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# Ethylene and α-Farnesene Metabolism in Green and Red Skin of Three Apple Cultivars in Response to 1-Methylcyclopropene (1-MCP) Treatment

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Relationships among α-farnesene synthesis and oxidation, ethylene production and perception, antioxidative enzyme activities, and superficial scald development in fruit of three commercial apple cultivars were investigated at the biochemical and gene transcriptional levels. Scald-susceptible Cortland and Law Rome and scald-resistant Idared apples were untreated or treated with the ethylene action inhibitor 1-methylcyclopropene (1-MCP) and stored for up to 25 weeks at 0.5 °C. Separate blushed (red) and unblushed (green) peel tissue samples were taken at harvest and after 2, 4, 6, 10, 15, 20, and 25 weeks of storage. Large increases in peel tissue concentrations of α-farnesene and its conjugated trienol (CTol) oxidation products occurred in untreated Cortland and Law Rome and were about 4−9-fold greater than those in Idared. In both Cortland and Law Rome, accumulation of CTols in green peel was nearly twice that in red peel. 1-MCP treatment delayed and attenuated α-farnesene and CTol accumulation in each cultivar. Activities of peroxidase (POX) and catalase (CAT) were lower in red peel than in green peel, with the exception of CAT in Law Rome, whereas no effects of 1-MCP on enzyme activities were detected except for Cortland. In control fruit, internal ethylene concentrations (IECs) increased during the first 4−6 weeks to reach highest levels in Cortland, intermediate levels in Law Rome, and low levels in Idared. In 1-MCP-treated fruit, IECs increased gradually to modest levels by 25 weeks in Cortland and Law Rome but were almost nil in Idared. Expression patterns of the α-farnesene synthase gene Md*AFS1*, the ethylene receptor gene Md*ERS1*, and the ethylene biosynthetic genes Md*ACS1* and Md*ACO1* were generally in accord with the patterns of α-farnesene and ethylene production. In particular, Md*AFS1* and Md*ACS1* showed similar patterns of expression in each cultivar. Among the controls, transcript levels increased more rapidly in Cortland and Law Rome than in Idared during the first few weeks of storage. In 1-MCP-treated fruit, transcript abundance in Cortland and Law Rome rose to untreated control levels after 10−15 weeks but remained low in Idared. Scald symptoms were restricted to unblushed skin, and the incidence in controls after 25 weeks was nearly 100% in Cortland and Law Rome compared with 1% in Idared. 1-MCP treatment reduced scald incidence to 14, 3, and 0% in Cortland, Law Rome, and Idared, respectively. Overall, the results support the proposed role of CTols in scald induction and indicate that α-farnesene synthesis is tightly regulated by ethylene. However, gene transcription alone does not account for the big differences in ethylene and α-farnesene production in Cortland, Law Rome, and Idared apples.

**KEYWORDS: Apple; *Malus domestica*; ethylene; α-farnesene synthesis; conjugated trienes; gene expression; superficial scald; physiological disorder**

## INTRODUCTION

Superficial scald is a physiological storage disorder of many apple and pear cultivars that develops during prolonged cold storage (*1*). The disorder, which may arise due to chilling injury (*2*, *3*), results from necrosis of the hypodermal cortical tissue (*4*) and is manifested as brown or black patches on the fruit skin. Scald susceptibility is influenced greatly by cultivar (*1*, *5*, *6*). Resistant apple cultivars include Gala, Empire, and Idared, whereas susceptible ones include Cortland, Delicious, Granny Smith, and Law Rome. Scald susceptibility is also influenced by preharvest factors such as growing climate and maturity (*5*, *7*, *8*).

The widely accepted hypothesis for scald development is that the disorder is induced or exacerbated by conjugated trienol

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(CTol) oxidation products of the sesquiterpene (*E*,*E*)-α-farnesene (*9*−*14*). α-Farnesene typically accumulates in the skin of fruit during storage, but usually declines after about 2−3 months. In scald-susceptible cultivars, CTols increase to a maximum concentration at about 4−6 months (*7*, *10*, *12*, *15*, *16*). The enzyme α-farnesene synthase (AFS), which catalyzes the rate-limiting final step in the α-farnesene biosynthetic pathway, has been characterized (*17*), and expression of the gene *AFS1* has been described in apples and pears (*18*−*21*).

Although Anet (*22*) showed variation of naturally occurring antioxidants in scald-susceptible and -resistant cultivars, further investigation into antioxidative systems, and in particular the associated enzymes, has been relatively recent (*6*, *23*−*27*). Catalase (CAT) and peroxidase (POX) activities were often higher in scald-resistant than in scald-susceptible cultivars (*6*, *28*), but overall the results of these studies have been variable.

In susceptible cultivars, scald is often restricted to, or is more intense on, the greener or unblushed side of the fruit. It is therefore noteworthy that despite the extensive literature on the accumulation of α-farnesene and CTols during cold storage, there is no information on differences between red and green tissues of bicolored apple cultivars. In the past, most studies involved dipping whole fruit into hexane to extract α-farnesene and its oxidation products (*10*, *16*, *29*). More recently, researchers have relied on headspace analysis by GC-MS (*30*) or hexane extraction from frozen peel tissue strips, with expression of the concentration per unit surface area (*27*, *31*) or unit mass (*12*, *18*, *20*, *25*, *32*). In the latter studies, peel tissue strips invariably have been excised from the entire equatorial zone around the fruit, with no attempt to collect and analyze separate blushed and unblushed tissue fractions. Thus, published data represent the averages of tissue types.

Commercially, the antioxidant diphenylamine (DPA) is widely used to control scald (*33*) via prevention of α-farnesene oxidation to CTols (*15*, *25*, *29*, *34*). However, a new technology based on 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception (*35*, *36*), has become available to apple industries in the United States and around the world. α-Farnesene production is linked with ethylene production (*3*, *12*, *37*), and it is well documented that inhibition of ethylene perception and production by 1-MCP treatment inhibits α-farnesene accumulation in the fruit skin, consequently limiting formation of its CTol oxidation products (*18*, *25*, *27*, *31*, *32*). The observation that scald development is inhibited by 1-MCP led to the substitution of DPA by 1-MCP in some storage operations. However, scald control by 1-MCP may be incomplete for some apple and pear cultivars (*31*, *38*−*40*). Cultivars vary in persistence of ethylene inhibition when treated with 1-MCP (*32*, *39*−*41*), and recovery of ethylene production from this inhibition may be linked to scald development.

Little information is available about the responses of genes and enzymes related to ethylene biosynthesis in 1-MCP-treated fruit during cold storage. However, studies of untreated and 1-MCP-treated fruit during short-term storage at higher temperatures (20−24 °C) indicate that feedback inhibition occurs in response to 1-MCP treatment. Accumulation of transcripts for, and/or activities of, 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and oxidase (ACO), key enzymes in ethylene biosynthesis, are suppressed by 1-MCP (*42*−*45*). Transcript levels increase when ethylene production in 1-MCP-treated fruit starts to recover from inhibition (*43*, *44*). Accumulation of transcripts for genes involved in ethylene signal transduction (ethylene receptor genes *ETR1* and *ERS1*, but not *CTR1*) is also markedly reduced in response to 1-MCP treatment (*44*).

In this study our objective was to investigate the responses to 1-MCP in fruit of three apple cultivars, Cortland, Law Rome, and Idared, which differ in their susceptibility to scald. Our focus was on the recovery of ethylene and α-farnesene metabolism from 1-MCP-induced inhibition and any differences between green and red (unblushed and blushed) skin.

## MATERIALS AND METHODS

**Plant Material and Treatments.** Cortland, Idared, and Law Rome apples were harvested from mature trees growing at the Cornell University Orchards at Lansing, NY, on October 1, 13, and 13, 2003, respectively. Twelve boxes of approximately 100 pieces of fruit were harvested to provide three replicates of 200 pieces of fruit for each of two treatments: untreated control and 1-MCP. Ten fruits per replicate were sampled for initial evaluations, and the remaining fruits were cooled overnight in a cold room maintained at 0.5 °C. 1-MCP was applied the following day using sealed 135-L plastic containers. 1-MCP as a powder (SmartFresh, AgroFresh, Springhouse, PA; 0.14% active ingredient by weight) was weighed into Erlenmeyer flasks to provide a final gas concentration of 1 $\mu$L L$^{-1}$. Water was added to liberate the 1-MCP from the powder. After 24 h, the containers were vented. All fruits were stored at 0.5 °C for the duration of the experiment.

**Sampling.** For each cultivar, the three 10-fruit harvest samples plus 10 fruits from each of the three replicates per treatment were used for analysis after 2, 4, 6, 10, 15, 20, and 25 weeks of storage. To avoid warming of the fruit, all subsequent steps were carried out on 10 fruit samples before the next group was analyzed. After the internal ethylene concentration of each fruit was measured, fruits were peeled carefully, avoiding excess flesh, with green, unblushed tissues and red, blushed tissues being immediately frozen in liquid nitrogen. The peel tissues were stored at −80 °C until used for the extraction and analysis of α-farnesene and CTols, assays of peroxidase and catalase activities, and isolation of RNA.

**Scald Assessment.** Scald incidence was assessed visually after 25 weeks in cold storage plus 7 days of poststorage at 20 °C.

**Internal Ethylene Concentration (IEC).** The IEC of each fruit was measured on 1 mL samples of internal gas from the core cavity (*32*). Ethylene was measured using a Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Wilmington, DE) equipped with a flame ionization detector and fitted with a stainless steel column packed with 60/80 mesh alumina F-1 (2 m × 2 mm, i.d.). Analyses were run isothermally with an oven temperature of 200 °C and injector and detector temperatures of 220 and 250 °C, respectively. The flow rates for nitrogen, hydrogen, and compressed air were 30, 30, and 230 mL min$^{-1}$. Samples were injected directly into the gas chromatograph. Ethylene was quantified by peak area, and external standards were used for calibration.

**Extraction and Quantification of α-Farnesene and CTols.** The method of Whitaker et al. (*12*) was used with modifications. Frozen peel samples were pulverized in liquid $N_2$, and 5 g of powder was transferred to 40 mL Teflon-lined screw-cap centrifuge tubes containing 20 mL of HPLC-grade hexane. The tubes were sealed and shaken continuously at 4 °C for 1.5 h. The supernatant was filtered through a glass fiber filter disk and the filtrate made up to 20 mL. Appropriate dilutions were made for spectrophotometric estimation of α-farnesene and CTol concentrations according to the method of Rao et al. (*28*). Absorbances of appropriate dilutions of the hexane extracts were measured at 232 nm (α-farnesene) and at 258, 269, 281, and 290 nm (CTols) using a Beckman diode array spectrophotometer (model DU 7400, Beckman Instruments, Columbia, MD). Concentrations of α-farnesene were calculated using a molar extinction coefficient of $\epsilon_{232} = 27700$ (*29*). Concentrations of CTols were calculated from $A_{258-290}$, $A_{269-290}$, and $A_{281-290}$, with a molar extinction coefficient of 25000 (*7*). The spectrometric method was validated by HPLC (*12*). Data are expressed as micromoles per gram of fresh weight of peel tissue.

**Enzyme Extraction and Assay.** Frozen peels (5 g) were ground under liquid nitrogen and placed in 40 mL screw-cap tubes containing 15 mL of extraction medium including 200 mM phosphate buffer (pH 7.8), 2 mM ethylenediaminetetraacetic acid (EDTA), 5% polyvinylpolypyrrolidone, and 1 mM phenylmethanesulfonyl fluoride. The

**Table 1.** Primer Sequences Used in 3′ RACE Gene Isolation Experiments[a]

| primer name | gene-specific primer sequence | gene | reaction |
|---|---|---|---|
| NEG082 | 5′-AAACTCAGCACCTTCTCTCCGCCATGC-3′ | Md*ACS1* | primary |
| NEG083 | 5′-ATTAGCTGCCTCAACGGCAATGCTGG-3′ | Md*ACS1* | nested |
| NEG086 | 5′-AAACACAACATCTCTTGGCTTCCATG-3′ | Md*ACS3* | primary |
| NEG087 | 5′-ATCGAGTGTTTGAAAGGAAATGCTGG-3′ | Md*ACS3* | nested |
| NEG088[b] | 5′-AASCCAGACCTGATCAAGGGWCTCC-3′ | Md*ACO* | primary |
| NEG089[b] | 5′-TSTTCCAGGATGACAAGGTCAG-3′ | Md*ACO* | nested |
| NEG168 | 5′-AAGCGAGGGTATCGACAGAGG-3′ | Md*ERS* | primary |
| NEG169 | 5′-TATCGTTGCATTCATCGTCC-3′ | Md*ERS* | nested |
| NEG170 | 5′-TTAGTGGCACTTACCGGAAGC-3′ | Md*ETR* | primary |
| NEG171 | 5′-ATGAGGGTTGGTGTGGATGGTG-3′ | Md*ETR* | nested |

[a] The first round of PCR was designated the "primary" reaction using cDNA as a template. A second round of PCR was designated the "nested" reaction using the "primary" reaction as a template. [b] Degenerate primers were designed to amplify any of the Md*ACO* family members expressed in apple peel tissue (degenerate nucleotides: W = A + T; S = C + G).

mixture was homogenized by a vortex mixer and then centrifuged at 14000*g* for 30 min at 4 °C. The resulting supernatant was divided into aliquots and kept at −80 °C for protein and enzyme assays (*26*). Total soluble proteins were measured according to the Bradford method using Bio-Rad protein assay dye with bovine serum albumin as the standard (*46*).

Total soluble peroxidase (POX) activity was measured using the method of Rao et al. (*28*) with some modifications (*26*). A 50 μL aliquot of the enzyme extract was added to 2.95 mL of assay solution including 100 mM phosphate buffer (pH 6.8), 2.7 mM guaiacol, and 4 mM $H_2O_2$, and the increase in absorbance at 470 nm was monitored at 24 °C on a spectrophotometer (Beckman-Coulter, DU 7400, Fullerton, CA). Activity is reported as change in absorbance ($A_{470}$) per minute per milligram of protein.

Catalase (CAT) activity was assayed according to the method of Aebi et al. (*47*). A 50 μL aliquot of the enzyme extract was added to 2.8 mL of assay solution (2 mM $H_2O_2$ and 1 mM EDTA in 50 mM phosphate buffer, pH 7.0). $H_2O_2$ oxidation was measured at 240 nm using the extinction coefficient of $H_2O_2$. Activity is reported as the change in $A_{240}$ per minute per milligram of protein.

**Gene Isolation.** For gene isolation using reverse transcriptase Polymerase Chain Reaction (RT-PCR), total RNA was isolated as described by Pechous et al. (*18*) with modification. Frozen peel tissue samples (0.2 g) were ground to a powder in liquid $N_2$ using a mortar and pestle and transferred to 15 mL screw-cap centrifuge tubes, followed by immediate addition of 0.25 mL of plant aid solution and 2 mL of lysis buffer from the RNAqueous RNA extraction kit (Ambion, Inc., Austin, TX). After vortexing and incubation for 10 min at 25 °C, the samples were processed according to the manufacturer's instructions. The resulting RNA preparations were treated with DNase (Promega, Madison, WI) to degrade genomic DNA and extracted with phenol/chloroform, 1:1 (v/v), and total RNA was precipitated by the addition of 1 volume of isopropyl alcohol plus 0.1 volume of 3 M sodium acetate (pH 5.5). The precipitated RNA was pelleted by centrifugation, washed with cold 70% ethanol, and resuspended in RNase-free water.

Total RNA isolated from peel tissue of Law Rome fruit stored for 4−10 weeks was pooled and cDNA produced by reverse transcription (RT) using the Thermoscript RT-PCR system (Invitrogen Life Technologies, Carlsbad, CA) with the 3′ RACE (random amplification of cDNA ends) adapter primer (Ambion) following the manufacturer's guidelines. Partial length nested 3′ RACE products were amplified by PCR for Md*ACS1*, Md*ACS3*, Md*ACO1*, Md*ERS1*, Md*ERS2*, and Md*ETR1* using the "forward" oligonucleotides presented in **Table 1**. Nested 3′ RACE was carried out using a 3′−5′ RACE kit (Ambion) to amplify the 3′ ends of Md*ACS1*, Md*ACS3*, Md*ACO1*, Md*ERS1*, Md*ERS2*, and Md*ETR1* using the "forward" gene-specific primers (GSPs) shown in **Table 1** with cDNA produced using the Ambion 3′ RACE adapter primer as the template. In the first round of PCR (primary reaction), GSPs were used with the Ambion 3′ outer primer, followed by a nested PCR reaction using a second GSP and the Ambion 3′ RACE inner primer. PCR was performed using 2 μL of cDNA as template in a 20 μL reaction volume for the primary reaction, and 5 μL of the first reaction was used as a template for the nested reaction. For both primary and nested reactions, 30 cycles of PCR were carried out using the following conditions: 95 °C for 30 s, 55 °C for 30 s, and 68 °C for 30 s, followed by a final extension of 7 min at 72 °C. The Roche FastStart High Fidelity PCR system (Roche, Indianapolis, IN) was used to amplify the Law Rome apple 3′ RACE products. All PCR products were isolated from agarose gels following electrophoresis and cloned into the 3kb pGEM-T Easy (Promega) cloning vector following the manufacturer's instructions. The resulting plasmids were sent for sequencing to validate the PCR products.

**DNA Sequencing.** Automated dideoxy sequencing was performed at the Iowa State University DNA Sequencing and Synthesis Facility. Reactions were set up using the Applied Biosytems (Foster City, CA) Prism BigDye Terminator v3.1 cycle sequencing kit with AmpliTaq DNA polymerase, and reactions were electrophoresed on an Applied Biosystems 3730 DNA analyzer. The programs BLASTN and BLASTP on the National Center for Biotechnology Information Website (www.ncbi/nlm/nih.gov) were used to search the nucleotide and protein sequence databases.

**Northern Analysis.** Total RNA was extracted from the peel tissues as described by Chang et al. (*48*). Northern analysis was carried out as described by Gapper et al. (*20*), using 5 μg of total RNA. RNA was transferred in 10× SSC (1.5 M NaCl, 150 mM $Na_3C_6H_5O_7 \cdot 2H_2O$, pH 7.0) to nylon membranes (Hybond XL, Amersham) by downward capillary transfer. After blotting, membranes were washed in 2× SSC and the RNA was fixed to membranes by UV cross-linking (GS Gene Linker UV Chamber, Bio-Rad). Double-stranded DNA probes for Md*AFS1* [AY182241 (*19*)], Md*ACS1*, Md*ACS3*, Md*ACO1*, Md*ERS1b*, *MdERS2*, Md*ETR1*, or *Pc18S* (*20*) were prepared by random primed labeling using the 5′−3′ polymerase activity of the Klenow fragment of T4 DNA polymerase (Roche) according to the manufacturer's instructions. Membranes were bathed in hybridization solution (*49*) and hybridized with [$^{32}$P]dATP-radiolabeled probes at 65 °C for 16 h. Membranes were washed for 20 min in 2× SSC, 1% SDS (w/v), 20 min in 1× SSC, 1% SDS (w/v), 20 min in 0.5× SSC, 1% SDS (w/v), and 20 min in 0.1× SSC, 1% SDS (w/v), at 65 °C. After washing, Kodak Biomax MR or MS film was exposed to membranes at −80 °C.

All Northern blots were repeated twice with similar results. All membranes were reprobed with a partial cDNA clone encoding a 'd'Anjou' pear ribosomal RNA (P*c18S*) to assess loading equality. All autoradiographs were scanned using the Fluor-S MultiImager (Bio-Rad), and transcript hybridization was measured using the software package Quantity One 4.2.0 (Bio-Rad) and then compared with the measured rRNA hybridization and plotted as relative intensity.

**Statistical Analysis.** All data were subjected to ANOVA using Minitab software v. 14.1 (Minitab, Inc., State College, PA). Three-way analyses were carried out for tissue type (red versus green), treatment (with and without 1-MCP), and storage time. Means were separated using least significant differences (LSD) at $P = 0.05$.

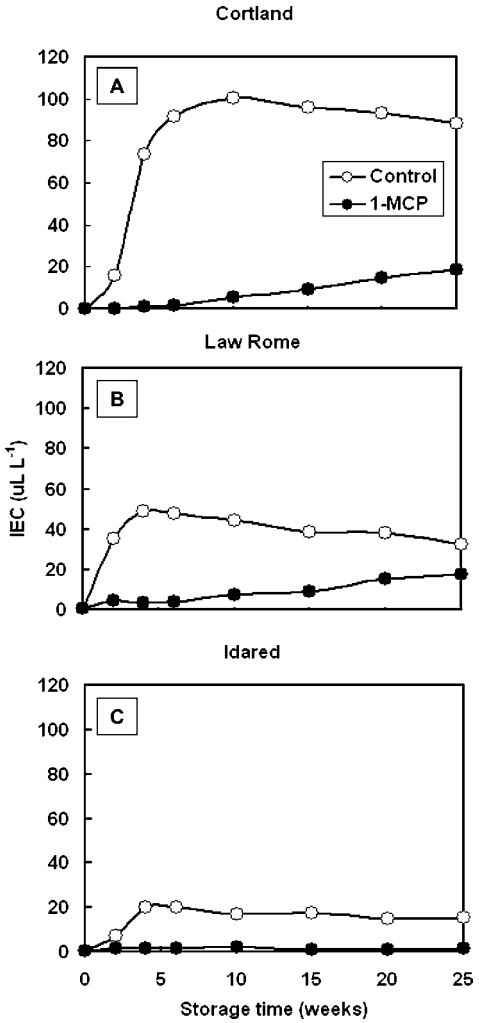


**Figure 1.** Internal ethylene concentration (IEC; $\mu$L L$^{-1}$) of Cortland (**A**), Law Rome (**B**), and Idared (**C**) apples either untreated (control) or treated with 1 $\mu$L L$^{-1}$ 1-MCP at harvest. Fruits were stored at 0.5 °C for up to 25 weeks and sampled at weeks 0, 2, 4, 6, 10, 15, 20, and 25. The LSD at $P = 0.05$ for comparison between treatment and storage time is 20.1, 11.7, and 4.3 $\mu$L L$^{-1}$ for Cortland, Law Rome, and Idared, respectively.

## RESULTS

**Superficial Scald.** Scald incidence was 96, 97, and 1% in untreated control fruit and 14, 3, and 0% in 1-MCP-treated fruit of Cortland, Law Rome, and Idared, respectively. In all cases, visible scald was restricted to the greener or unblushed side of fruit.

**Internal Ethylene Concentration.** The IEC of untreated control fruit increased during storage, with the greatest increase in Cortland and the least in Idared (**Figure 1**). 1-MCP-treated fruit had dramatically lower IECs, but in Cortland and Law Rome apples there was a gradual increase after 6 weeks in storage.

**α-Farnesene and Conjugated Trienol Concentrations.** The concentration of α-farnesene in peel tissues of untreated control Cortland and Law Rome fruit increased markedly during storage and reached maximum values at 10−15 weeks. The values obtained were dependent on the side of the fruit (blushed or unblushed) and the cultivar (**Figure 2A**,**B**). In contrast, α-farnesene concentrations in Idared fruit increased only gradually (**Figure 2C**) and to <25% of those in Cortland and Law Rome. Differences in α-farnesene concentrations between green and red peel tissues were not significant.

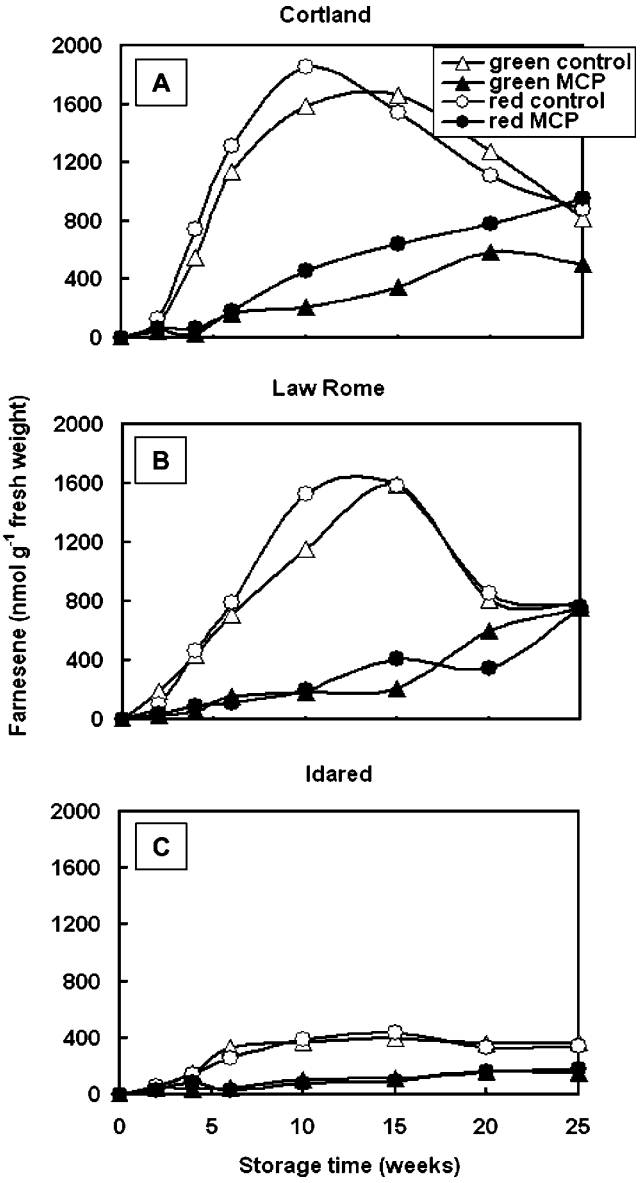


**Figure 2.** α-Farnesene concentrations (nmol g$^{-1}$ of fresh weight) in the red and green peel tissues of Cortland (**A**), Law Rome (**B**), and Idared (**C**) apples that were either untreated (control) or treated with 1 $\mu$L L$^{-1}$ 1-MCP at harvest. Fruits were stored at 0.5 °C for up to 25 weeks and sampled at weeks 0, 2, 4, 6, 10, 15, 20, and 25. The LSD at $P = 0.05$ for comparison between peel color, treatment, and storage time is 241, 179, and 87 nmol g$^{-1}$ of fresh weight for Cortland, Law Rome, and Idared, respectively.

α-Farnesene accumulation in peel tissues was initially inhibited by 1-MCP treatment but increased during storage (**Figure 2**). In 1-MCP-treated tissues, α-farnesene concentrations at the end of storage (25 weeks) were similar to those found in control fruit. By then, however, levels in control tissue had declined from maximal concentrations by about 50%. Little effect of peel tissue type was detected in any cultivar. CTol concentrations increased dramatically in untreated Cortland and Law Rome fruit after 4−6 weeks, but to a greater extent (about 2-fold) in green than in red peel tissue (**Figure 3A**,**B**). CTol concentrations increased during storage of 1-MCP-treated Cortland fruit after 10 weeks. There was no significant difference between tissue types, and CTol levels were roughly proportional to those of α-farnesene. CTol concentrations in Idared increased during storage but remained very low relative

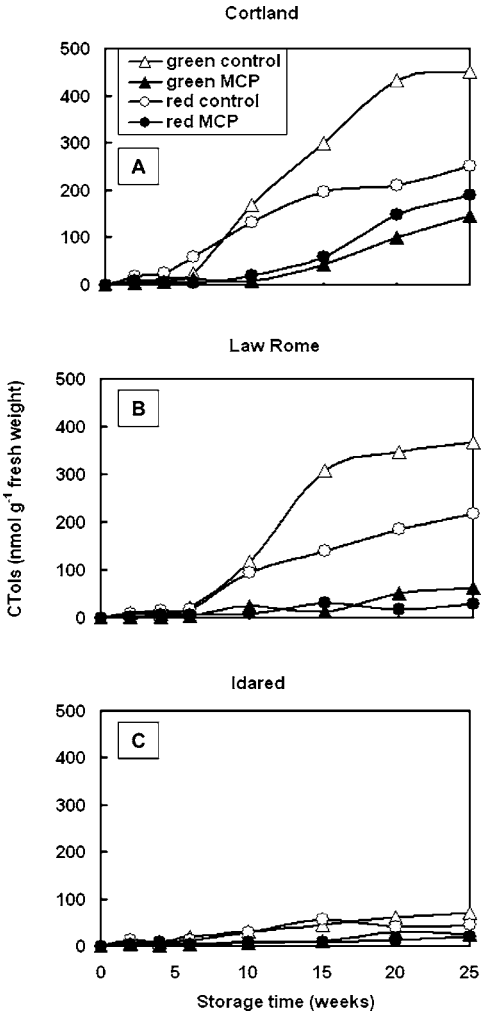


**Figure 3.** Conjugated trienol (CTol) concentrations (nmol $g^{-1}$ of fresh weight) in red and green peel tissues of Cortland (**A**), Law Rome (**B**), and Idared (**C**) apples that were either untreated (control) or treated with 1 $\mu$L $L^{-1}$ 1-MCP at harvest. Fruits were stored at 0.5 °C for up to 25 weeks and sampled at weeks 0, 2, 4, 6, 10, 15, 20, and 25. The LSD at $P = 0.05$ for comparison between peel color, treatment, and storage time is 51, 41, and 17 nmol $g^{-1}$ of fresh weight for Cortland, Law Rome, and Idared, respectively.

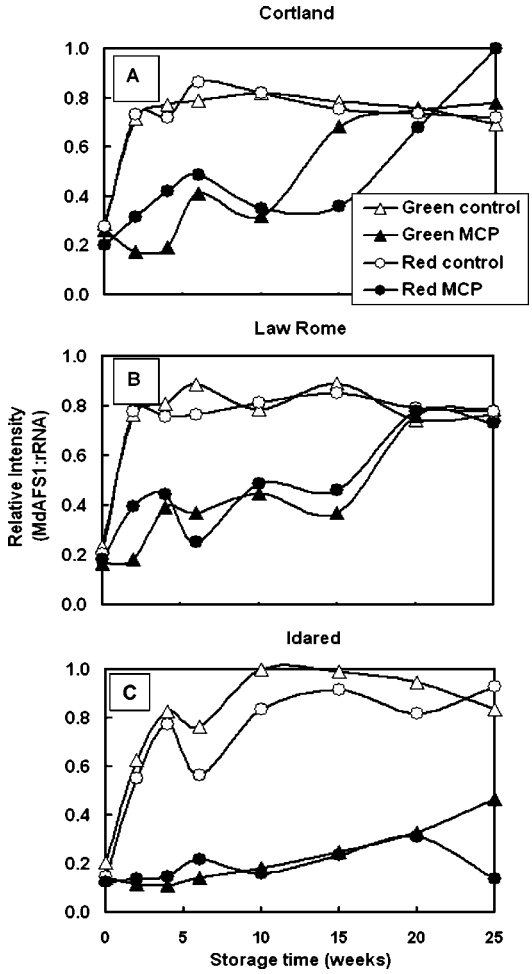


**Figure 4.** Changes in the relative abundance of α-farnesene synthase gene Md*AFS1* transcript in red and green peel tissues of Cortland (**A**), Law Rome (**B**), and Idared (**C**) apples that were untreated (control) or treated with 1 $\mu$L $L^{-1}$ 1-MCP at harvest and stored at 0.5 °C in air for up to 25 weeks. Transcript abundance was determined as the ratio of Md*AFS1* mRNA to 18S rRNA (Md*AFS1*) by densitometry scanning of Northern blots produced using total peel tissue RNA and $^{32}$P-labeled Md*AFS1* and *18S* gene fragments as probes. Values were normalized for the three cultivars and two tissues by arbitrarily setting the highest value at 1.0.

to those in Cortland and Law Rome (**Figure 3C**). 1-MCP treatment further reduced the CTol concentrations in Idared peel tissues.

**Md*AFS1* Transcript Levels.** Md*AFS1* transcript abundance increased rapidly in untreated Cortland and Law Rome fruit, reaching close to maximal values after 2 weeks of storage (**Figure 4A**,**B**), whereas in Idared controls it increased through 4 weeks, dipped slightly, and then increased to a maximum by 10 weeks (**Figure 4C**). 1-MCP treatment initially suppressed Md*AFS1* expression in all three cultivars. However, Cortland and Law Rome escaped from this suppression after 10−15 weeks, with Md*AFS1* transcript abundance increasing to maximal control levels by 15−20 weeks (**Figure 4A**,**B**). In Cortland, but not in Law Rome, this delayed increase in expression occurred sooner in green than in red tissue. Md*AFS1* transcript levels increased gradually in 1-MCP-treated Idared fruit, but throughout storage remained much lower than the maximum levels in untreated fruit (**Figure 4C**).

**Md*ERS* Transcript Levels.** In control fruit of all three cultivars, Md*ERS* transcript increased rapidly, reaching close to maximal values within the first 2 weeks of storage (**Figure 5**). For Cortland, after the initial sharp increase, Md*ERS1* transcript rose gradually in green tissue through 10 weeks and then declined, whereas in red tissue transcript abundance essentially leveled off. For untreated Law Rome and Idared, the initial rapid increase in Md*ERS1* transcript was followed by a small decrease through 4−6 weeks. Thereafter, more or less constant levels were maintained in Law Rome, whereas abundance slowly increased in Idared, more in green than in red tissue.

In general, the increase in Md*ERS1* transcript was delayed and more gradual in all fruit treated with 1-MCP, and levels were higher in green than in red tissue from 6 to 20 weeks of storage. Transcript levels in green tissue of Cortland started to rise after 2 weeks and continued to increase throughout 25 weeks of storage, whereas in red tissue a substantial increase occurred only after 15 weeks (**Figure 5A**). In green tissue of Law Rome, levels of Md*ERS1* mRNA slowly increased until 15 weeks but thereafter rose rapidly to maximal levels (**Figure 5B**). Changes in red tissue of Law Rome showed a similar trend except for a sharp dip in transcript abundance from 4 to 6 weeks of storage. For Idared, after a slight decline during the first 2 weeks, Md*ERS1* transcript in green tissue increased slowly between

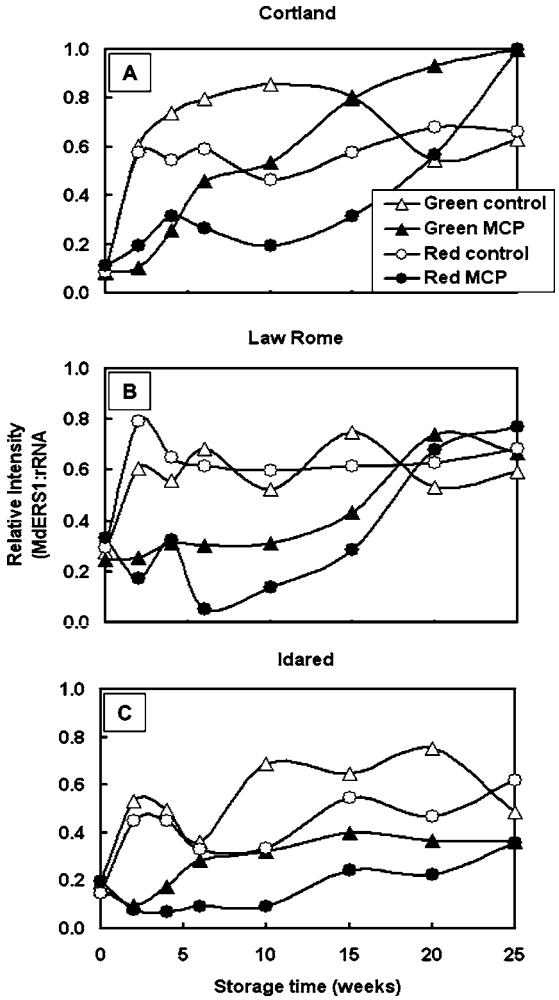


**Figure 5.** Changes in the relative abundance of the ethylene response sensor gene Md*ERS1* transcript in red and green peel tissues of Cortland (**A**), Law Rome (**B**), and Idared (**C**) apples that were untreated (control) or treated with 1 $\mu$L $L^{-1}$ 1-MCP at harvest and stored at 0.5 °C in air for up to 25 weeks. Transcript abundance was determined as the ratio of Md*ERS1* mRNA to 18S rRNA (Md*ERS1*) by densitometry scanning of Northern blots produced using total peel tissue RNA and $^{32}$P-labeled Md*ERS1* and *18S* gene fragments as probes. Values were normalized for the three cultivars and two tissues by arbitrarily setting the highest value at 1.0.

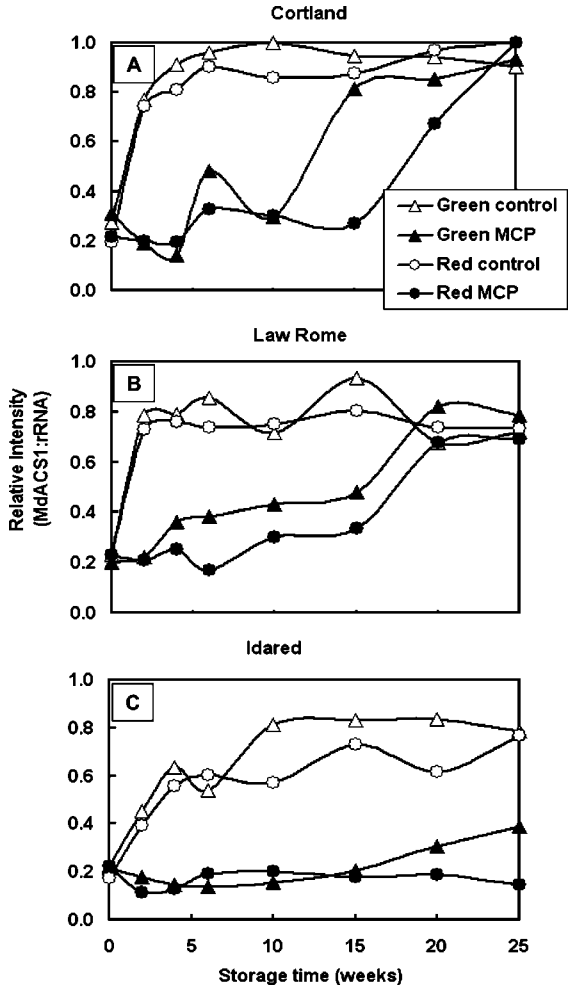


**Figure 6.** Changes in relative abundance of ACC synthase gene Md*ACS1* transcript in red and green peel tissues of Cortland (**A**), Law Rome (**B**), and Idared (**C**) apples that were untreated (control) or treated with 1 $\mu$L $L^{-1}$ 1-MCP at harvest and stored at 0.5 °C in air for up to 25 weeks. Transcript abundance was determined as the ratio of Md*ACS1* mRNA to 18S rRNA (Md*ACS1*) by densitometry scanning of Northern blots produced using total peel tissue RNA and $^{32}$P-labeled Md*ACS1* and *18S* gene fragments as probes. Values were normalized for the three cultivars and two tissues by arbitrarily setting the highest value at 1.0.

2 and 15 weeks of storage and then leveled off (**Figure 5C**). A similar moderate increase was observed in red tissue but was delayed, occurring after 10 weeks of storage.

**Md*ACS1* Transcript Levels.** Md*ACS1* transcript abundance increased rapidly in untreated Cortland and Law Rome fruit, reaching close to maximum values after 2 weeks of storage (**Figure 6A**,**B**). In Idared the increase was more gradual, reaching maximal levels after 10−15 weeks (**Figure 6C**). 1-MCP treatment markedly delayed the increase in Md*ACS1* transcript levels in Cortland and Law Rome, and in Idared it was all but eliminated. Accumulation of Md*ACS1* transcript in 1-MCP-treated Cortland and Law Rome increased abruptly after 10−15 weeks, and after 15−20 weeks, levels were close to the maximum abundance in control fruit. From 4 weeks to the end of storage, levels of Md*ACS1* transcript were generally higher in green than in red tissue of 1-MCP-treated Law Rome. In 1-MCP-treated Cortland, the rapid increase in transcript occurred at about week 10 in green tissue compared with week 15 in red tissue. In 1-MCP-treated Idared fruit, a modest increase in Md*ACS1* transcript abundance occurred only in green tissue after 15 weeks of storage.

**Md*ACO1* Transcript Levels.** Md*ACO1* transcript levels rose rapidly in untreated fruit of all three cultivars during the early stages of storage (**Figure 7**). For Cortland, levels of Md*ACO1* mRNA reached a plateau at 6−10 weeks and were higher in green than in red tissue at harvest and throughout the 25 week storage period. In Idared control fruit as well, Md*ACO1* transcript abundance was continuously higher in green than in red peel tissue. Maximum levels occurred at 4 and 6 weeks in green and red tissue, respectively, after which there was a steady decline in both tissues. By contrast with Cortland and Idared, Md*ACO1* transcript levels in untreated Law Rome were higher in red than in green peel tissue from 4 to 15 weeks of storage. A transient dip at 6−10 weeks was followed by an increase to maximum levels at 15 weeks.

1-MCP treatment delayed and reduced the increase in Md*ACO1* transcript abundance in red peel tissue of Cortland apples, which was always lower than that in the controls (**Figure 7A**). In green tissue of 1-MCP-treated Cortland, there was a sharp decline in Md*ACO1* transcript during the first 2 weeks

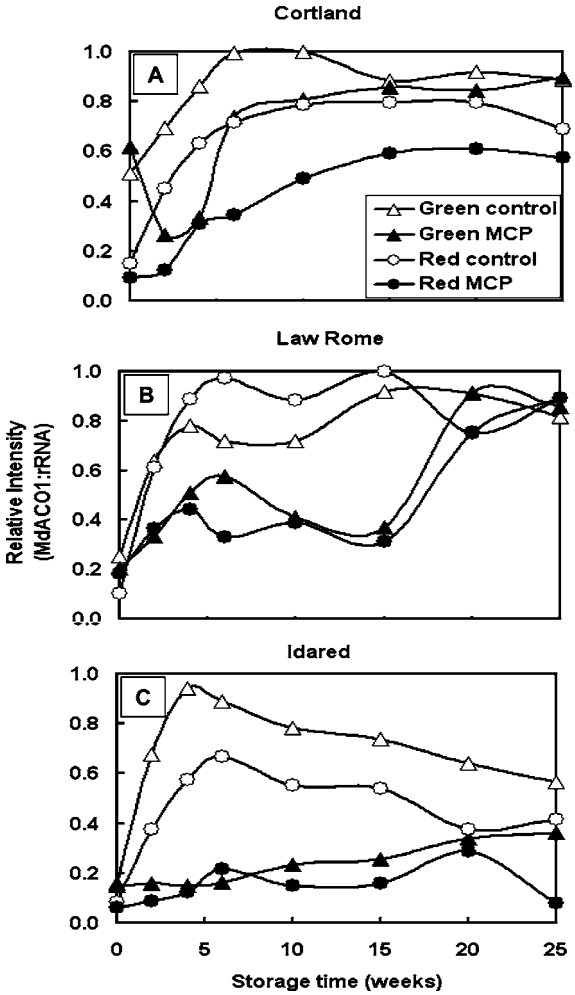


**Figure 7.** Changes in relative abundance of ACC oxidase gene Md*ACO1* transcript in red and green peel tissues of Cortland (**A**), Law Rome (**B**), and Idared (**C**) apples that were untreated (control) or treated with 1 $\mu$L $L^{-1}$ 1-MCP at harvest and stored at 0.5 °C in air for up to 25 weeks. Transcript abundance was determined as the ratio of Md*ACO1* mRNA to 18S rRNA (Md*ACO1*) by densitometry scanning of Northern blots produced using total peel tissue RNA and $^{32}$P-labeled Md*ACO1* and *18S* gene fragments as probes. Values were normalized for the three cultivars and two tissues by arbitrarily setting the highest value at 1.0.

of storage, followed by a rapid rise from 2 to 6 weeks. After 15 weeks, levels equaled those in control fruit. For Law Rome fruit treated with 1-MCP, Md*ACO1* mRNA levels increased slowly over the first 4−6 weeks of storage, followed by a gradual decrease through 15 weeks, and then a rapid increase to near maximal control levels by 20 weeks in both green and red tissues (**Figure 7B**). In contrast with Cortland and Law Rome, 1-MCP treatment of Idared apples strongly suppressed Md*ACO1* expression. Only a modest increase in the abundance of Md*ACO1* mRNA occurred in green and red peel tissues of Idared over the course of storage (**Figure 7C**).

**Peroxidase and Catalase Activities.** Activities of POX and CAT showed inconsistent patterns of change over time when expressed by specific activity or on a fresh weight basis (data not shown), and therefore only the main effects are shown (**Table 2**). Highest POX activity was measured in Cortland ($P < 0.001$), and highest and lowest CAT activities were measured in Idared and Law Rome, respectively ($P < 0.001$). With the exception of CAT activity in Law Rome, activities of both enzymes were lower in red than in green peel. The effects of 1-MCP were not significant except for Cortland, in which POX and CAT activities in peel tissues were lower and higher, respectively, in 1-MCP-treated compared with untreated control fruit.

**Table 2.** Peroxidase (POX) and Catalase (CAT) Activities in Green and Red Peel Tissues and in Peel from Untreated and 1-MCP-Treated Cortland, Law Rome, and Idared Apples

| | peel color | | treatment | |
|---|---|---|---|---|
| cultivar | green | red | control | 1-MCP |
| POX activity ($A_{470}$ $min^{-1}$ $mg^{-1}$ of protein) | | | | |
| Cortland | 2.31 | 2.10*** | 2.26 | 2.14* |
| Law Rome | 1.31 | 1.14*** | 1.22 | 1.23NS |
| Idared | 1.14 | 0.46*** | 0.82 | 0.79NS |
| CAT activity ($A_{240}$ $min^{-1}$ $mg^{-1}$ of protein) | | | | |
| Cortland | 7.43 | 3.92*** | 4.63 | 6.72* |
| Law Rome | 2.95 | 3.12NS | 2.62 | 3.46NS |
| Idared | 12.31 | 7.21*** | 9.38 | 10.14NS |

## DISCUSSION

1-MCP is a new technology used extensively by apple industries around the world to maintain postharvest fruit quality (*50*). A major commercial benefit of the technology is prevention of softening after storage, resulting in higher quality fruit throughout the marketing chain. Early experiments in which the effects of 1-MCP treatment were evaluated also showed that incidence of the physiological storage disorder superficial scald was decreased or eliminated (*30*−*32*). This suggested that 1-MCP treatment could replace a postharvest drench with the antioxidant DPA that is commonly used to control scald. In fact, substitution of DPA with 1-MCP has occurred in some storage operations. However, scald control by 1-MCP may be incomplete for some apple and pear cultivars (*31*, *38*−*40*), probably because new ethylene-binding sites are formed during storage. Thus, 1-MCP-induced inhibition of ethylene production and responsiveness, and consequently ripening, is overcome. To investigate the hypothesis that 1-MCP-induced inhibition is lost, the expression of key genes involved in, and the accumulation of products derived from, the biosynthesis of ethylene and α-farnesene were compared in untreated and 1-MCP-treated apples of three cultivars stored at 0.5 °C for up to 25 weeks. The comparison included Idared, an apple resistant to scald (*18*), and fruit of two scald-susceptible cultivars, Cortland and Law Rome. Cortland can recover relatively quickly from 1-MCP-induced inhibition of ethylene production, and 1-MCP-treated fruit can develop scald after long-term storage (*39*). In Law Rome, scald can be greatly inhibited by 1-MCP treatment (*32*), but effects can be variable under commercial conditions (unpublished data).

IECs of whole fruit, and α-farnesene accumulation in red and green peel tissues, increased after an initial delay during storage in untreated control fruit, but were inhibited in 1-MCP-treated fruit (**Figures 1** and **2**). Ethylene and α-farnesene concentrations were highest in Cortland and lowest in Idared and followed similar patterns, supporting the view that ethylene production is necessary for the induction of α-farnesene synthesis (*3*, *12*, *37*). The exact link between α-farnesene and ethylene is unknown, but Md*AFS1* expression is clearly inhibited by 1-MCP (**Figure 4**), and Md*AFS1* transcript abundance follows patterns similar to those of α-farnesene concentration (**Figure 2**) (*18*, *20*, *21*). There was little effect of tissue type on α-farnesene concentration or Md*AFS1* transcript accumulation (**Figures 2** and **4**).

1-MCP inhibited the early increase in IECs in all cultivars, but ethylene started to rise slowly at about week 10 in Cortland

and Law Rome (**Figure 1**). The increase in α-farnesene concentration was also inhibited by 1-MCP in all cultivars (**Figure 2**), but, as noted for IEC, levels of the sesquiterpene rose gradually in Cortland and Law Rome after several weeks of storage. However, the increase of α-farnesene was earlier and more rapid in Cortland (**Figure 2A**) than in Law Rome (**Figure 2B**). Consistent effects of tissue type on α-farnesene concentrations were detected only for Cortland, in which red tissues had higher concentrations than green tissues by week 10.

After a 4−6 week delay, CTol concentrations increased in untreated Cortland and Law Rome apples, and more in green than in red peel tissues by week 15 (**Figure 3A**,**B**). CTol concentrations increased only slightly in Idared (**Figure 3C**). CTol concentrations in 1-MCP-treated fruit remained very low in Idared and rose only slightly in Law Rome, but increased substantially in Cortland after week 10. The patterns of α-farnesene and CTol accumulation in the peel during storage are consistent with those shown for these and other cultivars, the usual patterns of change being an increase in α-farnesene early in storage and an increase in CTols later in storage (*3*, *7*, *10*, *11*, *15*). In general, CTol concentration is positively correlated with scald incidence, whereas the correlation between α-farnesene and scald is weak (*7*, *29*). 1-MCP appears to inhibit α-farnesene synthesis and thereby limits the precursor for oxidation to CTols (*18*, *20*, *21*, *25*). In contrast, DPA does not consistently affect α-farnesene production but inhibits its oxidation (*25*, *51*).

Comparison of changes in green and red tissues showed little difference in α-farnesene concentrations (**Figure 2**), but CTol accumulation was higher in green compared with red tissues (**Figure 3A**,**B**). However, one noteworthy point with respect to the role of CTols in scald induction is that although CTol concentrations in red tissues of untreated Cortland apples were higher than those in green tissues of 1-MCP-treated fruit, there was no visual evidence of scald on the blushed side of control fruit, whereas 14% of the 1-MCP-treated fruit developed scald symptoms on the unblushed side.

Our results indicate that dipping of whole fruit in hexane or use of peels from around the equatorial area of fruit to extract α-farnesene and CTols (*7*, *10*−*12*, *16*, *25*, *27*, *31*) accurately reflects α-farnesene concentrations. However, because of the difference in CTol concentrations in green and red tissues, combining tissues may result in underestimation of concentrations in the greener, scald-susceptible peel tissues of the fruit.

As scald development appears to involve oxidation of α-farnesene to CTols, disorder incidence should be related to antioxidative processes (*22*, *26*, *28*). CAT and POX activities were often higher in scald-resistant than in scald-susceptible cultivars (*6*, *28*). For example, POX activity was much lower in Law Rome than in Idared apples (*6*). In the present study, differences in the activities of antioxidative enzymes among the three cultivars were relatively small (**Table 2**). 1-MCP-treated fruit had lower POX activity and higher CAT activity in Cortland, but no effects of treatment were detected for other cultivars. Interestingly, however, the activities of POX and CAT were lower in red tissues compared with green tissues, with the exception of CAT activity in Law Rome. The presence or absence of individual isoenzymes may be a better indicator of scald susceptibility than total enzyme activities (*26*).

Information about antioxidant processes in blushed and unblushed peel tissues is limited. Ma and Cheng (*52*) compared fruits that were shaded or exposed to sunlight and found that sun-exposed peel had higher activities of ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase and a larger size and a higher reduction state of the ascorbate and glutathione pools. However, CAT activity was lower in the sun-exposed peel than in the shaded peel. Ascorbic acid concentrations were lower in flesh tissue from the green compared with the red side of Stayman Winesap apples (*53*). Currently, this kind of information is sparse and further research is warranted. One obvious difference between unblushed and blushed peel tissues is the abundance of anthocyanins and related phenylpropanoids in the latter. Although these compounds are typically potent antioxidants, they are generally water soluble and sequestered in the vacuole, so it is not readily apparent how they would limit the oxidation of α-farnesene. Possibly, blushed peel tissue also includes high concentrations of lipophilic phenolic antioxidant compounds such as those identified in epicuticular wax of scald-resistant 'Gala' apples (*54*).

One major aim of this study was to characterize the 1-MCP-induced inhibition of ethylene production and subsequent recovery from that inhibition in apple cultivars such as Cortland and Law Rome (**Figure 1A**,**B**) compared with Idared (**Figure 1C**). The inhibition of ethylene production by 1-MCP is thought to occur as a result of competitive, long-term binding of 1-MCP to ethylene receptor sites, although the exact mode of action is unknown (*35*, *55*). In various fruits, reduced or delayed ethylene production is associated with reduced expression of some, but not all, ETR and ERS genes (*36*, *56*) and negative feedback regulation of several *ACS* and *ACO* genes (*36*, *57*, *58*). In apple, transcript levels of genes involved in ethylene perception (ethylene receptor genes *ETR1* and *ERS1*, but not the signal transduction gene *CTR1*) and biosynthesis (*ACS* and *ACO*) are markedly reduced in response to 1-MCP treatment (*43*−*45*). Our results further indicate that the accumulation of Md*ERS1*, Md*ACS1*, and Md*ACO1* transcripts (**Figures 5**−**7**) is suppressed in 1-MCP-treated fruit compared with untreated fruit. In general, the effects of skin type were small, but accumulation of Md*ACO1* transcript was markedly higher in green compared with red tissues of Cortland apples. It is important to note that accumulation of transcripts involved in the biosynthesis of ethylene, Md*ACO1* and Md*ACS1*, was about the same in nontreated Idared as in nontreated Cortland and Law Rome fruits. However, IEC levels were much lower for this cultivar compared with both Cortland and Law Rome. The level of transcript in any given tissue does not always relate to the abundance of active encoded enzyme. Vogel et al. (*59*) reported that ACS5 in *Arabidopsis* undergoes post-transcriptional regulation during leaf senescence. Our seemingly contradictory results could be explained in part by post-transcriptional regulation of either ACO or ACS in Idared. Furthermore, the levels of *S*-adenosyl methionine might be low in Idared compared with other scald-susceptible cultivars, or this biosynthetic precursor to ethylene could be diverted away to some other biochemical pathway such as polyamine biosynthesis.

The recovery of ripening from 1-MCP-induced inhibition may result from production of new receptors (*35*, *55*). Studies of tomato show that delayed ethylene production in 1-MCP-treated fruit is associated with increased expression of some *ETR* and *ERS* genes, *ACS*, and to a lesser extent *ACO* (*36*). There appears to be no previous study on recovery of ripening from 1-MCP inhibition in apple under normal cold storage conditions. However, in apples held at room temperature, transcripts for genes encoding ERS, ACS, and ACO, but not ETR or CTR, started to increase when recovery of ethylene production occurred in 1-MCP-treated fruit (*43*, *44*). In this study, under

cold storage conditions we observed an accumulation of Md*ERS1* mRNA during storage as 1-MCP induced inhibition of ripening abated (**Figure 5**). The subsequent loss of feedback inhibition resulted in the accumulation of Md*ACS1* and Md*ACO1* transcripts (**Figures 6** and **7**) and, thus, initiation of ethylene production (**Figure 1**), albeit at much lower levels than those of untreated control fruit within the time frame of this study. Full recovery of ethylene production and attendant ripening processes will occur over time, the rate of recovery being dependent on cultivar and maturity at harvest (*30*, *32*, *41*).

In summary, we have evaluated the long-term responses to 1-MCP treatment in the fruit of three apple cultivars with different susceptibilities to scald, focusing on the recovery of ethylene and α-farnesene metabolism from 1-MCP-induced inhibition and comparing the effects in green and red (unblushed and blushed) skin. The cultivars varied in IEC, α-farnesene, and CTol accumulation, with lowest concentrations in Idared, which had negligible scald incidence. Accumulation of CTols was highest in Cortland and Law Rome, which had the highest scald susceptibility. 1-MCP inhibition of the increase in IEC was associated with delayed and reduced accumulation of Md*AFS1* transcript, α-farnesene, and CTols, but inhibition was transient for Cortland and Law Rome. The eventual loss of 1-MCP inhibition of ethylene and α-farnesene production appeared to result in increased susceptibility of these two cultivars to scald. CTol accumulation was greater in the green, more scald-susceptible skin than in the red skin of these fruit. Expression of key genes in ethylene signal transduction and biosynthesis was suppressed by 1-MCP, remaining low in Idared, but recovering in Cortland and Law Rome, with an associated increase in IEC.

Knowledge of how ethylene perception and production in fruits are inhibited by 1-MCP is necessary to achieve two main objectives: first, to be able to maintain inhibition of ripening in fruit such as apple, where it is desirable to consistently maintain inhibition of ripening associated phenomena (including scald development) during storage, and, second, to understand how release from 1-MCP inhibition of ripening is modulated to obtain ripening in the marketplace of fruits such as tomato and banana, where a delay but not a total inhibition of ripening is desirable.

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