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Effects of exogenous salicylic acid on physiological traits and CBF gene expression in peach floral organs under freezing stress

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Abstract: To elucidate the effects of exogenous salicylic acid (SA) treatment on the cold resistance of peach flower, the floral organs of two peach cultivars were treated with 20 mg/L SA and stored at 0°C for observation and sample collection. Water application was the control. After a treatment period, the anther relative water content of the control and SA-treated flowers decreased. The extent of the reduction was greater in the control, suggesting that the SA treatment significantly helped to maintain the anther water content of peach. Analysis of the stigma relative electric conductivity revealed that the SA treatment prevented membrane injury during the low temperature treatment. Additionally, we measured CBF gene expression at low temperature in the petal, stigma and ovary. The expression was markedly upregulated in the cold-treated floral organs. CBF gene expression after SA treatment was higher than in the control when cold conditions continued. These results suggest that the effects of SA on ameliorating the freezing injury to peach floral organs and on enhancing cold tolerance may be associated with the induction of CBF gene.

Key words: peach; floral organ; salicylic acid; freezing stress; physiological trait; CBF

INTRODUCTION

Plants grown in nature often face environmental stresses such as drought, salt, freezing, which are the most important factors limiting growth and productivity [1-3]. Among these, low temperature is one of the most harmful, especially in the early spring during the florescence of many horticultural crops [4,5]. Freezing injuries to these crops (apple, apricot, peach, etc.) represent a major economic loss in many countries. Currently, climatic events frequently lead to frost-damage injuries and can be a limiting factor in obtaining optimal agronomical and economic crop performances [6]. In China, regional low temperature extremes have occurred intermittently in recent years on the lower Yangtze River in the early spring [7]. These low temperatures can last half a month. The blooming period of peach (*Prunus persica* L.) is in the early spring and, therefore, often coincides with low temperature conditions. Peach buds are less resistant to low temperatures from swell to blossom, however, floral organs, such as the stamen, pistil and petal, are the most susceptible and lack resistance to freezing injuries [5,8].

SA is an endogenous regulator in plants and is involved in many plant physiological processes [9,10]. SA plays an important role not only in biotic stress [11] but also in abiotic stress [12-14]. Suitable concentrations of SA in plants can increase resistance to freezing injuries or even prevent such injuries [15-18]. Although a significant relationship between the activity of antioxidant enzymes and H_2O_2 metabolism have been reported [19], it is still unclear how SA affects plant metabolism through gene expression and molecular mechanisms under low temperature conditions in peach.

C-repeat binding factor (CBF) proteins, which belong to the CBF/DRE binding (DREB1) subfamily of the Apetala2/ethylene-responsive factor (AP2/ERF) superfamily of transcription factors [20], have vital roles in plant responses to abiotic stresses [21]. The CBF cold-response pathway is important for under-

standing plant freezing tolerance and has been demonstrated to exist in both herbaceous [22] and woody plants [23]. There is a lack of knowledge regarding the relationship between CBF gene expression and the tolerance of peach floral organs under freezing stress.

In the present study, flower buds in the big bud period of two peach cultivars treated with SA were chosen to study the flower physiological characteristic changes and CBF gene expression during low-temperature treatment. It is important to increase our understanding of the cold resistant mechanisms of fruit trees during the blooming period.

MATERIALS AND METHODS

Plant materials and experimental conditions

Experiments were conducted during the peach blooming season in the spring at the Experimental Orchard of the Jiangsu Academy of Agricultural Sciences, P. R. China. Two seven-year-old peach cultivars ('Xiahui 6' and 'Xiacui') were selected to study their sensitivity to SA and freezing stress. Branches that had medium growth vigor and had a length of 50~60 cm were brought to the laboratory immediately after being removed. Most of the flower buds were in the big bud period. SA (Sigma-Aldrich, St Louis, MO, USA) was dissolved in a small amount of ethanol and diluted to 20 mg/L, which is a suitable concentration according to the results of pre-experiments. The branches were sprayed with SA at 25°C, and the spraying was stopped as soon as the liquid dropped. The solution was absorbed for 2 h. Then, the branches were stored at 0°C (the moment the branches were put into 0°C was recorded as 0 h). Branches sprayed with water were used as controls. In this experiment, sixty branches of each treatment were used with three biological replications.

In a preliminary experiment, we observed that the floral organs of 'Xiahui 6' and 'Xiacui' exhibited low temperature tolerance differences. Flower buds on the upper part of 'Xiahui 6' and 'Xiacui' branches were picked at 0, 3, 10, 20 and 30 h. The petal, stigma, ovary and anther were separated after the flower buds were removed. Images of floral organs of each peach cultivar were taken at the end of the experiment. Sixty buds of each treatment were taken for the measurement with three replications. The petal, stigma and ovary were stored at -70°C after being dipped in liquid nitrogen for CBF gene expression analysis. The test was repeated three times.

Anther relative water content (RWC) measurement

The anther RWC was measured using a conventional method [24]. Fresh anthers were placed in Petri dishes after being weighed and then put into an artificial climate box at 25°C for 5 h. The anther RWC was calculated after the pollen had dispersed completely. RWC (%) = (FW − DW) / (TW − DW) × 100. (FW: fresh weight; DW: dry weight; TW: turgor weight).

Stigma relative electric conductivity (REC) measurement

To evaluate stigma plasma membrane integrity, we determined the REC of stigma picked from flowers under different treatments [25]. The stigmas were washed with distilled water to remove adsorbed electrolytes and then put into 10 mL of distilled water and a vacuum was applied for 30 min. After the vacuum was slowly released, the initial electric conductance (R_1) was directly measured using an electrical conductivity meter (DDSJ-318, Leici Instrument Co., Shanghai, China) at 25°C. The mixture was boiled in a 100°C water-bath for 30 min and then cooled to room temperature (25°C) to determine the final electric conductance (R_2). REC was evaluated as: REC (%) = $R_1/R_2 \times 100\%$.

qRT-PCR

Total RNA was isolated using a modified cetyltrimethylammonium bromide protocol [26]. Total RNA (0.5 μg), extracted from each sample was used for synthesis of a cDNA library using a Prime Script RT reagent kit with gDNA Eraser (TaKaRa Bio, Kyoto, Japan). Using the first-strand cDNA as templates, the *CBF*-cDNA (GenBank accession number, JX846908.1) was PCR-amplified using a forward primer (5' GGAGGAACAATGACAAGTGGG 3') and a reverse primer (5' TCTCAGCCGTCGGATAAGTC 3'). The housekeeping gene encoding for β-Actin, which was amplified using the forward primer 5' GTTATTCTTCATCGGCGTCTTCG 3' and the reverse primer 5' CTTCAC-

Fig. 1. Influence of exogenous SA on morphological changes in peach floral organs under freezing stress.

CATTCCAGTTCCATTGTC 3', was used as the internal control. qRT-PCR was performed using a My-IQ 2 (Bio-Rad, Hercules, CA, USA) and SYBR *Premix Ex Taq*™ (TaKaRa Bio, Kyoto, Japan). The qRT-PCR volume was 20 μL, containing 2 μL of diluted cDNA, 0.4 μL of each primer, 10 μL Master Mix and 7.2 μL ddH_2O. Thermocycling conditions were set with an initial polymerase activation step for 2 min at 95°C, followed by 40 cycles for 15 s at 95°C for template denaturation, 15 s at 60°C for annealing, and 20 s at 72°C for extension and fluorescence measurement. Each assay was replicated three times with an organ replicate. Relative quantitative expression was determined using $2^{(-\Delta\Delta Ct)}$ method [27].

Statistical analysis

The experiment was arranged as a completely randomized design with three replications. All the samples were analyzed at least three times. Data were analyzed using ANOVA, and the treatment means were compared using Duncan's multiple range test at 5% of probability. The standard deviation is plotted in all the figures.

RESULTS

Influence of exogenous SA on morphological changes in peach floral organs under freezing stress

Fig. 1 shows the morphological changes in peach floral organs under freezing stress due to the SA application. The freezing stress caused serious damage to the floral organs of flowers that were treated with water only (controls). Symptoms of freezing injury were found on the different floral organs. From the images, petal wilting and red color fading were documented. Additionally, freezing injuries occurred on both the stigmas and the ovaries, and most of the ovaries became darker. Compared with the control group, the petals of flowers that were treated with SA maintained their red color and freezing injuries were rarely observed on the stigmas and ovaries.

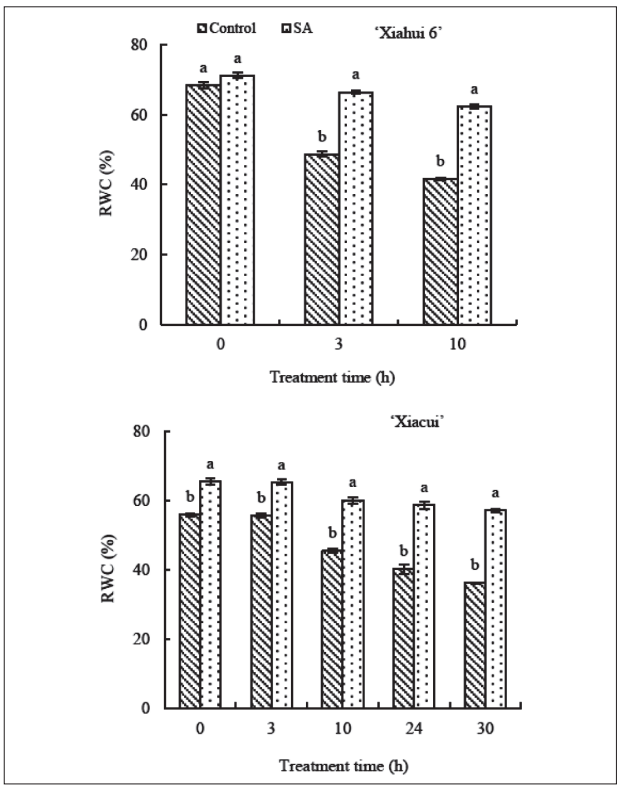

Fig. 2. Influence of exogenous SA on peach anther RWC under freezing stress. Values are means±standard error. For each treatment time, bars with different letters indicate significantly different values at $P<0.05$.

Influence of exogenous SA on peach anther RWC under freezing stress

The anther RWC for both the control and SA of the two peach cultivars had similar downward trends during the whole experiment. For the 'Xiahui 6' anther RWC, there was no difference between the control and SA treatment ($P>0.05$); however, the control RWC was lower than that of the SA treatment at 3 h and 10 h ($P<0.05$) (Fig. 2). During the experiment, the anther RWC of 'Xiacui' under control conditions was always

lower than that of the SA treatment ($P<0.05$) (Fig. 2). For 'Xiahui 6', the anther RWC decreased by 39.08% (control) and 12.51% (SA treatment) from 0 h to 10 h; however, for 'Xiacui', it decreased by 18.76% (control) and 8.14% (SA treatment). After 30 h of the freezing stress treatment, the anther RWC of the 'Xiacui' control decreased to 36.18% with the decrease in an amplitude of 35.35% compared with the SA treatment, which decreased to 57.16% with the decrease in amplitude of 12.73%.

Influence of exogenous SA on peach stigma REC under freezing stress

The peach stigma REC was measured to determine the degree of low-temperature damage to the stigma that was caused by freezing stress and the ameliorating effects of SA applications. Increasing trends of stigma REC were observed in each treatment of the two peach cultivars. There was no difference between the control and SA treatment at 0 h for the two cultivars. However, the stigma REC of the SA treatment was lower than that of the control at the following detection periods. The increasing amplitude of the SA treatment from 0 h to 10 h on 'Xiahui 6' was 51.01% compared with the control, which was 133.21% throughout the experiment (Fig. 3). As for 'Xiacui', the stigma REC increasing amplitude of the SA treatment was 12.54% compared with the control, which was 80.42% from 0 h to 10 h (Fig. 3). After 30 h of the freezing treatment, the stigma REC of 'Xiacui' under control conditions increased to 60.13% with a rising amplitude of 88.97% compared with the SA treatment, which was 39.28% with a rising amplitude of 27.57% (Fig. 3).

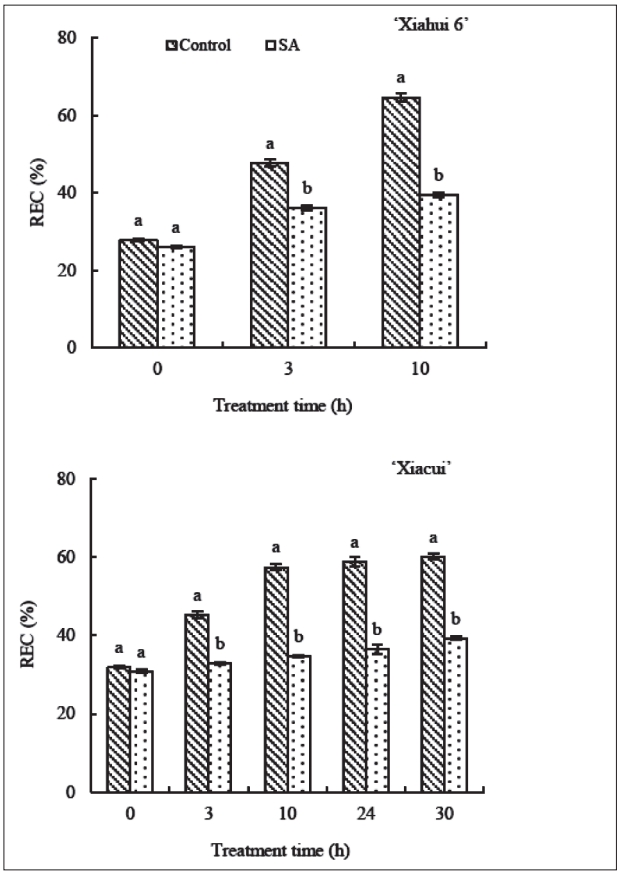

Fig. 3. Influence of exogenous SA on peach stigma REC under freezing stress. Values are means±standard error. For each treatment time, bars with different letters indicate significantly different values at $P<0.05$.

Influence of exogenous SA on CBF relative expression levels in peach floral organs under freezing stress

CBF-relative expression levels in peach floral organs caused by SA application under freezing stress are shown in Fig. 4. There was no difference in the the level of *CBF* expression in 'Xiahui 6' petals between the control and SA treatment at 0 h; however, the level of the control was lower than that of the SA treatment in the stigmas and ovaries (Fig. 4). *CBF* expression levels in the petal and stigma under control conditions were higher than those under the SA treatment at 3 h, but the opposite was true in the ovary. At the end of the experiment, the *CBF* expression in the control was lower than in the three floral organs of SA-treated plants. The overall trend of *CBF* expression in the three floral organs under the two treatment conditions were different from each other. *CBF* expression in the petals of the control increased during the experiment; however, the SA treatment first decreased and then increased expression (Fig. 4A). In the stigma, gene expression in the control first increased and then decreased, but an opposite trend was observed under SA treatment (Fig. 4B). In the ovary, the gene expression during both treatments had an increasing trend (Fig. 4C).

CBF expression in the petals of 'Xiacui' under freezing stress increased after 3 h of SA treatment, and was higher than in the control (Fig. 4D). At 10 h, the gene expression under SA treatment decreased and was much lower than in the control. No significant

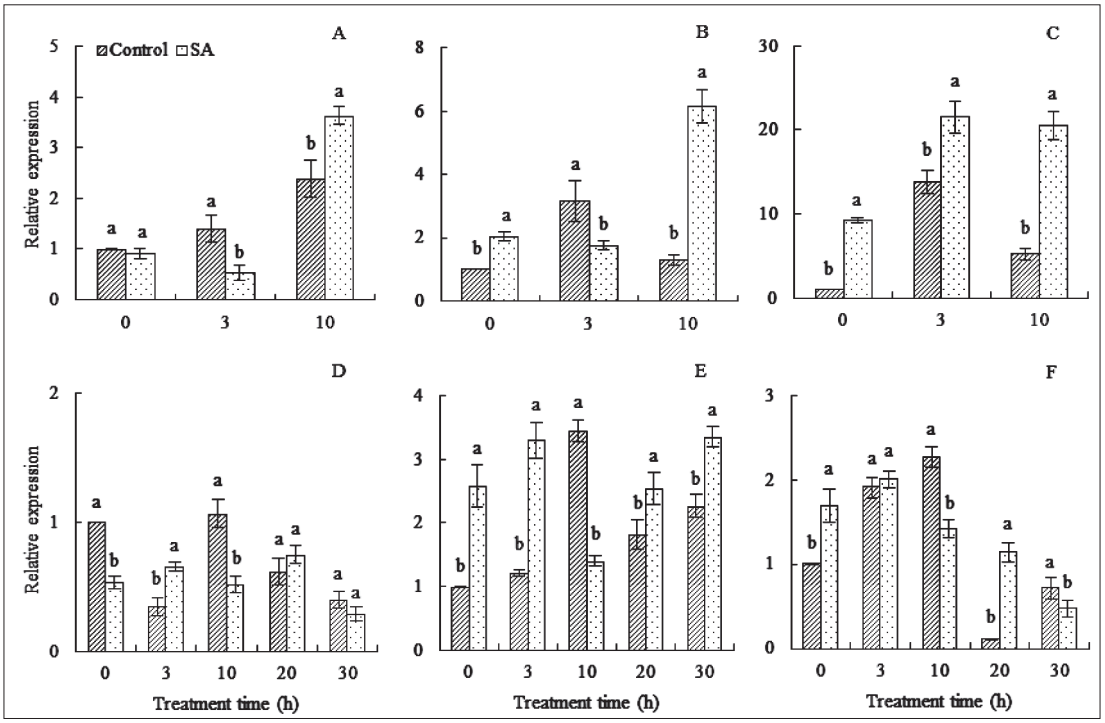

Fig. 4. Influence of exogenous SA on *CBF*-relative expression levels in peach floral organs under freezing stress. 'A', 'B' and 'C' in the figure represent the petals, stigma and ovaries of 'Xiahui 6', respectively. 'D', 'E' and 'F' in the figure represent the petals, stigma and ovaries of 'Xiacui', respectively. Values are means±standard error. For each treatment time, bars with different letters indicate significantly different values at $P<0.05$.

difference was observed between the SA treatment and control at 20 h and 30 h. However, *CBF* expression under both treatments at 30 h was lower than at 20 h (Fig. 4D). SA application led to higher *CBF* expression levels in the stigma and ovaries than were found in the control at 0 h (Fig. 4E, Fig. 4F). After 3 h of freezing stress, *CBF* expression in the floral organs increased not only in the control but also in the SA treated samples. Gene expression under SA treatment decreased at 10 h and became lower than in the control. At the end of the experiment, the gene expression level in the stigma was higher under the SA treatment than in the control (Fig. 4E). However, the opposite was found for the ovaries (Fig. 4F).

DISCUSSION

Isolated peach flowers that were treated with water at a low temperature showed severe freezing injury symptoms. Floral organs were damaged by different degrees. When comparing the two peach cultivars and when comparing SA-treated flowers with controls, the degree of visible freezing injuries showed close correlations with the anther RWC and stigma REC. With the passage of time, the anther RWCs under SA treatment and control conditions in both cultivars showed a decreasing trend. In contrast, the stigma REC showed an increasing trend. The optimal anther RWC is important to maintain pollen activity when flowers are exposed to temperature stress during the blooming period. Our results showed that the anther RWC of SA-treated flowers decreased more slowly, and the decrease in amplitude was less than that of the control group. The result that SA could maintain RWC is corroborated by the findings of previous studies [28,29]. This suggested that SA application could maintain the normal development of pollen better than water application under freezing stress.

Frost often accompanies low temperatures. In addition, the status of the stigma affects peach pollination and fertilization. In this study, the stigma REC of the control groups increased with time under freezing stress. Remarkably, although the stigma REC of the SA treatments also increased over time, the amplitude

was much lower than that of the control groups. An increase in electrolyte leakage is a reliable indicator of membrane injury [30]. REC is used as an index of injury and generally correlates with the extent of visible freezing injury [31-34]. The visible injuries to peach floral organs by freezing stress are dead cells which easily lose water. Thus, there is a correlation between electrolyte leakage and freezing injury symptoms. This result is consistent with previous research [35]. However, an SA application could ameliorate the effects of low temperature on peach floral organs and protect the floral organs from low-temperature damage.

SA is a signal molecule that is involved in the induction of defense mechanisms in plants [35]. Pretreatments of plants with low concentrations of SA might have an effect similar to that of acclimation, causing an increased tolerance to most kinds of abiotic stresses [36]. In the present study, not only SA treatments, but also control groups of both peach cultivars altered the floral organs' *CBF* expression in response to the low temperature.

In many plants, the *CBF* expression level may be a crucial factor in freezing tolerance, but the CBF genes are more or less specific to cold stress depending on the plant species [37-40]. The constitutive overexpression of *PpCBF1* in apple results in a 4-6°C increase in freezing tolerance in both the non-acclimated and acclimated states [41]. In grape, *CBF* expression increases at the beginning of cold stress and then decreases [42]. In this study, *CBF* expression in different floral organs at both 3 h for 'Xiahui 6' and 10 h for 'Xiacui' significantly increased (Fig. 4). However, freezing injury symptoms still existed. This indicated that although *CBF* expression increased in the different peach floral organs and freezing injury was relieved to some extent, freezing injury could not be avoided under freezing stress.

Many kinds of abiotic stresses, such as low temperature, drought and salt, can induce *CBF* expression, which is a normal plant response [42-44]. In addition to the abiotic stresses, SA can also enhance *CBF* expression. The combined effects of SA and low temperature have a greater effect on *CBF* expression. Our data showed that *CBF* expression levels in the petals and stigmas of SA-treated 'Xiahui 6' flowers were the highest at 10 h compared with at 3 h in the ovaries. This indicated that the highest SA-induced *CBF* expression in ovaries appeared earlier than in both petals and stigma under low-temperature conditions. The increasing trend of the *CBF* expression levels in the different floral organs of SA-treated 'Xiacui' began at an earlier stage of the experiment. This indicated that SA application could increase *CBF* expression in the floral organs of 'Xiacui' in the early stage under freezing stress.

CONCLUSION

This study reports the positive effects of SA treatment on the maintenance of normal physiology and enhanced the cold resistance of peach floral organs during cold storage. The SA treatment significantly decreased the reduction in anther RWC and increased the stigma REC. Additionally, both SA treatments and control groups had increased expression levels of the CBF gene. SA treatment with freezing stress had an enhancing effect on *CBF* expression. Taken together, all the physiological, morphological and molecular evidence suggests that SA treatments are of significant practical value to peach flower storage under low temperatures.

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Authors' contribution: Binbin Zhang and Ruijuan Ma conceived and designed the study; Binbin Zhang performed the experiments; Lei Guo analyzed the data; Binbin Zhang and Ruijuan Ma wrote the paper; Zhizhong Song and Mingliang Yu revised the paper. All authors read and approved the final manuscript.

Conflict of interest disclosure: The authors have declared that no conflict of interests exists.

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