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Alcohol Acetyltransferase Activity, Ethylene Production and Aroma Formation of Muskmelon during Fruit Development

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ABSTRACT

Muskmelon (*Cucumis melo* L.) varieties differ in a range of physical and chemical attributes. Aroma is one of the most important factors determining fruit quality and consumer preference. Volatile esters, a major class of compounds contributing to the aroma of muskmelon fruit, are synthesized by alcohol acyl-transferases (AATs). We demonstrate here that volatile aldehydes are most abundant in unripe 'Shannong Golden1' fruit, a climacteric muskmelon but volatile esters are the major components of ripe fruit. In contrast, both ripe and unripe 'Sweet Delight' fruit, a non-climacteric muskmelon, had an abundance of volatile aldehydes. Unripe 'Takami' fruit, another climacteric muskmelon, had an abundance of alcohols and aldehydes whereas ripe fruit of this cultivar had an abundance of esters and aldehydes. Ripe 'Takami' fruit produced ethylene at an intermediate concentration relative to that of 'Shannong Golden1' and 'Sweet Delight'. Interestingly, the levels of AAT of all three cultivars mirrored ethylene concentrations and again were highest in ripe 'Shannong Golden1' fruit and lowest in 'Sweet Delight' fruit. These data suggest that the volatiles differ between fruit types and higher ethylene concentrations result in higher volatile ester concentrations in muskmelon. Furthermore, AAT activity appears to be positively correlated with volatile esters produced during muskmelon ripening and thus may play an important role in aroma formation.

Keywords: AAT, climacteric type, *Cucumis melo*, volatile components

INTRODUCTION

Physicochemical and sensory attributes are important quality parameters of muskmelon varieties. Among these, volatiles are secondary metabolites that play a major role in fruit quality. In previous papers, more than 200 volatile compounds have been reported and over 100 of these compounds have been identified in the volatile fraction of muskmelon (Wyllie and Leach 1990; Wyllie *et al*. 1994; Jordan *et al.* 2001; Castro-Vazquez *et al*. 2006; Tang *et al.* 2007). Volatile C6 and C9 aldehydes, alcohols, and especially large quantities of esters present in their headspace are likely to be the key contributors to the unique aromas of muskmelon (Flores *et al*. 2002; Senesi *et al.* 2002; Aubert and Bourger 2004; Flores *et al.* 2005).

Ethylene production plays an especially important role in fruit ripening (Theologis 1992; Flores *et al*. 2002; Defilippi *et al.* 2005). The climacteric mode of ripening is characterized by a significant increase in the levels of respiration and release of ethylene, triggering profound and rapid changes in a range of attributes, such as flavor (sweetness and aroma), as well as in other physical parameters such as firmness of the fruit and a typical slip area produced around the peduncle in climacteric muskmelons. Some of the climacteric varieties of muskmelon are considered to be highly aromatic, whereas the non-climacteric varieties are normally considered to be less aromatic.

Many of the volatile aldehydes present in the headspace are formed by degradation of fatty acids (Croteau and Karp 1991), whereas the respective alcohols are derived from aldehydes by the action of alcohol dehydrogenases or as a result of degradation of amino acids (Treesl and Drawet 1973). Volatile esters, important contributors to the aroma of muskmelon fruit, are formed by esterification of alcohols and carboxylic acids, normally utilizing a CoA ester as previously noted in many muskmelon varieties by incubating fruit slices with isobutyl alcohol. The non-aromatic types had low esterification potential, and other cucurbits such as cucumber (*Cucumis sativus* L.) and watermelon [*Citrullus lanatus* (Thunb.) Matsum and Nakai] lacked the ability to esterify isobutyl alcohol (Ueda *et al.* 1997). The mechanism of ester formation has been studied in microorganisms, where a group of enzymes termed AAT have been identified (Yamakawa *et al*. 1978; Yoshioka and Hashimoto 1981). AAT catalyzes the transfer of an acetyl moiety from acetyl-CoA into the corresponding alcohol, forming an ester and free CoA. Alcohol acyl-transferases have been studied in some detail in ripe fruit, including apple (*Malus* × *domestica*) (Echeverría *et al*. 2004), banana (*Musa acuminata* L.) (Harada *et al*. 1985), muskmelon (Shalit *et al*. 2001; El-Sharkawy *et al*. 2005), and especially strawberry (*Fragaria xananassa* Duch.) (Pérez *et al*. 1996; Beekwilder *et al*. 2004) where the enzyme had been purified and characterized, and the gene had been cloned. Experiments performed with banana and strawberry fruit indicate a correlation between substrate specificity and volatile esters present in each fruit's aroma, suggesting a significant role of AAT enzyme in flavor biogenesis in these species (Pérez *et al*. 1993; Dixon and Hewett 2000). In preliminary experiments performed in muskmelons, the AAT enzyme activities have been described on broad substrate specificity toward the alcohol acceptor substrate in muskmelon fruit. However, despite the importance of AAT as a key enzyme in aroma synthesis in fruit many aspects such as the mechanism of action and physiological relevance, remain unclear (Shalit *et al*. 2001; Yahyaoui *et al*. 2002).

In this study, we monitored AAT activities during muskmelon fruit development and described a correlation between AAT activity and formation of esters in muskmelon cultivars. In addition, we measured the potential of each cultivar to produce esters from their respective alcohols to attempt to bio-chemically rationalize the differences in the volatile profiles of these cultivars.

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MATERIALS AND METHODS

Plant material and tissue sampling

Muskmelons ['Shannong Golden1', 'Sweet Delight' and 'Takami' (seeds provided by the College of Horticultural Science and Engineering, Shandong Agricultural University, Tai'an, China)] were grown in a greenhouse on an experimental farm at Shandong Agricultural University in Tai'an, China from Mar. through June 2007, with a spacing of 50 cm between plants, and 120 cm between rows. The average day/night temperatures were about 30°C/20°C. The average daylight was about 13 h. Fertilizer was applied at two stages, a preplant broadcast application of 900 kg/ha of 14N-6.1P-29.9K, followed by a side-dress application of 150 kg/ha N at the flowering stage. Irrigation by furrows was applied as needed. Freshly opened female flowers were tagged on the day of hand-pollination to identify fruit of known age and one fruit per plant was allowed to develop. Different developing stages [15, 30, and 45 d after pollination (DAP)] and mature fruit were harvested. Fifty-five DAP was considered commercial maturity for these three cultivars. Ethylene concentrations, AAT activity assay and volatile compound concentrations were determined for each sample. The experiments were repeated three times. For each replicate, five fruit were used.

Extraction of volatile compounds

Solid Phase Microextraction (SPME) was used to extract the volatile components of muskmelon. Fresh fruit were peeled, sectioned radially and the seeds were removed. The mesocarp was then homogenized in a food processor (Hymix Co., Ltd, Shanghai, China), juiced in a Juicer (Hymix Co., Ltd, Shanghai, China) and 8 ml aliquots were decanted into 15 ml vials with 2 g NaCl, which were then sealed. Headspace sampling was conducted immediately by inserting a 75 μm fused silica fiber syringe (Supelco Corp., St. Louis, MO) coated with polydimethylsiloxane (PDMS) inserted into tightly closed 15 ml vials containing the muskmelon homogenates. After gentle stirring for 40 min at 40°C, the SPME syringe was introduced into the injector port of the gas chromatography-mass spectrometry (GC-MS) for further analysis.

Gas Chromatography-Mass Spectrometry analysis

Volatile compounds were analyzed by GC-MS (Finnigan Corp., San Jose, Calif.) with a PEG-20 M, column (30 m × 0.25 mm, 0.25 μm film thickness) for 40 min runs. The injection port was operated in split-less mode and set at a constant flow of 0.8 $ml \cdot min^{-1}$ for the sample of ultrahigh-purity Helium (>99.99%). The initial oven temperature was held at 40°C for 4 min and then increased by $5°C \cdot min^{-1}$ for 10 min to 90°C followed by an increase of $8°C \cdot min^{-1}$ for 17.5 min to 230°C where it was held for an additional 8 min. The injection port and ionizing source were kept at 250°C and 200°C, respectively. The split ratio was 10:1 and 10 μl of sample was injected. The mass spectrometer was operated in the electron ionization mode at 70 eV, emission current 200 μA, detector voltage 350 V. The mass range was recorded from 29~600 atm. Identification of the components was done by comparison of mass spectra and retention time data with those of authentic standards from the NIST (v.1.5) and WILEY (v.7NIST98) libraries. The experiment was repeated three times and five fruit were used for each replication.

Ethylene measurement

Fresh fruit were placed in a 8 L jar, which was sealed with a rubber serum cap. Headspace gas was allowed to accumulate for 2 to 3 h at 25°C; Headspace samples (1 ml) were withdrawn and analyzed using a GC-9A Gas Chromatogram (Shimadzu Corp, Kyoto, Japan), equipped with a Flame Ionization Detector. The experiment was repeated three times and five fruit were used for each replication.

Analysis of AAT activity

Alcohol acyl-transferase (AAT) was extracted and assayed according to the method described by Pérez *et al.* (1996) with slight modifications. Mesocarp tissue was ground into the powder in a mortar, and 1 g of powdered tissue was homogenized in 0.75 ml of extraction solution containing 0.5 M Tris-HCl, pH 8.0, 0.1% (v/v) Triton X-100 (Sigma-Aldrich Corp., St. Louis, MO), 3 $mg \cdot g^{-1}$ PVPP, set on ice for 20 min at 4°C. The homogenate was then centrifuged at 12,000 × *g* for 20 min at 4°C.

AAT activity was assayed by mixing 2.5 ml $MgCl_2$ solution (5 mM $MgCl_2$ in 0.5 M Tris-HCl, pH 8.0), 150 μl of acetyl-CoA solution (5 mM acetyl-CoA in 0.5 MTris-HCl, pH 8.0), 50 μl butanol solution (200 mM butanol in 0.5 M Tris-HCl, PH 8.0), 150 μl enzyme extract. The mixture was incubated at 35°C for 15 min, and then 100 μl of 10 mM 5, 5'-dithiobis (nitrobenzoic acid) (DTNB) was added and allowed to stand at room temperature for 10 min. AAT activity was measured spectrophotometrically as increased absorbance at 412 nm over time, due to the formation of a yellow thiophenol product from DTNB and the free CoA liberated during the catalytic reaction. One activity unit (U) was defined as the increase in one unit of absorbance at 412 nm per minute, and results were expressed as specific activity ($mU \cdot mg^{-1}$ protein). The experiment was repeated three times and five fruit were used for each replication.

Total protein in the enzyme extract was determined according to the method described by Bradford (1976) using bovine serum albumen as a standard.

RESULTS

Compositions of the volatile during muskmelon fruit ripening

The SPME GC-MS analysis method was used to identify the volatiles present in developing and mature fruit in the three cultivars. A total of 156 volatiles were separated and the main compounds were shown in **Table 1**. In unripe 'Shannong Golden1' fruit, (30 and 45 DAP), contained predominately aldehydes (71.65% and 74.9% of the total volatiles, respectively), some alcohols (15.06 and 11.38%, respectively), and low levels of lactones (4.83% and 3.14%, respectively), but in 'Shannong Golden1' ripe fruit, 83.89% of the total volatiles were comprised of volatile esters, mostly acetates (80.5%), low levels of alcohols (3.4%), aldehydes (1.18%) and lactones (0.05%).

In unripe fruit (30 DAP) of 'Sweet delight', 43.28% of the volatiles were short- and medium-chain alcohols, 44.53% were C6 and C9 aldehydes and 1.06% were lactones. More than 30% volatiles were alcohols and more than 50% were aldehydes in 45 DAP and ripe fruit, and many c9 aldehydes and alcohols decreased, many c6 aldehydes increased with fruit ripening. Only a very low level of esters (such as acetic acid phenylmethyl ester) was separated until fruit ripening.

In unripe fruit (30 DAP) of 'Takami', 28.2% and 56% volatiles were alcohols and aldehydes, and 2.54% were esters, respectively. The levels of alcohols and aldehydes decreased obviously, and esters increased significantly in fruit of 45 DAP. Esters and aldehydes were the major volatile compounds in fruit ripening.

Ethylene production

Ethylene production was very low ($0.73 \mu l/Kg^{-1} \cdot hr^{-1}$ FW) in 30 DAP fruit of 'Shannong Golden1', and then increased notably with fruit ripening. However, ethylene production in 'Sweet Delight' fruit was still very low in developing and mature fruit. And ethylene production in 'Takami' fruit kept intermediate between the above two cultivars, such as the ripe 'Takami' fruit produced ethylene at intermediate concentrations (~2.70 μL/kg/hrFW) to that of 'Shannong Golden1' (~4.75 μL/kg/hrFW) and 'Sweet Delight' (~0.81 μL/kg/hrFW) (**Fig. 1**).

AAT activity during muskmelon fruit maturation

AAT activities was low in 15 and 30 DAP fruit of 'Shannong Golden1', but then increased significantly in 45 DAP

Table 1 Main aroma compounds in muskmelon fruit identified by Gas Chromatography-Mass Spectrometry.

Volatile[a]	Volatile relative content (%)								
	'Shannong Golden1'[b]			'Sweet Delight'			'Takami'		
	30 DAP	45 DAP	55 DAP	30 DAP	45 DAP	55 DAP	30 DAP	45 DAP	55 DAP
Aldehydes									
Pentanal	1.63	2.98	Nd	0.37	1.83	2.12	2.18	0.17	1.48
Hexanal	29.32	19.50	Nd	7.69	18.08	22.26	26.17	24.07	10.76
(*E*)-2-Pentenal	1.51	1.10	Nd	0.75	0.94	1.20	Nd	Nd	Nd
Heptanal	0.60	1.85	Nd	0.20	1.40	1.43	0.80	2.04	1.41
2-Hexenal	1.89	22.45	Nd	1.02	1.42	2.29	0.91	1.48	0.88
Octanal	0.93	1.44	Nd	0.38	0.90	0.42	1.34	0.68	0.68
(*Z*)-2-Heptenal	6.06	3.07	Nd	1.31	1.38	1.84	3.68	1.16	0.95
Nonanal	3.71	4.14	0.14	3.98	1.79	1.05	2.85	Nd	1.26
(*E*)-2-Octenal	4.31	2.54	Nd	0.71	0.21	0.43	2.91	0.49	0.28
(*E*)-6-Nonenal	Nd	Nd	Nd	6.52	Nd	2.94	Nd	Nd	Nd
(*Z*)-6-Nonenal	8.60	7.80	0.17	1.47	3.88	Nd	Nd	1.70	1.11
(*E*,*E*)-2,4-Heptadienal	2.10	0.60	Nd	1.78	0.96	1.36	0.73	0.28	Nd
Decanal	Nd	1.37	Nd	Nd	Nd	Nd	1.67	0.47	0.41
Benzaldehyde	0.60	0.56	0.81	0.36	0.80	1.07	0.50	1.95	2.42
(*E*)-2-Nonenal	3.47	1.87	Nd	6.87	0.42	0.68	4.32	0.47	0.32
(*E*,*Z*)-2,6-Nonadienal	4.19	2.39	0.06	11.12	0.61	1.60	5.41	0.41	0.33
Alcohols									
1-Butanol	0.64	Nd	Nd	Nd	Nd	Nd	6.29	Nd	Nd
1-Pentanol	1.82	0.99	Nd	0.75	2.09	3.22	1.69	2.85	1.57
Nonanol	1.87	1.15	Nd	6.99	3.37	1.63	1.27	0.44	0.42
(*Z*)-3-Nonen-1-ol	0.85	0.98	0.24	2.41	1.47	1.21	2.90	1.15	1.13
(*E*)-6-Nonen-1-ol	Nd	Nd	Nd	Nd	0.14	2.59	0.78	0.22	0.18
(*E*)-2-Nonen-1-ol	0.50	Nd	Nd	7.90	Nd	Nd	3.86	Nd	Nd
(*6Z*)-Nonen-1-ol	2.27	0.77	0.29	9.22	5.16	Nd	Nd	Nd	Nd
(*E*,*Z*)-3,6-Nonadien-1-ol	0.97	1.21	0.54	3.40	1.64	1.42	3.08	0.91	0.95
(*E*,*E*)-2,6-Nonadien-1-ol	Nd	Nd	Nd	10.24	Nd	Nd	Nd	Nd	Nd
Esters									
Acetic acid, methyl ester	Nd	Nd	9.08	Nd	Nd	Nd	Nd	Nd	Nd
Ethyl acetate	Nd	Nd	2.08	Nd	Nd	Nd	Nd	Nd	Nd
Acetic acid, 2-methylpropyl ester	Nd	Nd	1.38	Nd	0.75	0.07	Nd	6.00	2.91
Acetic acid, butyl ester	Nd	Nd	3.3	Nd	Nd	Nd	Nd	0.18	Nd
Ethanethioic acid, S-methyl ester	Nd	Nd	5.11	Nd	Nd	1.09	Nd	2.50	1.38
3-Methyl-1-butanol, acetate	Nd	Nd	5.50	Nd	1.11	0.17	Nd	8.67	2.66
Pentanethioic acid, S-ethyl ester	Nd	Nd	0.16	Nd	Nd	Nd	Nd	Nd	Nd
Acetic acid, hexyl ester	Nd	Nd	4.55	Nd	0.19	0.07	Nd	1.15	0.72
Methyl,2-(methylthio)acetate	Nd	Nd	0.55	Nd	Nd	Nd	Nd	Nd	Nd
2,4-Diacetoxypentane	Nd	Nd	6.41	Nd	Nd	0.09	Nd	0.30	0.27
Acetic acid, phenylmethyl ester	Nd	1.01	31.05	Nd	1.36	5.59	Nd	14.69	19.49
Acetic acid, 2-phenylethyl ester	Nd	3	3.69	Nd	0.23	0.27	Nd	1.29	3.34

[a] Volatile compounds from fresh melons were sampled using SPME and analyzed by GC-MS as described in the Materials and Methods. Identification was confirmed by comparison of mass spectra and retention times with those of authentic compounds analyzed under similar conditions, as indicated. [b] Different development stages. 'Nd' means not detected.

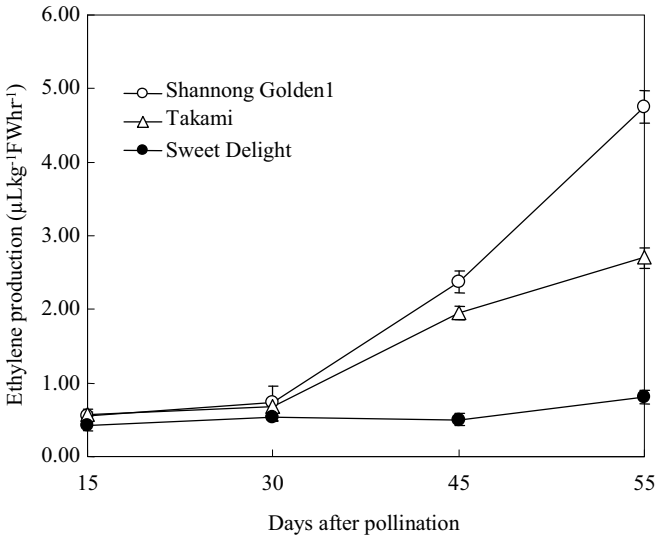

Fig. 1 Ethylene production by three muskmelon cultivars during fruit development. Fifteen, 30, 45 and 55 d after pollination (DAP) fruit were harvested, respectively. Fifty-five DAP is considered commercial maturity. The experiment was repeated three times and five fruit were used for each replicate. Bars indicate SE.

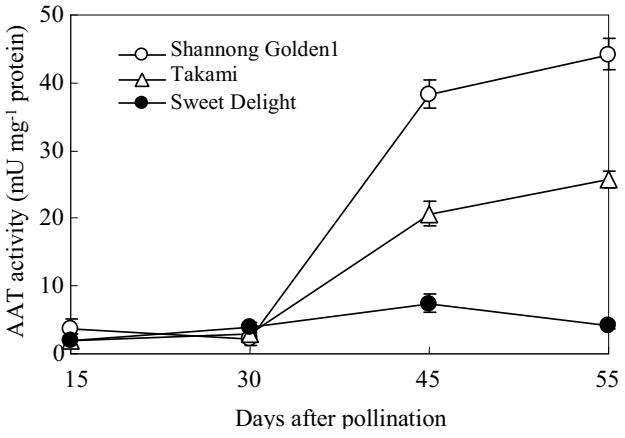

Fig. 2 Alcohol acyl-transferase activity in three muskmelon cultivars during fruit development. Fifteen, 30, 45 and 55 d after pollination (DAP) fruit were harvested, respectively. Fifty-five DAP is considered commercial maturity. The experiment was repeated three times and five fruit were used for each replicate. Bars indicate SE.

fruit and reached its highest level in mature fruit. In contrast, very low AAT activities still appeared in 'Sweet Delight' muskmelon during the different developing and mature fruit phases. AAT activities were low in 15 and 30 DAP fruit of 'Takami', and then increased gradually with fruit maturation. AAT activities of 'Takami' fruit kept increasing during fruit development, but their level was lower than that in 'Shannong Golden1' fruit and higher than that in 'Sweet Delight' fruit (**Fig. 2**).

DISCUSSION

Ethylene production and the formation of volatiles of muskmelon during fruit development

The plant hormone ethylene plays an important role in fruit ripening. Ripening is genetically regulated and includes both molecular and biochemical changes such as color changes, softening, development of characteristic aroma and flavor (Barry and Giovannoni 2007). In our work, we observed that the level of ethylene production increased significantly with fruit ripening in 'Shannong Golden1'. In contrast, it was very low and keeping almost constant during 'Sweet delight' fruit development. In addition, ethylene production was intermediate in 'Takami' with fruit ripening when compared to the other two cultivars (**Fig. 1**). The data suggests that 'Shannong Golden1' could be a climacteric type muskmelon, 'Sweet Delight' could be a non-climacteric type muskmelon, and 'Takami' could be a gradually climacteric type muskmelon.

Volatiles are considered to be one of the most important components to determine the quality level and consumer preferences of fruit (Lester 2006). In this study, we detected that the major volatile components were C6 and C9 aldehydes, alcohols and low levels of lactones (>3%) in the three varieties immature fruit (**Table 1**). These findings were in agreement with the results in immature muskmelon fruit of the previous reports (Beaulieu and Grimm 2001; Shalit *et al*. 2001; Lamikanra and Richard 2002; Aubert and Pitrat 2006). Mature fruit of 'Shannong Golden1' had higher levels of esters than those of 'Sweet Delight'. The latter contained mainly aldehydes and alcohols when fruit ripened. But esters and aldehydes were the major volatile compounds when 'Takami' fruit ripened (**Table 1**), a gradually climacteric type melon. The results showed that the kinds of volatiles differed in different climacteric types when fruit ripened: esters were major in climacteric type muskmelon, while aldehydes and alcohols were most abundant in non-climacteric muskmelon. Esters and some C9 aldehydes, alcohols [(*Z*)-6-nonenal, 3-nonenal, and 3, 6-nonadienal] recovered in the Cucurbitaceae family have been reported to be characteristic flavor/aroma compounds (Kemp *et al.* 1972). Cucumber flavor has been attributed mainly to aldehydes and to a lesser extent to certain corresponding alcohols. The pleasant odor was attributed to (*E*, *Z*)-2, 6-nonadienal, and two unsaturated aldehydes [(*E*)-2-hexenal and (*E*)-2-nonenal] and three saturated aldehydes (acetaldehyde, propanal, and hexanal) were considered to contribute secondarily to overall flavor (Fross *et al.* 1962). However, Fleming *et al*. (1968) demonstrated that some of the characteristic flavor compounds in cucumber fruit such as (*E*, *Z*)-2, 6-nonadienal, (*E*)-2-nonenal, (*E*)-2-hexenal, and acetaldehyde were generated enzymatically as a consequence of cutting or mechanical rupturing. Only propanal and hexanal attributed to cucumber flavor are included among those compounds. The level of hexanal was high in 'Sweet Delight' ripe fruit (22.26%) and this may be the reason why the aroma of 'Sweet Delight', a non-climacteric type muskmelon, is absent when fruit ripens.

AAT activity and the formation of volatiles in muskmelon during fruit development

AAT activities acting on benzyl and phenylethyl alcohol were higher in ripening 'Arava' muskmelon fruit, a climacteric type muskmelon, but absent in 'Rochet', a non-climacteric type muskmelon (Shalit *et al*. 2001). We also detected higher AAT enzyme activity in 'Shannong Golden1' ripe fruit (**Fig. 2**). This suggested that alcohols were rapidly derived from the respective aldehydes by the action of alcohol dehydrogenases or as a result of the degradation of amino acids, and then esterified by the action of AAT enzymes. Furthermore, AAT activities significantly increased after 30 DAP in fruit, but esters significantly formed until fruit ripened in 'Shannong Golden1'. The results implied that AAT forms and accumulates at an early stage of fruit development and that esters esterified very rapidly depending on the action of AAT activity. In contrast to 'Shannong Golden1', 'Sweet Delight', a non-climacteric type muskmelon, had lower AAT activities but higher alcohol concentrations during fruit maturation, and only some esters were esterified (**Fig. 2, Table 1**). The results suggest that AAT activity may be involved in the biogenesis of muskmelon fruit aroma.

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