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Impact of Suppression of Ethylene Action or Biosynthesis on Flavor Metabolites in Apple (*Malus domestica* Borkh) Fruits

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To understand the role of ethylene in regulating the overall flavor of apple fruits, ethylene production or action was reduced using transgenic apple trees suppressed for ACC-synthase or ACC-oxidase enzyme activity or by the addition of 1-methylcyclopropene (1-MCP), an ethylene action inhibitor. Flavor components were differentially regulated in response to the suppression of both ethylene biosynthesis and action. Headspace analysis of aroma production, an ethylene-associated event, showed a reduction in ester and alcohol production in the ethylene-suppressed lines and in the apples treated with 1 μL L^{-1} 1-MCP for 20 h at 20 °C. However, no major differences were observed in the concentrations of aldehyde volatiles. Other flavor metabolites that showed an ethylene-dependent pattern were organic acids and sugars. Malic acid degradation was significantly reduced under ethylene-suppressed conditions, showing a recovery after the fruit was exposed to ethylene. Sucrose and fructose concentrations were influenced by suppression or enhancement of ethylene. Total phenolics as well as individual phenolic compounds showed an ethylene-dependent regulation only in response to the suppression of ethylene biosynthesis, but not when ethylene action was inhibited.

KEYWORDS: Aroma; fruit flavor complex; phenolic compounds; sugars; *Malus domestica*; organic acids; transgenic apple fruit

INTRODUCTION

Among the many quality parameters defining the desirability of fruits and vegetables, there are important nonvisual characteristics such as texture, nutritional value, and flavor, which influence the final acceptance by the consumer (*1*). Flavor composition has been defined as a complex attribute of quality, in which the mix of sugars, acids, and volatiles plays a primary role (*2*). Another interesting group of metabolites are phenolic compounds, which are important secondary metabolites contributing to overall fruit quality, including flavor, nutritional value, and appearance (*3*).

During fruit development there are many changes in flavor metabolites caused by their synthesis, transport, or degradation. In climacteric fruits, ethylene plays an important role as a modulator of ripening. All of these fruit quality related metabolites may be directly regulated by ethylene (ethylene-dependent processes) or by other signals (ethylene-independent process) (*4*).

In addition to the flavor metabolites mentioned above, sugars and organic acids measured through total soluble solids (TSS) and titratable acidity (TA), respectively, are most commonly associated with fruit taste (*5*). The sugars sucrose, glucose, and fructose are responsible for the sweetness, with some minor contribution of sorbitol in apple (*2, 6*). Sugars are transported from source organs and accumulate in fruit during their development, where they form starch. The hydrolysis of starch in the fruit is an important source of sugars in the last stages of fruit development and starts before the climacteric peak (*6*). The effect of ethylene on sugar accumulation has been studied mainly using TSS as an indicator of sweetness, and it has been observed that the suppression of ethylene action or biosynthesis does not alter TSS accumulation during ripening; therefore, it is considered an ethylene-independent event (*7*, *8*). However, what remains unclear is the behavior of individual sugars in response to ethylene regulation.

Regarding acidity, malic acid is the most abundant organic acid in apples, followed distantly by citric acid (*6*, *9*). Because organic acids are substrates of respiration, their levels typically decrease during ripening. The use of inhibitors of ethylene biosynthesis and action have shown a reduction in the loss of acidity during ripening, which suggests that organic acids metabolism is an ethylene-dependent process (*7*).

In flavor, phenolic compounds are major substances responsible of bitterness and astringency, in which tannins, catechins, and epicatechins have been identified as astringent molecules (*10*). On the other hand, during enzymatic browning in which there is a development of undesirable colors and flavors, catechins and chlorogenic acid are the main substrates for the reaction (*11*). In general, there is a reduction in the total phenolic composition through apple fruit development; however,

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this behavior during maturation and storage varies according to cultural conditions, cultivar (*12*), environmental factors (*1*), and physiological stage at harvest (*13*). The evidence for the relationship between ethylene and phenolic composition in fruits is derived from the effect of storage conditions on ethylene biosynthesis and action, including low temperatures, low oxygen concentration, and high carbon dioxide concentration (*3*, *13*, *14*). In general, results indicate that phenolic metabolism in apple is stable under these conditions, and few changes are significant, suggesting that phenolic metabolism and turnover are low during long-term storage.

In addition to the four basic flavors (sweet, sour, salty, and bitter) that humans can recognize in fruits and vegetables, aroma has an important influence on the final consumer acceptance of the commodity (*15*). Fruit aroma is determined by a complex mixture of a large number of volatile compounds including alcohols, aldehydes, carboxylic esters, and ketones (*16*). Esters are among the most important classes of volatiles in apple fruit flavor, with more than 20 esters that have been detected in apple cultivars (*17*, *18*). In apple, the typical flavor develops during ripening with a maximum endogenous ester concentration occurring at the climacteric peak (*16*, *18*, *19*). In this paper we demonstrate that aroma production is under ethylene regulation using both biotechnological and chemical approaches for suppressing ethylene biosynthesis or action.

MATERIALS AND METHODS

Plant Material and Treatments. Transgenic Greensleeves apple fruits suppressed for ethylene biosynthesis were obtained from different lines grown in an experimental orchard in northern California. The lines used in these experiments were transformed using binary vectors that express the cDNAs corresponding to ACC-synthase (ACS) and ACC-oxidase (ACO) enzymes in either a sense or antisense pattern (*20*). Fruits of selected Greensleeves apple lines including a nontransformed fruit (GS), 68G (ACO-antisense), and 103Y (ACS-sense) were harvested at a preclimacteric stage and stored at 20 °C in air for 14 days. Relative humidity was maintained at 90−95% in all cases. A second approach for reducing ethylene metabolism was the use of the ethylene action inhibitor 1-methylcyclopropene (1-MCP). Greensleeves apples harvested from a different orchard were treated with 0 (control) and 1 μL L^{-1} 1-MCP, and each group of fruit was treated in a 20 L sealed glass jar for 20 h at 20 °C before storage at 20 °C for 14 days. Enhancement of ethylene production was done by exogenous application of ethylene using fruits from lines 68G and 103Y. Half of the fruit was stored at 20 °C in an ethylene-free atmosphere, and the other half was stored at 20 °C under a flow of air containing 80 μL L^{-1} ethylene during storage. A similar approach was used for the MCP-treated fruit.

Ethylene and Respiration Rate Measurements. Within each experiment ethylene production and respiration rates were determined every other day during storage for individual fruits using a static system. Five fruits from each replicate (three replicates per treatment) were weighed and placed in 0.5 L jars at 20 °C. The jars were sealed for 30 min before measurements. Carbon dioxide and ethylene concentrations were determined by an infrared gas analyzer (Horiba, Irvine, CA) and a Carle gas chromatograph (Hach Carle, Loveland, CO) equipped with a flame ionization detector, respectively.

Maturity and Quality Parameters. As maturity and quality indices, external color (Minolta Chromameter), TSS (refractometer), TA (automatic titration system), firmness (penetrometer), and starch pattern (IKI staining pattern, based on the rating scale for Granny Smith apples) were measured at harvest and at the end of storage.

Sugars and Organic Acids Determination. Samples for sugars and other chemical analyses were prepared from five fruits per replicate (three replicates per line or treatment). Sugars and organic acids were analyzed according to the method of Pérez et al. (*21*). Briefly, 10 g of tissue was homogenized in a Polytron with 25 mL of cold 95% ethanol for 3−5 min. The sample was centrifuged at 12000 rpm for 20 min and vacuum filtered through two layers of Whatman no. 1 paper. The solution was made up to 50 mL with 80% ethanol. Then, an aliquot of 10 mL was taken and dried under a nitrogen stream at 50 °C. The residue was dissolved in 2 mL of 0.2 N H_2SO_4 with 0.05% EDTA. The sample was loaded onto an activated Sep-Pak C18 cartridge and the eluate collected. The sample was washed through with a further 4 mL of the solution. The eluate was filtered through a 0.45 μm filter and analyzed by HPLC (Hewlett-Packard model 1050 pump) connected to a photodiode array detector (HP model 1040M series II) with an autosampler (HP model 1050), operated by HP ChemStation software (Hewlett-Packard, Menlo Park, CA). In this case, a refractive index (RI) monitor (model 1750; Bio-Rad Laboratories Inc., Hercules, CA) was connected in series with the DAD. Sugars and organic acids were separated by a stainless steel ION-300 column (300 mm × 7.8 mm, 10 μm) (Interaction Chromatography, San Jose, CA) using 0.0085 N H_2SO_4 at a flow rate of 0.4 mL min^{-1}. Detection was at 210 nm for organic acids and at 250 nm for sugars using the RI detector.

Total Phenolics. Total phenolic compounds were quantified by using the Folin−Ciocalteu spectrophotometric method (*22*). Data are reported as milligrams of *p*-coumaric acid/100 mL of juice. Because differences in phenolic composition have been determined among fruit tissues (*13*), 5 g samples were taken containing either 20% of peel or no peel.

Individual Phenolic Compounds. Individual phenolics were identified according to the procedure of Gil et al. (*23*). After extraction of 5 g of frozen tissue with methanol, juice was then filtered through a 0.45 μm filter for analysis by HPLC. Separations were carried out using the system described above. A reverse phase C_{18} Nucleosil column (150 × 4.6 mm, particle size = 5 μm), with a guard column of the same material (MetaChem Technologies Inc., Torrance, CA) was used. Acetic acid (2.5%) was added to water and methanol to increase peak resolutions before preparation of the following mobile phases: water (A); 88% water + 12% methanol (B); 20% water + 80% methanol (C); and methanol (D). Elution started with 100% A, which remained isocratic until 5 min. A gradient was then used to reach 100% B at 10 min, holding it isocratic for an additional 3 min. From 13 to 35 min a linear gradient was used to reach 50% B and 50% C and then 100% C at 40 min. The column was then washed with 100% D at 42 min. The flow rate was 1 mL min^{-1}, and chromatograms were recorded at 510, 350, and 280 nm. Whenever possible, peaks were identified and compared using known standards.

Determination of Volatiles. Apple cortical and skin tissue were ground using liquid nitrogen and kept at −80 °C until analysis. Six grams of the 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 (ref *24*, with modifications). Prior to sealing of the vials, 600 μL of the internal standard (IS) solution (1-octanol) was added, to get a 500 ppb final concentration of the IS. A poly(dimethylsiloxane)/divinylbenzene (PDMS/DVB, 65 μm thickness) SPME fiber was used. For manual SPME sampling conditions, an equilibration (position 2) at 50 °C for 30 min and desorption (position 4) for 4 min were used. A GC-MS system equipped with a DB-Wax column (J&W Scientific, 30 m, 0.32 mm i.d., 0.25 μm film thickness) was used for analysis. Conditions for chromatography were as follows: injector at 250 °C; initial oven temperature, 40 °C held for 5 min, increased to 50 °C at 2 °C min^{-1}, increased to 200 °C at 5 °C min^{-1}, and held for 5 min. Linear velocity of the carrier gas was 35 cm s^{-1} (*25*). Mass spectra were obtained by electron ionization at 70 eV, and a spectra range of 40−250 *m*/*z* was used (*17*). Identification of compounds was confirmed by comparison of collected mass spectra with those of authenticated reference standards and spectra in the National Institute for Standards and Technology (NIST) mass spectra library.

RESULTS AND DISCUSSION

Ethylene and Respiration Rate. As expected according to Dandekar et al. (*20*), fruit obtained from the transformed apples trees showed a major reduction in ethylene production relative to the nontransformed line (**Figure 1**). An inhibition of >95% was observed in all of the lines, and with the complete absence

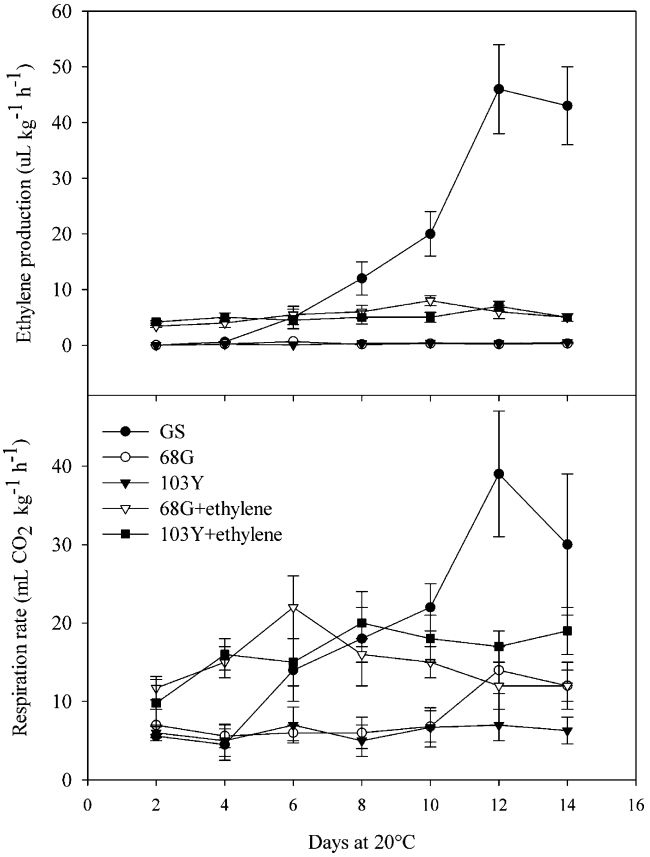

Figure 1. Ethylene evolution and respiration rate of three transgenic lines of Greensleeves apple stored at 20 °C for 14 days with or without exposure to 80 μL L^{-1} ethylene (means of three replicates ± SD of five fruits each). GS, nontransformed line; G, ACO antisense; Y, ACS sense.

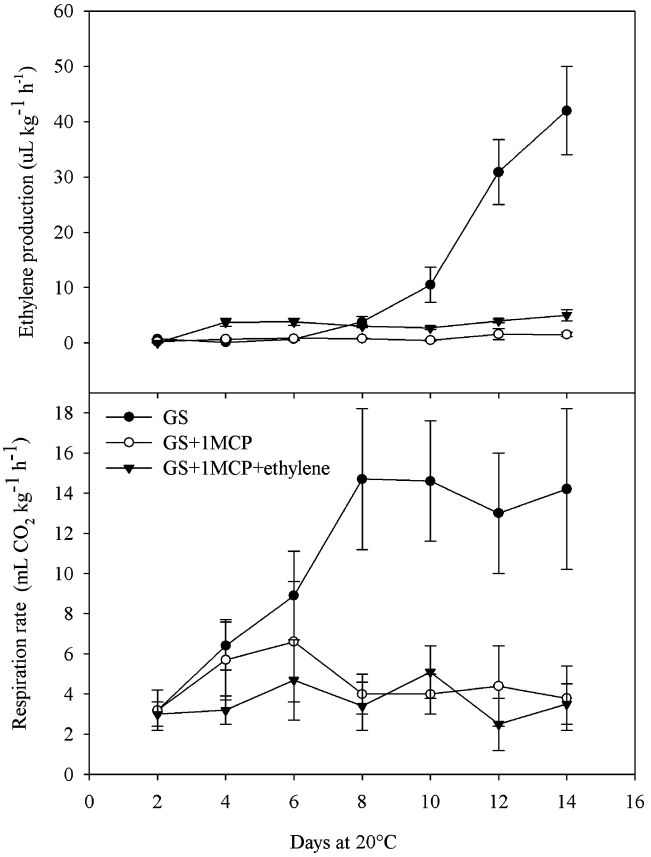

Figure 2. Ethylene evolution and respiration rate of Greensleeves apples treated with 1 μL L^{-1} 1-MCP and stored at 20 °C with or without exposure to 80 μL L^{-1} ethylene (means of three replicates ± SD of five fruits each).

or delay of the climacteric peak. Similarly, a remarkable inhibition of ethylene production was also observed in the fruit treated with 1-MCP, with values of inhibition of 70% at the end of storage (**Figure 2**). The permanent exposure to ethylene produced only a slight increase in ethylene biosynthesis in both experimental approaches. The respiration rate of fruits derived from both the transformed lines and the 1-MCP-treated fruit followed a pattern similar to ethylene production rate, especially with the block of ethylene action.

Maturity and Quality Parameters. When ethylene biosynthesis or action was suppressed or reduced, expectedly, these resulted in corresponding changes in maturity and quality parameters, including firmness, external color, and TA (**Tables 1** and **2**). A delay in softening, retention of green color, and reduction in acid degradation rate were observed and are representative of features that have previously been shown to be ethylene-dependent (*26*, *27*). On the other hand, another parameter, like TSS, was slightly affected by a reduction in ethylene biosynthesis or action and can be considered an ethylene-independent parameter (*28*). The supplementation of exogenous ethylene in transgenic lines suppressed for ethylene biosynthesis produced only a slight increase in ethylene biosynthesis, causing a differential enhancement of ethylene-dependent processes, such as a change in color and loss of firmness, without affecting significantly loss of TA (**Table 1**). However, in the 1-MCP-treated fruit under ethylene during storage only color development recovered to the levels observed in control fruit (GS) (**Table 2**). These data confirm the importance of ethylene action after harvest and the efficiency of 1-MCP in blocking its action and also may indicate that ethylene-dependent processes may have a differential threshold response to ethylene, as has also been suggested earlier (*4*, *7*). In contrast, parameters that have been considered to be slightly dependent or independent of ethylene such as TA and TSS,

Table 1. Quality Indices of Selected Lines of Greensleeves Apples Stored for 14 Days at 20 °C with or without Exposure to 80 μL L^{-1} Ethylene[a]

	firmness (N)		total soluble solids (%)		titratable acidity[b] (%)		color (hue angle)	
line[c]	at harvest	after storage	at harvest	after storage	at harvest	after storage	at harvest	after storage
GS	82 ± 6 a	45 ± 6 a*	12.5 ± 0.6 a	16.5 ± 0.6 b*	0.73 ± 0.12 a	0.52 ± 0.02 a*	113 ± 0.4 a	103 ± 2.1 a*
68G	94 ± 6 b	93 ± 7 b	12.4 ± 1.4 a	15.5 ± 0.7 ab*	0.80 ± 0.11 a	0.77 ± 0.02 b	112 ± 1.0 a	111 ± 0.6 b
68G + ethylene		79 ± 7 b		16.8 ± 0.4 b*		0.69 ± 0.11 ab		104 ± 2.7 a*
103Y	97 ± 9 b	85 ± 9 b	12.2 ± 1.0 a	14.8 ± 0.4 a*	0.78 ± 0.10 a	0.78 ± 0.04 b	114 ± 0.2 a	111 ± 1.1 b
103Y+ ethylene		60 ± 7 ab*		16.1 ± 0.4 ab*		0.70 ± 0.13 ab		103 ± 2.6 a*

[a] Values are means ± SD of three replicates of five fruits each. Means followed by different letters within the same column are significantly different relative to the control treatment at $P = 0.05$. Means follow by an asterisk are significantly different relative to the evaluation at harvest within individual lines at $P = 0.05$. [b] Titratable acidity as malic acid. [c] GS, nontransformed line; G, ACO antisense; Y, ACS sense.

Table 2. Quality Indices of Greensleeves Apples Treated with 1 μL L^{-1} 1-MCP and Stored for 14 Days at 20 °C with or without Exposure to 80 μL L^{-1} Ethylene[a]

days at 20 °C	treatments 1-MCP	treatments ethylene	firmness (N)	total soluble solids (%)	titratable acidity[b] (%)	color (hue angle)
0			95 ± 5.3 b	13.9 ± 0.8 a	0.79 ± 0.07 a	113 ± 1.4 b
14	no	no	57 ± 6.3 a	15.7 ± 0.2 b	0.62 ± 0.04 b	105 ± 0.9 a
	yes	no	89 ± 9.8 b	14.6 ± 0.7 a	0.71 ± 0.05 ab	110 ± 2.0 b
	yes	yes	84 ± 5.0 b	15.0 ± 0.4 ab	0.68 ± 0.10 ab	106 ± 1.4 a

[a] Values are means ± SD of three replicates of five fruits each. Means followed by different letters within the same column are significantly different relative to the control treatment at $P = 0.05$. [b] Titratable acidity as malic acid.

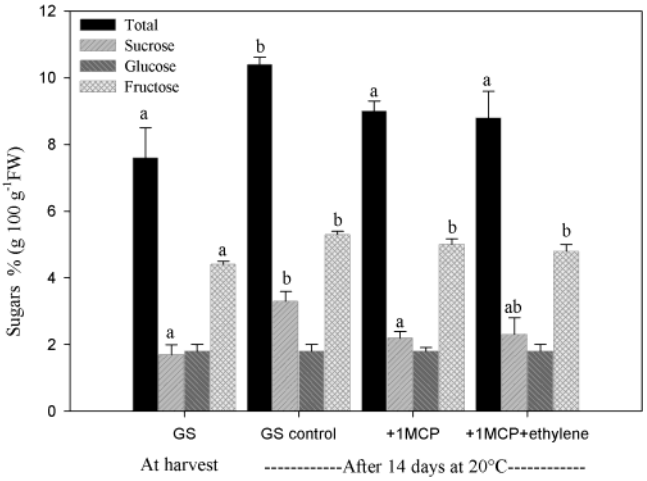

Figure 3. Changes in sugar composition of Greensleeves apples treated with 1 μL L^{-1} 1-MCP and stored at 20 °C with or without exposure to 80 μL L^{-1} ethylene. Bars with different letters are significantly different within an individual compound at $P = 0.05$.

respectively, seem to be differentially affected by ethylene and include a broad group of compounds such as sugars (sucrose, glucose, and fructose), phenolics, and organic acids; these should be analyzed separately.

Sugar Composition. Despite the importance of TSS as an indicator of fruit sweetness, the only metabolites determining its sweetness are the sugars glucose, fructose, sucrose, and sorbitol in apple. As can be seen in **Table 3**, in the transgenic apples with a down-regulation of ethylene biosynthesis, total sugars did not accumulate to the levels observed in wild-type control GS, in which a 25% increase was observed between harvest and the end of storage. However, when the lines were exposed to ethylene, they reached the levels observed in GS at the end of storage. In terms of individual sugars, sucrose and fructose showed the same trend as total sugars, with glucose

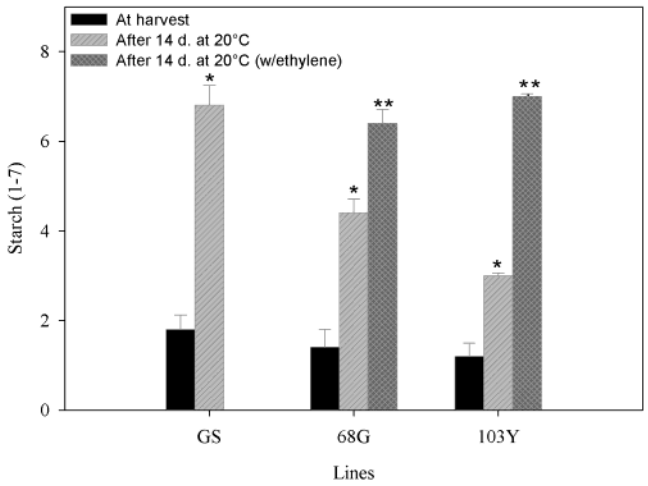

Figure 4. Starch index of transgenic lines of Greensleeves apples stored at 20 °C with or without exposure to 80 μL L^{-1} ethylene. Bars with one or two asterisks indicate significant differences within individual lines at $P = 0.05$. GS, nontransformed line; G, ACO antisense; Y, ACS sense.

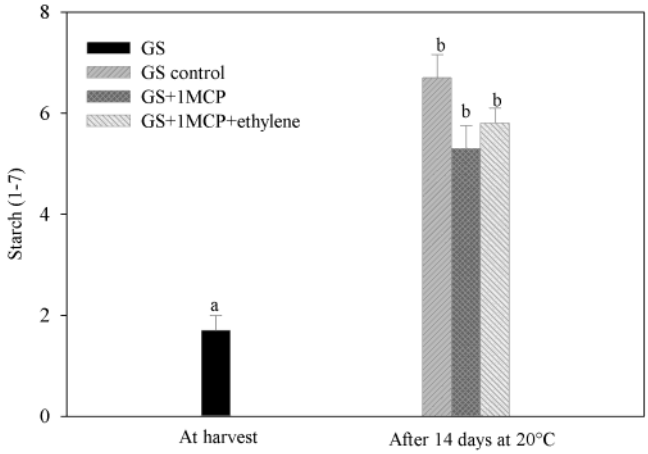

Figure 5. Starch index of Greensleeves apple treated with 1 μL L^{-1} 1-MCP and stored at 20 °C with or without exposure to 80 μL L^{-1} ethylene. Bars with different letters are significantly different at $P = 0.05$.

not being affected at all. The block of ethylene action caused similar effects on total sugars and sucrose accumulation, with the 1-MCP-treated fruit reaching lower levels of sugars than GS at the end of storage (**Figure 3**). No effects of ethylene application on the 1-MCP-treated fruit were observed, which suggests that 1-MCP blocked efficiently the binding sites for ethylene during the period in which metabolism of sugars could have been affected, confirming the importance of the transgenic

Table 3. Changes in Sugars (Percent) in Greensleeves Apples Stored for 14 Days at 20 °C with or without Exposure to 80 μL L^{-1} Ethylene[a]

	total sugars		sucrose		glucose		fructose	
line[b]	at harvest	after storage	at harvest	after storage	at harvest	after storage	at harvest	after storage
GS	9.3 ± 0.78 a	11.6 ± 0.60 a*	2.2 ± 0.41 a	3.7 ± 0.29 a*	2.2 ± 0.76 a	2.5 ± 0.11 a	4.2 ± 0.61 a	5.4 ± 0.4 a*
68G	9.0 ± 0.51 a	9.6 ± 0.73 a	2.0 ± 0.22 a	2.3 ± 0.21 b	1.9 ± 0.21 a	2.3 ± 0.22 a	5.1 ± 0.22 a	5.0 ± 0.4 a
68 G + ethylene		11.1 ± 0.50 a*		3.5 ± 0.38 a*		2.1 ± 0.15 a		5.5 ± 0.5 a*
103Y	7.8 ± 1.20 b	9.7 ± 0.87 a	1.6 ± 0.51 a	2.3 ± 0.56 ab	1.9 ± 0.31 a	2.4 ± 0.31 a	4.3 ± 0.65 a	5.0 ± 0.1 a
103Y +ethylene		10.3 ± 0.67 a		3.0 ± 0.22 a*		2.6 ± 0.15 a		4.7 ± 0.5 a

[a] Values are means ± SD of three replicates of five fruits each. Means followed by different letters are significantly different within the same row for an individual compound at $P = 0.05$. Means follow by an asterisk are significantly different relative to the evaluation at harvest within individual lines at $P = 0.05$. [b] GS, nontransformed line; G, ACO antisense; Y, ACS sense.

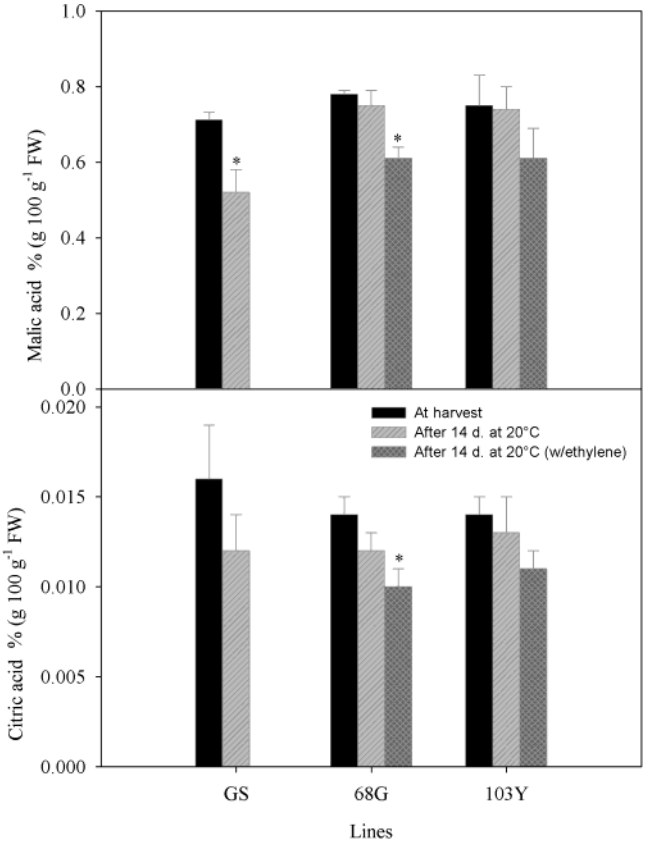

Figure 6. Acids content of transgenic lines of Greensleeves apple stored at 20 °C with or without exposure to 80 μL L^{-1} ethylene. Bars with an asterisk are significantly different relative to the evaluation at harvest within individual lines at $P = 0.05$. GS, nontransformed line; G, ACO antisense; Y, ACS sense.

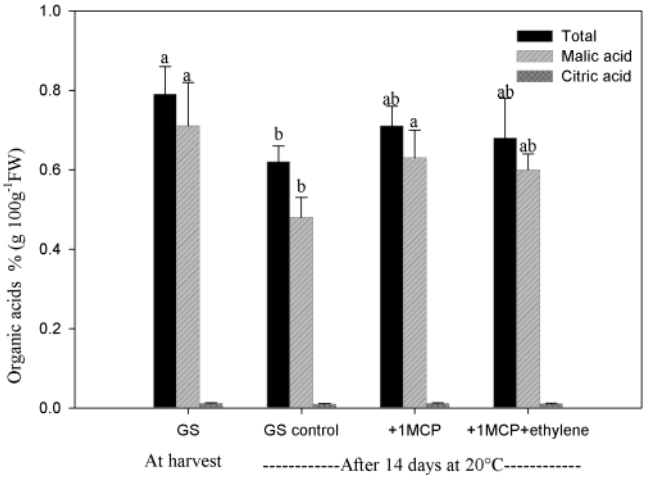

Figure 7. Changes in acids content of Greensleeves apples treated with 1 μL L^{-1} 1-MCP and stored at 20 °C with or without exposure to 80 μL L^{-1} ethylene. Bars with different letters are significantly different for an individual compound at $P = 0.05$.

lines for understanding ethylene regulation. Therefore, these results indicate that sugar accumulation is under ethylene regulation during ripening. It has been shown that the hydrolysis of starch plays a role as a source of sugars in the last stages of fruit development (*6*), and prior research has concluded that loss of starch did not appear to be related with ethylene (*7*, *28*, *29*). In our study we have observed consistently a reduction in starch loss, based on starch−iodine rating, in the ethylene-suppressed lines, which may explain the changes in sugar levels (**Figure 4**); however, no changes in starch−iodine rating were observed in the 1-MCP-treated fruit (**Figure 5**). These results suggest a complex interaction between ethylene and sugar metabolism in fruits and may be similar to the complexity observed in vegetative tissues (*30*).

Organic Acid Content. A reduction in organic acids was observed between harvest and the end of storage, which was reflected in both the value of TA (**Tables 1** and **2**) and the concentrations of individual acids, that is, malic and citric acids (**Figures 6** and **7**). Down-regulation of ethylene biosynthesis and action significantly reduced this loss of acids, keeping the levels close to ones measured at harvest. The exposure of the fruit to ethylene enhanced the degradation of acids only in the transgenic lines, having a partial effect in the 1-MCP-treated fruit. It has been concluded that the main cause of organic acids degradation in climacteric fruit is the large increase in respiration rate that occurs during ripening (*6*). With the suppression of ethylene biosynthesis and action we did notice a reduction of respiration rate, mainly with the use of 1-MCP, in which the levels were 20% of that observed in nontreated fruit (**Figures 1** and **2**). Exogenous ethylene application in the transgenic lines increased the respiration rate close to that of the GS fruit, resulting in an increase in acid degradation relative to the nontreated fruit. However, in the 1-MCP-treated fruit, exogenous ethylene caused only a slight increase in respiration rates, reaching levels 10% higher than in the non-ethylene-treated fruit (**Figure 2**). Because changes of 0.08% in TA in apples are noticed by trained panelists, the effect of down-regulation of ethylene on acid regulation will be reflected in the final overall flavor (*31*).

Phenolic Compounds. In general, minor changes in both total and individual phenolics were measured between harvest and the end of storage, with only a slight increase in total phenolics (close to 20%) in the nontransformed line and in the nontreated with 1-MCP fruit (**Table 4**; **Figure 8**). The main phenolic compounds identified were chlorogenic acid, epicatechin, and

Table 4. Changes in Phenolic Content (Micrograms per Gram) in Greensleeves Apples Stored for 14 Days at 20 °C with or without Exposure to 80 μL L^{-1} Ethylene[a]

	total phenolics		chlorogenic acid		phloridzin		epicatechin	
line[b]	at harvest	after storage	at harvest	after storage	at harvest	after storage	at harvest	after storage
GS	1023 ± 83 a	1359 ± 140 a*	158 ± 8 a	187 ± 8 a*	23 ± 3 a	28 ± 6 a	78 ± 15 a	123 ± 19 a*
68G	1100 ± 72 a	1150 ± 156 a	159 ± 12 a	153 ± 15 b	42 ± 14 b	37 ± 7 a	101 ± 16 a	100 ± 20 a
68 G + ethylene		1180 ± 84 a		169 ± 9 ab		39 ± 8 a		122 ± 6 a
103Y	1021 ± 57 a	1170 ± 150 a	137 ± 10 a	138 ± 17 b	27 ± 6 a	26 ± 5 a	43 ± 8 b	53 ± 10 b
103Y + ethylene		1436 ± 231 a		155 ± 7 b*		38 ± 8 a		60 ± 7 b*

[a] Values are means ± SD of three replicates of five fruits each. Means followed by different letters are significantly different within the same column for an individual compound at $P = 0.05$. Means follow by an asterisk are significantly different relative to the evaluation at harvest within individual lines at $P = 0.05$. [b] GS, nontransformed line; G, ACO antisense; Y, ACS sense.

Table 5. Aroma Composition (Nanoliters per Liter) in Transgenic Lines of Greensleeves Apples Evaluated at Harvest and after 14 Days at 20 °C[a]

compound	GS[b] at harvest	GS after storage	68G at harvest	68G after storage	68G + ethylene	103Y at harvest	103Y after storage	103Y + ethylene
hexanal	271 ± 26	237 ± 17	198 ± 27	290 ± 77	295 ± 20	230 ± 17	320 ± 52	300 ± 19
(2*E*)-hexenal	57 ± 7	391 ± 47	48 ± 15	282 ± 51	315 ± 16	61 ± 25	254 ± 41	295 ± 10
total aldehydes	328 ± 19	628 ± 54	246 ± 41	572 ± 29	610 ± 35	291 ± 42	574 ± 93	595 ± 29
butanol	4 ± 1	16 ± 4	4 ± 0	10 ± 2	7 ± 1	3 ± 2	9 ± 2	10 ± 1
2-methylbutanol	4 ± 1	20 ± 7	ND[c]	ND	13 ± 1	ND	ND	9 ± 3
hexanol	9 ± 2	20 ± 7	7 ± 1	17 ± 4	62 ± 11	9 ± 1	27 ± 4	42 ± 10
total alcohols	17 ± 4	56 ± 18	11 ± 1	27 ± 6	82 ± 13	12 ± 3	36 ± 6	61 ± 14
butyl butanoate	ND	97 ± 14	ND	15 ± 6	67 ± 3	ND	20 ± 4	77 ± 3
butyl 2-methylbutanoate	ND	47 ± 9	ND	ND	41 ± 8	ND	ND	61 ± 10
hexyl butanoate	9 ± 7	558 ± 80	6 ± 1	120 ± 8	360 ± 40	10 ± 1	90 ± 9	300 ± 60
hexyl 2-methylbutanoate	ND	321 ± 79	ND	10 ± 3	198 ± 37	ND	11 ± 1	220 ± 47
hexyl hexanoate	ND	23 ± 5	5 ± 1	5 ± 1	14 ± 3	6 ± 3	7 ± 1	32 ± 9
total esters	9 ± 7	1046 ± 33	11 ± 3	150 ± 13	680 ± 57	16 ± 4	128 ± 7	690 ± 136

[a] Values are means ± SD of three replicates of five fruits each. [b] GS, nontransformed line; G, ACO antisense; Y, ACS sense. [c] ND, not detected.

Table 6. Aroma Composition of Greensleeves Apple Fruit Treated with 1 μL L^{-1} 1-MCP and Stored for 14 Days at 20 °C with or without Exposure to 80 μL L^{-1} Ethylene[a]

	concentration (nL L^{-1})			
	at harvest	after 14 days at 20 °C		
aroma compound	GS control	GS control	GS 1 μL L^{-1} 1-MCP	GS + 1 μL L^{-1} 1-MCP + ethylene
hexanal	233 ± 26	295 ± 63	295 ± 11	336 ± 50
(2*E*)-hexenal	50 ± 9	345 ± 25	301 ± 11	295 ± 47
total aldehydes	282 ± 35	640 ± 41	596 ± 26	621 ± 98
butanol	4 ± 1	19 ± 3	10 ± 2	15 ± 8
2-methylbutanol	6 ± 1	11 ± 4	9 ± 2	11 ± 2
hexanol	9 ± 2	30 ± 12	12 ± 3	16 ± 4
total alcohols	19 ± 4	60 ± 19	31 ± 7	41 ± 14
butyl butanoate	ND[b]	102 ± 18	30 ± 10	9 ± 2
butyl 2-methylbutanoate	ND	33 ± 9	ND	5 ± 3
hexyl butanoate	10 ± 2	471 ± 90	25 ± 2	50 ± 8
hexyl 2-methylbutanoate	ND	194 ± 31	ND	45 ± 7
hexyl hexanoate	ND	26 ± 4	ND	ND
total esters	10 ± 2	826 ± 135	55 ± 14	109 ± 20

[a] Values are means ± SD of three replicates of five fruits each. [b] ND, not detected.

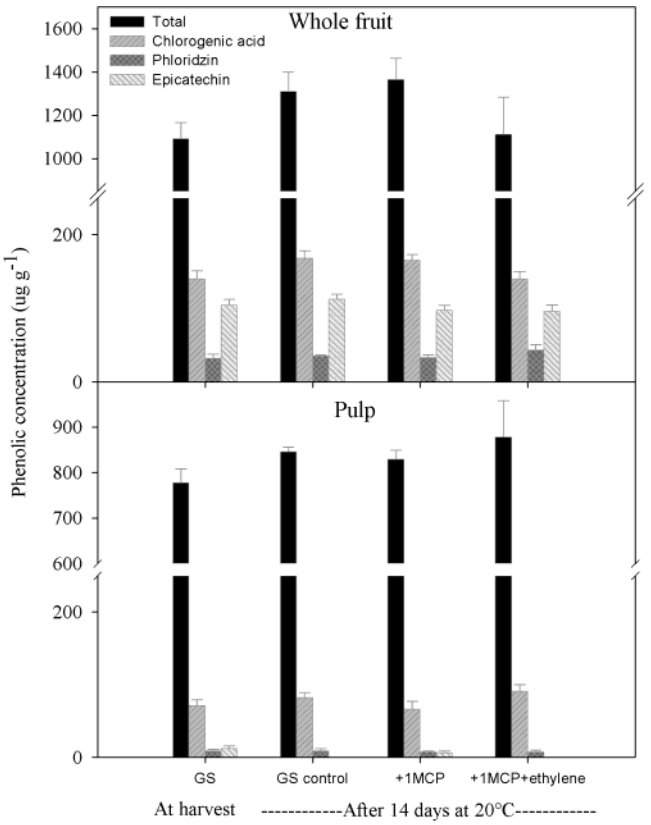

Figure 8. Changes in phenolic composition of Greensleeves apple treated with 1 μL L^{-1} 1-MCP and stored at 20 °C with or without exposure to 80 μL L^{-1} ethylene.

phloridzin, with higher levels in samples containing skin (**Figure 8**). In terms of ethylene regulation, a differential effect of ethylene suppression was observed depending on the mechanism of suppression used. The transgenic lines showed a different pattern relative to GS with no significant changes between harvest and the end of storage at 20 °C for either total or individual phenolics. In contrast, when ethylene action was blocked, fruit from this treatment behaved similarly to the nontreated fruit. Moreover, applications of ethylene after harvest could not recover the levels of phenolics in the transgenic lines, which indicates that the accumulation of phenolics during storage is an ethylene-independent process as has been previously suggested in experiments using controlled atmosphere (*12*, *14*). In addition, these differences may be also explained by the differential effect of the mechanism of suppression on different stages of fruit development; that is, in the transgenic lines ethylene biosynthesis is being affected from early stages of fruit development when most of the changes in phenolic concentration occurred (*13*), but with the use of 1-MCP, ethylene perception is affected only after harvest in the last stages of fruit development. The mechanism by which ethylene regulates phenolic compounds is not clear in fruits, and preliminary work suggests that it involves complex interactions (*32*, *33*). Moreover, most of the information available concluding that phenolic metabolism is stable was generated with experiments performed on fruit after harvest and during commercial storage at 0 °C, without considering earlier stages of fruit development (*3*, *12*, *14*) or higher temperatures. Additionally, because of the effect of varietal differences and environmental conditions, it is very difficult to make useful comparisons of compositional differences across different varieties.

Volatile Compounds. Aroma profile of Greensleeves apple was characterized with the presence of more than 40 compounds, but only the most important in terms of abundance are reported in this paper. The effect of ethylene suppression in both the transgenic lines and the 1-MCP-treated apples was dramatic,

resulting in a remarkable reduction or delay in the accumulation of ester compounds reaching levels of 12−15% in the transgenic lines and <10% in the 1-MCP-treated fruit (**Tables 5** and **6**, respectively) relative to GS. Similar levels of ester inhibition were observed previously in transgenic ACO antisense lines of melon (*34*) and in apple fruit treated with the ethylene inhibitors AVG and 1-MCP (*35*, *36*). Therefore, these results confirm that ester production is under ethylene regulation in apples. No major effects were observed in the levels of aldehyde compounds. It is possible that the lipoxygenase pathway acts independently in the disrupted tissue, resulting in constant levels of aldehydes, which differs from data on intact fruit in which ethylene suppression reduced aldehyde accumulation (*36*). In addition, a reduction/delay in the accumulation of alcohols was observed in the transgenic lines and in the 1-MCP-treated fruit, which suggests that in Greensleeves fruit the steps upstream in the ester biosynthetic pathway are under ethylene coordination (*18*, *36*). Further support for this idea is demonstrated by the recovery of alcohol and ester accumulation in the ethylene-treated transgenic lines, in which the levels of these compounds were significantly higher than in the nontreated fruit. Similar to sugars and organic acids, ethylene was not able to recover levels of esters, which supports the idea that a continuous presence of ethylene is required for volatile synthesis (*34*).

Conclusions. It is clear that the regulation of flavor metabolites in apple by ethylene is complex. However, the availability of the transgenic fruits suppressed in ethylene biosynthesis and the efficient ethylene action inhibitor (1-MCP) present a unique opportunity for understanding the underlying regulatory mechanisms. Results from this research indicate that compounds contributing to the TSS value showed a differential level of ethylene dependence in apple; that is, sugars (mainly sucrose and fructose) and organic acids were under ethylene regulation. On the other hand, phenolic compounds metabolism may be considered an ethylene-independent process at least in the last stages of fruit development. Among aroma compounds, ester accumulation was clearly regulated by ethylene, but the mechanism of ethylene control of specific metabolites remains to be elucidated.

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