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# Molecular cloning and expression of a gene encoding alcohol acyltransferase (*MdAAT2*) from apple (cv. Golden Delicious)

Dapeng Li [a,c], Yunfeng Xu [b], Gangming Xu [b], Lingkun Gu [b], Dequan Li [b,*], Huairui Shu [c]

[a] *College of Food Sciences, Shandong Agricultural University, Tai'an, Shandong 271018, PR China*
[b] *College of Life Sciences, Shandong Agricultural University, Tai'an, Shandong 271018, PR China*
[c] *College of Horticulture Sciences, Shandong Agricultural University, Tai'an, Shandong 271018, PR China*

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**Abstract**

Volatile esters are major aroma components of apple, and an alcohol acyltransferase (AAT) catalyzes the final step in ester biosynthesis. The gene *MdAAT2*, which encodes a predicted 51.2 kDa protein containing features of other acyl transferases, was isolated from *Malus domestica* Borkh. (cv. Golden Delicious). In contrast to other apple varieties, the *MdAAT2* gene of Golden Delicious is exclusively expressed in the fruit. The MdAAT2 protein is about 47.9 kDa and mainly localized in the fruit peel, as indicated by immunoblot and immunolocalization analysis. Northern blot and immunoblot analysis showed that the transcription and translation of *MdAAT2* have a positive correlation with apple AAT enzyme activity and ester production, except in the later ripening stage, suggesting that MdAAT2 is involved in the regulation of ester biosysthesis and that a post-translation modification may be involved in regulation of AAT enzyme activity. Tissue disk assays of fruit peel revealed that using extraneous alcohols can recover the corresponding ester formation. Transcription and translation of *MdAAT2* were both depressed by 1-methylcyclopropene (1-MCP) treatment and subsequent ester production was also prevented. These results suggest that: (1) ester production is mainly regulated by MdAAT2; (2) ethylene is also involved in this regulatory progress and (3) ester compounds rely principally on the availability of substrates.
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*Keywords:* *Malus domestica* Borkh.; Rosaceae; Apple; Gene expression; Development; Ripening; Alcohol acyltransferase; MdAAT2; Esters

## 1. Introduction

Aromatic volatile compounds are among the factors that determine fruit quality and influence final consumer acceptance of the product (Baldwin, 2002; Shalit et al., 2003). Volatile esters, derived from amino and fatty acids, are important aromatic components in many ripened fruits (Aharoni et al., 2000; Dixon and Hewett, 2000; Beekwilder et al., 2004). Alcohol acyltransferase (AAT), a member of the BAHD superfamily (St. Pierre and De Luca, 2000), is a key enzyme in ester biosynthesis, catalyzing the final step in ester formation by linkage of an acyl moiety from acyl-CoA to the appropriate alcohol (Fig. 1).

In previous studies, the activity of AAT enzymes was investigated from various fruit species, including banana, strawberry, apple and melon (Wyllie and Fellman, 2000; Shalit et al., 2003; Echeverría et al., 2004; Olı'as et al., 2002) . In each of these studies, AAT activity was shown to be ripening-induced. The gaseous plant hormone, ethylene, plays an important role in the regulation of fruit ripening and senescence (Lelièvre et al., 1997; Jiang and Fu, 2000; Alexander and Grierson, 2002; Chang and Bleecker, 2004) and is also involved in regulation of ester biosynthesis (Yahyaoui et al., 2002; Defilippi et al., 2005). However, little is known about the molecular mechanism of this regulatory process.

In recent years, several full-length cDNAs encoding enzymes with AAT activity have been isolated and characterized from strawberry, melon, banana and apple (Aharoni et al., 2000; Yahyaoui et al., 2002; Beekwilder et al., 2004;

* Corresponding author. Tel.: +86 0538 8241937; fax: +86 0538 8249608.
*E-mail address:* dqli@sdau.edu.cn (D. Li).

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Fig. 1. Formation of volatile esters by AAT enzyme. AAT enzyme catalyzes the formation of esters using acyl-CoA and alcohols as substrates.

Souleyre et al., 2005). In these studies, the substrate specificity of AAT enzyme appears to be broad and displays different preferences towards alcohols and acyl-CoAs. Beekwilder et al. (2004) suggested that predicting substrate usage, based on sequence grouping, remains difficult. These cases illustrate that, between plant species, the function and characteristics of AATs are complex and various.

Esters are the most significant contributors to apple aroma (Rowan et al., 1999; Fellman et al., 2003), accounting for up to 80% of total volatiles in the 'Golden Delicious' apple (López et al., 1998), but ester compounds vary between apple varieties (Young et al., 2004). Volatile compounds in apples are primarily synthesized in the fruit peel (Fan et al., 1997; Rudell et al., 2002) and GC–MS analysis indicated that the capacity for volatile ester production was greater in the peel than in the pulp (Defilippi et al., 2005).

In this paper, we report the isolation of the *MdAAT2* gene from apple. Immunolocalization showed that the MdAAT2 protein is mainly localized in peel tissue cells. Transcription and translation of *MdAAT2*, and the relationship between the AAT enzyme and ester production in fruit peel were also investigated.

## 2. Results and discussion

### 2.1. Characterization of the Malus domestica Borkh. MdAAT2 cDNA clone

RNA used for the reverse transcription (RT) reaction was isolated from fruit of the apple cultivar 'Golden Delicious',

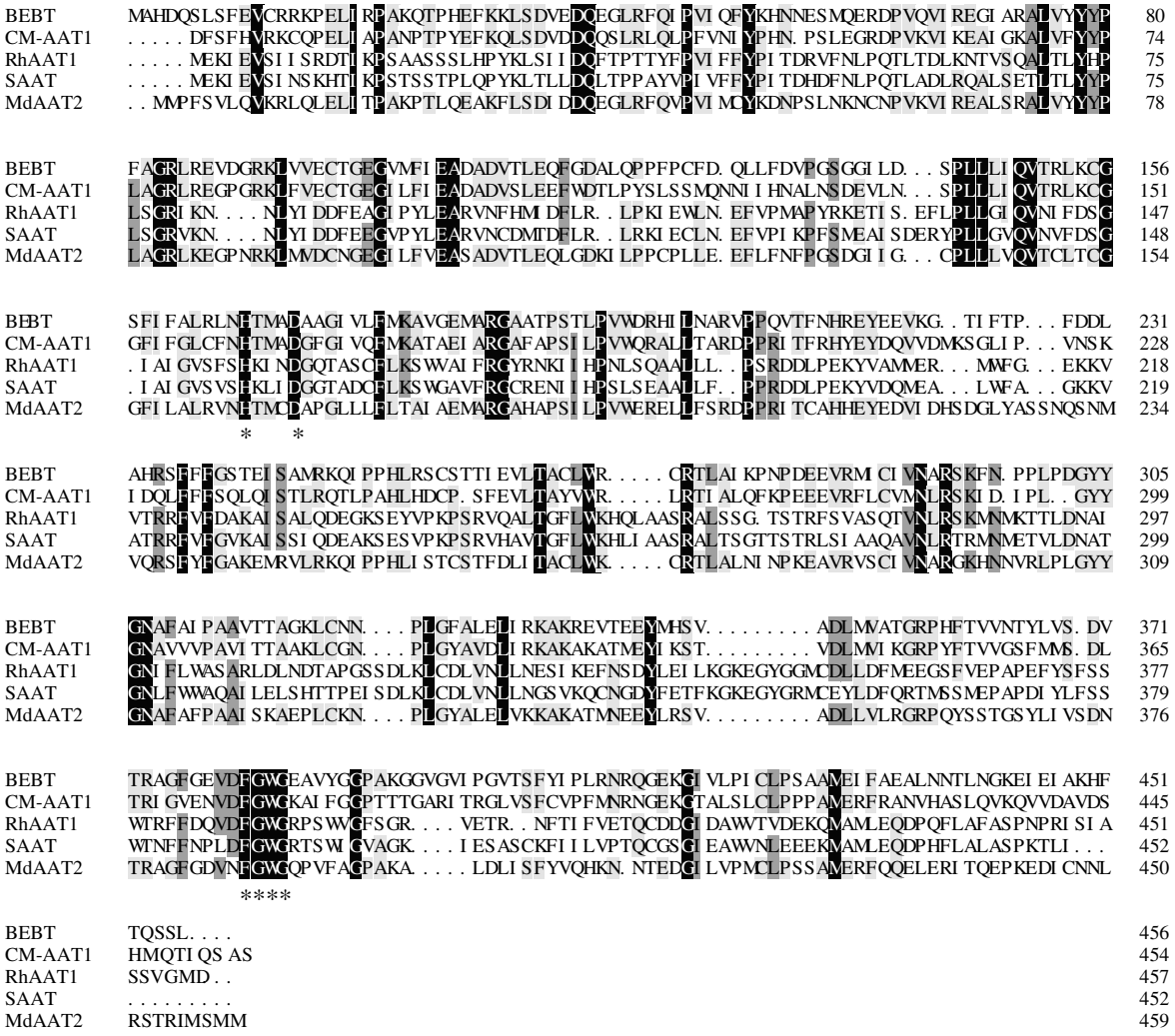



Fig. 2. Amino acid sequence alignment of MdAAT2 with other plant flowers and fruit acyl transferases. BEBT (*Clarkia breweri* benzoyl-CoA: benzyl alcohol benzoyl transferase; **AF500200**); CM-AAT1 (*Cucumis melo* alcohol acyltransferase; **CAA94432**); RhAAT1 (*Rosa* hybrid cultivar alcohol acyl transferase; **BQ106456**); SAAT (strawberry alcohol acyltransferase; **AF193789**). MdAAT2 (apple alcohol acyl transferase; **AY517491**). Asterisks indicate the positions of the conserved amino acids in active site regions of plant acyl transferases.

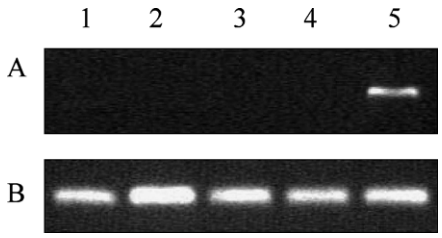



Fig. 3. Expression of the *MdAAT2* gene in apple fruit tissues. RT-PCR using (A) MdAAT1 specific primers amplifying a fragment of 330 bp for 31 cycles. (B) 18S rRNA-specific primers amplifying a fragment of 87 bp for 23 cycles. Lanes: (1) root; (2) stem; (3) leaf; (4) flower; (5) fruit (20 DAH).

which were stored at 20 °C in air for 20 days. On the basis of highly conserved regions, two degenerate primers were designed from a comparison of known plant AAT amino acid sequences (Fig. 2.). Using 5′ and 3′ RACE PCR, a 1628 bp fragment, named MdAAT2 (GenBank Accession No. **AY517491**), was amplified, cloned and sequenced to reveal an open reading frame (ORF) of 1377 nucleotides, a 5′-UTR of 67 nucleotides and a 3′-UTR of 183 nucleotides.

By RT-PCR, transcripts of the *MdAAT2* gene were detected only in apple fruit (Fig. 3). Souleyre et al. (2005) reported that MpAAT1 from Royal Gala was detected not only in apple fruit but also in apple leaves and flowers. We also detected transcripts of the AAT gene in flowers of the apple cultivar 'Red Star' (date not shown). Thus, it was concluded that apple AAT genes are expressed in different organs depending on the apple variety. Interestingly, it is also likely that some other AAT may contribute to volatile ester biosynthesis in Royal Gala apple (Souleyre et al., 2005). These facts indicated that, in apple, there may be several AAT enzymes with different characteristics.

In contrast to MpAAT1 (Souleyre et al., 2005), the predicted MdAAT2 protein has a molecular mass of 51.2 kDa and p*I* of 7.46, which also shares a cluster with other BAHD family members previously characterized in fruit and flowers, and the sequence identities to SAAT, RhAAT, CM-AAT1, and BEBT are 15.9%, 18.9%, 45.8% and 51.8%, respectively. A previously isolated apple AAT cDNA clone (GenBank Accession No. **AX025508**) and the *MpAAT1* showed 96.2% and 93.4% identity, respectively, to the *MdAAT2* cDNA clone described in this paper. The H-Xaa-Xaa-Xaa-D (Xaa indicates a variable identity amino acid) motif, corresponding to residues 164–168 in MdAAT2, is the most conserved consensus sequence and active site motif present in BAHD family members. A second highly conserved motif is FGWG (corresponding to

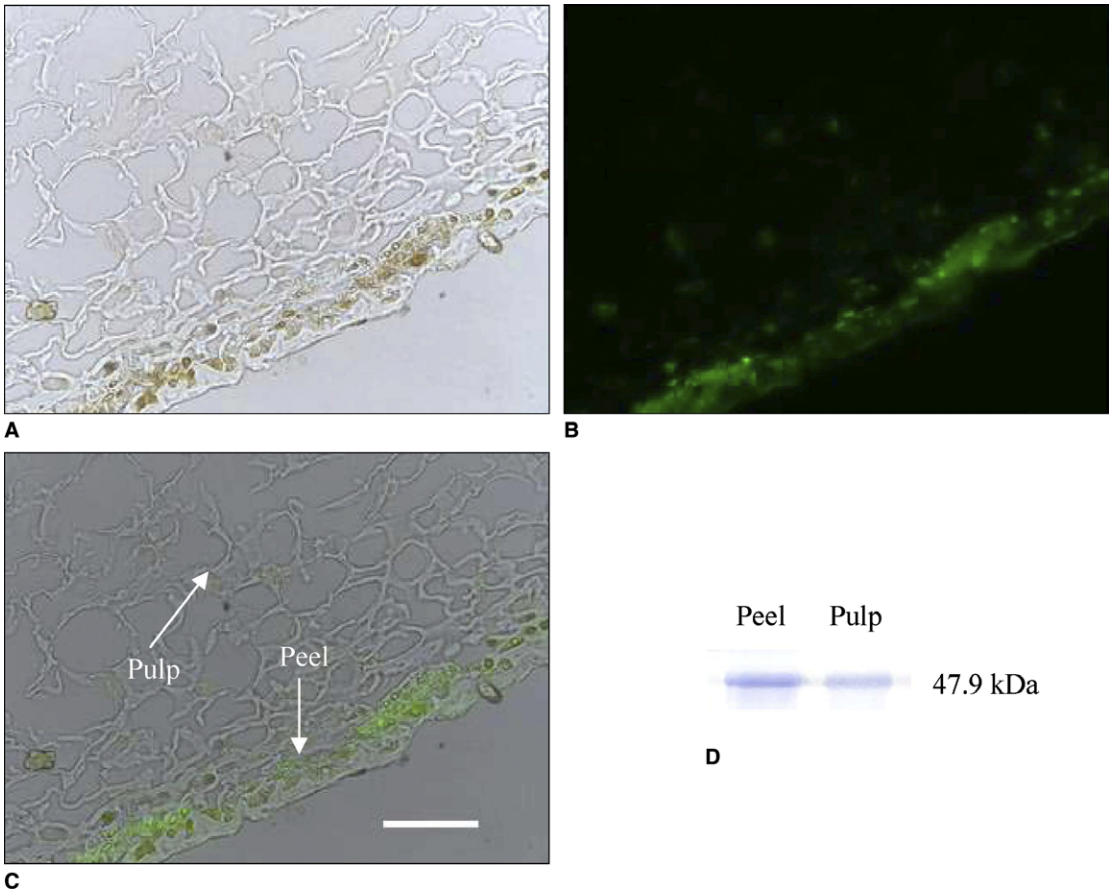
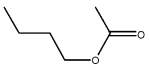
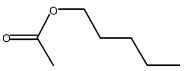
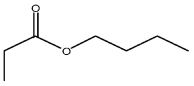
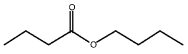
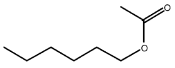
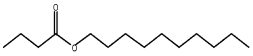
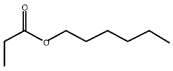
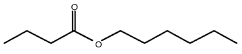
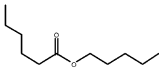
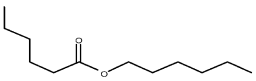
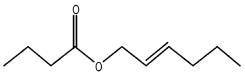
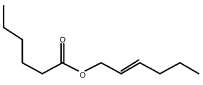
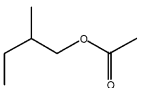


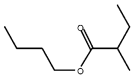
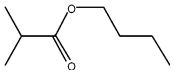
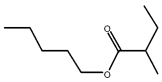
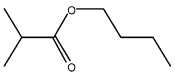
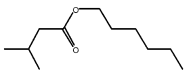

Fig. 4. Immunolocalization and immunoblot analysis of MdAAT2 protein from apple fruit tissues on 20 DAH. (A) Optical microscope observation of apple fruit tissues. (B) Fluorescence emitted by FITC. (C) Merged images of (A) and (B). The scale bar indicates 100 μm. (D) Total proteins from apple fruit peel and pulp were separated by SDS–PAGE, transferred onto PVDF membranes and probed with anti-MdAAT2 antiserum.

Table 1
Ester composition and concentration (nl $L^{-1}$, means of three replicates ± S.D.) of apple fruit peel during development and ripening

| Ester compounds | Development[a] (days after full bloom, DAFB) | | | Postharvest (days after harvest, DAH) | | | Molecular structure |
|---|---|---|---|---|---|---|---|
| | 95 | 115 | 135 | 20 | 25 | 35 | |
| Butyl acetate, **1** | 3 ± 1 | 5 ± 1 | 9 ± 1 | 48 ± 3 | 70 ± 2 | 43 ± 1 |  |
| Pentyl acetate, **2** | 2 ± 0.4 | 2 ± 0.2 | 4 ± 0.4 | 11 ± 1 | 5 ± 0.3 | – |  |
| Butyl propanoate, **3** | –[b] | – | 3 ± 0.2 | 9 ± 0.4 | 11 ± 1 | 4 ± 0.4 |  |
| Butyl butanoate, **4** | 2 ± 0.2 | 5 ± 1 | 10 ± 0.4 | 48 ± 2 | 66 ± 1 | 14 ± 2 |  |
| Hexyl acetate, **5** | 63 ± 1 | 25 ± 1 | 53 ± 2 | 196 ± 3 | 100 ± 3 | 71 ± 2 |  |
| Decyl butanoate, **6** | – | – | 5 ± 0.6 | – | – | – |  |
| Hexyl propanoate, **7** | 5 ± 0.3 | 7 ± 0.4 | 17 ± 1 | 54 ± 1 | 19 ± 1 | ND |  |
| Hexyl butanoate, **8** | ND | 41 ± 1 | 96 ± 3 | 68 ± 3 | 40 ± 1 | 25 ± 2 |  |
| Pentyl hexanoate, **9** | – | – | 9 ± 1 | 55 ± 1 | – | – |  |
| Hexyl hexanoate, **10** | 11 ± 0.4 | 17 ± 1 | 64 ± 2 | 25 ± 0.6 | 47 ± 2 | 17 ± 1 |  |
| *trans*-2-Hexenyl butanoate, **11** | 3 ± 0.2 | – | – | – | – | – |  |
| *trans*-2-Hexenyl hexanoate, **12** | 2 ± 0.4 | – | – | – | – | – |  |
| *Total straight-chain esters* | 91 ± 3.9 | 102 ± 5.6 | 174 ± 11.6 | 513 ± 15 | 358 ± 11.3 | 174 ± 8.4 | |
| 2-Methylbutyl acetate, **13** | 15 ± 1 | 8 ± 2 | 8 ± 1 | 57 ± 3 | 46 ± 1 | 25 ± 1 |  |

(*continued on next page*)

Table 1 (*continued*)

| Ester compounds | Development[a] (days after full bloom, DAFB) | | | Postharvest (days after harvest, DAH) | | | Molecular structure |
|---|---|---|---|---|---|---|---|
| | 95 | 115 | 135 | 20 | 25 | 35 | |
| Butyl 2-methylbutanoate, **14** | 3 ± 0.2 | 7 ± 0.4 | 9 ± 0.1 | 20 ± 1 | 14 ± 0.4 | – |  |
| Butyl isobutanoate, **15** | 2 ± 0.3 | – | 7 ± 1 | – | 2 ± 1 | – |  |
| Pentyl 2-methylbutanoate, **16** | 20 ± 1 | 3 ± 0.3 | 3 ± 0.5 | 5 ± 1 | 2 ± 0.6 | – |  |
| Hexyl 2-methylbutanoate, **17** | 18 ± 0.3 | 37 ± 2 | 85 ± 2 | 43 ± 1 | 26 ± 0.6 | 12 ± 1 |  |
| Hexyl 3-methylbutanoate, **18** | 2 ± 0.4 | – | – | – | 6 ± 0.5 | – |  |
| *Total branched-chain esters* | 60 ± 3.2 | 55 ± 4.7 | 112 ± 4.6 | 125 ± 6 | 96 ± 4.1 | 37 ± 2 | |
| *Total esters* | 151 ± 7.1 | 157 ± 10.3 | 286 ± 16.2 | 638 ± 21 | 454 ± 15.4 | 211 ± 10.4 | |

[a] No ester composition was detected on 65 DAFB (data not listed).
[b] Not detected.

residues 386–389 in MdAAT2), located near the C terminus, whose function is still unknown (St. Pierre and De Luca, 2000). We also found that expression of MdAAT2, using different prokaryotic expression vectors and *E. coli* strains, tended to produce recombinant protein that precipitated as inactive inclusion bodies (Souleyre et al., 2005) and had little AAT enzyme activity. The enzyme activity that could be detected in a pET32a-MdAAT2 construct expressed in Origami B (DE3) showed that the MdAAT2 enzyme is involved in volatile ester biosynthesis.

### 2.2. Immunolocalization of the MdAAT2 protein in apple fruit tissues

To investigate the localization of apple AAT protein, the *MdAAT2* gene was cloned into pET-30a-c(+) and expressed in BL21(DE3)pLysS strain, and the recombinant MdAAT2 protein was purified by Ni-NTA spin column. Then, polyclonal antisera against recombinant MdAAT2 were prepared and used to determine the abundance and localization of the MdAAT2 protein in fruit by immunoblot and immunofluorescence microscopy, respectively. Apple fruit (20 days after harvest, DAH) tissues were prepared as described by Dixon and Klessig (1995), incubated with the primary antisera and then with FITC-conjugated second antibody (rabbit serum as negative control). Fig. 4C shows that MdAAT2 was mainly observed in the peel tissue cells. Fig. 4D also indicates that the MdAAT2 protein is expressed mainly in the fruit peel, more so than in the pulp, and its molecular weight is about 47.9 kDa, which is lower than the predicted 51.2 kDa according to the protein marker using Quantity One software (Bio-Rad). Defilippi et al. (2005) suggested that the capacity for volatile ester production in apple fruit is greater in the peel than in the pulp. These results indicate that the abundance of AAT protein is significantly correlated with the concentration of esters in different apple fruit tissues.

### 2.3. Apple AAT enzyme activity and volatile ester biosynthesis during fruit development and postharvest ripening

To investigate the relationship between ester biosynthesis and AAT enzyme activity, the ester compounds and concentration in apple fruit peel were identified by GC–MS at different development and postharvest ripening stages. No ester compounds were detected in headspace by GC–MS before 65 days after full bloom (DAFB; Table 1), while AAT enzyme activity was slightly detected in this stage (Fig. 5). Ester concentration increased during the investigated stages but decreased during the later stage of postharvest ripening, which presented a positive correlation with AAT enzyme activity. This indicated that the apple AAT enzyme may be involved in the regulation of ester biosynthesis.

The ester components of apple fruit varied, while butyl acetate **1**, butyl butanoate **4**, hexyl acetate **5**, hexyl hexanoate **10**, 2-methylbutyl acetate **13** and hexyl 2-methylbutanoate **17** could be detected during all investigated stages (see Table 1). In a recent report (Souleyre et al., 2005), a recombinant apple AAT enzyme, whose gene was isolated from *Malus pumila* (cv. Royal Gala) and expressed in *E. coli*, prefers to produce hexyl esters. We also found that the ratio of hexyl esters always tended towards high levels in all investigated stages. This indicated that the AAT enzyme from Golden Delicious might be similar to that from Royal Gala in substrate preference.

To determine whether the various ester compounds depended on precursor substrates, hexanol **20** and pentanol **19** were administered to peel tissue disks of 65 and 115 DAFB, respectively, and *trans*-2-hexenol **22** and *cis*-2-hexenol **21** were fed to peel tissue disks of 115 DAFB (Table 2). Table 2 shows that pentyl acetate **2** and hexyl acetate **5** were detected in the peel tissue disks, while AAT enzyme activity was also detected in the fruit peel of 65 DAFB (Fig. 5); however, no esters were detected in this stage (Table 1.). This indicated that the lack of alcohol precursors resulted in the absence of corresponding esters on 65 DAFB. *cis*-2-Hexenyl hexanoate **26**, *trans*-2-hexenyl acetate **27**, *trans*-2-hexenyl butanoate **11** and *trans*-2-hexenyl hexanoate **12** were detected in peel tissue disks of 115 DAFB, which suggested that the corresponding esters could be produced if the *cis*/*trans*-2-hexenol **21**/**22** and suitable acyl-CoAs were available during these stages. While pentyl, hexyl and *cis*/*trans*-2 hexenyl ester production were recovered with administered alcohols, the variety of recovered esters was still limited; for example, pentyl acetate **2** could be detected on 65 and 115 DAFB but pentyl propanoate **23** and pentyl hexanoate **9** could only be detected on 115 DAFB. These results indicated that the variety or quantity of acyl-CoA also plays an important role in ester biosynthesis.

Table 2
Ester compounds of peel tissue disks recovered by using extraneous alcohols from 65 DAFB and 115 DAFB apple fruit

| Alcohols | Esters | 65 DAFB | 115 DAFB |
|---|---|---|---|
| 1-Pentanol, **19** | Pentyl acetate, **2** | +[a] | + |
| | Pentyl propanoate, **23** | –[b] | + |
| | Pentyl hexanoate, **9** | – | + |
| 1-Hexanol, **20** | Hexyl acetate, **5** | + | + |
| | Hexyl butanoate, **8** | – | + |
| | Hexyl hexanoate, **10** | – | + |
| *cis*-2-Hexenol, **21** | *cis*-2-Hexenyl acetate, **24** | NT[c] | – |
| | *cis*-2-Hexenyl butanoate, **25** | NT | + |
| | *cis*-2-Hexenyl hexanoate, **26** | NT | + |
| *trans*-2-Hexenol, **22** | *trans*-2-Hexenyl acetate, **27** | NT | + |
| | *trans*-2-Hexenyl butanoate, **11** | NT | + |
| | *trans*-2-Hexenyl hexanoate, **12** | NT | + |

[a] Detected.
[b] Not detected.
[c] Not tested.

### *2.4. Expression of MdAAT2 mRNA and protein of apple fruit peel during development and postharvest ripening*

Volatile compounds in apples are primarily synthesized in the fruit peel (Fan et al., 1997; Rudell et al., 2002), and immunolocalization and immunoblot assays of apple AAT have provided new evidence to confirm the above result at the protein level. Thus, investigations on MdAAT2 expression have focused on apple fruit peel in this study. The accumulation of MdAAT2 mRNA dramatically increased during development but slightly decreased during later postharvest ripening; MdAAT2 protein presented a similar expression pattern to its mRNA. Although AAT enzyme activity was consistent with the level of MdAAT2 protein during development and the early ripening stages, after 25 DAH it decreased dramatically while MdAAT2 protein abundance still maintained high levels (Figs. 5 and 6A). These results indicate that post-translational modification may be involved in the regulation of AAT enzyme activity.

Inhibition of ethylene production suppresses the biosynthesis of fruity volatiles in climacteric fruits (Fan and

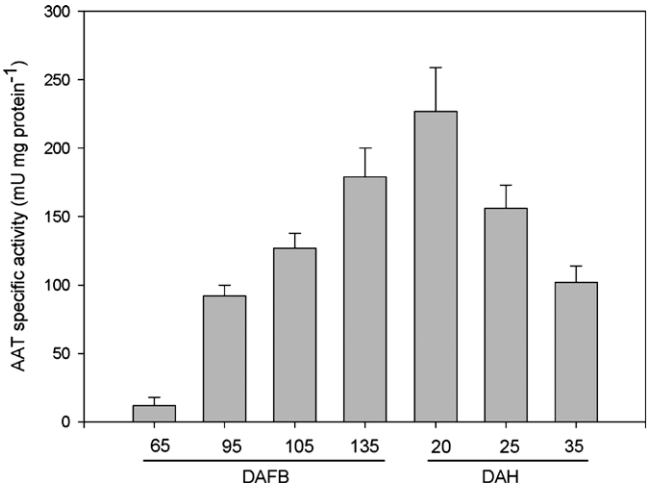



Fig. 5. AAT activities (means of three replicates ± S.D.) of apple fruit peel during development and ripening.

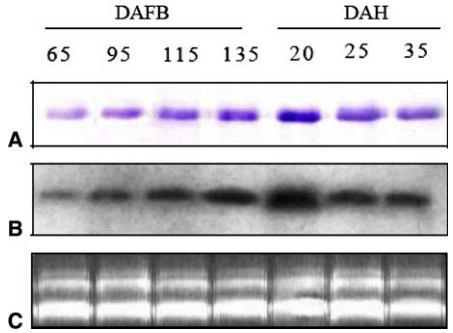



Fig. 6. Expression of *MdAAT2* mRNA and protein in apple fruit peel during development and ripening. (A) The total proteins (20 μg per lane) samples were separated by SDS–PAGE, transferred onto PVDF membranes and probed with anti-MdAAT2 antiserum. (B) The total RNA (20 μg per lane) samples were probed with labeled DNA fragments specific for *MdAAT2* gene. (C) Total RNA.

Mattheis, 1999; Lurie et al., 2002; Botondi et al., 2003; Mattheis et al., 2005). To investigate the effect of ethylene on MdAAT2 transcription and translation, apple fruit were treated with 1-methylcyclopropene (1-MCP) immediately after harvest. Fig. 7A shows that ethylene production had a 94% inhibition and a 74% recovery in 1-MCP- and 1-MCP+ETH-treated apple fruit on 20 DAH, respectively. Similar to the report of Defilippi et al. (2005), transcription of the *MdAAT2* gene was strongly depressed (Fig. 7D) and the ester biosynthesis was also prevented by 1-MCP, but partially recovered by ETH on 20 DAH (Fig. 7B). Interestingly, the translation of *MdAAT2* was also depressed by 1-MCP treatment (Fig. 7C), suggesting that it is regulated by ethylene at the transcription level.

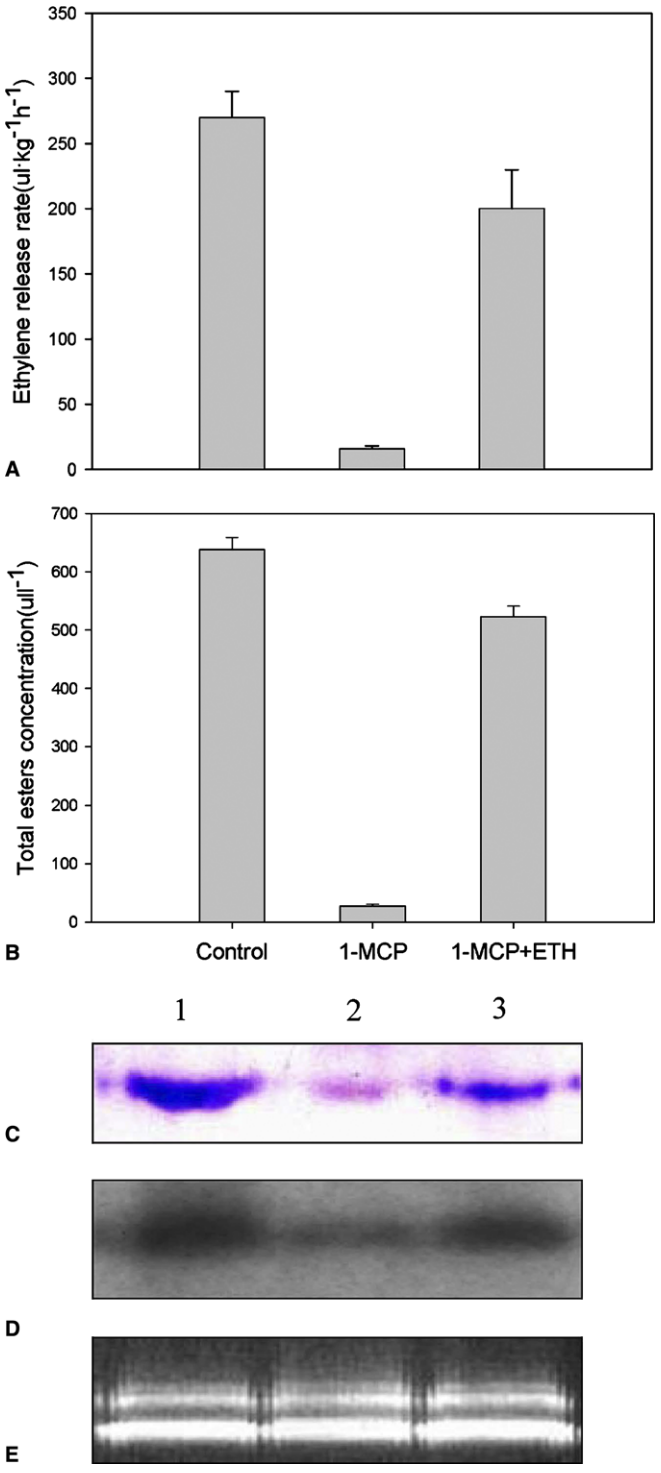



Fig. 7. Effect of application of 1-MCP and ETH on ethylene production, total ester concentration and the *MdAAT2* gene transcription and translation in apple fruit peel on 20 DAH. (A) Ethylene production. (B) Total ester concentration (mean of three replicates ± S.D.) (C) MdAAT2 protein. (D) *MdAAT2* mRNA. (E) Total RNA. Lanes: (1) Untreated apple fruit as control; (2) 1-MCP-treated apple fruit peel; (3) 1-MCP+ETH-treated apple fruit peel.

## 3. Conclusions

We have isolated and characterized MdAAT2 from apple fruit, containing the HXXXD and FGWG motifs of alcohol acyltransferase known from other plants. Sequence analysis of the *MdAAT2* gene indicates that it has lower sequence identities with other fruit or flower AAT genes. In contrast to other apple varieties, the MdAAT2 of Golden Delicious was exclusively expressed in the fruit. The molecular weight of apple AAT protein was found to be about 47.9 kDa by immunoblot, which is lower than the predicted 51.2 kDa. Immunolocalization of apple fruit tissues indicated that the MdAAT2 protein is mainly localized in fruit peel tissue cells.

In this study, expression analysis of the *MdAAT2* gene at mRNA, protein and enzyme activity levels is reported. Data suggest that the expression of MdAAT2 protein is regulated at the transcription level in the fruit peel. AAT enzyme activity and *MdAAT2* gene transcription and translation are strongly depressed by 1-MCP, and the ester concentration is also suppressed in 1-MCP-treated apple fruit. This indicates that ethylene plays a crucial role in the regulation of MdAAT2 transcription and translation, and the subsequent regulation of ester biosynthesis. The pattern of AAT enzyme activity complements that of the MdAAT2 protein during fruit development and ripening, except that, after 25 DAH, AAT enzyme activity strongly decreased while MdAAT2 protein abundance still maintained a relatively high level. This indicates that post-translational modification might also involve a loss of activity of unmodified enzyme during fruit storage.

In the present study, we found that AAT activity correlates with total volatile esters, but volatile ester production also depends on substrate availability, as reported previously (Aharoni et al., 2000; Yahyaoui et al., 2002; Beekwilder et al., 2004).

## 4. Experimental

### 4.1. Plant material and treatment

'Golden Delicious' apple (*Malus domestica* Borkh.) fruits were harvested on 65, 95, 115 and 135 DAFB (days after full

bloom) from a research orchard near Tai'an, Shandong Province. After harvest, the peel and pulp were frozen in liquid nitrogen and stored at −80 °C. The fruit harvested on 135 DAFB were randomly separated into three groups for the following treatments: (1) 1-MCP; (2) 1-MCP + ETH; (3) untreated control. For groups 1 and 2, fruit were treated with 1-MCP (0.75 μl $l^{-1}$) generated from SmartFresh (AgroFresh Inc., Spring House, PA, USA) for 20 h at 20 °C in a sealed steel chamber; group 3 was treated as above, but without 1-MCP. After 1-MCP treatment, group 2 was dipped into 2 mM ETH (Sigma, St. Louis, MO, USA) in 0.02% (v/v) Tween 20 and 1% (v/v) ethanol for 2 min; groups 1 and 3 were treated as above, but without ETH. After treatment, the fruit were held at 20 °C in air.

### 4.2. Isolation of alcohol acyltransferase cDNA clone from apple fruit peel

Two degenerate primers, MDAAT51 (5′-TCTATT(CT)-TACCAGT(AG)TGG-3′) and MDAAT31 (5′-AGCA-TA(AT)CC(AC)AGTGG(AG)TT-3′), were designed from the comparison of known plant AAT amino acid sequences. The cDNA amplification products were subcloned into the vector pGEM-T Easy (Promega, Madison, WI, USA) and sequenced. According to the sequence of clones mentioned above, two different reverse primers, MU3 (5′-GCCATAG-TATCCCAAGGGAAGACGT-3′) and MU1 (5′-ATGTT-TGACTGGTTACTGGATGCGT-3′), were designed and the 5′-region amplified according to the instruction manual (5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0; Invitrogen, Carlsbad, CA, USA). The 3′-region of this gene was amplified by using the primers 5′-TCTATTCTACCAGTGTGG-3′ and $B_{26}$. PCR was carried out as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 50 s.

### 4.3. Semi-quantitative RT-PCR

Two micrograms total RNA was denatured at 70 °C for 5 min and 2 μl AMV reverse transcriptase (Promega) added. After briefly mixing, the transcription reaction was incubated at 42 °C for 1 h and terminated at 85 °C for 10 min (Sambrook et al., 1989). Amplimers were designed with the program Primer Premier (Ver. 5.0). Sequence, expected fragment size and annealing temperature (ta) of primers used in the semi-quantitative RT-PCR analysis were as follows: MdAAT2 (5′-ATACGCATCCAGTAACCA-3′ and 5′-TCCAAAGCATATCCCAGT-3′; 330 bp; 55 °C), 18S rRNA (5′-GGGTTCGATTCCGGAGAGG-3′ and 5′-CCGTGTCAGGATTGGGTAAT-3′; 87 bp; 60 °C).

### 4.4. RNA extraction and Northern blot analysis

Total RNA was extracted from apple peel tissue following the method described by Chang et al. (1993) for tissues with high concentrations of polyphenols and polysaccharides. RNA samples (20 μg) were fractionated in a 1.2% (w/v) agarose gel containing formaldehyde and blotted onto Hybond N+ membranes (Amersham, Buckinghamshire, UK). The blots were hybridized in a solution containing 0.26 M $Na_2PO_4$, 7% (w/v) SDS, 1 mM EDTA and 1% (w/v) bovine serum albumin at 60 °C with $^{32}$P-labeled MdAAT2 cDNA probe (Promega). The membranes were washed twice in 2× SSC and 0.1% (w/v) SDS at 60 °C for 20 min each and then exposed to X-ray film (Kodak, USA).

### 4.5. Preparation of enzyme crude extract from apple fruit peel

Peel tissue cut from four apples was frozen in liquid nitrogen and homogenized with a mortar and pestle in 0.75 ml $g^{-1}$ (tissue) of 0.5 M Tris–HCl, pH 8.0, buffer containing 0.1% Triton X-100 and 0.3 mg $g^{-1}$ of polyvinyl polypyrrolidone (PVPP). After filtration and centrifugation (12,000*g* for 20 min), the supernatant was used for enzyme assay.

### 4.6. Assay of AAT activity

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 (2.5 mM acetyl-CoA in 0.5 M Tris–HCl, pH 8.0), 50 μl butanol solution (200 mM butanol in 0.5 M Tris–HCl, pH 8.0) and 150 μl enzyme extract. The mixture was incubated at 35 °C for 15 min, then 100 μl of 10 mM 5,5-dithiobis (nitrobenzoic acid) (DTNB) was added and allowed to stand at room temperature for 10 min. The increase in absorbance at 412 nm over time, due to a yellow thiophenol product formed by the reaction of DTNB with the free CoASH liberated during the catalytic reaction, was measured with a spectrophotometer (SCINCO S3150, Korea). One activity unit (U) was defined as the increase in one unit of absorbance per minute and results were expressed as specific activity (mU $mg^{-1}$ protein).

### 4.7. Analysis of volatile ester compounds and ethylene

Apple peel tissue (6 g) was homogenized in a Polytron homogenizer in the presence of $H_2O$ (12 ml) with 2 mM NaF. The homogenized tissue was filtered through four layers of cheesecloth and centrifuged (20,000*g* for 20 min) at 4 °C. Ten milliliters of the supernatant was placed into crimp-sealed 40-ml vials containing 2 g of NaCl. Prior to sealing of the vials, 600 μl of the internal standard (IS) solution (1-octanol) was added, to get a final concentration of 500 ppb. A poly(dimethylsiloxane)/divinylbenzene (PDMS/DVB, 65 μm thickness) SPME fiber was used. For manual SPME sampling conditions, an equilibration at 50 °C for 30 min and desorption for 2 min were used. GC–MS system (HP 5890, 5971 A, Hewlett–Packard) equipped with a DB-1 column (J&W Scientific) was used for analysis. Conditions for chromatography were as follows: injector at

250 °C; initial oven temperature, 40 °C held for 5 min, increased to 50 °C at 2 °C $min^{-1}$, increased to 200 °C at 5 °C $min^{-1}$ and held for 5 min. Linear velocity of the carrier gas was 35 cm $s^{-1}$. Mass spectra were obtained by electron ionization at 70 eV and a spectra range of 40–250 $m/z$ was used. Identification of compounds was confirmed by comparison of collected mass spectra with those of authenticated reference standards and spectra in the National Institute for Standards and Technology (NIST ver.2.0a) mass spectra library.

Ethylene production of fruit was determined in three replicates each of four fruit. Each replicate was sealed in a 2000-ml flask for 1 h. Gas samples were collected by syringe to measure the ethylene concentrations by a gas chromatograph of model GC17A (Shimadzu, Kyoto Japan) using a flame ionization detector fitted with an activated alumina column.

### 4.8. Tissue disk assay of apple fruit peel

Tissue disk assay was modified from that of Rudell et al. (2002). Apple fruit peel disks were prepared by peeling and excised from the slices using a 9-mm (diameter) stainless steel cork borer. Apple peel disks were placed in 25-ml Erlenmeyer flasks, treated with 5 μl of extraneous alcohols (Sigma) after 15 min and sealed with a serum stopper. Each flask (three flasks/treatment) contained a bulk tissue sample from four different fruit. The SPME (PDMS/DVB, 65 μm thickness) device was inserted into the flask through the serum stopper and the fiber extended from its protective sheath to expose the sorption surface for a period of 40 min. The following GC–MS analysis was the same as described above.

### 4.9. Protein content, antibody production, immunoblot and immunolocalization analysis

Protein content was determined by the Bradford method (Bio-Rad) using bovine serum albumin (BSA) as standard (Bradford et al., 1976).

The MdAAT2-6His fusion protein was expressed in *E. coli* strain BL21(DE3)pLysS from the pET-30a-c(+) vector according to the manufacturer (Novagen) and then purified by Ni-NTA spin column (Novagen), as prescribed in the pET System Manual. The purified fusion protein was used as an immunogen to raise polyclonal antisera in rabbits (Proteintech Group).

Protein extract was the same as preparation of enzyme crude extract. Twenty micrograms of total proteins per lane were separated by SDS–PAGE as described by Laemmli (1970). Polyacrylamide gels (12%) were electro-blotted onto supported PVDF membranes (Millipore) by a semi-dry Transfer Unit (Amersham, T70). Membranes were incubated overnight at 4 °C with the primary antibody (1:1000 diluted), washed and incubated with goat anti-rabbit (AP-conjugated) secondary antibody (1:2000 diluted; Proteintech Group). Then, AP chromogenic substrate (BCIP and NBT; Roche) was added and color development monitored.

Immunolocalization of apple fruit tissues: Apple fruit tissues were prepared as described by Dixon and Klessig (1995) and the paraffin-embedded samples were sectioned to a thickness of 9–10 pm using a rotary microtome. After removing paraffin by xylenes, the samples incubated with the primary antisera (1:200), then with fluorescein isothiocyanate (FITC)-conjugated second antibody (1:1000). Sections were visualized and photographed using a fluorescence microscope equipped with an FITC filter (Olympus BX51, Tokyo, Japan).

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## References

Aharoni, A., Keizer, L.C., Bouwmeester, H.J., Sun, Z., Alvarez-Huerta, M., Verhoeven, H.A., Blaas, J., van Houwelingen, A.M., De Vos, R.C., van der Voet, H., Jansen, R.C., Guis, M., Mol, J., Davis, R.W., Schena, M., van Tunen, A.J., O'Connell, A.P., 2000. Identification of the SAAT gene involved in strawberry flavor biogenesis by use of DNA microarrays. Plant Cell 12, 647–662.

Alexander, L., Grierson, D., 2002. Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. J. Exp. Bot. 53, 2039–2055.

Baldwin, E., 2002. Fruit flavor, volatile metabolism and consumer perceptions. In: Knee, M. (Ed.), Fruit Quality and its Biological Basis. CRC Press, Boca Raton, FL, pp. 89–106.

Beekwilder, J., Alvarez-Huerta, M., Neef, E., Verstappen, F.W., Bouwmeester, H.J., Aharoni, A., 2004. Functional characterization of enzymes forming volatile esters from strawberry and banana. Plant Physiol. 135, 1865–1878.

Botondi, R., DeSantis, D., Bellincontro, A., Vizovitis, K., Mencarelli, F., 2003. Influence of ethylene inhibition by 1-methylcyclopropene on apricot quality volatile production and glycosidase activity of low- and high-aroma varieties of apricots. J. Agric. Food Chem. 51, 1189–1200.

Bradford, M.M., 1976. A refined and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. 72, 248.

Chang, C., Bleecker, A.B., 2004. Ethylene biology. More than a gas. Plant Physiol. 136, 2895–2899.

Chang, S., Puryear, J., Cairney, J., 1993. A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Rep. 11, 113–116.

Defilippi, B.G., Kader, A.A., Dandekar, A.M., 2005. Apple aroma: alcohol acyltransferase, a rate limiting step for ester biosynthesis, is regulated by ethylene. Plant Sci. 168, 1199–1210.

Dixon, DC, Klessig, DF, 1995. Immunolocalization of proteins in fixed and embedded plant tissues. In: Methods in Plant Molecular Biology: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Dixon, J., Hewett, W.E., 2000. Factors affecting apple aroma/flavour volatile concentration: a review. NZ J. Crop Hortic. Sci. 28, 155–173.

Echeverría, G., Graell, J., López, M.L., Echeverría, I.L., 2004. Volatile production, quality and aroma-related enzyme activities during maturation of 'Fuji' apples. Postharvest Biol. Technol. 31, 217–227.

Fan, X., Mattheis, J.P., 1999. Impact of 1-methylcyclopropene and methyl jasmonate on apple volatile production. J. Agric. Food Chem. 47, 2847–2853.

Fan, X., Mattheis, J.P., Fellman, J.K., Patterson, M.E., 1997. Effect of methyl jasmonate on ethylene and volatile production by summerred apples depends on fruit developmental stage. J. Agric. Food Chem. 45, 208–211.

Fellman, J.K., Rudell, D.R., Mattinson, D.S., Mattheis, J.P., 2003. Relationship of harvest maturity to flavor regeneration after CA storage of 'Delicious' apples. Postharvest Biol. Technol. 27, 39–51.

Jiang, Y., Fu, J., 2000. Ethylene regulation of fruit ripening: molecular aspects. Plant Growth Regul. 30, 193–200.

Laemmli, U.K, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lelièvre, J., Latché, A., Jones, B., Bouzayen, M., Pech, J., 1997. Ethylene and fruit ripening. Physiol Plant. 101, 727–739.

López, M.L., Lavilla, M.T., Riba, M., Vendrell, M., 1998. Comparison of volatile compounds in two seasons in apples: Golden Delicious and Granny Smith. J. Food Quality 21, 155–166.

Lurie, S., Pre-Aymard, C., Ravid, U., Larkov, O., Fallik, E., 2002. Effect of 1-methylcyclopropene on volatile emission and aroma in cv. Anna apples. J. Agric. Food Chem. 50, 4251–4256.

Mattheis, J.P., Fan, X., Argenta, L.C., 2005. Interactive responses of gala apple fruit volatile production to controlled atmosphere storage and chemical inhibition of ethylene action. J. Agric. Food Chem. 53, 4510–4516.

Olı'as, R., Perez, A.G., Sanz, C., 2002. Catalytic properties of alcohol acyltransferase in different strawberry species and cultivars. J. Agric. Food Chem. 50, 4031–4036.

Rowan, D.D., Allen, J.M., Fielder, S., Hunt, M.B., 1999. Biosynthesis of straight-chain ester volatiles in red delicious and granny smith apples using deuterium-labeled precursors. J. Agric. Food Chem. 47, 2553–2562.

Rudell, D.R., Mattinson, D.S., Mattheis, J.P., Wyllie, S.G., Fellman, J.K., 2002. Investigations of aroma volatile biosynthesis under anoxic conditions and in different tissues of "Redchief Delicious" apple fruit (*Malus domestica* Borkh). J. Agric. Food Chem. 50, 2627–2632.

Sambrook, J., Fritsch, E., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shalit, M., Guterman, I., Volpin, H., Bar, E., Tamari, T., Menda, N., Adam, Z., Zamir, D., Vainstein, A., Weiss, D., Pichersky, E., Lewinsohn, E., 2003. Volatile ester formation in roses. Identification of an acetyl-coenzyme A Geraniol/citronellol acetyltransferase in developing rose petals. Plant Physiol. 131, 1868–1876.

Souleyre, E.J., Greenwood, D.R., Friel, E.N., Karunairetnam, S., Newcomb, R.D., 2005. An alcohol acyl transferase from apple (cv. Royal Gala), MpAAT1, produces esters involved in apple fruit flavor. FEBS J. 272, 3132–3144.

St. Pierre, B, De Luca, V., 2000Evolution of Acyltransferase Genes: Origin and Diversification of the BAHD Superfamily of Acyltransferases Involved in Secondary Metabolism, vol. 34. Elsevier, Oxford, pp. 285–315.

Wyllie, S.G., Fellman, J.K., 2000. Formation of volatile branched chain esters in bananas (*Musa sapientum* L.). J. Agric. Food Chem. 48, 3493–3496.

Yahyaoui, F.E.L., Wongs-Aree, C., Latche', A., Hackett, R., Grierson, D., Pech, J.-C., 2002. Molecular and biochemical characteristics of a gene encoding an alcohol acyl-transferase involved in the generation of aroma volatile esters during melon ripening. Eur. J. Biochem. 269, 2359–2366.

Young, J.C., Chu, C.L., Lu, X., Zhu, H., 2004. Ester variability in apple varieties as determined by solid-phase microextraction and gas chromatography-mass spectrometry. J. Agric. Food Chem. 52, 8086–8093.