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Effects of 1-MCP on chlorophyll degradation pathway-associated genes expression and chloroplast ultrastructure during the peel yellowing of Chinese pear fruits in storage

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ABSTRACT

The peel yellowing is an important pigment physiological process of green fruit ripening, which mainly results from chlorophyll degradation in the fruit peel. In this work, two typical cultivars with different ripening speed, a slow ripening pear 'Emerald' (*Pyrus bretschneideri* Rehd. cv. Emerald) and a fast ripening 'Jingbai' (*Pyrus ussuriensis* Maxim. cv. Jingbai) were used to investigate the molecular mechanism of chlorophyll degradation in pear yellowing/ripening during postharvest storage. The fruits after harvest were treated with 1-methylcyclopropene (1-MCP), an ethylene action inhibitor at 1.0 $\mu L l^{-1}$ to determine its effect on chloroplast ultrastructure and the expression of chlorophyll degradation associated genes in peel tissues. Our results show that the pears treated with 1-MCP had a lower ethylene production rate and higher chlorophyll content compared to those of untreated fruit. The more intact chloroplasts with well-organised grana thylakoids and small plastoglobuli were maintained in the peel of 1-MCP treated fruit for up to 30 and 15 d in 'Emerald' and 'Jingbai', respectively. The expression of chlorophyll degradation associated genes: pheophorbide a oxygenase (*PAO*), non-yellow colouring (*NYC*), NYC1-like (*NOL*), stay-green 1(*SGR1*), was suppressed, while no significant change was found in chlorophyllase 1 (*CHL1*) and red chlorophyll catabolite reductase (*RCCR*) in both cultivar fruits treated with 1-MCP. These results suggest that 1-MCP can delay chlorophyll degradation by inhibiting ethylene production and suppressing the gene expression of *PAO*, *NYC*, *NOL* and *SGR1*, which are closely associated with chlorophyll catabolic pathway.

1. Introduction

The yellowing of the peel is an important physiological metabolism including pigment chlorophyll during fruit ripening as a complex and programmed process, manifesting the dramatic changes in colour, texture, flavor, and aroma of the fruit (Alexander & Grierson, 2002). Over yellowing often causes its commercial devaluation in some cultivars. Chlorophyll degradation is an important catabolic process of both leaf senescence and green-peel fruit ripening. It is well known that dissociation of grana, increase in size and number of plastoglobuli and disruption of the chloroplast envelope are the main characteristics of chloroplast breakdown (Bonora, Pancaldi, Gualandri, & Fasulo, 2000; Matile, Hörtensteiner, Thomas, & Kräutler, 1996; Zavaleta-Mancera, Thomas, Thomas, & Scott, 1999). The molecular mechanism of chlorophyll breakdown in the leaf has been well studied in recent years (Hörtensteiner, 2006; Kräutler, 2008; Matile, Hörtensteiner, & Thomas, 1999). However, less work has been done on the mechanism of chlorophyll breakdown in peel tissue of green-peel fruits.

Chlorophyll breakdown is a multi-step enzymatic process. The initial step in chlorophyll *a* degradation is the removal of the phytol tail by chlorophyllase (CLH), producing the first green breakdown product, chlorophyllide (Matile et al., 1999; Tsuchiya et al., 1999). After the central Mg^{2+} of chlorophyllide is removed by Mg-dechelatase, pheophorbide as the product is converted to primary fluorescent chlorophyll catabolite (FCC) by pheophorbide a oxygenase (PAO) and red chlorophyll catabolite reductase (RCCR) (Rodoni et al., 1997). Then FCC is converted to the final non-fluorescent chlorophyll catabolites (NCCs). The conversion of

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pheophorbide to FCC has been thought to be the key step, and PAO is the only up-regulated gene involved in this way during chlorophyll breakdown (Hörtensteiner, Vicentini, & Matile, 1995; Thomas, Ougham, Canter, & Donnison, 2002).

Chlorophyll *b*, however, is degraded only after conversion to chlorophyll *a*, and this process is executed by two enzymes: chlorophyll *b* reductase and hydroxymethyl chlorophyll *a* reductase (Ito, Tanaka, Tsuji, & Tanaka, 1993; Rüdiger, 2002; Scheumann, Schoch, & Rüdiger, 1998). Previous reports have shown that non-yellow colouring 1 (NYC1) which participated in chlorophyll degradation was similar to a short-chain dehydrogenase/reductase, and NYC1-like (NOL) protein showed chlorophyll *b* reductase activity *in vitro* (Kusaba et al., 2007). Recently, the stay-green (SGR) genes have been cloned from different plant species (Armstead et al., 2006; Barry, McQuinn, Chung, Besuden, & Giovannoni, 2008; Jiang et al., 2007; Park et al., 2007; Sato, Morita, Nishimura, Yamaguchi, & Kusaba, 2007). Further analyses indicate that the expression of SGR is closely associated with leaf senescence and fruit ripening (Alós et al., 2008; Armstead et al., 2006; Barry et al., 2008; Hörtensteiner, 2006; Sato et al., 2007; Yang et al., 2009). Despite the fact that the primary pathway of leaf chlorophyll degradation has been well known, the factors that initiate chlorophyll degradation have still not been understood.

Previous studies have shown that ethylene was involved in chlorophyll degradation in fruit peel (Porat et al., 1999; Purvis & Barmore, 1981; Trebitsh, Goldschmidt, & Riov, 1993). Exogenous ethylene could accelerate the chlorophyll degradation of mature green citrus and avocado fruit (Amir-Shapira, Goldschmidt, & Altman, 1987; Iglesias, Tadeo, Legaz, Primo-Millo, & Talon, 2001; Jeong, Huber, & Sargent, 2002). Current data have shown that 1-methylcyclopropene (1-MCP), an effective inhibitor of ethylene action, reduces the chlorophyllase activity and causes retention of the green peel colour in vegetables and fruit (Chen, Zhang, & Wang, 2009; Gong & Mattheis, 2003; Hershkovitz, Saguy, & Pesis, 2005). However, it is still unclear how 1-MCP affects on chlorophyll degradation at the sub-cellular and molecular level in harvested fruits.

The peel colour of many varieties of pear is green at harvest. The 'Emerald' pear (*Pyrus bretschneideri* Rehd. cv. Emerald) and the 'Jingbai' pear (*Pyrus ussuriensis* Maxim. cv. Jingbai) are two typical pear cultivars in China which are green at harvest. The process of peel yellowing and ripening in 'Emerald' pear is slow. In contrast, the 'Jingbai' pear shows a rapid peel yellowing and has shorter ripening process than 'Emerald' pear (Wei, Ma, Guan, Yuan, & Zhu, 2009; Zhou & Li, 2006). The aim of this work is to explore the molecular mechanism of chlorophyll degradation in the pear peel yellowing by comparing the yellowing processes of two typical green pear cultivar fruits during storage. Quantitative RT-PCR analysis for chlorophyll degradation associated genes and transmission electron microscopy for the changes of chloroplast ultrastructure, ethylene production and the chlorophylls quantification were performed to determine the effect of 1-MCP on fruit ripening and senescence.

2. Materials and methods

2.1. Materials and treatment

Pears were harvested at Xinji County (Hebei, China) at fruit commercial mature stage (July 31, 2010) for 'Emerald' pear (weight 254.42 ± 15.23 g, firmness 60.37 ± 11.04 N, soluble solids content 9.34 ± 0.07%), and at Daxing District (Beijing, China) at fruit commercial mature stage (September 10, 2010) for 'Jingbai' pear (weight 133.50 ± 13.24 g, firmness 120.13 ± 4.56 N, soluble solids content 12.26 ± 0.47%). The harvested fruits were transported to the laboratory within 4 h.

The fruits were selected for the uniformity of weight, shape and colour without any visual defects, and then randomly divided into two lots. One lot was carefully put into plastic box (60 × 40 × 30 cm each box) and then sealed tightly with plastic film bag (85 × 65 × 90 cm). One plastic film bag contained about 400 fruits of 'Emerald' or 750 fruits of 'Jingbai' pear, then exposed to 1.0 $\mu L l^{-1}$ 1-MCP (Rohm and Haas China Inc., Beijing) for 24 h at 25 ± 2 °C. Another lot was sealed with air as control. After treatment, each group was stored at 25 ± 2 °C, 75% relative humidity. For measurement of ethylene production rate, twelve fruits of each replicate were taken at 3-day intervals till 39 days in 'Emerald' and 18 days in 'Jingbai' pear, respectively, and the assay was performed in three replicates. For measurement of the chlorophyll content and observation of transmission electron microscopy five replicates were performed and ten fruits of each replicate were taken at 10-day and 5-day intervals till 40 days in 'Emerald' and 20 days in 'Jingbai' pear, respectively. The peel samples were quickly frozen in liquid nitrogen and stored at -80 °C freezer for RNA extraction.

2.2. Ethylene production rate measurement

The treated and control pears with similar size were randomly selected and sealed in glass desiccators (9.35 l) for 8 h at 25 ± 2 °C. A 1 ml sample of the headspace gas was withdrawn by a gas-tight syringe from each desiccator through a septum stopper, and injected into a gas chromatograph (GC-9800, Shanghai, China) that was equipped with a GDX-502 column and a flame ionisation detector (FID). The column temperature was 78 °C and injection temperature 120 °C. The carrier gas was N_2 with a rate of 40 ml min^{-1}, and the rate of ethylene production was expressed as $\mu l\ kg^{-1}\ h^{-1}$.

2.3. Chlorophyll content measurement

Twenty peel discs (1 cm^2 each disc) of ten fruits in each replicate were extracted in 15 ml of 80% (v/v) acetone in the dark at 25 °C for 24 h. Absorbance (A) was read at 645 and 663 nm on a spectrophotometer (UNICO UV-2100, USA). Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and chlorophyll *a* + *b* (Chl *a* + *b*) were calculated according to Arnon's equations (1949). The results were expressed as mg chlorophyll per g of fresh weight tissue.

2.4. Chloroplast ultrastructure

According to Bonora's method (2000), the peel tissues, 2 mm^3 in volume, were fixed in 2% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 0.1 M PBS (pH 7.2) for 24 h at 4 °C. The tissue was post-fixed in 1% (w/v) OsO_4 for 3 h at 4 °C, and then dehydrated by a gradual concentration of ethanol (30–100%). The samples were embedded in the Epon812 resin (SPI Supplies Division of Structure Probe, Inc., USA). Ultra-thin sections (70–80 nm thickness) were cut with a diamond knife on a Leica Ultracut R ultramicrotome (Leica, Germany), counter-stained with 5% (w/v) uranyl acetate for 20 min followed by a 2% (w/v) lead citrate solution for 3 min, and examined with the transmission electron microscope (H-7650, Hitachi, Japan) at 80 kV.

2.5. RNA isolation and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using 'easyspin plant RNA extraction' reagent (Biomed, China) according to the manufacturer's instructions, all RNA extracts were treated with DNase I (TaKaRa Biomedicals, Japan), and then purified following the manufacturer's instructions.

First-strand cDNAs were synthesised from DNase-treated RNA (0.5 μg) using Takara RNA PCR Kit (AMV) Version 3.0 (TaKaRa

Table 1
Primers for quantitative PCR analysis.

Gene	Accession No.	Forward	Reverse	Product (bp)
PbCLH1 (chlorophyllase 1)	JN168001	5′-CAACGTTCATAGACTTCCCCTACG-3′	5′-GGAGAGTACAAGGCAAGAGCTGC-3′	111
PbNYC (non-yellow coloring)	JN167997	5′-AATTTCTTCTTTCTGGAGATCGTGTAG-3′	5′-TGCATGTGTCAGGTTCTTGGA-3′	140
PbNOL (NYC1-like)	JN167996	5′-CCGTGAGGCAATAAAAATGATG-3′	5′-CGCTTTGTTGCCCCGTAT-3′	120
PbPAO (pheophorbide a oxygenase)	JN167998	5′-AGCTAATGCCACCAAGCCTCC-3′	5′-AGCCATAGAACAGATCACGCTGG -3′	92
PbSGR1 (stay-green1)	JN168000	5′-ATTCAACAAGGTGGAGTGCTGG-3′	5′-GCCGCTGTTGTTTTCCTGG-3′	97
PbRCCR (red chlorophyll catabolite reductase)	JN168002	5′-TTCATAGACTTCCCCTACGTGTCG-3′	5′-GGAGAGTACAAGGCAAGAGCTGC-3′	106
PbACT2 (actin 2)	GU830958	5′-GGACATTCAACCCCTCGTCT-3′	5′-ATCCTTCTGACCCATACCAACC-3′	145

Biomedicals, Japan). Quantitative PCR was performed using SYBR Premix Ex Taq™ (Perfect Real Time) Kit (TaKaRa Biomedicals, Japan) on a 7500 Real-Time PCR system (Applied Biosystems, USA). PCR primers for *CLH1*, *NYC*, *NOL*, *PAO*, *SGR1* and *RCCR* were designed using OMIGA 2.0 based on the sequences of *Pyrus bretschneideri* published database in NCBI. Sequences for the primers were listed in Table 1. The qRT-PCR reaction was carried out in a final volume of 25 μL containing 12.5 μL SYBR Green PCR Premix Ex Taq™,1 μM forward and reverse primers, and 10 ng cDNA, and afterward the cycles was performed as follows: 10 s at 95 °C, 40 cycles of 95 °C for 5 s, and 60 °C for 34 s. To confirm the quality of product and primer specificity, the Tm (melting temperature) of the amplification products was analysed in a dissociation curve. *Actin2* (*ACT2*) was used as a reference gene. And all qRT-PCR reactions were normalised using Ct value corresponding to the *Actin2* gene, the relative expression levels of target genes were calculated with formula $2^{-\Delta\Delta CT}$, and the experiment was performed with four replicates.

2.6. Statistical analysis

Experiments were performed using a completely randomized design. All data were analysed by one-way analysis of variance (ANOVA). All values are expressed as mean ± SE. Differences were considered significant at $P < 0.05$. All analyses were performed with OriginPro 8.0 software (Origin Lab Corporation, MA).

3. Results

3.1. Ethylene production rate in fruits of the 'Emerald' and the 'Jingbai' pear

1-MCP treatment reduced the ethylene production during the peel yellowing of both 'Emerald' and the 'Jingbai' pear (Fig. 1). For the 'Emerald' pear, the ethylene production rate in the control fruit increased gradually and had two peaks, a lower peak at the 15th day and a highest peak at the 33rd day after storage. However, pears treated with 1-MCP had a low rate of ethylene production for the first 9 days, and then a gradually increasing rate afterwards but significantly lower than in the control fruit (Fig. 1A). For the 'Jingbai' pear, the ethylene production rate of the control fruit immediately increased and reached a peak at the 6th day, and then decreased sharply. However, the ethylene production of the pears treated with 1-MCP was almost inhibited in the first 12 days, and then slightly increased but less than the control afterwards (Fig. 1B). The results in Fig. 1 clearly show that 1-MCP inhibited the ethylene production and delayed their climacteric peaks in both of the cultivars and the 'Jingbai' pear was much sensitive to such inhibition.

3.2. The chlorophyll content in fruit peel of the 'Emerald' and the 'Jingbai' pear

The speed of peel yellowing in the 'Emerald' pear was much slower than the 'Jingbai' pear under the same storage conditions without 1-MCP treatment (unpublished data not shown). The 'Emerald' pear fruit peel became fully yellow at 30th d, while the 'Jingbai' pear fruit peel turned yellow much earlier at the 5th day of storage. In agreement with the above results, the 'Jingbai' pear compared with those of the 'Emerald' pear had lower chlorophyll content in peel which decreased much faster during storage (Fig. 2). In the 'Emerald' pear, there was a small difference in the chlorophyll content between control and 1-MCP treated fruits at 10 and 20 days of storage, while in the 'Jingbai' pear, the chlorophyll content was obviously different between control and 1-MCP treated fruits after 5 days storage (Fig. 2). 1-MCP treatment slowed down the decrease in chlorophyll *a*, chlorophyll *b* and chlorophyll *a* + *b* content in the fruit peel of both pear cultivars (Fig. 2), thus showing the delay in the process of peel yellowing.

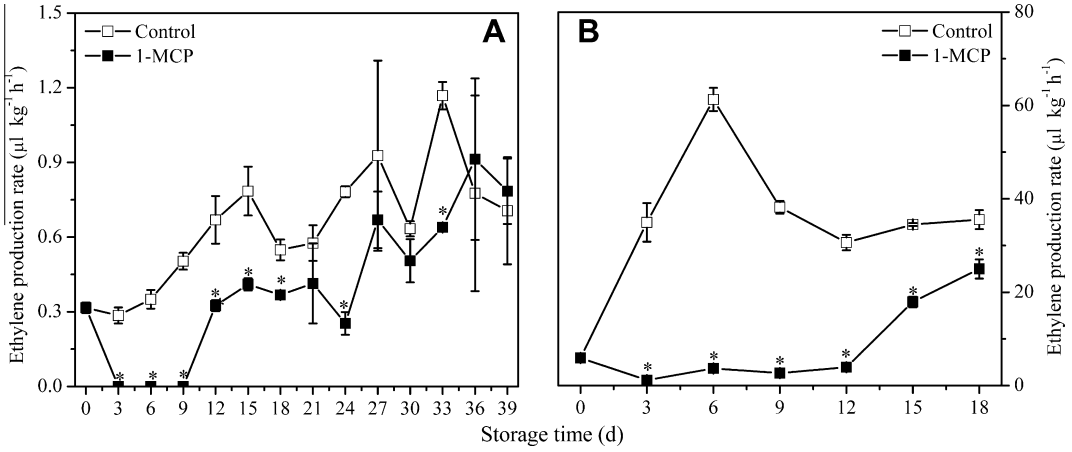

Fig. 1. Ethylene production rate of the 'Emerald' (A) and 'Jingbai' (B) pear fruit with 1-MCP (■) treatment and control (□) stored at 25 ± 2 °C. Values are the means ± SE ($n = 3$). Star represents significant difference ($P < 0.05$).

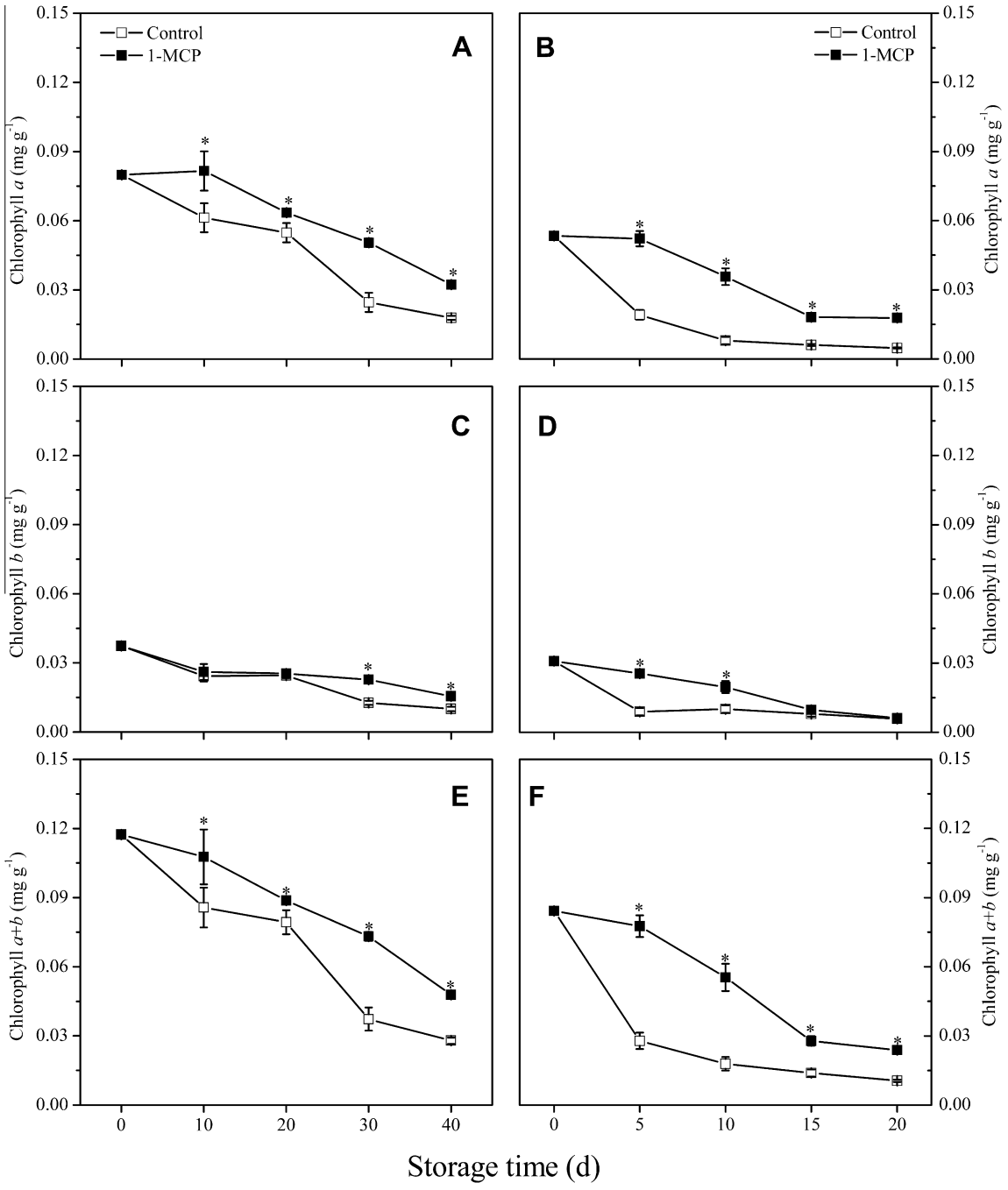

Fig. 2. Changes of chlorophyll *a*, chlorophyll *b* and chlorophyll *a* + *b* in the 'Emerald' (A, C and E) and 'Jingbai' (B, D and F) pear fruit peel with 1-MCP treatment (■) and control (□) stored at 25 ± 2 °C for 40 and 20 d, respectively. Values are the means ± SE (n = 5). Star represents significant difference (P < 0.05).

3.3. The chloroplast ultrastructure in fruit peel of the 'Emerald' and the 'Jingbai' pear

In the 'Emerald' pear at harvest, chloroplasts of the peel showed a typical oval shape, with an intact double envelope. Also, it was observed that the chloroplasts remained a well-organised grana thylakoids and stroma membranes, high electron opacity of the stroma and small plastoglobuli up to 20 days after storage in both the control and the 1-MCP treated pears. The chloroplasts became smaller and rounder after the 30th day of storage, with less electron opacity of the stroma in the control. The chloroplasts were swollen and their thylakoids were dispersed and numerous and large plastoglobuli were formed. In contrast, the fruits treated with 1-MCP appeared to have more structure intact chloroplasts after 30 days of storage. After the 40th day, chloroplasts were completely degraded and dispersed into cytoplasm in the control fruit. However, chloroplasts in the 1-MCP treated fruit peel remained intact but began to form a large number of vesicles (Fig. 3).

In the 'Jingbai' pear the change of chloroplast structure was more dramatic. After only 5 days storage, the chloroplast became smaller and rounder, with less electron opacity of the stroma and swollen and dispersed thylakoids and numerous large vesicles. Then, the chloroplast membrane gradually broke down and was mixed with the cytoplasm at the end in the control. However, the chloroplast in the 1-MCP treated fruit was still kept until the 15th day after storage and then started to form a large number

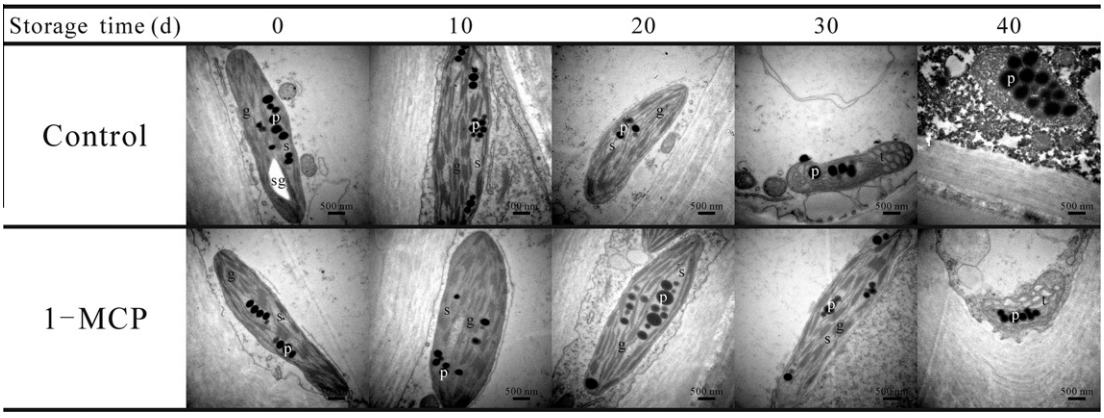

Fig. 3. Structure of chloroplasts from control and 1-MCP treatment of 'Emerald' pear fruit peel stored at 25 ± 2 °C for 40 d. g, grana, p, plastoglobuli, sg, starch grain, s, stroma, t, thylakoids. Scale bar: 500 nm.

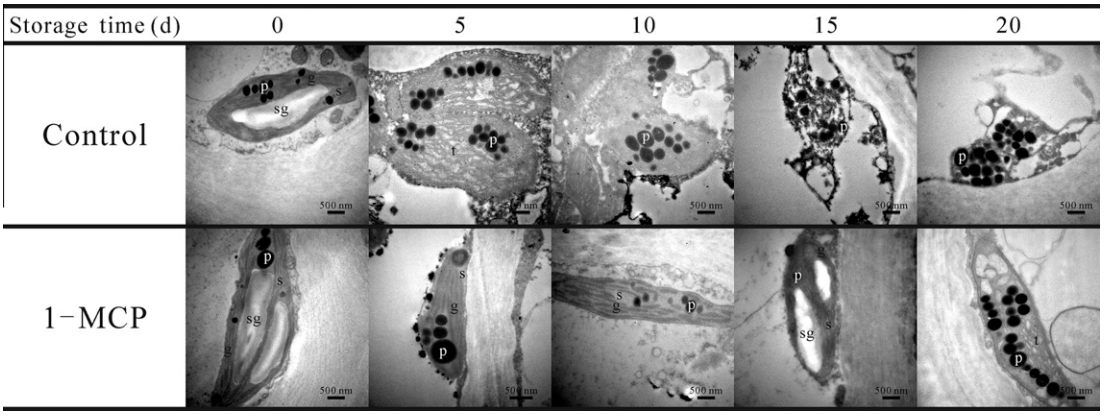

Fig. 4. Structure of chloroplasts from control and 1-MCP treatment of 'Jingbai' pear fruit peel stored at 25 ± 2 °C for 20 d. g, grana, p, plastoglobuli, sg, starch grain, s, stroma, t, thylakoids. Scale bar: 500 nm.

of vesicles (Fig. 4). These results show that 1-MCP treatment delayed the chloroplast breakdown of the peel tissue in both the 'Emerald' and the 'Jingbai' pear.

3.4. The expression pattern of chlorophyll-degradation associated genes in peel of the 'Emerald' and the 'Jingbai' pear

In the 'Emerald' pear, the mRNA level of *SGR1*, *NYC* increased until the 30th d of storage, and then it declined in control. 1-MCP treatment massively decreased the mRNA levels of *NYC* and *SGR1*, especially the increasing expression of *SGR1* was nearly stopped after 20 days of storage (Fig. 5E and K). The *NOL* and *PAO* expression decreased in the first 10 days after storage, and then increased and reached a maximum level at the 30th day and afterwards decreased again during storage without 1-MCP treatment. However, 1-MCP treatment also suppressed the expression of *NOL* and *PAO*, especially in the later stage of storage (Fig. 5C and G). This was also the case for *CLH1* and *RCCR* though their expression was not increased but slightly decreased during storage (Fig. 5A and I).

In the 'Jingbai' pear, *NOL*, *NYC*, *PAO*, *SGR1* mRNA increased during storage, reaching a peak at the 15th day, much earlier than in the 'Emerald' pear and then decreased in both of 1-MCP treated and control, while their respective mRNA levels were dramatically suppressed by 1-MCP treatment (Fig. 5D, F, H and L). The expression of *CLH1* and *RCCR* showed no significant change until 15 days of storage, and then decreased in both treatment groups (Fig. 5B and J).

4. Discussion

The mechanism of chlorophyll degradation in fruit peel may differ from these in leaf senescence since the process of chlorophyll degradation in fruit peel may be influenced by fruit flesh and core during ripening. Previous studies have suggested that exogenous or endogenous ethylene released from fruit flesh and core may accelerate chlorophyll degradation in fruit peel (Garcia-Luis, Fornes, & Guardiola, 1986; Jacob-Wilk, Holland, Goldschmidt, Riov, & Eyal, 1999; Porat et al., 1999; Purvis & Barmore, 1981; Trebitsh et al., 1993). In this work, we observed that the ethylene production pattern in 'Jingbai' pear was exactly matched to the pattern of peel yellowing or the decrease in chlorophyll *a*, *b* and *a* + *b* content in fruit peel during storage (Fig. 1). On the other hand, in the 'Emerald' pear, ethylene production was related to chlorophyll degradation only in the second half of the storage period in which the peak of ethylene production appeared (Fig. 1). Therefore, the faster chlorophyll degradation in the 'Jingbai' than in the 'Emerald' pear (Fig. 2) might be explained due to its faster rate of endogenous ethylene production (Fig. 1). 1-MCP massively reduced the ethylene production of the fruit (Fig. 1) and retained the higher chlorophyll content (Fig. 2) of peel for a longer time. These results may imply that the increasing of endogenous ethylene could act as a signal to stimulate chlorophyll catabolism in fruit peel.

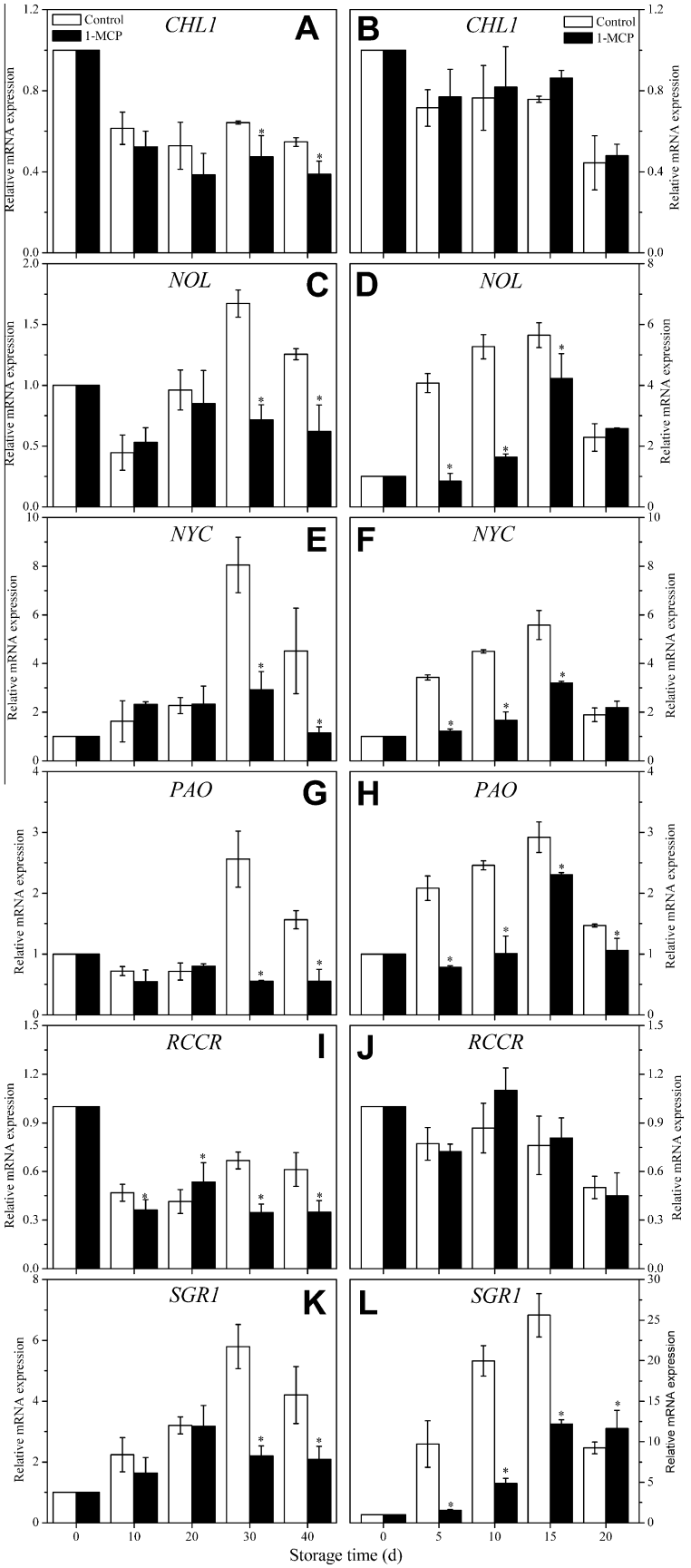

Fig. 5. Expression of chlorophyll degradation-associated genes, *CHL1*, *NOL*, *NYC*, *PAO*, *RCCR* and *SGR1* in the 'Emerald' (A, C, E, G, I and K) and 'Jingbai' (B, D, F, H, J and L) pear fruit peel with 1-MCP (■) treatment and control (□) stored at 25 ± 2 °C for 40 and 20 d, respectively. Expression value of chlorophyll degradation associated genes in 0 d storage was set to 1. Values are the means ± SE (n = 4). Star represents significant difference (P < 0.05).

Chlorophyllase is the first enzyme in a multi-step enzymatic process of the chlorophyll catabolic pathway, and acts as a rate-limiting enzyme in chlorophyll catabolism controlled via post-translational regulation (Harpaz-Saad et al., 2007), since the expression of *CLH1* gene did not increase during chlorophyll degradation (Jacob-Wilk et al., 1999). Our results are consistent with the above conclusions, showing no up-regulation of *CHL1* expression during storage of two cultivar pears (Fig. 5A and B) and 1-MCP had very little influence on its gene expression. Previous research had shown that two other enzymes, PAO and RCCR, participated in the chlorophyll catabolic pathway. PAO is a key enzyme and up-regulated during leaf senescence (Ginsburg, Schellenberg, & Matile, 1994; Hörtensteiner et al., 1995; Pruzinská, Tanner, Anders, Roca, & Hörtensteiner, 2003). By contrast, the expression of RCCR is constitutive (Rodoni et al., 1997; Trebitsh et al., 1993; Tsuchiya et al., 1999). In this study a similar expression pattern of PAO and RCCR was observed in fruit peel of both pear cultivars. The expression of *PAO* was up-regulated whereas the expression of RCCR in peel was not up-regulated during chlorophyll degradation in both pear cultivars (Fig. 5I and J). It would be very interesting to see if RCCR is also subjected to post-translational regulation as *CHL1*. 1-MCP treatment suppressed all aforementioned gene expression (Fig. 5A, G, H and I) except *CHL1* and *RCCR* in 'Jingbai' pear with unknown reasons, but it is still unclear whether such inhibition was a direct result from its inhibition on ethylene production. As these genes were responsive to 1-MCP and 1-MCP delayed chlorophyll degradation, thereby the peel yellowing during storage, thus we may suggest that *PAO*, *CHL1* and *RCCR* are seemly involved in the process of pear peel yellowing. Again it is still unknown whether 1-MCP has any effects on *CLH1* and *RCCR* post-translational modification.

Chlorophyll *b* is degraded only after conversion to chlorophyll *a*. NYC and NOL protein have chlorophyll *b* reductase activity *in vitro*, and regulate the thylakoid membrane degradation (Kusaba et al., 2007). Compared with the expression of chlorophyll degradation associated genes, our data showed that expressions of *NCY* and *NOL* had a striking increase in 1-MCP untreated fruits during storage and were greatly inhibited by the 1-MCP treatment in both pear cultivars (Fig. 5C–F). The change of chlorophyll *b* content was exactly corresponded to the expression of *NOL* and *NCY* gene (Fig. 2C and D), suggesting that *NOL* and *NCY* might play a main role in the process of chlorophyll *b* degradation in the two pear cultivars.

In green fruit, the chloroplast structure breaks down during ripening and senescence (Bonora et al. 2000; Ding, Ahmad, Razak, Saar, & Mohamed 2007). By examining the change in the chloroplast structure during storage, it was obviously found that chloroplasts developed a typical gerontoplast appearance, characterised by disassembled grana thylakoids and deterioration of internal chloroplast membranes in control of both pear cultivars, while 1-MCP treatment slowed chloroplast breakdown (Figs. 3 and 4). Recent studies suggested that SGR may take part in the chloroplast breakdown process (Kusaba et al., 2007; Park et al., 2007). SGR, as a chloroplast protein, plays a role in chlorophyll degradation by catabolic enzymes and proteases through inducing LHCPII disassembly in the thylakoid membrane (Aubry, Mani, & Hörtensteiner, 2008). Our results support this notion, since when chloroplast breakdown occurred during the peel yellowing, the expression of the *SGR* gene in both pear cultivars was dramatically up-regulated and was inhibited with 1-MCP treatment (Fig. 5K and L).

Taking into account the above results, it was concluded that endogenous ethylene production increased gradually in the early ripening stage, and then activated the expression of chlorophyll degradation associated genes during fruit storage. The expression of a few of chlorophyll degradation associated genes (*PAO*, *NYC*, *NOL* and *SGR1*) and the activation of their respective enzymes coded by these genes may lead to chloroplast breakdown and chlorophyll degradation, thus the peel change to yellow. 1-MCP restrained the ethylene production, and thereby delayed a series of downstream events in the chlorophyll catabolic pathway. Thus, the fruit retained its green peel colour for a longer time. However, further study is required to unravel the biochemical and molecular factors that link the ethylene signal transduction and the chlorophyll breakdown pathway.

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References

Alexander, L., & Grierson, D. (2002). Ethylene biosynthesis and action in tomato: A model for climacteric fruit ripening. *Journal of Experimental Botany, 53*(377), 2039–2055.

Alós, E., Roca, M., Iglesias, D. J., Mínguez-Mosquera, M. I., Damasceno, C. M., Thannhauser, T. W., et al. (2008). An evaluation of the basis and consequences of a stay-green mutation in the navel negra citrus mutant using transcriptomic and proteomic profiling and metabolite analysis. *Plant Physiology, 147*(3), 1300–1315.

Amir-Shapira, D., Goldschmidt, E. E., & Altman, A. (1987). Chlorophyll catabolism in senescing plant tissues: *In vivo* breakdown intermediates suggest different degradative pathways for citrus fruit and parsley leaves. *Proceedings of the National Academy of Sciences USA, 84*(7), 1901–1905.

Armstead, I., Donnison, I., Aubry, S., Harper, J., Hörtensteiner, S., James, C., et al. (2006). From crop to model to crop: Identifying the genetic basis of the staygreen mutation in the Lolium/Festuca forage and amenity grasses. *New Phytologist, 172*(4), 592–597.

Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiology, 24*(1), 1–15.

Aubry, S., Mani, J., & Hörtensteiner, S. (2008). Stay-green protein, defective in Mendel's green cotyledon mutant, acts independent and upstream of pheophorbide a oxygenase in the chlorophyll catabolic pathway. *Plant Molecular Biology, 67*(3), 243–256.

Barry, C. S., McQuinn, R. P., Chung, M. Y., Besuden, A., & Giovannoni, J. J. (2008). Amino acid substitutions in homologs of the stay-green protein are responsible for the *green-flesh* and *chlorophyll retainer* mutations of tomato and pepper. *Plant Physiology, 147*(1), 179–187.

Bonora, A., Pancaldi, S., Gualandri, R., & Fasulo, M. P. (2000). Carotenoid and ultrastructure variations in plastids of Arum italicum Miller fruit during maturation and ripening. *Journal of Experimental Botany, 51*(346), 873–884.

Chen, S., Zhang, M., & Wang, S. (2009). Physiological and quality responses of Chinese 'Suli' pear (*Pyrus bretschneideri* Rehd) to 1-MCP vacuum infiltration treatment. *Journal of the Science of Food and Agriculture, 90*(8), 1317–1322.

Ding, P., Ahmad, S. H., Razak, A. R. A., Saar, N., & Mohamed, M. T. M. (2007). Plastid ultrastructure, chlorophyll contents, and colour expression during ripening of Cavendish banana (*Musa acuminata* 'Williams') at 18 and 27 °C. *New Zealand Journal of Crop and Horticultural Science, 35*(2), 201–210.

Garcia-Luis, A., Fornes, F., & Guardiola, J. L. (1986). Effects of gibberellin A_3 and cytokinins on natural and post-harvest, ethylene-induced pigmentation of Satsuma mandarin peel. *Physiologia Plantarum, 68*(2), 271–274.

Ginsburg, S., Schellenberg, M., & Matile, P. (1994). Cleavage of chlorophyll–porphyrin (requirement for reduced ferredoxin and oxygen). *Plant Physiology, 105*(2), 545–554.

Gong, Y., & Mattheis, P. (2003). Effect of ethylene and 1-methylcyclopropene on chlorophyll catabolism of broccoli florets. *Plant Growth Regulation, 40*, 33–38.

Harpaz-Saad, S., Azoulay, T., Arazi, T., Ben-Yaakova, E., Metta, A., Shibolethc, Y. M., et al. (2007). Chlorophyllase is a rate-limiting enzyme in chlorophyll catabolism and is posttranslationally regulated. *The Plant Cell, 19*(3), 1007–1022.

Hershkovitz, V., Saguy, S. I., & Pesis, E. (2005). Postharvest application of 1-MCP to improve the quality of various avocado cultivars. *Postharvest Biology and Technology, 37*(3), 252–264.

Hörtensteiner, S. (2006). Chlorophyll degradation during senescence. *Annual Review of Plant Biology, 57*, 55–77.

Hörtensteiner, S., Vicentini, F., & Matile, P. (1995). Chlorophyll breakdown in senescent cotyledons of rape, *Brassica napus* L.: Enzymatic cleavage of phaeophorbide a in vitro. *New Phytologist, 129*, 237–246.

Iglesias, D. J., Tadeo, F. R., Legaz, F., Primo-Millo, E., & Talon, M. (2001). *In vivo* sucrose stimulation of colour change in citrus fruits epicarps: Interactions between nutritional and hormonal signals. *Physiologia Plantarum, 112*(2), 244–250.

Ito, H., Tanaka, Y., Tsuji, H., & Tanaka, A. (1993). Conversion of chlorophyll *b* to chlorophyll *a* by isolated cucumber etioplasts. *Archives of Biochemistry and Biophysics, 306*(1), 148–151.

Jacob-Wilk, D., Holland, D., Goldschmidt, E. E., Riov, J., & Eyal, Y. (1999). Chlorophyll breakdown by chlorophyllase: Isolation and functional expression of the

Chlase1 gene from ethylene-treated *Citrus* fruit and its regulation during development. *The Plant Journal, 20*(6), 653–661.

Jeong, J., Huber, D. J., & Sargent, S. A. (2002). Influence of 1-methylcyclopropene (1-MCP) on ripening and cell-wall matrix polysaccharides of avocado (*Persea americana*) fruit. *Postharvest Biology and Technology, 25*(3), 241–256.

Jiang, H., Li, M., Liang, N., Yan, H., Wei, Y., Xu, X., et al. (2007). Molecular cloning and function analysis of the stay green gene in rice. *The Plant Journal, 52*(2), 197–209.

Kräutler, B. (2008). Chlorophyll breakdown and chlorophyll catabolites in leaves and fruit. *Photochemical & Photobiological Sciences, 7*(10), 1114–1120.

Kusaba, M., Ito, H., Morita, R., Iida, S., Sato, Y., Fujimoto, M., et al. (2007). Rice non-yellow colouring1 is involved in light-harvesting complex II and grana degradation during leaf senescence. *The Plant Cell, 19*(4), 1362–1375.

Matile, P., Hörtensteiner, S., & Thomas, H. (1999). Chlorophyll degradation. *Annual Review of Plant Physiology and Plant Molecular Biology, 50*, 67–95.

Matile, P., Hörtensteiner, S., Thomas, H., & Kräutler, B. (1996). Chlorophyll breakdown in senescent leaves. *Plant Physiology, 112*(4), 1403–1409.

Park, S. Y., Yu, J. W., Park, J. S., Li, J., Yoo, S. C., Lee, N. Y., et al. (2007). The senescence-induced staygreen protein regulates chlorophyll degradation. *The Plant Cell, 19*(5), 1649–1664.

Porat, R., Weiss, B., Chen, L., Daus, A., Goren, R., & Droby, S. (1999). Effects of ethylene and 1-methylcyclopropene on the post-harvest qualities of 'Shamuti' oranges. *Postharvest Biology and Technology, 15*(2), 155–163.

Pruzinská, A., Tanner, G., Anders, I., Roca, M., & Hörtensteiner, S. (2003). Chlorophyll breakdown: Pheophorbide a oxygenase is a Rieske-type iron–sulfur protein, encoded by the *accelerated cell death 1* gene. *Proceedings of the National Academy of Sciences USA, 100*(25), 15259–15264.

Purvis, A. C., & Barmore, C. R. (1981). Involvement of ethylene in chlorophyll degradation in peel of citrus fruits. *Plant Physiology, 68*(4), 854–856.

Rodoni, S., Muhlecker, W., Anderl, M., Krautler, B., Moser, D., Thomas, H., et al. (1997). Chlorophyll breakdown in senescent chloroplasts (cleavage of pheophorbide a in two enzymic steps). *Plant Physiology, 115*(2), 669–676.

Rüdiger, W. (2002). Biosynthesis of chlorophyll *b* and the chlorophyll cycle. *Photosynthesis Research, 74*, 187–193.

Sato, Y., Morita, R., Nishimura, M., Yamaguchi, H., & Kusaba, M. (2007). Mendel's green cotyledon gene encodes a positive regulator of the chlorophyll-degrading pathway. *Proceedings of the National Academy of Sciences USA, 104*(35), 14169–14174.

Scheumann, V., Schoch, S., & Rüdiger, W. (1998). Chlorophyll *a* formation in the chlorophyll *b* reductase reaction requires reduced ferredoxin. *Journal of Biological Chemistry, 273*(52), 35102–35108.

Thomas, H., Ougham, H., Canter, P., & Donnison, I. (2002). What stay-green mutants tell us about nitrogen remobilization in leaf senescence. *Journal of Experimental Botany, 53*(370), 801–808.

Trebitsh, T., Goldschmidt, E. E., & Riov, J. (1993). Ethylene induces de novo synthesis of chlorophyllase, a chlorophyll degrading enzyme, in Citrus fruit peel. *Proceedings of the National Academy of Sciences USA, 90*(20), 9441–9445.

Tsuchiya, T., Ohta, H., Okawa, K., Iwamatsu, A., Shimada, H., Masuda, T., et al. (1999). Cloning of chlorophyllase, the key enzyme in chlorophyll degradation: Finding of a lipase motif and the induction by methyl jasmonate. *Proceedings of the National Academy of Sciences USA, 96*(26), 15362–15367.

Wei, J. M., Ma, F. W., Guan, J. F., Yuan, J. W., & Zhu, X. Q. (2009). Cell wall metabolism and its regulation in harvested Pyrus ussuriensis Maxin. cv. Jingbaili fruit during ripening. *Scientia Agricultura Sinica, 42*(8), 2987–2996.

Yang, X., Pang, X., Xu, L., Fang, R., Huang, X., Guan, P., et al. (2009). Accumulation of soluble sugars in peel at high temperature leads to stay-green ripe banana fruit. *Journal of Experimental Botany, 60*(14), 4051–4062.

Zavaleta-Mancera, H. A., Thomas, B. J., Thomas, H., & Scott, I. M. (1999). Regreening of senescent Nicotiana leaves. I. Reappearance of NADPH-protochlorophyllide oxidoreductase and light-harvesting chlorophyll a/b-binding protein. *Journal of Experimental Botany, 50*, 1683–1689.

Zhou, J., & Li, Y. W. (2006). Comparison of the performance of four introduced pear varieties. *Journal of Anhui Agricultural Sciences, 34*, 3664–3665.