



CsSAD: a fatty acid desaturase gene involved in abiotic resistance in *Camellia sinensis* (L.)

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ABSTRACT. Tea (*Camellia sinensis* L.) is a thermophilic evergreen woody plant that has poor cold tolerance. The *SAD* gene plays a key role in regulating fatty acid synthesis and membrane lipid fluidity in response to temperature change. In this study, full-length *SAD* cDNA was cloned from tea leaves using rapid amplification of cDNA ends and polymerase chain reaction (PCR)-based methods. Sequence analysis demonstrated that *CsSAD* had a high similarity to other corresponding cDNAs. At 25°C, the *CsSAD* transcriptional level was highest in the leaf and lowest in the stem, but there was no obvious difference between the root and stem organs. *CsSAD* expression was investigated by reverse transcription-PCR, which showed that *CsSAD* was upregulated at 4° and -5°C. At 25°C, *CsSAD*

was induced by polyethylene glycol, abscisic acid, and wounding, and a similar trend was observed at 4°C, but the mean expression level at 4°C was lower than that at 25°C. Under natural cold acclimation, the 'CsCr05' variety's *CsSAD* expression level increased before decreasing. The *CsSAD* expression level in variety 'CsCr06' showed no obvious change at first, but rapidly increased to a maximum when the temperature was very low. Our study demonstrates that *CsSAD* is upregulated in response to different abiotic conditions, and that it is important to study the stress resistance of the tea plant, particularly in response to low temperature, drought, and wounding.

Key words: *Camellia sinensis* (L.); *SAD*; Abiotic stress; Gene expression; Polyethylene glycol; Abscisic acid

INTRODUCTION

Stearoyl-acyl carrier protein (ACP) desaturase (*SAD*, EC1.14.99.6) is a key enzyme in fatty acid synthesis. It is the main product of plant fatty acid synthesis, and the *SAD*-mediated catalysis of stearoyl-ACP to oleoyl-ACP is the starting point for the formation of unsaturated fatty acids (Byfield and Upchurch, 2007). Therefore, to a great degree, *SAD* determines the ratio of saturated to unsaturated fatty acids in higher plants, and this ratio is closely associated with many functions in plants (Somerville and Browse, 1996; Los and Murata, 1998), particularly in plants acclimated to low temperatures (Kodama et al., 1995; Lindqvist et al., 1996).

The *SAD* gene was originally isolated and purified from *Carthamus tinctorius* and *Persea americana*, and the primary structure of *C. tinctorius SAD* has been described (Thompson et al., 1991). The Fe-O-Fe cluster structure of *Ricinus communis SAD* was detected using resonance Raman spectroscopy (Fox et al., 1994), and a crystal structure was revealed (Lindqvist et al., 1996). Although a *fab2* mutant of *Arabidopsis thaliana* had an inactive *SAD* and substantially increased stearic acid content, it still performed unsaturated fatty acid synthesis; the mutant exhibited a dwarf phenotype, which can tolerate high temperatures (Lightner et al., 1994). Therefore, *SAD* may play a key role in regulating fatty acid synthesis and membrane lipid fluidity in response to temperature change. *SAD* expression in *Brassica napus* cultivated at low temperature was upregulated, which resulted in elevated *SAD* protein levels (Tasseva et al., 2004). *SAD* overexpression in plants increases cold tolerance by increasing the desaturation of fatty acids, and membranes are less damaged (De Palma et al., 2008). The results of these studies indicate that it is possible to modify the composition of plant fatty acids by manipulating *SAD*.

SAD cDNA has been extracted from a variety of plants, including *Jatropha curcas* (Tong et al., 2006), *Helianthus annuus* (Salas et al., 2008), *Arabidopsis thaliana* (Cao et al., 2010), *Elaeis guineensis* (Saed Taha et al., 2012), *Chlorella zofingiensis* (Liu et al., 2012), and rice (Shelley et al., 2013). Over 70 *SAD* sequences, or sequence fragments, are registered on GenBank, but *SAD* from the tea plant, *Camellia sinensis* (L.) O. Kuntze, has not been characterized.

C. sinensis is thermophilic and has poor cold tolerance. The plant faces three main problems during the overwintering period: chilling or frost damage caused by chronic low temperatures, and snow or frost damage caused by heavy snowfall and glazed frost; water deficit caused by low rainfall and subnormal relative air humidity in winter; and cold or frost damage and wind damage caused by monsoons. The cell membrane is the most sensitive plant tissue to stress, particularly

low temperature. Cell membrane damage caused by low temperature is the root cause of plant cold injury and death. Therefore, it is important to study the stress resistance of the tea plant, particularly in response to low temperature, drought, and wounding.

Several studies have been conducted in order to better understand the physiological characteristics and molecular regulation mechanisms of the tea plant (Zhang et al., 2010; Ma et al., 2012; Wang et al., 2012; Pan et al., 2013). In the present study, we cloned the *CsSAD* gene, analyzed its sequence characteristics, and studied its expression under low temperatures, abscisic acid (ABA) treatment, drought stress, and wounding at low temperature. We also analyzed *CsSAD* expression in different tissues, and examined the effects of *CsSAD* expression in two varieties that exhibited different resistance to low temperature. This is the first study that has characterized *CsSAD*, and its results increase our understanding of the mechanisms involved in abiotic stress.

MATERIAL AND METHODS

Plant materials and treatments

Healthy, uniform 1-year-old clonal tea (*C. sinensis* cv. Longjing43) seedlings were procured from the Chengyang Tea Research Station (36°19' N, 120°23' E), Qingdao Agricultural University, China, and grown in a growth chamber maintained at 25° ± 3°C for 16 h during the day and at 20° ± 3°C for 8 h at night with 75% relative humidity for 2 weeks. For the low-temperature stress treatment, the plants were subjected to either 4° or -5°C. The plants that received the ABA treatment were sprayed with 0.1 mM ABA. Polyethylene glycol (PEG) is often used to study drought resistance in plants (Song and Wang, 2002); therefore, for the drought treatment, the plants were sprayed with 20% PEG-6000. For the wounding treatment, plants were cut into approximately 5-15- mm long sections with a sterile razor blade. For the natural cooling process, two varieties (one with large leaves named 'CsCr05', the other with small leaves named 'CsCr06') were chosen. Leaf samples were harvested and photographs taken from October 17, 2013 until January 16, 2014. The third mature leaf from the top was harvested at different periods (0, 1, 3, 6, 12, and 24 h) after treatment, immediately frozen in liquid nitrogen, and stored at -70°C for analysis.

Isolation of total RNA and preparation of cDNA

Total RNA was isolated using an Easy-Spin™ Rapid Plant Total RNA Extraction Kit (Yuan-pinghao Biotechnology Co. Ltd., Beijing, China) following the manufacturer instructions. RNA was quantified by electrophoresis on a 1.2% (w/v) agarose gel, which was visualized under ultraviolet light after staining with ethidium bromide. First-strand cDNA was obtained using a ReverTAid™ First-Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer instructions. The RNA was stored at -20°C.

CsSAD cloning

Alignments of the cDNA sequences of *Vernicia fordii* (GenBank accession No. GU363502), *Jatropha curcas* (DQ084491), *Vernicia montana* (EU072353), *Lotus corniculatus* (DQ020280), and *Glycine max* (L34346) identified several conserved domains. The amino acid sequences were obtained from the National Center for Biotechnology Information (NCBI) GenBank database (<http://www.ncbi.nlm.nih.gov/>). Two conserved regions were used to design sense and antisense degen-

erate primers for *SAD* amplification. The amplification of the middle fragment was performed using a pair of degenerated primers, S1 and S2. The 3'- and 5'-polymerase chain reaction (PCR) primers were designed based on the middle fragment sequences. A 3'-rapid amplification of cDNA ends (RACE)-PCR of *SAD* cDNA was initially conducted using the primer pair B26 and S5, and then by nested PCR using the primers B25 and S6. A 5'-RACE-PCR amplification was conducted by using the primers AAP and S3, followed by AUAP and S4. Based on the nucleotide sequences of the 5'- and 3'-RACE products, the primers S7 and S8 were used for the amplification of the complete coding sequence of *CsSAD*. The primers and their annealing temperatures are listed in Table 1.

PCRs were performed in a total reaction volume of 25 μ L that contained 2 μ L DNA template, 2 μ L of each primer, 12.5 μ L PCR Master Mix, and 6.5 μ L ddH₂O. The PCR conditions were 94°C for 5 min, 94°C for 30 s, 57°C for 45 s, 72°C for 1 min for 35 cycles, and a final extension at 72°C for 10 min. The PCR products were separated on 1.2% agarose gels and the targeted DNA fragments were recovered and cloned into a pTZ57R/T vector using a TA cloning® kit (Promega). The ligated products were transformed into *Escherichia coli* (DH5 α) cells and sequenced by the Sangon Biotech Co., Shanghai, China.

Table 1. Primers used in the study.

Primer	Sequence (5'→3')	Annealing temperature (°C)	Usage
S1	AAGAAGCCWTTYAYKCCTCC	55.1	for middle fragments
S2	TACATSARGTGTGCHGGCAT	54.3	
S3	ATACATCATTCCAAGAGCGG	57.6	for 5' RACE
S4	CTTTGAGATTGACCTGATG	55.6	for 3' RACE
AAP	GGCCACGCGTCCGACTAGTACGGGGGGGGGGGGGGGG	81.4	for full-length
AUAP	GGCCACGCGTCCGACTAGTAC	63.9	for RT-PCR
S5	CCTTTGCCCTCTCCCTTAGT	59.7	
S6	GTTGCCAACATTTCTCCACT	57.6	
B25	GACTCTAGACGACATCGA	48.6	
B26	GACTCTAGACGACATCGATTTTTTTTTTTTTTTTT	66.1	
S7	GCCTTTTAGCCCTCCTCATG	61.1	
S8	CTTCGCACAACAGTATTAAG	51.3	
S9	GCTAAGGACCACGCAGATAT	56.9	
S10	CTTTCCTCAAGCCGTCTAAT	56.2	
GAPDH-F	TTGGCATCGTTGAGGGTCT	61.6	
GAPDH-R	CAGTGGGAACACGGAAGC	61.7	

RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction.

Bioinformatic analysis

The Primer Premier 5 software (<http://www.Premierbiosoft.com>) was used for all of the primer designs. Sequences of *SAD* proteins from a variety of plant species were obtained from the NCBI database for phylogenetic analysis. Multiple-sequence alignments and a phylogenetic tree were generated based on the neighbor-joining method in DNAMAN (Lynnon Biosoft). Subcellular localization was predicted using the PSORT tool (<http://www.psort.org/>), and EXPASY (<http://www.expasy.cn/tools/>) was used to predict the properties and structure of the protein (Gasteiger et al., 2005).

Real-time quantitative reverse transcription (qRT)-PCR

The primers S9 and S10 were synthesized based on a conserved region of the *SAD* sequences obtained. The universal primers *GAPDH-F* and *GAPDH-R* were used as internal constitutively expressed controls (reference genes) for calculating the relative transcript abundance.

All of the primers used for the qRT-PCR are listed in Table 1. The qRT-PCR was conducted using a Maxima™ SYBR® Green qPCR Master Mix (2X) Kit following the manufacturer protocol. A total of 2 µL cDNA was used as a template for the PCR. The PCR cycling conditions consisted of an initial polymerase activation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 57°C for 1 min. The internal reference gene *GADPH* and target gene *SAD* were analyzed on one plate, and the reactions were run in triplicate for each sample to ensure that the results were reproducible. An amplification curve was generated after analyzing the raw data and adjusting the cycle threshold (Ct) value. The model $2^{-\Delta\Delta Ct}$ for comparing relative expression results under different treatments was used (Livak and Schmittgen, 2001). The number of targets, normalized to the reference control and relative to a calibrator, was given by $R = 2^{-\Delta\Delta Ct}$, where $2^{-\Delta\Delta Ct} = 2^{-\Delta Ct \text{ sample} - \Delta Ct \text{ control}}$. The final value obtained was a measure of the fold change in gene expression for the particular gene of interest between the treated samples and the untreated samples.

RESULTS

Characterization of CsSAD

The composite nucleotide sequence of the full-length *SAD* cDNA was determined from cDNA clones by 3'- and 5'-RACE-PCR (Figure 1). The 1591-bp *SAD* cDNA contained a coding domain sequence of 1191 bp and was flanked by 5'- and 3'-untranslated regions (UTRs) of 442 and 881 nucleotides, respectively. Its GenBank accession number is KC242133. The 999-bp coding domain sequence began with ATG at position 75 and ended with the termination codon TAA at position 1262. Based on the results of the multiple alignment of *CsSAD* with other *SAD* genes, the deduced amino acid sequence had two conserved domains: one belonged to the acyl-ACP desaturase family and the other to a ferritin-like family (Figure 2), which suggests that *CsSAD* belongs to both the acyl-ACP desaturase family and the ferritin-like family (Davydov et al., 2005; Tong et al., 2006). The phylogenetic analysis revealed that the *CsSAD* protein sequence had 80% identity with the *A. thaliana SAD* (*AtSAD*), 93% identity with the *Camellia oleifera SAD* (*CoSAD*), and 86-90% identity with other *SAD* genes (Figure 3). *CsSAD* had the greatest similarity to *CoSAD*, and a lower similarity to *AtSAD* and the *B. napus SAD*.

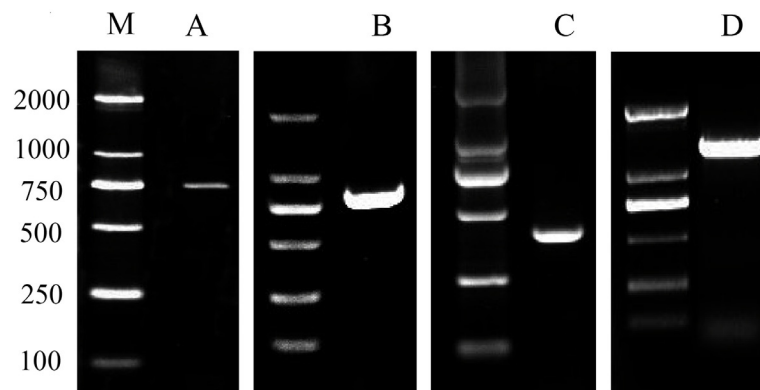


Figure 1. Isolation of *SAD* from tea (*Camellia sinensis*). Lane M, marker; lanes A-D, middle fragment, 3'-fragment, 5'-fragment, and full-length cDNA, respectively.

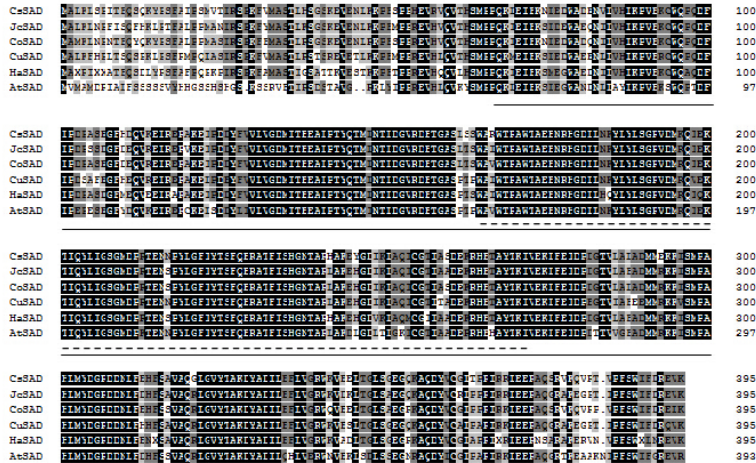


Figure 2. Alignments of predicted amino acid sequences of SAD from different plant species. The acyl-ACP desaturase and ferritin-like family domains are underlined by solid and dashed lines, respectively. *JcSAD*, *Jatropha curcas* SAD; *CoSAD*, *Camellia oleifera* SAD; *CuSAD*, *Cucumis sativus* SAD; *HaSAD*, *Helianthus annuus* SAD; *AtSAD*, *Arabidopsis thaliana* SAD.

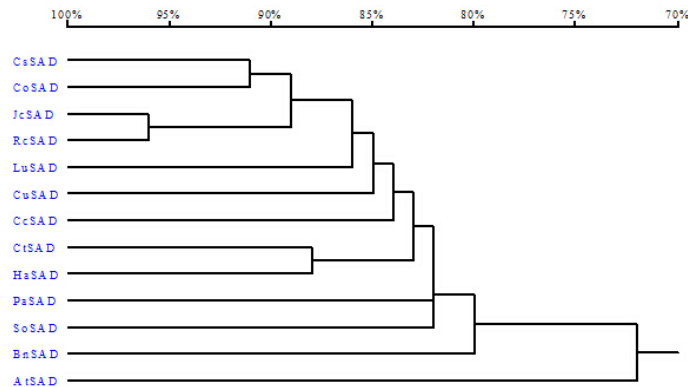


Figure 3. Phylogenetic tree of deduced SAD amino acid sequences in different plant species. The percentage values represent the putative protein sequence similarity between *Camellia sinensis* and other plants. *CoSAD*, *Camellia oleifera* SAD; *JcSAD*, *Jatropha curcas* SAD; *RcSAD*, *Ricinus communis* SAD; *LuSAD*, *Linum usitatissimum* SAD; *CuSAD*, *Cucumis sativus* SAD; *CcSAD*, *Camellia chekiangoleosa* SAD; *CtSAD*, *Carthamus tinctorius* SAD; *HaSAD*, *Helianthus annuus* SAD; *PaSAD*, *Persea americana* SAD; *SoSAD*, *Spinacia oleracea* SAD; *BnSAD*, *Brassica napus* SAD; *AtSAD*, *Arabidopsis thaliana* SAD.

Sequence analysis of the CsSAD protein

The deduced SAD protein was composed of 396 amino acids with a calculated molecular mass of 45.1 kDa, a theoretical isoelectric point of 6.42, and an instability index of 30.98. Based on the results of hydrophilic/hydrophobic analysis, we found that the hydrophilic part was higher than the hydrophobic part (Figure 4). Therefore, the *CsSAD* encoding product may be a hydrophilic protein. Subcellular localization of the deduced *CsSAD* amino acid sequence using the PSORT tool revealed that it was localized in the chloroplast. The secondary structure of the SAD protein

was analyzed using SOPMA (Ullah et al., 2012), which revealed that it contained an alpha helix, an extended chain, a beta turn, and a random curl. These four structures accounted for 53.79, 8.33, 6.31, and 31.57% of the total.

CsSAD expression patterns in different organs

We analyzed CsSAD expression patterns in different organs at different temperatures and periods (Figure 5A-C). At 25°C, the CsSAD transcriptional level was the highest in the leaf and lowest in the stem, and there was an obvious difference between them. Differences in CsSAD expression were found between three organs at 4° and -5°C (Figure 5B and C). At 4°C, root CsSAD expression increased before decreasing within 3 h, while it gradually increased in the leaf and stem. At -5°C, CsSAD expression increased before decreasing in each organ.

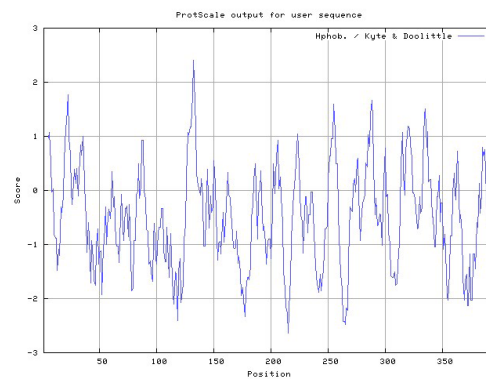


Figure 4. Hydrophilic analysis of the SAD cDNA encoding region product from *Camellia sinensis*.

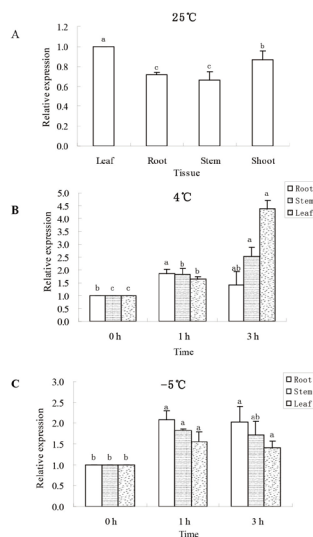


Figure 5. Relative CsSAD expression levels. **A.** CsSAD expression levels in different tissues at 25°C. **B.** CsSAD expression levels in different tissues and at different time points at 4°C. **C.** CsSAD expression levels in different tissues and at different time points at -5°C.

Leaf *CsSAD* expression in response to stress

At 4°C, *CsSAD* expression rapidly increased at 1 h after treatment, dramatically decreased at 3 h, increased again at 6 h, and then decreased to the previous level, with no significant change in the subsequent periods (Figure 6A). At -5°C, *CsSAD* expression increased at 1 h, decreased slightly at 3 h, remained at a stable level until 12 h, before slightly increasing again at 24 h. In general, the *CsSAD* expression level at 4°C was higher than that at -5°C, except at 24 h, suggesting that the dark period between 16 and 24 h was involved in the upregulation of *CsSAD*.

At 25° and 4°C after ABA treatment, *CsSAD* expression increased at first and then decreased, reaching its maximum at 6 h at 25°C and at 12 h at 4°C, which suggests that low temperature could delay the response to ABA (Figure 6B).

After PEG treatment, *CsSAD* expression had increased after 1 h at 25°C, decreased at 3 h, and dramatically increased to a maximum at 12 h (Figure 6C). At 4°C, *CsSAD* expression dramatically increased after 1 h, gradually decreased by 12 h, and increased again at 24 h. Therefore, tea plants take different times to respond to drought depending on the temperature, and a relatively low temperature could accelerate drought damage.

After wounding at 25°C, *CsSAD* expression increased for 3 h, decreased, then increased again at 24 h (Figure 6D). At 4°C, its expression increased for 6 h, immediately decreased to its original level, and was barely discernible at 24 h, suggesting that there was an interaction between wounding and low temperature.

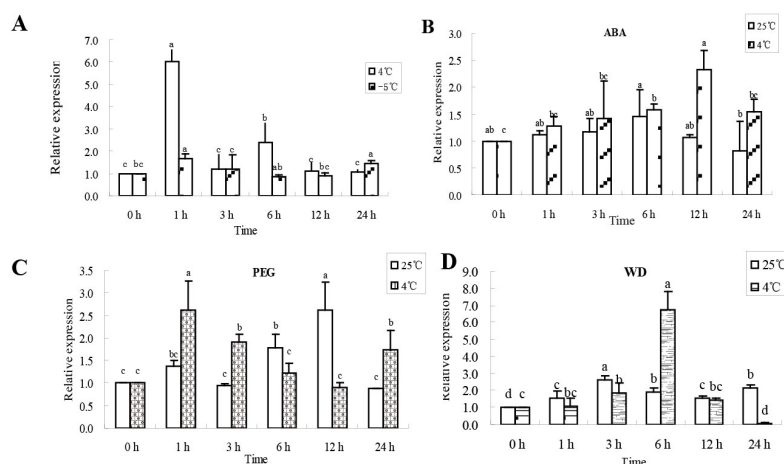


Figure 6. Relative *CsSAD* expression levels at different time points in response to different treatments. **A.** Low temperature. **B.** Abscisic acid (ABA). **C.** Polyethylene glycol (PEG). **D.** Wounding (WD).

CsSAD expression during natural cold acclimation

The temperature decreased from October 17, 2013 to January 16, 2014 (Figure 7A and B), and *CsSAD* expression in 'CsCr05' increased at first before decreasing with the temperature; it reached its highest value on December 4. *CsSAD* expression in 'CsCr06' remained relatively stable, but dramatically increased to its maximum value on January 16. These results suggest that *CsSAD* is upregulated in both 'CsCr05' and 'CsCr06' at low temperature, but they differ in their re-

sponses over time, possibly because they have different sensitivities to low temperature. Changes in leaf color were similar in the two varieties (Figure 7C). The leaves showed no visible signs of cold injury until November 13, when the leaves had lost their luster and had darkened. During the cold acclimation process, 'CsCr05' leaves became swollen and crinkled, before they turned red from the edge. Meanwhile, 'CsCr06' leaves turned yellow and brown, but cold damage occurred later than in 'CsCr05'.

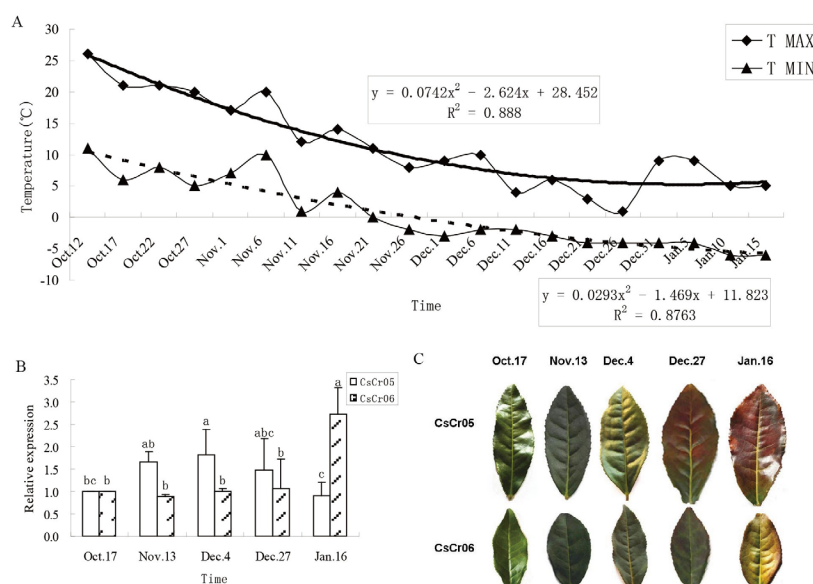


Figure 7. Transcription analysis of CsSAD during the natural cooling process. **A.** Changes in temperature and humidity during the natural cooling process. **B.** CsSAD transcription accumulation during the natural cooling process. **C.** Leaf-color changes in two varieties of *Camellia sinensis* during the natural cooling process.

DISCUSSION

SAD is involved in many plant processes that are based on catalyzing the desaturation of stearyl-ACP, such as resistance to fungi and bacteria (Moche et al., 2003), maintenance of the crystalline liquid membrane (Los and Murata, 1998), regulation of growth, and the formation of storage oils (Zhang et al., 2008). In this study, a novel full-length SAD gene was isolated from developing leaves of *C. sinensis*. The deduced CsSAD peptide sequence was highly homologous (79-93% sequence identity) to other SAD genes, which suggests that SAD genes have been highly conserved during evolution.

We found that CsSAD expression is tissue-specific at the chilling temperature of 4°C and the freezing temperature of -5°C. At 25°C, the CsSAD transcriptional level was highest in the leaf and lowest in the stem, and there were obvious differences between the organs, except between the stem and root. At 4°C, different organs of the plants exhibited different responses at the same temperature. At -5°C, CsSAD expression in the roots was greater than that in the leaves, possibly because the quantity of synthesized unsaturated fatty acids in the roots was greater than in the leaves. This is consistent with the fact that the cold resistance of roots and stems is greater than that of leaves. Previous studies have also found that the expression of some SAD genes is tissue-

specific. Zaborowska et al. (2002) found that *SAD* is moderately expressed in the leaves and stems of yellow lupine (*Lupinus luteus*). In lima bean (*Phaseolus lunatus*) plants, RT-PCR analysis revealed a high level of *PISAD* expression in the leaves, but few transcripts were found in the stems and roots (Zhang et al., 2011). In wild-type *Arabidopsis* plants, transcripts of most *SAD* genes have been reported in all of the tissues, and almost all *SAD* genes (except for one) exhibit tissue specificity (Kachroo et al., 2006). They are expressed at different levels in the root, stem, and leaf tissues, suggesting tissue specificity expression abundance of the *SAD* gene family. Other mechanisms, such as transcriptional and post-transcriptional regulation or substrate inhibition, might also be involved (Wang et al., 2013).

We found that *CsSAD* was upregulated by different degrees by ABA, drought, and wounding at low temperature and at 25°C; however, at 4°C, the maximum expression level occurred later than at 25°C after ABA treatment or wounding, whereas it occurred earlier after PEG treatment. This result indicates that low temperature may delay the response to ABA and wounding, but accelerates the response to drought stress. A large body of research suggests that temperature regulates fatty acid desaturase expression at both the transcriptional and post-transcriptional levels (Upchurch, 2008). Vega et al. (2004) reported an increase in *SAD* transcript levels in *Solanum commersonii* after cold acclimation, and suggested that this increase may be associated with an increase in freezing tolerance in the potato. *SAD* transcript accumulation increased in the leaves of cold-acclimated *S. commersonii* leaves, but not in cultivated, non-acclimated varieties (Vega et al., 2004). The upregulation of *CsSAD* transcripts may be an important factor that contributes to the susceptibility to chilling that characterizes tea plants (Byfield and Upchurch, 2007). In this study, *CsSAD* expression patterns were investigated under stress conditions at low temperature, with ABA being the most important stressor. ABA is found extensively in plants (Lui and Li, 2001), plays a pivotal role in adaptive stress responses to environmental stimuli, and has been shown to induce the expression of a variety of genes (Liu et al., 2014). ABA increases in response to water deficit, and regulates root growth in order to maintain an adequate water supply during drought (Sharp et al., 2004). Several transgenic studies have improved drought tolerance by increasing ABA levels through the alteration of ABA biosynthetic and catabolic pathways (Sreenivasulu et al., 2012). It is not known which step in ABA biosynthesis is activated by mechanical damage. These findings indicate that in the plant kingdom, a variety of defense networks may function as a combination of different signals with a set of defense-related genes. Our results suggest that the ABA pathway does indeed play a significant role in the regulation of *CsSAD* expression.

Frost damage is caused by meteorological conditions such as low temperature, drought, and strong winds, which often occur together. Low temperature is the main reason for frost damage, while drought and wind often exacerbate the damage. Frost damage can also cause mechanical stress. We studied *CsSAD* expression levels in 'CsCr05' and 'CsCr06' during natural cold acclimation, and found that they increased and then decreased in 'CsCr05'. In 'CsCr06', they exhibited no obvious change at first, but increased to a maximum when the temperature was very low. Regarding the plants' leaves, frost damage started within the tip and edge of the uppermost leaves and then extended to the middle part of the blade, which turned yellow and brown. The mature leaves lost their luster and curled, before withering. Large-leaf varieties usually have thin leaves with a single layer of palisade tissue and thick, spongy tissue, and often suffer frost damage at -5°C, while medium- and small-leaf varieties can endure -15°C for a short period; therefore, the cold and drought resistance of large-leaf varieties is poor. Because the leaves of small-leaf varieties contain multilayer palisade tissue, most of them have strong cold and drought resistance. This difference in cold resistance between different varieties is important when selecting cold-resistant germplasm resources.

To the best of our knowledge, this is the first report on the cloning and expression of *SAD* in *C. sinensis*. Our data demonstrate that *CsSAD* participates in stress-response pathways induced by ABA, drought, wounding, and low temperature. Abiotic stress altered gene expression both positively and negatively, in a regulatory network with synergistic and antagonistic effects that has an important function in improving stress resistance in the tea plant. Future studies should investigate the mechanisms involved in this regulatory network, and how different regulating pathways interact.

Conflicts of interest

The authors declare no conflict of interest.

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