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Salicylic Acid Regulates Sugar Metabolism that Confers Freezing Tolerance in *Magnolia wufengensis* During Natural Cold Acclimation

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Abstract
To improving the understand of the accumulation pattern of soluble sugars in *Magnolia wufengensis* during natural cold acclimation, the dynamics of freezing tolerance, the content of various soluble sugars in the shoots of *M. wufengensis* seedlings treated with salicylic acid, and the correlation between them were analyzed from September 2017 to March 2018. Salicylic acid advanced the natural cold acclimation and ultimately enhanced mid-winter hardiness by promoting the accumulation of total soluble sugars. Improved freezing tolerance highly correlated with enhanced glucose, fructose, and raffinose accumulation. The enhanced accumulation of these sugars by elevated amylase, sucrose synthase, and sucrose-P-synthase activities may contribute to advanced natural cold acclimation.

Keywords Cold acclimation · Salicylic acid · Freezing tolerance · *Magnolia* · Soluble sugar

Introduction

Freezing injuries adversely affect the growth, productivity, and geographical distribution of horticultural plants (Weiser 1970; Pearce 2001). The susceptibility of plants to winter injury is due to mid-winter hardiness, and the rate of natural cold acclimation (Suojala and Lindén 1997). Cold acclimation, the process by which plants transit from a cold-sensitive to cold-hardy state, is essential for woody plants growing in temperate regions (Teets et al. 1989; Arora et al. 1992). Accumulation of soluble sugars is an important biochemical mechanism to increase freezing tolerance during cold acclimation. Soluble sugar can influence freezing tolerance via facilitating the deep super-cooling of plant tissues (Kasuga et al. 2007), decreasing the freezing point of intracellular water (Morin et al. 2007), and preventing membrane and macromolecule injuries from freeze-induced dehydration (Shao et al. 2006; Krasensky and Jonak 2012). However, the major sugar that is associated with freezing tolerance is species-dependent (Flinn and Ashworth 1995; Imanishi et al. 1988; Palonen et al. 2000; Morin et al. 2007).

Salicylic acid (SA), an important plant hormone, is a critical signal molecule in local defenses systems and systemic acquired resistance (Gaffney et al. 1993; Vlot et al. 2009). During recent years, there has been an increase in the number of studies on the beneficial roles of exogenously applied SA under abiotic stresses were published, such as water (Singh and Usha 2003), salinity (Nazar et al. 2011), high temperature (Wang and Li 2006) and cold stresses (Kang and Saltveit 2002; Tasgínet al. 2003). However, compared with the comprehensive signaling pathways in SA-mediated tolerance to biotic stresses, data on the action mechanisms of SA during abiotic stresses are limited. Salicylic acid may cause reactive oxygen species (ROS) production, which activates the antioxidative capacity of plants and induces the synthesis of protective compounds (Janda et al. 2007). However, proteomic studies have revealed that exogenous application of SA can upregulate or downregulate some proteins

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and enzymes involved in plant carbohydrate/energy metabolism (Rajjou et al. 2006; Chan et al. 2008; Tarchevsky et al. 2010). This indicates that SA may have a substantial effect on sugar metabolism in plants in response to cold stress.

Magnolia wufengensis, a new Magnolia species (Magnoliaceae), was discovered growing with other native species in natural secondary forests in Wufeng County, Hubei Province, southern China (Ma et al. 2006a, b). Considering its primitive evolutionary position in angiosperm (Ma et al. 2019) and variations in the number (9–25, 32, or 46) and color (pure, dark, or pale red) of tepals (Sang et al. 2011), *M. wufengensis* would be an ideal species for both floral development research in ancient angiosperms and forestry greening if it could be introduced to northern China successfully where types of ornamental arbor are quiet limited. Therefore, *M. wufengensis* is regarded as a valuable arbor species owing to its exceptional ornamental value (Fig. 1), and it has been introduced to northern China in recent years. However, freezing injuries have restrained the sustainability and profitability of *M. wufengensis* seedling production and have caused substantial economic losses in northern China (Yang et al. 2015a, b, c, 2016; Duan et al. 2019). We found exogenous SA application at appropriate concentrations could successfully enhance the freezing tolerance of *M. wufengensis* under natural conditions in the winter of 2016 (data not shown, preliminary study). But it is not clear if soluble sugars and what kind of sugars when accumulated in excess are responsible for improved freezing tolerance or/and promoted cold acclimation in both deciduous and evergreen woody plants. Therefore, in this study we analyzed the correlation between freezing tolerance and major sugar content and key enzyme activities in *M. wufengensis*, to (1) determine if soluble sugars are involved in enhancing freezing tolerance with SA application; and (2) identify key sugars and enzymes that contribute to improved natural cold acclimation in *M. wufengensis* seedlings subjected to SA application. The results could elucidate the subsequent precise molecular mechanism and carbohydrate regulation involved in enhancing cold stress in *M. wufengensis* even other species.

Fig. 1 *Magnolia wufengensis* in Wufeng County, Hubei Province, P. R. China

Materials and Methods

Plant Materials and Experimental Treatments

Dongdadi Experimental Base in Beijing University of Agriculture, Changping District, Beijing, China (39° 48′ N, 116° 28′ E) was used in this study. The data of air temperature conditions in the site during our experimental period (from September 2017 to March 2018) were obtained from the Dongdadi Experimental Base (Fig. 2a). The soil type was cinnamon soil.

In early April 2017, 1-year-old *M. wufengensis* seedlings were randomly planted three plots in the experimental site each 10 m × 3 m plot contained 60 plants. The planting space was 0.5 m × 0.5 m in each plot. From April to August, the seedlings were well-irrigated and protected from bacterial pathogens and weed competition. Artificial irrigation was stopped after late August.

In pre-experiment of 2016, September was found to be the optimum time for SA application, and 300 mg/L as the optimum concentrations (data not shown). Thus, the seedlings were subjected to water (controlled treatment) and SA application at 300 mg/L between 16:00 and 18:00 h on September 1, 11, and 21. Salicylic acid powder with 5% effective components was provided by Sichuan Guoguang Agrochemical Co., Ltd. (Sichuan, China). The other 95% components mainly contain surfactant. The powder was dissolved in water. There were three plots (three replications). Whole seedlings were sprayed with the solutions to runoff with a 5-L handheld sprayer averaging a spray volume of 0.5 L/seedling.

Determination of the Freezing Tolerance of Shoots

The freezing tolerance of shoots, assessed as the low temperature at which 50% injury occurred (LT50), was determined monthly from September 30, 2017 to March 30, 2018. Ten representative seedlings were selected and one healthy upper-crown shoot on each representative seedling was selected for LT50 measurement. There are seven designed temperatures, 0, −5, −10, −15, −20, −25, and −30 °C in our freezing test. The shoots were cooled at a rate of 5 °C/h

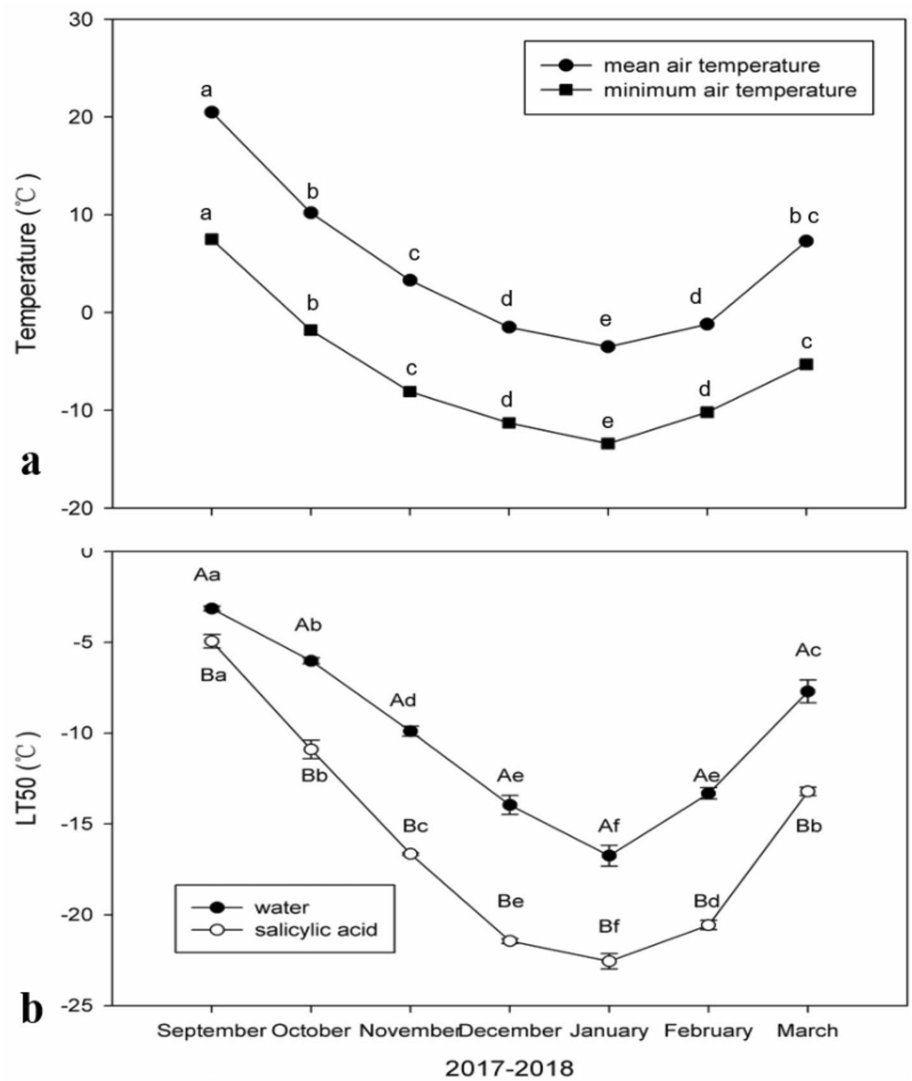

Fig. 2 Dynamics of air temperature (**a**) and freezing tolerance (**b**) of shoots in *Magnolia wufengensis* under water and SA treatments during natural overwintering period. Different lowercase letters indicate significant differences ($p < 0.05$) among months. Different capital letters indicate significant differences ($p < 0.05$) between treatments. The same below

until the target temperatures were reached, and were maintained at the target temperature for 2 h. LT50 was estimated from an asymmetric sigmoid curve constructed using the Gompertz function and fitted to the percent injury data calculated based on electrolyte leakage according to the method of Jun et al. (2012). The LT50 was measured according to the method of Lim (1998).

Determination of Soluble Sugars and Starch in the Shoots

The content of total soluble sugars in the shoots was determined monthly from September 30, 2017 to March 30, 2018. Ten representative seedlings were selected and one healthy upper-crown shoots from each representative seedling was selected for the measurements. The soluble sugar content was measured according to the method described by Li (2000). The content of glucose, fructose, sucrose, and raffinose content were measured according to the method of Liu et al. (2004). Starch content was enzymatically determined using the Quantitative Glc Determination kit (Sigma Chemical, St. Louis, MQ, USA), with glucose as equivalent.

Determination of Enzymes Activities

Sucrose-P-synthase (SPS) activity was measured according to the method of described by Cardemil and Varner (1984) and Dong et al. (2011). Sucrose synthase (SS) activity was determined according to the method of Dong et al. (2011).α- and β-amylase activities were determined using alkaline dinitro salicylic acid as described by Doehlert et al. (1982). We defined 1 U of amylase activity as the amount of enzyme required to generate 1 mg of maltose per min per g fresh weight of sample.

Statistical Analysis

A one-way analysis of variance was performed to determine the main effects of different months and a correlation analysis was performed to determine the correlation between freezing tolerance and the sugars content and enzymes activities. Graphs were constructed using SigmaPlot 10. Excel 2013 (Microsoft, USA) was used for the data collation. All data were analyzed using SPSS Statistics 18.0 (SPSS Inc., Chicago, IL, USA).

Results

Effect of SA on the Freezing Tolerance in Shoots

The LT50 values of shoots treated with water and SA initially decreased during cold acclimation, reached the minimum level in January (with values of − 16.75 and − 22.55 °C, respectively), then increased during the de-acclimation period (Fig. 2b). The decreased rates of LT50 values during cold acclimation were 3.4 and 4.4 °C per month under water and SA treatment, respectively.

Effects of SA on Starch and Total Soluble Sugar Content in Shoots

The starch content initially increased, reached the maximum level in November (32.69 and 29.97 mg/g FW, respectively), then decreased until February (25.93 and 23.24 mg/g FW, respectively), subsequently increased again in March (Fig. 3a). Throughout the overwintering period, starch content in SA treatment was lower than that in controlled. The total soluble sugar content under both treatments kept increasing throughout cold acclimation, and reached the maximum in December (62.24 and 91.64 mg/g FW, respectively), then decreased (Fig. 3b). During overwintering period, the content of total soluble sugars in SA was significantly higher than that in the controlled treatment, and increased rate of total soluble sugar content were 9.6 and 11.2 mg/g FW per month under water and SA treatment, respectively.

Effects of SA on Major Soluble Sugars in Shoots

Similar to the dynamic of total soluble sugar content (Fig. 3b), the content of both glucose and fructose content in both treatments increased sharply during cold acclimation, peaked in December (with values of 15.74, 25.30, 20.61 and 28.47 mg/g FW, respectively), and then decreased (Fig. 4a, b).

On the contrary, the sucrose content in water and SA treatment initially declined during early cold acclimation period, then reached minimum in November, and the decreased rate of sucrose content was 0.53 and 0.98 mg/g FW per month under water and SA, respectively. Subsequently gradually increased, the increased rate of sucrose content was 1.69 and 2.26 mg/g per month in water and SA, respectively (Fig. 4c).

A similar change pattern was observed for raffinose content in water and SA, that is, it slightly declined in early cold acclimation, then sharply kept increased until December, then decreased again during de-acclimation. The raffinose content in SA was higher than that in water throughout overwintering period (Fig. 4d).

Effects of SA on Major Enzyme Activities in Shoots

The amylase activity in the shoots of both treatments rapidly increased during cold acclimation, and reached maximum in December (18.28 and 22.47 μmol/(min*g FW)), then decreased. Moreover, the amylase activity in SA was higher during wintering, and the increased rate of amylase activity in SA was 33.79% per month higher than that in controlled treatment (Fig. 5a).

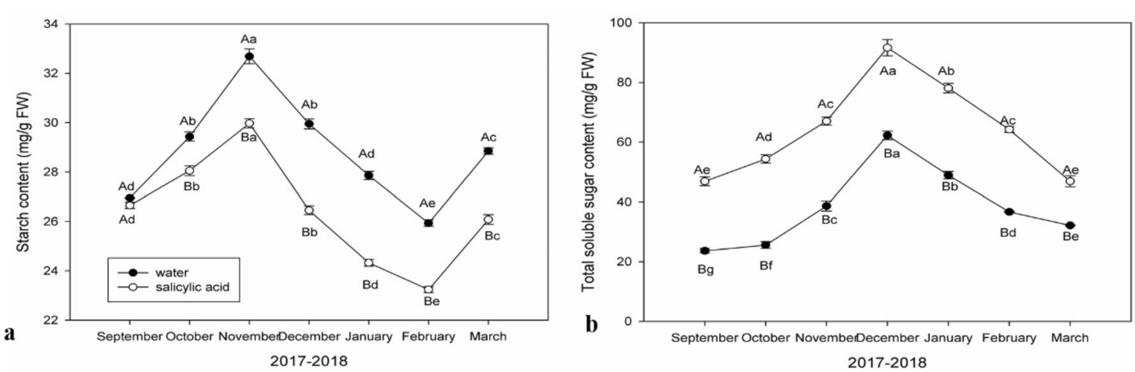

Fig. 3 Dynamics of the total soluble sugar content in *Magnolia wufengensis* under water and SA treatments during natural overwintering period

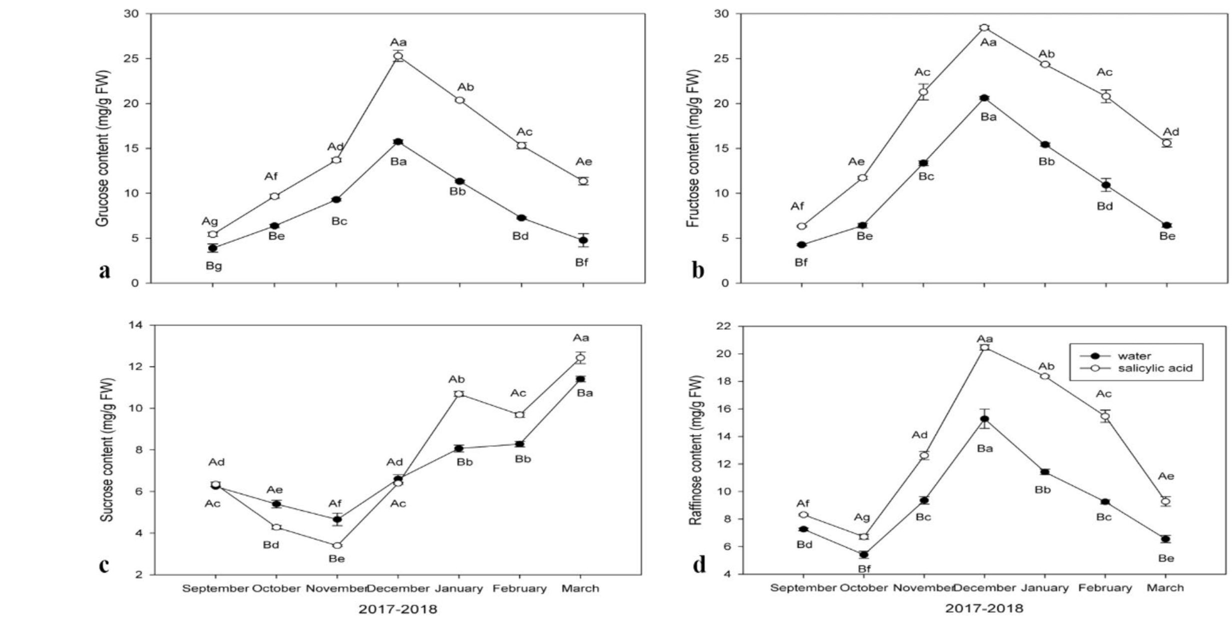

Fig. 4 Dynamics of glucose (**a**), fructose (**b**), sucrose (**c**), and raffinose (**d**) contents in the shoots in *Magnolia wufengensis* under water and SA treatments during natural overwintering period

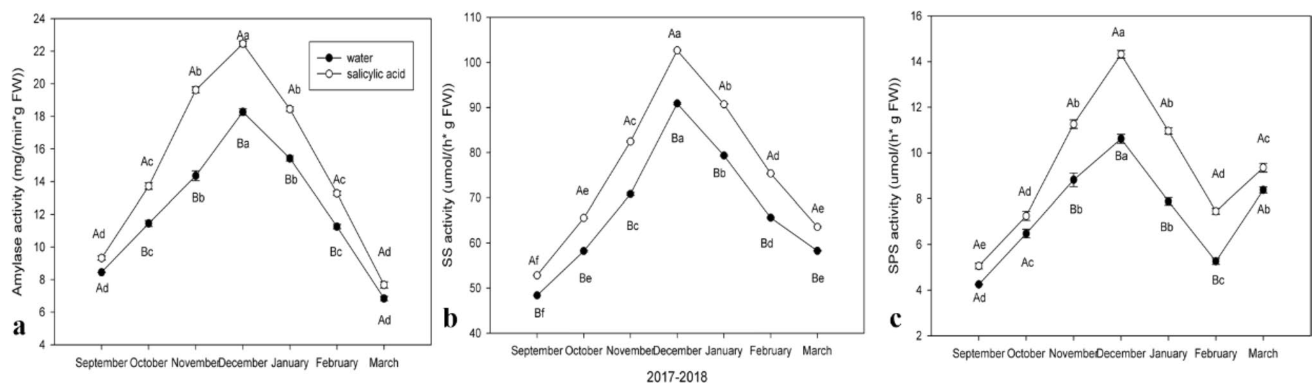

Fig. 5 Dynamics of amylase activity (**a**), SS activity (**b**), and SPS activity (**c**) in the shoots in *Magnolia wufengensis* under water and SA treatments during natural overwintering period

As shown in Fig. 5b, change trend of SS activity in controlled and SA treatment was similar and consistent with tendency of amylase activity, SS activity showed a dramatic increase for SA-treated and water-treated shoots. Compared with water treatment, increasement rate in SA was 17.25%.

The SPS activity displayed dramatical increase, especially in SA-treated shoots, reached maximum in December (with values of 10.62 and 14.32 μmol/(h*g FW)), then decreased till February, then rose again. The SPS activity of shoots in SA was always higher than that in controlled treatment during whole experimental period (Fig. 5c).

Correlation Between Freezing Tolerance and Carbohydrate Contents

For both treatments, there were significant negative correlation between LT50 values and total soluble sugar, glucose and fructose content ($p < 0.01$), LT50 values was significantly negative related to amylase activity and SS activity ($p < 0.05$). In controlled and SA treatment, LT50 values was negatively significantly with raffinose content and SPS activity ($R = -0.752^*$, -0.852^*, -0.890^{**}, and -0.884^{**} respectively). However, LT50 values were not

Table 1 Correlation coefficients between freezing tolerance (estimated as LT50) and carbohydrate and key enzymes activities in the shoots of *Magnolia wufengensis* in salicylic acid treatments

Variable	Correlation coefficient (R)	
	Water treatment	SA treatment
Starch	NS	NS
Total soluble sugar	−0.737**	−0.819**
Glucose	−0.796**	−0.937**
Fructose	−0.835**	−0.953**
Sucrose	NS	NS
Raffinose	−0.752*	−0.890**
Amylase	−0.722*	−0.734*
SS	−0.717*	−0.739*
SPS	−0.852*	−0.884**

NS, * and ** indicate not significant, significant at $p < 0.05$ and significant at $p < 0.01$, respectively

significant with starch content and sucrose content for both treatments (Table 1).

Discussion

In the present study, the freezing tolerance of *M. wufengensis* was strengthened during natural cold acclimation, similarly to other woody perennials (Lim and Arora 1998; Yang et al. 2015a, b, c, 2016; Duan et al, 2019). The LT50 values revealed that the freezing tolerance of shoots was improved by SA treatment compared with controlled. Exogenous SA application have previously been reported to benefit many plants in enhancing the freezing tolerance (Kang and Saltveit 2002; Tasgínet al. 2003; Wang et al. 2006; Pociecha et al. 2010; Mutlu et al. 2013; Yu et al. 2016). In addition, relatively more and severer freezing damage was observed in one-year shoots of the seedlings without SA application in mid-winter January (visual observation), indicating the effectiveness of SA for increasing the freezing tolerance of *M. wufengensis*.

Starch is degraded to soluble sugars under cold stress (Kaplan et al. 2006; Yue et al. 2015). It has been reported that starch degradation and sugar accumulation are elevated during cold acclimation, and they might be vital for enhancing cold tolerance of plants (Yue et al. 2015). However, in this study, the starch content of shoots in both treatments initially increased during early cold acclimation, the increase in starch content during this period might result from the transfer of compounds from leaves to overwintering tissues such as shoots (Charrier and Ameglio 2011). The decrease in starch content between late November and late December may be attributed to the increase in soluble sugar at the same time (Fig. 3a, b), and this strengthened freezing tolerance by hydrolyzing starch into soluble sugars. The starch content rose from February to late March, whereas the soluble sugar content fell at the same time, which might result from the transition from soluble sugar to starch in preparation for re-growth (Fig. 3a, b). However, during whole overwintering period, the lower starch content and higher total soluble sugar content in SA-treated shoots might be related to higher amylase activity and SS activity. Amylase functions a positive role in enhancing cold tolerance of plants by hydrolyzing starch to maltose (Kaplan et al. 2006; Zeeman et al. 2010). SS could digest sucrose into a monosaccharide or derivative (Yue et al. 2015). SA application improved amylase and SS activities and accelerate starch and sucrose degradation, which was consistent with Dong et al. (2011).

Our results indicated starch was not significantly correlated with LT50 values (Table 1), in spite of its recognized role in promoting cold hardiness in plants. However, the amylase activity was significantly correlated with LT50 values (Table 1). Hence, this could indirectly prove key role of starch in clod acclimation of *M. wufengensis*. A more significant role of SA in accumulation of total soluble sugars (Fig. 2b) and a significant negative correlation between total soluble sugars and LT50 (Table 1) were found in our study, which was consistent with the findings of previous studies (Dong et al. 2011; Lee et al. 2012). Thus, higher accumulation of total soluble sugars induced by SA treatment might have caused greater cold hardiness, as soluble sugars can influence freezing tolerance.

Among the major soluble sugars, glucose and fructose act to maintain membrane phospholipids in the liquid-crystalline state and to prevent structural changes in soluble proteins when cells suffer from frost-induced water deficit (Bravo et al. 1998). In this study, both glucose and fructose content increased in SA-treated shoots (Fig. 4a, b), and their content was highly correlated with LT50 values (Table 1). Thus, the more accumulation of glucose and fructose content may at least partially explain the stronger freezing tolerance in SA application, which was consistent with Guy et al. (1992), Gusta et al. (2004), Dong et al. (2011), Yang et al. (b, c, 2016, , 2015a).

In addition, sucrose together with glucose and fructose acted as not only an important osmo-protectant against injuries to the membrane (Bravo et al. 1998), but also an important signal substance (Rekarte-Cowie et al. 2008; Ruan 2014). Moreover, sucrose might be involved in ROS (especially hydroxyl radical) scavenging in response to stress (Keunen et al. 2013; Cao et al. 2014). The decrease in sucrose content between September and October may be associated with the increase in glucose and fructose content at the same time (Fig. 4a, b), suggesting that sucrose was hydrolyzed into the glucose and fructose during this period. In the same way, less sucrose content in SA might be involved in the more accumulation of glucose and fructose during cold acclimation (Fig. 4a, b), which might due to the considerably higher SS activity in SA treatment. The SS activity in SA treatment was also much elevated compared to controlled treatment (Fig. 5b, c), which was similar to Dong et al. (2011). Despite the increased sucrose content during late cold acclimation, the glucose and fructose content increased (Fig. 4a, b), that could be related to higher amylase activity (Fig. 5a). In addition, correlation analysis indicated there was a significant correlation between sucrose and freezing tolerance (Table 1), in spite of its recognized role on promoting cold hardiness in plants. Hence, the key enzymes involved in interconversion of sucrose and glucose and fructose may be the vital factor determining the dynamic changes in the contents of sugars in the shoots of woody perennials.

Raffinose has been shown to play a cryoprotective role by protecting cell membranes, stabilizing proteins and retaining enzyme activities (Nishizawa et al. 2008; Yue et al. 2015). However the increased rate in SA treatment was much higher than that in water treatment, whereas decreased rate displayed the opposite trend, these results suggest the more and quicker accumulation of raffinose in SA application partly explains better freezing tolerance of *M. wufengensis*. Dong et al. (2011) also reported SA application could accelerate accumulation of soluble sugar such as raffinose.

Conclusion

To sum up, our results showed the salicylic acid could improve the mid-winter hardiness of *M. wufengensis*, and SA application did had a dramatic effect on sugars metabolism in *M. wufengensis*, SA promoted the accumulation of total soluble sugar during natural cold acclimation process. Among the major sugars, SA application accelerated accumulation of glucose, fructose and raffinose by elevating amylase, SPS and SS activities during natural cold acclimation.

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Author Contributions XD designing the experiments, doing the experiments, running data analyses and writing the paper; ZZ designing the experiments partly, running data analyses and supervising the work; YY designing the experiments and supervising the work; JD, ZJ and FC coordinating and supervising the work; ZS coordinating, supervising and partly financially supporting work; LM conceiving and designing the experiments, supervising the work; All the authors read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflicts of interest.

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