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Full Length Article

Differential Expression of Resistance Genes and Physiological Indices in Rosaceae Family Plants under Freezing Stress

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Abstract

This study analyzed changes in physiological indices of four species of Rosaceae family plants under low temperature stress and to assess differential expression of heat shock protein gene (HSP90). The results showed that the ranking of LT50 (median lethal temperature) of four species of Rosaceae family plants at -19.62 ~ -11.38°C was (from low to high) *Rosa omeiensis* Rolfe, *R. longicuspis* Bertal., *R. banksiae* Ait., and *R. laevigata* Michx. Importantly, at low temperature, the soluble protein (SP), free proline (Pro) content, superoxide dismutase (SOD) and peroxide dismutase (POD) activities in leaves of tested four species of Rosaceae family plants were first increased and then decreased. In addition, the soluble sugar (SS) and malondialdehyde contents (MDA) were continuously accumulated. Upon genetic analysis, the differential expression analysis of HSP90 gene indicated a gradually increasing trend in all the plants analyzed. Taken together, our results indicated that species with stronger freezing resistance can activate response mechanisms to freezing and expression of relevant resistance genes faster, and adjust physiological and biochemical activities to resist and adapt to freezing stress. These findings provide a theoretical basis for the freezing resistance mechanism of Rosaceae family plants and the breeding of freezing-resistant varieties.

Keywords: Freezing stress; Gene differential expression; Rosaceae; Physiological changes

Introduction

Rosa hybrida var. *Minimum* is a perennial woody plant belonging to the Rosaceae family. It is a short and concentrated plant with diverse colors and low temperature resistance. It is mostly used in potted plants, ground plants among others with huge market potentials. Its long florescence and strong adaptability make it one of the top flowers in China, and it is becoming increasingly popular. In recent years, the winter season is having increasing impacts on the quality of *R. hybrida var. Minimum* where it is anticipated that freezing stress hinders the growth of branches and causes the formation of abnormal petals, which is a great loss to the producers. In the current stage, freezing in winter is directly affecting the distribution of *R. hybrida*, and the selection of freezing-tolerant rose species is a key method to expand its distribution (Xu *et al.* 2012).

Low temperature stress affects the growth, development and geographical distribution of plants and its impact on plants is significantly greater than other environmental stress factors (Yang *et al.* 2016). Low temperature stress interferes with important factors such as plant biomass, leaves and stems and causes metabolic disorders. The low temperature will even directly cause the death of plants (Li *et al.* 2012). Previous studies have shown that after detecting low temperature stress signals, plants actively activate their defense mechanisms, improve cell membrane permeability, reduce the production of reactive oxygen species, rapidly increase protective enzymes synthesis, increase the concentrations of substances such as soluble protein (SP), soluble sugar (SS) and free proline (Pro) to adjust osmotically (Moellering *et al.* 2010) and MDA (Jena *et al.* 2010), and inhibit metabolic-related physiological activities (Winfield *et al.* 2010). In the process of studying low temperature stress resistance in plants, sub-lethal low temperature (LT50) and related physiological indicators are often taken as key reference indicators for assessing freezing stress resistance in plants (Skinner *et al.* 2008).

At low temperatures, the physiological and biochemical functions of plants change due to the regulation of various genes, which further induce the expression of

specific genes to prevent adversity (Baxter *et al.* 2014). The second generation of high-throughput sequencing technology can facilitate rapid identification and functional study of a large number of transcripts, detection of differential expression of genes, and comprehensively display the expression of genes under stress. As a major approach for exploring the function of plant genes, it has made great progress in the fields of low temperature stress response mechanism and key gene mining, and is widely used in the study of plants such as *Arabidopsis thaliana* (Fowler and Thomashow 2002), wheat (Xiong *et al.* 2017), etc. Most of the studies on the low temperature stress resistance of *Rosaceae* family plants at home and abroad are aimed at the comparative study of low temperature stress resistance physiological indicators (Zhu *et al.* 2017; Liu *et al.* 2018), while there are few studies on the freezing stress resistance mechanism of Rosaceae family plants at the molecular level.

Besides, it has been found that the HSP gene plays a decisive role in the transcriptional regulation in plant response to abiotic stress (Wu *et al.* 2018). To investigate the freezing stress resistance mechanisms of plants in Rosaceae family at greater details, four *Rosa* species namely *R. omeiensis Rolfe*, *R. longicuspis Bertal.*, *R. banksiae Ait.*, and *R. laevigata Michx*, were selected to deeply explore the specific expression of low temperature stress resistance differential genes. The real-time quantitative PCR technology was used to assess the differentiation of HSP90 gene among four Rosaceae family species, and to measure the physiological indices of leaves of each species under various differential treatment temperatures, such as soluble protein and free proline content. These investigations provide sufficient foundational data to further understand the freezing stress resistance mechanisms of Rosaceae family plants and the selection of freezing stress-resistant varieties. These finding would facilitate the adaptation of these factors to be applied in gardens for plants longevity and better ornament.

Materials and Methods

Research materials and treatment

Potted seedlings of four species of *Rosa* species namely *R. omeiensis* (S1), *R. longicuspis Bertal.* (S2), *R. banksiae Ait.* (S3) and *R. laevigata Michx* (S4), were studied. In December 2017, 54 cut seedlings with normal and similar growth were planted into pots and cultivated in a greenhouse (temperature: 25°C, humidity: 65%, light intensity: 200 μE/m^2, photoperiod: 14 h light /10 h dark) for 2 weeks. A total of four treatment steps were carried out during the procedure, with 3 plants in each step and repeated 3 times. They were exposed to freezing stress at 0, -5, -10, -15°C for 2 h in artificial low temperature climate boxes, and mature leaves on the annual shoot were selected from each treated plant of each tree species to complete the measurement of conductivity and various physiological indexes. Young leaves at the top of each plant were quickly taken and placed in liquid nitrogen in the centrifuge tube for freezing, and then stored in a -80°C refrigerator for extraction and qPCR experiment of RNA. The experiments were repeated three times and data was presented accordingly.

Test methods

Determination of physiological and biochemical indexes: The method of Gao (2005) was applied to determine the specific values of each physiological parameter. Malondialdehyde (MDA) was measured using thiobarbituric acid (TBA) developing process, free proline (Pro) was measured using the acid ninhydrin method, superoxide dismutase (SOD) activity was measured using NBT photo-reduction method, the peroxide dismutase (POD) activity was measured using guaiacol method, and the content of soluble sugar (SS) was measured using anthrone colorimetry (Gao 2005).

Total RNA extraction and cDNA synthesis: Total RNA was extracted from leaves of each plant using TaKaRa MiniBEST Plant RNA Extraction Kit (Dalian Takara Biotech. Co., Ltd.). The RNA purity, integrity, and RIN values were measured using NanoDrop 2000 (Thermo Scientific, USA). The cDNA was synthesized using qPCR reverse transcription specificPrimeScript TMRT reagent Kit (Dalian Takara Biotech. Co. Ltd., China), as recommended by the manufacturers.

qPCR candidate genes and primer design: The differential expression analysis of the freezing resistance gene was performed as conducted by Li Ruixue (Li *et al.* 2019). The qPCR analysis was carried out strictly according to the steps in the instructions using TB Green Premix Ex Taq II (Dalian Takara Biotech Co, Ltd, China).

Data processing and analysis

According to the method of Xu and Chen (2008), the logistic equation is fitted according to the conductivity: $y= K/ (1+ ae^{-bx}$ where y represents the relative conductivity under different freezing temperature treatment, K represents the saturation value of conductivity (the maximum value is 100), X represents the temperature under stress treatment, a and b represent the regression coefficient, the values of a and b are obtained by linearizing the equation, and the half lethal temperature (LT 50): lt 50 = lna/b.

According to Li *et al*. (2017), the relative expression of the gene in qPCR test was calculated by $2^{-\Delta\Delta CT}$ method. The formula was: relative expression of gene = $2^{-\Delta\Delta CT}$. Among them, ΔΔCT calculation formula is: ΔCT = ΔCT (test group) - ΔCT (control group); ΔCT calculation formula of test group or control group is: ΔCT = CT (objective gene) - CT (internal reference gene). Excel 2007 was used to process and map the

data, and spss19.0 statistical software was used to analyze the significance of physiological indexes.

Results

Semi-lethal freezing temperature for four *Rosa* species

The LT50 of four species of *Rosaceae* family plants ranged from -19.62 to -11.38°C, the LT50 of *R. omeiensis Rolfe* and *R. longicuspis Bertal*. was both below -15°C, and that of *R. banksiae* and *R. laevigata* was between -10 and -15°C (Fig. 1). The ranking of LT50 of four species of *Magnoliaceae* plants was: *R. omeiensis* < *R. longicuspis* < *R. banksiae* < *R. laevigata*

Impact of freezing stress on soluble protein (SP) content

As shown in Fig. 2, the difference in SP contents in leaves of four species of Rosaceae family plants was expectedly 0°C, and slowly increased as the temperature decreased. The peaks of *R. omeiensis*, *R. longicuspis* and S3 were at -15°C, and the peak of S4 was at -10°C. At the peak, SP contents were ranked as *R. omeiensis* > *R. longicuspis* > *R. banksiae* > *R. laevigata*, which was 127.05, 135.42, 142.17 and 142.41%, respectively higher than that at 0°C. Therefore, it can be concluded that the lower the LT50, the greater the increase in the SP content corresponding to the tree species.

Impact of freezing stress on soluble sugar (SS) content

The SS contents in leaves of four species of *Rosaceae* family plants continuously increased with a decrease in temperature (Fig. 3). At 0°C, SS contents in various leaves were significantly different (P<0.05), while the contents of *R. omeiensis* were significantly higher than that in the rest of the samples. Below 0°C, the SS content in *R. omeiensis* leaves was continuously increased to the highest level, followed by *R. longicuspis*, and the SS contents in *R. laevigata* leaves were always at the lowest level. At -15°C, the SS contents in four species of plants was ranked as *R. omeiensis* > *R. longicuspis* > *R. banksiae* > *R. laevigata* which was 47.37, 33.78, 21.03 and 19.10% higher than that at 0°C. It can, therefore, be concluded that the lower the LT50 of the sample, the more SS was produced at a freezing.

Impact of freezing stress on free proline (Pro) content

Under freezing stress, Pro contents in leaves of four species of *Rosaceae* family plants showed an increasing trend (Fig. 4). Peaks of Pro contents in four species of plant were static at -15°C. At the peak, Pro contents in leaves were ranked as *R. omeiensis* > *R. longicuspis* > *R. banksiae* > *R. laevigata*, which were 381.82, 338.10, 294.50 and 298.78% higher than 0°C. These data revealed that there were significant differences among the four species of plants at different freezing temperatures (P<0.05).

Impact of freezing stress on MDA content

The MDA content in each plant carried an overall increasing trend (Fig. 5). At freezing temperatures, the lower the LT50 of species, the lesser the MDA content in the leaves was observed. All four species of Rosaceae are significantly different at 0°C (P<0.05), and the MDA content continued to increase with the decrease in freezing temperature, peaking at -15°C. It can be found that the higher the LT50, the higher the membrane lipid peroxidation concentration of the Rosaceae plants after freezing stress, and more MDA was produced at freezing temperatures. Throughout the application of freezing stress, the ranking of MDA content was in the orders: *R. longicuspis* < *R. omeiensis* < *R. banksiae* < *R. laevigata*.

Impact of cold Stress on superoxide dismutase (SOD) and preoxidase (POD) activities

Based on the data (Fig. 6-7), it was observed that during the cold stress period, changes in SOD and POD activities of four kinds of plant leaves were remained the same, with the following trends; *R. omeiensis* and *R. longicuspis* showed an increasing trend, while in *R. banksiae* and *R. laevigata* SOD and POD increased first and then decreased. At 0°C, the SOD and POD activities of *R. omeiensis* leaves were significantly smaller than those of the other tree species (P<0.05), and differences between four species were relatively higher. At -5°C, similar change in trend were still observed; the SOD activity of *R. longicuspis* and *R. banksiae* was the same at 10°C, which was 61.63 and 46.68% higher than that at 0°C, and the POD activity was 105.84 and 75.38% higher than that at 0°C. The SOD and POD activities of *R. omeiensis* and *R. longicuspis* leaves were still very high at -15°C, the SOD and POD activities in the leaves of *R. banksiae* and *R. laevigata* were decreased (P<0.05). The difference among four species of plants were obvious, the SOD activity was 4.57 and 17.66% lower than that at -10°C; the POD activity of *R. omeiensis*, *R. longicuspis*, *R. banksiae*, and *R. laevigata* showed an increasing trend with decrease in freezing temperature. At -15°C, the POD activity of *R. omeiensis* was the highest, which was 206.21% higher than that at 0°C; the POD activity of *R. banksiae* and *R. laevigata* was still lower than that at -10°C by 13.01% and 31.26%, respectively.

Differential expression analysis of HSP90 gene under freezing stress

The cold-tolerant gene (HSP90) from four species of plant leaves showed an increasing trend under continuous cold stresses (Fig. 8). At 0°C, the relative expression of the gene was significantly different (P<0.05). Thus, it is plausible

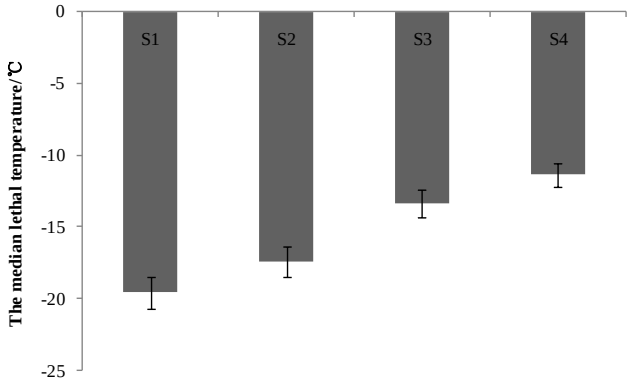

Fig. 1: Semi-lethal temperature of different Rosaceae family plants
Note: In this and subsequent Fig., S1: *R. omeiensis*, S2: *R. longicuspis*, S3: *R. banksiae*, S4: *R. laevigata*

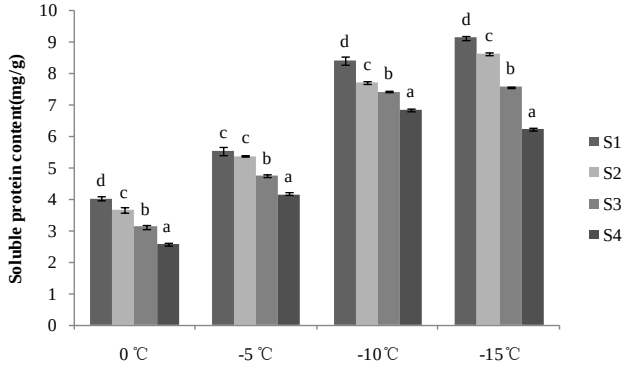

Fig. 2: Changes in soluble protein content in four species of Rosaceae family plants at different low temperatures

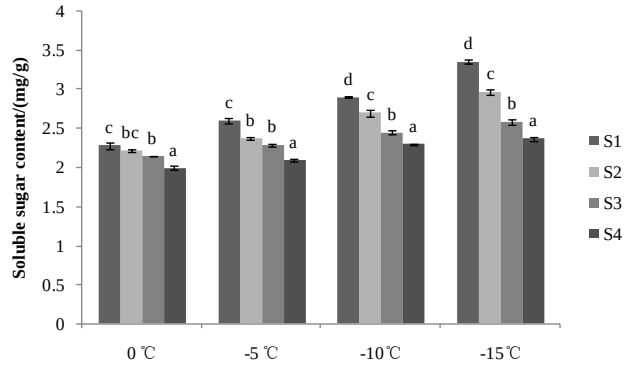

Fig. 3: Changes in soluble sugar content in four species of Rosaceae family plants at different low temperatures

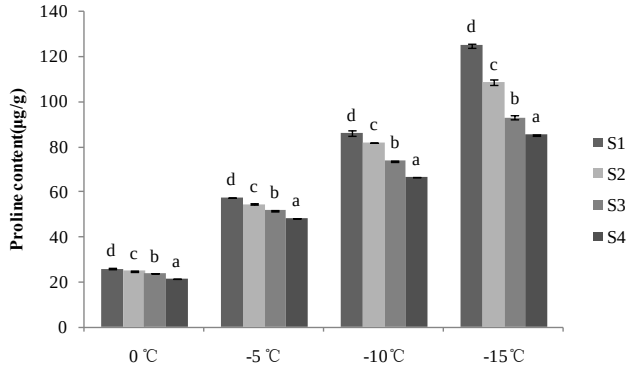

Fig. 4: Changes in free proline contents in four species of Rosaceae family plants at different low temperatures

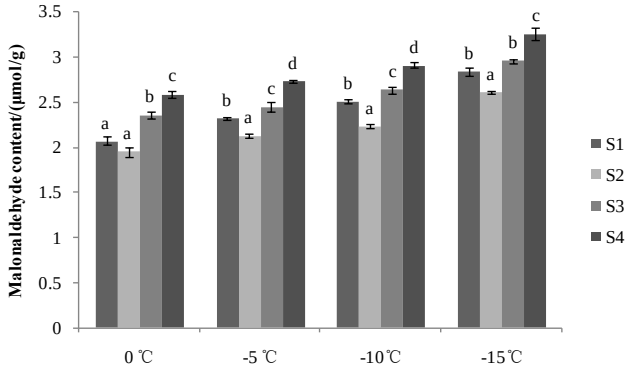

Fig. 5: Changes of malondialdehyde contents in four species of Rosaceae family plants at different low temperatures

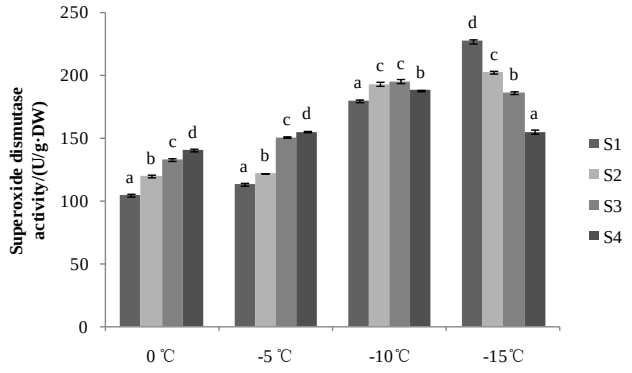

Fig. 6: Changes in superoxide dismutase activity in four species of Rosaceae family plants at different low temperatures

that at -5°C, to better reduce the damage caused by freezing stress, plants actively stimulated the cold response mechanism by activating the cold-tolerant gene. However, when approaching to -10°C, the expression of all four species of plants was reactivated; at -15°C, the gene was activated in depth. In the cold stress, the ranking of HSP90 expression was in the order; *R. omeiensis* > *R. longicuspis* > *R. banksiae* > *R. laevigata*. This indicated that these plants can better resist cold stress by enhancing the expression of HSP90 gene and the larger the relative expression of HSP90 gene, the higher was the cold resistance of the plant.

Discussion

Plants like other eukaryotes carry physiological and biochemical adaptations to resist cold stresses (Nasef 2018).

The semi-lethal low temperature (LT50) can accurately reflect the cold resistance in plants. Lower was the LT50, higher the cold resistance (Ling *et al.* 2015). When the temperature was below LT50, plant cells froze rapidly, physiological metabolism will change, and freezing damage will occur, and the damage suffered by the plants themselves cannot be recovered (Ren *et al.* 2014). Therefore, the LT50 can be used as a threshold temperature at which the damage of plant tissue cannot be reversed. Our results showed that the LT50 values of four species of *Rosaceae* family plants were between -19.62 and -11.38℃, and the ranking was *R. omeiensis* < *R. longicuspis* < *R. banksiae* < *R. laevigata*. Analysis of plants' gene expression under cold stress, especially before and after the LT50 temperature, played a key role in understanding their cold resistance mechanism.

The SP is an essential osmotic adjustment substance in plants and is closely related to the cold tolerance in plants (Yao *et al.* 2016). Studies have shown that with the differential expression protein genes related to both cold stress and energy metabolism, such as malate dehydrogenase, and heat shock protein (HSP), the soluble proteins change, which play a critically important role in the process of plants responding to cold stress signals, and participate in the primary metabolism and secondary metabolism in plants' response to stress (Heidarvand *et al.* 2017). The increase of SP promotes the synthesis of downstream substances, and provides the corresponding substances and energy to plants in response to cold stress (Rasheed *et al.* 2010). It also promote the change of extracellular metabolic behavior, thereby restoring the stability of biosynthesis and carbohydrate metabolism, and effectively improving survival rate at freezing temperatures (Wang *et al.* 2010). At freezing temperatures, carbohydrate metabolism is often used to regulate the balance between biosynthesis and protein breakdown. The hydrolysis of soluble proteins requires a large number of proteolytic enzymes such as Clp protease and ATP protease, and to make the polypeptide irreversibly generate soluble sugar (Wang *et al.* 2014). This study revealed that soluble protein content showed a continuously increasing trend under freezing stress. The plant with higher freezing resistance showed a greater increase in the corresponding protein and higher efficiency of breaking sugar after stimulation. This implied that samples with higher cold resistance can quickly increase the concentration of cell sap after receiving the low temperature signal, and continuously convert the protein into sugar, and drived the corresponding physiological reaction through soluble protein. The transfers of soluble protein play a crucial role in the synthesis of other materials, which allows plants to quickly respond and adapt to freezing temperatures. This is consistent with the results of previous studies on plants such as Rhododendron (Liu *et al.* 2016), Iris (Wang *et al.* 2014), and Magnolia (Li *et al.* 2019).

Plants can effectively improve the low temperature resistance by reducing the free Pro content at low

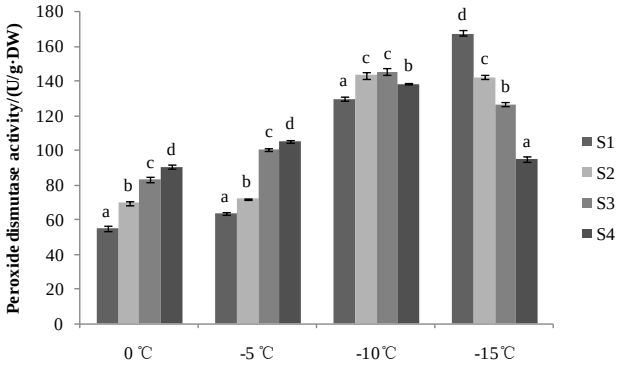

Fig. 7: Changes in superoxide dismutase activity in four species of Rosaceae family plants at different low temperatures

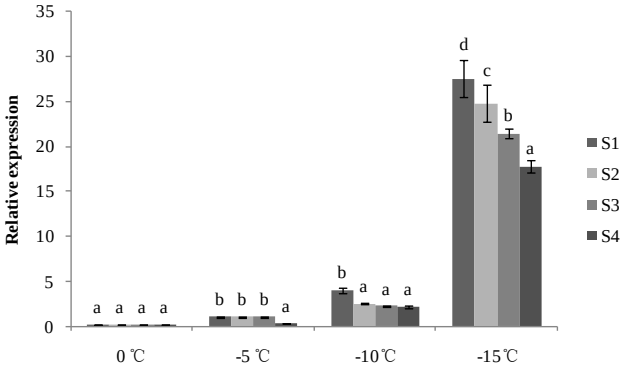

Fig. 8: Differential expression in HSP90 gene in four species of Rosaceae family plants at different low temperatures

temperatures (Yue *et al.* 2010). After the plant is subjected to low temperature stress, free Pro undergoes hydration of the protein, and can effectively ensure that the cell spatial structure is not adversely affected, thereby effectively protecting the plant (Wang 2014). Previous study on Arabidopsis showed that high-concentration of proline is a core cause of increased freezing resistance (Xin and Browes 1998). The proline contents of four species of Rosaceae plants showed an increasing trend with the decrease of temperature. The Pro peaked in *R. omeiensis* and *R. longicuspis* with the lowest LT50 was at about -20°C, and the free proline content was significantly higher than other tree species at freezing temperatures, which was consistent with the conclusion on plants' physiological behavior changes at freezing temperatures in the study of Li *et al.* (2019). This means that the later the LT50 is produced; the more Pro is accumulated in the plant and the higher the cold resistance. The increase of cold stress, the enzyme system is damaged, the Pro synthesis rate of leaves decreases, and the degradation rate enhances, reducing the Pro concentration (Li *et al.* 2019).

At freezing temperatures, plants produce soluble sugars to enhance the osmotic potential of cellular tissues, thereby reducing the freezing point, avoiding excessive dehydration and protecting from low

temperatures (Li *et al.* 2019). This study showed that with the continuous decline of temperature, the SS content of four species of plants continues to increase, which as reported for corn and tomato (Jian *et al.* 2005) and sugar cane (Zhang *et al*. 2011). The SS content corresponding to the *R. omeiensis* with the lowest LT50 was significantly higher than the other tree species, which indicates it has the highest freezing stress resistance. The next was *R. longicuspis* and while SS in *R. laevigata* was the smallest, which means that the species with lower LT50 generate more SS at and have higher freezing stress resistance as reported earlier on Magnoliaceae plants (Li *et al.* 2019).

Cold stress can cause the fast generation of intracellular reactive oxygen species (ROS), which induced membrane lipid peroxidation and caused the accumulation of MDA (Wang *et al.* 2018). Campos *et al.* (2003) have pointed out that the MDA content can be measured to understand the membrane lipid peroxidation, the degree of cell membrane damage and the level of freezing resistance. Therefore, MDA content can be used as a key indicator for determining the degree of plant damage to cold (Ye *et al.* 2017). In this study, the MDA content of four selected Rosa species showed a continuously increasing trend with freezing stress, which means that the degree of plant membrane peroxidation was gradually increasing; the lower the LT50, the slower the MDA content in the corresponding leaves the *Rosa* species. The *R. omeiensis* leaves with the lowest LT50 maintained a relatively low level of MDA content at freezing temperatures, which means that the membrane lipid peroxidation level of *R. omeiensis* was weak under cold stress. At the same time, increase in MDA has an inverse relationship with the freezing stress resistance on plants similar to the observations made earlier (Deng *et al.* 2014; Wang *et al.* 2016; Liu *et al.* 2018). Both SOD and POD can effectively remove free radicals, thus ensuring the physiological functions of plants and improving their cold resistance (Ameglio *et al.* 2003). In this study, leaves of four *Rosa* species showed an overall enhancement. However, increase in SOD and POD activities could effectively reduce the damage of freezing temperatures to biofilm, which means that the plants respond to freezing temperatures. An increase in MDA content indicates that the membrane lipid peroxidation level of leaves increases with the decline in temperature. At -5°C to -10°C, SOD and POD activities in the leaves of *R. omeiensis* and *R. longicuspis* with low LT50 were smaller than those of the other two samples, which may be because the plants themselves could resist a freezing temperature, and did not need to improve protection with protective enzymes; with the continuous decline of temperature, SOD and POD activities of *R. omeiensis* and *R. longicuspis* leaves were activated to protect against freezing stress damage. This indicates that severe freezing can hinder the antioxidant enzyme activity of plants, and then damage the cells. However, an increase of SOD and POD activities in the specific interval of cold stress can effectively prevent the damage caused by cold to the cells.

Cold is an important factor interfering with plant growth and development and the plants activate different defense mechanisms to protect against cold damages (Wang *et al.* 2016). Transcription factor-mediated signal transduction is one of the most efficient defense responses, which constitutes a key part of the complex regulatory network of plants (Pelham 1982). Many studies have shown that various heat shock proteins (HSP) have an irreplaceable key role in temperature sensing and signal processing. HSPs recognize and combine the heat shock elements (HSE) under stress, and thus promote the transcription and expression of downstream gene HSP (Pelham 1982). HSP90, as a part of HSPs, is an important part of steroid receptor, which can promote the accumulation of target proteins and the activation of kinases, integrate plastid signaling pathways, and regulate CBF and COR expression (Noren *et al.* 2016). Studies have shown that at low temperatures, HSP90 gene expression was effectively promoted, which regulated the growth and development of Arabidopsis seedlings (Wang *et al.* 2016). At low temperatures, the HSP gene is over-expressed to remove excess of ROS, thereby reducing the oxidative stress (Park *et al.* 2015). In this study, the HSP90 gene showed an overall increasing trend, and this expression was consistent with the changes of SP, Pro, SOD and POD in species of studied plants, which means that HSP90 joins the accumulation process of the target protein. At 0°C, the expression of HSP90 gene was not significantly different among the four *Rosa* species; with the continuous increase of freezing stress, the expression of this gene in the leaves was also continuously increased. At freezing temperatures, the expression of HSP90 in cold-tolerant germplasm *R. omeiensis* and *R. longicuspis* was always at the highest level. This means that in terms of gene expression, the *Rosa* species plants have different cold-resistance potential, and their cold response mechanisms were also different. They will rapidly activate cold response mechanisms and HSP90 expression to prevent damage of cold, further make downstream cold-tolerant genes complete expression, improve ROS clearance, and reduce the impact of oxidative stress (Yang *et al.* 2010).

Conclusion

The LT50 of studied four species of Rosaceae family plants ranged from -19.62 to -11.38°C, and the soluble protein in leaves increased slowly with a decline of temperature. Under cold stress, the Pro content in leaves showed an increasing trend; the lower the LT50, the less the MDA content in the leaves of the species. The changes of SOD and POD activities remained the same, which were continuously increased in *R. omeiensis* and *R. longicuspis*. Freezing temperature enhanced the enhanced expression of HSP90 gene to resist and adapt to freezing stress. The results highlight the certain reference value for enriching the

molecular and physiological mechanisms of *Rosa* species adaptation to freezing. However, to comprehensively analyze the cold resistance molecular mechanism in *Rosa* species, additional cold-resistant candidate genes need to be analyzed.

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Author Contributions

Chunyan Guo measured the SOD and POD activities and cold-related gene differential analysis, and wrote the paper; Xiang Wang analyzed the soluble protein and soluble sugar; Haijun Xie estimated the free proline and MDA; Jinjia Liu controlled ssub-lethal freezing temperature system.

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