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A Cu/Zn Superoxide Dismutase Gene From *Saussurea involucreata* Kar. et Kir., *SiCSD*, Enhances Drought, Cold and Oxidative Stress in Transgenic Tobacco

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Abstract: Superoxide dismutase (SOD) plays an important role in stress-tolerance in higher plants. In the present study, a novel CuZn superoxide dismutase gene, named as *SiCSD* (accession no. KC912564) was cloned from *Saussurea involucreata* Kar. et Kir. The deduced amino acid sequence shared 85% identity with CuZn-SOD of *Solanum tuberosum* and *Solanum lycopersicum*. Quantitative real-time polymerase chain reaction (qRT-PCR) showed that *SiCSD* was upregulated by treatments with cold, drought and oxidative stresses. *SiCSD* transgenic tobacco plants improved tolerance to drought, freezing and oxidative stresses and exhibited a higher survival rate, relative water content (RWC), photosynthesis efficiency, and higher activities of superoxide dismutases (SOD), catalases (CAT) and ascorbate peroxidase (APX), but lower ion leakage (IL) and malondialdehyde (MDA) contents compared with wild type. These data demonstrate that *SiCSD* may act as a positive regulator in drought and cold stress by reducing oxidant injury.

Key words: *Saussurea involucreata* Kar. et Kir., CuZn-SOD, *SiCSD* gene, transgenic tobacco, abiotic stress

Introduction

Abiotic stresses such as drought and cold do seriously harm to plant growth, development, and crop productivity. One of the amazing responses of plants to abiotic stresses is the formation of reactive oxygen species (ROS) in their cells, which affect plant growth and production (Jaspers and Kangasjarvi 2010). In order to protect against damaging effects of ROS, plants have developed a complex antioxidative system such as superoxide dismutases (SOD), catalases (CAT), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Foyer and Noctor 2005; Mittler 2002). Of the antioxidant enzymes, SOD is considered as the first line of cellular defense against ROS by early scavenging of superoxide radicals and converting them to hydrogen peroxide (Fridovich 1995; Kim et al. 2010).

Plant SODs are commonly classified according to their active site cofactors into manganese SOD (MnSOD), iron SOD (FeSOD) and copper/zinc SOD (CuZn-SOD). Three types of CuZn-SODs (CSD1, CSD2 and CSD3) are reported in plant cell, which are located in the cytoplasm, chloroplast and peroxisome, respectively (Kliebenstein et al. 1998). Several SOD genes have been cloned in plant species like *Arabidopsis thaliana* (Kliebenstein et al. 1998), tobacco (Hérouart et al. 1993), pea (Scioli and Zilinskas 1988), tomato (Perl-Treves et al. 1990), rice (Sakamoto et al. 1995) and wheat (Wu et al. 1999). These genes are thought to be involved in plants's response to abiotic stresses. For instance, overexpression of CuZn-SOD in tobacco plants confer increased resistance to oxidative stress (Gupta et al. 1993) and partial resistance to ozone-induced foliar necrosis (Pitcher et al. 1996). Overexpression of CuZn-SOD from *Arachis hypogaea* alleviates salinity and drought stresses in tobacco (Negi et al. 2015). Overexpression of CuZn-SOD improves tolerance to oxidative and chilling stresses in cassava (*Manihot esculenta* Crantz) (Xu et al. 2014). These efforts are important for developing stress-resistant crops that are much needed for sustain growth and productivity in extreme environment.

Saussurea involucrata Kar. et Kir., popularly known as snow lotus, belongs to family Asteraceae and distributes in the mountains at heights of 2800~3400 m of Tianshan in Xinjiang province of China, where almost all the other flowering plants difficult to grow. The special growth habits and environment make it possess special functions for the treatment of many diseases, including rheumatic arthritis, gynecological disorders etc. (Li and Zhao 1989; Jia et al. 2005). To survive in extreme environment, *S. involucrata* might have formed stable physiological and biochemical mechanisms to increase the resistance ability. But at present, only a few stress resistant genes had been cloned and functionally identified in *S. involucrata* (Guo et al. 2012, Qiu et al. 2014). Therefore, it is important to understand how *S. involucrata* maintains the normal physiological functions in such extreme conditions.

In a previous study, we created a cDNA library for *S. involucrata* to characterize genetic factors associated with the cold stress. A DNA fragment which has high homology to plant CuZn-SOD was found in the library, named as *SiCSD*. Here, we report the cDNA cloning and expression of *SiCSD* under drought, cold and oxidative stresses, and establish the role of *SiCSD* in conferring abiotic stress tolerance in transgenic tobacco.

Materials and methods

Plant materials and growth conditions

Mature seeds of *Saussurea involucrata* from Tianshan Mountain (Xinjiang, China) were used for obtaining of aseptic seedling. Seeds were surface-sterilized by treating with 2% HgCl₂ and Triton X 100 (in 100 ml H₂O) for 20 min with gentle agitation. The seeds were then thoroughly washed with sterile distilled water twice prior to use for *in vitro* germination studies. Sterilized seeds were cultured on 1/2 MS medium. The culture conditions were: temperature, 20 ± 1 °C; light intensity, 13 klux; and light period, 16/8 h (light/dark). Tobacco (*Nicotiana tabacum* L. cv. NC89) was used for transgenic studies. Seeds were germinated on MS medium plates. Plants were grown at 23/16 °C day/night cycles under a 16/8 h (light/dark) photoperiod.

Cloning and sequence analysis of *SiCSD* cDNA

Total RNA was extracted from leaves of *S. involucrata* using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using Superscript II (Invitrogen). The open reading frame (ORF) of *SiCSD* was amplified by using the specific primers *SiSOD1* and *SiSOD2* (Table 1). DNA sequencing was performed by Invitrogen in Shanghai, China. The multiple sequence alignments were analyzed by DNAMAN version 6.0 (Lynnon BioSoft, USA). The phylogenetic tree was constructed with MEGA software (version 5.0, Biodesign Institute, Tempe, AZ, USA) using the Neighbor-Joining (NJ) method and the bootstrap test carried out with 1000 iterations.

Expression analyses by real-time quantitative PCR

Total RNA was extracted from leaves of *S. involucrata* treated for 0, 1, 3, 6, 12 and 24 h with drought, cold or oxidative stresses. Then RNA was treated with DNase I (Fermentas, Burlington, ON, Canada). 1 µg of total RNA was used for reverse transcription. QRT-PCR was performed on each cDNA template using SYBR Green I Master Mix on LightCycler® 480 II instrument (Roche Biochemicals, Indianapolis, IN, USA). *S. involucrata* *GADPH* gene (accession no. KF563904.1) was used as an internal control. Primers used for qRT-PCR are listed in Table 1. The qRT-PCR was done as follows: 94°C for 5 min, 40 cycles of 94°C for 15 s, 58°C for 20 s, and 72°C for 20 s. The results were analyzed using the comparative Ct method and quantified relative to the wild type ($2^{-\Delta\Delta C_t}$) (Livak, et al. 2001).

Plasmid construction and tobacco transformation

SiCSD was amplified by PCR using specific primers SiSOD3 and SiSOD4 (Table 1). The plasmid pBI121 (Clontech, Palo Alto, CA) was digested with *Bam*H I and *Sac* I to obtain the expression plasmid 35S: *SiCSD*. The *SiCSD* ORF was located between the CaMV35S promoter and the NOS 3'poly (A) signal. The construct was transformed into *Agrobacterium tumefaciens* (strain GV3101). Tobacco (*Nicotiana tabacum* L. cv. NC89) was transformed through *Agrobacterium tumefaciens*-mediated (strain GV3101) T-DNA transformation (Gallois et al. 1995). Transformants were selected on 1/2 MS medium containing kanamycin (100 μ M).

PCR and Southern blot analysis of transgenic tobacco

Genomic DNA was isolated from the leaves of one-month-old tobacco plants of putative transgenic and wild type (WT) type plants using the CTAB method (Edwards et al. 1991). Gene specific primers SiSOD5 and SiSOD6 (Table 1) were used for PCR analysis. The program was done as follows: 94°C for 3 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and 72°C for 10 min. The final PCR products (107 bp) were resolved on a 1.0% agarose gel and visualized by staining with ethidium bromide (EtBr). T1 generation plants were used in all experiments unless otherwise indicated.

Southern blot analysis was also used to detect the transgene copy number. 25 μ g of tobacco genomic DNA was digested with *Bam*H I, electrophoresed on a 0.8% agarose gel, and blotted onto a nylon membrane (Amersham, UK) according to the standard procedures (Sambrook et al. 1989). α -³²P-dCTP-labeled complete *SiCSD* cDNA was used as a probe for hybridization. The membrane was exposed to X-ray film (Eastern Kodak) at -80°C for 5 d.

Gene-expression assays of transgenic tobacco

One-month-old seedlings of WT and transgenic tobacco plants grown in soil were used to determine the *SiCSD* expression. The seedlings were treated with 10% PEG 6000, 4°C and 5 μ M paraquat, respectively. Then tobacco leaves were collected at 0, 1, 3, 6, 12 and 24 h after treatments for RNA extraction and qRT-PCR. Gene specific primers SiSOD5 and SiSOD6 were used for qRT-PCR, with tobacco *actin* gene (accession no. X69885) as internal reference (Table 1). The thermal cycle used was 94°C for 5 min and 40 cycles of 94°C for 15 s, 58°C for 20 s, and 72°C for 20 s. The results were analyzed using the comparative Ct method and quantified relative to the wild type ($2^{-\Delta\Delta C_t}$) (Livak, et al. 2001).

Abiotic stress treatments

WT and transgenic tobacco seeds were germinated on 1/2 MS media with 100 μ M kanamycin or without kanamycin. After 3 weeks, seedlings were transferred into pots with vermiculite-peat (1:1, v/v) medium. One month later, seedlings were used for abiotic stress. For drought treatment, WT and transgenic tobacco seedlings were withheld from water for 0, 5, 10 and 15 d and then rewatering for 2 d. For cold treatment, plants were incubated at 4, 0, -2 or -4°C for a maximum of 2 h. For oxidative treatment, the whole tobacco leaves were sprayed with 200 mL paraquat (0, 200, 400 or 600 μ M) once per day for 4 d. All of the treatments above were repeated three times.

Assay of RWC, PSII activity, MDA and relative ion leakage

Tobacco leaves were collected after drought, cold and oxidative treatments for physiological measurement. The relative water content (RWC) was measured as described by Aroca et al. (2003). The photosynthetic activity was recorded via chlorophyll fluorescence determinations of the maximum quantum yield of photosystem II photochemistry (F_v/F_m), using an imaging chlorophyll fluorometer (Walz Imaging PAM, Walz GmbH, Effeltrich, Germany) after 30 min of dark adaptation. The relative ion leakage (IL) was assayed following the method of Cao et al. (2007). The malondialdehyde (MDA) content was determined according to a modified thiobarbituric acid (TBA) method (Kim and Nam 2013). These experiments were repeated at least three times.

Measurement of SOD, CAT and APX activities

Leaf tissue (0.5 g) were cut into pieces and homogenized (1:5 m/v) in an ice-cold mortar using 50 mM sodium phosphate buffer, pH 7.8 containing 1% polyvinylpyrrolidone and 10 mM β -mercaptoethanol. After centrifugation (13 000 g, 15 min, 4°C), the supernatant was used for the determination of SOD, CAT and APX activities. SOD activity was assayed with the method of Beauchamp and Fridovich (1971). Absorbance was read at 560 nm. CAT activity was determined according to Cakmak and Marschner (1992). APX activity was estimated according to the method of Nakano and Asada (1981). Enzyme activity was determined as the decrease in absorbance of ascorbate at 290 nm.

Statistical analyses

All statistical analyses were performed with the SPSS 13.0 statistical software, and all data were evaluated with one-way ANOVA. Data were expressed as mean $\pm$ SD, $p < 0.05$ was taken as significant, and $p < 0.01$ was taken as extremely significant.

Results

Cloning of *SiCSD* cDNA

A full-length cDNA, named *SiCSD* (accession no. KC912564), was obtained from a cDNA library of *S. involucrata* leaves induced by cold stress. The sequence of *SiCSD* contained an open reading frame encoding a polypeptide of 157 amino acids with a calculated molecular mass of 16.02 kDa. Sequence alignment of the deduced amino acid sequence (Fig. 1A) showed that it was approximately 85% identical to its homologues in CuZn-SODs of *Solanum tuberosum* and *Solanum lycopersicum*. Various potential copper-binding (His-48, His-50, His-65, His-121) and zinc-binding (His-65, His-73, His82, Asp-85) sites (Bannister et al. 1987; Tainer et al. 1983), two Cys residues that may participate in disulphide bond formation (Cys-59 and Cys-148) (Deng et al. 1993) were found in *SiCSD* protein (Fig. 1A). In addition, phylogenetic analysis based on a neighbor-joining (NJ) bootstrap method indicated that *SiCSD* was most closely related to CSD3 of *Arabidopsis thaliana* which located in peroxisomes (Fig. 1B).

Expression profile of *SiCSD*

To determine whether the expression of *SiCSD* is regulated by drought, cold and oxidative stress, *S. involucrata* aseptic seedlings were kept at 4°C, 10 % PEG6000 or 5 μ M paraquat. Detached leaves were used for RNA extraction and qRT-PCR analysis at 0, 1, 3, 6, 12, and 24 h after cold, drought and oxidative treatments. The results showed that drought stress caused up-regulation in *SiCSD* expression at 1 h and 6 h (Fig. 2). The same expression profile was found in cold stress and higher *SiCSD* expression was detected at 1 h compared with drought stress (Fig. 2). For paraquat-induced oxidative stress, *SiCSD* expression was declined at 1 h, but up-regulated at 12 h and peaked at 24 h (Fig. 2). The results showed that *SiCSD* expression was sensitive to drought and cold, but had a slow response to paraquat-induced oxidative stress.

Over-expression of *SiCSD* in Tobacco

The *SiCSD* induced expression by drought, cold and oxidative stress prompted us to analyze its function in abiotic stress resistance through overexpression in tobacco. Using genomic DNA, PCR and Southern blot methods were used to identify transgenic plants. A 107 bp band corresponding in size to the *SiCSD* product was obtained from transgenic tobacco, whereas no bands found in WT (Supplementary Fig. S1A), and two transgenic lines (line3 and 5) were finally selected for southern blot to detect the transgene copy number. While a single-copy transgene insertion was seen in the line5, two copies of the transgene was found integrated into the genome of the transgenic

line3 and none were detected in the WT plants (**Supplementary Fig. S1B**). The qRT-PCR analysis showed that mRNA of *SiCSD* was successfully expressed in the transgenic line5 under drought, cold and oxidative stresses (**Supplementary Fig. S1C**), whereas it was absent in the WT plant.

***SiCSD* enhances the resistance of transgenic tobacco plants to drought, cold and oxidative stress**

In our study, no difference between transgenic lines and WT in phenotype was observed under normal conditions (Fig. 3A), while a significant difference between transgenic lines and WT was found in phenotype after withheld of water for 5 d. Leaf rolling was substantially delayed in *SiCSD* overexpressing plants (line3 and 5) compared with WT (Fig. 3A). Ten days after drought stress, all plants were wilting, whereas the transgenic lines showed faster recovery after 2 d of rewatering (Fig. 4A). Eleven days later, the average survival rate of transgenic lines were 66.7%, which was 7 times higher than that of WT ($p < 0.05$) (Figs. 4A, D). These results indicated that overexpression of *SiCSD* enhanced tolerance to drought stress in transgenic plants.

For cold stress analysis, two-month-old plants were exposed to different low temperatures treatment (4°C , -2°C and -4°C). After 4°C for 2 h, the morphological appearances of transgenic lines and WT were no significant difference. Foliar tissue drooping was more evident in the WT plants relative to the two transgenic lines after 2 h of exposure at -2°C . After -4°C for 1 h, WT seedling was serious injured, whereas the leaves of two transgenic lines were slightly damaged (Fig. 3B). After -10°C for 2 h and recovery for 3 d, the average survival rate of WT was 22.3%, whereas the average survival rate of transgenic lines were higher (64.9%) (Figs. 4B, D). These results indicated that overexpression of *SiCSD* could enhance cold tolerance in transgenic plants.

For oxidative stress analysis, two-month-old seedlings growing in soil were sprayed with various concentrations of paraquat (200, 400 and 600 μM). After 4 d of spraying, leaves of WT plants were monitored for bleaching at 200 μM paraquat and died at 600 μM paraquat treatment, whereas the transgenic plants still alive at 600 μM paraquat treatment (Fig. 3C). To compare the average survival rate between WT and transgenic plants, 1-month-old WT and two transgenic lines (3 and 5) growing on MS medium were used for analysis. After exposed to 2 μM paraquat treatment for 4 d with continuous light, the average survival rate of transgenic plants was 4 times higher than WT ($p < 0.05$) (Figs. 4C, D). These results showed that transgenic plants overexpressing *SiCSD* gene had enhanced tolerance to oxidative stress.

Overexpression of *SiCSD* improves the RWC and decreases malonaldehyde (MDA) and relative ion leakage (IL) under drought and cold stress

The expression of *SiCSD* enhanced drought and cold tolerance led us to determine the effects of the physiological status caused by *SiCSD* expression. Result of relative water contents (RWC) analysis indicated that water loss rate under drought stress was less reduced in transgenic lines after 5, 10 and 15 d of drought stress, respectively ($p < 0.05$) (Fig. 5A). Relative Ion leakage (IL), an important indicator of membrane injury, was significantly higher in WT under drought and cold stresses (Figs. 5B, C). MDA content is thought to be a marker of lipid peroxidation (Gao et al. 1999). During drought and cold stress, both WT and transgenic lines were increased. However, the MDA content was much higher in WT (Figs. 5D, E). These indicate that drought and cold stress caused more pronounced oxidative damage in WT than in transgene plants.

Overexpression of *SiCSD* transgenic plants had higher F_v/F_m after drought, cold and oxidative stress

To further evaluate the increased drought, cold and oxidative tolerance of transgenic tobacco overexpressing *SiCSD*, the chlorophyll fluorescence (F_v/F_m) were determined (Figs. 6A–C). Under normal conditions, there were no significant ($P > 0.05$) difference of F_v/F_m among leaves of WT and transgenic plants. However, after drought, cold and oxidative treatments, the transgenic plants maintained significantly higher levels of F_v/F_m compared with WT, indicating that the photosystem of transgenic plants was less affected than that of WT

during abiotic stresses.

Enhanced antioxidant enzyme activity in *SiCSD* transgenic tobacco plants under drought, cold and oxidative stress

SiCSD may reduce H_2O_2 production through the activation of the antioxidative system. SOD, CAT and APX belong to this system, and are involved in H_2O_2 elimination. In this research, two-month-old tobacco seedlings were treated with drought, cold or oxidative stress for measurement of enzyme activities. Under normal conditions, there was no significant difference ($P>0.05$) in SOD activity between transgenic plants and WT (Fig. 7A), but CAT and APX activity was 3 to 4-fold higher than WT ($p<0.05$) (Figs. 7B, C). Under drought stress, SOD activity declined rapidly in WT, but increased in transgenic plants ($p<0.05$) (Fig. 7A). After 10 d of drought, APX activity in transgenic plants was 15-fold higher than WT (Fig. 7C). After -2°C for 2 h, SOD, APX and CAT activities were all significantly enhanced in transgenic plants compared with WT, which had a 9-, 25- and 10-fold higher than WT plants, respectively ($p<0.05$) (Figs. 7D-F). After paraquat-induced oxidative stress (600 μM) for 4 d, SOD activity was 2.5-fold higher compared with WT (Fig. 7G). CAT and APX activity in transgenic plants increased first and then decreased with large amount of variation, but still higher than WT (Figs. 7H, I). These results suggest that *SiCSD* may promote drought, colds and oxidative tolerance through the elevation of the activities of antioxidative enzymes.

Discussion

Abiotic stress, such as drought and cold, restricts the plant growth and therefore causes major threat to plant productivity. It has been reported that increased SOD activity is positively correlated with the tolerance to various abiotic stress (Ueda et al. 2013). In this paper, we isolated a *SiCSD* from a *S. involucrata* cDNA library. Sequence alignment showed that *SiCSD* has highest homology to CuZn-SOD of *S. tuberosum* and *S. lycopersicum* with 85% identity. However, although this gene shares characteristics common to other plant CuZn-SOD, it may also have unique features. *S. involucrata* grows in a snow-covered alpine environment where most of other plants have hard to survive, its genetic and evolutionary processes have been relatively independent from other species and may have evolved with some genes associated with environmental stresses.

The expression of *SiCSD* revealed higher accumulation of its transcript in leaves rapidly by cold (4°C) and drought stress, which may form an important mechanism to detoxify ROS and protect against environmental stress. These observations generally agree with those other studies reporting increased CuZn-SOD expression in plants exposed to drought and cold stress (Mittler and Zilinskas 1994; Negi et al. 2015; Xu et al. 2014). However, the paraquat stress induced a slow response of *SiCSD* mRNA expression which indicated that the regulation mechanism of this gene in *S. involucrata* might be not the same in drought, cold and oxidative stress, and these need to be further investigated.

To elucidate the contribution of *SiCSD* to abiotic stress, transgenic tobacco plants overexpressing *SiCSD* were generated. The transgenic plants maintained higher tolerance ability and survival rate than WT under stress conditions. This finding is consistent with previous reports of transgenic tobacco plants overexpressing *CuZn-SOD* and *APX* genes (Lee et al. 2013). PS II photochemical efficiency (F_v/F_m) changes are also indicator of abiotic stress (Chen et al. 2006). Studies have shown that environmental stresses, such as cold and oxidative stress, inhibit the repair of PSII and cause the photodamage of the reaction center (Takahashi et al. 2008; Mauro et al. 1997). Our study showed that abiotic stresses could reduce F_v/F_m values in tobacco leaves, and much little decrease of F_v/F_m was found in transgenic tobacco than in WT, indicating that the *SiCSD* gene could effectively protect the PS II reaction center.

Abiotic stresses such as cold and drought can enhance membrane peroxidation, increase cell membrane permeability and electrolyte leakage (Gao et al. 1999). MDA is one of the final products of oxidative modification

of lipids and is responsible for membrane damage leading to cell death (Sharma et al. 2012). Here, we found that the MDA content of transgenic plants were significantly lower compared to WT when exposed to drought and cold stresses. This indicates that the expression of *SiCSD* gene can enhance the protection of cell membrane.

The increase of SOD activity in each transgenic line was remarkably higher than WT under drought, cold and oxidative stresses. These results indicate that the ability of eliminating of O_2^- in transgenic tobacco is increased significantly. The CAT and APX activities also increased significantly in transgenic plants after abiotic stresses. Du et al (2008) showed that the increase of antioxidase activities under abiotic stresses can rescue the plant from ROS. Hence the increased activity of SOD, CAT and APX in *SiCSD* overexpressing plants may be useful for scavenging ROS.

In conclusion, we isolated, cloned and characterized a gene *SiCSD* from *S. involucrata*. The *SiCSD* gene showed up-regulation by different abiotic stresses. Further more, under drought, cold and oxidative stress, *SiCSD* transgenic tobacco plants have higher relative survival rate, relative water content, antioxidant enzyme activity, and higher photosynthesis efficiency compared with WT. Our findings suggest that *SiCSD* is an important antioxidant enzyme in engineering crops to improve tolerance to abiotic stresses.

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Fig. 1 Sequence alignment and phylogenetic analysis.

(A) Multiple sequence alignment of the deduced amino acid sequence of SiCSD with CuZn-SOD of other plants. Gaps are introduced to maximize alignment. The putative amino acid residues required for coordinating copper and zinc are marked by asterisks. The Arg residue that is necessary to guide the superoxide anion to the active site is labeled by closed triangle. The two cysteine residues which form a disulfide bond are labeled by closed circles. Plant sources and GenBank accession No. are indicated. *Solanum lycopersicum* (CSD, XP004234809), *Solanum tuberosum* (CSD, XP006349575), *Arabidopsis thaliana* (CSD1, CAA43270; CSD3, AAC24833), *Oryza sativa* (CSD1, BAA00800) and *Saussurea involucrata* Kar.et Kir. (SiCSD, KC912564).

(B) Phylogenetic analysis was conducted by Mega 5.0 program based on a multiple alignment of amino acid sequences retrieved from the GenBank database. Numbers at the nodes are bootstrap values (1000 replications). The scale bar is 0.05

Fig. 2 The level of *SiCSD* transcripts induced by drought, cold and oxidative stresses.

Total RNA were isolated from one-month-old leaves of *S. involucrata* treated with 10% PEG 6000, 4 $\square$ or paraquat (5 μ M), and then analyzed by qRT-PCR. The transcripts of *GAPDH* were used as a control, and the amount of mRNA expression under normal conditions at 0 h was set as 1. Error bars indicate the standard deviation of the mean (N = 3). Three replicates were analyzed.

Fig. 3 Comparison of drought, cold and oxidation tolerance of *SiCSD* overexpressing transgenic and WT plants.

(A) Morphology of WT and transgenic tobacco lines (line3 and 5) growing under drought stress (5 d and 15 d without irrigation, and recover for 2 d) conditions.

(B) The simulated cold stress (4 $^{\circ}$ C, -2 $^{\circ}$ C for 2 h, or -4 $^{\circ}$ C for 1 h).

(C) Oxidative stress caused by paraquat (0, 200, 400 or 600 μ M) for 4 d.

Fig. 4 Comparison of survival rate between *SiCSD* overexpression transgenic plants and WT under various stresses.

(A) Photograph of WT and transgenic plants under drought stress. Seeds of WT and transgenic lines (3 and 5) were germinated and grew on soil for one month, and then water was withheld for 11 d.

(B) The simulated cold stress (-10 $^{\circ}$ C for 2 h).

(C) Oxidative stress caused by paraquat. Plants were first floated on MS solution for one month, and then treated with 2 μ M paraquat. Photographs were taken 4 d after incubation in continuous light (300 μ M/m 2 /s).

(D) Survival rate of WT and transgenic tobacco plants under drought, cold and oxidative stress.

WT: wild type; line3 and line5: independent *SiCSD* transgenic lines. Data are means of three replications.

* indicates significant difference among materials at the 0.05 level

Fig. 5 Analysis of relative water contents (RWC), IL and MDA content in transgenic plants under drought and cold stress.

WT and transgenic tobacco plants were grown in soil with sufficient water for 3 weeks. The water was withheld for 5, 10 or 15 d (Drought), or temperature was dropped to 4, 0, -2 or -4 $^{\circ}$ C for 2 h (Cold) before pictures were taken. Tobacco leaves were sampled to detect RWC (A), IL (B and C) and MDA (D and E).

WT: wild type; line3 and line5: independent *SiCSD* transgenic lines. Data are means of three replications.

* indicates significant difference among materials at the 0.05 level

Fig. 6 Analysis of Chlorophyll fluorescence (F_v/F_m) changes under drought, cold and oxidative stress.

WT and *SiCSD*-overexpressing transgenic tobacco lines (3 and 5) were used for stress analysis.

(A) Drought stress (without irrigation) for 0, 5, 10 and 15 d.

(B) Cold stress (4°C, 0°C, -2°C and -4°C) for 2 h.

(C) Oxidative stress caused by paraquat (0, 200, 400 and 600 µM) for 4 d.

WT: wild type; line3 and line5: independent *SiCSD* transgenic lines. Data are means of three replications.

* indicates significant difference among materials at the 0.05 level

Fig. 7 Activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) in leaves of WT and transgenic tobacco seedlings under drought, cold and oxidative stress.

(A- C) SOD, CAT and APX activity in tobacco seedlings growing under normal conditions (control) or after withheld of water for 5, 10, and 15 d.

(D-F) The simulated cold stress (4°C, 0°C, -2°C for 2 h) .

(G-I) Oxidative stress caused by paraquat (0, 200, 400 and 600 µM) for 4 d.

WT: wild-type tobacco plants; Line3 and line5: *SiCSD* over-overexpressing transgenic tobacco lines. Data are means of three replications. * indicates significant difference among materials at the 0.05 level

Supplementary Fig. S1 Detection of the transgene from kanamycin-resistant lines.

(A) Confirmation of *SiCSD* transgenic tobacco plants by PCR. WT, wild type plants; L1~L7, independently transformed plant lines; M, DNA marker III.

(B) Southern blot analysis of the transgenic plants was done after digestion of genomic DNA with BamH I, probed with full length *SiCSD* cDNA. WT plants showed no detectable hybridization.

(C) QRT-PCR analysis confirming expression of *SiCSD* in young leaves of transgenic tobacco plants (line5) after drought (10% PEG6000), cold (4 °C) and 5µM paraquat induced oxidative stresses. Leaves were collected at 0, 1, 3, 6, 12 and 24 h after treatments. Tobacco actin gene was used as an internal control.

Table 1 Primers used in the experiments.

Primer	Sequences (5'-3')	Purpose
SiSOD1	ATGGGTGGTTTGAAAGCGGT	Clone and Southern blot
SiSOD2	TTACACTGAAGATTGAAGCC	Clone and Southern blot
SiSOD3	<u>GGATCC</u> ATGGGTGGTTTGAAAGCGGT (<i>Bam</i> H I site underlined)	Tobacco expression
SiSOD4	<u>GAGCTC</u> TTACACTGAAGATTGAAGCC (<i>Sac</i> I site underlined)	Tobacco expression
SiSOD5	TGTGAGGGGTAGGATAGTAGGGC	PCR and qRT-PCR
SiSOD6	TTCTTTAGTGGATTGAAATGAGGC	PCR and qRT-PCR
GAPDH-F	TTCAACATTATTCCCAGCAGCAC	qRT-PCR
GAPDH-R	TAAGTAGCCTTCTTCTCAAGTCTCACA	qRT-PCR
Actin-F	CCTGAGGTCCTTTTCCAACCA	qRT-PCR
Actin-R	GGATTCCGGCAGCTTCCATT	qRT-PCR

Fig. 1 Sequence alignment and phylogenetic analysis.
(A) Multiple sequence alignment of the deduced amino acid sequence of SiCSD with CuZn-SOD of other plants.

244x177mm (85 x 83 DPI)

Phylogenetic analysis was conducted by Mega 5.0 program based on a multiple alignment of amino acid sequences retrieved from the GenBank database. Numbers at the nodes are bootstrap values (1000 replications). The scale bar is 0.05

228x176mm (150 x 150 DPI)

The level of SiCSD transcripts induced by drought, cold and oxidative stresses.

182x103mm (150 x 150 DPI)

Fig. 3 Comparison of drought, cold and oxidation tolerance of SiCSD overexpressing transgenic and WT plants.

242x126mm (150 x 150 DPI)

Fig. 4 Comparison of survival rate between SiCSD overexpression transgenic plants and WT under various stresses.

238x155mm (150 x 150 DPI)

Fig. 5 Analysis of relative water contents (RWC), IL and MDA content in transgenic plants under drought and cold stress.

264x282mm (150 x 150 DPI)

Fig. 6 Analysis of Chlorophyll fluorescence (F_v/F_m) changes under drought, cold and oxidative stress.

246x178mm (150 x 150 DPI)

Fig. 7 Activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) in leaves of WT and transgenic tobacco seedlings under drought, cold and oxidative stress.

311x453mm (150 x 150 DPI)