Available online at www.sciencedirect.com
SCIENCE DIRECT®
Plant Science 168 (2005) 1199–1210
PLANT SCIENCE
www.elsevier.com/locate/plantsci

Apple aroma: alcohol acyltransferase, a rate limiting step for ester biosynthesis, is regulated by ethylene

Bruno G. Defilippi [a,b], Adel A. Kader [a], Abhaya M. Dandekar [a,*]

[a] *Department of Plant Sciences, University of California, One Shields Avenue, Davis, CA 95616, USA*
[b] *Institute for Agricultural Research (INIA), La Platina Experimental Station, Casilla 439/3, Santiago, Chile*

Received 16 November 2004; received in revised form 21 December 2004; accepted 21 December 2004
Available online 12 January 2005

Abstract

The role of ethylene in aroma biosynthesis of apple fruits was investigated using transgenic 'Greensleeves' apple trees suppressed for ACC-oxidase or ACC-synthase enzyme activity, and an ethylene action inhibitor (1-methycyclopropene, 1-MCP). In the transgenic lines and 1-MCP treated fruit, reductions higher than 90% in ethylene biosynthesis and respiration rates were observed in apples held at 20 °C for 14 days. We observed a major reduction in ester production in the ethylene-suppressed lines and in the 1-MCP treated fruit, with only slight differences in the levels of alcohol and aldehyde volatiles under these conditions. The activity of alcohol acyl-CoA transferase (AAT), a key enzyme in ester biosynthesis, showed an ethylene dependent pattern of regulation. Additionally, gene expression levels of specifically an AAT clone were highly regulated by ethylene. In contrast, activity and expression levels of alcohol dehydrogenase (ADH) were not affected by changes in the levels of endogenous ethylene. These results suggest that ethylene is involved in ester biosynthesis in apple via regulation of AAT.

Keywords: Fruit flavor complex; Fruit ripening; *Malus domestica*; Aroma; Transgenic apple; 1-MCP

1. Introduction

Key components of the fruit flavor complex are the volatile compounds that constitute aroma. These include a broad group of metabolites that are important components of flavor in fruit and vegetables and in addition regulate the interactions of plants with other organisms [1]. In addition to the four basic tastes that the human palate can recognize, aroma exerts an important influence on the final consumer acceptance of a fruit/vegetable commodity [2]. The aroma properties of fruits depend upon the combination of volatiles and the concentration, and threshold of individual volatile compounds. In apple, the typical aroma compounds are the fruity esters that develop during ripening with a maximum endogenous ester concentration occurring at the climacteric peak [3,4]. The gaseous plant hormone ethylene is associated with many physiological processes in plants, and plays an especially important role in the ripening process of climacteric fruit, initiating and enhancing ripening-related changes including decreased firmness, increased soluble solids content and enhanced flavor [5–7]. The association between ethylene and aroma production has been shown through the use of both ethylene action and ethylene biosynthesis inhibitors that result in a reduction in levels of ester volatiles in apple fruit [8,9]. Similarly, in climacteric ACC-oxidase antisense transgenic melons, ripening parameters including color of the rind and aroma (especially esters) production were strongly reduced at low levels of endogenous ethylene [10,11], suggesting that these parameters are physiologically regulated by ethylene during fruit development. However, little is known of the underlying mechanisms that regulate this relationship between ethylene biosynthesis and ester biosynthesis. It is also not clear if the enzymes responsible for aroma components are constitutive or induced during the climacteric response [4].

Earlier studies have established that the beta-oxidation of fatty acids is the primary biosynthetic process that provides

* Corresponding author. Tel.: +1 530 752 7784; fax: +1 530 752 8502.
E-mail address: amdandekar@ucdavis.edu (A.M. Dandekar).

0168-9452/$ – see front matter © 2005 Published by Elsevier Ireland Ltd.
doi:10.1016/j.plantsci.2004.12.018

alcohols and acyl co-enzyme A (CoA) for ester formation [12]. Acyl CoAs are reduced by acyl CoA reductase to aldehydes, which are in turn reduced by the alcohol dehydrogenase (ADH) enzyme to form alcohols that are converted to esters via the action of alcohol acyltransferase (AAT) enzyme [13]. The AAT enzyme catalyzes the linkage of an acetyl moiety from acetyl CoA to the appropriate alcohol. This enzyme has been studied in some detail in ripe fruit, including apple [3,14], banana [15], melon [16,17], and especially strawberry where the enzyme has been purified and characterized, and the gene cloned [18,19]. 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 [4,20]. In preliminary experiments performed in apples, the activity of AAT appears to increase with the onset of ripening followed by a decrease in extractable activity [3]. However, despite the importance of AAT as a key enzyme in aroma synthesis in fruits, many aspects, such as the mechanism of action, substrate specificity, and physiological relevance, remain unclear [16,20]. Ester biosynthesis can also be limited by the concentration of precursor alcohols [21], which suggests that critical steps for ester formation may be located upstream in the pathway. The enzyme ADH has been associated with fruit ripening and has been shown to be responsible for the interconversion of aldehyde and alcohol forms of flavor volatiles [22,23]. Mature green tomato contained lower levels of *ADH2* transcripts as compared to ripened fruits, which was correlated with lower levels of alcohols and higher levels of aldehydes [24]. Similarly, differences in the accumulation of lipoxygenase-derived volatiles were observed in an *Arabidopsis ADH* mutant that lacked ADH activity, which resulted in the accumulation of C6 aldehydes and a reduction of alcohols [25]. This evidence suggests that ethylene plays an important role in apple as a regulator of ester biosynthesis with AAT and/or ADH enzymes as key enzymes that modulate the biochemical steps in flavor biogenesis. The main objective of this research was to outline the underlying mechanism of ester biosynthesis and its regulation in apple fruit.

2. Material and methods

2.1. Plant material and treatments

Transgenic Greensleeves (GS) apple fruits suppressed for ethylene biosynthesis were obtained from different lines grown in an experimental orchard in Northern CA [26]. Fruits of selected GS apple lines (GS, 67G, 68G, 103Y, and 130Y) were evaluated in 2001 and 2002, and the ones with the highest level of ethylene suppression (67G and 68G) were selected for further studies in 2003. Fruits were harvested at a preclimacteric stage relative to the non-transformed line, prior to initiation of autocatalytic ethylene production, and held at 20 °C in air for up to 14 days with a 90–95% relative humidity in all cases. A second approach for reducing ethylene availability was the use of the ethylene action inhibitor 1-MCP. For this treatment, GS apples were harvested and treated with 0 (control) or 1 $\mu L\ L^{-1}$ 1-MCP. Each group of fruit was treated in a 20 L sealed glass jar for 20 h at 20 °C before storage at 20 °C for 14 days. Enhancement of ethylene production was done by exposure to 80 $\mu L\ L^{-1}$ ethylene. All the treatments were performed during at least two fruiting seasons.

2.2. Ethylene and respiration rate measurements

Within each experiment ethylene production and respiration rates were determined every other day during storage for individual fruits using a static system [7].

2.3. Determination of volatiles

Apple cortical tissue with and without skin tissue was ground using liquid nitrogen and kept at −80 °C until analysis. Six grams of the tissue were homogenized in the presence of 12 mL of sodium fluoride (2 mM) solution. After filtration and centrifugation, 10 mL of the supernatant was used for headspace gas analysis by using a solid phase microextraction procedure [7]. Conditions for GC–MS were as described by Defilippi et al. [7]. 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) mass spectra library. The quantitative determination of individual compounds was done using the peak of internal standard (1-octanol) as relative value.

2.4. Ethylene biosynthesis

Apple cortical tissue, with or without the associated skin tissue, was homogenized, filtered, and centrifuged at 4 °C. ACC synthase and ACO activities were assayed as described earlier [27–29]. Similarly, concentrations of ACC were determined by the method of Lizada and Yang [30].

2.5. Alcohol acetyl CoA transferase activity

Apple cortical tissue (3 g), with or without the associated skin tissue, was frozen in liquid nitrogen and homogenized with mortar and pestle in 2 mL g^{-1} (tissue) of 100 mM potassium phosphate buffer (pH 7.0) and 0.33 mg g^{-1} of PVPP. After filtration and centrifugation, the supernatant was recovered and used for the enzyme assay [31]. 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) [14].

2.6. Alcohol dehydrogenase activity

For each replicate, cortical tissue (3 g), with or without the associated skin tissue, was obtained and homogenized in a Polytron homogenizer in 10 mL of 100 mM 2-(*N*-morpholino)ethane-sulfonic acid (MES) buffer (pH 6.5) containing 2 mM DTT and 1% (w/v) polyninyl pyrrolidone. After filtration and centrifugation, the supernatant was recovered and used for the enzyme assay as described earlier [32,33].

2.7. Protein content

Protein content of enzyme extracts used to determine in vitro activity was determined by the method of Bradford [34] using a Bio-Rad protein assay kit (BioRad Laboratories, Hercules, CA) and bovine serum albumin (BSA) as a standard.

2.8. Cloning and sequencing of AAT and ADH genes

First strand cDNA was synthesized using ProStar First-Strand RT-PCT kit (Stratagene) from 1 μg of total RNA extracted from a pooled sample of non-transformed Greensleeves apple at different maturity stages. Specific primers for PCR amplification of Greensleeves *AAT* and *ADH* were derived from the apple sequence (GenBank accession numbers AX025508 and Z48232, respectively). For *AAT* the primers selected were: AAT2·forward 5′GATGTCATTCTCAGTACTTCAGGTGAAACGATTGC3′ and AAT2·reverse 5′TCATTGACTAGTTGATCTAAGGTTGTTACATATATCCTCC3′. For a*dh* primers were: ADH1·forward 5′CAGGAGGGATTGTGGAGAGTGTTGGTG3′ and the reverse ADH2·reverse 5′GCATTTGAATGTGTCCATGATGGTTGGGGTG3′. PCR conditions were as follows for both genes: denaturation at 94 °C for 1 min; annealing at 60 °C for 1 min and primer extension at 68 °C for 1 min; 30 cycles. The expected fragment was cloned by TA cloning strategy into a pCR4 vector and propagated in *Escherichia coli* (TOPO TA Cloning kit; Invitrogen Corp., Carlsbad, CA). Clones were screened for the inserts by colony PCR using the specific primers, and then purified using QIA prep Miniprep kit (Qiagen). Positive clones were submitted for sequencing using an automated DNA sequencer at Davis Sequencing (Davis, CA) and compared with sequences deposited at National Center for Biotechnology Information (NCBI) using BLAST alignment programs.

2.9. Real time quantitative TaqMan PCR systems

For each target gene, PCR primers and a TaqMan® probes were designed using the Primer Express (Applied Biosystems, Foster City, CA). Each probe was labeled with a reporter dye FAM (6-carboxyfluoroscein) at the 5′-end, and with the quencher dye TAMRA (6-carboxytetramethylrhodamine) at the 3′-end, the probe was phosphate-blocked at the 3′-end to prevent extension by AmpliTaq Gold DNA polymerase [35]. In order to prevent co-amplification of contaminating genomic DNA (gDNA), TaqMan PCR primers were designed to cover exon–exon junctions where possible (Table 1).

Table 1
Sequences of PCR primers and TaqMan probes

Target	Primer	Sequence (5′→3′)	Length	Probe	Probe sequence (5′→3′)
Housekeeping genes					
18S rRNA	a18S-373f	GGGTTCGATTCCGGAGAGG[b]	87	395p	CCTGAGAAACGGCTACCACATCCAAGGA
(multiple)[a]	a18S-459r	CCGTGTCAGGATTGGGTAATTT	104		
GAPDH	mGADPH-141f	GCTGCCAAG**GC**TGTTGGAA		176p	TAATGGAAAATTGACCGGAATGTCC
(CN494000)	mGADPH-244r	ACAGTCAGGTCAACAACGGAAAC			
S19 (CN580829)	mS19-196f	CTTGCGAGGTTG**TA**CGAGGTGA	82	231p	CATCTTCGTATTCAAGTTCCGCACCCA
	mS19-277r	TWGACTTCCCCCCTCCAA			
Target genes					
ACS1 (AF312737)	ACS1-233f	GTTCAAAAAG**GC**AATGGTAGATTTC	118	293p	TCCCAACCACTTAGTGCTCACCGCC
	ACS1-350r	GAAAATAAAGGTCTCATTCGCTGAAG			
ACS2 (L31347)	ACS2-1171f	AATATATCTCCTGGATCGTCTTGTCATT	74	1200p	CACGGAACCTGGTTGGTTCCGTGTC
	ACS2-1244r	TCGGGCAAGTTGGCAAA			
ACO1 (AF030859)	ACO1-655f	GACCAGATTG**AG**GTGATCACCA	104	688p	AAAAGTGTGATGCACCGGGTGATAG
	ACO1-758r	GGGTTGTAGAACGAGGCTATCG			
AAT2	AAT2-1259f	GGTACCAATGTGTTTGCCATTCT	132	1301p	TAGAGAGGATTACTCAGGAACCTAAGGAGGA
(own sequence)	AAT2-1390r	AACACTTACATCATTGACATGAT**CC**TAGTT			
ADH1 (Z48234)	ADH1-165f	GGAAGCCAAG**GG**ACAAAACC	85	382p	CCTAGAATTTATGGTCATGAGGCAGGAGGG
	ADH1-319r	CACGCCCTCACCAACACTCT			

[a] Brackets indicate GenBank accession number.
[b] Exon boundaries are in bold and underlined.

Total RNA was isolated from the stored tissue by the method of Wan and Wilkins [36] with modifications [37,38]. Genomic DNA contamination in the total RNA fraction was digested using RNase-free DNase I (Invitrogen, Carlsbad, CA) for 15 min at 37 °C and inactivated at 95 °C for 5 min following chilling on ice. Absence of gDNA contamination was confirmed using a universal 18S TaqMan PCR system on digested total RNA. Complementary DNA (cDNA) was synthesized using 50 units of SuperScript III (Invitrogen), 600 ng random hexadeoxyribonucleotide ($pd(N)_6$) primers (random hexamer primer) 10 U RNaseOut (RNase inhibitor), and 1 mM dNTPs (all Invitrogen) in a final volume of 40 μL [35]. The reverse transcription reaction proceeded for 120 min at 50 °C. After addition of 60 μL of water, the reaction was terminated by heating for 5 min to 95 °C and cooling on ice. Each PCR reaction contained 20× Assay-on-Demand primer and probes for the respective TaqMan system and commercially available PCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems). The samples were placed in 96-well plates and amplified in an automated fluorometer (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). Standard amplification conditions were used: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 60 s at 60 °C. Fluorescent signals were collected during the annealing temperature and CT values extracted with a threshold of 0.04 and baseline values of 3–10. In order to determine the most stably transcribed housekeeping gene, a housekeeping gene validation experiment was run on a representative number of samples from all tissue types. Three commonly used housekeeping genes were used for this experiment: a TaqMan PCR system recognizing plant 18S rRNA (ssrRNA), apple GAPDH, and apple ribosomal protein S19 (Table 1). 18S rRNA was found to show the least standard deviation across all tissues and was therefore transcribed most stably. 18S rRNA CT values served to normalize against the target gene CT values. Final quantitation was done using the comparative CT method (User Bulletin #2, Applied Biosystems) and is reported as relative transcription or the *n*-fold difference relative to a calibrator cDNA (i.e. lowest target gene transcription). In brief, the housekeeping gene 18S rRNA was used to normalize the CT values of the target genes (ΔCT). The ΔCT was calibrated against the weakest signal within each target gene. The relative linear amount of target molecules relative to the calibrator was calculated by $2^{-\Delta\Delta Ct}$.

3. Results and discussion

3.1. Ethylene biosynthesis

Fruit obtained from the selected transgenic lines showed a 95% reduction in ethylene production relative to the non-transformed line, with an absence or delay of the climacteric peak (Fig. 1).

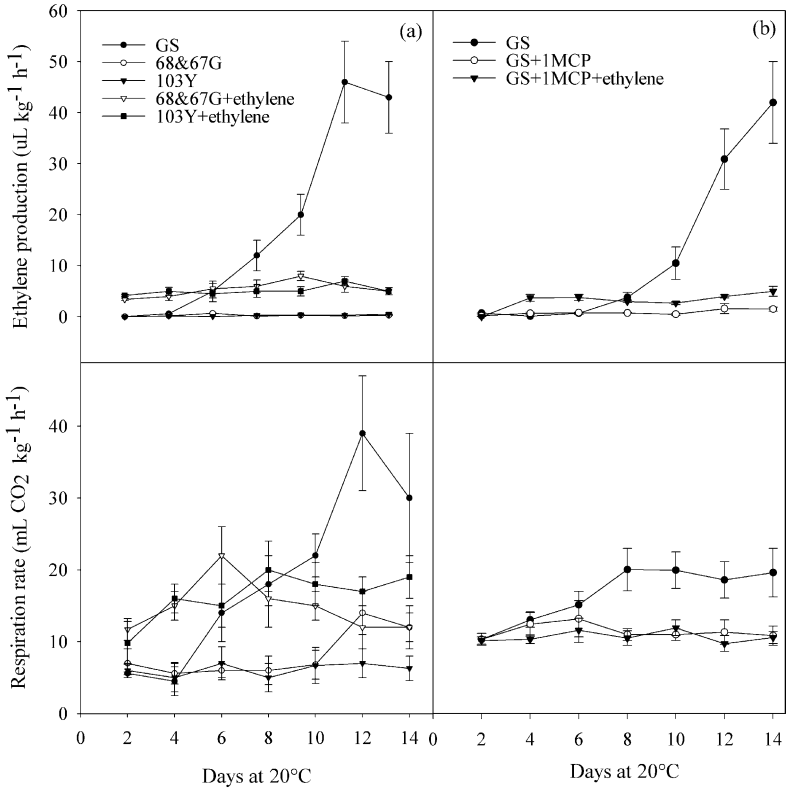

Fig. 1. Ethylene production and respiration rates (means of three replicates ± S.E.) of three Greensleeves apple lines held at 20 °C for 14 days with or without exposure to 80 μL L^{-1} ethylene (a), and GS apples treated with 1 μL L^{-1} 1-MCP and held at 20 °C with or without exposure to 80 μL L^{-1} ethylene (b).

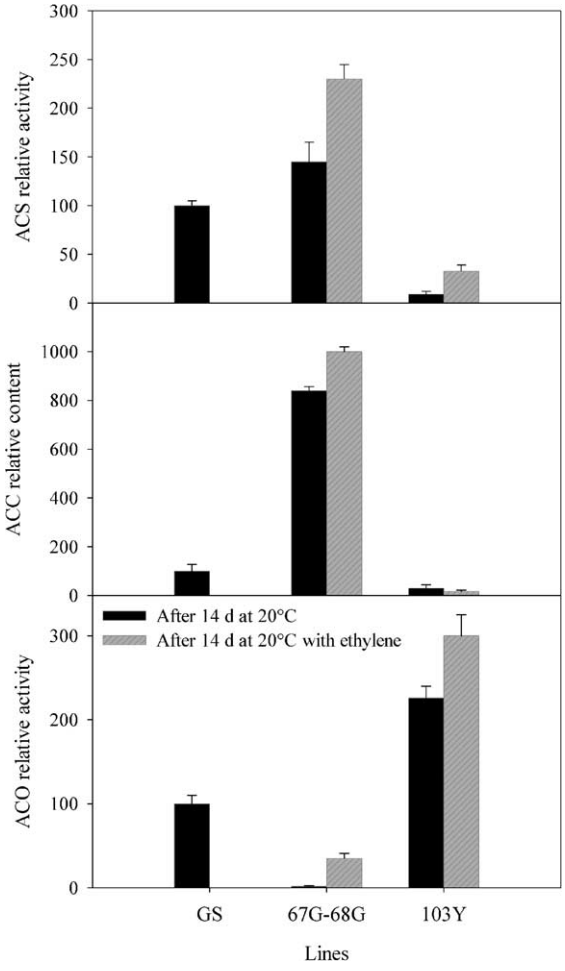

Fig. 2. Relative ACS activity, ACC content and ACO activity of three Greensleeves apple lines held at 20 °C for 14 days with or without exposure to 80 μL L^{-1} ethylene. Data shown are means of three replicates ± S.E., and are expressed as a percentage of that observed for the untransformed line GS (ACS activity = 2.4 nmol mg^{-1} (protein) h^{-1}, ACC content = 5 nmol ACC g^{-1} (tissue) and ACO activity = 143 nL C_2H_4 mg^{-1} (protein) h^{-1}).

This decrease was related to a reduction in either ACS enzyme activity in the ACS-silenced line (103Y) or ACO enzyme activity in the ACO-silenced lines (67G and 68G) (Fig. 2). In the ACS-silenced line there was a 90% reduction in ACS enzyme activity relative to that of the control line. This resulted in a lower accumulation of the immediate ethylene precursor ACC. On the other hand, in the ACO-silenced lines the activity of ACO enzyme was almost completely suppressed, which resulted in a massive accumulation of ACC (more than 10 times that of the control line). The application of ethylene to fruits from line 67G and 68G did not activate autocatalytic ethylene production, and induced only a slight increase in both ethylene production and ethylene biosynthesis (Figs. 1 and 2).

Similarly, in the fruit treated with 1-MCP there was a major reduction of ethylene production, with 70% inhibition at the climacteric peak (Fig. 1). At the enzyme level we observed 40% lower activities of ACS and ACO than that of the non-treated fruit after 14 days at 20 °C, and the

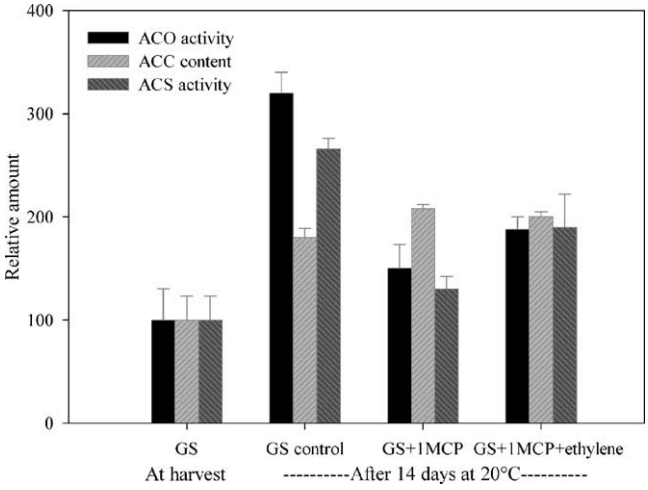

Fig. 3. Relative ACS activity, ACC content, and ACO activity of three Greensleeves apples treated with 1 μL L^{-1} 1-MCP and held at 20 °C with or without exposure to 80 μL L^{-1} ethylene. Data shown are means of three replicates ± S.E. and are expressed as a percentage of that observed for the fruit at harvest (ACS activity = 1.0 nmol mg^{-1} (protein) h^{-1}, ACC content = 2.1 nmol ACC g^{-1} (tissue), and ACO activity = 40 nL C_2H_4 mg^{-1} (protein) h^{-1}).

exogenous application of ethylene only produced a minor increase in this level, suggesting an inhibition of the autocatalytic ethylene production (Fig. 3). The respiration rate of fruits from both the transformed lines and the 1-MCP-treated fruit followed a pattern similar to that of the ethylene production rate (Fig. 1).

At the molecular level, there was a massive accumulation of *ACS* and *ACO* gene transcripts between harvest and after 14 days at 20 °C in the non-transformed lines (Fig. 4). In the ACO suppressed lines, both genes followed the same pattern of enzyme activity, with a major reduction of *ACO* expression levels in these lines (at least 70-fold lower compared with the non-transformed line). In these lines, supplementation of ethylene only induced the expression of *ACS* genes (*ACS*1 and *ACS*2). There were different levels of *ACS* gene expression in samples with or without peel tissue, suggesting that the levels of *ACS* transcripts (4–10-fold higher in the peel) may be an important factor in determining the capacity of ethylene production in different fruit tissues [39]. However, the high levels of induction of *ACS* genes were not concomitant with the increase in ACS activity, which may suggest an important function of other *ACS* genes present in apple [40]. As observed in the transgenic lines, the application of 1-MCP caused a remarkable down-regulation in the expression levels of *ACS* and *ACO* genes. There was no change, however, in these levels with the application of ethylene (Fig. 5), which can be explained by the significant effect of 1-MCP in blocking the receptors for ethylene, resulting in a suppression of ethylene responses [41].

3.2. Overall aroma production in Greensleeves fruit

The aroma production of Greensleeves apples was assessed during two fruiting seasons. From the lines

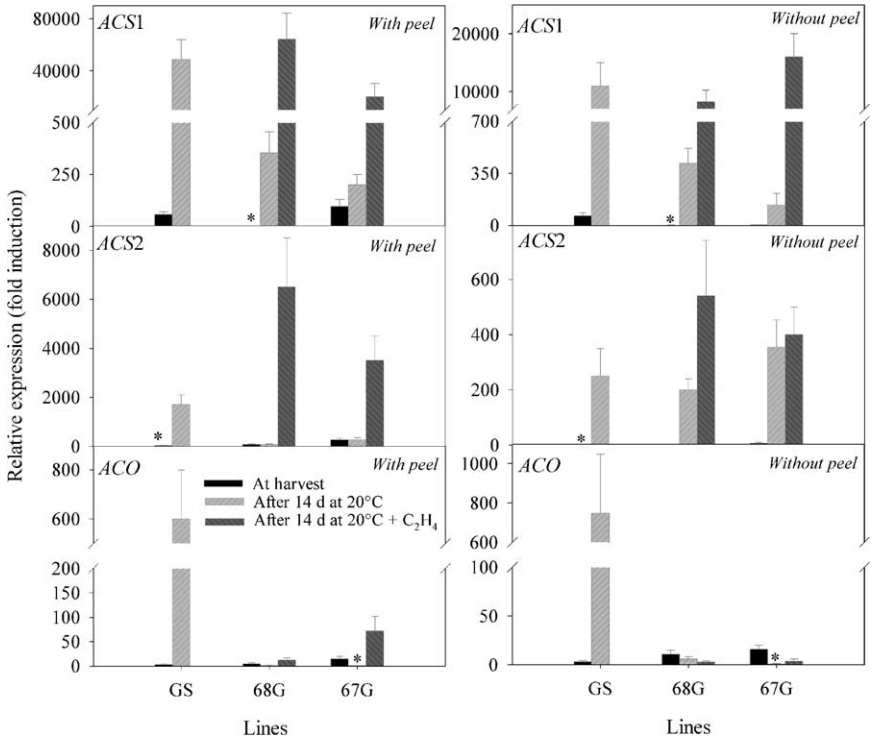

Fig. 4. Relative expression levels of ethylene biosynthesis genes in three Greensleeves apple lines held at 20 °C for 14 days with or without exposure to 80 μL L^{-1} ethylene. Values were normalized with respect to the internal control *18S rRNA*. The line with the lowest level of expression was set as one (*). Data shown are means of three replicates ± S.E.

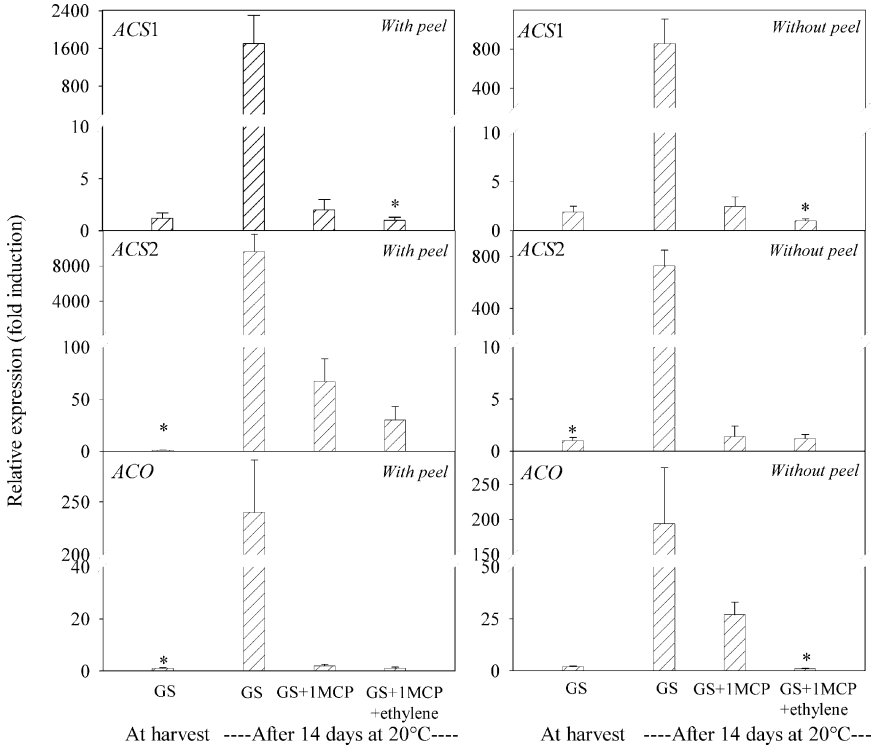

Fig. 5. Relative expression levels of ethylene biosynthesis genes in Greensleeves apples treated with 1 μL L^{-1} 1-MCP and held at 20 °C with or without exposure to 80 μL L^{-1} ethylene. Values were normalized with respect to the internal control *18S rRNA*. The treatment with the lowest level of expression was set as one (*). Data shown are means of three replicates ± S.E.

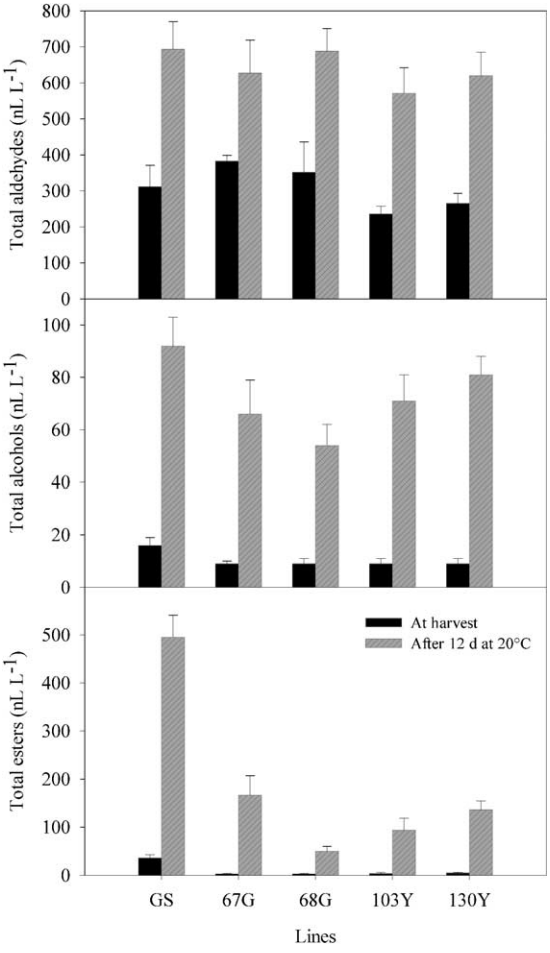

Fig. 6. Total aroma composition (means of three replicates ± S.D.) in several lines of Greensleeves apple lines held at 20 °C for 12 days.

evaluated in the first year (Fig. 6), only 67G and 68G were considered for further studies.

In general, aroma production was characterized by the presence of more than 14 compounds that were identified and quantified in the headspace of apple tissue (Tables 2 and 3). In the non-transformed lines the aroma profile was first dominated by aldehydes at harvet (close to 90%). Finally, esters dominated representing more than 60% of total volatiles, which means an increase of more than 90% relative to that at harvest. In terms of individual compounds, hexanal and (2E)-hexenal were the main aldehydes present in mature and ripe fruit, with a change in favor of (2E)-hexenal through the progress of ripening (Table 2). This group of volatiles contributes to the ''green note'' in overall aromas, which explains their higher abundance in early stages of development [3]. In the case of alcohols, hexanol was the predominant compound and it accumulated as ripening progressed, providing substrate for ester formation as will be discussed later. Esters were abundantly present at the end of the holding period, with hexyl butanoate comprising at least 50% of the total ester compounds, followed by hexyl 2-methylbutanoate and butyl butanoate. All of these volatiles have been identified in other apple cultivars, with important difference in terms of abundance among cultivars [3,6,9,42].

3.3. Effect of ethylene suppression on aroma production

The effect of ethylene suppression on ester production in both the transgenic lines and the 1-MCP-treated apples was significant, resulting in an important reduction or delay in the accumulation of ester compounds. Levels of 10–13% in the transgenic lines and less than 10% in the 1-MCP-treated fruit were observed relative to the non-transformed and non-treated fruit, respectively. These results suggest that ester production is significantly affected by ethylene regulation in Greensleeves apples, as also observed in other varieties and species [8–10]. No major effects were observed in the levels of total aldehydes, with a significant change only in the hexanal/(2E)–hexenal ratio, which was higher in both the ethylene suppressed lines and 1-MCP-treated fruit as compared to the control fruit (Tables 2 and 3). This suggests a possible regulation in early stages of β-oxidation or lipoxygenase pathway. For example, it is possible that the lipoxygenase pathway acts independently in the disrupted tissue resulting in constant levels of aldehydes. Alternatively, upstream steps involving precursor availability may determine aldehyde accumulation under these conditions [43]. A reduction/delay in alcohol accumulation was also measured in the transgenic lines and 1-MCP-treated fruit, with levels close to 50% of the control line, which can be important in terms of substrate limitation for ester production [8].

These results suggest that not only the formation of esters is under ethylene control, but also steps upstream in the biosynthetic pathway of ester biosynthesis are under ethylene regulation [3,9]. Additionally, a massive accumulation of all groups of volatiles occurs between harvest and after 14 days at 20 °C, suggesting an increase in the supply of primary precursors for aroma, including fatty acids and amino acids [4,43].

With the exogenous application of ethylene, ester levels of the transformed fruit partially recovered, attaining values of 70% of that of the non-transformed line (Tables 2 and 3). These levels were only reached with continuous exposure to 80 μL L^{-1} ethylene during the 14 days at 20 °C and not with a partial exposure as we observed in the first year (data not shown). This indicates that a continuous presence of ethylene is required for volatile synthesis, particularly ester compounds as supported by other investigations [7,8]. A similar trend was observed for total alcohols but with some variation among individual compounds. In the 1-MCP-treated fruit, the exogenous application of ethylene caused only a marginal increase in alcohols and esters, which can be attributed to the effectiveness of 1-MCP in blocking ethylene action. These data are consistent with our previous work on ethylene-dependent flavor metabolites [7]. These findings indicate that not only the presence of ethylene is

Table 2
Aroma composition (nanoliters per liter; means of three replicates ± S.D.) in lines of Greensleeves apples evaluated at harvest and after 14 days at 20 °C

Aroma compounds	GS At harvest	GS After 14 days at 20 °C	68G At harvest	68G After 14 days at 20 °C	68G After 14 days at 20 °C + C_2H_4	103Y At harvest	103Y After 14 days at 20 °C	103Y After 14 days at 20 °C + C_2H_4
Hexanal	271 ± 26	237 ± 17	198 ± 27	290 ± 77	295 ± 20	230 ± 17	320 ± 52	300 ± 19
(E) 2-hexenal	57 ± 7	391 ± 47	48 ± 15	282 ± 51	315 ± 16	61 ± 25	254 ± 41	295 ± 10
Total aldehydes	328 ± 19	628 ± 54	246 ± 41	572 ± 29	610 ± 35	291 ± 42	574 ± 93	595 ± 29
Butanol	4 ± 1	16 ± 4	4 ± 0	10 ± 2	7 ± 1	3 ± 2	9 ± 2	10 ± 1
2-Methylbutanol	4 ± 1	20 ± 7	ND[a]	ND	13 ± 1	ND	ND	9 ± 3
Hexanol	9 ± 2	20 ± 7	7 ± 1	17 ± 4	62 ± 11	9 ± 1	27 ± 4	42 ± 10
Total alcohols	17 ± 4	56 ± 18	11 ± 1	27 ± 6	82 ± 13	12 ± 3	36 ± 6	61 ± 14
Butyl butanoate	ND	97 ± 14	ND	15 ± 6	67 ± 3	ND	20 ± 4	77 ± 3
Butyl 2-methylbutanoate	ND	47 ± 9	ND	ND	41 ± 8	ND	ND	61 ± 10
Hexyl butanoate	9 ± 7	558 ± 80	6 ± 1	120 ± 8	360 ± 40	10 ± 1	90 ± 9	300 ± 60
Hexyl 2-methyl butanoate	ND	321 ± 79	ND	10 ± 3	198 ± 37	ND	11 ± 1	220 ± 47
Hexyl hexanoate	ND	23 ± 5	5 ± 1	5 ± 1	14 ± 3	6 ± 3	7 ± 1	32 ± 9
Total esters	9 ± 7	1046 ± 33	11 ± 3	150 ± 13	680 ± 57	16 ± 4	128 ± 7	690 ± 136

[a] ND: not detected.

required for ester biosynthesis but also a level of ethylene above a threshold that can activate the ethylene signaling pathway is essential for triggering and modulating ester production during fruit ripening [11].

3.4. Biochemical basis of ester biosynthesis under ethylene regulation

Since there was a major effect of ethylene suppression and enhancement on ester production, we focused our analysis on the last two stages of ester formation in Greensleeves apples. In vitro AAT enzyme activity, the main enzyme in ester biosynthesis showed 40–60% increase between harvest and the end of storage, levels also observed for other apple varieties during ripening [31]. In the transgenic lines lower levels of enzyme activity at harvest relative to the non-transformed line were measured, and consistently we observed no significant changes in activity until the end of the holding period (Fig. 7). As with ester production, only an exposure to 80 μL L^{-1} ethylene during 14 days at 20 °C recovered levels of enzyme activity close to those observed in the non-transformed line, which was not achieved with a partial application of 20 μL L^{-1} ethylene during the first year (data not shown). These results suggest that ethylene has an important role, direct or indirect, in modulating AAT enzyme activity in Greensleeves apples. This observation is also supported by the use of 1-MCP, in which we observed an inhibition of enzyme activity relative

Table 3
Aroma composition (means of three replicates ± S.D.) of Greensleeves apples treated with 1 μL L^{-1} 1-MCP and held at 20 °C for 14 days with or without exposure to 80 μL L^{-1} ethylene

Aroma compound	Concentration (nL L^{-1})			
	At harvest	After 14 days at 20 °C		
	Control	Control	1-MCP	1 μL L^{-1} 1-MCP + C_2H_4
Hexanal	233 ± 26	295 ± 63	295 ± 11	336 ± 50
(2E) hexenal	50 ± 9	345 ± 25	301 ± 11	295 ± 47
Total aldehydes	282 ± 35	640 ± 41	596 ± 26	621 ± 98
Butanol	4 ± 1	19 ± 3	10 ± 2	15 ± 8
2-Methylbutanol	6 ± 1	11 ± 4	9 ± 2	11 ± 2
Hexanol	9 ± 2	30 ± 12	12 ± 3	16 ± 4
Total alcohols	19 ± 4	60 ± 19	31 ± 7	41 ± 14
Butyl butanoate	ND[a]	102 ± 18	30 ± 10	9 ± 2
Butyl 2-methylbutanoate	ND	33 ± 9	ND	5 ± 3
Hexyl butanoate	10 ± 2	471 ± 90	25 ± 2	50 ± 8
Hexyl 2-methyl butanoate	ND	194 ± 31	ND	45 ± 7
Hexyl hexanoate	ND	26 ± 4	ND	ND
Total esters	10 ± 2	826 ± 135	55 ± 14	109 ± 20

[a] Not detected.

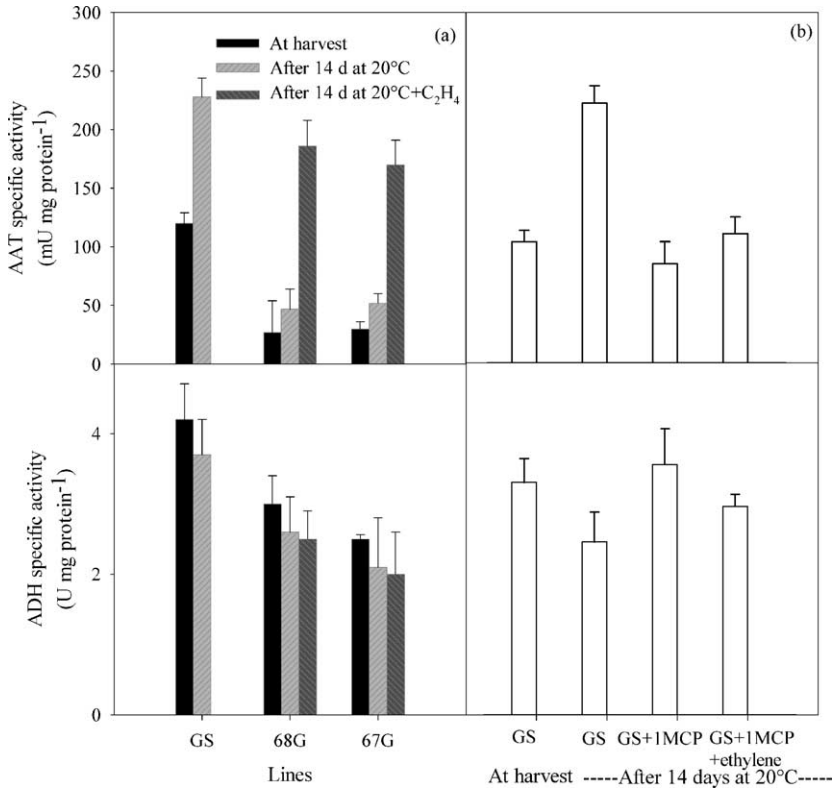

Fig. 7. Alcohol acyl transferase (AAT) and alcohol dehydrogenase (ADH) activities (means of three replicates ± S.E.) of three Greensleeves apple lines stored at 20 °C for 14 days with or without exposure to 80 μL L^{-1} ethylene (a), and GS fruit treated with 1 μL L^{-1} 1-MCP and stored at 20 °C with or without exposure to 80 μL L^{-1} ethylene (b).

to the non-treated fruit; however, the exogenous application of ethylene did not recover the levels of the control treatment, which increased more than 30% between harvest and at the end of the holding period (Fig. 7). The changes caused by ethylene regulation of AAT enzyme activity were more significant in the transgenic lines than the ones observed during ripening of non-transformed fruit, which suggests that AAT enzyme may have a significant role in ester production in early stages of ripening when the fruit has a reduced flux through the ethylene biosynthesis pathway. As explained earlier, aldehydes showed ethylene regulation only for (2E)-hexenal with hexanal levels being independent of this regulation. These findings indicate that hexyl esters, which are derived from hexanal and showed an ethylene-dependent pattern, further support the importance of the last steps of ester formation. Taken together, these data suggest that the significant reductions in ester compounds observed under ethylene suppression conditions may be also caused by a reduction in AAT enzyme activity levels, and not only by a limitation of precursor [43,44].

Although we observed a relatively high AAT enzyme activity at harvest in the non-transformed fruit, we only measured a small amount of ester in comparison to the evaluation after 14 days at 20 °C, which may indicate the importance of other biochemical steps contributing to ester production. In this context, a minor increase in the availability of alcohol substrates for ester formation was observed after 14 days at 20 °C in all the lines, and its production was slightly stimulated by ethylene. Measurements of ADH enzyme activity did not show any significant change between the measurements done at harvest and after 14 days at 20 °C under any condition (Fig. 7), and only a trend to a reduced level of ADH activity by the end was noticed [14]. However, a significant difference was observed in the total activity of the transgenic lines which was lower than the non-transformed fruit, both at harvest and at the end of the holding period, which may explain the lower levels of alcohols measured in the transgenic lines; but this does not explain the low level of alcohols obtained either in the 1-MCP-treated fruit or in the recovery of alcohols after the use of ethylene in the transgenic fruit. Therefore, it seems ADH enzyme is not limiting substrate availability for ester production under these conditions. Unfortunately, no measurements were done at intermediate intervals during the holding period, which is important considering that ADH activity has shown the highest activity prior to ripening [45].

3.5. Cloning of genes and expression of AAT and ADH

Single clones for each gene, *AAT* and *ADH*, were isolated and sequenced using RT-PCR with gene specific primers

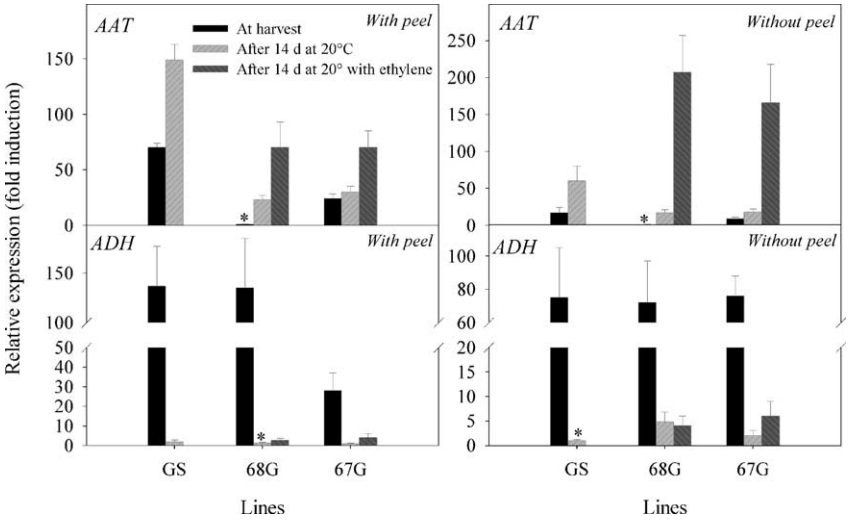

Fig. 8. Relative expression levels of aroma biosynthesis genes in three Greensleeves apple lines held at 20 °C for 14 days with or without exposure to 80 μL L^{-1} ethylene. Values were normalized with respect to the internal control *18S rRNA*. The line with the lowest level of expression was set as one (*). Data shown are means of three replicates ± S.E.

from Greensleeves apple tissue. Total RNA was extracted from fruit kept at 20 °C for 12 days. The RNA was used to obtain cDNA, which was used as a template in a PCR reaction with specific primers. For AAT, an *AAT* clone showing a higher expression in ripe fruit was isolated. The 1.3 kb partial sequence was 96% identical at the nucleotide level to the sequence registered in the GenBank database for apple (AX025508), and 100% identical to an *AAT* clone isolated from 'Red Delicious' fruit (provided by Randy Beaudry, Michigan State University). Relative to ADH, a 600 bp *ADH* clone was obtained with >98% similarity to the sequence available in the GenBank for 'Granny Smith' apple (Z48234).

To characterize the changes in transcript levels, real time quantitative TaqMan PCR was used. As shown in Fig. 8, the levels of expression of *AAT* in the non-transformed line were higher in fruit evaluated at the end of the holding period, with three to six-fold increase relative to the expression levels at harvest, and these increases were more significant in cortical tissue containing peel. A significant reduction in *AAT* transcript accumulation for *AAT* was observed relative to the non-transformed fruit and only with slight increases after 14 days at 20 °C. The exogenous application of ethylene in these lines showed a massive accumulation of *AAT* transcript, concomitant with AAT enzyme activity levels, reaching a maximum of 20-fold increase for cortical tissue. Similarly, the suppression of ethylene action by 1-MCP inhibited or delayed *AAT* transcript accumulation especially in the cortical tissue, and could not be recovered by the application of ethylene providing the same levels

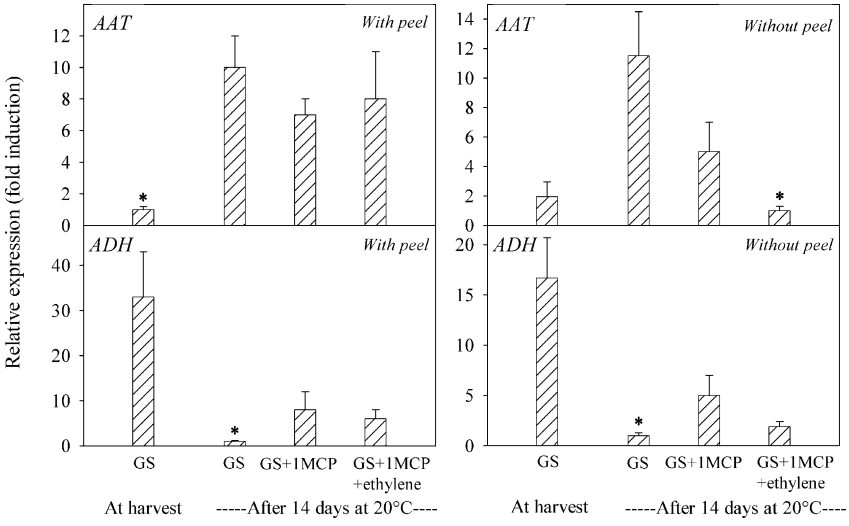

Fig. 9. Relative expression levels of aroma biosynthesis genes in Greensleeves apples treated with 1 μL L^{-1} 1-MCP and stored at 20 °C with or without exposure to 80 μL L^{-1} ethylene. Values were normalized with respect to the internal control *18S rRNA*. The treatment with the lowest level of expression was set as one (*). Data shown are means of three replicates ± S.E.

observed at harvest (Fig. 9). This reduction in *AAT* transcript levels in the 1-MCP-treated fruit was also concomitant with a reduction in AAT enzyme activity, which may indicate a role of *AAT* in the regulation of AAT enzyme activity and in determining ester formation under these conditions. Despite the fact that there was a high inhibition of *AAT* transcript levels in the transgenic lines, we did observe a minor increase in the expression levels through the progress of ripening. Since these lines were held in an atmosphere free of ethylene, other regulatory mechanisms may be important during fruit ripening [17]. The findings showing important differences in the expression pattern in apple tissues, may also suggest that the *AAT* gene studied in this work is not the only *AAT* gene involved in ester production and may be part of a gene family as observed in other fruit species [17,46]. Our results only showed that the changes in ethylene regulation are concomitant with *AAT* transcript levels; however, we do not know whether the modulating effect of ethylene on *AAT* transcripts is done directly by ethylene or through the interaction with other signaling molecules as demonstrated for other metabolic processes [47,48].

For the *ADH* clone, there was a major reduction in transcript levels (>100-fold difference) during the experimental period with changes higher in cortical tissue with peel than without peel (Fig. 8). These dramatic changes in *ADH* transcripts were not correlated with the relatively stable levels of ADH enzyme activity that only showed a slight reduction after 14 days at 20 °C. This result suggests that ADH regulation occurs at the post-transcriptional and translational level, as supported by [49,50]. Contrasting *AAT* transcript levels, ethylene suppression and enhancement did not affect *ADH* transcript levels. This suggests that the dramatic changes in *ADH* transcripts are not regulated by ethylene, and other plant hormones or metabolites may have a role [51,52]. On the other hand, it is important to mention that *ADH* gene is normally present in many species as a gene family with two to seven members; therefore, further work will be required in order to elucidate the presence and function of other *ADH* genes in apples.

4. Conclusions

The availability of the transgenic Greensleeves apples suppressed in ethylene biosynthesis and the ethylene inhibitor 1-MCP allowed the identification and characterization of an important biochemical step modulating aroma via ester production during fruit ripening. Under these conditions, the last step of ester biosynthesis seems to be an important control point, in which AAT enzyme activity and transcripts were highly modulated by ethylene. However, the molecular and functional characterization of the *AAT* clone studied here and its role in ester biosynthesis remain to be elucidated. Another factor determining ester accumulation during ripening was the availability of alcohol substrates, which accumulate following a similar pattern as that observed for the esters; however, our results suggest that ADH enzyme activity is not a limiting step.

Acknowledgements

We acknowledge the Washington Fruit Tree Research Commission for their support of this research. We also thank Christian M. Leutenegger, DVM, Ph.D., FVH, Lucy Whittier Molecular and Diagnostic Core Facility, UC Davis, for expert real-time TaqMan PCR analysis.

References

[1] E. Pichersky, J. Gershenzon, The formation and function of plant volatiles: perfumes for pollinator attraction and defense, Curr. Opin. Plant Biol. 5 (2002) 237–243.
[2] E. Lewinsohn, F. Schalechet, J. Wilkinson, K. Matsui, Y. Tadmor, K.-H. Nam, O. Amar, E. Lastochkin, O. Larkov, U. Ravid, W. Hiatt, S. Gepstein, E. Pichersky, Enhanced levels of the aroma and flavor compound S-Linalool by metabolic engineering of the terpenoid pathway in tomato fruits, Plant Physiol. 127 (2001) 1256–1265.
[3] J.K. Fellman, T.W. Miller, D.S. Mattinson, J.P. Mattheis, Factors that influence biosynthesis of volatile flavor compound in apple fruits, HortScience 35 (2000) 1026–1033.
[4] J. Dixon, E.W. Hewett, Factors affecting apple aroma/flavour volatile concentration: a review, N. Z. J. Crop Hort. Sci. 28 (2000) 155–173.
[5] A. Theologis, One rotten apple spoils the whole bushel: the role of ethylene in fruit ripening, Cell 70 (1992) 181–184.
[6] N.A. Mir, R. Perez, P. Schwallier, R. Beaudry, Relationship between ethylene response manipulation and volatile production in Jonagold variety apples, J. Agric. Food Chem. 47 (1999) 2653–2659.
[7] B.G. Defilippi, A.M. Dandekar, A.A. Kader, Impact of suppression of ethylene action or biosynthesis on flavor metabolites in apple (*Malus domestica Borkh*) fruits, J. Agric. Food Chem. 52 (2004) 5694–5701.
[8] X. Fan, J.P. Mattheis, D. Buchanan, Continuous requirement of ethylene for apple fruit volatile synthesis, J. Agric. Food Chem. 46 (1998) 1959–1963.
[9] S. Lurie, C. Pre-Aymard, U. Ravid, O. Larkov, E. Fallik, Effect of 1-methylcyclopropene on volatile emission and aroma in cv. Anna apples, J. Agric. Food Chem. 50 (2002) 4251–4256.
[10] A.D. Bauchot, D.S. Mottram, A.T. Dodson, J. John, Effect of aminocyclopropane-1-carboxylic acid oxidase antisense gene on the formation of volatile esters in cantaloupe Charentais melon (cv. Vedrandais), J. Agric. Food Chem. 46 (1998) 4787–4792.
[11] F. Flores, M. Ben Amor, B. Jones, J.C. Pech, M. Bouzayen, A. Latché, F. Romojaro, The use of ethylene-suppressed lines to assess differential sensitivity to ethylene of the various ripening pathways in cantaloupe melons, Physiol. Plant. 113 (2001) 128–133.
[12] N.M.M. Paillard, Biosynthese des produits volatils de la pomme formation des alcohols et des esters a partir des acids gras, Phytochemistry 18 (1979) 1165–1171.
[13] I.M. Bartley, P.G. Stoker, A.D.E. Martin, S.G.S. Hatfield, M. Knee, Synthesis of aroma compounds by apples supplied with alcohols and methyl esters of fatty acids, J. Sci. Food Agric. 36 (1985) 567–574.
[14] G. Echeverría, J. Graell, M.L. López, I. Lara, Volatile production, quality and aroma-related enzyme activies during maturation of 'Fuji' apples, Postharv. Biol. Technol. 31 (2004) 217–227.
[15] M. Harada, Y. Ueda, T. Iwata, Purification and some properties of alcohol acyltransferase from banana fruit, Plant Cell Physiol. 26 (1985) 1067–1074.
[16] M. Shalit, N. Katzir, Y. Tadmor, O. Larkov, Y. Burger, F. Shalekhet, E. Lastochkin, U. Ravid, O. Amar, M. Edelstein, Z. Karchi, E. Lewin-

sohn, Acetyl Co-A: alcohol acetyltransferase activity and aroma formation in ripening melon fruits, J. Agric. Food Chem. 49 (2001) 794–799.
[17] F.E.L. Yahyaoui, C. Wongs-Aree, A. Latché, R. Hackett, D. Grierson, J.C. Pech, 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 (2002) 2359–2366.
[18] A.G. Pérez, C. Sanz, J.M. Olías, Partial purification and some properties of alcohol acyltransferase from strawberry fruits, J. Agric. Food Chem. 41 (1993) 1462–1466.
[19] A. Aharoni, L.C.P. Keizer, H.J. Bouwmeester, Z. Sun, M. Alvarez-Huerta, H.A. Verhoeven, J. Blaas, A.M.M.L. van Houwelingen, R.C.H. De Vos, H. van der Voet, R.C. Jansem, M. Guis, J. Mol, R.W. Davis, M. Schena, A.J. van Tunen, A.P. O'Connell, Identification of the SAAT gene involved in strawberry flavor biogenesis by use of DNA microarray, Plant Cell 12 (2000) 647–661.
[20] A.G. Pérez, C. Sanz, R. Olias, J.J. Rios, J.M. Olias, Evolution of strawberry alcohol acyltransferase activity during fruit development and storage, J. Agric. Food Chem. 44 (1996) 3286–3290.
[21] R.G. Berger, F. Drawert, Changes in the composition of volatiles by post-harvest application of alcohols to red delicious apples, J. Sci. Food Agric. 35 (1984) 1318–1325.
[22] A.R.S. Chen, T. Chase, Alcohol dehydrogenase 2 and pyruvate decarboxylase induction in ripening and hypoxic tomato fruit, Plant Physiol. Biochem. 31 (1993) 875–885.
[23] J. Speirs, E. Lee, K. Holt, K. Yong-Duk, N.S. Scott, B. Loveys, W. Schuch, Genetic manipulation of alcohol dehydrogenase levels in ripening tomato fruit affects the balance of some flavor aldehydes and alcohols, Plant Physiol. 117 (1998) 1047–1058.
[24] S. Prestage, R.S.T. Linforth, A.J. Taylor, E. Lee, J. Speirs, W. Schuch, Volatile production in tomato fruit with modified alcohol dehydrogenase activity, J. Sci. Food Agric. 79 (1999) 131–136.
[25] N.J. Bate, J.C.M. Riley, J.E. Thompson, S.J. Rothstein, Quantitative and qualitative differences in C-6-volatile production from the lipoxygenase pathway in an alcohol dehydrogenase mutant of *Arabidopsis thaliana*, Physiol. Plant. 104 (1998) 97–104.
[26] A.M. Dandekar, G. Teo, B.G. Defilippi, S.L. Uratsu, A.J. Passey, A.A. Kader, J.R. Stow, R.J. Colgan, D.J. James, Effect of down-regulation of ethylene biosynthesis on fruit flavor complex in apple fruit, Transgenic Res. 13 (2004) 373–384.
[27] W.K. Yip, X.Z. Jiao, S.F. Yang, Dependence of in vivo ethylene production rate on 1-aminocyclopropene-1-carboxylic acid content and oxygen concentrations, Plant Physiol. 88 (1991) 553–558.
[28] J.G. Dong, J.C. Fernandez-Maculet, S.F. Yang, Purification and characterization of 1-aminocyclopropane-1-carboxylate oxidase from apple fruit, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 9789–9793.
[29] J.C. Fernandez-Maculet, S.F. Yang, Extraction and partial characterization of ethylene forming enzyme from apple fruit, Plant Physiol. 99 (1992) 751–754.
[30] M.C.C. Lizada, S.F. Yang, A simple and sensitive assay for 1-aminocyclopropene-1-carboxylic acid, Anal. Biochem. 100 (1979) 140–145.
[31] J.K. Fellman, J.P. Mattheis, Ester biosynthesis in relation to harvest, maturity and controlled atmosphere storage of apples, in: M. Leahy, R. Rouseff (Eds.), Fruit Flavor: Biogenesis, Characterization, and Authentication, ACS Symposium Series 596, American Chemical Society, Washington, DC, 1995, pp. 149–162.
[32] D. Ke, L. Zhou, A.A. Kader, Mode of oxygen and carbon dioxide action on strawberry ester biosynthesis, J. Am. Soc. Hort. Sci. 119 (1994) 971–975.
[33] I.M. Bartley, S.J. Hindley, Alcohol dehydrogenase of apple, J. Exp. Bot. 121 (1980) 449–459.
[34] M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[35] C.M. Leutenegger, C.N. Mislin, B. Sigrist, M.U. Ehrengruber, R. Hofmann-Lehmann, H. Lutz, Quantitative real-time PCR for the measurement of feline cytokine mRNA, Vet. Immunol. Immunopathol. 71 (1999) 291–305.
[36] C.Y. Wan, T.A. Wilkins, A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.), Anal. Biochem. 223 (1994) 7–12.
[37] D.A. Hunter, B.C. Steele, M.S. Reid, Identification of genes associated with perianth senescence in Daffodil (*Narcissus pseudonarcissus* L. 'Dutch Master'), Plant Sci. 153 (2002) 13–21.
[38] R. Chairat, Biochemical and molecular responses of 'Thompson Seedless' grapes to insecticidal controlled atmosphere, Ph.D. dissertation, University of California, Davis, 2003, p. 88.
[39] D.R. Rudell, D.S. Mattinson, J.K. Fellman, J.P. Mattheis, The progression of ethylene production and respiration in the tissues of ripening 'Fuji' apple fruit, HortScience 35 (2000) 1300–1303.
[40] T. Sunako, W. Sakuraba, M. Senda, S. Akada, R. Ishikawa, M. Niizeki, T. Harada, An allele of the ripening-specific 1-aminocyclopropane-1-1carboxylic acid synthase gene (*ACS1*) in apple fruit with a long storage life, Plant Physiol. 119 (1999) 1297–1303.
[41] X. Fan, J.P. Mattheis, Impact of 1-methylcyclopropene and methyl jasmonate on apple volatile production, J. Agric. Food Chem. 47 (1999) 2847–2853.
[42] J. Song, F. Bangerth, The effect of harvest date on aroma compound production from golden delicious apple fruit and relationship to respiration and ethylene production, Postharv. Biol. Technol. 8 (1996) 259–269.
[43] A.A. Saquet, J. Streif, F. Bangerth, Impaired aroma production of CA-stored 'Jonagold' apples as affected by adenine and pyridine nucleotide levels and fatty acid concentration, J. Hort. Sci. Biotechnol. 78 (2003) 695–705.
[44] J. Song, F. Bangerth, Fatty acids as precursors for aroma volatile biosynthesis in pre-climacteric and climacteric apple fruit, Postharv. Biol. Technol. 30 (2003) 113–121.
[45] T.J. Longhurst, H.F. Tung, C.J. Brady, Developmental regulation of the expression of alcohol dehydrogenase in ripening tomato fruits, J. Food Biochem. 14 (1990) 421–433.
[46] J. Beekwilder, M. Alvarez-Huerta, E. Neef, F.W.A. Verstappen, H.J. Bouwmeester, A. Aharoni, Functional characterization of enzyme forming volatile esters from strawberry and banana, Plant Physiol. 135 (2004) 1865–1878.
[47] S. Gazzarrini, P. McCourt, Genetic interactions between ABA, ethylene and sugar signaling pathways, Curr. Opin. Plant Biol. 4 (2001) 387–391.
[48] K. Grossmann, C. Rosenthal, J. Kwiatkowski, Increases in jasmonic acid caused by indol-3-acetic acid and auxin herbicides in cleavers (*Galium aparine*), J. Plant Physiol. 161 (2004) 809–814.
[49] M.E. Vayda, C. Webster, Translation regulation during periods of environmental stress, in: J. Bailey-Serres, D.R. Gallie (Eds.), A Look Beyond Transcription: Mechanisms Determining mRNA Stability and Translation in Plants, American Society of Plant Physiologists, Rockville, MD, 1998, pp. 102–114.
[50] C. Tesnière, C. Verriès, Molecular cloning and expression of cDNAs encoding alcohol dehydrogenase from *Vitis vinifera* L. during berry development, Plant Sci. 157 (2000) 77–88.
[51] H.K. Noguchi, Induction of alcohol dehydrogenase by plant hormones in alfalfa seedlings, Plant Growth Regul. 30 (2000) 1–3.
[52] P.K. Macnicol, J.V. Jacobsen, Regulation of alcohol dehydrogenase gene expression in barley aleurone by gibberellin and abscisic acid, Physiol. Plant. 111 (2001) 533–539.