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Overexpression of AtCBF1 in *Sinningia speciosa* Enhances Chilling Stress Tolerance

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Abbreviations: APX: Ascorbate Peroxidase; AtCBF1: *Arabidopsis Thaliana* C-Repeat Binding Factor 1; 6-BA: 6-Benzylaminopurine; CAT: Catalase; H₂O₂: Hydrogen Peroxide; Kan: Kanamycin; MDA: Malondialdehyde; MS medium: Murashige and Skoog Medium; NAA: Naphthalene Acetic Acid; O₂•⁻: Superoxide Radical; OE: Overexpression Plant; POD: Peroxidase; ROS: Reactive Oxygen Species; SOD: Superoxide Dismutase; WT: Wild Type.

ABSTRACT

Chilling is a common environmental stress strongly limits the survival and quality of plants. As tropical flower, *Sinningia speciosa* can hardly survive below 5°C. Breeding new chilling-tolerant *Sinningia speciosa* varieties by transgenic approaches would expand its geographical distribution. As a transcriptional factor, C-Repeat Binding Factor 1 (CBF1) could increase chilling tolerance of many plants. In this study, *Arabidopsis thaliana* CBF1 (AtCBF1) gene was successfully transformed into *Sinningia speciosa*. Compared with wild-type plants (WT), the overexpressing plants (OE) exhibited low levels of hydrogen peroxide (H₂O₂) and superoxide radical (O₂•⁻) under 4°C chilling stress. Similarly, less malondialdehyde (MDA) content accumulated in OE than in WT plants. Consistent with the accumulation of reactive oxygen species, the activities of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POD), were apparently higher in OE than in WT plants. The changes of physiological indexes were consistent with the phenotype analysis under chilling stress. Overexpression of AtCBF1 could also increase the resistance of osmotic stress in detached leaves. This will be very helpful for expanding the planting of *Sinningia speciosa* in low-temperature areas.

INTRODUCTION

Abiotic stresses, especially chilling and drought stresses are the major environmental factors that affect the growth and geographical distribution of chilling-sensitive plants^[1,2]. Transgenic breeding is an important mechanism for improving yields, qualities, and other traits of commercial value.

Sinningia speciosa, also known as gloxinia, originates from tropical South America and is widely cultivated as ornamental flower around the world because of its large oval leaves and gorgeous flowers^[3,4]. *Sinningia speciosa* is a tropical chilling-sensitive flower that can hardly survive below 4°C^[5]. Chilling stress not only limits the geographical distribution of *Sinningia speciosa*

but also reduces its quality and the market productivity. Therefore, cultivating cold-resistant varieties would expand the indoor flower's growth distribution and would be suitable to grow outdoors in some area. Transgenic breeding provides a powerful tool for developing new *Sinningia speciosa* traits that conventional breeding can rarely achieve. Although *Sinningia speciosa* has been transformed in other studies, no study has attempted to improve the chilling tolerance of *Sinningia speciosa* [6-8].

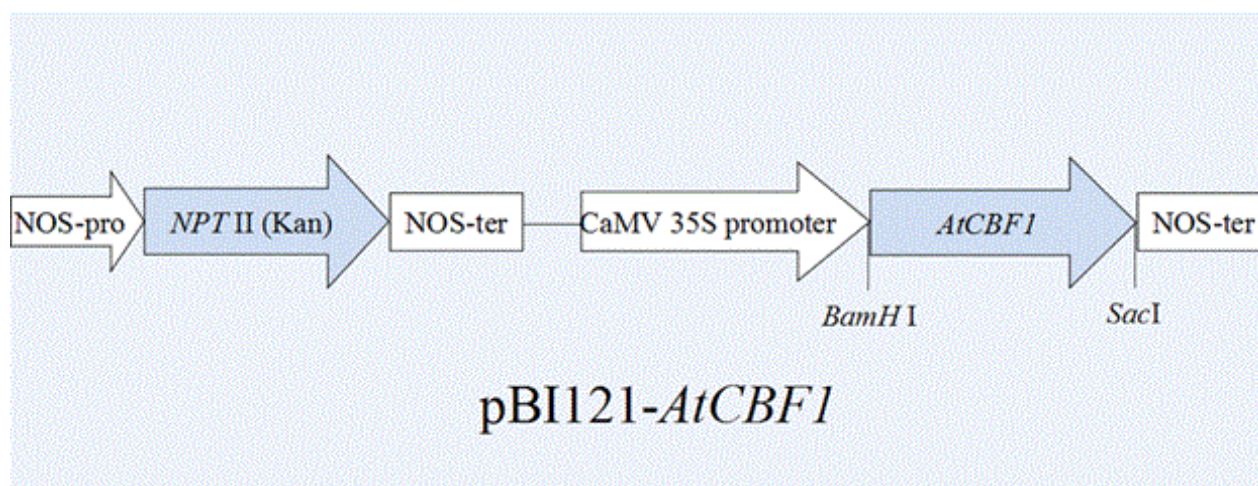
As a transcriptional factor, CBF1 plays an important role in resistance to cold and dehydration in *Arabidopsis*[9-11]. Moreover, CBF1 has been transformed into many plants, including tomato, rice and potato, and so on. It may enhance tolerance to low temperature, drought, and salt [12-14]. However, whether overexpressing CBF1 has a function in tropical ornamental flowers has not been explored. We wondered whether *AtCBF1* could increase the chilling stress of *Sinningia speciosa*, without affecting its ornamental value.

In the present study, to increase the tolerance of chilling stress of *Sinningia speciosa*, *AtCBF1* gene was successfully transformed into *Sinningia speciosa* and screened out overexpression plants (OE). The phenotype analysis of the transgenic plant showed an obvious higher chilling tolerance than that of the WT. Lower accumulation of H_2O_2 , $O_2^{\bullet-}$ and MDA were accumulated in OE than in WT plants under chilling stress. Moreover, higher antioxidant enzyme activities were observed in transgenic *Sinningia speciosa*. Transgenic *Sinningia speciosa* also had increased resistance to salt and drought stress. All results confirmed that overexpressing *AtCBF1* in *Sinningia speciosa* increased abiotic stress tolerance.

MATERIALS AND METHODS

Plasmid Construction

The CBF1 gene was isolated by PCR from 4-week-old *Arabidopsis thaliana* seedlings with the forward primer (5'-CCGGATCCATGAACTCATTTTCAGCTT-3') and the reverse primer (5'-CCCGAGCTCTTAGTAAGTCCAAAGCGA-3') and was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in the pBI121 expression plasmid (designated as pBI121-*AtCBF1*) carrying the selectable marker neomycin phosphotransferase II (npt II), as shown in **Figure 1**.



NPTII: Neomycin PhosphoTransferase II; CaMV 35S: Cauliflower Mosaic Virus 35S promoter; NOS-ter: Terminator Of Itric Oxide Synthase; NOS-pro: Promoter Of Nitric Oxide Synthase

Figure 1. Diagram of binary vector pBI121 with the *AtCBF1* gene.

The recombinant plasmid was sequenced by Sunny biotechnology company (Shanghai China) and confirmed through alignment with the sequence in NCBI. The recombinant vector was transformed into *Agrobacterium tumefaciens* strain LBA4404.

Agrobacterium Mediated Transformation of *Sinningia Speciosa*

Leaf discs (10 mm in diameter) and petioles (7-10 mm in length) of *Sinningia speciosa* were pre-cultured on inducing medium, Murashige and Skoog (MS, pH 5.8) + 1.0 mg • L⁻¹ 6-BA + 0.1 mg • L⁻¹ NAA + 15 g • L⁻¹ sucrose + 7.5 g • L⁻¹ agar, for 1 day prior to inoculation with bacteria. Cultures of the *Agrobacterium tumefaciens* strain LBA4404 carrying pBI121-*AtCBF1* grown for five hours (OD600=0.4-0.6) were centrifuged at 4,000 rpm for 8 min and were re-suspended in inducing medium. The pre-cultured explants were infected with bacterial culture for 3-5 min. After being blotted dry, the infected explants were placed on the same medium for 2 days in the dark. Then, the explants were washed 4 times with sterile water with 250 mg • L⁻¹ cephotaxime and were placed on selection medium, inducing medium + 20 mg • L⁻¹ kanamycin (Kan) + 250 mg • L⁻¹ cephotaxime, to prevent bacterial overgrowth. Sub-culturing was performed every 7 days until shoots appeared. After 2 months, adventitious shoots generated from the infected explants (>1.0 cm) were excised and cultured in rooting medium, MS + 0.2 mg • L⁻¹ NAA + 30 g • L⁻¹ sucrose + 10 mg • L⁻¹ Kan, for further growth and selection. After 30 days, the rooted seedlings were transferred to a greenhouse at 25°C for further study. In comparison to previous studies about transformation of *Sinningia speciosa*, we mainly shortened the infection

time from 10-15 min to 3-5 min and decreased the sucrose added in the inducing medium to $15 \text{ g} \cdot \text{L}^{-1}$ instead of $30 \text{ g} \cdot \text{L}^{-1}$, in order to reduce the browning of explants.

DNA and RNA Extraction, Polymerase Chain Reaction (PCR) Analysis and Real - Time Quantitative Reverse Transcription - PCR (qRT-PCR)

Fresh leaves from the independent putative seedlings were ground for genomic DNA extraction, using the modified Cetyl Trimethyl Ammonium Bromide (CTAB) method of Doyle and Doyle [15]. The total RNA was extracted from the leaves and flowers using the EASYspin Plant RNA Kit of Aidlab Biotech (Beijing, China). The cDNA synthesis was performed according to standard procedures of RevertAid First Strand cDNA Synthesis Kit using total RNA as template (Fermentas, Canada).

To identify the transgenic seedlings, PCR analysis was performed using the genome DNA as template. To check the expression of AtCBF1, the PCR analysis was performed using the cDNA as template. The gene-specific primers for AtCBF1 were 5'-CCGGATCCATGAACATTTTCAGCTT-3' and 5'-CCCAGCTCTTAGTAATC CAAAGCGA-3'.

To determine the copy numbers in genome and the transcriptional expression level of AtCBF1 in transgenic plants, The mean Cycle Threshold (Ct) values versus different copies (10^3 to 10^8 copies $\cdot \mu\text{L}^{-1}$) of plasmid containing AtCBF1 gene or *Actin-1* were determined by qRT-PCR as the standard curves for AtCBF1 and for *Actin-1*, according to previous studies. Using the DNA or cDNA as template, the Ct value of AtCBF1 in transgenic lines were determined by qRT-PCR and plugged into the standard curves to confirm the copy numbers and expression level of AtCBF1 [16-18]. The primers for absolute quantification were designed by the Beacon Designer software as followed: 5'- GATACGACGACCACGAAT -3' and 5'-GCTCTGTTCCGGTGTATA -3' for AtCBF1. 5'-CAATAAATTGCGTGTGCTCCTGAG -3' and 5'-TGTTCCGTACCGATCCTTTCTGATA-3' for *Actin-1* [19].

Plant Materials and Treatments

The vegetative propagated *Sinningia speciosa* were grown in a greenhouse at $24 \pm 1^\circ\text{C}$ with a 16 h light/8 h dark cycle and ambient humidity (>84%) for 30 days. For chilling stress, the temperature was reduced to 4°C , and the plants were photographed routinely. The leaves and flowers were collected after 1 day, 2 days and 3 days chilling treatment and used to measuring the oxidative damage. For osmotic stress, the 1 cm^2 leaf-discs excised from the plants were floated in 200 mM NaCl or 20% (w/v) PEG6000 for 48 h [20]. Then, the chlorophyll content of the leaf-discs was calculated according to Wu and Li using 95% ethanol [21]. In every experiment, at least three leaves from three different plants were measured, and three independent experiments were conducted.

Determination of H_2O_2 , $\text{O}_2 \cdot^-$ and MDA Contents

Oxidative damage to the cell membrane was determined by measuring the content of H_2O_2 and $\text{O}_2 \cdot^-$ according to prior methods with slight modifications [22,23]. The content of Malondialdehyde (MDA) from the leaves of the OE and WT plants was assayed according to the existing methods [24] the leaves were prepared in 10% trichloroacetic acid containing 0.65% 2-Thiobarbituric Acid (TBA) and heated at 100°C for 15 min; then the absorbance at 532 nm, 600 nm and 450 nm were measured.

Extraction and Activity Assay of Antioxidant Enzymes

To assay the activities of antioxidant enzymes, liquid nitrogen-ground leaves (0.5 g) were suspended in 5 mL ice-cold buffer (25 mM, pH 7.8) containing 4% PVP and 0.3 mM EDTA. The homogenate was centrifuged at 4°C and 12,000 g for 20 min, and the supernatant was used to determine the activities of antioxidant enzymes [25]. Catalase (CAT) activity was assayed at 240 nm via the decomposing of H_2O_2 according to an existing method [26]. To analyse the activity of APX at 290 nm, H_2O_2 -dependent oxidation of ascorbic acid (AsA, $\epsilon = 2.8 \text{ mM} \cdot \text{cm}^{-1}$) was used [27]. The SOD activity was measured by recording the rate of p-nitro blue tetrazolium chloride (NBT) reduction at an absorbance of 560 nm [28]. The activity of peroxidase (POD) was analysed according to previous studies [29].

RESULTS

Transgenic Plants with High Expression of AtCBF1 were Generated

Total 120 kanamycin (Kan) resistant seedlings were screened out from about 300 explants after transformation and 18 independent transgenic lines were further confirmed by PCR using genome DNA as template. The successful transformants ratio was near 6%. The integration of AtCBF1 into nuclear genome and the expression were confirmed by PCR analysis using genome DNA or cDNA as template **Figure 2**.

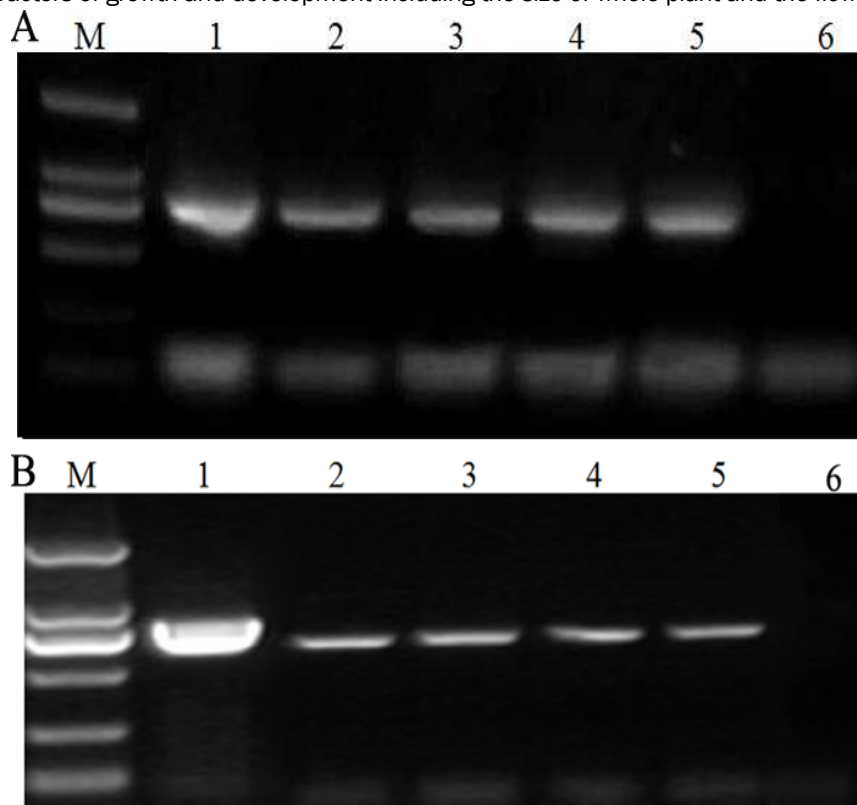
The copy numbers in genome and the transcriptional expression level of AtCBF1 were confirmed by the real-time quantitative reverse transcription-PCR (qRT-PCR) **Figure 3**.

High transcriptional expression level of AtCBF1 both in leaves and flowers suggested that AtCBF1 may play roles in stress response. The transgenic plants were further used for vegetative propagation and following studies.

Overexpression of AtCBF1 conferred transgenic plants higher chilling resistance

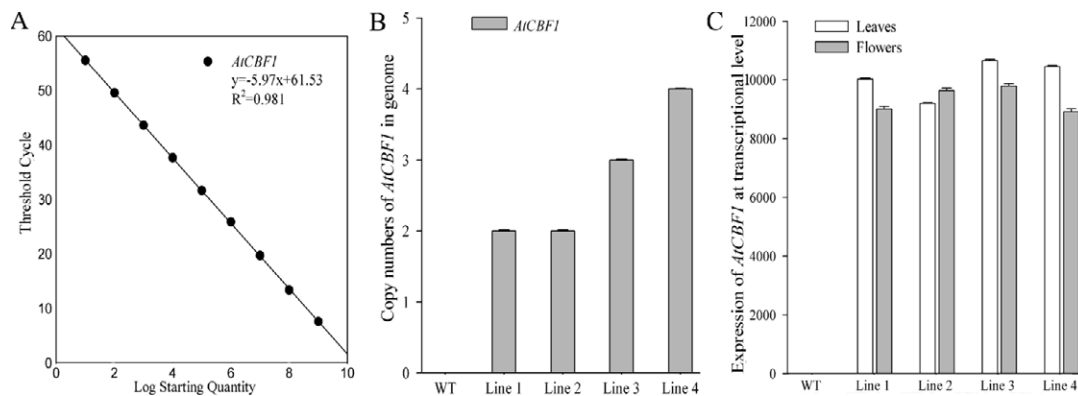
To reveal whether overexpression of AtCBF1 increased the chilling tolerance of *Sinningia speciosa*, 3-month-old WT and OE

plants were cultivated under chilling stress at 4°C. As shown in, before chilling treatment, little difference between WT and OE plants could be found in characters of growth and development including the size of whole plant and the flower time as well **Figure 4**.



A: PCR amplification of *AtCBF1* gene (642 bp) in genome of candidate transgenic plants; M: 2K DNA Marker
Lane 1: Positive Plasmid Control; Lane 2-5: Transgenic Plants; Lane 6: Untransformed WT Plants
B: PCR Amplification of *AtCBF1* gene in transcriptional level of candidate transgenic plants; M: 2K DNA Marker
Lane 1: Positive Plasmid Control; Lane 2-5: Transgenic Plants; Lane 6: Untransformed WT Plants

Figure 2. Identification of transgenic plants.



A: Standard curves used for quantifying *AtCBF1*; B: Copy number of *AtCBF1* in genome of 4 transgenic Plants;
C: Transcriptional expression level of *AtCBF1* in leaves and flowers of 4 transgenic plants. The error bars indicated the SD. Values with the different letters are significantly different at $P < 0.05$, as determined by SAS software version 9.1

Figure 3. Copy numbers in genome and transcriptional expression level of *AtCBF1* in transgenic *Sinningia speciosa*

After 1 day of chilling treatment, one or two leaves of WT plant were injured and became grey, while OE leaves were almost unchanged. After 2 days of chilling treatment, four leaves except for the tender leaves of WT plant were wilted; however, only one or two leaves of OE plant were slightly wilted. After 3 days of chilling treatment, six leaves of WT plant were all injured and 50% of the WT plants died, while the OE plants were all alive. This phenotype strongly confirms that overexpression of *AtCBF1* in transgenic *Sinningia speciosa* increases chilling tolerance.

Accumulation of ROS was Decreased by Overexpression of *AtCBF1* under Chilling Stress

Under chilling stress, the accumulation of reactive oxygen species (ROS) aggravates the damage to the cell membrane. Malondialdehyde (MDA) was commonly used as an indicator of membrane lipid peroxidation. To gain insight into the differences

in membrane damage between WT and OE plants under 4°C chilling stress, we detected the H₂O₂, O₂^{•-} and MDA content. Under normal conditions, the transgenic plants had a similar H₂O₂ level compared with the WT plants. After chilling treatment, H₂O₂ content increased in both the WT and OE plants; however, the increase in the WT plants was obviously higher than that in the OE plants **Figure 5A**. Similarly, the contents of O₂^{•-} and MDA were obviously lower in the OE than in the WT plants **Figure 5B and 5C**.

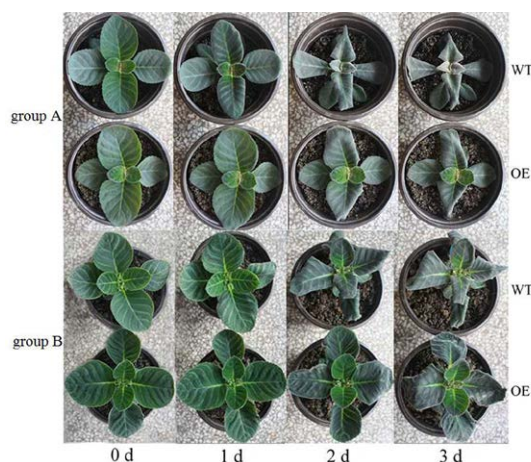
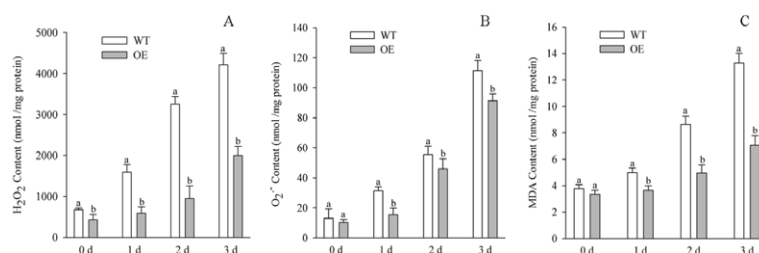


Figure 4. Phenotypic comparison of WT and OE *Sinningia speciosa* plants treated at 4°C for 0, 1, 2 and 3 days



The error bars indicated the SD. Values with the different letters are significantly different at P<0.05, as determined by SAS software version 9.1

Figure 5. Accumulation of H₂O₂, O₂^{•-} and MDA in leaves under 4°C treatment for 0, 1, 2 and 3 days

The lower accumulation of MDA, H₂O₂ and O₂^{•-} in the OE plants suggested that they experienced less oxidative injuries than the WT plants under chilling stress.

Activities of Antioxidant Enzyme were Increased by Overexpression of AtCBF1 Under Chilling Stress

ROS accumulation under chilling stress is often related with the activity of antioxidant enzymes [30]. Thus, the activities of antioxidant enzymes in WT and OE *Sinningia speciosa* were assayed. As shown in Figure 6A, under normal conditions, the activity of SOD was not different between the WT and OE plants. After 1 day of 4°C chilling treatment, SOD activity was upregulated, and reached its peak. The activity of SOD in OE plants was always apparently higher than that in the WT plants. However, after 3 days of treatment, SOD activity in the WT plants was lower than that at 0 day, suggesting serious damage in the WT but not in the OE plants. Similarly, CAT, POD and APX activities showed a similar trend for SOD activity **Figure 6B-D**.

These results indicate that the overexpression of AtCBF1 in *Sinningia speciosa* improved the activities of antioxidant enzymes and further increased chilling stress tolerance.

Overexpression AtCBF1 also Enhanced the Tolerance of Salt and Drought Stresses.

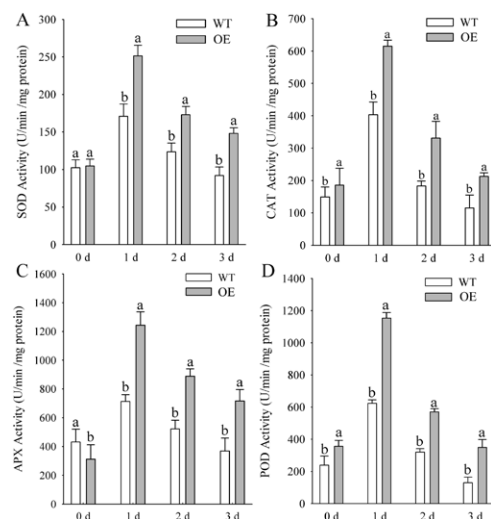
CBF1 is known to have multiple effects on abiotic stresses. We examined the osmotic tolerance of transgenic *Sinningia speciosa* by floating leaf-discs in 200 mM NaCl or 20% (w/v) PEG6000 for 48 h. As shown in **Figure 7**, the leaf-discs treated with distilled water retained almost the same percentage of chlorophyll in the WT and OE plants.

However, after 48 h of the salt or drought treatment, the leaf-discs of the WT plants were bleached to some degree, while the OE plants retained a higher percentage of chlorophyll content compared to the WT plants. This result suggests that transgenic *Sinningia speciosa* not only has increased chilling stress tolerance but also increased osmotic stress tolerance.

DISCUSSION

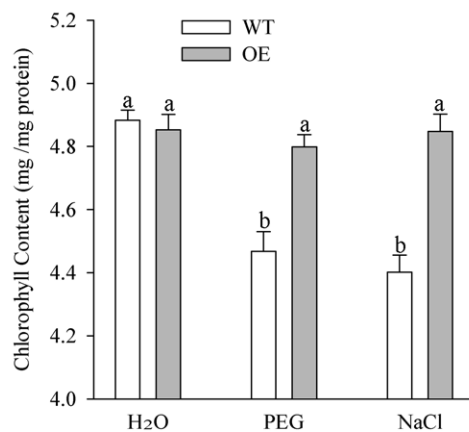
The quality of plants is strongly influenced by abiotic stress conditions, such as low temperature, high salt and drought. Transgenic breeding is an effective method for developing new varieties with high quality. In this study, a new chilling tolerant *Sinningia speciosa* variety overexpressing AtCBF1 was generated by the transgenic breeding method. The increased chilling

tolerance proved that overexpression of CBF1 has a function in ornamental flowers. Chilling stress results in the overproduction of ROS, which damages cell membrane stability [31]. Chilling stress also increases the activities of an array of ROS scavenger enzymes to reduce the damage [32]. Previous studies indicated that CBF1 was related to the ROS and ROS-eliminating system through multiple interactions [33]. In this study, the expression of *AtCBF1* in *Sinningia speciosa* affected the overproduction of ROS and the activities of ROS scavenger enzymes. In OE plants, H_2O_2 , $O_2 \bullet^-$ and MDA were all obviously lower than in WT plants under 4°C chilling stress. Also, the activities of the ROS scavenging enzymes in OE plants were much higher than those in the WT plants under 4°C chilling treatment. The elevated antioxidant enzyme activities were consistent with the decreased level of H_2O_2 and $O_2 \bullet^-$. These results indicate that *AtCBF1* in *Sinningia speciosa* conferred high chilling resistance by reducing the accumulation of ROS and increasing antioxidant enzyme activities under chilling stress.



The error bars indicated the SD. Values with the different letters are significantly different at $P < 0.05$, as determined by SAS software version 9.1

Figure 6. Antioxidant enzyme activity assays of leaves after treatment at 4°C for 0, 1, 2 and 3 days.



The error bars indicated the SD. Values with the different letters are significantly different at $P < 0.05$, as determined by SAS software version 9.1

Figure 7. Leaf-disc bioassay of WT and OE plants under salt and drought stresses. Chlorophyll content analysis of leaf-discs treated with 200 mM NaCl or 20% PEG for 48 h

Salt and dehydration stress influence the growth and chlorophyll content of plants [34]. In this study, *AtCBF1* increases, not only the chilling stress tolerance but also the salt and drought stress tolerance of *Sinningia speciosa*. Similarly, the overexpression of *Arabidopsis* CBF1 in a transgenic potato enhanced both chilling and drought tolerance [35]. Our experimental results demonstrated that the *Arabidopsis* CBF1 gene had similar functions in *Sinningia speciosa* with in other plants to improve abiotic stress tolerance.

Previous works have shown that a transgenic tomato overexpressing the CBF gene was stunted in growth, delayed in flowering and reduced in fruit production [36]. In contrast, transgenic *Solanum tuberosum* with *Arabidopsis* DREB genes driven by either a constitutive CaMV 35S or a stress-inducible *Arabidopsis* rd29A promoter reach the same level of freezing tolerance and grow similarly to WT plants [37]. Additionally, a transgenic grape overexpressing *AtCBF1* showed no phenotypic difference from wild-type plants in its young stage [38]. In this experiment, no observable influence on plant size or flowering time was found by overexpression of the *AtCBF1* gene in *Sinningia speciosa*, but it significantly increased the resistance to chilling stress in transgenic plants as shown in **Figure 4**.

Previous researches show that CBF gene could induce the expression of COR (cold-regulated) genes, like COR15a and

COR78 and so on, in response to low temperature and dehydration stress^[39,40]. In many plants, like Arabidopsis, cucumber and potato, overexpression of CBF1 can significantly activate COR genes, which in turn increases the degree of cold resistance^[41-44]. Since the specific sequence of relative genes in *Sinningia speciosa*, such as COR15a and COR18, cannot be searched, no direct evidence provided here. However, the increased chilling resistance in transgenic *Sinningia speciosa* in this investigation suggested that AtCBF1 may regulate the expression of genes related to abiotic stress.

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