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Polyamines in plasma membrane function in melatonin-mediated tolerance of apricot fruit to chilling stress

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Abstract: Polyamines are closely associated with environmental stresses and melatonin pretreatment enhances the resistance of fruit to chilling stress. However, a mechanism underlying melatonin-mediated chilling resistance remains to be answered. This research aimed to illuminate whether polyamines would be involved in melatonin-mediated chilling resistance. Therefore, in the experiment, the polyamines conjugated to the plasma membrane from the melatonin-pretreated apricot flesh cell were examined under chilling stress. Chilling resistance was judged by four parameters: fruit browning degree, plasma membrane permeability, malondialdehyde content and plasma membrane protein sulfhydryl level. Results showed melatonin pretreatment led to obvious rises in the levels of non-covalently conjugated spermine and spermidine, and covalently conjugated putrescine and spermidine in the plasma membrane. Methylglyoxyl-bis (guanylhydrazone) pretreatment could inhibit the melatonin-induced increases of non-covalently conjugated spermidine and spermine by inhibiting *S*-adenosylmethionine decarboxylase (SAMDC) activity and free spermidine and spermine contents in flesh, coupled with the decrease in chilling resistance. Similarly, phenanthroline pretreatment could inhibit the melatonin-induced increases in covalently conjugated putrescine and spermidine in the plasma membrane through inhibiting transglutaminase (TGase) activity and simultaneously could aggravate chilling damage. The results suggested melatonin pretreatment could enhance chilling resistance by increasing non-covalently conjugated spermidine and spermine, as well as covalently conjugated putrescine and spermidine in the plasma membrane of apricot fruit.

Keywords: postharvest; fruit quality; chilling resistance; conjugated polyamines; melatonin; mechanism

It is increasingly important and attractive to preserve the nutritional quality of fruit for a long time. For example, cold storage has been developed for maintaining fruit quality after harvest (Cao et al. 2016), while dehydration transforms the fresh product into a different one (Karaaslan and Ekinci 2022). Low temperature storage sometimes is regarded as one of the most effective methods for keeping postharvest fruit fresh. However, cold storage can bring about some changes in physiological and biochemical indicators, such as malondialdehyde content, relative plasma membrane permeability, degree of flesh browning and sulfhydryl group content in proteins, which are generally called chilling damage (Lurie and Crisosto 2005; Cao et al. 2016). The main symptoms of fruit chilling injury are changes in skin colour, gel breakdown, flesh woolliness and colour, etc. (Koushesh Saba et al. 2012). Among the symptoms, flesh browning is typical in apricot fruit. Therefore, some modern

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effective bio-techniques, such as treatment with an exogenous regulator, are applied to alleviate chilling damage and elevate the resistance of fruit against cold stress (Palma et al. 2015; Min et al. 2020; Jiao 2021). Recently, the application of melatonin (*N*-acetyl-5-methoxytryptamine) has been increasingly attractive. Melatonin pretreatment can enhance the resistance of summer fruit against low temperatures (Cao et al. 2016). However, the mechanism underlying melatonin-induced chilling resistance remains to be answered.

Polyamines are plant growth regulators that mainly consist of putrescine, spermidine and spermine. Putrescine can be converted into spermidine and spermine through being linked with one and two aminopropyls, respectively. The biosynthesis of spermidine or spermine is mainly regulated by *S*-adenosylmethionine decarboxylase (SAMDC) (Tiburcio et al. 1993). Slocum (1991) reported that methylglyoxal-bis (guanylhydrazone) (MGBG) was the potent inhibitor of SAMDC. Besides free forms, polyamines, which carry positive charges at physiological pH, are able to be non-covalently conjugated to acidic proteins and phospholipid in cell membranes, which carry negative charges at physiological pH, through ionic and hydrogen bond (Sood and Nagar 2003). Galston and Kaur-Sawhney (1995) reported that non-covalently conjugated polyamines play key roles in maintaining cell membrane integrity under environmental stresses. Additionally, polyamine can be covalently conjugated to protein glutamine residues through transglutaminase (TGase) action, of which phenanthroline is a strong inhibitor. Covalently conjugated polyamines function in modifying proteins at the post-translating stage (Del Duca et al. 1995). Even though polyamines are involved in some studies on the mechanism of chilling resistance (Koushesh Saba et al. 2012; Jahan et al. 2019a), so far, the significance of polyamines conjugated to the plasma membrane in melatonin-induced chilling resistance has not been revealed yet.

The plasma membrane is an important site where plant cells first sense environmental stress information, including a chilling stress signal. Under cold conditions, the plasma membrane is damaged and the damage is performed by increasing the membrane permeability and the content of malondialdehyde, which is a by-product of peroxidation of membranes, as well as decreasing the content of protein sulfhydryl in the plasma membrane. Therefore, it would be anticipated that melatonin pretreatment could enhance apricot fruit chilling resistance through increasing the levels of non-covalently conjugated spermidine and spermine, as well as covalently conjugated putrescine and spermidine in the plasma membrane, which might maintain normal conformation and function of plasma membrane and proteins.

To explore the mechanism underlying melatonin-induced chilling resistance, postharvest apricot fruit was used as experimental material. Non-covalently and covalently conjugated polyamines in the plasma membrane of fruit flesh were detected and a relationship between these forms of conjugated polyamines and chilling resistance was discussed.

MATERIAL AND METHODS

Material selection and treatment

In the orchard (latitude: 33°38'28", longitude: 114°40'52") of Zhoukou Normal University, the fruit of apricot (*Prunus armeniaca* L.) cultivar 'Yuxuan 5', which were bred by Henan Academy of Agricultural Sciences, China, were selected and harvested when the volume of the fruit stopped increasing and the colour was becoming yellow. After fruits were picked, they were shortly transferred to a laboratory. For uniformity in volume and colour, the fruits were selected without any damage. Then, they were divided into four groups and treated with chilling, melatonin, inhibitors MGBG or phenanthroline.

Apricot fruit were immersed in de-ionised water (control group), melatonin solution (0.1 mM) [treatment 1 group (T1)], melatonin (0.1 mM) + MGBG (0.2 mM) solution [treatment 2 group (T2)] and melatonin (0.1 mM) + phenanthroline (0.1 mM) solution [treatment 3 group (T3)] for 100 min, then shifted into a chamber with relative humidity of 80% and suffered chilling storage at 4 °C for 30 days. According to our preliminary experiment, the concentrations of melatonin at 0.1 mM, MGBG at 0.2 mM and phenanthroline at 0.1 mM were appropriate, and the three reagents were from Sigma Chemical Co. (US). During 30 days of storage at 4 °C, the fresh fruits were sampled on the 0^{th}, 6^{th}, 12^{th}, 18^{th}, 24^{th}, and 30^{th} day. The experiments were repeated in 2019, 2020, and 2021. Every year, 180 fruit were selected as materials for every treatment group (including the control), so 720 fruits for four groups were used in one year. During the time-course for 30 days of storage, 180 fruits were sampled 6 times. So at each time, 30 fruits were sampled for 3 replicates.

Assessment of physiological parameters

The degree of apricot flesh browning was assessed by the method of Cao et al. (2016) with modifica-

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tions by observed browning surface with a range from 0 to 4: degree 0, no browning area; degrees 1, 2, 3, and 4 meant the browning area < 20%, ≥ 20% and < 50%, ≥ 50% and < 70%, and ≥70% of the cut surface, respectively. The chilling injury index indicated the browning degree and was calculated by the formula in Equation 1.

Plasma membrane permeability was assessed by referring to the means of Jahan et al. (2019b) with a few adjustments. The fruit flesh tissue was immersed into de-ionised water and incubated for 2 h at 25 °C. Original electrical conductivity (OEC) of the water containing the flesh tissue was determined with a conductivity meter. Afterwards, the samples were boiled and cooled. The terminal electrical conductivity (TEC) was determined. De-ionised electrical conductivity (DEC) represented electrical conductivity (EC) of de-ionised water. Relative plasma membrane permeability was assessed by the formula: $(OEC - DEC)/(TEC - DEC) \times 100$.

Malondialdehyde content was determined through the method of Alexieva et al. (2001) with some modifications. After the flesh sample was ground (Model JJ-2; Shanghai Shuaideng Instrument Equipment Co., Ltd., China) in trichloroacetic acid solution and centrifuged (Model TGL-16; Hunan Xiangyi Instrument Equipment Co., Ltd., China), aliquot trichloroacetic acid was added to the supernatant. It was boiled, cooled down and centrifuged. The content of malondialdehyde was determined by detecting absorbance at 532 nm and 600 nm.

A part of the prepared plasma membrane vesicle solution was treated with the disulphide reagent. Sulfhydryl group content in the plasma membrane of flesh cells was detected following the method described by Ellman et al. (1959). One unit was defined as nmol mg^{-1} protein.

Detection of biochemical parameters

Free polyamines in the tissue of apricot fruit flesh. Extraction and quantification of free polyamines were conducted by the method of Sharma and Rajam (1995) with minor adjustments. An aliquot of 0.5 g of flesh tissue was homogenised (Model JJ-2; Shanghai Shuaideng Instrument Equipment Co., Ltd., China) in 5 mL perchloric acid (5%, v/v), and the homogenate was left to stand for 1 h in a refrigerator at 4 °C. Then, it was centrifuged at 21 500 × g for 30 min (Model TGL-16; Hunan Xiangyi Instrument Equipment Co., Ltd., China). The supernatant was collected for a free polyamine assay. After benzoylation with benzoyl chloride, free putrescine, spermidine and spermine were quantified by high performance liquid chromatography (HPLC) (Model 2695; Waters, US).

Purification of the plasma membrane. Apricot fruit flesh tissue (2 g) was ground (Model JJ-2; Shanghai Shuaideng Instrument Equipment Co., Ltd., China) in sorbitol Tris-Hepes buffer (70 mM, pH 7.5). After filtering (diameter 9 cm filter paper; Shanghai Shunyou Technology Co., Ltd., China), the sample was centrifuged at 15 000 × g for 15 min. The supernatant was then centrifuged at 75 000 × g for 40 min. After microsomal precipitate was suspended in sucrose Tris-Hepes buffer (3.0 mM, pH 7.8), it was further purified (Model XPN-100; Beckman Culter Co., Ltd., US) through the method of Qiu and Su (1998) to obtain the isolated plasma membrane solution.

Isolation and detection of proteins in the plasma membrane. The isolated plasma membrane solution was added 10% Triton X-100 (v/v) drop-wise until the triton concentration being lowered to 1% and then ultrasonicated with an ultrasonic disintegrator (Model 200-W; Shanghai Guanter Co., Ltd., China). Afterwards, it was centrifuged at 20 000 × g for 30 min. The supernatant consisted of the proteins in the plasma membrane. Protein content was detected by the Bradford method of protein-dye binding (Bradford 1976); bovine serum albumin was used as standard.

Determination of non-covalently and covalently conjugated polyamines. They were determined through the method of Sharma and Rajam (1995) with a few modifications. Perchloric acid (w/v: 5%) was added into the aforementioned isolated plasma membrane solution. The sample was centrifuged for 40 min at 30 000 × g. The supernatant contained non-covalently conjugated polyamines. Perchloric acid (w/v: 5%) was added into the aforementioned plasma membrane protein solution. The mixture was centrifuged for 50 min at 30 000 × g at 4 °C. The precipitate was hydrolysed with 12 N HCl. Then, two forms of polyamines were benzoylated and quantified by HPLC through the method of Sharma and Rajam (1995).

Determination of SAMDC and TGase activities in apricot flesh. SAMDC activity was assayed by examining the release of $^{14}CO_2$ using substrates labelled with isotope ^{14}C, through the method of Kaur-Sawhney and Shin (1982). TGase activity was assessed via detecting the incorporation rate of putrescine labelled with $^{3}[H]$

$$Chilling\ injury\ index = \frac{\sum\left[\left(browning\ degree\right)\times\left(fruit\ number\ of\ the\ browning\ degree\right)\right]}{\left[4\left(the\ highest\ browning\ degree\right)\times 10\left(fruit\ number\ of\ each\ treated\ group\right)\right]} \quad (1)$$

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into proteins according to the method of Icekson and Apelbaum (1987).

Statistical analysis

The data shown in the paper are averages of 9 values ± standard error (SE). Microsoft Excel and SPSS 16.0 (SPSS Inc., US) were used for analysing data. The deviation of the averages was statistically evaluated by analysis of variance (ANOVA) and Duncan's method was used to compare multiple group means at $P < 0.05$ level. Significant differences between multiple groups were indicated by different letters above the columns in the figures.

RESULTS AND DISCUSSION

Chilling damage was alleviated by melatonin and aggravated by polyamine biosynthesis inhibitors. The parameter of flesh browning degree showed that apricot fruit suffered chilling damage. After 6 days of chilling storage, apricot fruit began to develop damage symptoms as displayed by flesh browning degree, and the damage degree increased with the prolonged time of chilling storage (Figure 1A). Reactive oxygen species induced by chilling stress could lead to the peroxidation of lipids and proteins in the plasma membrane

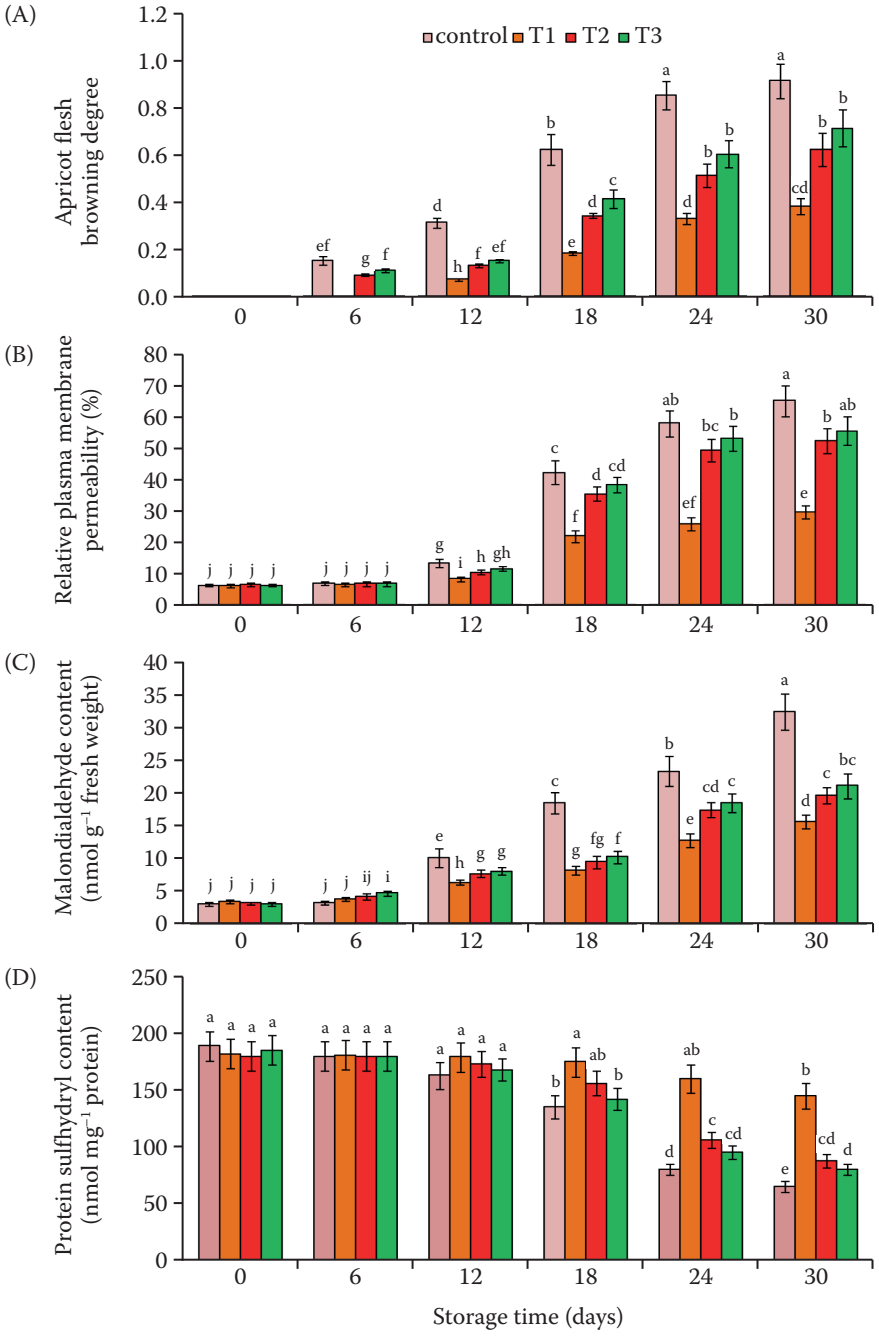

Figure 1. Changes in apricot (A) flesh browning degree, (B) relative plasma membrane permeability, (C) malondialdehyde, and (D) protein sulfhydryl contents

[a–j]Significant differences between multiple groups are indicated by different letters above the columns in the figure ($P < 0.05$); T1 – treatment 1 group; T2 – treatment 2 group; T3 – treatment 3 group

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(Koushesh Saba et al. 2012; Cao et al. 2016, 2018), which might result in increasing the plasma membrane permeability and malondialdehyde content and decreasing the protein sulfhydryl content. Therefore, to provide more evidence for chilling damage to apricot fruit, some latent parameters, such as plasma membrane permeability (Figure 1B), malondialdehyde (Figure 1C) and protein sulfhydryl (Figure 1D) contents, were examined in the research. All of the changes in these parameters indicated that apricot fruits were suffering from chilling damage, since plasma membrane permeability and malondialdehyde content increased, and sulfhydryl content decreased.

However, 0.1 mM melatonin treatment led to a decrease in browning degree and no marked browning was found on the 6th day. On the 12th day, browning merely began to appear mildly and on the 30th day, damage degree only got to less than 50%, compared with the control. Besides the browning degree index, the changes in the other parameters collectively showed that exogenous melatonin treatment authentically enhanced the resistance of apricot fruit to chilling stress. In the research, the roles which conjugated polyamines in the plasma membrane might play in melatonin-mediated resistance were illuminated.

Free spermidine and spermine were involved in melatonin-mediated resistance. To explore the function of conjugated polyamines in the plasma membrane, it was necessary to examine free polyamines in apricot flesh. In the apricot flesh tissue, three major free polyamines, putrescine, spermidine and spermine could be detected. Under chilling conditions at 4 °C, free putrescine increased sharply during the storage for 30 days (Figure 2A). Free spermidine (Figure 2B)

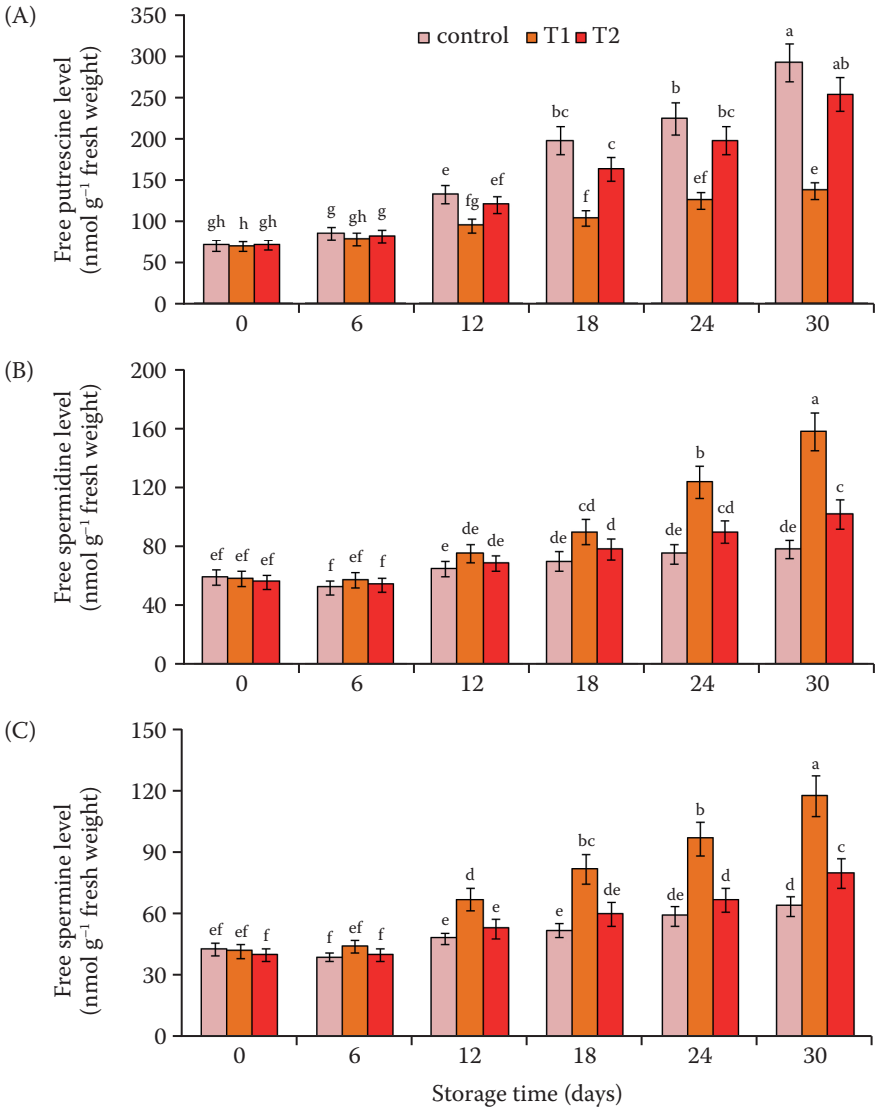

Figure 2. The dynamic course of free polyamine content: (A) putrescine, (B) spermidine, and (C) spermine in apricot flesh

$^{a-h}$Significant differences between multiple groups are indicated by different letters above the columns in the figure ($P < 0.05$); T1 – treatment 1 group; T2 – treatment 2 group

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and free spermine (Figure 2C) levels remained almost unchanged throughout the whole chilling storage at 4 °C for 30 days. Melatonin pretreatment significantly ($P < 0.05$) affected the free polyamine contents. After chilling storage at 4 °C for 6 days, free spermidine and free spermine levels of the melatonin-pretreated group increased significantly ($P < 0.05$), coupled with the marked decrease in putrescine content during the 30-day storage period.

Min et al. (2020) reported that free polyamines in fruit played an important function in methyl jasmonate-mediated resistance of tomato fruit to chilling. In the present study, we found that free spermidine and spermine might be involved in melatonin-mediated resistance. The finding was supported by melatonin and MGBG treatments. Firstly, exogenous melatonin pretreatment markedly ($P < 0.05$) enhanced chilling resistance, which was judged by the parameters of flesh browning degree (Figure 1A), plasma membrane permeability (Figure 1B), malondialdehyde (Figure 1C) and protein sulfhydryl (Figure 1D) contents. Meanwhile, exogenous melatonin enhanced the increases in SAMDC activity (Figure 3A) and the contents of free spermidine and spermine (Figures 2B, 2C), and inhibited the increase in putrescine (Figure 2A), compared with the control. Secondly, MGBG treatment inhibited melatonin-induced resistance (Figure 1), coupled with inhibiting melatonin-induced increases in SAMDC activity (Figure 3A) and the contents of the polyamines (Figures 2B, 2C). The study of Palma et al. (2015) showed free putrescine played roles in the resistance. Therefore, we speculated that the increase in putrescine content might only be a premise for chilling resistance and a key issue was whether chilling-induced free putrescine could be subsequently transformed to free spermidine and spermine. The speculation was testified by our study results on the free putrescine profile (Figure 2A) in apricot flesh under chilling, melatonin and MGBG treatments. Chilling stress induced the increase in free putrescine and melatonin inhibited the chilling-induced increase, while MGBG reversed the melatonin effect. Then, why could free spermidine and free spermine play an important function in the resistance induced by melatonin? They carried more positive charges and thereby could be non-covalently conjugated more easily to cell components with negative charges. The plasma membrane was mainly involved in chilling stress, so non-covalently conjugated polyamines in the plasma membrane were detected next.

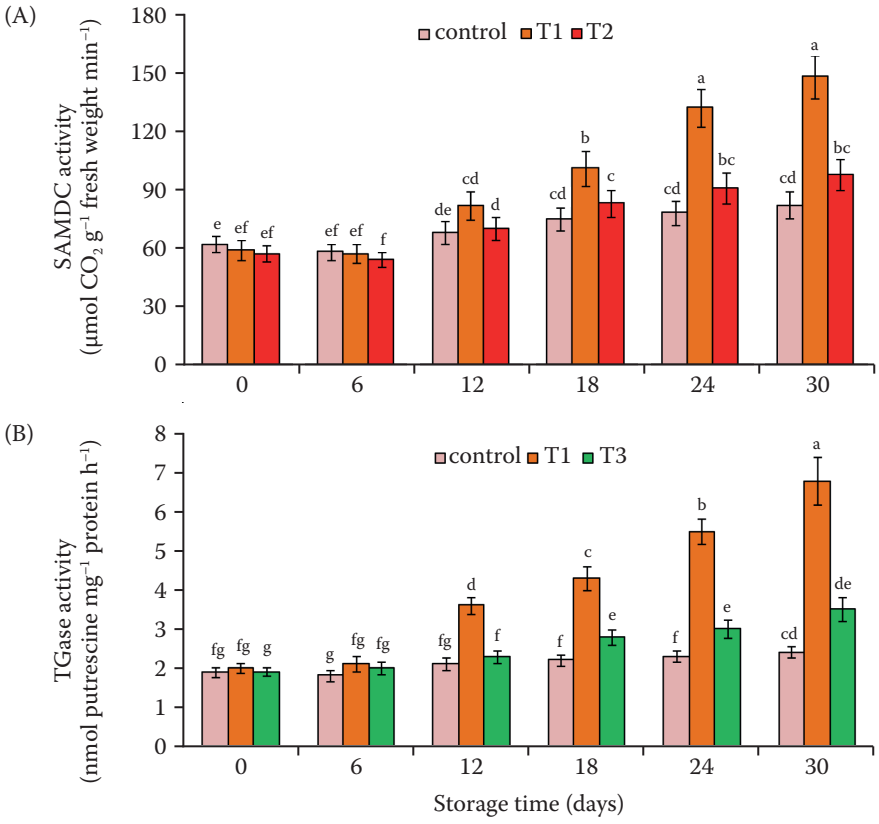

Figure 3. The dynamic course of enzyme activity: (A) SAMDC and (B) TGase in apricot flesh during chilling storage

[a–g]Significant differences between multiple groups are indicated by different letters above the columns in the figure ($P < 0.05$); T1 – treatment 1 group; T2 – treatment 2 group; T3 – treatment 3 group; SAMDC – *S*-adenosylmethionine decarboxylase; TGase – transglutaminase

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Function of spermidine and spermine non-covalently conjugated to plasma membrane. Chilling stress led to an increase in the non-covalently conjugated putrescine level in the plasma membrane from the apricot flesh tissue, whereas non-covalently conjugated spermidine and spermine contents were maintained nearly unchanged throughout the entire storage period of chilling at 4 °C (Figure 4). Melatonin pretreatment inhibited markedly ($P < 0.05$) the chilling-induced increase in the non-covalently conjugated putrescine level after storage for 18 days at 4 °C, and meanwhile melatonin pretreatment induced markedly ($P < 0.05$) the increases in non-covalently conjugated spermidine and spermine levels after storage for 12 days and 18 days at 4 °C, respectively. Treatment with MGBG reversed the effects of melatonin treatment on non-covalently conjugated spermidine and spermine contents markedly ($P < 0.05$) (Figures 4B, 4C). From these interesting results, it could be concluded that under chilling, melatonin and MGBG treatments, the changes in non-covalently conjugated spermidine and spermine contents were in parallel with the change in chilling resistance (Figure 1) during the entire storage. The finding was supported by Dutra et al. (2013), who suggested that free spermidine and spermine, which carry more positive charges than putrescine, could be non-covalently conjugated to the plasma membrane more easily and maintain the plasma membrane configuration and function.

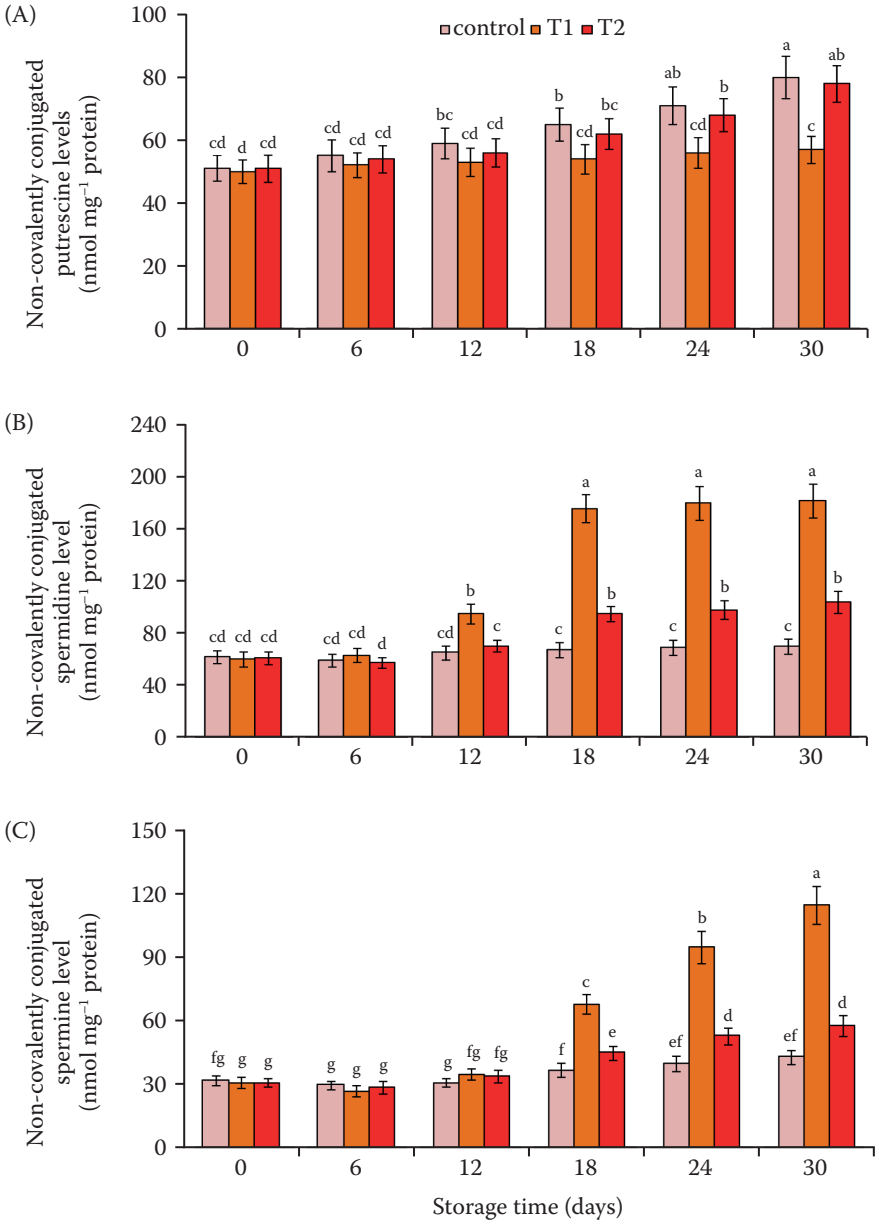

Figure 4. The dynamic course of non-covalently conjugated polyamine content: (A) putrescine, (B) spermidine, and (C) spermine in the membrane

[a–g]Significant differences between multiple groups are indicated by different letters above the columns in the figure ($P < 0.05$); T1 – treatment 1 group; T2 – treatment 2 group

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Why were non-covalently conjugated polyamines related to plant resistance? The interaction of ion channels and conjugated polyamines might be involved in the issue. For instance, it was suggested that non-covalently conjugated polyamines regulated ion channels in bio-membranes (Williams 1997), and in guard cells, a stomatal movement was modulated by the polyamines through the KAT_1-like (K^{+1} transporter 1) channels (Liu et al. 2000). Additionally, the activity of the proton pump in plasma membrane could be enhanced by the polyamines (Athwal and Huber 2002). All the researches have suggested that non-covalently conjugated polyamines can play an important role in maintaining the conformation and function of the plasma membrane and membrane proteins.

Significance of putrescine and spermidine covalently conjugated to the plasma membrane. Besides free and non-covalently conjugated polyamines, by TGase catalysing, free polyamines might be conjugated covalently to protein glutamine residues (Del Duca et al. 1995). Cell membranes are rich in proteins, such as proton pumps, ion channels, transporters, and protein enzymes. So in the study, the contents of the polyamines conjugated covalently to protein were also assessed. The melatonin-mediated resistance was closely associated with TGase activity in apricot flesh and covalently conjugated polyamines (Figure 5) in the plasma membrane, because melatonin pretreatment markedly ($P < 0.05$) increased TGase activity (Figure 3B) and the contents of covalently conjugated putrescine (Figure 5A) and spermidine (Figure 5B). More interestingly, pretreatment with phenanthroline (TGase inhibitor) decreased significantly ($P < 0.05$) not only the melatonin-mediated chilling resistance (Figure 1), but also TGase activity (Figure 3B), resulting in the drastic decreases in the contents of covalently conjugated putrescine and spermidine. Sulfhydryl in plasma membrane protein was oxidised by reactive oxygen species induced by chilling stress, and transformed into disulphide bonds, resulting in protein structure destruction and denaturation. Free putrescine and spermidine might be covalently conjugated to proteins in the plasma membrane to inhibit the oxidation induced by chilling stress. Our suggestion was in good accordance with the results of Del Duca et al. (1995) on covalently conjugated polyamines in the thylakoid membrane of the chloroplast. Collectively, it could be inferred that covalently conjugated putrescine and spermidine in the plasma membrane should play a similarly important role to non-covalently conjugated spermidine and spermine in maintaining the normal conformation and function of the plasma membrane and membrane proteins.

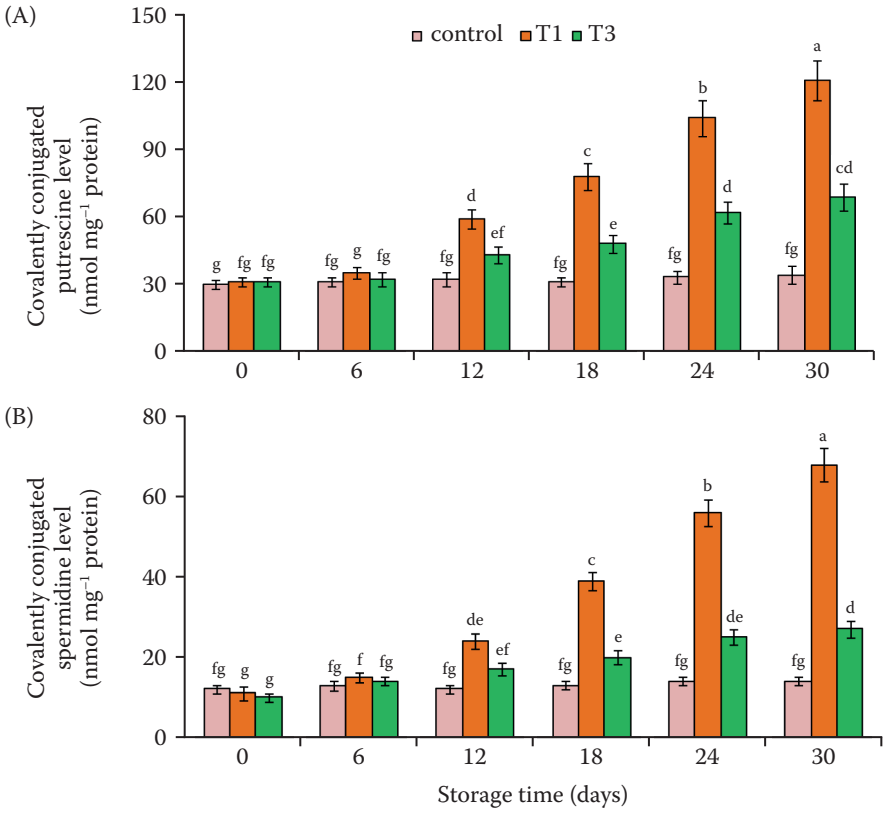

Figure 5. The dynamic course of covalently conjugated polyamine content: (A) putrescine and (B) spermidine in the membrane

[a–g]Significant differences between multiple groups are indicated by different letters above the columns in the figure ($P < 0.05$); T1 – treatment 1 group; T3 – treatment 3 group

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CONCLUSION

In summary, this work was the first to illuminate that melatonin pretreatment enhanced chilling resistance via increasing non-covalently conjugated spermidine and spermine, as well as covalently conjugated putrescine and spermidine in the plasma membrane of apricot fruit. Although the present study was just regarded as a modest spur, it revealed a novel mechanism underlying melatonin-mediated chilling resistance. In the future, the research on conjugated polyamines in bio-membranes would be increasingly attractive. By gene knockout and recombination technology, new comprehensive data in prospect would shed more interesting light on mechanisms underlying conjugated polyamines enhancing the resistance of fruit to chilling stress.

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