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Salicylic Acid, Ethephon, and Methyl Jasmonate Enhance Ester Regeneration in 1-MCP-Treated Apple Fruit after Long-Term Cold Storage

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Volatile esters, primarily synthesized in peel tissues, are major aromatic components of apple fruits [*Malus sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.]. The use of cold storage combined with 1-methylcyclopropene (1-MCP) treatment prolongs the life of apples but represses the regeneration of esters during poststorage ripening. In this study, the regeneration of total esters was significantly increased in apple fruits treated with salicylic acid (SA) and Ethephon (ETH) that had been treated once or twice with 1-MCP. However, methyl jasmonate (MeJA) treatment resulted in regeneration of total esters after a single 1-MCP treatment. To determine the mechanism by which SA, ETH, and MeJA regulate ester regeneration, the apple alcohol acyltransferase gene (*MdAAT2*) was investigated at the mRNA, protein, and enzyme activity levels. Genes associated with ethylene perception were also investigated by RT-PCR. The results suggest that *MdAAT2* controls ester regeneration and that *MdETR1* plays a key role in ethylene perception and regulation of downstream *MdAAT2* gene expression during poststorage. Ester compounds and concentrations differed in peels treated with different signal molecules, indicating that regulation of the pathway upstream of straight-chain ester biosynthesis depended on the regulation of lipoxygenase (LOX) and alcohol dehydrogenase (ADH) activity by SA, ETH, and MeJA during poststorage ripening.

KEYWORDS: Alcohol acyltransferase; apple [*Malus sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.]; ester regeneration; ethylene; poststorage ripening

INTRODUCTION

Volatile aroma compounds are important factors that determine fruit quality and influence final consumer acceptance of the commodity (*1*, *2*). Esters, derived from amino acids and fatty acids, are important components of volatile aroma compounds in many ripening fruits (*3*−*5*). Aroma profiles of apples are complex, and the number and profile of volatile compounds are cultivar-specific (*3*, *6*). Esters are the most significant contributors to apple aroma (*7*, *8*), accounting for up to 80% of total volatiles in the "Golden Delicious" apple variety (*9*).

1-Methylcyclopropene (1-MCP), a very important quality maintenance tool, can effectively prolong storage and shelf life (*10*), while reducing the volatile production of apple fruit (*11*). Long-term cold storage can also completely suppress the principal headspace components and block their subsequent regeneration during poststorage at room temperature (*12*−*14*). In our previous study, cold storage combined with 1-MCP treatment could effectively prolong the storage life of apples. However, after long-term storage, the capacity to recover volatile emission, especially esters, was reduced. Decreased production of volatiles by apple fruits, which greatly influences consumer acceptability, is usually a consequence of substrate availability and related enzyme activity (*3*, *15*). Therefore, the ability to regenerate esters after 1-MCP treatment and long-term storage is of great importance in maintaining apple eating quality.

In ester biosynthesis, alcohol acyltransferase (AAT) is the key enzyme that catalyzes the last step in ester formation by linking an acetyl moiety from acetyl CoA to the appropriate alcohols (*4*). In recent years, several full-length cDNAs encoding enzymes with AAT activity have been isolated and characterized from strawberries, melons, bananas, and apples (*4*, *5*, *16*, *17*). In these studies, the AAT enzyme appears to have broad substrate specificity, with a preference for alcohols and acyl-CoAs. Pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), and lipoxygenase (LOX) are also volatile-ester-related enzymes that can regulate the precursors of ester synthesis (*15*, *18*).

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Ethylene, a gaseous phytohormone, affects many physiological processes in plants (*19*). Production of volatile compounds (especially esters) was reduced in ACC-oxidase or ACC-synthase antisense transgenic fruits (*20*−*22*). This suggests that ester production is physiologically regulated by ethylene during fruit development and ripening. 1-MCP acts by binding irreversibly to ethylene receptors (*23*) and inhibiting ethylene production, which then suppresses the biosynthesis of fruity volatiles in climacteric fruits (*24*−*27*). Previous studies have provided extensive evidence that ethylene signaling is mediated by a family of copper-containing receptors (similar to the bacterial two-component histidine kinase receptors, including ETR1, ETR2, ERS1, ERS2, and EIN4 in *Arabidopsis*). Ethylene signaling also appears to include a MAP kinase cascade and a transcriptional cascade that regulate ethylene response genes (*28*−*30*). In addition to ethylene, jasmonates (JAs, including methyl jasmonate and its free-acid jasmonic acid) and salicylic acid (SA) are also important signaling molecules in fruit ripening that influence the production of plant secondary metabolites (*31*). Early investigations indicated that stimulation of apple fruit ripening by JA most likely occurs through ethylene production (*32*) and that methyl jasmonate (MeJA) influences the production of volatile compounds depending on the cultivar and harvest stage (*33*−*35*). SA and its derivative, acetylsalicylic acid (ASA), have been shown to inhibit ethylene production in cultured disks of apple fruit tissue (*36*), while increased endogenous ethylene biosynthesis at low SA concentrations has been reported in carrot suspension cultures (*37*). In this study, we have analyzed AAT gene transcription, translation, and aroma-related enzyme activity to investigate the effects of ETH, MeJA, and SA on ester regeneration in 1-MCP-treated apples after long-term cold storage.

MATERIAL AND METHODS

Plant Materials and Treatments. Golden Delicious apples [*Malus sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.] were harvested on Sept 22, 2004, from a research orchard near Tai'an, Shandong province. Fruit was treated on the day of harvest with 1-MCP (1 mL of 40 °C water was added to dissolve the 1-MCP from powder and provide a final gas concentration of 0.75 μL L^{-1}) generated from SmartFresh (AgroFresh Inc., Spring House, PA, 0.14% active ingredient by weight) for 20 h at 20 °C in a sealed 190-L steel chamber. Untreated control fruit was sealed in an identical container for the same duration without 1-MCP. After treatment, the fruits were stored for 14 weeks at 0 °C. Then, the 1-MCP-treated fruits were separated into two groups and held at 20 °C for the following treatments.

Group A, "Single 1-MCP". The fruit was divided into four groups: For control A, the fruit was dipped into 0.02% (v/v) Tween 20 and 1% (v/v) ethanol in water for 2 min; for SA treatment, the fruit was dipped into 2.0 mM SA (Sigma, St. Louis, MO) in 0.02% (v/v) Tween 20 and 1% (v/v) ethanol for 2 min; for ETH treatment, the fruit was dipped into 2 mM Ethephon (Sigma, St. Louis, MO) in 0.02% (v/v) Tween 20 and 1% (v/v) ethanol for 2 min; and for MeJA treatment, the fruit was dipped into 2.0 mM MeJA (Sigma, St. Louis, MO) in 0.02% (v/v) Tween 20 and 1% (v/v) ethanol for 2 min. Following treatment, fruit from all groups was stored at 20 °C in the air.

Group B, "Dual 1-MCP". The fruit was treated with 1-MCP a second time and then divided into four groups, namely, control B, SA, ETH, and MeJA, which were treated as described above.

At each sampling date, apple fruit peel tissue (including the skin and approximately 2 mm of hypodermal tissue) from 8 kg of apples (2 kg/replicate × 4 replicates) was excised, frozen in liquid nitrogen, and stored at −80 °C for analysis of volatiles. Four replicates of four fruit each were sampled randomly and used to analyze enzyme activity.

Assay of AAT Activity. For each replicate, peel tissue cut from four fruits 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 (12000*g* for 20 min at 4 °C), the supernatant was used in enzyme assays.

AAT activity was assayed by mixing 2.5 mL of $MgCl_2$ 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), and 50 μL of butanol solution (200 mM butanol in 0.5 M Tris-HCl, pH 8.0) with 150 μL of enzyme extract. The mixture was incubated at 35 °C for 15 min, and then 100 μL of 10 mM 5,5-dithiobisnitrobenzoic acid (DTNB) was added and allowed to stand at room temperature for 10 min. The absorbance of the yellow thiophenol product formed by the reaction of DTNB with the free CoASH liberated during the catalytic reaction was measured at 412 nm over time using a spectrophotometer (SCINCO S-3150, Seoul, Korea). One activity unit (U) is defined as an increase in one unit of absorbance per minute, and results are expressed in terms of specific activity (mU mg^{-1} protein).

Assay of ADH and PDC Activities. The method used to extract and analyze ADH and PDC activities was described by Echeverría et al. (*15*). For each replicate, 3 g of peel tissue was obtained and homogenized with a mortar and pestle in 10 mL of extraction solution containing 85 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0), 5 mM dithiothreitol (DTT), and 1% (w/v) PVPP. After filtration and centrifugation (25000*g* for 15 min at 4 °C), the supernatant was used for enzyme assays.

PDC activity was analyzed together with the ADH reaction by mixing 2.1 mL of thiamine pyrophosphate (TPP)/$MgCl_2$ solution (5 mM TPP, 50 mM $MgCl_2$, in 85 mM MES, pH 6.0), 300 μL of NADH/ADH solution (0.85 mM NADH, 1600 units ADH, in 85 mM MES, pH 6.0), 300 μL of pyruvate solution (50 mM pyruvate in 85 mM MES, pH 6.0), and 300 μL of enzyme extract. ADH activity was assayed by combining 2.55 mL of NADH solution (0.15 mM NADH in 85 mM MES, pH 6.0), 150 μL of acetaldehyde solution (80 mM acetaldehyde in 85 mM MES, pH 6.0), and 300 μL of enzyme extract. For both PDC and ADH assays, the absorbance at 340 nm, due to NADH oxidation, was measured as a function of time with a spectrophotometer (SCINCO S-3150, Seoul, Korea). One activity unit (U) is defined as the increase in one unit of absorbance per minute, and results are expressed in terms of specific activity (U mg^{-1} protein).

Assay of LOX Activity. The method used to extract and analyze LOX activity was described by Echeverría et al. (*15*). For each replicate, 3 g of peel tissue was obtained and homogenized with a mortar and pestle in 5 mL of extraction solution containing 0.1 M phosphate (pH 7.5), 2 mM DTT, 1 mM EDTA, 0.1% (v/v) Triton X-100, and 1% (w/v) PVPP. After filtration and centrifugation (25000*g* for 15 min at 4 °C), the supernatant was used for enzyme assay.

LOX activity was assayed by mixing 2.5 mL of 0.1 M phosphate (pH 8.0), 400 μL of substrate solution (8.6 mM linoleic acid, 0.25% (v/v) Tween-20, 10 mM NaOH, in 0.1 M phosphate, pH 8.0), and 100 μL of enzyme extract. The formation of hydroperoxides from linoleic acid during the catalytic reaction was measured by absorbance at 234 nm over time using a spectrophotometer (SCINCO S-3150, Seoul, Korea). One activity unit (U) is defined as the increase in one unit of absorbance per minute, and results are expressed as specific activity (U mg^{-1} protein).

Analysis of Volatiles and Internal Ethylene. The method used to analyze volatiles was described by Defilippi et al. (*13*). Six grams of apple peel tissue was homogenized in a Polytron homogenizer in the presence of 12 mL of water with 2 mM NaF. The homogenized tissue was filtered through four layers of cheesecloth and centrifuged (20000*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 an internal standard (IS) solution (1-octanol) was added to obtain a final concentration of 500 nl L^{-1}. A poly(dimethylsiloxane)/divinylbenzene (PDMS/DVB, 65-μm thickness) solid-phase microextraction (SPME) fiber was used. For manual SPME, samples were equilibrated at 50 °C for 30 min and desorbed for 2 min. A GC/MS system (HP 5890, 5971A, Hewlett-Packard) equipped with a DB-1 column (0.32 mm, 30 m, 0.25 μm, J&W Scientific) was used for analysis. Chromatography conditions were as follows: injector at 250 °C; initial oven temperature 40 °C held for 5 min, increased by 2 °C min^{-1} to 50 °C, increased by 5 °C min^{-1} to 200 °C and held for 5

Table 1. Ester Composition[a] (nl L^{-1}) of Golden Delicious Apple Fruit Peel in Two 1-MCP Treatments and Control, Held for 8 Days at 20 °C after Removal from 14 Weeks of Cold Storage

	single 1-MCP				dual 1-MCP				
ester compound	SA	ETH	MeJA	control A	SA	ETH	MeJA	control B	control
2-methylbutyl acetate	23.3 ± 1.7	12.2 ± 2.0	6.6 ± 1.2	3.9 ± 1.2	18.0 ± 1.1	7.4 ± 1.8	3.1 ± 1.6	2.8 ± 1.4	6.1 ± 0.3
hexyl acetate	20.1 ± 1.2	13.6 ± 2.3	11.0 ± 0.9	5.6 ± 0.8	17.9 ± 1.3	11.8 ± 2.0	1.9 ± 0.9	1.2 ± 1.1	9.9 ± 1.2
hexyl butanoate	15.7 ± 0.8	7.8 ± 1.5	11.7 ± 2.3	ND[b]	12.4 ± 1.2	12.4 ± 2.7	ND	ND	7.7 ± 0.8
butyl butanoate	36.9 ± 2.1	ND	20.9 ± 3.7	ND	29.9 ± 3.2	ND	ND	ND	6.8 ± 1.8
nonly butanoate	15.2 ± 2.2	ND	ND	ND	13.1 ± 2.2	ND	ND	ND	ND
hexyl benzoate	35.7 ± 2.1	ND	35.7 ± 3.1	ND	29.7 ± 4.1	ND	ND	ND	ND
total esters	146.9 ± 10.1[A]	33.6 ± 5.8[D]	85.9 ± 11.2[C]	9.5 ± 2.0[E]	121.0 ± 13.1[B]	31.6 ± 6.5[D]	5.0 ± 2.5[E]	4.0 ± 2.5[E]	28.5 ± 4.1[D]

[a] Values represent means ± SE of four replicates per treatment. [b] ND = not detected. Means with the same letter are not significantly different at the 5% level (Duncan's multiple range test). After harvest, fruit were exposed to air (as the control) or 0.75 μL L^{-1} 1-MCP for 20 h at 20 °C. After treatment, all fruits were stored for 14 weeks at 0 °C. Fruits treated with 1-MCP were separated into two groups and then held at 20 °C for the following treatments. Group A, single 1-MCP: The fruits were divided into four groups: for control A, the fruits were dipped into 0.02% (v/v) Tween 20 and 1% (v/v) ethanol in water for 2 min; for SA treatment, the fruits were dipped into 2.0 mM SA in 0.02% (v/v) Tween 20 and 1% (v/v) ethanol for 2 min; for ETH treatment, the fruits were dipped into 2 mM Ethephon in 0.02% (v/v) Tween 20 and 1% (v/v) ethanol for 2 min; and for MeJA treatment, the fruits were dipped into 2.0 mM MeJA in 0.02% (v/v) Tween 20 and 1% (v/v) ethanol for 2 min. Then, the fruits were stored at 20 °C in the air. Group B, dual 1-MCP: The fruits were treated with 1-MCP a second time and then divided into four groups: control B, SA, ETH, and MeJA. The treatment of each group proceeded as above.

min. The linear velocity of the carrier gas was 35 cm s^{-1}. Mass spectra were obtained by electron ionization at 70 eV using a spectrum range of 40−250 *m*/*z*. Identification of compounds was confirmed by comparing 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. Quantitative determination of ester compounds was done using the peak of an internal standard (1-octanol) as a relative value.

Internal ethylene from 20 apple fruits was measured as described by Song and Bangerth (*38*). An internal gas sample (1 mL) was removed from the apple core using a gastight syringe fitted with a stainless steel needle. The ethylene concentration was determined using a GC system (HP 5890, Hewlett-Packard) equipped with a flame ionization detector and a 1.7-m glass column (4.0-mm i.d.) packed with 80/100 mesh Porapak Q (Alletech Associates Inc., Deerfield, IL). The gas flows for N_2, H_2, and air were 30, 30, and 300 mL min^{-1}, respectively, and the oven, injector, and detector temperatures were 80, 100, and 140 °C, respectively. Quantification was by comparison of peak height with a known ethylene standard.

RNA Extraction and Northern Blot Analysis. Total RNA was extracted from apple peel tissue as described by Chang et al. (*39*) 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, Bucks, U.K.). 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 SSC and 0.1% (w/v) SDS at 60 °C for 20 min each and then exposed to X-ray film (Kodak, Rochester, NY).

Semiquantitative RT-PCR. Two micrograms of total RNA was denatured at 70 °C for 5 min, and 2 μL of AMV reverse transcriptase (Promega) was added. After brief mixing, the transcription reaction was incubated at 42 °C for 1 h and terminated at 85 °C for 10 min. Amplimers were designed with the Primer Premier (ver. 5.0) program. The GenBank accession number, sequence, expected fragment size, and annealing temperature (t_a) of primers used in the semiquantitative RT-PCR analysis were as follows: *MdETR1* (DQ137848, 5′-TGTTGGGAATGCTGTGAAGT-3′ and 5′-ATGATTTGCTTGCAGCTTGG-3′; 418 bp; 55 °C), *MdERS1* (DQ137851, 5′-TCTGGCTGTGGTGTTCTCCC-3′ and 5′-GCAACGATACTCCCTCTGTC-3′; 194 bp; 55 °C), *MdCTR1* (AY670703, 5′-AACCTCCCATTGTTCATCGT-3′ and 5′-CTTCTGGTGCCATCCATTCA-3′; 159 bp; 56 °C), and 18S rRNA (CV826359, 5′-GGGTTCGATTCCGGAGAGG-3′ and 5′-CCGTGTCAGGATTGGGTAAT-3′; 87 bp; 60 °C).

Protein Content, Antibody Production, and Immunoblot Analysis. Protein content was determined by the Bradford method (Bio-Rad) using bovine serum albumin (BSA) as the standard.

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

Protein extracts were prepared as described for crude enzyme extracts. Twenty micrograms of total protein per lane was separated by SDS−PAGE as described by Laemmli (*40*). Polyacrylamide gels (12%) were electroblotted onto supported PVDF membranes (Millipore) by a semidry Transfer Unit (Amersham, T70). Membranes were incubated overnight at 4 °C with the primary antibody (1:1000 dilution), washed, and incubated with goat anti-rabbit (AP-conjugated) secondary antibody (1:2000 dilution; Proteintech Group). Alkaline phosphate (AP) chromogenic substrate [5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT); Roche] was then added and color development monitored.

Statistical Analysis. Statistical analyses were conducted using SAS software (SAS Institute, Cary, NC). Statistical differences between treatments and their respective controls were determined by one-way analysis of variance (ANOVA) followed by the Duncan's New Multiple Range Test. The level of significance was set at $p < 0.05$. Correlations between internal ethylene and total esters were analyzed by Pearson correlations using SPSS12.0 (SPSS, Chicago, IL).

RESULTS AND DISCUSSION

Volatile Ester Regeneration in Apple Peel. Volatile compounds in apples are synthesized primarily in the fruit peel (*33*, *41*), and GC/MS analysis indicates that the capacity for ester production is greater in the peel than in the pulp (*22*). In our previous work, immunoblot and immunofluorescence microscopy showed the AAT protein to be localized mainly in cells of the fruit peel (*42*). Therefore, our investigation of ester regeneration in apple fruit focused on the peel. 1-MCP is an inhibitor of ethylene action and ester biosynthesis, but the duration of the response to 1-MCP was determined by treatment conditions and 1-MCP concentration (*43*). To determine whether the effects of SA, ETH, and MeJA on ester regeneration were due to a diminished response to 1-MCP, apples were separated after 14 weeks storage into two groups, of which one group was treated again with 1-MCP while the other was not.

The compositions and concentrations of esters were significantly different among tissues treated with SA, ETH, and MeJA in the two 1-MCP treatment groups (**Table 1**). Among the esters detected, 2-methylbutyl acetate, hexyl acetate, hexyl butanoate, and butyl butanoate contribute significantly to the flavor of ripe

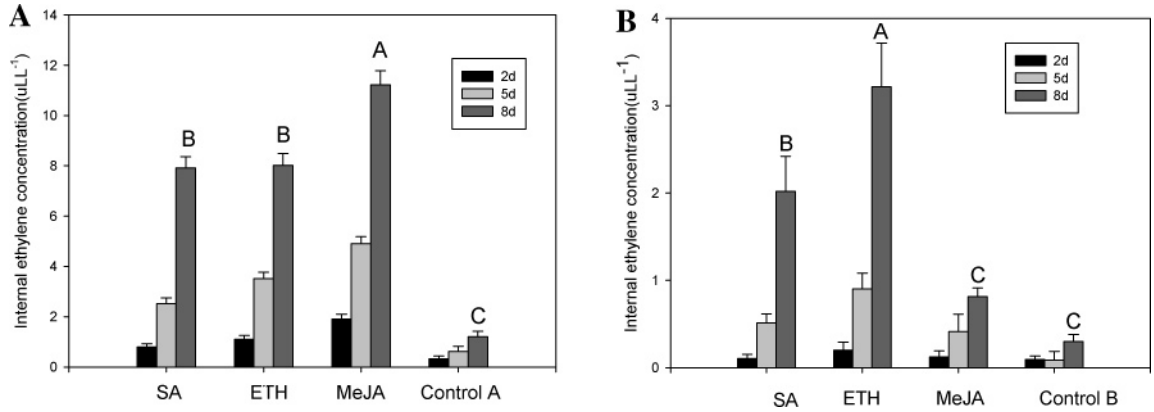

Figure 1. Internal ethylene concentration in (**A**) single 1-MCP treatment and (**B**) dual 1-MCP treatment of Golden Delicious apples held for 8 days at 20 °C after removal from 14 weeks of cold storage. Means with the same letter are not significantly different at the 5% level (Duncan's multiple range test). For an explanation of groups and treatments, see footnote *b* to **Table 1**.

fruit (*3*). Of these, hexyl butanoate and butyl butanoate were not detected in controls A and B, but they had been regenerated by SA in both 1-MCP treatment groups and by MeJA in the single 1-MCP treatment group. However, butyl butanoate was not detected after ETH treatment, suggesting that the capacity of ETH to regenerate esters is lower than that of SA and MeJA during poststorage under these treatment conditions. In contrast to the controls, ethylene suppression significantly repressed total ester regeneration in the single and dual 1-MCP controls, resulting in a very low concentration of total esters in apple fruit peel during poststorage ripening. Although significant regeneration of total esters was observed in the SA- and ETH-treated groups from both 1-MCP treatments and in the MeJA-treated group that received a single 1-MCP treatment, statistically significant differences were observed among the three signal molecule treatments and controls (**Table 1**). Previous studies showed that ethylene might play an important role in the biosynthesis of volatile compounds (*16*, *22*, *44*). To determine whether differences between total esters among these treatments were due to ethylene, GC was used to investigate the internal ethylene concentrations (IECs) of the fruits. The IECs of the fruits were regenerated significantly (**Figure 1**) by the three signal molecules in both 1-MCP treatments, and the esters were regenerated correspondingly, except for the MeJA treatments in the dual 1-MCP group (**Table 1**). A previous study showed that SA can delay the rise in ethylene production (*45*, *46*), while another study showed that SA could stimulate ethylene biosynthesis at low concentrations (<0.1 mM) after 5 days of treatment (*37*). In addition, ethylene production from disks of apple flesh and peel was suppressed after treatment with 0.1−20.0 mM SA for 6 h (*47*). In our study, treatment with 2.0 mM SA stimulated the production of internal ethylene in apple fruit, which might be due to the relatively shorter time (2 min) of the treatment. The mechanism of differing effects of SA on ethylene biosynthesis remains unknown. IEC is used to indicate onset of the climacteric (*48*), and in this study, we found that ester generation could be initiated only by a relatively high IEC compared to the three controls. This indicates that ethylene might regulate ester regeneration. Although the esters were regenerated, the correlation between IEC and total esters was not significant ($p > 0.5$) among the SA, ETH, and MeJA treatments. Additionally, more ester compounds were detected in the SA- and MeJA-treated apple fruits, suggesting that differences in ester concentration and composition among these treatments might be affected by internal ethylene, as well as by other factors such as aroma-related enzymes and genes.

MdAAT2 Gene Transcription, Translation, and Enzyme Activity. Recent studies have suggested that the AAT enzyme plays an important role in ester biogenesis in ripening apple fruit (*15*, *22*, *49*). In our previous study, the *MdAAT2* gene (GenBank accession no. AY517491), encoding the apple AAT enzyme, was cloned and characterized. *MdAAT2* expression was previously shown to be regulated by ethylene (*42*). To determine possible effects of the three signal molecules on ester regeneration and apple AAT gene expression, *MdAAT2* was investigated at the mRNA, protein, and enzyme activity levels. Compared to the two controls, the accumulation of MdAAT2 mRNA was strongly induced by SA or ETH treatment in the dual and single 1-MCP groups for 8 days at 20 °C after long-term cold storage (**Figure 2**). Similarly, the concentration of total esters was enhanced in SA- and ETH-treated apple fruit (**Table 1**). However, strong induction of *MdAAT2* mRNA transcript accumulation by MeJA was seen after the single 1-MCP treatment (**Figure 2B**), whereas neither total esters nor *MdAAT2* mRNA was increased by MeJA treatment in the dual 1-MCP-treated groups (**Table 1** and **Figure 2E**). This suggests that MeJA-induced transcription of MdAAT2 might be depressed by repeated 1-MCP treatment and that the *MdAAT2* gene might control ester regeneration. The pattern of MdAAT2 protein expression was consistent with *MdAAT2* mRNA transcription (**Figure 2A**,**D**). This indicates that translation of the *MdAAT2* gene is regulated at the transcriptional level during this stage. Further, AAT enzyme activity was consistent with MdAAT2 protein concentration for 8 days during poststorage (**Figure 2G**,**H**). Interestingly, transcription of the *MdAAT2* gene was enhanced as the IECs increased (**Figures 1** and **2**). Hence, high IECs might induce *MdAAT2* gene expression.

Effect of SA, ETH, and MeJA on Ethylene Perception. In the present study, the inductive effects of MeJA on *MdAAT2* and ester regeneration differed between single and dual 1-MCP treatments. As an inhibitor of ethylene action (*43*), 1-MCP might regulate ethylene signaling genes. We also found that expression of *MdAAT2* might be regulated by IEC. Therefore, genes related to ethylene perception and transduction were isolated from apple fruit, and their expression patterns were investigated by semi-quantitative RT-PCR.

In this study, expression of the *MdETR1* gene was extremely low in the two control groups (**Figure 3A,E**). With dual 1-MCP treatment, the accumulation of *MdETR1* mRNA was strongly induced by SA and ETH after 8 and 5 days, respectively, whereas it remained low in the MeJA-treated group (**Figure 3D**). In contrast, *MdETR1* mRNA transcripts were strongly

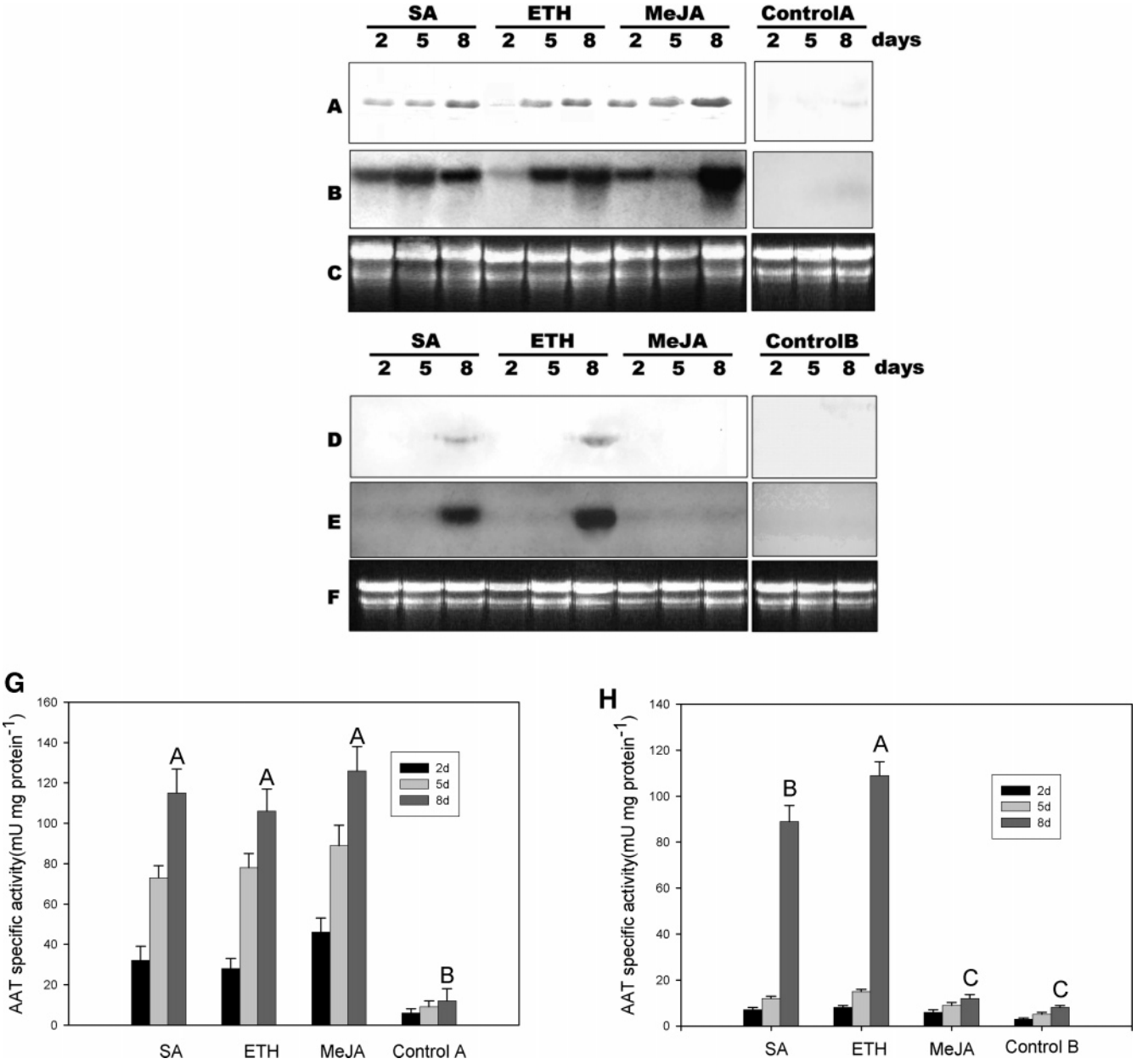

Figure 2. Apple fruits treated without/with a second 1-MCP treatment (single/dual 1-MCP treatment), after 14 weeks of cold storage as control A/control B, to which SA, ETH, and MeJA, respectively, were then applied. (**A**,**D**) Western blot analysis of MdAAT2 protein, (**B**,**E**) Northern blot analysis of MdAAT2 mRNA, (**C**,**F**) total RNA, (**G**,**H**) apple AAT activities. Means with the same letter are not significantly different at the 5% level (Duncan's multiple range test). For an explanation of groups and treatments, see footnote *b* to **Table 1**.

induced by MeJA, SA, and ETH in the single 1-MCP-treated group (**Figure 3A**). In previous studies, ETR1 did not change in Granny Smith apples at different stages of ripeness (*50*) or in Braeburn apples over 3 weeks of storage (*51*). In the present study, significant *MdETR1* expression was not detected in the two controls, but it was strongly increased together with increased ethylene production in two 1-MCP-treated groups, suggesting an ethylene-dependent pattern of regulation. A similar study showed that *ETR1* mRNA in melon is increased by treatment with ACC and ethylene, but is slightly decreased by treatment with AVG (*52*). However, ETR1 might not exist in *Delphinium* (*53*). We propose that ETR1 is differentially expressed in diverse plant species, tissues, and developmental stages. Accumulation of NR (the tomato *MdERS1* orthologue) mRNA was prevented in tomato fruit treated with 1-MCP during ripening (*54*). In the present study, the accumulation of *MdERS1* mRNA was slightly influenced after dual 1-MCP treatment (control A of **Figure 3B** and control B of **Figure 3F**). However, the abundance of *MdERS1* mRNA was inhibited by SA and slightly suppressed by ETH and MeJA after 5 days in the dual 1-MCP treatment group (**Figure 3F**).

In the present study, the accumulation of *MdCTR1* mRNA was prevented by 1-MCP treatment (**Figure 3C**, control A, and **Figure 3G**, control B) and was induced by SA, ETH, and MeJA after a single 1-MCP treatment (**Figure 3C**) and by SA and ETH after dual 1-MCP treatment (**Figure 3G**) for 8 days. This indicates that *MdCTR1* mRNA levels can be regulated by internal ethylene (**Figures 2** and **3**). In the rose, RhCTR1 also increases during flower development and is up-regulated in response to exogenous ethylene (*55*). Tomato LeCTR1 mRNA is up-regulated by ethylene during fruit ripening (*56*, *57*). These facts suggest that MdCTR1 might be involved in the ethylene signal transduction and might relate to ester regeneration during poststorage ripening. In addition, *MdCTR1* mRNA was induced

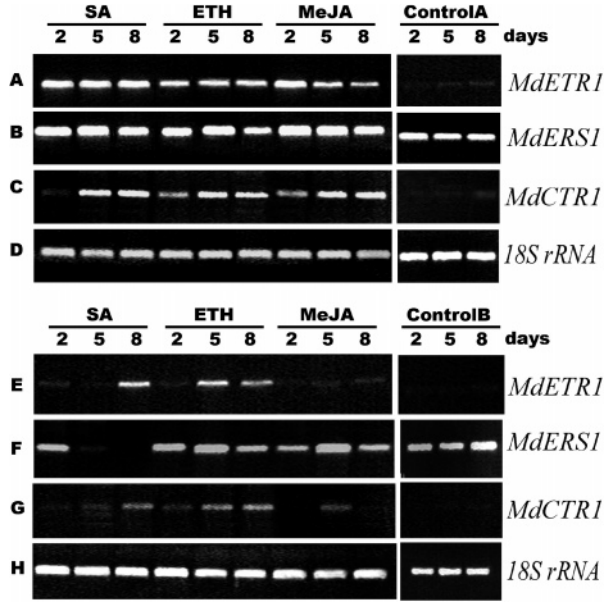

Figure 3. Expression of genes related to ethylene perception after (**A**–**D**) single or (**E**–**H**) dual 1-MCP-treated Golden Delicious apples for 8 days at 20 °C after removal from 14 weeks of cold storage. All RT-PCR reactions were from the same batch of cDNA and were repeated at least three times with identical results, only one image of which is shown here. 18S rRNA expression is shown below as a control. (**A**,**E**) *MdETR1* amplified for 31 cycles. (**B**,**F**) *MdERS1*amplified for 31 cycles. (**C**,**G**) *MdCTR1* amplified for 31 cycles. (**D**,**H**) 18S rRNA amplified for 23 cycles. For an explanation of groups and treatments, see footnote *b* to **Table 1**.

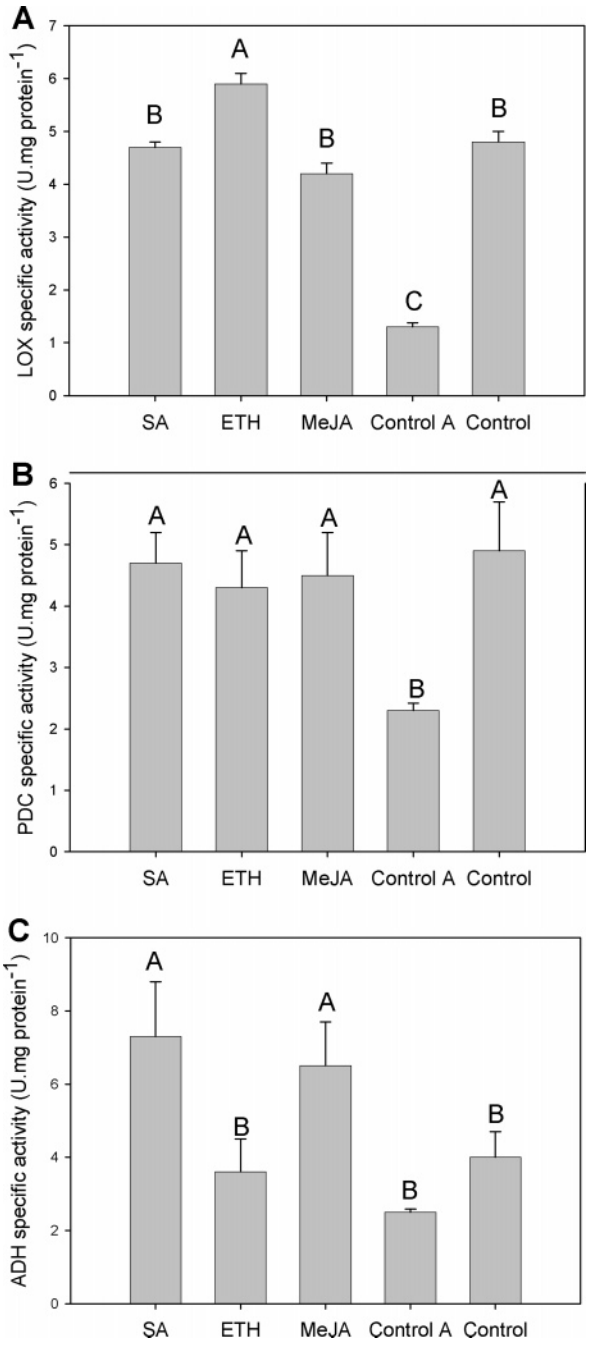

Figure 4. Specific activity of (**A**) LOX, (**B**) PDC, and (**C**) ADH in apple fruit peel of Golden Delicious apples held for 8 days at 20 °C after removal from 14 weeks of cold storage in group A (single 1-MCP) and control. Means with the same letter are not significantly different at the 5% level (Duncan's multiple range test). For an explanation of groups and treatments, see footnote *b* to **Table 1**.

by MeJA after dual 1-MCP treatment only at 5 day (**Figure 3G**). Adams-Phillips et al. provided evidence of a multigene family of tomato CTR1-like genes that display differential expression patterns regulated by ethylene during development (*58*). Thus, expression of other members of the CTR1 multigene family might be associated with MeJA.

In this study, *MdAAT2* mRNA levels were associated with *MdETR1* transcripts. This suggests that *MdAAT2* is downstream in the ethylene signal pathway and that *MdETR1* might play a key role in ethylene perception, while *MdERS1* and *MdCTR1* are also involved in regulating ester regeneration during poststorage.

Effect of SA, ETH, and MeJA on Aroma-Related Enzyme Activity. Although the abundance of *MdAAT2* mRNA, protein, and enzyme activity was strongly induced by SA, ETH, and MeJA after the single 1-MCP treatment, the concentration and composition of the esters were diverse after the different treatments. Hence, other factors might influence the level of ester regeneration. Straight-chain esters, the most abundant compounds during poststorage ripening (**Table 1**), are derived from fatty acids catabolized through β-oxidation and the lipoxygenase pathway (*59*). To distinguish the difference between these pathways, the LOX, ADH, and PDC activities and the concentrations of aldehydes and alcohols were investigated in the single 1-MCP treatment group after 8 days.

Although the LOX activity was strongly induced by SA (**Figure 4A**), the total aldehyde concentration was lower than in peel treated with MeJA or ETH (**Figure 5A**). Products of the LOX reaction can be converted to different compounds, including aldehydes, through at least six pathways (*60*). This indicates that SA might enhance other pathways, while reducing the aldehyde concentration. There was no notable difference in PDC activity among the three treatments and the control (**Figure 4B**). This suggests that PDC is not the limiting factor for ester regeneration. It has been reported recently that ADH is not a limiting factor for alcohol accumulation during ripening of "Fuji" and "Greensleeves" apples (*15*, *61*) or other fruits (*62*, *63*). In the present study, ADH activity was strongly induced by SA and MeJA (**Figure 4C**), and total alcohol and ester concentrations were higher than observed in the ETH and control treatment groups (**Figure 5B** and **Table 1**). Therefore, the induction of ADH by SA or MeJA treatment might be an important factor in alcohol accumulation during these stages. Further, regulation of the upstream pathway of straight-chain esters depends on the regulation of LOX and ADH activity by SA, ETH, and MeJA during poststorage ripening.

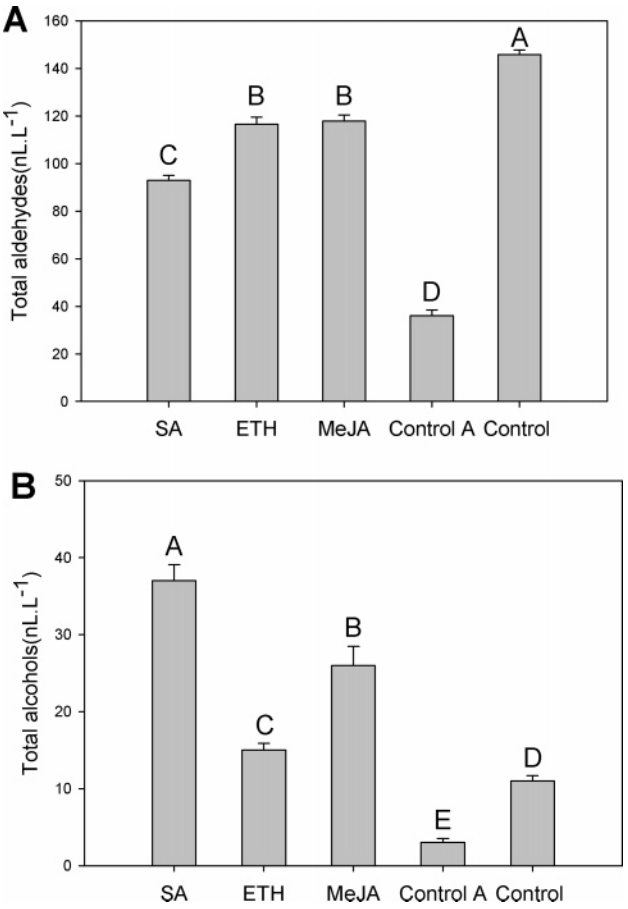

Figure 5. Production of (**A**) total aldehydes (hexanal, nonanal) and (**B**) total alcohols (butanol, hexanol) in single 1-MCP-treated apple fruit peel after 8 days. Means with the same letter are not significantly different at the 5% level (Duncan's multiple range test). For an explanation of groups and treatments, see footnote *b* to **Table 1**.

It should be noted that hexyl benzoate (**Table 1**), the product of benzoylation of hexanol, was not reported in apple fruit in previous studies (*3*, *64*) and was detected only in SA- and MeJA-treated groups in this study. Benzenoid compounds are derived from phenylalanine via phenylpropanoid pathways (*65*). Microarray analysis showed that genes encoding many enzymes in phenylpropanoid pathways are induced in response to SA and MeJA (*66*). This indicates that SA and MeJA also enhance phenylpropanoid pathways in apple peel during poststorage and that the apple AAT has the capacity to catalyze the transacylation from benzoyl-CoA to hexanol. The C6- and C9-aldehydes and alcohols are derived from linoleic and α-linolenic acids via the LOX pathway (*67*). Nonyl butanoate (**Table 1**), detected only in SA-treated apple peel, was produced through butyrylation of nonylol. These results indicate that SA might also regulate the LOX pathway and, accordingly, enhance ester regeneration. Because of limitations on the extraction and SPME sampling methods, there might be other undetected volatiles. Thus, it is possible that the three molecular signals might induce more esters than shown in this study, the effect of which needs further investigation.

In conclusion, MdAAT2, governed by the *MdAAT2* gene, is the key enzyme controlling ester regeneration and is regulated by ethylene. However, the quantity and variety of esters depend on the availability of upstream substrates, resulting in diverse differences in total esters following different treatment and control protocols. Further, SA appears to be the most effective signal molecule for enhancing the ester regeneration capacity, and some new esters arise; this has not been reported previously in apple. Whether these newly produced esters contribute to the aroma of apple fruit needs further study.

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