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# Over-Expression of *ScMnSOD*, a *SOD* Gene Derived from Jojoba, Improve Drought Tolerance in *Arabidopsis*

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## Abstract

Jojoba (*Simmondsia chinensis*) is mainly distributed in desert, and the molecular mechanisms of jojoba in response to abiotic stress still remain elusive. In this paper, we cloned and characterized a *SOD* gene from jojoba named as *ScMnSOD*, and introduced into *Arabidopsis* to investigate its functions of responding to drought stress. The transgenic *Arabidopsis* showed an improvement in drought tolerance. Moreover, under a water deficit condition, the accumulation of reactive oxygen species (ROS) was remarkably decreased in the transgenic lines compared to the WT. Furthermore, the *ScMnSOD* promoter was cloned to the 5′-upstream of GUS coding region in a binary vector, and introduced into *Arabidopsis*. And results showed that *ScMnSOD* expression can be induced by drought, salt, ABA, and low temperature. In conclusion, *ScMnSOD* plays an important role in drought tolerance which is, at least partially, attributed to its role in ROS detoxification.

Key words: drought, jojoba, promoter, ROS, *ScMnSOD*, stress tolerance.

## INTRODUCTION

Jojoba (*Simmondsia chinensis*), is a dioecious evergreen shrub native to Sonoran Desert in southwest United States and northern Mexico, which is very tolerant to drought and heat and is of great importance with regard to soil conservation and combating desertification. In the past years, most research had been focused on its immense economic importance due to its seeds, which store liquid wax with similar properties to sperm whale oil (Le Dreau *et al*. 2009; El-Mallah and El-Shami 2009). The liquid wax, derived from the seeds, is widely used as a lubricant, in the cosmetic (Mbah 2007), pharmaceutical (Touitou and Godin 2008), and plastic industries. However, Jojoba's molecular mechanism in response to biotic and abiotic stress still remains elusive.

Although the mechanism of jojoba's tolerance to extreme drought is still unknown, there is a well-documented compensatory mechanism in the drought stress, which leads to reactive oxygen species (ROS) accumulation in other plant species (Mittler 2002; Apel and Hirt 2004; Foyer and Noctor 2005). Under normal growth conditions, the production of reactive oxygen species (ROS) in plant is usually low, but has been shown to be dramatically enhanced under both biotic and abiotic stress conditions. ROS enhancement negatively affects many normal cellular functions by damaging DNA bases, oxidizing proteins and causing lipid peroxidation (Polle 2001). Stress-

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induced ROS accumulation is counteracted by intrinsic antioxidant systems in plants including a variety of enzymatic scavengers, such as superoxide dismutase (SOD), ascorbate peroxidase, glutathione peroxidase, glutathione S-transferase, and catalase. In addition, non-enzymatic, low molecular weight molecules, such as *L*-ascorbate, tocopherols, carotenoids, and glutathione, may also be important antioxidants (Mittler 2002; Mittler *et al*. 2004).

SODs constitute the first line of defense against highly toxic superoxide radicals by rapidly converting superoxide to hydrogen peroxide ($H_2O_2$) and molecular oxygen (Fridovich 1995). Based on the metal cofactor, SODs are classified into three groups: copper-zinc SOD (Cu/Zn-SOD), manganese SOD (MnSOD) and iron SOD (FeSOD), which are localized in different cellular compartments (Mittler 2002). Prompt scavenging of ROS by a group of participating enzymes is necessary for normal plant biogenesis and plant cell growth (Rizhsky *et al*. 2003; Miller *et al*. 2007). There have many reports on the roles of SODs in plant tolerating abiotic stresses. When the thylakoid-attached *Cu/ZnSOD* was knocked down, *Arabidopsis* showed growth retardation and abnormal chloroplasts (Rizhsky *et al*. 2003). Over-expression of cytosolic *Cu/ZnSOD* from a mangrove plant *Avicennia marina* in *indica* rice var *Pusa Basmati-1* confers abiotic stress tolerance (Prashanth *et al*. 2008). Expression of a pea *MnSOD* in rice confers drought tolerance (Wang *et al*. 2005). Over-expression of a *MnSOD* gene derived from *Tamarix androssowii*, enhances salt tolerance in transgenic poplar plants (Wang *et al*. 2010). *FeSOD* expression in transgenic alfalfa increases winter survival (McKersie *et al*. 2000). Phospholipid membranes are impermeable to $O_2^-$, SODs function for the removal of $O_2^-$ in various compartments of plant cells where $O_2^-$ radicals are formed (Takahashi and Asada 1983). All the results suggest that SODs play an important role in plants tolerating abiotic stress.

To investigate the mechanism of jojoba in response to water deficit stress, we cloned water stress-induced expressed sequence tags (ESTs) from jojoba mature leaves by suppression subtractive hybridization (SSH) (Geng *et al*. 2008). One EST was highly homologous with *MnSODs* isozymes. Although a large number of *MnSOD* genes that link to abiotic stress tolerance have been identified in many plant species, little is known about the *MnSOD* gene from jojoba, which we named it as *ScMnSOD*. This is the first study on the function of *MnSOD* in jojoba to our knowledge. We found that the expression of *ScMnSOD* can be induced by drought, salinity and abscisic acid (ABA). And overexpression of *ScMnSOD* in *Arabidopsis* can lead to great improvement of plant resistance to water deficit stress.

## RESULTS

### Isolation and sequence analysis of *ScMnSOD* cDNA

Full-length *ScMnSOD* was cloned by RACE (full name!) and the cDNA is 1 118 bp in length, including 696 bp ORF, encoding a SOD protein of 231 amino acids. Sequence alignment analysis indicated that the ScMnSOD protein shows high homology with other MnSODs. In all those SOD proteins, the amino acids which linked with the Mn and involved in the active site formation are identical (Fig. 1-A), suggesting that the MnSOD was evolutionarily conserved.

To detect the evolutionary relationships of ScMnSOD from Jojoba with other plant MnSOD proteins, twelve plant MnSOD proteins were selected from GenBank for phylogenetic analysis. These MnSOD proteins obviously split into three subgroups. ScMnSOD from jojoba was located on the 2nd subgroup, and shares high sequence homology with those from *Nicotiana plumbaginifolia*, *Prunus persica* and *Tamarix androssowii* (Fig. 1-B).

### SOD activities of the ScMnSOD protein

To investigate the SOD activity of ScMnSOD, ScMnSOD was expressed in *Escherichia coli* and purified using Ni-NTA column. The purified ScMnSOD showed a single protein band with a molecular weight (*MW*) of about 30 kD on SDS-PAGE. Since the *MW* of His6 is about 6 kD, the *MW* of the ScMnSOD should be estimated to be 24 kD, which was identical with the *MW* of the purified ScMnSOD (Fig. 2-A). The purified ScMnSOD protein concentration was measured by Bradford assay. The SOD activity of the purified

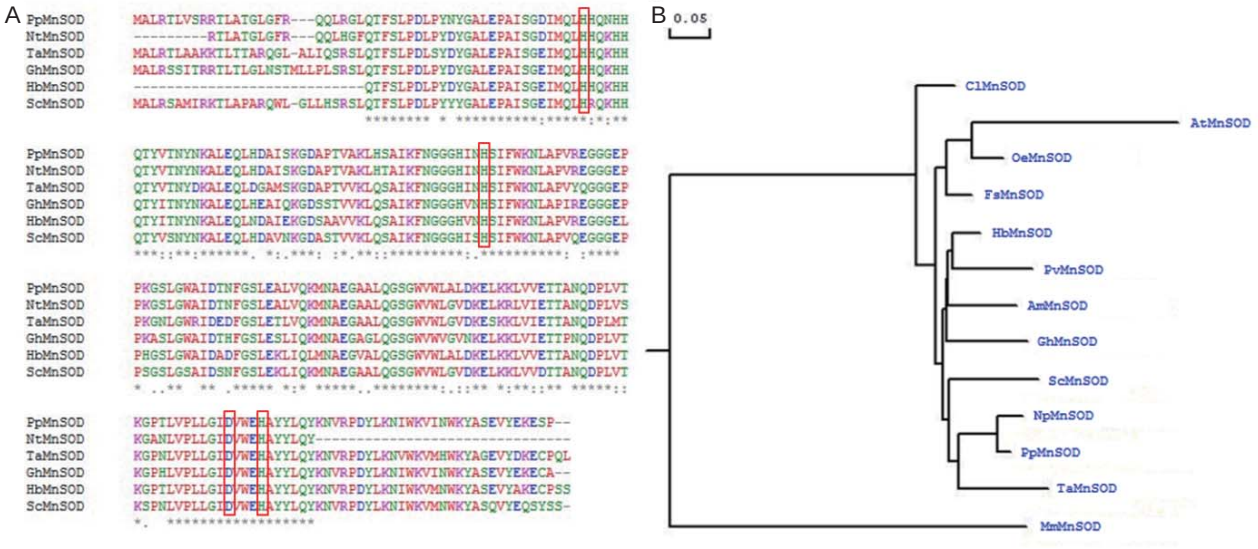


**Fig. 1** Sequence and structural features of *ScMnSOD*. A, sequence and structural features of ScMnSOD. Alignment of *Prunus persica* MnSOD PpMnSOD (CAB56851.1), *Nicotiana tabacum* MnSOD NtMnSOD(BAC75399.1), *Tamarix androssowii MnSOD* Ta*MnSOD* (AAS77885.2), *Gossypium hirsutum* GhMnSOD (ABA00455.1), *Hevea brasiliensis* Hb*MnSOD* (CAB53458.1), and *ScMnSOD* (http://www.ebi.ac.uk/Tools/msa/clustalo/). *, identical residues; :, conserved substitutions; ., semi conserved substitutions; the red box, the amino acids which linked with the Mn and involved in the active site. B, phylogenetic relationship of *ScMnSOD* with other known MnSOD proteins. The Minimum Evolution tree was constructed in DNAMAN. The accession numbers of the MnSOD proteins in GenBank are: *Citrullus lanatus* MnSOD ClMnSOD (AAS48178.1), *Arabidopsis thaliana MnSOD* AtMnSOD (AAC24832.1), *Olea europaea* MnSOD OeMnSOD (AAL24044.1), *Fagus sylvatica* MnSOD FsMnSOD (ABI26729.1), *Hevea brasiliensis* MnSOD HbMnSOD (CAB53458.1), *Pistacia vera* MnSOD PvMnSOD(ABR29644.1), *Avicennia marina* MnSOD AmMnSOD (AAN15216.1), *Gossypium hirsutum* MnSOD GhMnSOD (ABA00455.1), *Nicotiana plumbaginifolia* MnSOD NpMnSOD (CAA32643.1), *Prunus persica* MnSOD PpMnSOD (CAB56851.1), *Tamarix androssowii* MnSOD TaMnSOD(AAS77885.2), *Mus musculus* MnSOD MmMnSOD (NP_038699.2).

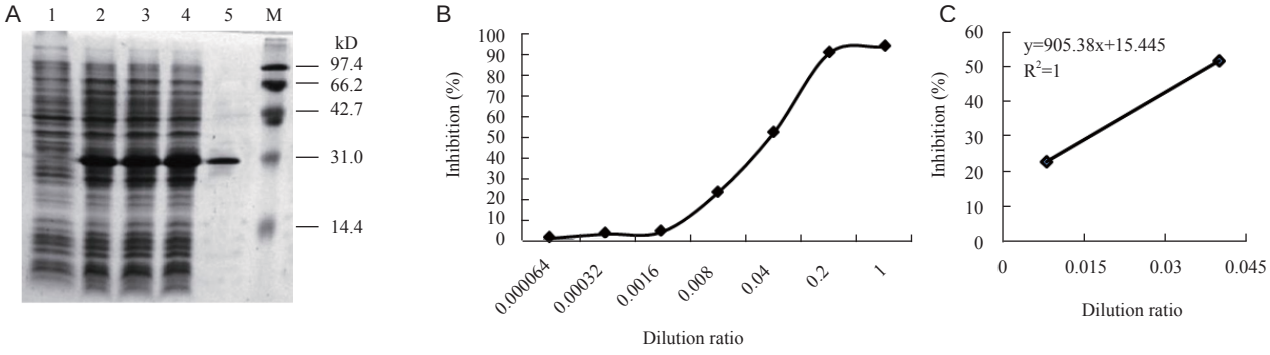


**Fig. 2** SOD activity assay of ScMnSOD. A, ScMnSOD prokaryotic expression and purification (lane 1 is sample before induction, lanes 2-4 were samples induced for 1, 2 and 3 h, respectively, lane 5 was the purification proteins with Ni-NTA column, M was the protein marker). B, Inhibition curve of ScMnSOD. C, trend equation of ScMnSOD. The inhibition curve and trend equation of ScMnSOD were drawn according to the Kit.

ScMnSOD protein was determined according to SOD Assay Kit-WST (Fig. 2-B and C). According to Kit, one unit of SOD is defined as the amount of the enzyme in 20 μL of sample solution that inhibits the reduction reaction of WST-1 with superoxide anion by 50%. The SOD activity of the purified protein is 1 189.91 U $mg^{-1}$. The results suggested that ScMnSOD protein has SOD activity.

## Over-expression ScMnSOD enhanced drought tolerance in *Arabidopsis*

To further investigate how *ScMnSOD* functions in stress

signaling, we compared the growth of control plants (WT) and *ScMnSOD* over-expression plants under water stress conditions. 4-wk-old control plants and *ScMnSOD* overexpression plants were deprived of water for 18 d and then re-watered once. Plant survival rates were scored 1 wk after re-watering. It was apparent that under normal conditions, the transgenic plants (35S: *ScMnSOD*) displayed similar morphological phenotypes as that of WT. After exposure to drought conditions for 2 wk, some WT wilted, and 18 d later, almost all the WT wilted. Conversely, the *ScMnSOD* over-expression plants maintained normal morphological phenotypes (Fig. 3-A). After being re-watered the *ScMnSOD* over-expression plants recovered and grew much better than the controls. Under the above conditions, the survival rate of *ScMnSOD* over-expression plants was consistently above 40%, while the survival rate for WT controls was generally below 20% (Fig. 3-B) (pls rewrite!).

To illustrate the underlining mechanism of over-expression of *ScMnSOD* enhancement to drought tolerance, we measured the leaf water content and water loss in both *ScMnSOD* over-expression plants and the control plants. 4-wk-old WT and *ScMnSOD* over-expression plants rosette leaves were put into natural dehydration condition for 6 h. The leaf water content assay results showed that the leaf water content in both treatments were all declined, and the WT leaf water content is lower than *ScMnSOD* over-expression plants although there was no significant difference between two groups ($P$>0.05) (Fig. 4-A).

These results suggested that there is no significant difference in water absorbing capacity under normal conditions, but the transgenic plants might have a stronger leaf water-holding capacity than WT. Therefore, we measured the water loss rate of these two genotypes leaves, showing that after 6 h natural dehydration the WT leaves lost about 53% water, *ScMnSOD* over-expression leaves lost about 42% water (Fig. 4-B). This result confirmed that the transgenic plants have the better leaf water-holding capacity.

Next, we analyzed the ROS content of 4-wk-old

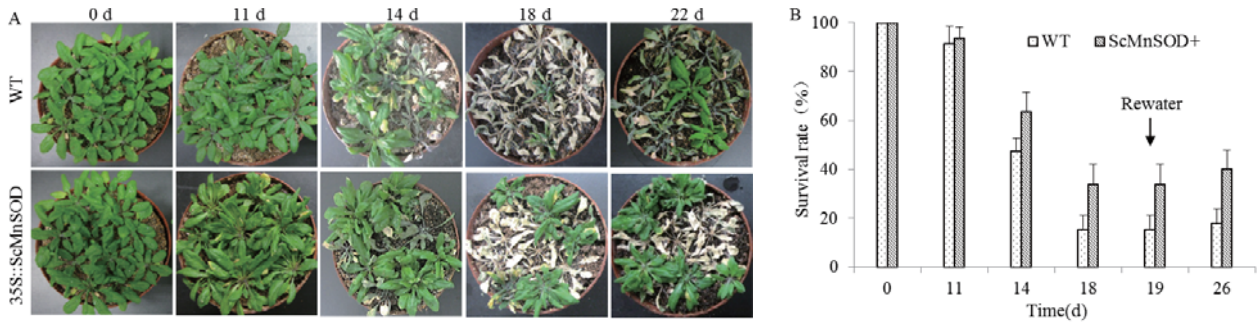


**Fig. 3** Performance of WT and ScMnSOD transgenic plants under drought stress. A, the phenotype of ScMnSOD overexpression plants and control plants under the drought stress, and after re-watering at 19 d. B, the survival rate of WT and *ScMnSOD* transgenic plants under the drought stress. Standard deviations (error bars) were calculated from results of three independent experiments (n>90 for each experiment).

WT and ScMnSOD over-expression leaves. The results revealed that there was no significant difference ($P$>0.05) in leaf ROS content between these two genotypes leaves, but after 6 d drought stress the ROS content increased significantly in WT leaves. While the transgenic leaves ROS content only increased slightly (Fig. 4-C). This result indicated *ScMnSOD* overexpression transgenic plants have a more efficient ROS scavenging capacity.

## The analysis of *ScMnSOD* promoter

The sequence of *ScMnSOD* promoter was isolated by Genome Walking Kit and analyzed using PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). There are some *cis*-acting elements in the promoter sequence (Appendix), including ABRE (ACGT-containing abscisic acid response element), LTR (low-temperature responsiveness),

TATC-box (gibberellin-responsiveness), TGACG-motif (the MeJA-responsiveness), and TGA-element (auxin-responsive element). Those *cis*-acting elements have all been reported in adverse stress responsiveness (Fujita *et al*. 2005; Hu *et al*. 2011; Xie *et al*. 2012), indicating that the ScMnSOD might play similar role in responding to adverse conditions. In addition, in the promoter region, we identified some *cis*-acting elements involving in light responsiveness, such as ATCT-motif, Box 4, GA-motif, and MRE, indicating that light may be involved in regulating expression of *ScMnSOD* (Appendix).

To validate the promoter activity of *ScMnSOD* promoter, we cloned the *ScMnSOD* promoter DNA fragment to the 5′-upstream of GUS coding region in a binary vector and transformed into *Arabidopsis*. GUS histochemical assays revealed that the *ScMnSOD* promoter could modulate GUS express in multiple tissues in the transgenic *Arabidopsis*, including seed, rosette leaves and inflorescence, but not in hypocotyl and silique at different developmental stages (Fig. 5-A). Under the stress of drought, salinity and ABA, GUS expression in transgenic *Arabidopsis* seedlings can be enhanced (Fig. 5-B), suggesting that *ScMnSOD* expression can be up-regulated by drought, salinity and ABA. We concluded that the *ScMnSOD* promoter sequence contains specific *cis*-acting elements response to drought, salinity and ABA.

To determine the core elements of the *ScMnSOD* promoter region, we constructed a sequential 5′-end

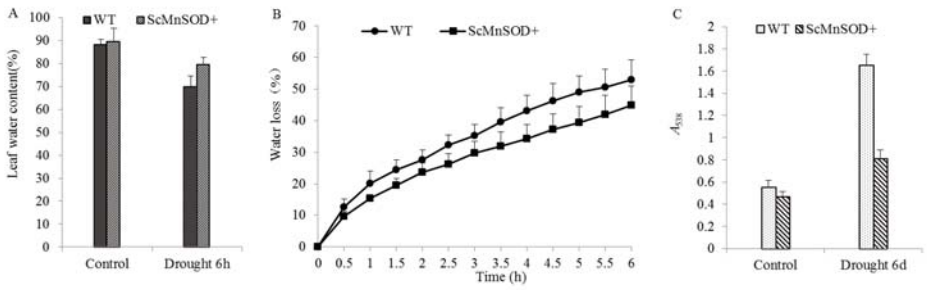


**Fig. 4** Physiological characterization of WT and ScMnSOD transgenic plants. A, leaf water content of WT and ScMnSOD overexpression plants. B, water loss rate of detached rosette leaves of WT and ScMnSOD overexpression plants. C, leaf ROS content of WT and ScMnSOD overexpression plants. Standard deviations (error bars) were calculated from results of three independent experiments (n>120 for each experiment).

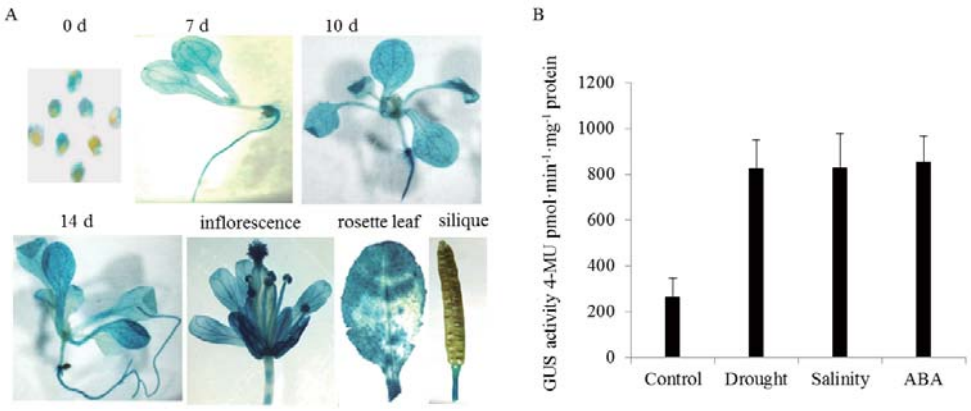


**Fig. 5** GUS staining of Promoter-GUS transgenic plants at different development stages and activity assay of ScMnSOD promoter. A, GUS staining of Promoter-GUS transgenic plants at different development stages. B, quantitative GUS assay of promoter activity after drought, salinity and ABA treatments. Control, MS liquid nutrient medium for overnight; drought stress, MS liquid nutrients medium containing 0.5 mol $L^{-1}$ Mannitol for overnight; salinity stress, MS liquid nutrients medium containing 200 mmol $L^{-1}$ NaCl for overnigh; ABA, MS liquid nutrient medium containing 100 μmol $L^{-1}$ ABA for overnight. Standard deviations (error bars) were calculated from results of three independent experiments (n>30 for each experiment). The same as below.

deletion fragments, and fused the fragments to a GUS reporter gene (Fig. 6-A). These 5′ deletion constructs were introduced into *Arabidopsis*. More than five homozygous transformants for each deletion were screened and used for GUS staining and quantitative fluorometric GUS assay. Under the conditions of drought, salinity and ABA treatments, the promoter activities of P380 was only 25% of that of $P_{ScMnSOD}$ and P591 (Fig. 6-B, C and D). Based on the bioinformatics analysis, the ABRE (full name!) element between -591 and -380 may be involved in response to those three adverse stresses.

Bioinformatics analysis indicated that there are two LTRs between -1 827 and -1 590. Under the conditions of 4°C, the promoter activities of P1590 were approximate 70% of that of $P_{ScMnSOD}$ and P1827, but the promoter activities of P380 were only 20% of that of $P_{ScMnSOD}$ (Fig. 6-E), demonstrating that plants may regulate *ScMnSOD* expression in response to low-temperature stress through LTR (full name!) and ABRE coordinately.

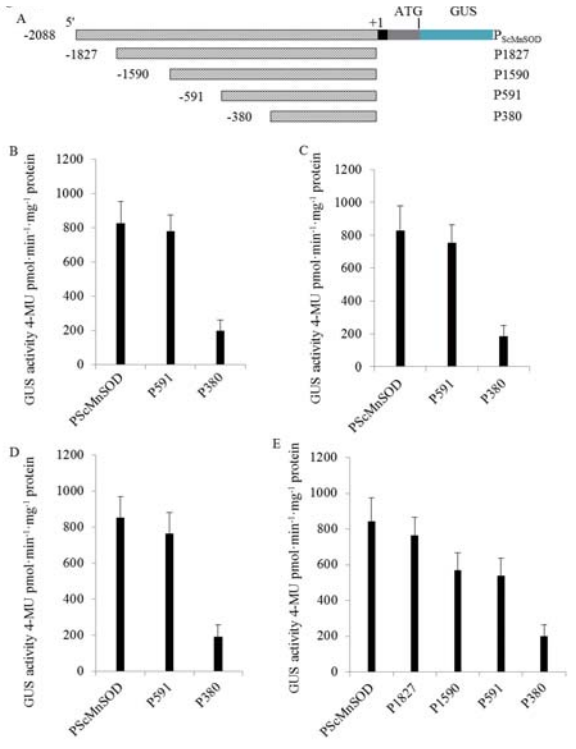


**Fig. 6** Analysis of *cis*-acting elements in the ScMnSOD promoter. A, schematic diagram of the progressive deletion constructs of the ScMnSOD promoter. B, C, D, and E are quantitative GUS assay of promoter activity after drought, salinity, ABA and low temperature treatment, respectively. The treatments were conducted as described above, and the low temperature was conducted at 4°C for overnight.

## DISCUSSION

Drought has been, and is becoming an acute problem most constraining plant growth, terrestrial ecosystem productivity, in many regions all over the world, particularly in arid and semi-arid areas. Plant would response to water stress by dramatically complex mechanisms from genetic molecular express, biochemical metabolism through individual plant physiological processes to ecosystem levels. Plants can tolerant drought stress *via* overproduce SODs (Xu *et al*. 2010). In this study, we identified the jojoba *MnSOD* gene, designated as *ScMnSOD*.

In general, the ability of plants tolerant drought stress is mainly reflected in two aspects: one is the water-absorbing capacity, the other is water-holding capacity, that can be estimated by the water loss rate. Results showed that the transgenic *Arabidopsis* has higher water-absorbing and water-holding capacities (Fig. 4-A and B). Combined with the phenotype of *ScMnSOD* overexpression plants and control plants under the drought stress (Fig. 3-A and B), the transgenic *Arabidopsis* has a strong drought tolerance may be due to its decreased water lose rate and stronger leaf water-holding capacity. Leaf ROS content assay demonstrated that the ROS content of transgenic leaves is much lower than WT's under the drought stress (Fig. 4-C), indicating that the over-expression of *ScMnSOD* enables the plants to enhance ROS scavenging mechanisms. Taken together, all the results suggested that the overproduced *ScMnSOD* make the transgenic *Arabidopsis* has a sturdy antioxidant enzyme system, which can scavenging ROS effectively. So the transgenic *Arabidopsis* would suffer less ROS stress compare with WT, and the transgenic plants obtained a higher water-absorbing and water-holding capacities, then its drought tolerance ability was improved.

ABRE can involve drought and low temperature stress responsiveness (Yamaguchi-Shinozaki and Shinozaki 1994; Kim *et al*. 2011). Under water deficit stress, the promoter activities dropped to 25% as the ABRE was deleted (Fig. 6-B, C and D). And ABRE is widely participated in various kinds of abiotic

stress processes, and ABRE could regulate ABA-dependent gene expression to abiotic stress by binding bZIP transcription factors (Yamaguchi-Shinozaki and Shinozaki 2005). Under 4°C condition, the promoter activities fell below 20% as the ABRE deleted (Fig. 6-E). LTR element was involved in low-temperature responsiveness (Dunn *et al*. 1998). In this study, when the promoter lacking the LTR, its activities dropped to 70% under 4°C condition (Fig. 6-E). The function of the LTR element also has been verified in the low temperature response of blt4.9 in barley (Dunn *et al*. 1998). It can assume that jojoba maybe can regulate *ScMnSOD* expression to tolerate extreme environment stress in desert. Bioinformatics analyses showed that there were other elements existed in *ScMnSOD* promoter, such as TGACG-motif (involved in the MeJA-responsiveness), Skn-1_motif (required for endosperm expression), TATC-box (involved in gibberellin responsiveness), and elements involved in light responsiveness (ATCT-motif, Box 4, GA-motif and MRE), suggesting that *ScMnSOD* may participate in other developmental processes as well.

In conclusion, *ScMnSOD* expressed in multiple tissues at different developmental stages, and its promoter contains some abiotic stress responsive *cis*-acting elements such as ABRE and LTR. Our results suggested that *ScMnSOD* functions as a positive regulator in the drought stress response in *Arabidopsis*, may provide new insights into adaption mechanisms of jojoba to extreme environment stress.

## CONCLUSION

The *ScMnSOD* expressed in multiple tissues at different developmental stages, and can be induced by drought, salinity, ABA and low temperature. Cloning and functional analysis of *ScMnSOD* would facilitate understanding of the molecular mechanism involved in tolerating drought stress process in jojoba.

## MATERIALS AND MEHTODS

### Cloning of jojoba *ScMnSOD* cDNAs

*ScMnSOD* sequence was obtained from the EST database of jojoba subtracted cDNA library. Total RNA was extracted from jojoba leaves using Plant RNeasy Kit (QIAGEN, country!). Reverse transcription-polymerase chain reaction (RT-PCR) was used to clone full-length SOD cDNAs with primers derived from SOD EST sequences. The 3′ ends of SOD cDNA were obtained using 3′ rapid amplification of cDNA ends (3′-RACE) and the 5′-ends were obtained using 5′-RACE. The 3′- and 5′-RACE products were purified and sequenced for further analysis.

### *in vitro* SOD activities of the ScMnSOD protein

The ScMnSOD was cloned in the pET28a vector in between *Eco*R I and *Sal* I restriction sites, The recombinant construct is named as pET28-ScMnSOD. After verified by sequencing, the pET28-ScMnSOD was transformed into the *E. coli* strain BL21 (DE3) for expression. His-tagged ScMnSOD was expressed in *E. coli* according to standard procedures (Thangadurai *et al*. 2008), and purified with Ni-NTA column (QIAGEN). SOD activity was determined according to Assay Kit-WST (Dojindo, country!).

### Generation of transgenic plants

The *ScMnSOD* was cloned in the pCAMBIA-1302 vector in between *Bln* I and *Nco* I restriction sites. The vector was transformed into *Agrobacterium tumefaciens strain* GV3101. The transgenic *Arabidopsis* were produced by Agrobacterium-mediated floral dip method (Clough and Bent 1998). *Arabidopsis thaliana* ecotype Columbia (Col-0) was used in the study. Plants were grown in a controlled culture room temperature at 22 to 24°C with relative humidity (RH) of 60% under long-day conditions (16 h of light and 8 h of dark).

### Drought tolerance assay

T3 homozygous plants were grown aseptically in petri dishes containing selective agar germination medium for 3 wk, then transferred to 8-cm pots filled with a 1:1 mixture of perlite and vermiculite, and grown for one more week before exposure to drought stress. Drought stress was imposed by withholding water for 18 d in a growth chamber (22°C, 50 to 60% RH, continuous 55 μmol $m^{-2}$ $s^{-1}$ photon flux density) until the lethal effect of dehydration was observed on most of the control plants. After rewatering for 3 d, the numbers of plants that survived and continued to grow were counted. Survival rates and standard deviations (error bars) were calculated from results of three independent experiments (n>90 for each experiment) (Tran *et al*. 2004).

Leaf water content and leaf water loss were determined as previously described (Wan and Li 2006; Han *et al*. 2012).

And the leaf ROS content was determined according to Reactive Oxygen Species Assay Kit (Nanjing, Jiancheng Bioengineering Institute, China).

## Promoter analysis

Approximately 2.3-kb-long sequences upstream of the ATG start codon of *ScMnSOD* was amplified from genomic DNA isolated from jojoba leaves adopting Genome Walking Kit (TaKaRa, country!). 5′-flanking deletion was used to analyze the promoter, all forward primers have *Hind* III added in the 5′ end, reverse primer has *Spe* I added in the 5′ end, in order to directional recombination of the PCR product into the pCAMBIA1305.1.

GUS staining was performed as previously described (Jefferson *et al*. 1987). For quantitative fluorometric GUS assay, T3 homozygous samples were collected from at least five seedlings and frozen immediately in liquid nitrogen and ground in GUS extraction buffer. After centrifugation, the clear supernatants were used for GUS assays. GUS activity was measured fluorometrically using 4-methylumbelliferyl glucoronide as a substrate accordingly (Jefferson *et al*. 1987). Standard deviations (error bars) were calculated from results of three independent experiments (n>30 for each experiment).

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Appendix associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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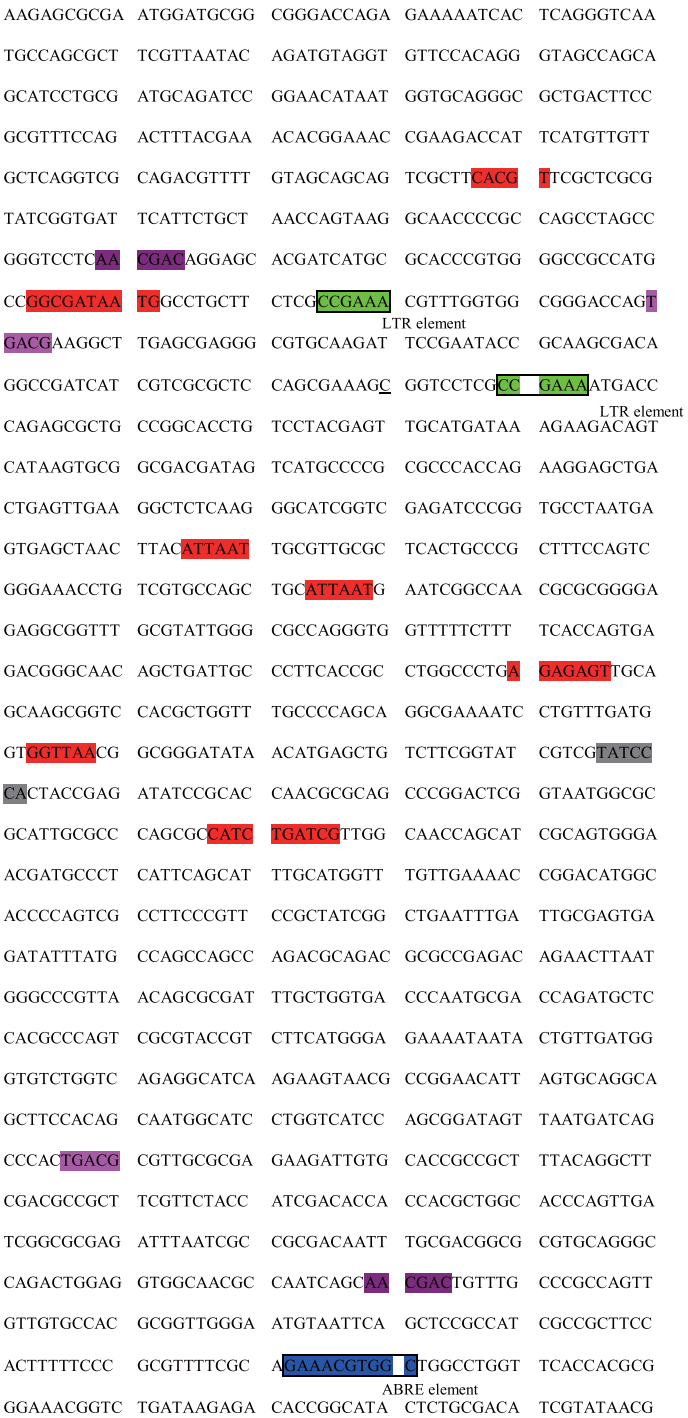


**Suppl. Fig. 1** Sequence analysis of ScMnSOD promoter. G: predicted transcription start site; *ATG*: initiator codon; Green region: LTR element; Blue region: ABRE element; Gray region: TATC-box; Purple region: TGA-element; Pink region: TGACG-motif; Red region: elements involved in light.