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The involvement of phenolic metabolism in superficial scald development in 'Wujiuxiang' pear

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Summary

Superficial scald often occurs after a long term of cold storage in apples and pears. In this study, the superficial scald index, the contents of major phenolic compounds, polyphenol oxidase (PPO) activity and its related genes expression in peel was investigated during cold storage period and at shelf life in 'Wujiuxiang' pear (*Pyrus communis* L. cv. Wujiuxiang) with or without 1-MCP treatment. It showed that arbutin, chlorogenic acid, catechin and epicatechin were the main phenolic compounds in the peel, and 1-MCP treatment significantly inhibited scald development while altering the composition of phenolic compounds, inhibited PPO activity and the expression of phenylalanine ammonia ligase (*PAL1*, *PAL2*), cinnamate 4-hydroxylase (*C4H1*, *C4H2*) and PPO (*PPO1*, *PPO5*) and up-regulated the expression of hydroxycinnamoyl-CoA shikimate/quinate hydroycinnamoyltransferase (*HCT1*), *p*-coumarate-3-hydroxylase (*C3H*) and PPO (*PPO4* and *PPO6*) in the peel. These results suggested that the phenolic metabolism iss closely related to the scald development, and several genes related to phenolic metabolism were involved in scald development.

Keywords: superficial scald; 1-methylcyclopropene; polyphenol; polyphenol oxidase; gene expression

Introduction

Superficial scald, as a physiological disorder causing brown or black patches on fruit peel, often occurs after a long term of cold storage in apples and pears (Lurie and Watkins, 2012). The development of the scald was associated with α-farnesene metabolism (Yazdani et al., 2011; Zhou et al., 2017), and antioxidant system (Gao et al., 2015; Larrigaudière et al., 2016, 2019; Li et al., 2016; Sabban-Amin et al., 2011; Zhou et al., 2016), which could be dependent on ethylene action (Feng et al., 2018; Xie et al., 2016; Zhi et al., 2018). However, a full understanding about the mechanism of scald development remains elusive.

It has been showed that phenolic metabolism may be associated with the scald development (Abbasi et al., 2008; Busatto et al., 2018; Busatto et al., 2014; Lu et al., 2014; Piretti et al., 1996). It is not only because most phenolic compounds have antioxidant activity which could resist against scald development (Andrés-Lacueva et al., 2010; Lee et al., 2003), but also phenolic oxidation products catalyzed by polyphenol oxidase (PPO) most likely results in browning reaction during the development of scald symptom (Mayer and Harel, 1991; Nishimura et al., 2003). In general, after a long term of cold storage, loss of membrane integrity allows the contacting of phenolic substrates with PPO, in which phenolic substrates could be catalyzed into oxidized forms by PPO, such as quinones, and then, quinones could react with thiol and amines groups, finally leading to the formation of brown or black pigments in peel (Valentines et al., 2005). However, in pears few evidence confirmed the involvement of phenolic metabolism in scald development, and multiple *PPO* gene members existed which usually exhibited various expression patterns in the process of growth and development or in response to stress (Thipyapong et al., 2007), which specific *PPO* members might play important role in scald development is unknown.

It shows that chlorogenic acid may be main substrate for PPO-catalyzed reaction during scald development (Busatto et al., 2014). Previous researches indicated that chlorogenic acid is synthesized via phenylpropanoid pathway, which is initiated from the deamination of phenylalanine to cinnamic acid by phenylalanine ammonia ligase (PAL), followed by hydroxylation of cinnamic acid by cinnamate 4-hydroxylase (C4H) and methylation by 4-hydroxycinnamoyl-CoA ligase (4CL), respectively, to produce *p*-coumaric acid, and then enters hydroxycinnamoyl-CoA shikimate/quinate hydroycinnamoyltransferase (HCT/HQT) pathway, finally leading to chlorogenic acid synthesis, which was hydroxylated by *p*-coumarate-3-hydroxylase (C3H), (Mahesh et al., 2007; Niggeweg et al., 2004; Zhao et al., 2013). However, the involvement and regulation of chlorogenic acid synthesis in scald development was not fully understood in pears.

Therefore, the objective of this study is to reveal the involvement and regulation of phenolic metabolism in scald development, and which genes may function in scald development in 'Wujiuxiang' pear (*Pyrus communis* L. cv. Wujiuxiang).

Materials and methods

Materials and treatments

'Wujiuxiang' pears were harvested in a commercial orchard in Jinzhou County (Hebei, China) at the commercial maturity stage (September 2, 2012). Fruit were transported to the laboratory in 2 h. Uniform fruit (average weight each fruit: 289.0 g) without any visual defects were selected and randomly divided into two lots of 150 fruit each. One lot was exposed to 1.0 μL/L 1-MCP (Rohm and Haas China Inc., Beijing) for 24 h at 25 ± 2 °C, and the other lot was exposed to air as control. After treatment, all fruit were stored at 0 °C. After 90 and 120 days of cold storage, fruit were removed for biochemical measurements and gene expression analysis. After 120 days of cold storage, all fruit left were transferred to 20 ± 2 °C for 7 days of shelf life. Each treatment contained three replicates of 10 fruit each at each sampling time.

Scald index measurement

The scald index was measured based on the percentage of the fruit surface area affected (Zanella, 2003), where no scald = 0, <25% = 1, 25-50% = 2, and >50% = 3. The scald index = ∑ (Score level × number of fruit at the level) / [3× (total number of fruit)].

Phenolic compounds extraction and content analysis

Phenolic compound was determined as described by Awad et al. (2000) with some modifications. A 2.0 g ground frozen peel tissue

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were used to extract phenolic compound with 10 mL of 80% (v/v) methanol for 20 min under ultrasonic condition, and then centrifuged at 10,000 g for 10 min at 4 °C. A 1.0 mL aliquot of supernatant was first filtered through a C_{18}-SPE (Bonna-Agela Technologies Inc., Tianjin, China) and then filtered via a 0.45 μm microporous membrane before being injected into the high performance liquid chromatograph (HPLC, Hitachi L-2000 system, Hitachi, Tokyo, Japan), which consisted of a Hitachi pump L-2130, a Hitachi automatic sample injector L-2200, and a Hitachi UV detector L-2400 equipped with a C_{18} column. The flow rate was maintained at 1 mL min^{-1} and the injection volume was 10 μL. The phenolic content was determined at wavelength of 280 nm. The integrated peaks were calculated by comparison with standard solutions of different known phenolic compounds. Data are expressed in mg g^{-1} FW.

Extraction and assay of PPO activity

The extraction and assay of PPO activity were performed as described by SERRADELL et al. (2000). A 1.0 g ground frozen peel was homogenized in 3 ml of phosphate buffer (0.1 mol/L, pH 7.8) with 1% polyvinylpyrrolidone (PVP), and then centrifuged at 20,000 g for 15 min at 4 °C. The supernatant was collected as a crude PPO extract. The reaction mixture contained 0.1 mol/L catechol containing in 0.05 mol/L phosphate buffer (pH 6.0). Changes in the absorbance at 410 nm were measured. One unit of PPO activity was defined as a change of 0.01 at 410 nm in the absorbance per min.

RNA isolation and quantitative RT-PCR analysis

Total RNA was isolated by CTAB (cetyltrimethylammonium bromide) (Sangon Biotech, Shanghai, China) method (GASIC et al., 2004). After isolation, 1.0 μg of total RNA was reverse-transcribed into first-strand cDNA using PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa Biomedicals). Quantitative RT-PCR was performed using the TB Green® Premix Ex Taq™ (Tli RNaseH Plus) Kit (TaKaRa Biomedicals) with the 7500 Real-Time PCR System (Applied Biosystems, USA). The PCR primers were designed using primer premier 6.0. The gene name, GenBank accession number, forward and reverse primers were shown in Tab. 1. The qRT-PCR reaction was performed in a final volume of 20 μL, containing 10 μL of TB Green® Premix Ex Taq™ mix, 0.4 μL of ROX II dye, 0.4 μL of forward and reverse primer each, and cDNA equivalent to 10 ng of RNA. The reaction conditions were carried out as follows: 10 s at 95 °C, 40 cycles of 95 °C for 5 s and 60 °C for 34 s. The melting temperature of the amplification products was analyzed using a dissociation curve to confirm the specificity of amplification. All quantitative RT-PCR reactions were normalized using a Ct value corresponding to the *PcActin* gene. The amplification efficiency of primers was between 95 and 105%, which was calculated by serial dilutions of cDNA samples. The relative expression levels of target genes were calculated with the formula $2^{-\Delta\Delta CT}$ (LIVAK and SCHMITTGEN, 2001), with samples harvested at day 0 used as a calibrator (assigned an arbitrary value of 1.0).

Statistical analysis

All values are expressed as the means ± standard errors (SE) of three replicates. The significance of the differences between means was calculated by Student t test using SPSS software (Version 19.0, SPSS Inc., Chicago, IL, USA). Differences were considered to be significant at $P < 0.05$.

Results

Scald development during cold Storage and shelf life

In the control fruit, scald symptom was appeared after 120 days of cold storage, and after 7 days of shelf life, scald developed more severely, while no scald was found in 1-MCP-treated fruit during all of cold storage and shelf life (Fig. 1).

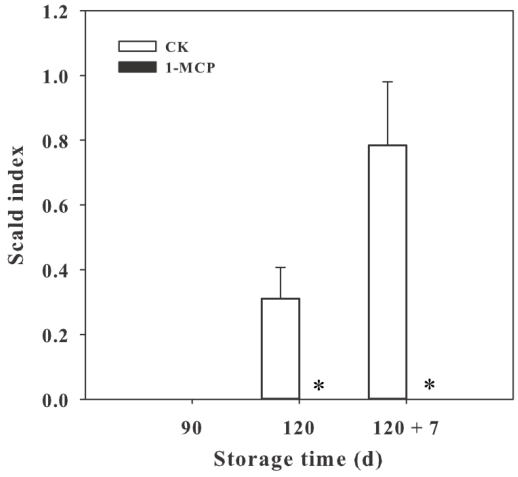

Fig. 1: Scald index in 'Wujiuxiang' pear during cold storage and shelf life. The error bars represent SE of the means. The asterisk "*" indicates a significant difference ($P<0.05$).

The content of phenolic compounds during scald development

The phenolic compounds, including arbutin, chlorogenic acid, gallic acid, catechin, epicatechin, coumaric acid, and caffeic acid, were detected in peel after 90, 120 days of cold storage and 7 days of shelf life. It showed that arbutin, chlorogenic acid, catechin, and epicatechin were the main phenolic compounds in the peel of 'Wujiux-

Tab. 1: Primers for quantitative RT-PCR analysis.

Gene	GenBank NO.	Forward (5'-3')	Reverse (5'-3')
PcPAL1	GU906268	GCAAAGAGGACTTTAACAACTGG	TACTCCCTATCGACAACTTTAAGC
PcPAL2	GU906269	CCGGGAAATAACCAAACC	ATGGCTCCCTTTCATTGC
PcC4H1	XM_009376113	AACTTCGAGCTTCTGCCTCC	CCCCAAGCATCAATCTACGC
PcC4H2	XM_009356593	CAAGCACACGGGCTACAAC	GATCGACCACAACGTGGTTT
PcHCT1	JQ280303	CCCCCTCCAGTCTGACCA	CCAATGAAAACACAAACACGTC
PcC3H	XM_009357051	GGTGCAACAAAAGGCTCAAG	TTAGTGGTGTTGGAGGGTGC
PcPPO1	HQ729709	TCCCTACTCACAAAGCCCAAG	GACCTCCAAGACCAAGAAGCA
PcPPO4	GU906265	AAGGTGACAATGATAACCCAGAC	TGCCGCACCGTAGAGACC
PcPPO5	GU906266	ACCAAAATAAAAACCCTTCCAC	CAGCCACTCCACCATACAGG
PcPPO6	GU906267	AGAAGGCGGAACGAGAGGA	GGTCTGGCTGGGCTGACTT
PcActin	AB190176.1	GCTGAGAGATTCCGGTGCCC	TTGACCCACCACTGAGCACG

iang' pear. In general, the content of arbutin, chlorogenic acid, gallic acid, epicatechin, and caffeic acid were increased during cold storage and shelf life, while catechin and coumaric acid were decreased. For arbutin, chlorogenic acid, gallic acid, epicatechin, and caffeic acid, 1-MCP treatment could significantly inhibit the accumulation of these phenolic compounds, especially after 7 days of shelf life (Fig. 2).

PPO activity during scald development

The PPO activity in peel showed no significant changes after 90 and 120 days of cold storage in both control and 1-MCP-treated fruit. However, it increased in control at shelf life, while it was still stable and significantly lower than control in 1-MCP-treated fruit (Fig. 3).

The expression of genes associated with phenolic compounds synthesis during scald development

In this study, the expression levels of phenolic compounds synthesis-related genes, including *PcPAL1*, *PcPAL2*, *PcC4H1*, *PcC4H2*, *PcHCT1*, and *PcC3H* were detected during cold storage and shelf life with and without 1-MCP treatment in 'Wujiuxiang' pear. The expression levels of *PcPAL1* and *PcPAL2* increased constantly during cold storage and shelf life in both control and 1-MCP-treated fruit, while 1-MCP treatment significantly inhibited their expression (Fig. 4A, B).

The expression of *PcC4H1* was slightly increased after 90 days of cold storage, and then declined, while 1-MCP-treatment inhibited the expression except for day 120 (Fig. 4C). However, no significant change was observed in the expression of *PcC4H2* (Fig. 4D).

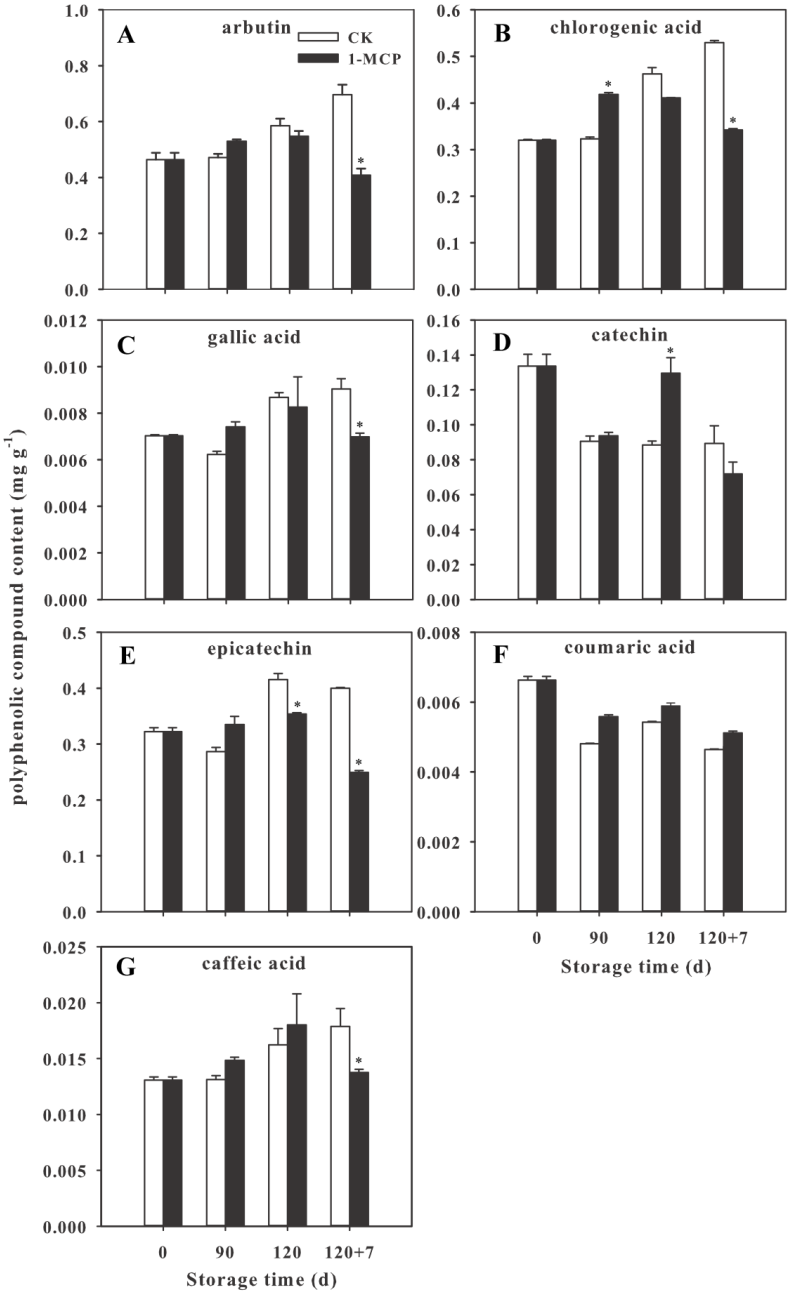

Fig. 2: The content of phenolic compounds during cold storage and shelf life with and without 1-MCP treatment in 'Wujiuxiang' pear: arbutin (A), chlorogenic acid (B), gallic acid (C), catechin (D), epicatechin (E), coumaric acid (F), and caffeic acid (G). The error bars represent SE of the means. The asterisk "*" indicates a significant difference (P<0.05).

The expression of *PcHCT1* and *PcC3H* were slightly inhibited during cold storage in both control and 1-MCP-treated fruit, while it was increased to a higher level after 7 days of shelf life in 1-MCP-treated fruit (Fig. 4E, F).

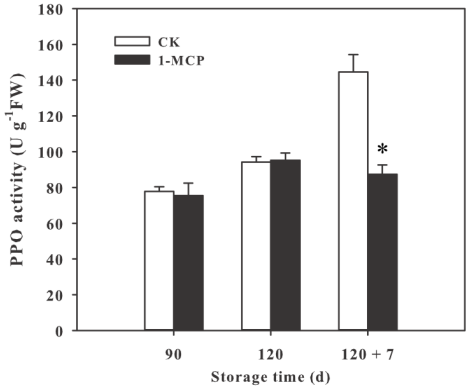

Fig. 3: PPO activities in peel of 'Wujiuxiang' pear during cold storage and shelf life. The error bars represent SE of the means. The asterisk "*" indicates a significant difference (P<0.05).

The expression of *PPO* gene members during scald development

The expression of *PcPPO1* and *PcPPO5* increased dramatically during cold storage and afterwards declined at shelf life in control, whereas they were significantly suppressed by 1-MCP treatment (Fig. 5A, C). The expression levels of *PcPPO4* and *PcPPO6* were decreased during cold storage and shelf life in control, while 1-MCP-treatment could up-regulate their expression. (Fig. 5B, D).

Discussion

Superficial scald could result in severe fruit quality loss in apples and pears, and many postharvest methods have been established to inhibit scald development (LURIE and WATKINS, 2012). In this study, 1-MCP treatment significantly inhibited scald development in 'Wujiuxiang' pear (Fig. 1), in good agreement with our previous reports (GAO et al., 2015; ZHOU et al., 2016, 2017), confirming the crucial role of ethylene action during scald development in 'Wujiuxiang' pear.

Phenolic compounds are particularly important in fruit, not only because they contribute to color and flavor, but also they could be antioxidant function in fruit, which may involve in multiple stress and senescence-related processes (ANDRÉS-LACUEVA et al., 2010). In this study, it was found that arbutin, chlorogenic acid, catechin, and epicatechin were main phenolic compounds in peel of 'Wujiuxiang' pear (Fig. 2). Rich and varied phenolic compounds make 'Wujiu-

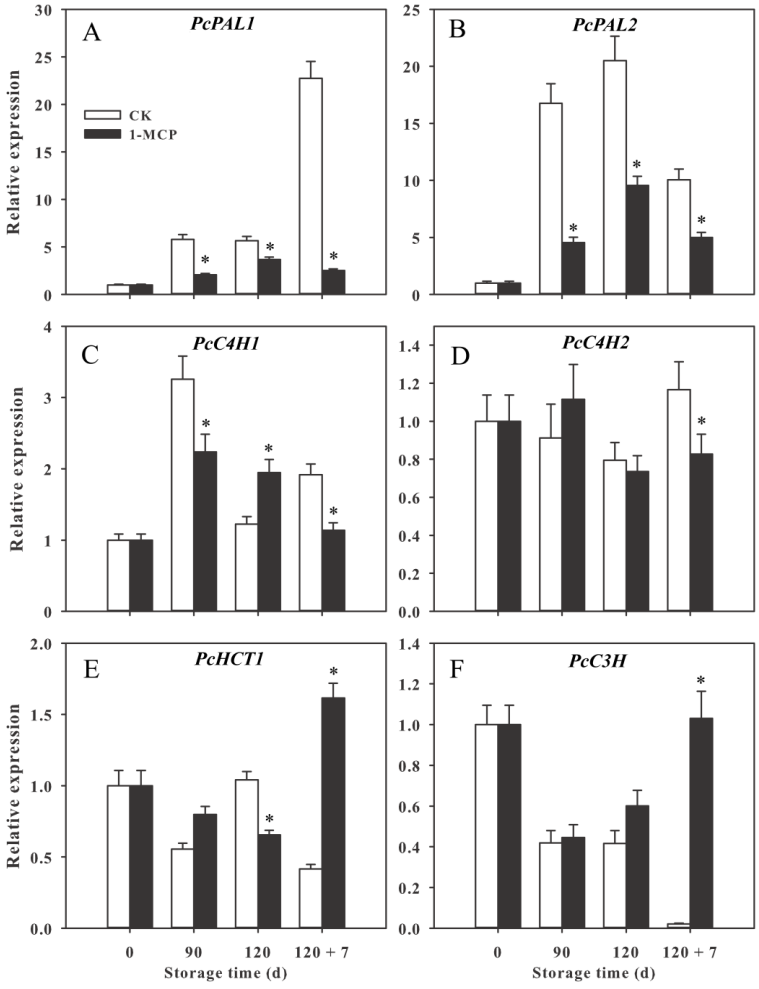

Fig. 4: Expression levels of chlorogenic acid synthesis-related genes during cold storage and shelf life with and without 1-MCP treatment in 'Wujiuxiang' pear: *PcPAL1* (A), *PcPAL2* (B), *PcC4H1* (C), *PcC4H2* (D), *PcHCT1* (E), and *PcC3H* (F). The error bars represent SE of the means. The asterisk "*" indicates a significant difference (P<0.05).

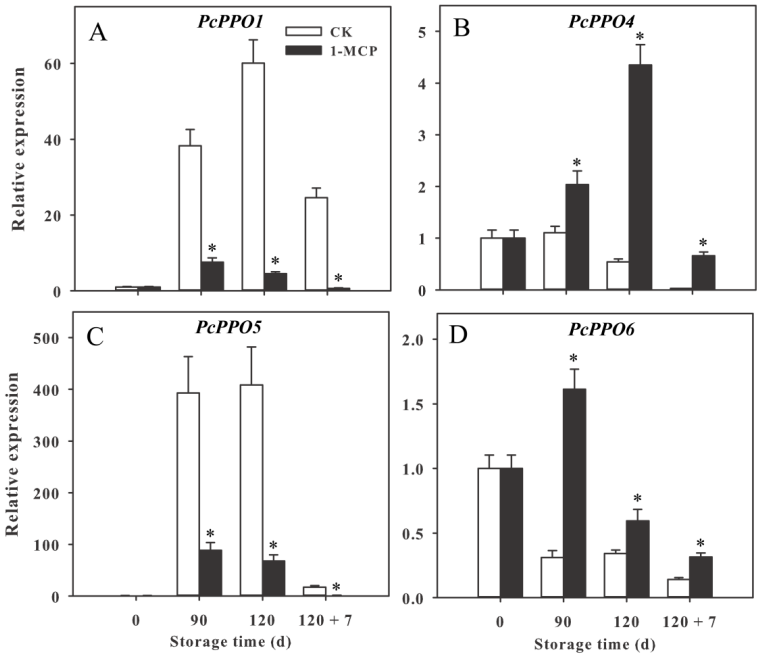

Fig. 5: Expression levels of *PPO* genes during cold storage and at shelf life with and without 1-MCP treatment in 'Wujiuxiang' pear: *PcPPO1* (A), *PcPPO4* (B), *PcPPO5* (C), and *PcPPO6* (D). The error bars represent SE of the means. The asterisk "*" indicates a significant difference (P<0.05).

xiang' pear more nutrient, however, they may also contribute to high scald incidence (Busatto et al., 2014; Piretti et al., 1996).

Some studies have suggested that the phenolic metabolism could be involved in scald development and could be altered by 1-MCP treatment in apples (Abbasi et al., 2008; Busatto et al., 2014; Pesis et al., 2007). In this study, the content of arbutin, chlorogenic acid, and epicatechin increased constantly in control fruit during cold storage and shelf life (Fig. 2A, B, E), while the scald symptom appeared and developed (Fig. 1). It suggested the involvement of phenolic compounds in cell senescence after a long cold storage and providing more substrates for PPO-catalyzed reaction during scald development. In contrast, 1-MCP effectively inhibited the accumulation of most phenolic compounds during cold storage and shelf life (Fig. 2A, B, E), while retarding the scald development (Fig. 1). These results suggested that arbutin, chlorogenic acid, and epicatechin may be associated with scald development in 'Wujiuxiang' pear. It was in agreement with the results in apples, which implied the accumulation of chlorogenic acid was positively correlated with the scald development (Busatto et al., 2014).

Phenolic biosynthesis pathway is initiated by PAL, in which by converting the phenylalanine to cinnamic acid. The expression of *PcPAL1* and *PcPAL2* dramatically increased during scald development and was effectively inhibited by 1-MCP treatment (Fig. 4A, B), confirming the involvement of phenolic metabolism in scald development. However, other phenolic synthesis-related genes, such as *PcC4H1*, showed a slight up-regulation (Fig. 4C), and *PcHCT1* and *PcC3H*, showed down-regulated expression pattern (Fig. 4E, F), while *PcC4H2* expression had no significant change during cold storage and shelf life in control (Fig. 4D), which were not coincident with scald development (Fig. 1). It suggested the *PAL* genes were the key regulator of phenolic metabolism during cold storage in pear. Interestingly, higher expression levels of *PcHCT1* and *PcC3H* were observed in 1-MCP treated fruit at shelf life (Fig. 4E, F), implying a negative feedback mechanism on phenolic biosynthesis and the scald development (Fig. 2B).

In this study, the increase of PPO activity was correlated with scald development (Fig. 1, 5). Considering higher content of chlorogenic acid during late cold storage and shelf life (Fig. 2), it confirmed the conclusion that after a long term cold storage, phenolic compounds released from the vacuole can get in contact with PPO enzyme and activate it, being oxidized which results in tissue browning (Abbasi et al., 2008). The expression levels of *PcPPO1* and *PcPPO5* showed a significant up-regulation in control during cold storage, and were effectively inhibited by 1-MCP treatment (Fig. 5A, C). It suggests that *PcPPO1* and *PcPPO5* may involve in browning reactions during scald development. In contrast, the expression levels of *PcPPO4* and *PcPPO6* were down-regulated in control, while they were up-regulated slightly in 1-MCP-treated fruit (Fig. 5B, D), implying *PcPPO4* and *PcPPO6* may play positive role in cold acclimation and senescence process in fruits. Our results are analogous to other reports, which showed *MdPPO* was linked with scald development in apple (Busatto et al., 2018; Busatto et al., 2014; Sabban-Amin et al., 2011). However, only one *PPO* gene member was analyzed in their reports. Our results indicate that different member of *PPO* genes may have different functions during scald development in pears.

In summary, these results showed the involvement of phenolic metabolism in the scald development in pears, in which arbutin, chlorogenic acid, and epicatechin might be the main phenolic compounds, and *PcPAL1*, *PcPAL2*, *PcPPO1* and *PcPPO5* were the key genes related with scald development. Meanwhile, 1-MCP inhibited the scald development by regulating expression of above phenolic metabolism-related genes.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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