Calcium treatments promote the aroma volatiles emission of pear (*Pyrus ussuriensis* ‘Nanguoli’) fruit during post-harvest ripening process

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Abstract Aroma is an important factor affecting pear fruit quality. This study was undertaken to assess whether pre-harvest calcium sprays (15 d before harvest, applications at 4%, w/v, 120 d after full bloom) could improve aroma of *Pyrus ussuriensis* ‘Nanguoli’ pear fruit at harvest and post-harvest, and analyse the mechanism of metabolic regulation.

Most compounds contributing to overall flavor in ripe ‘Nanguoli’ fruit at harvest and post-harvest were enhanced in response to pre-harvest calcium applications. Ester, especially acetyl ester, content was enhanced by calcium treatment; however, contents of alcohols, aldehydes and hydrocarbons were little affected. These effects were consistent with higher pyruvate decarboxylase and alcohol dehydrogenase activities in treated fruit, suggesting that calcium treatment increased supply of alcohols and acyl CoAs for ester biosynthesis. We also found that activities of β -glucosidases (involved in the emission of bound volatile compounds) were improved and the contents of bound volatiles were reduced by calcium treatment. At the same time, relative contents of linoleic and linolenic acids were improved by calcium treatment, expression of genes encoding fatty acid desaturase involved in the biosynthesis of unsaturated fatty acid precursors (linoleic and linolenic acids) for volatile aroma were improved. Calcium

treatment increased content of volatile aromatic substances, both because calcium promoted the decomposition of bound aroma substances to free aromatic forms, and also promoted the synthesis of volatile aromatic substances in pear fruit.

1. Introduction

Calcium (Ca) is a major element of fruit, and also functions as a intracellular signal transduction molecule in many physiological processes. Ca has an important influence on fruit quality and storability, and is used to maintain the quality of fruit during postharvest, reduce decay and extend shelf life for apple (Morales and Duque, 2002), peach (Verdini et al., 2008), apricot (Liu et al., 2009), strawberry (Chen et al., 2010; Chyau et al., 2003) and pear (Omama et al., 2010; Wójcik et al., 2014). Thus, Ca plays a critical role in fruit quality and storability (Fallahi et al., 1997; Malakouti et al., 1999).

Recently, Ca treatment was also found to promote the formation of volatile aromatic substances in fruit. For instance, Ca treatment promoted biosynthesis of aroma substances in ‘Fuji’ apple fruit during storage (Ortiz et al., 2009); Ca sprays before harvest increased aroma formation of ‘Fuji Kiku-8’ apple fruit (Ortiz et al., 2011); and Ca treatment at commercial maturity of ‘Golden Reinders’ apples can increase the content of aroma substances in medium-term storage (Ortiz et al., 2010). However, the effect of Ca treatment on pear aroma emission is not clear, and the mechanism of improvement of fruit volatile aroma substances by Ca treatment requires analysis.

‘Nanguoli’ pear, a variety of *Pyrus ussuriensis* that is one of the four main cultivated species of pear in China, is widely planted in northern China for its attractive color, exquisite flesh and pleasant flavor. Esters are the important volatiles of ripe fruit, endowing a ‘fruity’ flavor note (Qin et al., 2012). ‘Nanguoli’ fruit is very hard and has little aromatic volatile production when harvested at commercial harvest; however, after a post-harvest ripening process of about 10 d at 20°C, the fruit has an intense aromatic production, improving final fruit quality. Thus ‘Nanguoli’ is a good material for aroma research.

The aim of this experiment was to examine the effect of Ca on pear fruit aroma emission and analyse the mechanism of metabolic regulation.

2. Materials and methods

2.1. Treatments of pear fruit

For the characterization of aroma emission, ‘Nanguoli’ (*Pyrus ussuriensis*) was selected for research (Table 1). Fruits were provided by the National Germplasm Repository of Pear in the Research Institute of Pomology, Chinese Academy of Agricultural Sciences (CAAS), Xingcheng, Liaoning Province, China. Fifteen days before harvest (September 5, 2013 and September 7, 2014), six uniform ten-year-old ‘Nanguoli’ trees were selected and divided into two groups (three trees of each group were treated as biological replicates): one was treated with Ca, while the second one was maintained as control. Fruit of about the same size and color from different sides of experimental trees were sprayed with calcium chloride 4% (W/V) on fruit surface, and the other three trees were sprayed with water as control (CK). Orchard management procedures, such

as fertilization and irrigation, were the same for all treatments. Healthy fruits were harvested in commercial ripening period (135 days after pollination) and five days before commercial ripening (130 days after pollination) by hand-picking, with all fruits picked from the southern or western crown, about 2m above the ground. Individually packed fruit were taken to the laboratory immediately after harvest. Half of the fruits harvested in commercial ripening fruits were cored and seeded and the skin and flesh were frozen with liquid nitrogen and stored in -80°C freezer. The other half of the fruits harvested in commercial ripening were kept in 20°C for five days of post-harvest ripening, at the end of which were cored and seeded, while the skin and flesh was frozen with liquid nitrogen and stored in a -80°C freezer. For each treatment, 20-30 pears were combined for each of three replicates.

2.2. Extraction and concentration of volatile aroma compounds

GC-MS and HS-SPME were used for the extraction and concentration of volatile aroma compounds (Table 2), following the methods reported by Qin (2014). SPME fibres coated with a 65 µm thickness of polydimethylsiloxane-divinylbenzene (65 µm PDMS/DVB; Supelco Co., Bellefonte, PA, USA) were used in this study. Fibres were activated before sampling according to the manufacturer's instructions. The core and seeds of each pear fruits were removed and discarded, the skin and flesh was ground with a commercial blender to pulp for the extraction and concentration of volatile aromatic compounds. For each extraction, 10g of pulp were placed into a 20 ml screw-cap vial containing 3.6 g of NaCl to facilitate the release of volatile compounds. Prior to sealing of the vials, 50 µl of 0.04 g ml⁻¹ 3-nonanone was added as internal standard, and mixed with a glass rod. A magnetic follower was added to each vial, which was placed into a constant-temperature water bath at 40°C with stirring. The SPME fibre was exposed to the head space of the sample for 30 min to adsorb the analyte, then introduced into the heated injector port of the chromatograph for desorption at 250 °C for 5 min in splitless mode.

The volatile constituents were analyzed with an Agilent 5973B mass selective detector coupled to an Agilent 7890A gas chromatograph, equipped with a 30 m× 0.25 mm×1.0 mm HP-5 MS (5% phenyl-polymethylsiloxane) capillary column. A constant column flow of 1.0 ml/min helium was used as carrier gas. The injector and detector temperature were 250 and 280 °C, respectively. The oven temperature program was 35 °C for 8 min, increased at 2 °C/min to 140 °C, 140 °C for 2 min, then increased at 10 °C/min to 260 °C and held for 5 min. Mass spectra were recorded at 70 eV in electron impact (EI) ionization mode. The temperature of quadrupole mass detector and ion source were 150 and 230 °C, respectively. The temperature of transfer line was 280 °C. Mass spectra were scanned in the m/z range 33-350 amu at intervals of 1 s. Tentative identification of the volatile components was done by comparing the mass spectra of the samples with the data system library (NIST 98). Whenever it was possible, MS identification was confirmed with authentic references.

Quantification was done by the internal standard method, where the concentration of each volatile aromatic compound was normalized to that of 3-nonanone.

2.3. *Extraction and assay of aroma-related enzyme activities*

Fruits harvested in commercial ripening period (135 days after pollination) , 5 days before commercial ripening (130 days after pollination) and post-harvest ripening for 5 days were extraction and assay of aroma-related enzyme activities (Table 3), following the methods reported by Ortiz (2010a). Samples of both peel and pulp tissue were taken separately from 15 individual pear fruit (from combined sample of two treatments), frozen in liquid nitrogen, lyophilized, powdered and kept at -80°C until processing. Of freeze-dried, powdered tissue, 100 mg was used for each determination. Extraction and assays of lipoxygenase (LOX), pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH) and alcohol *o*-acyltransferase (AAT) activities on crude enzyme extracts were performed as described by Lara et al. (2003). Hydroperoxide lyase (HPL) was extracted and activity assayed according to Vick (1991). Total protein content in the enzyme extract was determined with the method of Bradford (1976). One activity unit (U) was defined as the variation in one unit of absorbance per minute. Each determination was done in triplicate and results were expressed as specific activity (U mg protein^{-1}).

2.4. *Measurement of ethylene production rate and determination of Ca in fruit tissue*

Ethylene production rate was measured by gas chromatograph (GC). Fruit of known weight (g) was placed in a sealed desiccator (about 2 L volume) for 1 h at 25°C . 1.0 ml gas in head space was withdrawn with a syringe and injected into the injector port of the GC (GC5890C, Nanjing Kejie Analytical Instruments Co. Ltd., China) equipped with a flame ion detector. Activated alumina column($0.53\text{ mm}\times 30\text{ m}$, Nanjing Jianuo Instrument Co. Ltd., China) was used to separate ethylene; and N_2 was used as the carrier gas at a rate of $45\text{ ml}\cdot\text{min}^{-1}$. The column, injector and detector temperatures were 45, 150 and 220°C , respectively. Five repetitions were taken and results were expressed as means ($\mu\text{l}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$) of the repetitions. Ethylene samples of known concentration ($0.5\text{ nl}\cdot\text{L}^{-1}$) were routinely used for calibration. The external standard was used for calculating ethylene concentration.

According to Ortiz (2010a), for each treatment, freeze-dried pulp tissue was obtained from five individual fruit per batch (Ca treatment $\times$ storage period). This pulp was submitted to acid digestion and analyzed using inductively coupled plasma emission spectroscopy (ICP-OES). Analyses were carried out in triplicate and results expressed as $\text{mg } 100\text{ g FW}^{-1}$.

2.5. *β -glucosidase assay*

The β -glucosidase assays were performed according to the method of Orruño (2001) with the following modifications: flesh of pear fruit at the three ripening stages was chopped, frozen in liquid nitrogen and kept at -80°C until use. The crude enzymatic extract was prepared by homogenizing 5 g of pear fruit tissue with 5 ml of 0.1 M citrate–0.2 M phosphate buffer (pH 4.0), followed by centrifugation at 5000 rpm and 4°C for 20 min. The supernatant was filtered through muslin cloth and kept on ice.

For the enzymatic assay, 100 μl of crude extract was mixed with 500 μl of 40 mM *p*-nitrophenyl- β -D-glucopyranoside solution and 400 μl of 0.1 M citrate–0.2 M phosphate buffer (pH 4.0), followed by incubation at 40°C for 30 min. Next, 2 ml of 3 M sodium carbonate solution was added to stop the reaction. The resulting mixture was transferred into a 96-well microplate and read at 405 nm against 0.1 M citrate–0.2 M phosphate buffer as a blank, using a SPECTRAmax PLUS 384 spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA). The protein content of the extracts was determined with a NanoDrop ND-1000 spectrophotometer (Wilmington, DE, USA). Results (specific activities) were expressed as means of triplicate experiments.

2.6 Analysis of the bound volatile fraction

The bound volatile (Table 4) extracts were analyzed as described by Garcia et al. (2012). A Shimadzu QP2010 Plus gas chromatograph (GC)-mass spectrometer (Kyoto, Japan), fitted with a Stabilwax column (Restek, Bellefonte, PA, USA; $30\text{ m} \times 0.25\text{ mm} \times 0.25\text{ lm}$) was used. The GC oven program was as follows: initial temperature 50°C , kept for 2 min, increased at $5^{\circ}\text{C}/\text{min}$ to 150°C , $10^{\circ}\text{C}/\text{min}$ to 200°C and $20^{\circ}\text{C}/\text{min}$ to 247°C , and kept at 247°C for 10 min. A 1- μl sample was injected, and mass spectra were acquired over the range m/z 40–400. Identification of the compounds was achieved by comparison of mass spectra and retention indices with those in the literature, or by using authentic standards, when available. Semi-quantification was achieved by comparing the areas of the peaks to that of an internal standard of cyclohexanone.

2.7 qRT-PCR analysis

The RNA were extracted from the individual fruit samples using Plant RNA Isolation Kit (AutoLab), followed by RNA purification with RNeasy MiniElute Cleanup Kit (Qiagen), according to the manufacturer's instructions, 8 aroma synthesis related genes were analyzed using quantitative real-time PCR, qRT-PCR analysis was conducted using the Lightcycle-480 (Roche), the qPCR was performed on 3 biological sample replicates. The primers used for amplifying each structural gene and transcript factor are presented in Table 5. qRT-PCR amplification and analysis were performed using a Roche LightCycler480. All reactions were performed using a SYBR Green MasterMix (SYBR Premix EX Taq™, TaKaRa) according to the manufacturer's instructions. The PCR reaction conditions were as follows: preincubation at 95°C for 5 min, then 45 cycles of 94°C for 10 s, 60°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 3 min.

Fluorescence was measured at the end of each annealing step. Amplification was followed by a melting curve analysis with continual fluorescence data acquisition during the 56–61 °C melt. The raw data were analyzed with iQ5 software (version 2.0, Bio-Rad), and the relative expression levels were calculated with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Tubulin (AY338250) was used as the internal control (Chen et al., 2015). qPCR data are technical replicates (n=3) with error bars, representing means±standard error (SE). Statistical and correlation analyses were performed with SPSS for Windows NT (release 8.0.0). The data was log_e transformed and median centered by genes using the Adjust Data function of CLUSTER 3.0 software, then further analyzed with hierarchical clustering with average linkage (Eisen et al. 1998). Finally, we performed tree visualization using Java Treeview (Stanford University School of Medicine, Stanford, CA, USA). The figure of time course genes was analyzed by MevSOM software.

2.8 Statistical analysis

All data were generated by triplicate experiments and concentrations were reported as the average of three replications. The SPSS16.0 statistical software package (IBM Software Group) was used for all statistical analysis. Analysis of variance was performed and the significant differences were detected using Duncan's test ($P < 0.05$).

3. Results and discussion

3.1 Effect of Ca treatments on Ca concentrations and ethylene (C_2H_4) production of pear fruit

Significant ($P < 0.01$) increases of Ca content were found in Ca-treated fruit at harvest (Fig. 1A), showing that exogenous Ca was efficiently absorbed by 'Nanguoli' fruit tissue.

C_2H_4 plays an important role in regulating aroma metabolites and emission: for example, volatile levels of tomato fruit were changed by C_2H_4 treatment (McDonald et al., 1996); 1-methylcyclopropene (1-MCP) treated peach fruit 'Tardibelle' altered the supply of alcohol and acyl-CoA precursors, leading to significant changes in emission of some volatile esters, particularly of the straight-chain type (Ortiz et al., 2010b); and C_2H_4 also influenced several metabolic pathways (fatty acid and amino acid metabolic pathways) leading to volatile formation in mountain papaya fruit (Cristian et al., 2007). In the present study, pre-harvest Ca treatment suppressed C_2H_4 production (Fig. 1B)-in agreement with Wójcik et al (2014), who found that spray treatments of calcium chloride ($CaCl_2$) inhibited production rates of C_2H_4 of 'Conference' pear fruit. We found C_2H_4 production of 'Nanguoli' fruit T6 was 105.0 $\mu\text{l/kg/h}$, which was 22.22% less than for T5 (135.0 $\mu\text{l/kg/h}$). Peel color, soluble solids (SS), titratable acid (TA) of 'Nanguoli' fruit were influenced by Ca treatment (Table 7), especially, a significant difference in SS/TA were found (at $P < 0.01$).

3.2 Effects of Ca treatment on the free aroma composition of 'Nanguoli' fruit

Volatile esters are reportedly the most important contributors to ripe pear fruit aroma, which

endows them a 'fruity' flavor note (Chen et al., 2005). It was reported that pre-harvest Ca sprays enhanced the emission of key volatile esters in ripe fruit of 'Fuji Kiku-8' apples (Ortiz et al., 2011). In the present study, 32 kinds of esters were identified from Ca-treated 'Nanguoli' fruit, while 31 esters were identified from the control (Table 2). Acetic acid, isobutyl ester, methyl pentanoate and 2-methyl-1-butyl acetate were only detected in the Ca-treated fruit; while 4-octenoic acid, ethyl ether and propyl acetate were only found in control fruit.

Ca treatment significantly increased the total ester aroma (Table 2) content of 'Nanguoli' fruit: T6 was increased by 652.98 ng/g (18.37%) more than control, and T4 increased by 91.2 ng/g (5.59%), with the esters that mainly increased including ethyl caproate, hexyl acetate, hexyl butanoate and butyl acetate. The ethyl ester content of T6 'Nanguoli' fruit increased 26.37% more than T5, and T4 by 19.77% more than T3. Thus, Ca treatment promoted the formation of esters in 'Nanguoli' fruit-notably ethyl ester compounds significantly increased-which made the fruit flavor more concentrated.

Qin et al (2014) reported that hexanal and hexanol were important intermediates of the fatty acid metabolic pathway and were more efficient metabolic intermediates for volatile synthesis than linoleic and linolenic acids. Hexanol can be directly used to synthesize volatile esters, and hexanal is also an important substrate of volatile ester biosynthesis. In the present study, Ca treatment significantly increased the total aldehyde contents of 'Nanguoli' fruit compared with controls, for instance T6 increased by 520.38 ng/g (31.88%) more than T5, and T4 by 151.65 ng/g (13.28%) more than T3. Hexanal, acetaldehyde and (E)-2-hexenal were significantly increased by Ca treatment, while content of isobutyraldehyde and 2-methyl-butanal were lower than in controls. Ca treatment also significantly increased the content of alcohols in 'Nanguoli' fruit compared to controls: ethanol content of T6 was 2.02 times that of T5, the hexanol content was improved and (E)-2-hexen-1-ol and 1-pentanol were only detected in the Ca-treated 'Nanguoli' fruit.

3.3 Effects of Ca treatment on the content of bound aroma volatiles

There are two kinds of aroma compounds in plants: free aroma compounds and bound aroma volatiles, which are bound to sugars as glycosides or glucosinolates. The proportion of glycosidically bound volatiles is usually greater than that of free volatiles, making them an important potential source of flavor compounds. The odorous aglycones (Reineccius, 2006) may be released from sugar moieties during maturation, processing and storage, or by the action of enzymes, acids or heat. In the present study, we found seven bound aroma volatiles (Table 3), including benzyl alcohol, eugenol, 3,4-dihydro-2,2-dimethyl-2H-1-benzopyran, 4-hydroxyacetophenone and dibutylhydroxytoluene. Ca treatment significantly reduced the contents of 3,4-dihydro-2,2-dimethyl-2H-1-benzopyran and dibutylhydroxytoluene in 'Nanguoli' fruit, and the total content of bound aroma volatiles of Ca-treated 'Nanguoli' pear fruit peel was reduced by 26.74% compared to controls.

It was reported that β -glucosidase functions in release of bound aroma volatiles in plants (García-Carpintero et al., 2011; Sarry et al., 2004), and Ca treatment can also improve production of β -glucosidase (Gupte and Madamwar, 1997). In the present study, we found that β -glucosidase activity of 'Nanguoli' fruit was significantly enhanced by Ca treatment (Fig. 2A), the β -glucosidase activity of Ca-treated peel was improved by 27.08% compared to controls and β -glucosidase activity of pulp (Fig. 2B) was improved by 11.08% compared to controls, which would have contributed to hydrolysis of bound aroma substances.

This study showed that Ca enhanced the β -glucosidase activity of pear, likely because Ca^{2+} acts as a β -glucosidase activity modulator and increases β -glucosidase activity. The β -glucosidase activity of pear fruit was significantly enhanced by Ca treatment, and increased β -glucosidase activity would mean more glucoside-bound alcohol compounds were hydrolyzed to free forms or metabolic substrates for further participation in the reaction.

3.4. Effects of Ca treatments on content of esters of different origins in 'Nanguoli' pear

Sanz et al (1997) reported that fatty acids were major precursors of aroma volatiles in most fruit. Fatty acid-derived straight-chain esters (Schwab et al., 2002) and branched chain volatile esters arise from branched chain amino acids (Goff et al., 2006; Perez et al., 2002; Wyllie et al., 2000). In the present study (Fig. 3A,B), fatty acid-derived esters of T6 increased by 3235.48 ng/g more than T5, while amino acid-derived esters of T6 only increased by 36.42 ng/g more than T5. Esters of 'Nanguoli' fruit mainly arose from fatty acid metabolism pathways, and Ca treatment mainly increased the esters derived from fatty acids, such as ethyl acetate, butyl acetate, hexyl acetate, hexyl butanoate and ethyl caproate. Ca regulation of aroma of 'Nanguoli' fruit was mainly through fatty acid metabolic pathways, rather than amino acid pathways.

In pear fruit, amino acid metabolism is another important path for synthesis of volatile aromatic substances. Ca treatment slightly improved the content of amino acid-derived aroma in 'Nanguoli' fruit but not to a significant extent. This indicated that Ca treatment had little effect on amino acid metabolism in pear fruit, and that content of amino acids was not the main factor limiting the formation of volatile aroma esters derived from amino acids.

3.5. Effects of Ca treatments on key enzyme activities of aroma synthesis

Increased PDC and ADH activities levels were observed in CaCl_2 -treated 'Golden Reinders' apple (Ortiz et al., 2010a), which possibly led to improved substrate availability for AAT action during the shelf-life period. In the present study, Ca treatment significantly improved the ADH and PDC activities in 'Nanguoli' fruit (Table 4), the ADH and PDC activities of Ca treated peel were improved 639.77% and 182.66% compared to controls, which promoting the synthesis of ethanol and acetaldehyde in fruit, and also improving synthesis of hexanal and hexanol, eventually forming a large number of ester compounds. We also found that Ca treatment significantly

improved LOX activity in peel, but non-significantly improved AAT and HPL activities of 'Nanguoli' fruit. The results showed that Ca treatment may promote the content of esters in 'Nanguoli' fruit, especially for acetyl ester, which made the fruit flavor more concentrated.

The C₂H₄ production and key enzyme activity of aroma synthesis together showed that C₂H₄ production was reduced, ADH activity was improved and AAT activity was not significantly improved. Our data were in agreement with Defilippi et al. (2005) and Cristian (2007), who concluded that ADH was not affected by changes in the level of endogenous C₂H₄, whereas AAT was regulated by C₂H₄.

Biosynthesis of esters is subject to the combined effects of substrate availability, enzyme activity and substrate specificity of AAT isozymes. The isozymes of AAT have a wide range of substrate specificity on a series of alcohols and acetyl coenzyme A (acetyl CoA). Biochemical and genetic studies have shown that esters can be produced from fatty acids and amino acids (Song and Bangerth 2003; Wang et al., 2001), and ADH, PDC and LOX catalyze the upstream of fatty acid and amino acid metabolism-thus enzyme activity plays a very important role in fruit ester biosynthesis. The results showed that Ca could increase LOX activity, which is the first key enzyme catalyzing unsaturated fatty acid cleavage in the fatty acid metabolism pathway, indicating that Ca treatment could accelerate the start of the aroma fatty acid metabolism pathway.

AAT can catalyze conversion of acetyl groups of acetyl CoA to esters with a certain source of alcohols. In this study, Ca treatment had little impact on AAT activity of 'Nanguoli' pear fruit peel and pulp, but the content of ester substances significantly increased-this finding suggests that AAT activity could satisfy the immediate needs of ester synthesis in pear fruit. However, availability of alcohol may be the main limiting factor for ester formation, which is agreement with results of Lara et al (2008) and Zhu et al (2008). Improved ADH activity and ester content may be because Ca promoted ADH activity to produce more alcohols used for ester biosynthesis.

PDC is one key enzyme in the degradation of amino acids, it use pyruvic acid as catalytic precursor and removal its acid forming acetaldehyde, ADH acts in formation of ethanol, or acetyl CoA is formed by acetaldehyde dehydrogenase, which can be used for the formation of ester compounds. Ca treatment improved PDC activity, meaning that PDC had a very important role in formation of ethyl esters. In addition, the biosynthesis of ethanol and acetaldehyde were increased by Ca treatment, and ethyl esters also increased, further showing that precursor availability determined the biosynthesis of fruit volatile substances.

3.6. Effect of Ca treatment on relative content of fatty acids in 'Nanguoli' fruit

An important step in the biosynthetic pathway of aroma compounds is the availability of primary precursor substrates, including fatty acids, which are highly regulated during fruit development in terms of amount and composition (Song et al., 2003). Fatty acids are major precursors of aroma volatiles in most fruit (Sanz et al., 1997). Fatty acid deficiency in fruit is the

primary cause of poor aroma (Harb et al., 2000; Wang et al., 2001). It was also reported that increased fatty acid supply (Qin et al., 2014) could enhance biosynthesis of volatile esters in pear fruit. In the present study, content of linoleic and linolenic acids in ‘Nanguoli’ fruit was significantly increased by Ca treatment (Table 6), linoleic acid and linolenic acid fatty acid contents were improved 223% and 740% compared to controls at harvest, respectively. Increased linoleic and linolenic acid contents provided ample substrate for aroma synthesis, and may be one reason for Ca treatment improving the aroma synthesis of ‘Nanguoli’ fruit.

3.7. Expression of aroma synthesis related genes

As reported by Defilippi et al. (2005), AAT expression is highly regulated by C₂H₄, whereas ADH expression is unaffected by changes in the levels of endogenous C₂H₄. We analyzed the expression of aroma synthesis related genes with quantitative real-time PCR (Fig. 4; Table 6), and the results showed that the trends of gene expression variation were basically the same as for enzyme activities. Ca treatment resulted in up-regulated expression of genes for ADH, PDC, β -glucosidase 44 and omega-3 fatty acid desaturase (omega-3 FAD), significantly improved expression of genes for LOX5 and LOX21 only at 5 d before harvest, no significant change in expression of genes for AAT and HPL compared to controls, slightly higher expression of the gene for AAT compared to controls only at 5 d post-harvest and in contrast lower expression of the gene for HPL compared to controls at 5 d post-harvest.

3.8. Discussion

Aroma is the product of a complex mixture of aromatic volatile compounds, and many factors including calcium affect aroma volatiles. There are several pathways (Hadi et al., 2013) involved in aroma volatile biosynthesis starting from lipids, amino acids, terpenoids and carotenoids in plant. Fatty acid metabolism is an important metabolic pathway involved in the biosynthesis of aroma volatiles in fruits (Qin et al., 2014). Fatty acids liberated by lipase activity and those further metabolized by β -oxidative enzymes and/or lipoxygenase (LOX) are generally regarded as the initial precursors of straight-chain esters, alcohols, and aldehydes produced in fruits during development and maturation (Bartley et al., 1985; Fellman et al., 2000; Song et al., 2003). Previous studies have reported that the core reactions of the LOX pathway consist of the sequential action of LOX, hydroperoxide lyase (HPL) and alcohol dehydrogenase (ADH) (Matsui et al. 2000). The LOX biosynthetic pathway has the potential to provide substrates for ester production. Decreased LOX activity possibly leads to a shortage of lipid precursors for pear fruit ester biosynthesis (Lara et al. 2003). The majority of plant aroma volatiles originate on a quantitative and qualitative basis from saturated and unsaturated fatty acids (Schwab et al., 2008). According to Ortiz et al. (2011), pre-harvest calcium treatment can improve volatile emissions at commercial harvest of apple (*Malus × domestica* Borkh., also known as *M. pumila*) -most of the compounds contributing to overall flavor in ripe fruit were enhanced in response to calcium applications, with the acetate esters particularly favored. These effects may have arisen from

increased PDC and ADH activities, possibly leading to a better supply of alcohols and acyl CoAs for ester biosynthesis. In the present study, we found that the activities of ADH, PDC and LOX were significantly improved by calcium treatment, which was consistent with the results of Ortiz et al. (2011). Interestingly, we also found that the content of straight chain ester was significantly higher than branched chain esters in calcium treatment 'Nanguoli' pear fruit, which suggesting that calcium treatment to improve the 'Nanguoli' pear fruit aroma was mainly by fatty acid metabolic pathways.

4. Conclusion

Ca treatment increased 'Nanguoli' pear fruit aroma mainly in three ways. First, Ca increased β -glucosidase activity and released more bound aroma compounds. Second, Ca treatment significantly increased the activities of ADH, PDC and LOX in fruit, thereby promoting synthesis of ethanol and acetaldehyde in fruit (improved synthetic ester substrate availability), eventually forming a large number of ester compounds. Third, Ca treatment increased amounts of metabolic substrates, especially of unsaturated fatty acids, providing more precursors for ester metabolic process needs. Ca treatment increased the content of volatile aromatic substances, both because Ca promoted the decomposition of bound aroma substance into free forms, and the synthesis of volatile aromatic substances in 'Nanguoli' pear fruit.

Authors' contributions

SWW contributed to the experimental design and management, quantitative RT-PCR, data analysis and manuscript preparation. STT conducted the data analysis and organized and reviewed the manuscript. GHQ, HPZ contributed to data analysis. JW and SMW prepared the plant materials and tissue collection. SLZ designed and managed the experiments. All authors have read and approved the final manuscript.

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Data archiving statement

Gene sequences of pear were obtained from the Pear Genome Project website (<http://peargenome.njau.edu.cn/>).

Figure captions

Figure 1. A: Ca concentrations of pears (mg/g DW). B: C₂H₄ production of 'Nanguoli' fruit.

Note: ** denotes a significant difference between treatment and CK at P < 0.01.

Figure 2. A: Effects of Ca treatment on β -glucosidase activity (U/mg protein) of pears; B: Effects of different Ca concentrations on β -glucosidase activity (U/mg protein) of pears. Note: Different lowercase letters indicate a significant difference at $P < 0.05$ and capital letters at $P < 0.01$.

Figure 3. Effects of Ca treatments on esters of different origins in 'Nanguoli' pear fruit. A: Contents of esters. B: Numbers of esters.

Figure 4. Real-time quantitative PCR analysis of aroma-related genes. Representative genes selected for the analysis were genes encoding Alcohol dehydrogenase (ADH), β -glucosidase 44, PDC1, AAT, Omega-3 fatty acid desaturase, HPL, LOX5, LOX21 involved in the fatty acid pathway.

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