Effects of grafting on free fatty acid contents and related synthetic enzyme activities in peel and flesh tissues of oriental sweet melon during the different development period

J H Hao[1a], Z Y Qi[1a], J R Li[2], C J Liu[1], L Wang[1], W T Jin[1], X Li[3] and H Y Qi[4,5]

[1] Beijing Key Laboratory for Agricultural Application and New Technique, College of Plant Science and Technology, Beijing University of Agriculture, No. 7 Beinong Road, Changping District, Beijing, P. R. of China

[2] Administration Office for public service in Benxi city of Liaoning Province, No. 101 Jiefang North Road, Mingshan District, Benxi, P. R. of China

[3] Agricultural Bureau of Jing Country of Hebei Province, No.36 Jinghua Road, Jing Country, Hebei, P. R. of China

[4] Key Laboratory of Protected Horticulture of Education Ministry and Liaoning Province, College of Horticulture, Shenyang Agricultural University, No. 120 Dong Lin Road, Shenhe District, Shenyang, P. R. of China

[a] J H Hao and Z Y Qi are co-first authors.

E-mail: hyqiaaa@126.com

Abstract. In this experiment, white seed pumpkin 'Shengzhen No.1' was used as rootstock for the oriental sweet melon (*Cucumis melo L.*)'Yumeiren'. The fruits of own-rooted melons were used as control. The objective of this experiment was to study the effects of grafting on the following parameters: content of free fatty acids and activities of synthetic enzymes (LOX, ADH, and AAT). These parameters were measured in the peel and flesh tissues of oriental sweet melon at different time-points. Due to grafting, the content of oleic acid and linolenic acid decreased in peel tissues of melons at early stages of maturity. Therefore, total contents of free fatty acids were lower than those of control. However, there was no significant difference in the content of three free fatty acids in flesh tissue; this content was lower than that in peel tissue. Due to grafting, enzymatic activities of ADH and AAT decreased in peel tissue of melons; however, these enzymes showed different activities in flesh tissues of melons subjected to grafting. Nevertheless, there was no significant difference in enzymatic activities of flesh tissues; however, these activities were lower than that of peel tissue. These results indicate that free fatty acid content and related synthetic enzyme activities decreased when grafting was included in the cultivation of oriental sweet melons. Consequently, the aroma of these melons decreased due to grafting.

1. Introduction

Melon (*Cucumis melo L.*) is one of the most popular fruits in the world, and it is known for its unique flavor. Aromatic components are the most valuable fraction of volatile compounds in melon fruits, because they possess high sensory values and characteristic physiological values. Therefore, aromatic components have become one of the most important indices that define the quality of melon fruits [1-3]. Aroma is a quality characteristic that attracts a lot of attention from fruit growers, consumers, and quality breeders. In our previous study [4], we investigated oriental sweet melons and detected 40 types of aromatic compounds, including 32 different esters. Peak area of esters accounted for 70.14–95.77% of the total peak area, indicating that esters are the main aromatic components of melon fruits.

Fatty acids are the precursors of most straight-chain esters in aromatic volatile compounds [5-6]. In this study, we found that large amounts of free fatty acids accumulate in apples even before aromatic compounds develop in the fruit. Six-carbon aldehydes and alcohols were detected by crushing the leaf and fruit of tomato (Lycopersicon esculentum Mill.); these compounds result from the degradation of lipids and free fatty acids [7]. Collectively, these C-6 volatile compounds are considered as the most important aromatic compounds. As fruit aromas become stronger, the content of free fatty acids decreases rapidly [8]. In a study conducted by Francisc V Dulf, the dominating fatty acids of berry pulp/peel oils were arranged in a descending order: oleic (20–40%), palmitic (17–27%), palmitoleic (10–22%), linoleic (10–20%), and á-linolenic (4–9%) acids [9]. In addition, palmitoleic acid (0.1–0.5%) was found in trace amounts in berry seed oils. The proportions of oleic (13–21%) and linoleic (33–43%) acids showed relatively high deviations in different varieties of berry seed oils. Unlike pulp oils, seed oils contain higher amounts of polyunsaturated fatty acids (PUFAs) (65–72%) but relatively lower proportions of monounsaturated fatty acids (MUFAs) (16–21.5%) and saturated fatty acids (SFAs) (11–16%). The study was conducted by Schaffer *et al.* [10]. They showed that alcohol dehydrogenase (ADH) and alcohol acyltransferase (AAT) play a pivotal role in the synthesis of ester from amino acids. Moreover, only a handful of genes were associated with the production of esters in apple, including alcohol acyltransferase (AAT) and alcohol dehydrogenase (ADH). As the fruit develops, ADH activity gradually decreases but AAT activity steadily increases in the fruit [11-12].

Grafting is the effective method to prevent blight and successive cropping obstacle in protected cultivation [13-15].However, grafting leads to a decline in quality and fragrance of melon fruits. Krumbein and Schwarz [16] reported that total aromatic compounds and special esters decreased when grafting was implemented in tomato cultivation; there was a decline in the content of aromatic substances, which primarily attract people with their beautiful fragrance. Therefore, it is very important to determine the influence of grafting on the metabolism of volatile substances in melon fruits. With this strategy, we could improve the metabolism of volatile substances in melon fruits. However, very few studies have investigated how grafting affects the metabolism of aromatic substances in melon fruits. Therefore, we analyzed the content of free fatty acids and related activities of synthetic enzymes (LOX, ADH, AAT) in peel and flesh tissues of sweet melons at different development periods; both own-rotten and grafted varieties of oriental sweet melons were included in this study. Our main aim was to investigate the influence of grafting on aromatic substances and to elucidate the underlying physiological mechanism. This study served as the theoretical basis for regulating the quality of melons in the future.

2. Materials and methods

2.1. Plant material and growth conditions

Yumeiren is a cultivar of oriental sweet melon (*Cucumis melo L.*); it was used as scion with white-seeded pumpkin (*Cucurbita moschata Duch*.). Shengzhen1 was used as rootstock, and conventional methods were used to cultivate seedlings. The first two true leaves were produced on melon plants; these plants and a terminal bud were directly grafted onto white-seeded pumpkins by grafting (inarching) method. The own-rooted melon plants were used as controls (CK). Melons that underwent grafting treatment (G) were planted along with the controls in cultivation barrels (Meidolin agricultural equipment co. LTD), which were incubated in a greenhouse. Each barrel had a height of

30 cm and a diameter of 30 cm. These barrels were filled with a fermented potting substrate (soil: peat: chicken manure = 3:2:1). The bottoms of barrels were covered with a layer of perlite, which was 2 cm in thickness. Within-row plant spacing was 60 cm, whereas between-row spacing was 80 cm. Melon plants were pruned to single stems during the process of cultivation. We preserved all the melons that grew beyond the 10^{th} node of the main stem, so only 2–3 melons were excluded from each plant. We waited for the day when the female flowers bloomed completely. Then, growth regulator Fengchanji 2 (Shenyi Biology Co., Ltd.) was sprayed on these freshly bloomed flowers in the morning. Thereafter, they were pollinated and tagged for further analyses. After the blooming of grafted and own-rooted plants, we collected samples from the flesh and peels of melons. Following the blooming of plants, samples were collected on the following days: 20, 25, 30, and 35. These samples were quickly frozen in liquid nitrogen and then stored at –80 ℃. These samples were analyzed to determine free fatty acid content and the activity of enzymes (LOX, ADH, and AAH), which participated in the synthesis of esters.

2.2. Fatty acid determination

Fresh samples (5 g each) were ground into a homogeneous fine powder. Then, 10 mL was taken out from a solution mixture of petroleum and ether (4/3, v/v), and it was added to the fine powder and mixed thoroughly. This suspension was extracted for 24 h at 0–4 ℃. Then, 10 mL of 0.4 mol/L KOH methanol solution was added to the extract. Finally, methyl esterification was carried out for 2 h. Redistilled water was added to separate out the organic phase. Then, organic phase was concentrated or evaporated to dryness. Finally, it was resolubilized and filtered through an acetate filter (0.45 μm pore size, Nalgene).

Gas chromatography (VARIAN CP-3800, USA) was performed to characterize fatty acids in this solution. To perform analysis, CP 7485 column was used along with splitless injection in the gas chromatography instrument. Injection pore temperature was maintained at 240 °C, while detector temperature was maintained at 250 °C. Temperature programming was set at 150 °C for 1 min, and then it was raised to 230 °C at a rate of 10 °C/min; this temperature was again maintained for 1 min. Finally, it was raised to 240 °C at a rate of 3 ℃/min. This temperature was maintained for 10 min. The flow rate was 10 mL/min. Experimental related drugs were purchased from Sigma-Aldrich (St. Louis City, MO, USA).

2.3. Analysis of lipoxygenase (LOX) activity

Fresh samples (3 g each) were ground into a homogeneous fine powder. To prepare crude enzyme extracts, this powder was mixed thoroughly with 8 mL of 50 mmol/L phosphate buffer (pH 7.0). Then, it was precooled to 4 °C. This mixture was centrifuged (Thermo Scientific™, Sorvall™ ST 16R, USA) at 15000 g and 4°C for 15 min. The supernatant was used to determine the activity of LOX enzyme.

Analysis of enzyme activity: The reaction system (total volume 3 mL) contained 25 μl of sodium linoleate stock solution (substrate), 2.775 mL of 100 mmol/L acetate buffer (pH 5.5), and 0.2 mL of crude enzyme extracts. The reaction temperature was fixed at 30 ℃. Lysyl oxidase (LOX) activity was measured with a spectrophotometer at 234 nm. After adding crude enzyme solution, we first measured optical density (OD) value at 15 s. Then, OD values were continuously monitored and recorded for 1 min. Thus, changes in OD values were observed for 1 min. Enzyme activity was expressed as U/mg protein. Enzyme activity assay was repeated thrice.

2.4. Analysis of alcohol dehydrogenase (ADH) activity

Preparation of crude enzyme extracts: Fresh samples (3 g each) were placed in liquid nitrogen medium and ground thoroughly into a homogeneous fine powder. Six milliliters of enzyme extraction buffer was precooled to 4 °C. Then, it was added to the powder and mixed thoroughly. The composition of enzyme extraction buffer was as follows: 100 mM MES-tris buffer solution (pH 6.5), 2 mM dithiothreitol (DTT), and 1% polyvinylpolypyrrolidone (PVPP). After incubation, the mixture was centrifuged at 15000 g and 4 °C for 30 min. The supernatant was used to determine ADH activity.

Analysis of enzyme activity: The reaction system (total volume 3 mL) consisted of the following reagents: 2.4 mL of 100 mM MES-Tris buffer (pH 6.5), 0.15 mL of 1.6 mM nicotinamide adenine dinucleotide (reduced form) (NADH), 0.15 mL of 80 mM acetaldehyde, and 0.3 mL of crude enzyme extracts. The temperature of the reaction was fixed at 30 ℃. The OD values were measured with a spectrophotometer at 340 nm. After adding crude enzyme extracts, we first measured OD values at 15 s. Then, changes in OD values were continuously monitored and recorded for 1 min. The enzyme activity assay was repeated three times.

2.5. Analysis of alcohol acyltransferase (AAH) activity

Preparation of crude enzyme extracts: Fresh samples (3 g each) were placed in liquid nitrogen medium and ground thoroughly into a homogeneous fine powder. Then, 1 mg of polyvinylpolypyrrolidone (PVPP) and 6 ml 0.1M Tris-HCl (pH 8.0) were added to the powder and mixed thoroughly. After incubation, the mixture was centrifuged at 16000 g and 4 °C for 30 min. The supernatant was used to determine AAT activity.

Analysis of enzyme activity: The reaction system consisted of the following reagents: 0.5 M Tris-HCl buffer (pH 8.0), 5 mM $MgCl_2$, 5 mM acetyl coenzyme A, 200 mM butanol, and 0.6 mL of crude enzyme extracts. The temperature of reaction was fixed at 35 ℃ for 10 min. Then, 150 μl of 20 mM DTNB was added and mixed thoroughly with the reaction mixture. This mixture was allowed to stand at room temperature for 10 min. The OD values were measured at 412 nm. The enzyme activity assay was repeated three times.

2.6. Statistics and data analysis

The experiment was arranged in a randomized block design and performed in three replicates. Data were analyzed with DPS software (Refine Information Tech, Hangzhou, Zhejiang, China). Statistical significance of the difference was estimated by least significant difference (LSD) test at the 5% or 1% level [17]. Figures representing physiological parameters were drawn using OriginPro software (OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. The effects of grafting on the oleic acid, linoleic acid and linolenic acid contents in the peel and flesh tissues of melons at different stages of development

In the peel and flesh of oriental sweet melons, Oleic acid, linoleic acid, and linolenic acid content reached a peak on 20^{th} day after full bloom. The content of three free fatty acids decreased steadily with the gradual development of melon fruits. On 35^{th} day after blooming, there was significant increase in the content of three free fatty acids, except for the oleic acid in G-flesh and the oleic acid content in G-peel (Figure 1). In melon fruit, linolenic acid content was the highest among the three free fatty acids, followed by the linoleic acid. Oleic acid content was the lowest in melon fruits. Compared to the flesh of melon fruit, the content of three free fatty acids was significantly higher in the peel. At the early stage of maturity, grafting reduced the content of oleic acid and linolenic acid in the peel of melon fruit; however, grafting exerted little effect on the 30^{th} day after blooming. Grafting reduced linoleic acid content in the melon peel on 20^{th} and 35^{th} days after blooming. In the flesh of grafted and own-rooted melons, no significant differences were observed in the content of three free fatty acids.

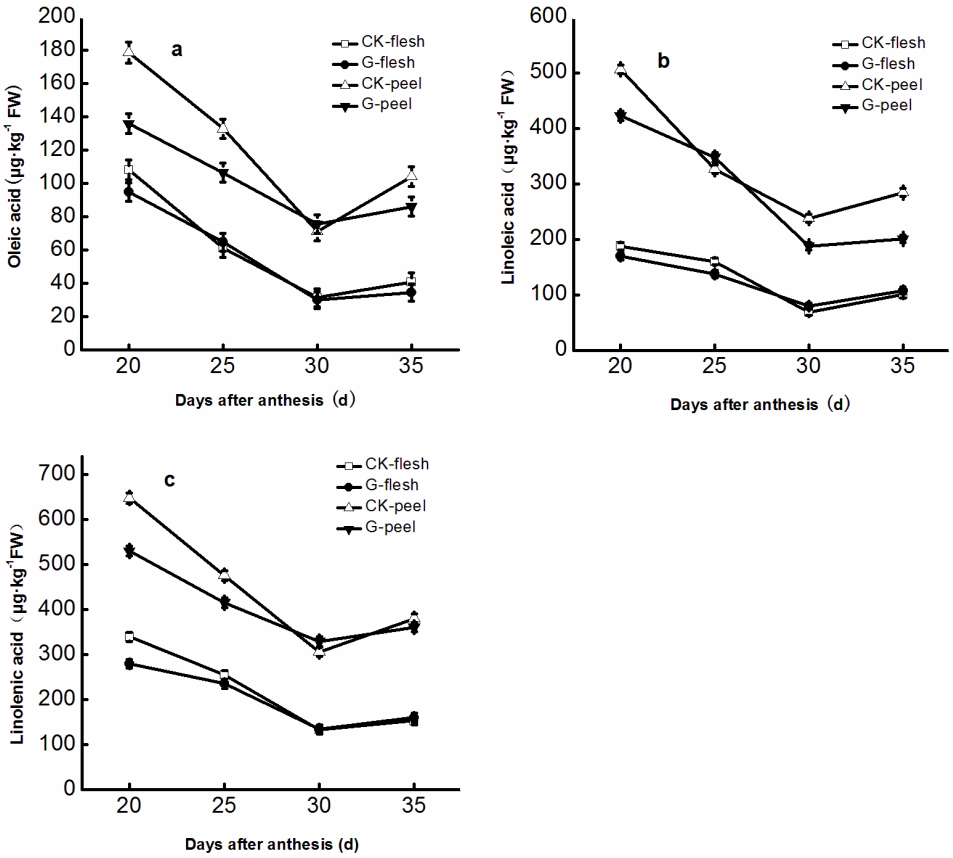

Figure 1. Effects of grafting on the content of three free fatty acids in the peel and flesh tissues of oriental sweet melon during fruit development(a, Content of oleic acid; b, Content of linoleic acid; c, Content of linolenic acid; bars, SE).

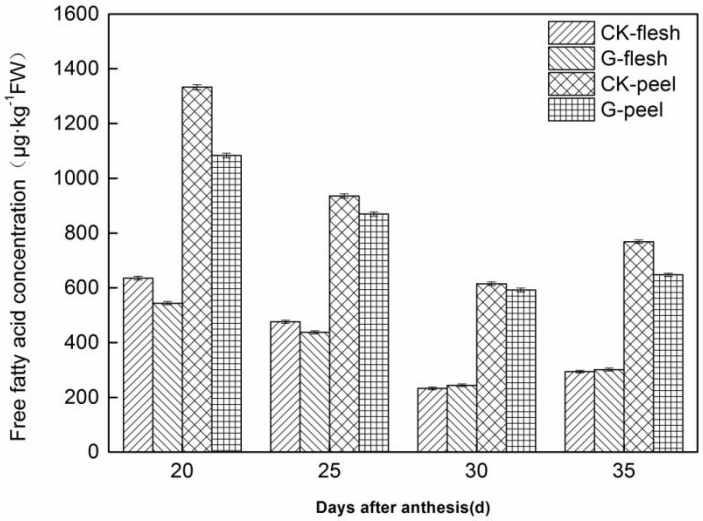

Figure 2. Effect of grafting on the total content of free fatty acid of oriental sweet melon during fruit development (bars, SE).

3.2. The effect of grafting on the total content of the three free fatty acids (TFFA) at different stages of development

It is now believed that oleic acid, linoleic acid, and linolenic acid are precursors in the synthesis of straight-chain fatty alcohols, fatty aldehydes, and fatty esters; the synthesis reaction proceeds through LOX pathway [6]. Figure 2 illustrates that changes in the total content of TFFA were essentially similar to those of three free fatty acids. The total content of TFFA reached a maximum on 20th day after blooming. The total content of TFFA decreased gradually with the development of melon fruits. The total content of TFFA was significantly higher in mature fruits. Compared to the flesh of melon fruit, the peel contained a significantly higher content of TFFA. Compared with own-rooted melons, the peel of grafted melons contained considerably lower content of TFFA at all stages of development. On 20th and 25th days after blooming, the flesh of grafted melons had sharply lower levels of free fatty acids than in own-rooted melons. On 30th and 35th day after blooming, no significant differences were observed in the total content of TFFA in the flesh of grafted melons and own-rooted melons. The results indicated that grafting reduced the total content of TFFA in the peel of oriental sweet melons. In the flesh of melons, grafting showed various effects on the total content of TFFA; these effects depended on the different stages of development.

3.3. The effect of grafting on the LOX, ADH and AAT activity levels in the peel of oriental sweet melon

In the peel of grafted and own-rooted melons, LOX activity decreased initially; however, it increased subsequently and decreased again (Figure 3). Interestingly, LOX activity reached a peak on 20th day after blooming. Moreover, LOX activity reached its peak before a large number of volatile esters developed in the peel of oriental sweet melons. The results indicated that LOX activity occurred prior to the production of volatile esters on a large scale. Furthermore, LOX activity gradually decreased with the development of melon fruits. At 25–30 days after blooming, LOX activity increased. After the fruit became fully mature, LOX activity decreased slightly. Grafting reduced LOX activity in the melon peel on 20th and 35th days after blooming; however, no significant changes were observed in the enzymatic activity levels on 25th and 30th days after blooming.

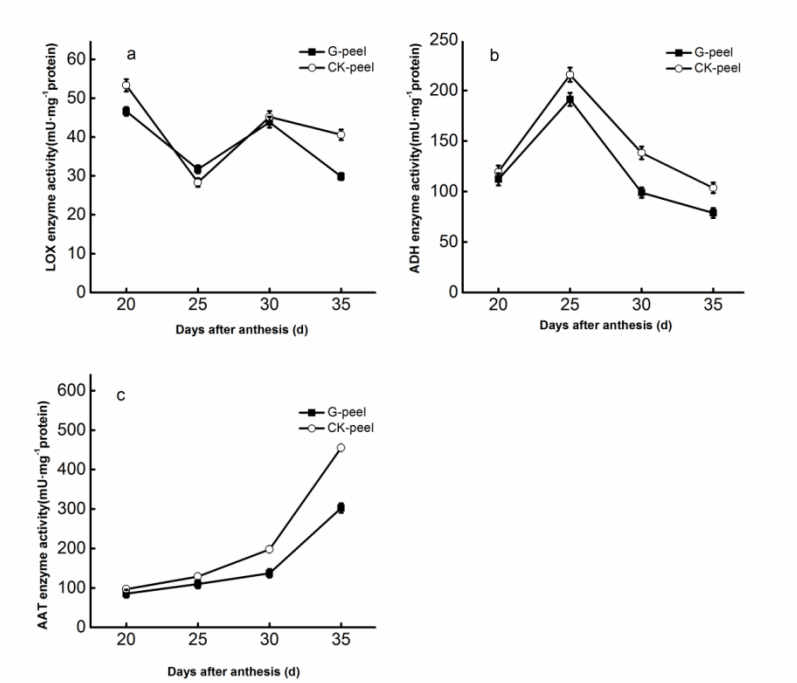

Figure 3. Effects of grafting on LOX, ADH, and AAT activities in peel tissue of oriental sweet melon during fruit development (a, activity of LOX enzyme; b, activity of ADH enzyme; c, activity of AAT enzyme; bars, SE).

As shown in Figure 3, ADH activity first increased and then decreased as the fruit matured gradually. Moreover, ADH activity reached its peak on 25th day after blooming. Consequently, a large number of intermediate products were available for esterification. However, ADH activity gradually declined with the development of melon fruits. Due to grafting, ADH activity decreased in melon peels at 25–35 days after bloom.

In the peel of oriental sweet melons, AAT activity increased steadily with the gradual maturation of fruit. Moreover, ATT activity increased slowly at 20–30 days after bloom; then, it increased rapidly at 30–35 days after bloom. Compared to controls, ATT activity was significantly reduced in grafted melons at 25–35 days after blooming (Figure 3).

3.4. The effect of grafting on the LOX, ADH and AAT activity levels in the flesh of oriental sweet melons

The activity of LOX, ADH, and AAT was lower in the flesh than that in peel of oriental sweet melons. In the flesh of melons, the activity of LOX, ADH and AAT enzymes underwent changes, which were similar to those observed in the peel of melons (Figure 4). Due to grafting, the LOX activity decreased in melon flesh on 20th and 35th days after bloom; however, no significant changes were observed in LOX activity at any other time point. Due to grafting, ADH activity decreased on 20th day after bloom; however, it hardly showed any significant changes at other time points. No significant differences were observed in ATT activity of grafted melons and own-rooted melons.

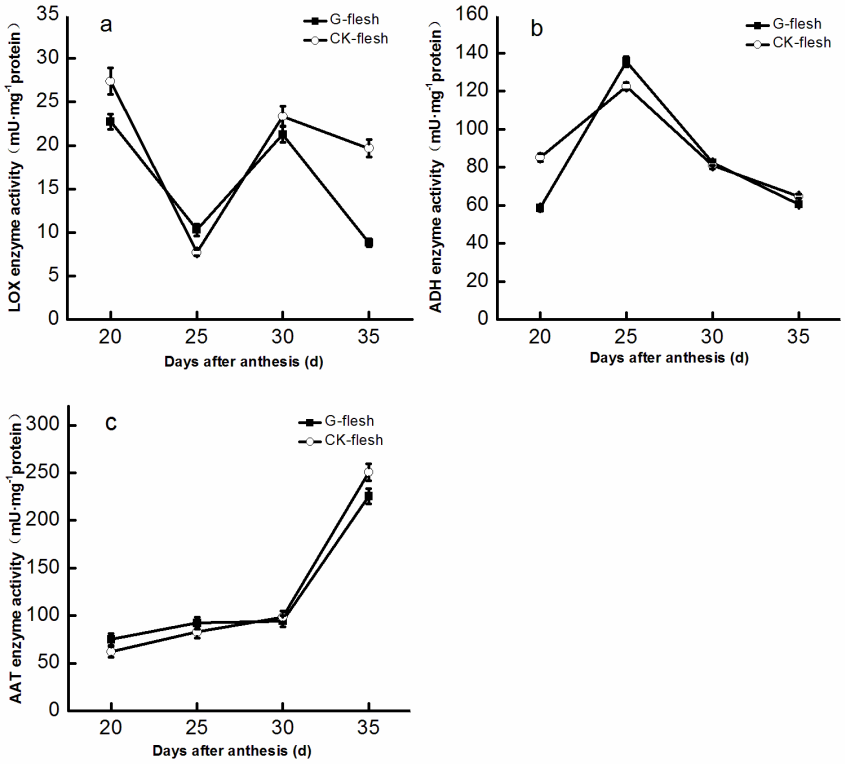

Figure 4. Effects of grafting on activities of LOX, ADH, and AAT enzymes in flesh tissues of oriental sweet melon during fruit development (a, activity of LOX enzyme; b, activity of ADH enzyme; c, activity of AAT enzyme; bars, SE).

4. Discussion

Fatty acids are precursors of most straight chain esters with aromas [5, 6]. Fatty acids are undergoing α-oxidation reaction to produce acetic acid, butyric acid, and hexanoic acid: these carboxylic acids are further reduced to their corresponding alcohols [18]. In the presence of acyl-CoA, alcohols are

esterified by AAT [19-21]. Certain alcohols are used in the synthesis of straight-chain esters; these alcohols are derived from LOX pathway. Due to the enzymatic activity of LOX, fatty acids get converted into aldehydes; these compounds are further reduced to alcohols with the help of ADH [22]. In the synthesis of esters, the following fatty acids act as precursors: oleic acid, linoleic acid, and linolenic acid.

In this study, a large amount of free fatty acids (oleic acid, linoleic acid, and linolenic acid) accumulate in melon fruits; they accumulate before aromatic volatiles are formed in melons. Fruit aromas become stronger as the fruit matures gradually. At the same time, the content of free fatty acids also decreases rapidly. These results comply with the findings of an earlier study on grapes; this study was conducted by Agudelo-Romero P *et al* [23]. They showed that immature apple fruits contain rather low levels of free fatty acids; these fruits produce little aroma as they are immature. As these fruits mature gradually, fatty acid content increases steadily. In particular, the content of unsaturated fatty acids (oleic acid, linoleic acid, and linolenic acid) increases sharply. Consequently, the aroma becomes stronger with the maturation of fruits [24]. α-linoleic acid and linolenic acid were the two essential fatty acids in banana peels; the content of α-linoleic acid and linolenic acid was 20.75% and 19.56%, respectively [25]. Khan et al. [26] analyzed and compared the ratio of linoleic acid to linolenic acid in 10 different varieties of cucumbers. They found a strong positive correlation between the flavor of cucumbers and the ratio of linolenic acid to linoleic acid. In addition, exogenous linoleic and linolenic acids were added as supplements during the cultivation of cucumbers. Consequently, contents of two flavor compounds increased sharply with these supplements. These compounds were characteristic of fresh cucumbers: trans-2, cis-6-nonadienal and trans-2-nonenal. Duan *et al*. [27] investigated the correlation between peach fruit firmness and fatty acid content. They found that the content of linolenic acid increased significantly with fruit ripening, but only oleic acid was positively correlated with fruit firmness. In addition, linoleic acid content increased initially but then decreased during fruit ripening [27].

In this study, the activity of LOX enzyme reached its peak value on 20th day after blooming. Moreover, LOX activity reaches its peak before the formation of several volatile esters in oriental sweet melons. The results indicate that LOX activity occurs prior to the production of volatile esters on a large scale. Moreover, ADH activity reached its peak value on 25th day after bloom. Owing to ADH activity, a large number of intermediate compounds participated in ester synthesis. In addition, ADH activity gradually decreased with the development of fruit melons. In contrast, AAT activity increased steadily with the maturation of fruits. Compared to flesh tissues, the three enzymes showed higher activity in melon peel. Owing to grafting, there was a sharp decrease in the activity of ADH and AAT enzymes in the peel. This adversely affected the conversion of aldehydes and biosynthesis of esters. However, future studies must be conducted to know whether grafting affects the expression of key enzyme genes. The investigation has to be carried out at transcriptional level of genes.

Previous studies have investigated the activity of AAT gene in melon fruits. The expression of AAT genes has been determined at the molecular level in these studies. Therefore, these studies are valuable references, which can be used to determine the expression of key enzyme genes. Yahyaoui *et al*. [28] and Islam *et al*. [29] cloned four genes from cantaloupe melon fruit: CM-AAT1, CM-AAT2, CM-AAT3, and CM-AAT4. All four genes are members of acyltransferase gene family of plants. These genes encode AAT proteins. Except for CM-AAT2, the three genes are expressed in the fruit. They encode proteins that display a strong preference for substrates. It is speculated that ATT activity decreases after grafting due to the expression of these four genes. Islam *et al*. [29] and Manri'quez *et al*. [30] studied the expression patterns of genes that encoded AAT enzymes in melon fruits. They also analyzed functional characteristics of Cm-AAT1, Cm-AAT2, Cm-AAT3, and Cm-AAT4 enzymes, which played a pivotal role in ester synthesis. To restore the activity of enzymes, alanine residue was mutated into threonine residues of AAT. The results indicate that threonine residues are essential for AAT activity, which governs the process of ester biosynthesis. The present study showed that AAT activity decreased in melon peel when grafting was conducted during cultivation.

Previous studies have also determined the expression of ADH gene in melon fruits. Manri'quez *et al.* [30] investigated the effects of CM-ADH1 and CM-ADH2, the two ADH genes cloned from melon fruit, on the aromas of the fruit [30]. In melon fruits, they found that ADH plays an important role in aroma development. This implies that CM-ADH1 and CM-ADH2 are highly specific ADH genes in melon fruits; these genes are expressed specifically in mature melons and their expression is regulated by ethylene. The enzyme aldehyde reductase was strongly preferred by proteins, which were encoded by the two genes. In our current study, ADH activity was strongly reduced in melon peel by including grafting technique during cultivation. However, further studies must be conducted to confirm whether grafting affects the expression of the ADH gene.

In summary, the peel of free fatty acids (oleic acid, linolenic and acid linoleic acid) content and related synthetic enzyme (LOX, ADH and AAT) activities are higher than those in flesh tissue of melons. By grafting method, free fatty acid (oleic acid, linolenic and acid linoleic acid) contents and related activities of synthetic enzymes were reduced in peel tissue of melons. Consequently, the aroma of melon fruits declined due to grafting. To elucidate the mechanism through which grafting brings about these changes, we have to further analyze the expression of genes and proteins during various stages of fruit development. These analyses must include both own-rooted and grafted melons. Moreover, the interaction between proteins and genes must be further elucidated in future studies.

Acknowledgements
This work was financially supported by the Fund of the National Natural Science Foundation of China (31372057), the 2017 Agriculture Education Teaching Reform Project of Beijing University of Agriculture (BUA2017JG030), 2017 Research Fund for Academic Degree & Graduate Education of Beijing University of Agriculture (5056516005/033), and the Innovation Team Construction of Leafy Vegetables of Beijing (blvt-02).

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