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Salicylic acid alleviates chilling injury in 'Huangguan' pear

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Summary

Chilling injury (CI) often occurs in 'Huangguan' pear (*Pyrus bretschneideri* Rehd) at low temperature storage, which is characterized by brown spot on the fruit surface. In this study, the 'Huangguan' pear fruit was soaked either with salicylic acid (SA) or distilled water (control) and subsequently stored at 0 °C. The results showed that 5 mM and 10 mM SA treatments significantly reduced the CI index of the fruit compared with the control, but had no significant effect on fruit firmness, soluble solids content (SSC) and titratable acid (TA) content. Further study on the mechanism of CI showed that 5 mM SA treatment increased the content of SA in peel, enhanced the activities of ascorbic acid peroxidase (APX), glutathione reductase (GR) and superoxide dismutase (SOD), reduced the accumulation of phenols in the later stage, decreased the activity of polyphenol oxidase (PPO) before the occurrence of CI, inhibited the expression of *PPO1* and *PPO5* genes in peel, and significantly down-regulated expression of *LOX1 and PLD4,* which code for lipoxygenase and phospholipase D, respectively. These results indicated that SA treatment increased the antioxidant capacity of the peel, inhibited the degradation of cell membrane lipids, reduced the appearance of brown spot on the fruit surface and alleviated CI during cold storage in 'Huangguan' pear.

Keywords: Salicylic acid; Pear; Antioxidant; Phenol; Chilling injury

Introduction

'Huangguan' pear (*Pyrus bretschneideri* Rehd) has become one of the main cultivars in China with the characteristics of early maturity, beautiful appearance and good quality. In order to extend its shelf life, low-temperature storage is often adopted after harvest for 'Huangguan' pear, however, it is sensitive to low temperature and easy to cause chilling injury (CI), mainly manifested by the brown spots on the fruit surface (Li et al., 2017), with an incidence of about 30% and a serious incidence of over 90%, resulting in huge economic losses (Li et al., 2011).

Salicylic acid (SA) is an endogenous small molecule phenolic compound, which plays an important role in regulating stress resistance, growth and development in plants (Han et al., 2017; Supapvanich et al., 2017). Studies have shown that SA enhanced CI in cucumber (Cao et al., 2009), tomato (Aghdam et al., 2014), pineapple (Lu et al., 2010), li (Luo et al., 2011), pomegranate (Sayyari et al., 2011) and peach (Cao et al., 2010; Yang et al., 2012) fruits.

Excessive reactive oxygen species (ROS) lead to the occurrence of CI caused by oxidative stress, and SA enhances the activities of superoxide dismutase (SOD), ascorbic acid peroxidase (APX), and glutathione reductase (GR) and therefore SA is involved in chilling resistance (Siboza and Bertling, 2013; Siboza et al., 2017). CI is also closely related to the damage of cell membranes and the oxidation of phenolic substances. Lipoxygenase (LOX) and phospholipase D (PLD) are involved in cell membrane lipid degradation. SA treatment reduces the activities of PLD and LOX, maintaining cell membrane integrity and slowing down CI in tomato fruit (Aghdam et al., 2014). Phenolic metabolism is related to the occurrence of brown spot (Li et al., 2011; Li et al., 2017), and polyphenol oxidase (PPO) catalyzes the phenolic oxidation and plays an important role in the process of tissue browning (Lu et al., 2011; Tareen et al., 2012).

So far, there is no relevant information on the effect of SA treatment on CI of pear fruit. Therefore, in this study, 'Huangguan' pear was treated with SA to study the changes of the activities of some enzymes and the expression of genes associated with CI, and to explore the mechanism of SA slowing down CI in 'Huangguan' pear fruit.

Materials and methods

Materials

'Huangguan' pear fruit were harvested from an orchard located in Jinzhou city (38.03356° N, 115.0441° E), Hebei Province on August 14, 2016. Fruit without pests, diseases and mechanical injury were selected. The fruit were soaked in 5 and 10 mM SA (Sangon Biotech Co. Ltd., Shanghai, China) solution for 30 minutes, the control was distilled water, and then naturally dried at room temperature overnight, and subsequently stored at 0 °C. After the determination of fruit quality, the peel tissue was sampled, immediately frozen in liquid nitrogen and then stored at -80 °C for use. Triplicate per treatment, 10 fruit per replicate.

Determination of fruit quality and CI Index

Firmness was determined by fruit hardness tester (GY-4, Zhejiang, China), soluble solids content (SSC) was determined by portable sugar tester (PAL-1, Atago, Japan), titratable acid (TA) content was determined by acid-base titration with reference to Cao et al. (2007). According to the method of Li et al. (2017), the classification of CI as brown spot area on fruit surface with 0, 0-25%, 25%-50% and > 50% was assigned as grade 0, 1, 2 and 3, respectively. The CI index = Σ (each CI grade × the corresponding fruit quantity) / (3 × the total number of fruit).

Determination of SA content

Frozen peel was ground using liquid nitrogen as coolant and a ground sample of 50 mg was extracted over night at -20 °C in 0.5 ml 80% (v/v) methanol. The supernatant, obtained after centrifugation at 10000 *g* for 60 min at 4 °C was filtered with a C-18 solid phase extraction column and subsequently dried with nitrogen gas. The dried sample was dissolved in 0.5 ml 0.01 M PBS (pH=7.2-7.4), then the supernatant was obtained after centrifuged at 10000 *g* for 15 min at 4 °C. The content of SA was determined by using plant SA ELISA kit (ml036342; Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) with a microplate reader (Rayto RT-6100, Shenzhen, China). The content of SA was calculated based on a standard curve. The SA content was expressed as $\mu g \cdot g^{-1}$ FW.

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Determination of enzyme activity

Frozen peel was ground using liquid nitrogen as coolant and a ground sample of 50 mg was extracted in 0.5 ml 0.01 M PBS (pH=7.2-7.4). The supernatant was obtained after centrifuged at 5000 *g* for 15 min at 4 °C and used as crude enzyme extract. The activities of SOD, APX, GR and PPO were determined by using plant SOD, APX, GR and PPO ELISA kits (ml397001, ml764550, ml764402 and ml763547; Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) with a microplate reader (Rayto RT-6100, Shenzhen, China), respectively. The unit of catalytic activity (1 U) of SOD, APX, GR and PPO was defined as conversion of 1 μmol substrates into products per minute, i.e. 1 U = 1 μmol·min^{-1}. The enzyme activity was expressed by the unit of enzyme activity in the crude enzyme extract per gram (U·g^{-1} FW).

Determination of phenolic content

The determination of total phenolic content refers to SINGLETON and ROSSI (1965), which is based on the Folin-Ciocalteau method. Frozen peel samples where ground using liquid nitrogen and subsequently 0.5 g aliquots were combined with 5 ml 80% (v/v) ethanol and extracted by ultrasonic treatment. The supernatant was obtained after centrifuged at 10000 *g* for 15 min at 4 °C. The absorbance was measured at 760 nm. The standard curve was carried out with gallic acid, and the phenol content was expressed as mg·g^{-1} FW.

RNA Extraction and Real-time Fluorescence Quantitative PCR (qRT-PCR) Analysis

RNA extraction: The frozen peel was ground with liquid nitrogen, and the total RNA was extracted from 100 mg ground sample according to the instruction of RNA prep Pure Plant kit (Tiangen Biotechnology Co., Ltd., Beijing, China).

qRT-PCR analysis: 500 ng total RNA was reverse transcribed into cDNA by referring to the instructions of the PrimeScript™ RT Reagent kit and subsequently applying the gDNA Eraser kit (TaKaRa, Dalian, China). The SYBR® Premix Ex Taq™ II kit (TliRNaseH Plus) (TaKaRa, Dalian, China) was used according to manufacturer's instructions to perform RT-PCR analysis with the 7500 qRT-PCR system (ABI, Applied Biosystems Inc., USA). With pear *PbActin2* as internal reference gene (LI et al., 2017), the gene expression was defined as 1.0 at initial value in the control, and the relative quantitative expression of gene was referred to the $2^{(-\Delta\Delta Ct)}$ method (ZHANG et al., 2017). Quantitative PCR primers were designed according to 'Dangshan Suli' pear genome (http://www.ncbi.nlm.nih.gov/genome/12793). The sequence of primers is shown in Tab. 1.

Statistical Analysis

Data were analyzed by SPSS software (Version 18.0, SPSS Inc., Chicago, USA). Results were expressed as the mean ± standard error (SE) of triplicate value. The Duncan's multiple range test of single factor was used to analyze the significant difference ($p < 0.05$).

Results

Effect of SA on fruit CI and quality in 'Huangguan' pear

CI occurred on the fruit surface after 10 days of cold storage at 0 °C in 'Huangguan' pear, and afterwards, the CI index increased slowly in SA treatment in contrast to that in control. This indicated that SA treatment significantly inhibited the occurrence of CI, but there was no significant difference between the two concentrations of SA treatment (Fig. 1).

It was found that firmness, SSC and TA content in SA treatment had no significant difference compared with the control at 0 °C during a short-term storage (30 days) in 'Huangguan' pear fruit (Tab. 2), which indicated that SA had no significant effect on fruit quality.

In order to further reveal the mechanism of SA inhibiting CI, the fruit treated with 5 mM SA were selected for further study.

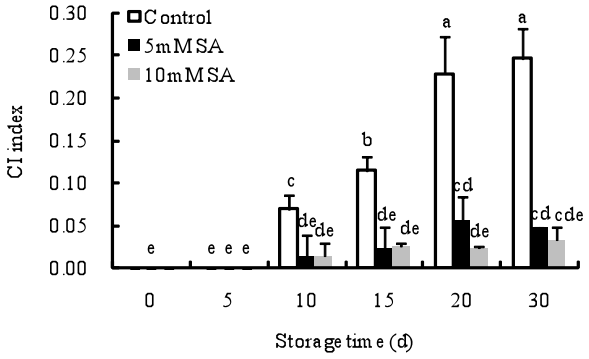

Fig. 1: Changes of CI index in 'Huangguan' pear by SA treatment during cold storage. Values are mean ± standard error (SE) of three replicates. Different letters represent significant differences at $P = 0.05$.

Tab. 2: Effect of SA on fruit quality in 'Huangguan' pear

Storage time (d)	Treatment	Firmness (N)	SSC (%)	TA (%)
0	Control	68.2 ± 3.50 a	12.2 ± 0.19 a	0.141 ± 0.011 b
	5 mM SA	68.2 ± 3.50 a	12.2 ± 0.19 a	0.141 ± 0.011 b
30	Control	69.2 ± 3.41 a	12.2 ± 0.38 a	0.163 ± 0.004 a
	5 mM SA	66.0 ± 3.00 a	12.4 ± 0.09 a	0.155 ± 0.008 ab

Values are mean ± SE of three replicates. Mean values within each column followed by the same letter are not significantly different at level $p = 0.05$.

Effect of SA on SA content in peel

The SA content in the peel showed a significant upward trend, and it was significantly higher in SA treatment than that in control (Fig. 2).

Effect of SA on antioxidant enzymes activities in peel

The SOD activity decreased temporarily after 5 days of cold storage at 0 °C, and afterwards, it was significantly higher in SA treatment

Tab. 1: The sequence of primers

Gene name	GenBank accession no.	Forward primer	Reverse primer
PbPPO1	HQ729709	5′-TCCCTACTCACAAAGCCCAAG-3′	5′-GACCTCCAAGACCAAGAAGCA-3′
PbPPO5	GU906266	5′-ACCAAAACAAAAACCATTCCAC-3′	5′-CAGCCACTCCACCATACAGG-3′
PbLOX1	XM_009378409	5′-CTTCCAAGTTTTCGATGGAATG-3′	5′-TGACACGCTTGAATCTTCAACC-3′
PbPLD4	XM_009369269.2	5′-TCCCCCTCATTCTCCTCATCA-3′	5′-TGATAAGGTATGCATCGTCCACT-3′
PbActin2	GU830959	5′-GGACATTCAACCCCTCGTCT-3′	5′-ATCCTTCTGACCCATACCAACC-3′

than that in control at day 10 and day 15, and significantly lower at day 20 (Fig. 3A). This indicated that SA treatment increased the SOD activity of peel at the early stage of cold storage.

The activities of APX and GR of peel in fruit treated with SA increased continuously after cold storage at 0 °C, and were significantly higher than in control samples during the whole period (Fig. 3B, 3C).

Effect of SA on phenolic content, PPO activity and its gene expression in peel

The phenolic content of peel increased slowly under cold storage in control, and it was significantly lower in SA treatment than that in control after day 20, but there was no significant difference from day 5 to day 15 (Fig. 4A).

The changing trend of PPO activity in SA treatment was basically the same as that in control. It increased significantly at day 5 and then decreased significantly in the control. The PPO activity was significantly lower in SA treatment at days 5 and 10 than that in control, and was significantly higher at days 15 and 20, but there was no significant difference at day 30 (Fig. 4B).

Further analysis of *PPO* gene expression showed that *PPO1* and *PPO5* gene expression greatly increased in the control under cold storage (Fig. 5). The relative expression of *PPO5* increased at a lower level (Fig. 5B), but a change of *PPO1* was not obvious in SA treatment (Fig. 5A). The expression level of *PPO* in SA treatment from day 10 to day 30 was significantly lower than that in control (Fig. 5).

Effect of SA on the expression of *LOX1* and *PLD4* genes in peel

The relative expression of *LOX1* was significantly up-regulated during cold storage. Compared with the control, the expression of *LOX1* was significantly lower in SA treatments at days 10 and 15, and afterwards there was no significant difference between the two treatments (Fig. 6A). The expression of *PLD4* gene showed an increasing trend in the control after day 5, and it was significantly lower in SA treatment compared with the control (Fig. 6B).

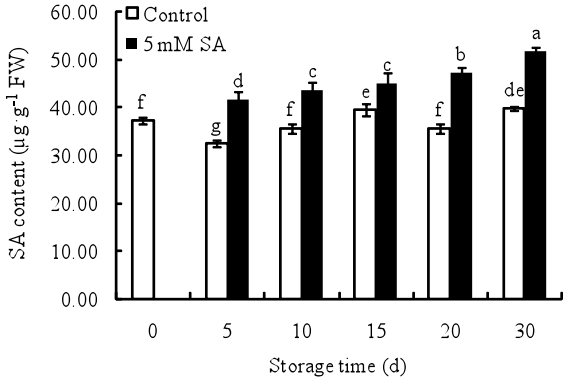

Fig. 2: Changes of SA content of peel in 'Huangguan' pear by SA treatment during cold storage. Values are mean ± SE of three replicates. Different letters represent significant differences at $P = 0.05$.

Discussion

This study showed that SA treatment significantly reduced the CI index and occurrence of brown spot in 'Huangguan' pear during low temperature storage (Fig. 1), which was consistent with previous studies on tomato, cucumber, pineapple, plum and peach fruits (Aghdam et al., 2014; Cao et al., 2009; Lu et al., 2010; Luo et al., 2011; Wang et al., 2006). This was closely related to the increase of SA content after SA treatment (Fig. 2).

Antioxidant enzymes can inhibit oxidative stress induced by excessive reactive oxygen species. Aghdam and Bodbodak (2013) stated that SA alleviated CI by improving antioxidant activity and reducing oxidative stress in fruit. The results showed that SA enhanced the activities of APX, GR and SOD in the peel during cold storage period (Fig. 3), it indicated that SA treatment increased the antioxidant level and thus helped to enhance the chilling resistance of the pear fruit. It

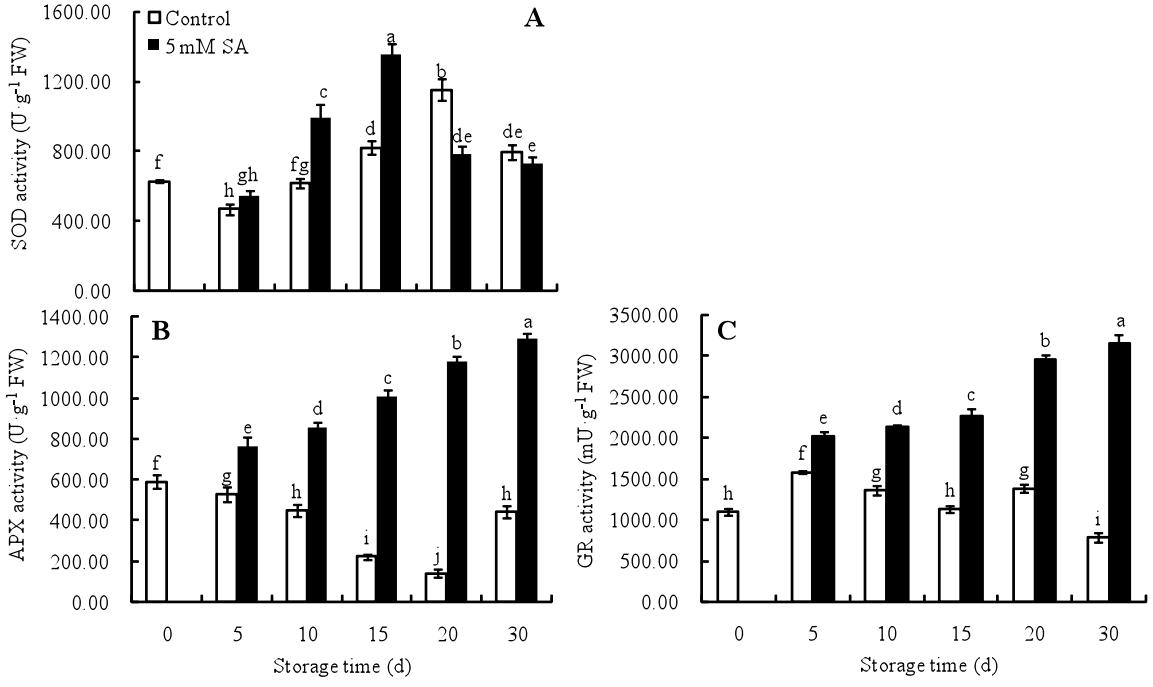

Fig. 3: Changes of activities of SOD (A), APX (B) and GR(C) of peel in 'Huangguan' pear by SA treatment during cold storage. Values are mean ± SE of three replicates. Different letters represent significant differences at $P = 0.05$.

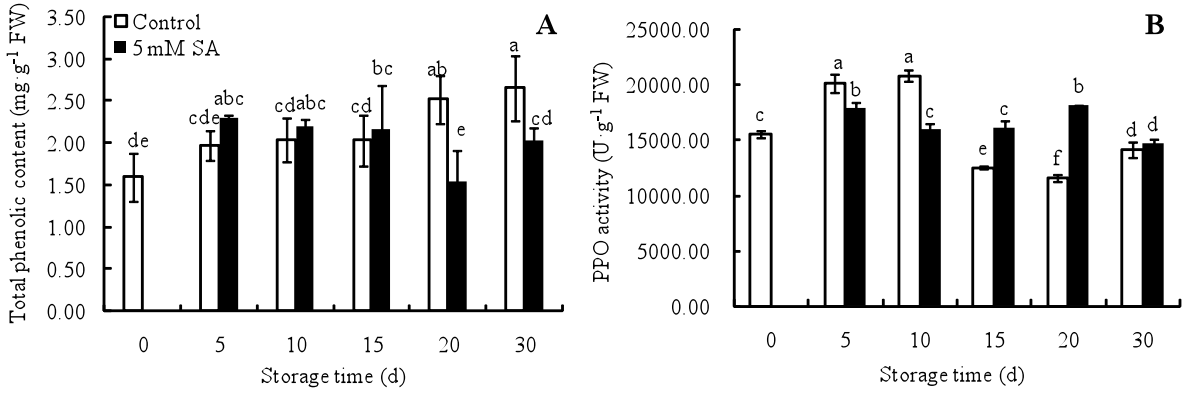

Fig. 4: Changes of total phenolic content (A), PPO activity (B) of peel in 'Huangguan' pear by SA treatment during cold storage. Values are mean ± SE of three replicates. Different letters represent significant differences at $P = 0.05$.

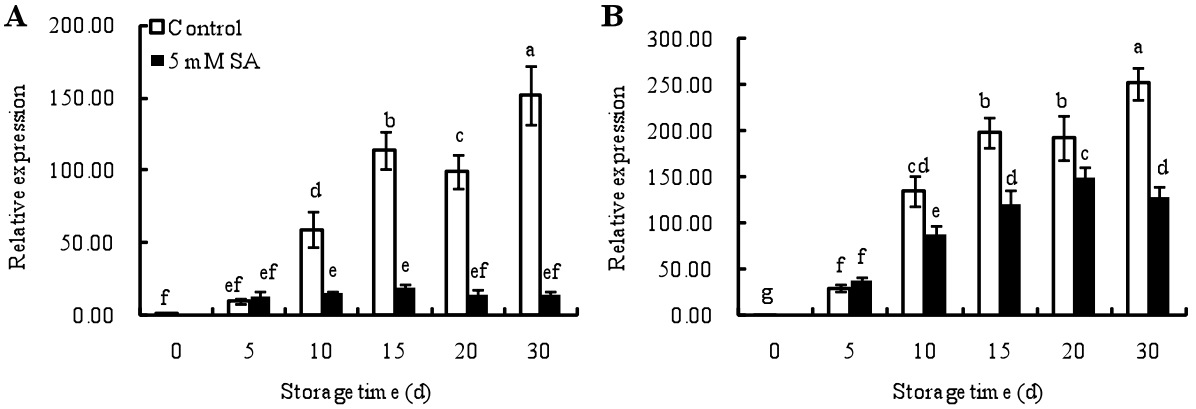

Fig. 5: Changes of relative expression level of *PPO1* (A) and *PPO5* (B) genes of peel in 'Huangguan' pear by SA treatment during cold storage. Values are mean ± SE of three replicates. Different letters represent significant differences at $P = 0.05$.

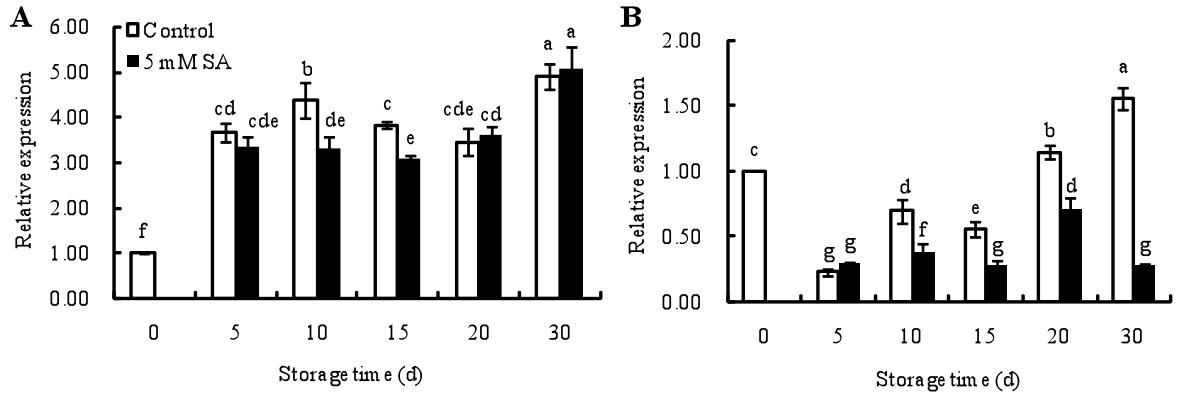

Fig. 6: Changes of relative expression level of *LOX1* (A) and *PLD4* (B) genes of peel in 'Huangguan' pear by SA treatment during cold storage. Values are mean ± SE of three replicates. Different letters represent significant differences at $P = 0.05$.

is consistent with the results on pineapple, papaya, apricot and peach fruits (Lu et al., 2010; Promyou and Supapvanich, 2016; Wang et al., 2015; Wang et al., 2006).

The CI is manifested as brown spot on the fruit surface in 'Huangguan' pear fruit. Phenolic substances are oxidized to quinones by PPO to result in browning (Lu et al., 2011). Low temperature conditioning could reduce CI of 'Huangguan' pear by decreasing PPO activity and inhibiting its gene expression (Li et al., 2017). This study showed that SA had no significant effect on the phenolic content of peel in the early stage (5-15 days), but significantly decreased PPO activity (Fig. 4) and significantly inhibited the expression of *PPO1* and *PPO5* genes (Fig. 5). Therefore, the lower PPO activity of peel reduced by SA contributed to slowing down the browning process, which was similar to the results on sponge gourd, pineapple and plum fruits (Han et al., 2017; Lu et al., 2010; Luo et al., 2011).

CI of fruit is closely related to PLD, LOX activity and their gene expression, which was associated with membrane lipid degradation (Aghdam and Bodbodak, 2013). The lower activities of PLD and LOX under SA treatment reduced CI (Cao et al., 2010; Rui et al., 2010). This study demonstrated that SA treatment significantly

decreased the expression of *LOX1* and *PLD4* genes at cold storage (Fig. 6). This indicated that SA helped to maintain the integrity of cell membranes and weakened the lipid peroxidation, thus alleviated the CI of 'Huangguan' pear fruit.

Conclusion

SA treatment mainly increased SA content and the activities of APX, GR, SOD, decreased the PPO activity, and down-regulated gene expression of *PPO1, PPO5*, *PLD4* and *LOX1* of peel, accompanied by the inhibition of brown spot on fruit surface, thus alleviated CI in 'Huangguan' pear.

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