https://doi.org/10.17221/87/2023-HORTSCI

Putrescine alleviates cold injury in peach fruit via elevating the conjugated polyamines in tonoplast and thereby maintaining vacuole conformation

Huaipan Liu[1], Dongxiao Liu[2,3]*

[1]College of Life Science and Agronomy/Henan Key Laboratory of Crop Molecular Breeding and Bioreactor, Zhoukou Normal University, Zhoukou Henan, P. R. China
[2]Jiangsu Provincial Key Laboratory of Crop Genetics and Physiology, Yangzhou University, Yangzhou Jiangsu, P. R. China
[3]College of Life Science and Engineering, Henan University of Urban Construction, Pingdingshan Henan, P. R. China

**Corresponding author: liuhuaipan2013@126.com*

Citation: Liu H.P., Liu D.X. (2024): Putrescine alleviates cold injury in peach fruit via elevating the conjugated polyamines in tonoplastand thereby maintaining vacuole conformation. Hort.Sci. (Prague), 51: 98–110.

Abstract: Exogenous polyamines can elevate postharvest fruit cold resistance and alleviate cold injury. However, the mechanism, by which polyamines mediate cold tolerance, is still to be explored. So in this paper, the conjugated polyamines in tonoplast and vacuole conformation were illuminated in the flesh cells of peach fruit subjected to cold stress, which were pretreated with exogenous putrescine. The results showed that under cold condition, fruit firmness decreased and flesh browning area increased, and vacuole conformation changed. The cold injury could be alleviated by pretreatment with exogenous putrescine, as judged by stabilization of the vacuole conformation, increased fruit firmness and reduced flesh browning area. Furthermore, the cold alleviation was coupled with the increases in the levels of covalently conjugated putrescine and non-covalently conjugated spermidine and spermine in the tonoplast. The results suggested that these conjugated polyamines in tonoplast and vacuole conformation might be involved in putrescine-mediated cold tolerance. The suggestion was further verified by applications with inhibitors, phenanthroline and methylglyoxal-bis (guanylhydrazone) (MGBG). Phenanthroline and MGBG could restrain the putrescine-induced increases in covalently and non-covalently conjugated polyamines mentioned above in the tonoplast, respectively, and increase flesh browning area, decrease fruit firmness and vacuole conformation stabilization. So, we can conclude that exogenous putrescine alleviates cold injury in peach fruit via elevating the contents of the polyamines conjugated to the tonoplast and thereby maintaining vacuole conformation.

Keywords: cold stress; conjugated polyamines; peach *(Prunus persica Batsch)*; tonoplast; vacuole conformation

The quality of postharvest fruit can be maintained by cold storage because some metabolic activities are decreased under cold condition. However, inappropriate low temperature treatment could induce some physiological and biochemical disorders and changes in surface appearance (Lurie, Crisosto 2005) and lead to low temperature damage in fruit. For example, the classic symptoms of fruit under cold con-

Supported by National Natural Science Foundation of China (Grant No. 31271627) and Natural Science Foundation of Henan province (Grant No. 242300421341).

https://doi.org/10.17221/87/2023-HORTSCI

dition often include the decrease in fruit firmness and the increase in flesh browning area (Cao et al. 2018). The appearance of cold damage is related to oxidative stress resulted from an elevated production of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radical, nitric oxide, and peroxynitrite. The dramatic increase in ROS leads to lipid peroxidation and membrane degradation (Gill, Tuteja 2010). Due to this phenomenon, fruit quality is deteriorated significantly during postharvest cold storage and the shelf life is limited in the fruit industry. Therefore, to find ways to effectively mitigate cold damage is one of the aims in studying fruit subjected to cold stress (Rodriguez et al. 2001). Exogenous polyamines, especially putrescine application can alleviate fruit cold damage during cold storage and prolong the shelf life (Palma et al. 2015; Ahmad, Ali 2019).

Polyamines are regarded as plant growth substances and closely associated with environmental stresses (Liu et al. 2004; Yamaguchi et al. 2007; Farooq et al. 2009; Gupta et al. 2013). In plants, putrescine, spermidine and spermine are three main polyamines, which play important roles in abiotic stress responses in maintaining conformation and function of biomacromolecules and organelles, scavenging directly active oxygen species, signaling, etc. (Gupta et al. 2013). Methylglyoxal-bis (guanylhydrazone) (MGBG) can inhibit effectively the conversion of putrescine to spermidine and spermine, because it can inhibit potently the activity of *S*-adenosylmethionine decarboxylase (SAMDC), which functions as a key enzyme catalyzing the conversion (Lutts et al. 2013). Polyamines are well reported in alleviating fruit cold injury (Palma et al. 2015; Ahmad, Ali 2019). For example, the exogenous putrescine application can regulate bioactive compounds, decrease the activities of cell wall softening enzymes and thereby preserve postharvest carambola quality (Ahmad, Ali 2019). For another example, putrescine treatment can maintain zucchini fruit quality by inducing the accumulation of betaine and proline, the expression of fatty acid desaturase, and the changes in the biochemical γ-aminobutyric acid shunt pathway during cold storage (Palma et al. 2015). So, it is increasingly interesting to explore the mechanism, by which exogenous polyamines enhance fruit resistance against cold stress. However, only endogenous free polyamine function is well reported (Koushesh Saba et al. 2012; Palma et al. 2014; Shi, Chan 2014; Palma et al. 2015; Cao et al. 2016; Sharma et al. 2017; Cao et al. 2018; Ahmad, Ali 2019). In the fruit pretreated with exogenous polyamine, the relationship between the cold resistance and the polyamines conjugated to tonoplast remains to be elucidated.

The plant vacuoles play pivotal functions in controlling main protein localization and transport, regulating substance storage and transport, maintaining cell turgor pressure, and responding to abiotic stresses (Jiang et al. 2021). As same as the other biomembranes, tonoplast is composed of phospholipids and proteins. The acidic proteins and phospholipids with negative electric charges in tonoplast can be non-covalently conjugated to free polyamines, which are positively charged at normal physiological pH (Lutts et al. 2013). Furthermore, glutamic acid residues of the proteins in tonoplast can be covalently conjugated to free polyamines with transglutaminase catalyzing and phenanthroline can inhibit strongly transglutaminase activity (Del Duca et al. 1995; Lutts et al. 2013). Under abiotic stresses, tonoplast can play an important role in determining stress levels (Dietz et al. 2001) and vacuole conformation often changes abnormally (Tanaka et al. 2007; Bak et al. 2013). However, in the fruit pretreated with exogenous polyamine plus cold stress, the relationship between the conjugated polyamines in tonoplast and vacuole conformation is still not answered.

So, this research aimed to explore the mechanism, by which exogenous putrescine pretreatment enhanced the fruit cold resistance. The novelty about this research was to reveal that putrescine-mediated cold tolerance might be related to the polyamines conjugated to the tonoplast and the vacuole membrane conformation. The hypothesis was that putrescine pretreatment could elevate the contents of non-covalently conjugated spermidine and spermine, and conjugated putrescine in tonoplast, and that the conjugated polyamines could maintain the normal vacuole conformation. This research could contribute not only to the fruit industry in preserving fruit quality by the exogenous putrescine application, but also to the theory knowledge of polyamine-mediated tolerance. In the performed experiments, the cold injury parameters, the free polyamine contents, the activities of SAMDC and transglutaminase, polyamines conjugated to tonoplast, and the vacuole conformation were detected step by step. Inhibitors, MGBG and phenanthroline, which could reduce the contents of the polyamines conjugated to tonoplast,

https://doi.org/10.17221/87/2023-HORTSCI

were also applied in the study to further verify the hypothesis.

MATERIAL AND METHODS

Cultivar selection, fruit harvest and treatment. Peach (*Prunus persica Batsch*) cultivar "Yuxian 3" cultivar was used in the experiment. This cultivar is bred by Academy of Agricultural Sciences of Henan Province of China and distributed in North China. The ripe fruit of the cultivar is large in size, tastes good and is popular with the consumer, but it is a little cold-sensitive and unfitted for cold storage. Furthermore, preliminary experiments showed that exogenous putrescine application could obviously prolong shelf life of the fruit under cold storage. Therefore, this cultivar was selected in the research. Trees were planted in the orchard of Zhoukou Normal University (33"38'N, 114"40'E) and subjected to standard horticultural practices. The experiment began in 2020 with the trees at age 5 years. In 2020, 2021 and 2022, although there were slight changes in temperature, humidity, precipitation, etc. in the experiment's location, they could not affect growth and development of the peach fruit. After the fruit were selected and treated, they were preserved in an environmental chamber with the same storage conditions. So, the experimental outcomes in the three years were not affected by the climatic conditions. This research was repeated in three years and the results showed repeatability, so that the conclusion would be reliable. Every year, the selected 300 peach fruit were to be uniform in color, shape and size, and free of pest or disease injury, and gently transported to the lab. Then the fruit were randomly divided into 5 groups (60 fruit/group), which were subject to the following pretreatment, respectively.

Control: Peach fruits were submerged into distilled water without any reagent for 100 minutes, and then sampled immediately.

Cold treatment (TR1): Peach fruits were submerged into distilled water without any reagent for 100 min, and then preserved for 20 days at 4 °C.

Putrescine pretreatment and cold treatment (TR2): Peach fruits were submerged into a solution of 1 mM putrescine for 100 minutes, and then preserved for 20 days at 4 °C.

Putrescine, MGBG pretreatment and cold treatment (TR3): Peach fruits were submerged into a solution of putrescine (1 mM) and MGBG (0.2 mM) for 100 min, and then preserved for 20 d at 4 °C.

Putrescine, phenanthroline pretreatment and cold treatment (TR4): Peach fruits were submerged into a solution of putrescine (1 mM) and phenanthroline (0.1 mM) for 100 min, and then preserved for 20 days at 4 °C.

Except the control group, the treated materials of the four groups mentioned above were preserved in an environmental chamber with 78% relative humidity and 4 °C temperature. After preservation for 20 days, the peach fruits were sampled and tested.

Index of peach flesh browning area (IPFBA) assesment. Peach fruits were selected at random, and cut near the fruit stone by two cut parallels. IPFBA was assessed by the way of Cao et al. (2016). By detecting browning area of cut surface, five browning grades from 1 to 5 were used in the study. The first grade: no browning area; the second grade: browning area < 25%; the third grade: browning area ≥ 25% and < 55%; the fourth grade: browning area ≥ 55% and < 75%; the fifth grade: browning area ≥ 70%. IPFBA was calculated with the formula: IPFBA = Σ [(the number of browning grade) × (the number of this grade)]/5 × (the number of total fruit tested).

Surface firmness detection. The surface firmness of peach fruit was detected by a texture analyzer (Shimadzu Co., Nakagyo, Kyoto, Japan, Model: EZ-Test/CE-346-51990). The texture analyzer could detect the penetration force, which was needed to push a plunger into a fruit. The value of the fruit surface firmness was the maximum penetration force to press a piston to puncture across fruit surface. The unit was expressed in Newtons (N) and determined for randomly selected fruit of every group.

Free polyamine detection. By the way of Du et al. (2022), free polyamine contents were estimated. 2 g of peach flesh tissue was ground with 5 mL perchloric acid (PCA, v/v, 6%), put in a refrigerator at 5 °C, preserved for 1 h and then centrifuged at 20 000 × *g* for 30 min. The supernatant was derived with benzyol chloride. The benzoylated free polyamines were extracted with diethyl ether, dried with warm air and re-dissolved with methanol. Free polyamines were detected by High Performance Liquid Chromatography (HPLC) (Waters 2695, USA) with C-18 reverse-phase column as separation column, 254 nm as detecting wavelength and 1, 6-Hexanediamine as an internal standard. Unit

https://doi.org/10.17221/87/2023-HORTSCI

of the polyamine content was expressed in nmol/g fresh weight.

Purification of the tonoplast and the proteins in the tonoplast of the peach flesh cells. 10 g peach flesh tissue (fresh weight) was ground with 20 mL hydroxyethyl piperazine ethiosulfonic acid-trismetyl aminomethane (50 mM) buffer containing phenylmethanesulfonyl fluoride (2 mM), dithiothreitol (1 mM), $K_2S_2O_5$ (1 mM), sorbitol (250 mM), KCl (125 mM), polyvinyl pyrrolidone (1.5%, w/v) and bull serum albumin (BSA, 0.1%, w/v). Then the samples were filtered and filtrates were differentially centrifuged step by step at different centrifugal force (800× *g*, 1 000 × *g*, 50 000 × *g*). Subsequently, the pellet was operated with the way of Suzuki et al. (1999) to get purified tonoplast vesicles. The proteins in the purified tonoplast vesicles of peach flesh cell were purified in depth according to the way Du et al. (2022) and were estimated by the way of Bradford (1976).

Assessment of the levels of polyamines conjugated to the tonoplast. By the way of Du et al. (2022), polyamines conjugated to the tonoplast were assessed. The tonoplast vesicles purified above were added into with PCA (5%, v/v) and centrifuged at 40 000 × *g* for 35 minutes. Non-covalently conjugated polyamines were contained in the supernatant. To get covalently conjugated polyamines, PCA was dropwise added into the part of above-purified protein extract of the tonoplast until PCA terminal concentration was up to 5%. Then, the solutions were centrifuged at 45 000 × *g* for 20 minutes. Discard the supernatant and re-suspend the precipitate with prepared 5% PCA. Under seal condition, the samples were hydrolyzed with 12 N HCl for 24 hours at 110 °C to release the covalently conjugated polyamines. Then, the hydrolysates were filtrated and the filtrates were dried with warm air. The sediment was dissolved in prepared 5% PCA. The solution contained the covalently conjugated polyamines. After non-covalently and covalently conjugated polyamines were derivatized with benzyol chloride, they were assessed by HPLC same as the free polyamines mentioned above. One unit of the conjugated polyamine content was indicated in nmol/mg protein.

Estimation of SAMDC and transglutaminase activities. By radioisotope labeling technique, activities of SAMDC and transglutaminase were estimated. SAMDC activitie was estimated via assessing the content of released $^{14}CO_2$ by the way of Kaur-Sawhney and Shin (1982) and its unit was indicated in μL $^{14}CO_2$/g fresh weight. Transglutaminase was estimated via assessing the content of converted putrescine by the way of Icekson and Apelbaum (1987) and its unit was expressed in nmol ^{3}[H] putrescine/(mg protein h).

Observation of vacuole conformation. Peach flesh tissue was immersed in phosphate buffer 80 mM (pH 7.3) and immobilized with glutaraldehyde (3.5%, w/v) for 30 minutes. Then the flesh tissue was immobilized again with osmium tetroxide (1.2%, w/v) prepared with the same phosphate buffer as that mentioned above for 10 hours. Subsequently, the flesh tissue was embedded with agar 100 resin. Afterwards, it was cut into 70 to 90 nm ultrathin sections with a cryoultra-microtome (EM UC6 + FC6, Leica). The ultrathin section was stained with uranyl acetate solution and lead citrate 3% (w/v) prepared with water. Vacuole conformation was observed with a transmission electron microscope (CM 100, Philips) by operation at 80 kV and the image was recorded with a Bioscan CCD camera using Digital Micrograph software.

Data analysis. In the research, the same sampling event was repeated in 2020, 2021 and 2022 and three samples were taken for the control and each treated groups every year. So, the data reported in the figures were indicated as mean of 9 values ± S.E. The data were statistically analyzed by SPSS 21.0 (SPSS Inc., Chicago, USA). The significant differences among the control and the treated materials were assessed with Duncan's tests ($P < 0.05$).

RESULTS

Changes in IPFBA and peach fruit surface firmness under cold, putrescine, MGBG and phenanthroline. Cold stress brought about a statistically significant increase in IPFBA from 0.22 to 0.98 (Figure 1A, from "e" to "a", $P < 0.05$) and a significant decrease in peach fruit surface firmness from 11.5 to 3.1 N (Figure 1B, from "a" to "d", $P < 0.05$), indicating that cold damage occurred in the peach fruit. Under cold stress, phenolic substances were oxidized into brown quinines by phenol oxidase. So, the IPFBA of peach fruit increased. Cold stress might activate cell wall softening enzymes resulting in the decrease in fruit surface firmness. However, IPFBA declined significantly from 0.98 to 0.39 (Figure 1A, from "a" to "d", $P < 0.05$, the same below) and surface firmness increased significantly from

https://doi.org/10.17221/87/2023-HORTSCI

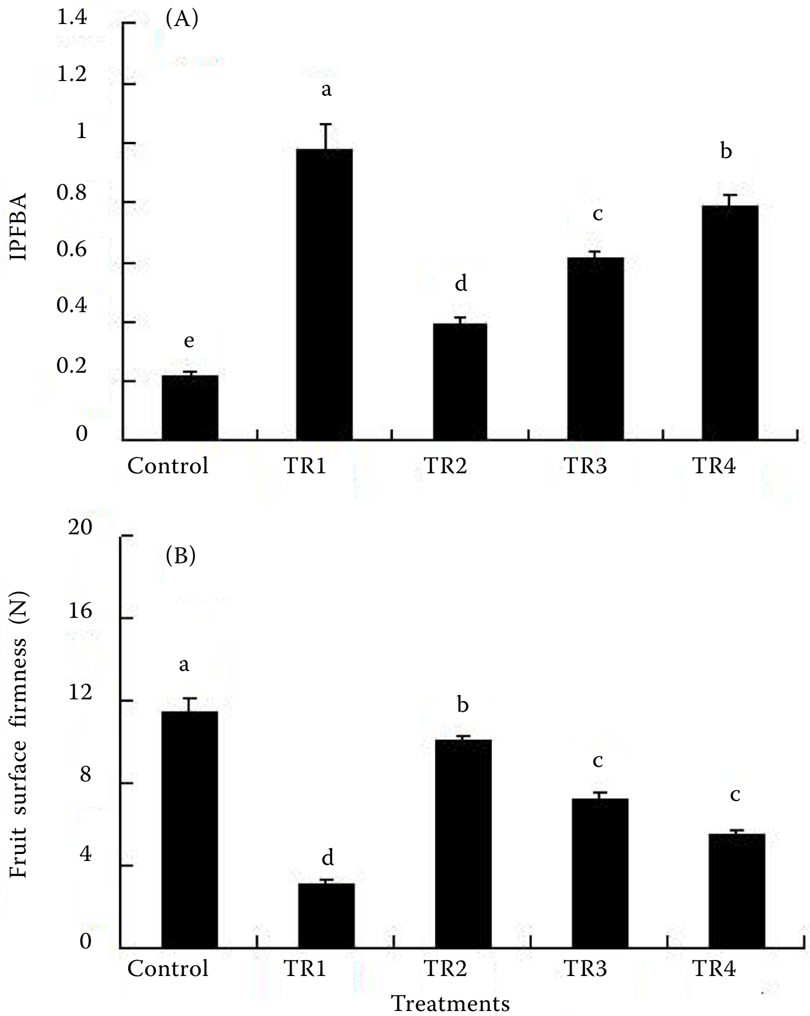

Figure 1. Changes in index of peach flesh browning area (IPFBA, A) and peach fruit surface firmness (B) under cold, putrescine, methylglyoxal-bis (guanylhydra zone) and phenanthroline treatment

Control – peach fruits were submerged into distilled water without any reagent for 100 minutes, and then sampled

Treatment 1: TR1 – peach fruits were submerged into distilled water without any reagent for 100 minutes, and then preserved for 20 days at 4 °C; treatment 2: TR2 – peach fruits were submerged into a solution of 1 mM putrescine for 100 minutes, and then preserved for 20 days at 4 °C; treatment 3: TR3 – peach fruits were submerged into a solution of 1 mM putrescine and 0.2 mM MGBG for 100 min, and then preserved for 20 days at 4 °C; treatment 4: TR4 – peach fruits were submerged into a solution of 1 mM putrescine and 0.1 mM phenanthroline for 100 minutes, and then preserved for 20 days at 4 °C

The significant differences among the control and the treated materials were assessed with Duncan's tests ($P < 0.05$) and were expressed over the columns by different lowercase letters. TR1, TR2, TR3 and TR4 in the following Figures mean the same as in Figure 1

3.1 to 10.1 N (Figure 1B, from "d" to "b") in the fruit pretreated with exogenous putrescine under cold stress, suggesting that exogenous putrescine could alleviate the cold damage. We speculated that exogenous putrescine pretreatment could alleviate cold stress in peach fruit by elevating the contents of conjugated polyamines in tonoplast, leading to the maintenance of normal vacuole conformation. Therefore, conjugated polyamines in tonoplast and vacuole conformation were elucidated subsequently. Additionally, inhibitors, MGBG and phenanthroline were applied in the research to verify the hypothesis. MGBG pretreatment could markedly reverse the effects of exogenous putrescine on IPFBA and fruit surface firmness from 0.39 to 0.61 (Figure 1A, from "d" to "c") and from 10.1

https://doi.org/10.17221/87/2023-HORTSCI

to 7.2 N (Figure 1B, from "b" to "c"), respectively. Pretreatment with phenanthroline exerted a little more reversed effects on the two parameters than that with MGBG.

Changes in free polyamines in peach flesh under cold, putrescine and MGBG. It was preferential to understand the changes in free polyamines in peach flesh before to explore the conjugated polyamines in tonoplast of flesh cells because the levels of conjugated polyamines are greatly affected by free polyamines. In the experiment, 3 main free polyamines, putrescine, spermidine and spermine, which are closely related to abiotic stresses, were assessed in peach fruit flesh. Compared with the control, cold stress could hardly bring about a change in free putrescine level (Figure 2A), while it increased free spermidine level from 53.2 to 83.2 nmol/g fresh weight (Figure 2B) and free spermine from 46.1 to 74.5 nmol/g fresh weight (Figure 2C). Furthermore, in the fruit pretreated with exogenous putrescine, the cold stress-induced increases of free spermidine and spermine were markedly enhanced by over 90% and 80%, respectively, indicating that free spermidine and spermine were closely related to fruit cold resistance. Because free spermidine and spermine carry more positive charges than free putrescine, they can non-covalently conjugate to the phospholipids and acidic proteins in tonoplast more easily than putrescine (Lutts et al. 2013). These non-covalently conjugated polyamines in tonoplast might play important roles in maintaining tonoplast conformation and function. However, the enhancement was substantially inhibited by MGBG, whereas free putrescine level was obviously elevated (Figure 2), indicating that MGBG inhibited the conversion from free putrescine to spermidine and spermine by inhibiting SAMDC activity.

Changes in the polyamines non-covalently conjugated to tonoplast of peach fruit cells under cold, putrescine and MGBG. In tonoplast, three non-covalently conjugated polyamines could be detected, with the highest level of spermidine, the second level of spermine and the lowest level of putrescine (Figure 3). Furthermore, the level of non-covalently conjugated putrescine kept almost constant under cold, putrescine and MGBG, indicating that non-covalently conjugated putrescine had little relationship with fruit cold resistance. Nevertheless, non-covalently conjugated spermidine and spermine increased under cold stress, and with the exogenous putrescine pretreatment, the cold-induced increases were markedly enhanced from 35.2 to 75.6 nmol/mg protein (Figure 3B, from "c" to "a") and from 26.9 to 62.5 nmol/mg protein (Figure 3C, from "c" to "a"), respectively. The result showed that non-covalently conjugated spermidine and spermine in tonoplast were closely associated with fruit cold resistance. The hypothesis was further verified by the result with inhibitor MGBG. The enhancement was substantially inhibited with MGBG application (Figure 3), coupled with the decrease in fruit cold resistance, as judged by the indexes of IPFBA and fruit surface firmness (Figure 1).

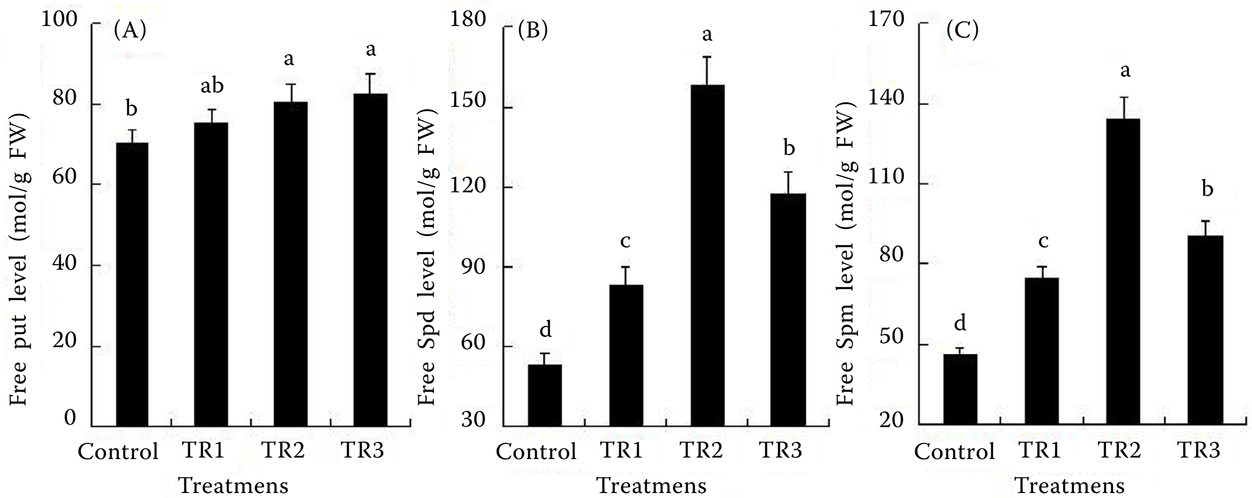

Figure 2. Changes in free polyamines in peach flesh under cold, putrescine and methylglyoxal-bis (guanylhydra zone)

(A) free putrescine; (B) free spermidine; (C) free spermine; for TR1–4 and "Control" explanation see Figure 1

https://doi.org/10.17221/87/2023-HORTSCI

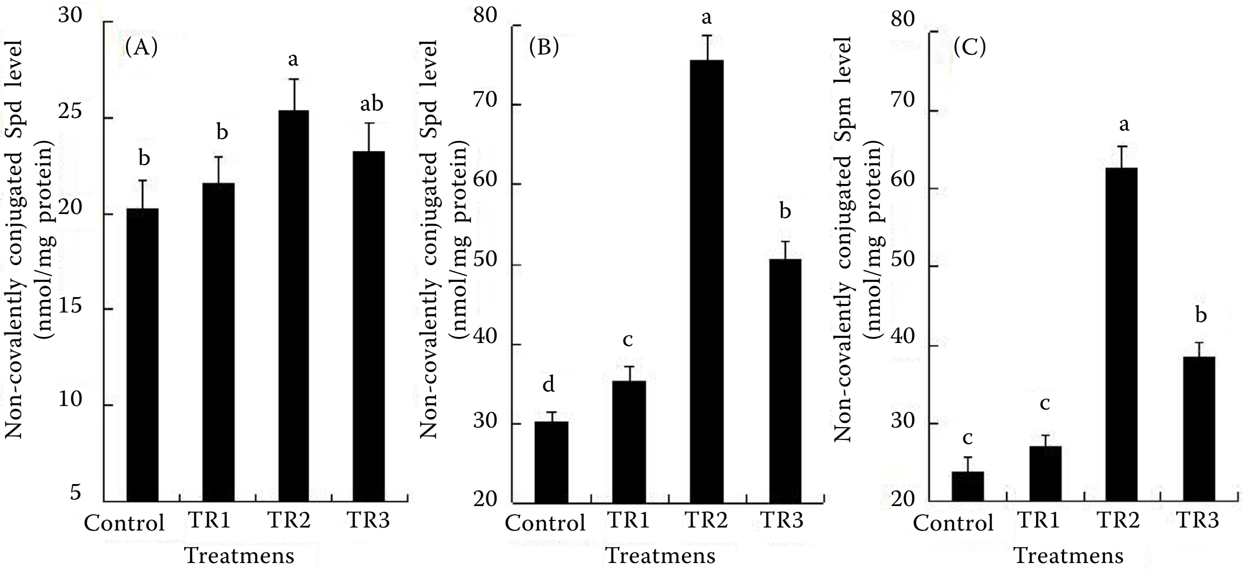

Figure 3. Changes in the polyamines non-covalently conjugated to tonoplast of peach fruit cells under cold, putrescine and inhibitor methylglyoxal-bis (guanylhydra zone)

(A) free putrescine; (B) free spermidine; (C) free spermine; for TR1–4 and "Control" explanation see Figure 1

The result implied not only that MGBG affected the levels of non-covalently conjugated spermidine and spermine by affecting the levels of free spermidine and spermine, but also that MGBG decreased cold resistance via decreasing the levels of non-covalently conjugated spermidine and spermine in tonoplast.

Change in covalently conjugated putrescine in tonoplast of peach fruit cells under cold, putrescine and phenanthroline. Only covalently conjugated putrescine in tonoplast could be detected (Figure 4), while the other conjugated polyamines could not be found because their quantities might be too little. Compared with the control, in tonoplast of peach fruit cells under cold stress, the level of covalently conjugated putrescine increased from 45.6 to 63.9 nmol/mg protein. Furthermore, the cold-induced increase was markedly enhanced from 63.9 to 120.1 nmol/mg protein (from "c" to "a") by the pretreatment with exogenous putrescine, indicating that covalently conjugated putrescine were closely related to fruit cold resistance. The notion was further supported by the result with inhibitor phenanthroline. Phenanthroline could potently inhibit transglutaminase activity and thereby decrease the content of covalently conjugated polyamines (Lutts et al. 2013). So, the inhibitor was used in the experiment. In this research, phenanthroline substantially inhibited the exogenous putrescine-induced increase in covalently conjugated putrescine in tonoplast (Figure 4), coupled with the decrease in fruit cold resistance, as judged by the indexes of IPFBA and fruit surface firmness (Figure 1).

Changes in SAMDC and transglutaminase activities under cold, putrescine and inhibitors. SAMDC, the pivotal enzyme in biosynthesis

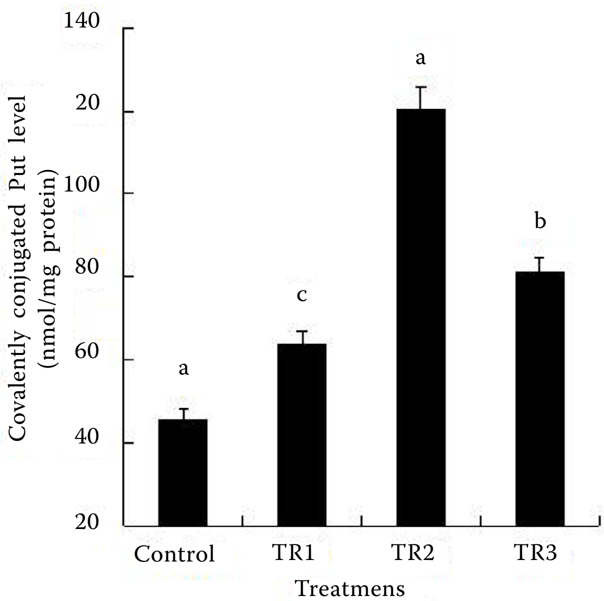

Figure 4. Change in covalently conjugated putrescine in tonoplast of peach fruit cells under cold, putrescine and inhibitor phenanthroline

For TR1–4 and "Control" explanation see Figure 1

https://doi.org/10.17221/87/2023-HORTSCI

of free spermidine/spermine, can affect the contents of the polyamines non-covalently conjugated to tonoplast. The biosynthesis of covalently conjugated polyamines in tonoplast proteins can be directly catalyzed by transglutaminase. So, the activities of the two enzymes were additionally assessed in this research. Compared with the control, in the fruit under cold condition, both SAMDC and transglutaminase activities increased slightly (Figure 5) and the increases were markedly enhanced by 89% (Figure 5A) and 172% (Figure 5B) by the pretreatment with exogenous putrescine, respectively. These results suggested that exogenous putrescine increased the levels of free spermidine and spermine, and non-covalently conjugated spermidine and spermine in tonoplast by elevating SAMDC activity, and thereby enhanced the fruit cold resistance. In addition, exogenous putrescine increased the level of covalently conjugated putrescine in tonoplast by elevating transglutaminase activity, and thereby enhanced the fruit cold resistance. The suggestions were supported by the application of inhibitors, MGBG and phenanthroline, The increases in SAMDC and transglutaminase activities, which were mediated by exogenous putrescine, were inhibited by 37% and 46% with MGBG and phenanthroline pretreatments, respectively (Figure 5), coupled with the decrease in the fruit cold resistance (Figure 1).

Change in vacuole conformation under cold, putrescine, MGBG and phenanthroline. In the control of peach flesh tissue, a large central vacuole could be observed in the cells (Figure 6A). The vacuole was characterized with regular oval appearance, clear and smooth tonoplast border and high transparency, indicating a normal fruit vacuole conformation. Nevertheless, under cold storage, the normal central large vacuole disappeared and some small vacuoles were observed. These vacuoles could be characterized with uneven and vague borders, different sizes, irregular shapes and low transparency (Figure 6B), suggesting that cold stress severely destroyed the normal central large vacuole conformation. Pretreatment with exogenous putrescine could prevent the normal vacuole conformation from being destroyed by cold stress to a great extent, because a central oval large vacuole appeared again with clear and smooth tonoplast border and high transparency (Figure 6C), implying that exogenous putrescine alleviated cold injury in vacuole conformation of peach fruit cells. This alleviation phenomenon might be attributed to the increases in non-covalently conjugated spermidine and spermine (Figure 3), and conjugated putrescine (Figure 4) in tonoplast, which were induced by exogenous putrescine. However, from figure 6D and figure 6E, it could be observed that the alleviation effect was suppressed to a different extent by the pre-

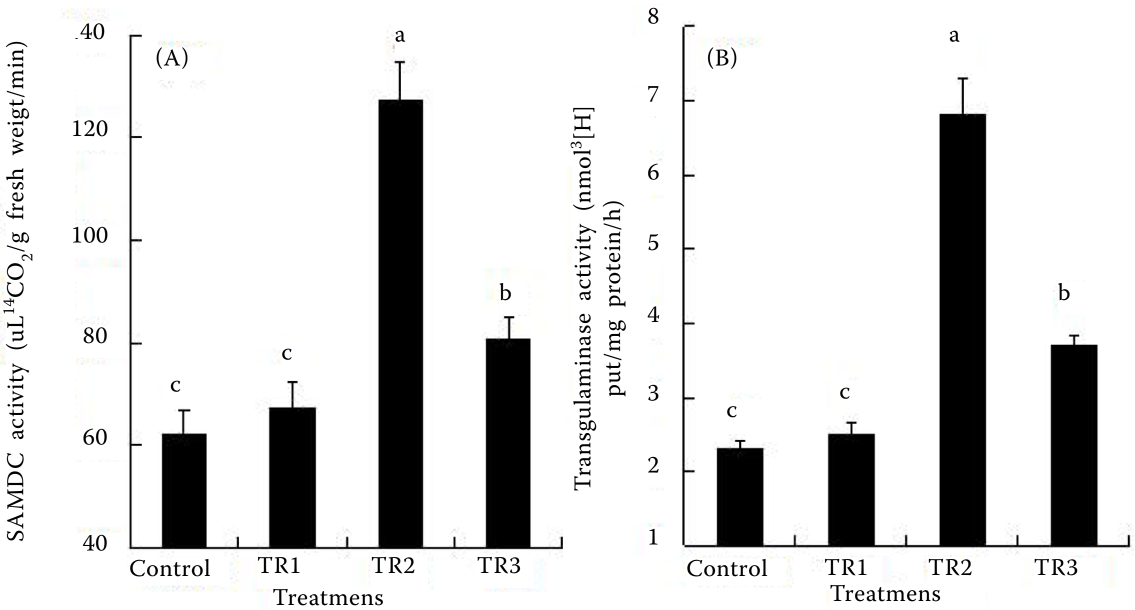

Figure 5. Changes in the activities of S-adenosylmethionine decarboxylase (SAMDC) and transglutaminase under cold, putrescine and two inhibitors

(A) SAMDC activity; (B) transglutaminase activity; for TR1–4 and "Control" explanation see Figure 1

https://doi.org/10.17221/87/2023-HORTSCI

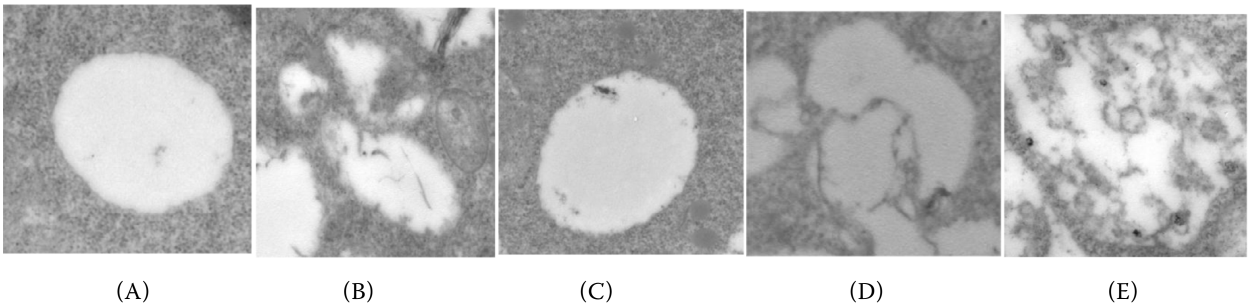

Figure 6. Change in vacuole conformation under cold stress, putrescine and two inhibitors
(A) – Control; (B) – TR1; (C) – TR2; (D) – TR3; (E) – TR4; for TR1–4 and "Control" explanation see Figure 1

treatment with MGBG or phenanthroline, hinting that these inhibitors aggravated cold injury in vacuole conformation, which might be attributed to the decreases in the activities of SAMDC and transglutaminase (Figure 5) and thereby the desreases in non-covalently conjugated polyamines (Figure 3) and conjugated putrescine (Figure 4) in tonoplast.

DISCUSSION

Exogenous putrescine alleviated peach fruit cold injury. Although low temperature storage can prolong fruit preservation, fruit are often subjected to cold stress (Lurie, Crisosto 2005). The decrease in fruit firmness and the increase in browning degree are performances for cold damage (Cao et al. 2016). So, from the changes in two parameters, IPFBA (Figure 1A) and fruit surface firmness (Figure 1B), it could be deduced that harvested peach fruit were definitely subjected to cold stress. From the decrease in fruit flesh browning area and increase in surface firmness, which were induced by exogenous putrescine pretreatment (Figure 1), it can be concluded that exogenous putrescine alleviate peach fruit cold damage. This finding is in a good agreement with the previous reports (Palma et al. 2015; Ahmad, Ali 2019; Hassan et al. 2020).

Many agronomic techniques, including pretreatment with exogenous plant growth regulator, are supplied to prolong the postharvest storage life of vegetable and fruit, such as tomato (Min et al. 2020), potato (Tosetti et al. 2021), zucchini (Palma et al. 2015), kiwifruit (Jiao 2021), carambola (Ahmad, Ali 2019), peach (Cao et al. 2018), etc. Among these regulators, polyamine application has been widely taken into attention (Palma et al. 2015; Ahmad, Ali 2019). Among three main polyamines, putrescine is more preferentially taken into consideration due to its lower price. Therefore, exogenous putrescine could be applied in fruit preservation practices. For a different peach cultivar with different cold resistance, putrescine dose and processing time would be worthy of further exploration and research. The present study can only provide theoretical support for horticulture practice. In the present study, only cold-sensitive peach cultivar was used as experimental material, so in the future, the effect of exogenous putrescine on cold-resistance peach cultivar should deserve to be explored, too.

The mechanism by which exogenous putrescine mitigates fruit cold injury deserves to be further explored. In this study, we found that exogenous putrescine pretreatment alleviated cold stress in peach fruit by elevating the contents of non-covalently conjugated spermidine and spermine, as well as conjugated putrescine in tonoplast, leading to the maintenance of normal vacuole conformation. The further discussion is as follows.

Free polyamines in flesh tissue and cold resistance. Free polyamines in flesh tissue should be elucidated before exploring the conjugated polyamines. No unanimous conclusion can be drawn regarding the significance of endogenous free putrescine accumulation under abiotic stresses (Du et al. 2023). It has well documented that endogenous free polyamines (for example, free putrescineare) are associated with cold tolerance (Galston 1983; Rodriguez et al. 2001). Endogenous free putrescine build-up can cause injury and decrease plant resistance to environmental stresses because oxidation and degradation products of putrescine, such as H_2O_2, amido aldehyde and propylene aldehyde, can be cross-linked with proteins and nucleic acids, leading to cell senescence and apoptosis (Rodriguez et al. 2001; Jing et al. 2020). The present result showed that endog-

https://doi.org/10.17221/87/2023-HORTSCI

enous putrescine level kept contant almost constant under cold, exogenous putrescine and MGBG. These differences might be attributed to different treatment periods and experimental materials, especially cultivar variation in tolerance.

In the present experiment, putrescine pretreatment increased the contents of endogenous free spermidine (Figure 2B) and spermine (Figure 2C), implying that the two free polyamines were involved in cold tolerance. The implication was further verified with the following test of inhibitor treatment. Inhibitor MGBG pretreatment inhibited the increases in free spermidine (Figure 2B) and spermine (Figure 2C) by restraining the activity of SAMDC (Figure 5A), and simultaneously decreased the fruit cold tolerance (Figure 1). Since Spd and Spm carry more positive charges, they could be linked to biomacromolecules, such as acidic proteins and membrane phospholipids, more easily than putrescine, and could play important roles under abiotic stresses (Dutra et al. 2013). Polyamines seem to regulate cell membrane proteins such as two major vacuolar cation channels and plasma membrane H^+-ATPase (Pottosin et al. 2021; Du et al. 2022). Previous research reveals that the conversions of free putrescine into free spermidine and spermine are the main pathway by which the spermidine and spermine levels are elevated (Du et al. 2023). Therefore, we can infer that endogenous free putrescine is converted into free spermidine and spermine by SAMDC under cold and exogenous putrescine treatment. In this sense, SAMDC should play a key function in the context of peach fruit cold resistance.

Conjugated polyamines in tonoplast and cold resistance. The polyamines in plants often function in the conjugated forms. Free polyamines with positive charges could be non-covalently conjugated to membrane phospholipids and acidic proteins with negative charges by electrostatic force (Lutts et al. 2013). Besides, free polyamines could be covalently conjugated to the proteins in bio-membranes (Del Duca et al. 1995; Lutts et al. 2013). In this research, the changes in the conjugated polyamines in peach tonoplast were illustrated. Interestingly, exogenous putrescine pretreatment increased synchronously the cold resistance (Figure 1) and the contents of conjugated polyamines in tonoplast, including non-covalently conjugated spermidine (Figure 3B) and spermine (Figure 3C), and covalently conjugated putrescine (Figure 4). From the results, it can be inferred that the three forms of polyamines might be involved in the tolerance of peach fruit to cold stress. The inference should be further verified by the following experimental testimonies with inhibitor pretreatments. Inhibitor MGBG pretreatment restrained the SAMDC activity (Figure 5A), thereby decreased non-covalently conjugated spermidine (Figure 3B) and spermine (Figure 3C) contents in tonoplast, and simultaneously decreased fruit cold tolerance (Figure 1). Similarly, inhibitor phenanthroline pretreatment restrained transglutaminase activity (Figure 5B), thereby decreased covalently conjugated putrescine content (Figure 4) in tonoplast, and simultaneously decreased fruit cold tolerance (Figure 1).

Pottosin et al. (2021) argued that non-covalently conjugated polyamines in the tonoplast played an important role by regulating the activities of transporters in tonoplast. Del Duca et al. (1995) have reported the function of covalently conjugated polyamines in chloroplasts. Therefore, we can infer that free spermidine and spermine are non-covalently conjugated to phospholipids and acidic proteins in tonoplast by electrostatic interaction, and that free putrescine is covalently conjugated to proteins in tonoplast by transglutaminase under cold and exogenous putrescine treatment. These conjugated polyamines play important roles in maintaining the conformation and function of the tonoplast and proteins in membrane to elevate the resistance of peach fruit to cold stress. Since transglutaminase catalyzes the biosynthesis of covalently conjugated putrescine, its function is obvious in the context of peach fruit cold resistance. In this study, the relationship between the conjugated polyamines and vacuole conformation was focused on and discussed as follows.

Vacuole conformation and cold resistance. Vacuoles of plant cells are important multifunctional organelles for regulating metabolism and reserving ions and metabolites, such as sugars and the other flavor-related substances in fruit. The investigation of vacuole is of great scientific significance and has potential applications in horticulture practice. Plant growing and developing are closely associated with the normal vacuole conformations. Plants are often subjected to abiotic stresses, for example, to drought (Hassan et al. 2020), salt (Lutts et al. 2013), high temperature (Zhang et al. 2023), etc. Vacuoles play pivotal roles in plant responses to abiotic and biotic stresses, and abiotic stresses often lead to the change in vacuole conformation (Tanaka et al. 2007; Bak et al. 2013; Jiang et al. 2021). So, it is regarded as a performance of stress resistance to keep the normal vacuole confor-

https://doi.org/10.17221/87/2023-HORTSCI

mation. In this research, the normal vacuole conformation was destroyed by cold stress (Figure 6B) and exogenous putrescine pretreatment could prevent the abnormal vacuole conformation to a great extent (Figure 6C) and elevating the fruit cold resistance (Figure 1), suggesting that it was vital to maintain the normal vacuole conformation in the context of peach fruit cold resistancewas required for peach fruit cold tolerance. The suggestion was further verified by the pretreatments with inhibitor, MGBG and phenanthroline, which reversed the effects of exogenous putrescine on maintaining vacuole conformation (Figure 6D, 6E) and on alleviating cold injury (Figure 1). The results of Hassan et al. (2020) have showed shown polyamine treatments could maintain vacuole conformation and thereby alleviate wheat seedling drought stress, which is consistent with the present findings. Then, the next question is why exogenous putrescine pretreatment can maintain the normal vacuole conformation under cold stress.

Vacuole conformation and conjugated polyamines to tonoplast. Dutra et al. report (2013) that non-covalently conjugated polyamines in membranes are related to the physical state of the membranes. Del Duca et al. (1995) have explored covalently conjugated polyamines in thylakoid membranes. However, the relationship between vacuole conformation and the conjugated polyamines to tonoplast remains to be answered. Interestingly, in the present study, we found that exogenous putrescine pretreatment not only prevented the abnormal vacuole conformation (Figure 6C), but also elevated markedly the contents of conjugated polyamines in tonoplast, including non-covalently conjugated spermiding (Figure 3B) and spermine (Figure 3C), and covalently conjugated putrescine (Figure 4). The result implied that these conjugated polyamines in tonoplast might be involved in maintaining vacuole conformation. To further verified the finding, inhibitors MGBG and phenanthroline were applied in the experiment. The two inhibitors inhibited exogenous putrescine-induced increases in non-covalently conjugated polyamines (Figure 3B, 3C) and covalently conjugated polyamine (Figure 4), respectively, and simultaneously reversed the effect of exogenous putrescine on vacuole conformation (Figure 6D, 6E). The normal conformation of vacuole and the proteins in tonoplast are important for fruit quality (Jiang et al. 2021). However, abiotic stresses often lead to peroxidation of membrane phospholipid and denaturation of membrane proteins, which result in the membrane phase transformation (Singer, Nicolson 1972). Del Duca et al. (1995) argued that conjugated polyamines in the large subunit of Rubisco could stabilize the protein conformation. Therefore, we can infer that exogenous putrescine pretreatment can maintain vacuole conformation by elevating the polyamines conjugated to tonoplast, which protect tonoplast phospholipid and proteins against peroxidation and denaturation in the context of peach fruit cold resistance.

CONCLUSION

Putrescine pretreatment alleviates cold stress via elevating the contents of non-covalently conjugated spermidine and spermine, and conjugated putrescine in tonoplast, and thereby maintains normal vacuole conformation. Therefore, putrescine-mediated fruit cold resistance involved in these conjugated polyamines in tonoplast and maintenance of membrane conformations. To elucidate more clearly the novel mechanism of exogenous putrescine-mediated cold resistance, a sketch image was displayed in Figure 7. Undoubtedly, the present finding broadens the understanding of polyamine function in horticulture

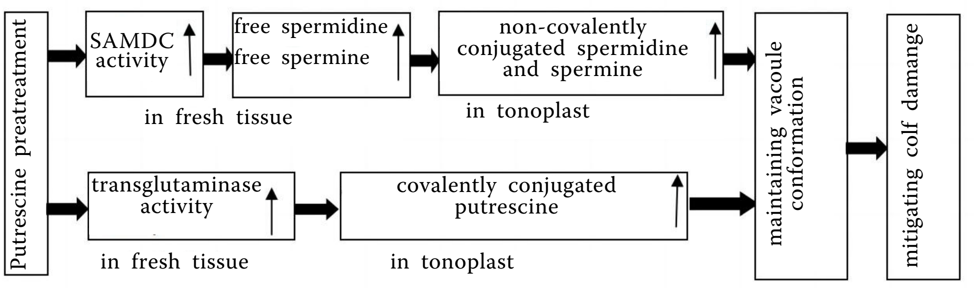

Figure 7. A sketch image of the novel mechanism of exogenous putrescine-induced cold resistance
The bold arrow indicates causal relationship and the thin arrow presents the level increasing

https://doi.org/10.17221/87/2023-HORTSCI

field. However, in the present research, we have done an only modest exploration from the aspects of vacuole morphology and conjugated polyamines in tonoplast. Therefore, three aspects of the study should be addressed in the future. The first involves in exploring proper dose and duration of exogenous putrescine treatment for a different peach cultivar in horticultural industry practice. The second is related to application of ^{14}C-isotope-labeled exogenous putrescine and electron microscope autoradiography technique, so that putrescine conversion would be exactly examined. The third aspect is associated with gene knockout and recombination technology. These new comprehensive data in prospect should shed more interesting light on the arena.

REFERENCES

Ahmad A., Ali A. (2019): Improvement of postharvest quality, regulation of antioxidants capacity and softening enzymes activity of cold-stored carambola in response to polyamines application. Postharvest Biology and Technology, 148: 208–217.

Bak G., Lee E.J., Lee Y., Kato M., Segami S., Sze H., Maeshima M., Wang J., Lee Y. (2013): Rapid structural changes and acidification of guard cell vacuoles during stomatal closure require phosphatidylinositol 3, 5-bisphosphate. Plant Cell, 25: 2202–2216.

Bradford M.M. (1976): A rapid and sensitive methods for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binging. Analytical Biochemistry, 72: 248–254.

Cao S., Shao J., Shi L., Xu L., Shen Z., Chen W., Yang Z. (2018): Melatonin increases chilling tolerance in postharvest peach fruit by alleviating oxidative damage. Scientific Reports, 8: 806. Available at https://doi.org/10.1038/s41598-018-19363-5

Cao S., Song C., Shao J., Bian K., Chen W., Yang Z. (2016): Exogenous melatonin treatment increases chilling tolerance and induces defense response in harvested peach fruit during cold storage. Journal of Agricultural and Food Chemistry, 64: 5215–5222.

Del Duca S., Beninati S., Serafini-Fracassini D. (1995): Polyamines in chloroplasts: identification of their glutamyl and acetyl derivatives. Biochemical Journal, 305: 233–237.

Dietz K.J., Tavakoli N., Kluge C. (2001): Significance of the V-ATPase for the adaption to stressful growth conditions and its regulation on the molecular and biochemical level. Journal of Experimental Botany, 52: 1969–1980.

Du H.Y., Chen B.X., Li Q., Liu H.P., Kurtenbach R. (2022): Conjugated polyamines in root plasma membrane enhanced the tolerance of peach seedling to osmotic stress by stabilizing membrane structure and therefore elevating H^+-ATPase activity. Frontiers in Plant Science, 12: 812360. Available at https://doi.org/10.3389/fpls.2021.812360

Du H.Y., Liu G.T., Liu D.X., Liu H.P., Kurtenbach R. (2023): Significance of putrescine conversion in filling grain embryos of wheat plants subjected to drought stress. Plant and Soil, 484: 589–610.

Dutra N.T., Silveira V., Azevedo I.G., Gomes-Neto L.R., Facanha A.R., Steiner N., Guerra M.P., Floh E.I.S., Santa-Catarina C. (2013): Polyamines affect the cellular growth and structure of pro-embryogenic masses in *Araucaria angustifolia* embryogenic cultures through the modulation of proton pump activities and endogenous levels of polyamines. Physiologia Plantarum, 148: 121–132.

Farooq M., Wahid A., Lee D.J. (2009): Exogenously applied polyamines increase drought tolerance of rice by improving leaf water status, photosynthesis and membrane properties. Acta Physiologiae Plantrum, 31: 937–945

Galston A.W. (1983): Polyamines as modulators of plant development. Bioscience, 33: 382–388.

Gill S.S., Tuteja N. (2010): Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry, 48: 909–930.

Gupta K., Dey A., Gupta B. (2013): Plant polyamines in abiotic stress responses. Acta Physiologiae Plantrum, 35: 2015–2036.

Hassan N., Ebeed H., Aljaarany A. (2020): Exogenous application of spermine and putrescine mitigate adversities of drought stress in wheat by protecting membranes and chloroplast ultra-structure. Physiology and Molecular Biology of Plants, 26: 233–245.

Icekson I., Apelbaum A. (1987): Evidence for transglutaminase activity in plant tissue. Plant Physiology, 84: 972–974.

Jiang Y.T., Yang L.H., Ferjani A., Lin W.H. (2021): Multiple functions of the vacuole in plant growth and fruit quality. Molecular Horticulture, 1: 4. Available at https://doi.org/10.1186/s43897-021-00008-7

Jiao C. (2021): IP3 mediates NO-enhanced chilling tolerance in postharvest kiwifruit. Postharvest Biology and Technology, 176: 111463. Available at https://doi.org/10.1016/j.postharvbio.2021.111463

Jing J.G., Guo S.Y., Li Y.F., Li W.H. (2020): The alleviating effect of exogenous polyamines on heat stress susceptibility of different heat resistant wheat (*Triticum aestivum* L.) varieties. Scientific Reports, 10: 7467. Available at https://doi.org/10.1038/s41598-020-64468-5

Kaur-Sawhney R., Shin M. (1982): Relation of polyamines synthesized titer to aging and senescence in oat leaves. Plant Physiology, 69: 405–410.

Koushesh saba M., Arzani K., Barzegar M. (2012): Postharvest polyamine application alleviates chilling injury and affects

https://doi.org/10.17221/87/2023-HORTSCI

apricot storage ability. Journal of Agricultural and Food Chemistry, 60: 8947–8953.

Lurie S., Crisosto C. (2005): Chilling injury in peach and nectarine. Postharvest Biology and Technology, 37: 195–208.

Lutts S., Hausman J., Quinet M., Lefèvre I. (2013): Polyamines and their roles in the alleviation of ion toxicities in plants. In: Ahmad P., Azooz M., Prasad M. (eds): Ecophysiology and Responses of Plants under Salt Stress. New York USA, Springer: 315–353.

Min D., Zhou J., Li J., Ai W., Li Z., Zhang X., Fu X., Zhao X., Li F., Li X., Guo Y. (2020): SlMYC2 targeted regulation of polyamines biosynthesis contributes to methyl jasmonate-induced chilling tolerance in tomato fruit. Postharvest Biology and Technology, 174: 111443. Available at https://doi.org/10.1016/j.postharvbio.2020.111443

Palma F., Carvajal F., Jamilena M., Garrido D. (2014): Contribution of polyamines and other related metabolites to the maintenance of zucchini fruit quality during cold storage. Plant Physiology and Biochemistry, 82: 161–171.

Palma F., Carvajal F., Ramos J.M., Jamilena M., Garrido D. (2015): Effect of putrescine application on maintenance of zucchini fruit quality during cold storage: Contribution of GABA shunt and other related nitrogen metabolites. Postharvest Biology and Technology, 99: 131–140.

Pottosin I., Olivas-Aguirre M., Dobrovinskaya O., Zepeda-Jazo I., Shabala S. (2021): Modulation of ion transport across plant membranes by polyamines: Understanding specific modes of action under stress. Frontiers in Plant Science, 11: 616077. Available at https://doi.org/10.3389/fpls.2020.616077

Rodriguez S., Lo´pez C.B., Chaves A.R. (2001): Effect of different treatments on the evolution of polyamines during refrigerated storage of eggplants. Journal of Agricultural and Food Chemistry, 49: 4700–4705.

Sharma P., Rajam M.V. (1995): Spatial and temporal changes in endogenous polyamine levels associated with osmotic embryogenesis from different hypocotyls segments of eggplant (*Solanum melongena* L.). Journal of Plant Physiology, 146: 658–664.

Sharma S., Pareek S., Sagar N.A., Valero D., Serrano M. (2017): Modulatory effects of exogenously applied polyamines on postharvest physiology, antioxidant system and shelf Life of Fruits: A Review. International Journal of Molecular Sciences, 18: 1789. Available at https://doi.org/10.3390/ijms18081789

Shi H., Chan Z. (2014): The cysteine2/histidine2-type transcription factor ZINC FINGER OF ARABIDOPSIS THALIANA 6-activated C-REPEAT-BINDING FACTOR pathway is essential for melatonin-mediated freezing stress resistance in Arabidopsis. Journal of Pineal Research, 57**:** 185–191.

Singer S.J., Nicolson, G.L. (1972): The fluid mosaic model of the structure of cell membranes. In: Day S.B., Good R.A. (eds): Membranes and Viruses in Immunopathology. New York, USA, Springer: 7–47.

Suzuki Y., Kanayama Y., Shiratake K., Yamaki S. (1999): Vacuolar H^+-pyrophosphatase purified from pear fruit. Phytochemistry, 50: 535–539.

Tanaka Y., Kutsuna N., Kanazawa Y., Kondo N., Hasezawa S., Sano T. (2007): Intra-vacuolar reserves of membranes during stomatal closure: the possible role of guard cell vacuoles estimated by 3-D reconstruction. Plant and Cell Physiology, 48: 1159–1169.

Tosetti R., Waters A., Chope G.A., Cools K., Alamar M.C., McWilliam S., Thompson A.J., Terry L.A. (2021): New insights into the effects of ethylene on ABA catabolism, sweetening and dormancy in stored potato tubers. Postharvest Biology and Technology, 173: 111420. Available at https://doi.org/10.1016/j.postharvbio.2020.111420

Yamaguchi K., Takahashi Y., Berberich T., Imai A., Takahashi T., Michael A.J., Kusano T. (2007): A protective role for the polyamine spermine against drought stress in Arabidopsis. Biochemical and Biophysical Research Communications, 352: 486–490.

Zhang Y.D., Yang Z.Q., Wang P.J., Xu C. (2023): Long-term high temperature stress decreases the photosynthetic capacity and induces irreversible damage in chrysanthemum seedlings. Horticultural Science (Prague), 50: 159–173.

Received: August 13, 2023
Accepted: December 7, 2023