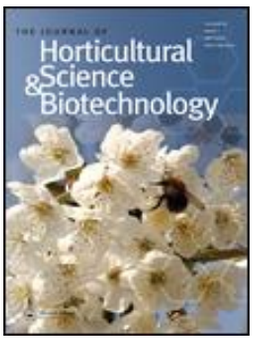

The Journal of Horticultural Science and Biotechnology

ISSN: 1462-0316 (Print) 2380-4084 (Online) Journal homepage: http://www.tandfonline.com/loi/thsb20

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To cite this article: Jamil Harb, Isabel Lara, Omar Saleh, Josef Streif & Basel Khraiwesh (2011) Treatments that suppress ethylene production or ethylene action modify ADH and AAT gene expression and aroma-related enzyme activities in 'Delbarde Estivale' apple: consequences for the aroma profiles of fruit, The Journal of Horticultural Science and Biotechnology, 86:2, 182-188, DOI: 10.1080/14620316.2011.11512745

To link to this article: http://dx.doi.org/10.1080/14620316.2011.11512745

Published online: 07 Nov 2015.

Journal of Horticultural Science & Biotechnology (2011) **86** (2) 182–188

Treatments that suppress ethylene production or ethylene action modify *ADH* and *AAT* gene expression and aroma-related enzyme activities in 'Delbarde Estivale' apple: consequences for the aroma profiles of fruit

By JAMIL HARB[1], ISABEL LARA[2], OMAR SALEH[3], JOSEF STREIF[4] and BASEL KHRAIWESH[5,*]

[1]Department of Biology and Biochemistry, Birzeit University, P.O. Box 7, Birzeit, West Bank, Palestine

[2]Departament de Química, Unitat de Postcollita-XaRTA, Universitat de Lleida, Rovira Roure 191, 25198 Lleida, Spain

[3]Plant Biotechnology, Faculty of Biology, University of Freiburg, Schänzlestraße 1, 79104 Freiburg, Germany

[4]Kompetenzzentrum Obstbau-Bodensee (KOB), Schuhmacherhof 6, 88213 Ravensburg, Germany

[5]Department of Plant Systems Biology, VIB and Department of Plant Biotechnology and Genetics, Ghent University, Technologiepark 927, 9052 Ghent, Belgium

(e-mail: bakhr@psb.vib-ugent.be) *(Accepted 4 November 2010)*

SUMMARY

The effects of aminoethoxyvinylglycine (AVG; ReTain®) and 1-methylcyclopropene (1-MCP; SmartFresh™) on the biosynthesis of aroma volatiles and overall quality in 'Delbarde Estivale', an early apple variety, are reported for the first time. Apple fruit were treated with AVG 3 weeks before commercial harvest, following standard procedures. 1-MCP was applied directly after harvest. Treated and untreated fruit were kept at room temperature and analysed periodically thereafter. The results indicated that both ethylene-suppressing treatments improved fruit firmness, total acidity, and preserved skin colour during shelf-life. However, both treatments had a negative impact on the biosynthesis of aroma volatiles, a major quality attribute for the sensory quality of apples. The production of straight-chain esters was particularly affected. The activities of lipoxygenase (LOX), alcohol dehydrogenase (ADH), and pyruvate decarboxylase (PDC) were reduced by both treatments, in some instances to values well below 50% of those in untreated controls; whereas the activity of alcohol *o*-acyltransferase (AAT) was higher in 1-MCP-treated fruit. Measuring the levels of expression of the genes encoding ADH and AAT confirmed these results and the importance of an adequate supply of substrates for the biosynthesis of volatile esters in apple fruit.

It is widely accepted that ethylene plays a crucial role in promoting fruit ripening, although it is well known that this physiological process includes both ethylene-dependent and ethylene-independent processes that occur simultaneously (Silverman *et al*., 2004). The biosynthesis of aroma volatiles, a major ripening-related event, has been reported to be partially dependent on ethylene, although some regulatory differences appear to exist among different fruit species and cultivars. For instance, continuous removal of ethylene during long-term storage of pre-climacteric 'Gloster 69' apples kept under 2.0 kPa O_2 at 15ºC caused a general depletion in the emission of aroma volatiles, although the levels of some important compounds (e.g., 2-methyl butyl acetate and hexyl acetate) were apparently not affected (Tsantili and Knee, 1991). In contrast, Defilippi *et al*. (2005) found that the activities of alcohol dehydrogenase (ADH; EC 1.1.1.1) and lipoxygenase (LOX; EC 1.13.11.12), two important enzymes that supply substrates for the biosynthesis of volatile esters, were ethylene-independent. Interestingly, the binding of straight chain, branched chain, aromatic, and terpene alcohol substrates to alcohol *o*-acyltransferase (AAT; EC 2.3.1.84) has been reported to be rate-limiting for the final step in ester biosynthesis (Souleyre *et al*., 2005). Moreover, the enzyme AAT1 from *Malus pumila* (MpAAT) was shown to produce hexyl esters of C3, C6, and C8 CoAs, preferentially, although its eventual preference for a substrate also depended upon substrate concentration.

The ethylene-dependence of the biosynthesis of aroma volatiles is important, because the increasing use of ethylene-suppressing chemicals, mainly aminoethoxy-vinylglycine (AVG) and 1-methylcyclopropene (1-MCP) could have negative consequences on the eating quality of fruit, as aroma is an important sensory attribute for acceptance by consumers. AVG, which is generally used to prevent fruit-drop (Robinson *et al*., 2006) and to delay harvest, blocks the production of ethylene through inhibition of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity, thereby retarding several key ripening-related events such as starch degradation and fruit softening (Silverman *et al*., 2004). AVG has also

*Author for correspondence.

been found to inhibit aroma volatile production in some apple cultivars (Fan *et al*., 1998; Mir *et al*., 1999), although not all compounds were apparently affected to the same extent. Conversely, 1-MCP binds to ethylene receptors with ten-fold higher affinity than ethylene itself (Blankenship and Dole, 2003). 1-MCP therefore acts by antagonising the action of ethylene, thus impairing ethylene-dependent events during fruit ripening. 1-MCP treatment is known to maintain the firmness and acidity of fruit flesh, and to extend the shelf-life of apples (Li *et al*., 2006), but 1-MCP has also been reported to suppress the production of flavour-related volatile esters (Kondo *et al*., 2005; Li *et al*., 2006), thus altering the aroma profile of the fruit.

Because of the quantitative relevance of esters in the volatile fraction emitted by apples (Dixon and Hewett, 2000) and other important fruit species, research on the effects of commercial procedures on the biosynthesis of aroma-active volatile compounds have focussed mainly on *AAT* gene expression and activity. However, because the supply of the alcohol precursor has consistently been shown to be essential for the production of volatile esters in different fruit species (Beekwilder *et al*., 2004; Lara *et al*., 2003; Ortiz *et al*., 2010a,b), it was thought necessary to widen these studies to include other key enzyme activities in order to gain a wider overview of the mechanisms underlying the development and modification of the aroma profile of fruit in response to different treatments.

In this work, the effects of pre-harvest (AVG) or post-harvest (1-MCP) treatments for the suppression of ethylene on the biosynthesis of volatile aroma compounds were assessed during the shelf-life of 'Delbarde Estivale' apple fruit at room temperature.

MATERIALS AND METHODS

Plant material and treatment with 1-MCP or AVG

'Delbarde Estivale' apple (*Malus* × *domestica* Borkh.) trees growing at the Kompetenzzentrum Obstbau-Bodensee (KOB) experimental orchard near Ravensburg (Germany) were selected for uniform growth, fruit set, and fruit size. AVG (ReTain®; Valent BioSciences, Libertyville, IL, USA) was applied at 125 µl l^{-1} a.i. 3 weeks before harvest. Non-treated and AVG-treated samples were harvested at commercial maturity and selected for their uniformity and absence of defects. After harvest, 50% of the untreated fruit (n = 500) were treated with 1-MCP at 0.625 µl l^{-1} for 24 h at 10ºC. Fruit (500 fruit treatment^{-1}) from each of the three treatments were then stored at room temperature (20ºC), and samples were taken periodically for analysis, as described below.

Analysis of fruit quality and maturity parameters

Fruit firmness (FF), total soluble solids (TSS) contents, and titratable acidity (TA) were measured each week on 20 individual fruit per treatment using a semi-automated penetrometer (Guess Fruit Texture Analyzer; Strand, South Africa), a digital refractometer (PR-1; Atago Co. Ltd., Tokyo, Japan), and by titration of 10% (v/v) juice to pH 8.1 with 0.1 M NaOH, respectively.

Skin colour was also measured using a colorimeter (CR-300; Minolta Co. Ltd., Osaka, Japan), applying CIE illuminant D65 and an 8 mm-diameter measuring aperture. L*, a*, and b* values were recorded.

Rates of respiration and ethylene production were measured each day at 08.00 h (in triplicate) for each treatment (ten fruit per replicate), using samples enclosed in tightly-closed jars incubated at room temperature. An infrared gas analyser (URAS3G; Hartmann & Braun AG, Frankfurt, Germany) was used for all CO_2 measurements. To measure ethylene production, 1 ml headspace-gas samples were taken from each jar and injected into a Carlo-Erba gas chromatograph (Fractovap 2150; Thermo-Finnigan, Egelsbach, Germany) equipped with a flame ionisation detector (FID) and an alumina oxide column (0.9 m × 1/8 steel, filled with 80-mesh activated Al-oxide), with an injector temperature of 150ºC, and an oven temperature of 100ºC.

Analysis of volatile aroma compounds

Sections of unpeeled fruit (five fruit per replicate; three replicates) were taken each week, shock-frozen in liquid nitrogen (N_2), and kept at –30ºC until analysis. Each frozen sample was ground to a coarse powder, from which 45 g were added to 15 ml saturated NaCl solution and mixed vigorously. The slurry was transferred to a 50 ml tube, and further homogenised using a IKA T 10 Basic Ultra-Turrax Homogeniser (T25 basic; IKA Labortechnik, Staufen, Germany) for 2 min at 20,000 rpm, then centrifuged at 18,400 × *g* for 15 min at 4ºC. Twelve ml of the clear supernatant was placed in a 25 ml vial to adsorb the volatile aroma compounds by means of headspace solid-phase micro-extraction (HS-SPME). One µl of a 1.92 mM cyclohexanone solution was added as an internal standard. The vials were stirred at constant speed for 50 min at 30ºC prior to the insertion of a fused-silica fibre coated with polydimethyl siloxane (100 µm-thick) which was exposed to the headspace for 60 min. A manual SPME Fibre Holder (Supelco Co., Bellefonte, PA, USA) was used to insert the fibre into the sample vial. For GC analysis, the volatile compounds were thermally desorbed from the SPME fibre for 10 min in the injection port of the gas chromatograph. GC/MS was performed using a gas chromatograph (GC-2010 Series; Shimadzu, Duisburg, Germany) equipped with a Zebron capillary column (ZB-WAX; 30 m × 0.25 mm i.d.; 0.25 µm film thickness; Phenomenex, Aschaffenburg, Germany) coupled to a QP2010 mass spectrometer (Shimadzu). The oven temperature was held at 35ºC for 5 min, then raised to 180ºC at 5ºC min^{-1}. The carrier gas (helium) inlet pressure was 64.3 kPa, at a linear velocity of 40 cm s^{-1}, a total flow rate of 2.2 ml min^{-1}, and a column flow rate of 1.24 ml min^{-1}, with an injection temperature of 220ºC. The splitless mode was used for injection. For MS analysis, the ion source temperature was 200ºC, the interface temperature was 190ºC, the solvent cut-time was 0.51 min, and the mass scan range was 40 – 250 m/z. Electron ionisation took place at 70 eV. The identification, quantification, and calibration of volatile aroma compounds were conducted by GC/MS under the conditions mentioned above. Authentic standard compounds were obtained from Sigma-Aldrich Chemie (Munich, Germany) and Carl-Roth (Karlsruhe, Germany). The mass spectra of the designated compounds were matched with the NIST Library of

TABLE I
Primers for PCR amplification of the gene-specific probes used for northern analysis of gene expression

Gene name		Primers
Alcohol dehydrogenase (*ADH*)	F	5'-GGAAGCCAAGGGACAAAACC-3'
	R	5'-CACGCCCTCACCAACACTCT-3'
Alcohol *o*-acyltransferase (*AAT*)	F	5'-GGTACCAATGTGTTTGCCATTCT-3'
	R	5'-ACACTTACATCATTGACATGATCCTAGTT-3'

standard compounds and their retention times. The volatile aroma compounds were quantified using external standards and calibration curves, after correction with the same internal standard (i.e., 1.0 µl of 1.92 mM cyclohexanone). The calibration curves were obtained using a series of dilutions of the standard mixture in undiluted juice of 'Delbarde Estivale' apple to overcome the matrix effect, since each specific juice has its own matrix. Matrix is the capacity of fruit juice to retain aroma-volatiles.

Analysis of gene expression

Total RNA was isolated as described by Chang *et al*. (1993), with slight modifications. Briefly, β-mercapto-ethanol was added [2% (v/v), final concentration] to the CTAB buffer, and pre-warmed to 65°C. Unpeeled apple samples were frozen in liquid N_2, finely ground, and 1.0 g was suspended in 10 ml extraction buffer and incubated for 10 min at 65ºC prior to extraction with 24:1 (v/v) chloroform:isoamyl alcohol followed by precipitation and recovery using 5.0 M lithium chloride and 70% (v/v) ethanol, respectively. Total RNA was purified using RNeasy spin columns (QIAGEN GmbH, Hilden, Germany). RNA quality and quantity were assessed by 1.0% (w/v) agarose gel electrophoresis and by A_{260}:A_{280} ratios, respectively.

Twenty µg of total RNA was dissolved in 20 µl of RNA-denaturing buffer [500 µl deionised formamide, 120 µl 37% (v/v) formaldehyde, 200 µl 10× MOPS (0.2 M 3-morpholinopropane-1-sulphonic acid, pH 7.0, 20 mM sodium acetate, 10 mM EDTA, in diethylpyrocarbonate (DEPC)-treated H_2O), and 1 µl ethidium bromide] and incubated for 10 min at 65°C. Total RNA was separated by electrophoresis in a 1% (w/v) agarose-formaldehyde gel [1.5 g agarose, 108 ml H_2O, 15 ml 10× MOPS buffer and 27 ml 37% (v/v) formaldehyde] and blotted onto a Hybond-N^+ nylon membrane (GE Healthcare, Munich, Germany) using a Turbo blotter (Schleicher & Schuell, Dassel, Germany). The RNA was fixed to the membrane by UV cross-linking. Membranes were pre-hybridised for 4 h at 67ºC, then hybridised overnight at 67ºC with [α-^{32}P] dCTP-labelled DNA probes derived from *ADH* (Accession No. Z48234) and *AAT* (Accession No. AY517491) gene sequences, amplified using the PCR primers described in Table I. DNA-labelling was carried out using the Rediprime II Random Prime Labeling Kit (GE Healthcare). Northern blots were washed three-times with 0.5× SSC containing 0.1% (w/v) SDS and once with 1× SSC containing 0.1% (w/v) SDS at 67°C. ^{32}P signals were detected using a Molecular Imager FX (Bio-Rad, Munich, Germany).

Assessment of aroma-related enzyme activities

Separate samples of unpeeled apple cortex were removed from each of five apples from each treatment, shock-frozen in liquid nitrogen, lyophilised, and powdered. Lyophilized powder (100 mg) was used for each determination.

Extraction and assays for lipoxygenase (LOX), pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), and alcohol *o*-acyltransferase (AAT) activities were performed on crude enzyme extracts as described elsewhere (Lara *et al*., 2003). Hydroperoxide lyase (HPL) activity was assayed according to Vick (1991). Total protein contents in the crude enzyme extracts were determined according to the Bradford (1976) method, using bovine serum albumin as the standard. In all cases, one Unit of enzyme activity was defined as causing a change of 1 absorbance unit min^{-1} at the appropriate wavelength. The results were expressed as specific activities (Units mg^{-1} protein).

Statistical analysis

All results were subjected to analysis of variance (ANOVA) using 1998 CoStat-software (CoHort Software, Monterrey, CA, USA). Means separation was done using Duncan's Multiple Range Test at $P \leq 0.05$.

RESULTS AND DISCUSSION

Both treatments (AVG or 1-MCP) resulted in a significant decrease in the rates of ethylene production and respiration (Table II). Treated fruit ripened more slowly, with improved preservation of FF, TSS content,

TABLE II
Influence of AVG or 1-MCP treatment on maturity and quality attributes of 'Delbarde Estivale' apple fruit kept at room temperature for 7, 14, or 21 d after harvest

	7 d			14 d			21 d		
Parameter	Control	AVG	1-MCP	Control	AVG	1-MCP	Control	AVG	1-MCP
Rate of ethylene production (µl kg^{-1} h^{-1})	16.6 a[†]	0 b	0 b	49.2 a	0.4 b	0.5 b	38.7 a	0.4 b	0 c
Rate of CO_2 production (ml kg^{-1} h^{-1})	33.3 a	12.8 b	15.2 b	31.7 a	11.8 b	11.5 b	28.5 a	11 b	9.7 b
Firmness (N)	5.8 b	7.0 a	6.8 a	5.3 b	6.5 a	6.8 a	5.4 c	6.1 b	7.2 a
TSS content (g l^{-1})	15.6 a	14.6 a	15.4 a	14.9 b	14.4 b	15.8 a	14.6 b	15.2 a	15.5 a
TA (g l^{-1})	9.1 a	9.5 a	10.2 a	7.5 c	8.5 b	9.5 a	6.8 b	7.7 b	9.2 a
L* value	76.5 a	72.9 b	75.8 a	78.5 a	72.9 c	74.4 b	79.0 a	77.4 a	75.5 a
a* value	–13.3 a	–16.4 b	–13.9 a	–10.0 a	–14.5 b	–14.2 b	–6.8 a	–9.8 b	–11.7c
b* value	42.7 a	42.0 a	39.2 b	46.9 a	45.3 ab	43.4 b	48.9 a	49.6 a	42.3 b

[†]All values are the means of 20 (standard quality parameters) or three [rates of ethylene production and respiration (CO_2 production)] replicates. Mean values followed by a different lower-case letter within each row for a given shelf-life period indicate significant differences at $P \leq 0.05$.

TABLE III
Influence of AVG or 1-MCP treatment on the aroma-active compounds (μg kg^{-1} FW) detected in the volatile fraction emitted by 'Delbarde Estivale' apple fruit kept at room temperature for 1, 7, 14, or 21 d after harvest

		1 d			7 d			14 d			21 d		
Aroma volatile	RT[‡]	Control	AVG	1-MCP	Control	AVG	1-MCP	Control	AVG	1-MCP	Control	AVG	1-MCP
Ethyl 2-methylbutanoate	5.80	0.000a[†]	0.000a	0.000a	0.000a	0.000a	0.000a	0.091a	0.000b	0.000b	0.122a	0.085b	0.000c
Butyl acetate	6.46	0.171a	0.046b	0.170a	0.586a	0.254b	0.181c	0.619a	0.469b	0.279c	0.426a	0.292c	0.366b
Hexanal	6.63	0.361b	0.197c	0.627a	0.504a	0.334b	0.272c	1.109a	1.189a	0.255b	0.961a	0.790b	0.531c
3-Methylbutyl acetate	7.58	0.854a	0.077b	0.975a	0.543b	0.340c	0.602a	0.965a	0.870a	0.670b	0.637b	0.868a	0.410c
Pentyl acetate	8.93	0.101a	0.053b	0.107a	0.123ab	0.139a	0.11b	0.118b	0.163a	0.086c	0.065c	0.106b	0.137a
Butyl propanoate	8.04	0.024c	0.098a	0.034b	0.155a	0.06b	0.05c	0.117b	0.193a	0.040c	0.104b	0.199a	0.081c
1-Butanol	8.84	0.020b	0.016c	0.080a	0.717a	0.129c	0.143b	0.852a	0.554b	0.153c	1.585a	0.666b	0.222c
Butyl butanoate	10.12	0.092a	0.037b	0.085a	0.195a	0.060c	0.095b	0.117a	0.121a	0.021b	0.211a	0.143b	0.105c
2-Methylbutanol	10.37	0.000c	0.010b	0.264a	0.247b	0.012c	0.396a	0.210b	0.114c	0.325a	0.000c	0.391a	0.031b
Butyl 2-methylbutanoate	10.45	0.057a	0.005b	0.047a	0.283a	0.022c	0.044b	0.000c	0.033a	0.005b	0.127a	0.073b	0.039c
1-Pentanol	11.49	0.157a	0.046c	0.135b	0.328a	0.177c	0.207b	0.317a	0.301a	0.219b	0.423a	0.355b	0.169c
Hexyl acetate	11.73	0.440b	0.189c	0.585a	2.080a	1.576b	0.738c	2.247b	2.701a	1.081c	1.377ab	1.396a	1.238b
3-Methylbutyl butanoate	12.91	0.003a	0.000c	0.001b	0.014a	0.003b	0.002b	0.000c	0.001b	0.002a	0.000b	0.001a	0.000b
Propyl hexanoate	13.15	0.000a	0.000a	0.000a	0.036a	0.003c	0.005b	0.071a	0.012b	0.007c	0.053a	0.037b	0.008c
Hexyl propanoate	13.56	0.028b	0.012c	0.068a	0.222a	0.089b	0.092b	0.183b	0.418a	0.083c	0.198a	0.090b	0.054c
1-Hexanol	14.21	0.086c	0.648a	0.144b	0.457a	0.238b	0.146c	0.576b	0.634a	0.179c	0.867a	0.468b	0.182c
Heptyl acetate	14.53	0.000b	0.000b	0.001a	0.001b	0.001b	0.000a	0.002a	0.001b	0.001b	0.001a	0.001a	0.001a
Butyl hexanoate	15.54	0.008a	0.002c	0.004b	0.119a	0.014b	0.008c	0.125a	0.034b	0.019c	0.100a	0.052b	0.019c
Hexyl butanoate	15.58	0.012b	0.002c	0.013a	0.345a	0.048b	0.035c	0.313a	0.116b	0.056c	0.290a	0.148b	0.058c
Hexyl 2-methylbutanoate	15.88	0.297c	0.611a	0.369b	0.391b	0.507a	0.405b	0.374b	0.402a	0.321c	0.218a	0.054b	0.211a
1-Heptanol	16.87	0.062a	0.016b	0.06a	0.000b	0.060a	0.000b	0.000b	0.060a	0.000b	0.142a	0.000c	0.054b
Octyl acetate	17.21	0.000a	0.000a	0.000a	0.003a	0.001b	0.000c	0.004a	0.002b	0.000c	0.000b	0.001a	0.001a
Decanal	17.77	1.861a	1.113b	1.051b	1.423b	1.418b	2.177a	1.768a	1.365b	1.390b	0.990b	0.955b	1.310a
(E)-2-Nonenal	18.68	0.005b	0.004c	0.011a	0.012a	0.009b	0.010b	0.010b	0.013a	0.010b	0.009b	0.009b	0.013a
1-Octanol	19.42	0.033b	0.017c	0.043a	0.121a	0.067b	0.039c	0.144a	0.115b	0.045c	0.184a	0.085b	0.048c
Hexyl hexanoate	20.51	0.013a	0.003b	0.011b	0.222a	0.116b	0.084c	0.237a	0.12b	0.101b	0.185a	0.125b	0.094b
Butyl heptanoate	22.86	0.000a	0.000a	0.000a	0.421a	0.105b	0.097b	1.21a	1.681b	1.561c	0.881a	0.678b	0.247c
α-Farnesene	23.70	0.012a	0.001b	0.009a	1.526a	0.077c	0.281b	3.944a	0.427b	0.707b	2.164a	1.74b	1.071c
Hexyl octanoate	25.06	0.001a	0.000b	0.000b	0.015a	0.003b	0.003b	0.043a	0.011b	0.004c	0.020a	0.018a	0.006b
Nonanoic acid	32.58	0.018a	0.014b	0.016ab	0.017b	0.018ab	0.02a	0.022a	0.017b	0.023a	0.019b	0.017b	0.025a
TOTAL	–	4.71a	3.22b	4.91a	11.23a	5.88b	6.22b	15.78a	12.14b	7.64c	12.36a	9.84b	6.73c

[†]All values are the means of three replicates (five fruit per replicate). Mean values followed by a different lower-case letter within the same row for a given shelf-life period indicate significant differences at $P \leq 0.05$.
[‡]RT, retention time (min) on a ZB-WAX column.

and colour attributes (a* and b* values). In addition, 1-MCP-treated fruit retained higher TA values after 2 and 3 weeks at 20ºC, while AVG treatment was not so effective. These effects on ripening-related fruit metabolism were also reflected in the production of volatile aroma compounds, that was negatively affected by both ethylene-suppressing treatments, especially 1-MCP (Table III). The effects of 1-MCP were generally more severe and long-lasting compared to AVG, particularly after 2 and 3 weeks of storage at 20ºC. Thirty compounds were identified in the volatile fraction, including 19 esters, among which 14 were of the straight-chain type that are generally believed to be fatty acid derivatives (Schwab *et al.*, 2008). When the production of volatile esters was considered, important changes were observed in the responses to both treatments, although not all esters were affected equally.

The emission of branched-chain esters did not differ significantly between treated and untreated fruit. No consistent trend could be observed, suggesting that both ethylene-dependent and ethylene-independent pathways exist for the production of this family of volatile esters. For example, the emission of ethyl 2-methylbutanoate was significantly reduced by both ethylene-suppressing treatments, in particular after 2 and 3 weeks of storage. In contrast, the production of 3-methylbutyl acetate, a major branched-chain ester in apple, was differentially affected by the application of AVG and 1-MCP. While AVG caused a transitory reduction in the production of this aroma-volatile, 1-MCP increased its production transiently (Table III).

In contrast, the biosynthesis of most straight-chain esters was significantly reduced by both treatments, especially during the first 2 weeks after harvest. Butyl and hexyl esters, which are prominent among the volatiles emitted by apple, were particularly affected (Table III). Reduced emissions of butyl and hexyl esters might have arisen from an inhibition of AAT activity, as ATT catalyses the final step in their biosynthetic pathway. However, our data indicate that treatment with AVG did not significantly affect AAT activity, while, in 1-MCP-treated samples, ATT activity was higher than in the controls (Table IV). *AAT* gene expression levels were also higher in AVG or 1-MCP treated than in untreated fruit (Figure 1). Therefore, the decrease in ester production detected in treated fruit was apparently independent of AAT activity. These observations, together with the limited effect of both ethylene-suppressing treatments on the emission of branched-chain esters (Table III), the final step of which is also catalysed by AAT, suggest that the specific decrease in straight-chain esters might have arisen from changes in the upstream regulation of the biosynthetic pathway. Thus, the reduced production of straight-chain esters might have arisen from a shortage of alcohol or acyl-CoA substrates for the AAT-catalysed reaction. Indeed, the production of alcohols was found to be inhibited in AVG or 1-MCP treated apples (Table III). For example, both treatments reduced the production of 1-butanol and 1-hexanol, in parallel with decreases in the emission of butyl and hexyl esters, which illustrates the importance of the availability of alcohols for ester production, in agreement with previous reports on apple fruit (Harb, 1994; Lara *et al.*, 2006; Berger and Drawert,

TABLE IV

Influence of AVG or 1-MCP treatment on different aroma volatile-related enzyme activities (Units mg^{-1} protein) extracted from 'Delbarde Estivale' apple fruit kept at room temperature for 1, 7, 14, or 21 d after harvest

Treatment	LOX‡				HPL				PDC				ADH				AAT			
	1 d	7 d	14 d	21 d	1 d	7 d	14 d	21 d	1 d	7 d	14 d	21 d	1 d	7 d	14 d	21 d	1 d	7 d	14 d	21 d
Control	11.91a†	08.46a	11.34a	15.17a	12.65b	13.63b	11.98a	09.95a	16.49a	14.60a	24.61a	19.07a	17.06a	17.21a	15.45a	14.17a	0.015b	0.014b	0.015b	0.015a
AVG	05.93b	03.85b	07.45b	11.01b	08.70c	06.78c	09.18b	10.59a	06.47b	07.29c	05.97c	05.51c	15.29b	08.29b	09.12c	07.74b	0.013b	0.016ab	0.014b	0.017a
1-MCP	04.86b	10.01a	09.44ab	12.56b	19.22a	21.17a	12.87a	10.23a	09.17b	07.97b	07.94b	07.63b	12.56c	08.95b	13.59b	09.10b	0.017a	0.018a	0.021a	0.017a

†All values are the means of five replicates. Mean values followed by a different lower-case letters within the same column indicate significant differences at $P \leq 0.05$.
‡LOX, lipoxygenase; HPL, hydroperoxide lyase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase; AAT, alcohol o-acyltransferase.

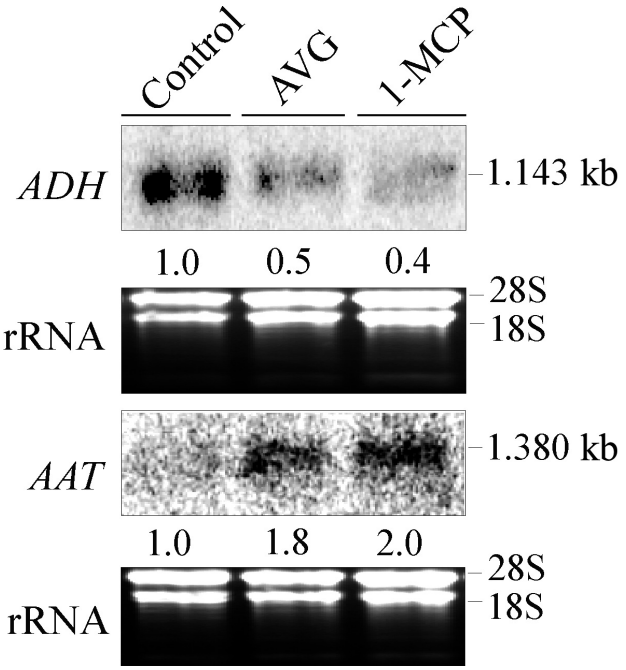

FIG. 1

Northern blot analysis of *ADH* and *AAT* gene expression in AVG or 1-MCP-treated 'Delbarde Estivale' apple fruit kept at room temperature for 7 d after harvest. Total RNA (20 µg) was loaded on each lane. The hybridisation signals were normalised to the rRNA bands (shown below to indicate equal loadings), and the levels of expression of *AAT* and *ADH* in untreated fruit were set at 1.0. Values indicate the relative levels of expression of *AAT* and *ADH* in AVG- or 1-MCP-treated samples.

1984). Moreover, because the substrate specificity of all AAT enzymes isolated so far from different fruit tissues seems to be generally broad towards alcohols and acyl CoAs (Souleyre *et al*., 2005; Olías *et al*., 2002; Wyllie and Fellman, 2000), the composition of the volatile esters that are emitted must be determined, to some extent, by the available precursors, although substrate preferences have been reported for some isoforms of AAT (Zabetakis and Holden, 1997; Beekwilder *et al*., 2004).

The inhibition of alcohol production was in agreement with the decrease in ADH activity (Table IV), the enzyme that catalyses the reduction of aldehydes to alcohols, as well as to reduced levels of *ADH* gene expression (Figure 1), in response to both treatments.

Other enzyme activities reportedly involved in the biosynthesis of volatile esters from fatty acids were also affected. For example, PDC, which produces aldehydes from oxo-acids and thus provides the substrates for further reduction by ADH, was strongly inhibited in AVG or 1-MCP treated apples (Table IV), indicating that it was ethylene-regulated. This result was also interesting in view of the major decrease in respiration rates found for treated apples (Table II). PDC is central to respiratory metabolism (Hübner *et al*., 1978). Reduced rates of respiration might therefore have influenced the biosynthesis of volatile esters by limiting the supply of high-energy compounds such as adenine (ATP) and/or pyridine nucleotides, which have been proposed to be a limiting factor for the availability of fatty acid-derived precursors (Tan and Bangerth, 2001). Similarly, reduced rates of respiration in treated fruit might have led to a decrease in pyruvate dehydrogenase (PDH; EC 1.2.4.1) activity, which contributes to the oxidative

decarboxylation of pyruvate and other oxo-acids into acyl-CoAs (Arjunan *et al*., 2002). Consequently, the reduced synthesis of acyl-CoA substrates might also have accounted for the decrease in the production of volatile esters.

Both ethylene-suppressing chemicals were also found to affect the activity of HPL that is responsible for the cleavage of fatty acid hydroperoxides to aldehydes and oxo-acids (Vancanneyt *et al*., 2001). Although similar values were achieved by the end of the experimental period, regardless of treatment (Table IV), AVG decreased HPL activity levels during the first 2 weeks at 20ºC, while 1-MCP-treated samples showed enhanced HPL activity after 1 week (Table IV). These observations suggest complex regulatory mechanisms for HPL enzyme activity, possibly at the post-transcriptional level, as hypothesised elsewhere (Vancanneyt *et al*., 2001).

The promotion of HPL activity by treatment with 1-MCP, despite the reduced emission of volatile esters (Table III), contradicts one possible role for HPL as a limiting factor in the capacity to synthesise aroma volatiles, and emphasises LOX as a key control point in the pathway, as supported by previous work (Villatoro *et al*., 2008). A substantial increase in the emission of esters during on-tree maturation of 'Pink Lady' apples was observed concomitantly with a sharp increase in LOX activity at the later stages of fruit development, while AAT activity did not vary significantly (Villatoro *et al*., 2008). Similarly, the promotion of fatty acid metabolism by over-expression of *LOX* genes resulted in an enhanced delivery of some straight-chain alcohols (Beekwilder *et al*., 2004). Conversely, apples in which the production of ethylene was suppressed, genetically, synthesised substantially lower amounts of hexyl and butyl esters than control fruit, although the levels of the aldehyde and alcohol precursors for these esters were inhibited by only 12 – 38%. The significant change in the hexanal/(2E)-hexenal ratio suggested that ethylene might be involved in the regulation of either *LOX* or *HPL* gene expression (Dandekar *et al*., 2004). Results reported herein consistently indicated that LOX activity decreased in response to both ethylene suppression treatments (Table IV), and therefore that this aroma-related enzyme activity was ethylene-dependent. The effects of AVG were detectable immediately after harvest and throughout the experimental period, while those of 1-MCP were more random and occurred gradually. LOX catalyses the addition of an oxygen molecule to polyunsaturated fatty acids to produce an unsaturated fatty acid hydroperoxide (Porta and Rocha-Sosa, 2002). Therefore, the supply of hydroperoxides for further reactions leading to the formation of hexenal and other metabolites is likely to be reduced when LOX activity is inhibited. Genes in the *LOX* family are regulated differentially (Chen *et al*., 2004), and certain LOX isoforms might be modulated in different ways in response to both the ethylene-suppressing treatments considered herein.

The activities of LOX, PDC, and ADH were significantly inhibited by both AVG and 1-MCP treatments, suggesting that ethylene is required for the expression or the activity of the corresponding gene or gene product, respectively. In contrast, AAT activity was not affected by AVG, but was enhanced by 1-MCP due, at least in part, to an increased level of *AAT* gene expression in 1-MCP-treated fruit (Figure 1). These results challenge the general view that *AAT* gene expression and/or enzyme activity are ethylene-dependent (Defilippi *et al*., 2005), although this is not the first report that AAT activity is unaffected or enhanced upon suppression of ethylene action (Schaffer *et al*., 2007; Ortiz *et al*., 2010b), which suggests differential regulation mechanisms among species or cultivars. This work also showed that ethylene-suppression treatments affect enzymes located upstream in the pathway, and demonstrated that the amounts of volatile esters decreased despite enhanced AAT activity and gene expression, clearly implying that ester production in ripening 'Delbarde Estivale' apple fruit is controlled, to a large extent, by upstream reactions that provide the alcohol and acyl CoA precursors. These data de-emphasise the role of AAT in the chain of events leading to the production of volatile esters, and suggest that changes in gene expression and/or in protein activity in response to specific post-harvest procedures might be largely irrelevant for ester-forming capacity, provided a minimum level of AAT activity is present in the tissues.

Financial support was provided, in part, by the Ministerio de Ciencia e Innovación (MICINN) of Spain (Project Ref. AGL2006-00345/ALI) and is gratefully acknowledged. The authors are indebted to Pilar Sopeña for technical assistance and thank Martine De Cock for help in reading the manuscript.

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