity over a temperature range of  $10$ – $50^\circ\text{C}$ , indicating that it may contribute to the evolutionary adaption of resistance to high temperature.

This study investigated the thermostability of *Larix* SOD proteins. Residual activities were determined after incubating the purified proteins at a range of temperatures ( $20^\circ\text{C}$ – $50^\circ\text{C}$ ) for 10, 30, 60 and 90 min, separately. Divergence in thermostability was observed among the four *Larix* SOD proteins. LkSOD1 and LkSOD4 displayed similar thermostability, and both retained  $> 80\%$  of their maximum activity after incubating at  $20^\circ\text{C}$  and  $30^\circ\text{C}$  for 90 min (Fig. 5). Except LkSOD2, all proteins lost their activity after incubating at  $50^\circ\text{C}$  for 10 min.

#### 3.5. NaCl tolerance of transgenic *Arabidopsis* expressing *Larix* SOD genes

To explore the physiological function of SOD gene in plants, the six LkSOD genes were transformed into *Arabidopsis thaliana* using the floral-dip method, respectively. After transformation, only three 3 genes (LkSOD2, 4 and 6) were successfully inserted into the genome of *Arabidopsis*, with at least 10 independent transgenic lines (T1) taken to T2 generation for each gene. The single copy insert lines were affirmed for by 3:1 segregation. Homozygous lines in T3 generation were confirmed on hygromycin plate. Semi-quantitative RT-PCR analysis confirmed over-expression of LkSOD in the transgenic line, and then 3 lines with higher expression level were selected for further analysis (Supplementary Fig. S5).

In *Arabidopsis*, salt sensitivity was most evident during seed germination and subsequent seedling growth (Gao et al., 2003). In this study, transgenic lines exhibited higher germination percentage as compared to the wild type (WT) under different concentration of NaCl treatment (Fig. 6). When growing on 1/2 MS plate without NaCl, no difference in germination percentage was observed between transgenic lines and WT. Followed by the supplementation with different concentrations of NaCl, a significant difference in the rate of germination was found between those two groups of *Arabidopsis* plants. Under 150 mM NaCl treatment, the germination rates of all the transgenic lines of LkSOD2, 4 and 6 were higher than those of the wild type. Follow 200 mM NaCl treatment, the germination rate of each transgenic line was significantly higher than that of the wild type. Follow 250 mM NaCl stress, the

germination rate of wild type was only about 2%, while the germination rate of each transgenic line was above 20%. Compared to the wild type, transgenic *Arabidopsis* exhibited longer root under 150 mM NaCl (Fig. 7). Follow 150 mM NaCl treatment, the root lengths of the LkSOD2, 4 and 6 transgenic lines were significantly increased when compared to that of the wild-type (*t*-test,  $P < 0.01$ ).

In previous study, *Arabidopsis* Fe-SOD (*AtFSD2*) was proved to play essential roles in early chloroplast development (Myouga et al., 2008). To explore the function of *Larix* Fe-SOD in vivo, *LkSOD5* was transformed into homozygous *atfsd2-2* mutant, which exhibited pale green and dwarf phenotype. *LkSOD5* and *AtFSD2* showed 43.5% sequence identity (Supplementary Fig. S6), and the transgenic plants expressing *LkSOD5* showed similar phenotype with wild type (Fig. 8). *LkSOD5* gene could rescue pale green and dwarf phenotype of *Arabidopsis atfsd2-2* mutant, indicating that *LkSOD5* may have similar function to *AtFSD2*.

#### 4. Conclusion

This study analyzed the expression profiles, subcellular location, biochemical characters of six SOD genes from *Larix kaempferi*. Six SOD proteins showed different subcellular location. Over expressions of three *Larix* SOD genes (*LkSOD2*, 4 and 6) in *Arabidopsis thaliana*, respectively, showed increased resistance to salt stress. *LkSOD5* gene could rescue pale green and dwarf phenotype of *Arabidopsis atfsd2-2* mutant. These data led to new insight into the gymnosperm SOD gene family.

#### Author contributions

QY and TL conceived the project. QXC and QY performed research. XMH analyzed the data. XMH and YJL wrote the article.

#### Funding

This study was supported by the Forestry Industry Research Special Funds for Public Welfare Projects (201504104) and the National Science Fund for Distinguished Young Scholars of China (31425006).

#### Conflict of interest

The authors declare that they have no conflict of interest.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gene.2018.10.089>.

#### References

- Attia, H., Karray, N., Msilini, N., Lachaâl, M., 2011. Effect of salt stress on gene expression of superoxide dismutases and copper chaperone in *Arabidopsis thaliana*. *Biol. Plant.* 55, 159–163.
- Bailey, T.L., Boden, M., Buske, F.A., Frith, M., Grant, C.E., Clementi, L., Ren, J., Li, W.W., Noble, W.S., 2009. MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res.* 37, W202.
- Boruc, J., Mylle, E., Duda, M., De, C.R., Rombauts, S., Geelen, D., Hilson, P., Inzé, D., Van, D.D., Russinova, E., 2010. Systematic localization of the *Arabidopsis* core cell cycle proteins reveals novel cell division complexes. *Plant Physiol.* 152, 553–565.
- Bose, J., Rodrigomoreno, A., Shabala, S., 2014. ROS homeostasis in halophytes in the context of salinity stress tolerance. *J. Exp. Bot.* 65, 1241.
- Brawn, K., Fridovich, I., 1981. DNA strand scission by enzymically generated oxygen radicals. *Arch. Biochem. Biophys.* 206, 414–419.
- Ewing, J.F., Janero, D.R., 1995. Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. *Anal. Biochem.* 232, 243–248.
- Gao, X.H., Ren, Z.Y., Zhang, H., 2003. Overexpression of SOD2 increases salt tolerance of *Arabidopsis*. *Plant Physiol.* 133, 1873–1881.
- Gill, S.S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.* 48, 909.
- Gill, T., Sreenivasulu, Y., Kumar, S., Ahuja, P.S., 2010. Over-expression of superoxide dismutase exhibits lignification of vascular structures in *Arabidopsis thaliana*. *J. Plant Physiol.* 167, 757–760.
- Gill, S.S., Anjum, N.A., Gill, R., Yadav, S., Hasanuzzaman, M., Fujita, M., Mishra, P., Sabat, S.C., Tuteja, N., 2015. Superoxide dismutase—mentor of abiotic stress tolerance in crop plants. *Environ. Sci. Pollut. Res.* 22, 10375–10394.
- Guindon, S., Gascuel, O., 2003. PhyML—a simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* 52, 696.
- Gupta, A.S., Heinen, J.L., Holaday, A.S., Burke, J.J., Allen, R.D., 1993. Increased resistance to oxidative stress in transgenic plants that overexpress chloroplastic Cu/Zn superoxide dismutase. *Proc. Natl. Acad. Sci. U. S. A.* 90, 1629–1633.
- Hasanuzzaman, M., Hossain, M.A., Fujita, M., 2012. Exogenous selenium pretreatment protects rapeseed seedlings from cadmium-induced oxidative stress by upregulating antioxidant defense and methylglyoxal detoxification systems. *Biol. Trace Elem. Res.* 149, 248–261.
- Huang, C.H., Kuo, W.Y., Weiss, C., Jinn, T.L., 2012. Copper chaperone-dependent and -independent activation of three copper-zinc superoxide dismutase homologs localized in different cellular compartments in *Arabidopsis*. *Plant Physiol.* 158, 737–746.
- Karuppanapandian, T., Juncheol, M., Changsoo, K., Manoharan, K., Wook, K., 2011. Reactive oxygen species in plants: their generation, signal transduction, and scavenging mechanisms. *Aust. J. Crop. Sci.* 5, 709–725.
- Kliebenstein, D.J., Monde, R.A., Last, R.L., 1998. Superoxide dismutase in *Arabidopsis*: an eclectic enzyme family with disparate regulation and protein localization. *Plant Physiol.* 118, 637.
- Layne, E., 1957. Spectrophotometric and turbidimetric methods for measuring proteins. *Methods Enzymol.* 3, 447–454.
- Liu, Z.B., Zhang, W.J., Gong, X.D., Zhang, Q., Zhou, L.R., 2015. A Cu/Zn superoxide dismutase from *Jatropha curcas* enhances salt tolerance of *Arabidopsis thaliana*. *Genet. Mol. Res.* 14, 2086–2098.
- Marques, A.C., Vinckenbosch, N., Brawand, D., Kaessmann, H., 2008. Functional diversification of duplicate genes through subcellular adaptation of encoded proteins. *Genome Biol.* 9, R54.
- Martin, M.V., Fiol, D.F., Sundaresan, V., Zabaleta, E.J., Pagnussat, G.C., 2013. *oiwa*, a female gametophytic mutant impaired in a mitochondrial manganese-superoxide dismutase, reveals crucial roles for reactive oxygen species during embryo sac development and fertilization in *Arabidopsis*. *Plant Cell* 25, 1573–1591.
- Miller, A.F., 2012. Superoxide dismutases: ancient enzymes and new insights. *FEBS Lett.* 586, 585–595.
- Mittler, R., Vanderauwera, S., Gollery, M., Van, B.F., 2004. Reactive oxygen gene network of plants. *Trends Plant Sci.* 9, 490–498.
- Morgan, M.J., Lehmann, M., Schwarzländer, M., Baxter, C.J., Sienkiewiczporzucek, A., Williams, T.C., Schauer, N., Fernie, A.R., Fricker, M.D., Ratcliffe, R.G., 2008. Decrease in manganese superoxide dismutase leads to reduced root growth and affects tricarboxylic acid cycle flux and mitochondrial redox homeostasis. *Plant Physiol.* 147, 101–114.
- Myouga, F., Hosoda, C., Umezawa, T., Izumi, H., Kuromori, T., Motohashi, R., Shono, Y., Nagata, N., Ikeuchi, M., Shinozaki, K., 2008. A heterocomplex of iron superoxide dismutases defends chloroplast nucleoids against oxidative stress and is essential for chloroplast development in *Arabidopsis*. *Plant Cell* 20, 3148.
- Niki, E., Yoshida, Y., Saito, Y., Noguchi, N., 2005. Lipid peroxidation: mechanisms, inhibition, and biological effects. *Biochem. Biophys. Res. Commun.* 338, 668–676.
- Wang, Y., Ying, Y., Chen, J., Wang, X., 2004. Transgenic *Arabidopsis* overexpressing Mn-SOD enhanced salt-tolerance. *Plant Sci.* 167, 671–677.
- Xing, Y., Cao, Q., Zhang, Q., Qin, L., Jia, W., Zhang, J., 2013. MKK5 regulates high light-induced gene expression of Cu/Zn superoxide dismutase 1 and 2 in *Arabidopsis*. *Plant Cell Physiol.* 54, 1217–1227.
- Yoo, S.D., Cho, Y.H., Sheen, J., 2007. *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat. Protoc.* 2, 1565–1572.
- Zelko, I.N., Mariani, T.J., Folz, R.J., 2002. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radic. Biol. Med.* 33, 337.
- Zhang, X., Henriques, R., Lin, S.S., Niu, Q.W., Chua, N.H., 2006. *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nat. Protoc.* 1, 641–646.