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Effect of down-regulation of ethylene biosynthesis on fruit flavor complex in apple fruit

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Abstract

The role of ethylene in regulating sugar, acid, texture and volatile components of fruit quality was investigated in transgenic apple fruit modified in their capacity to synthesize endogenous ethylene. Fruit obtained from plants silenced for either ACS (ACC synthase; ACC – 1-aminocyclopropane-1-carboxylic acid) or ACO (ACC oxidase), key enzymes responsible for ethylene biosynthesis, expectedly showed reduced autocatalytic ethylene production. Ethylene suppressed fruits were significantly firmer than controls and displayed an increased shelf-life. No significant difference was observed in sugar or acid accumulation suggesting that sugar and acid composition and accumulation is not directly under ethylene control. Interestingly, a significant and dramatic suppression of the synthesis of volatile esters was observed in fruit silenced for ethylene. However, no significant suppression was observed for the aldehyde and alcohol precursors of these esters. Our results indicate that ethylene differentially regulates fruit quality components and the availability of these transgenic apple trees provides a unique resource to define the role of ethylene and other factors that regulate fruit development.

Abbreviations: FFC – fruit flavor composition; ACC – 1-aminocyclopropane-1-carboxylic acid; ACO – ACC oxidase; ACS – ACC synthase; PTGS – post transcriptional gene silencing; DAFB – days after full bloom; Gm – gentamycin resistance; GS – Greensleeves

Introduction

A salient genetic attribute of tree fruits is the unique blend of sugar, acid and volatile components that determine the flavor of each individual kind of fruit (e.g., apple, peach, orange). The genetic regulation of this important trait recognized by consumers of fresh fruit occurs through a complex interaction of metabolic pathways and regulatory circuits that result in the unique fruit flavor composition (FFC). What determines fruit quality and what underlying processes regulate FFC during development, maturation?

One of the key players is the autocatalytic synthesis of the gaseous hormone ethylene that has been shown to influence the physiology and biochemistry of tomato fruit via the expression of specific genes involved in ripening (Theologis, 1994; Fluhr & Mattoo, 1996; Ciardi & Klee, 2001; Giovannoni, 2001). In climacteric fruit like apple the exponential increase in ethylene production coincides with a rise in respiration and correlates with the development of FFC (Yang & Hoffman, 1984; Knee, 1993). An important question is what components of FFC are specifi-

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cally regulated by ethylene. These components can be defined through the genetic manipulation of ethylene biosynthesis. The biosynthesis of ethylene occurs in two enzymatic steps; in the first reaction, *S*-adenosyl methionine is converted to ACC (1-aminocyclopropane-1-carboxylic acid) by ACC synthase (ACS), and in the second ACC is metabolized by ACC oxidase (ACO) to produce ethylene. During ripening, the expression of ACS and ACO genes and the activity of their encoded enzymes govern the rate of ethylene production. ACS regulates the rate-limiting step in ethylene biosynthesis (Yang & Hoffman, 1984), but ACO can also play a regulatory role (Gray et al., 1992). Both these enzymes have been well characterized (McKeon & Yang, 1987). The ACS and ACO enzymes of apple have also been purified, characterized, and their genes cloned facilitating our analysis (Dong et al., 1991; Yip et al., 1991; Dong et al., 1992; Fernandez-Maculet & Yang, 1992). This is the first in depth study on apples transformed with ethylene related genes. Almost all of the work on genetic modification of fruit has been carried out on tomato *Lycopersicon esculentum* (Hamilton et al., 1990, Grierson & Schuch, 1993; Grierson & Fray, 1994). Tomato with its relatively short life cycle, long history of genetic research and it's relative ease to transform genetically has been adopted as a model to study fruit ripening (Ciardi & Klee, 2001; Giovannoni, 2001; Alexander & Grierson, 2002). Expression of antisense ACS mRNA successfully inhibited ethylene production (<0.1 nl g^{-1} h^{-1}) and prevented tomato fruit ripening (Oeller et al., 1991). Another example was the cantaloupe charentais melon where a strong inhibition of ethylene production was also observed after transformation with antisense ACO (Guis et al., 1997). Apple, though physiologically well characterized is less attractive for transgenic studies, as it takes many years to obtain fruit. However, apple can be transformed (James et al., 1989, 1996) and thus has become a target for the genetic analysis of ethylene related genes because of its economical importance, very well studied ripening physiology and it's potential use as a model for perennial fruit species. In this paper, we report the successful silencing of ethylene biosynthesis in apple fruit via the suppression of ACS or ACO mRNA. Transgenic apple trees whose fruit made little ethylene were identified. Our results demonstrate that FFC is differentially regulated in apple.

Experimental procedures

Binary vector construction

Plasmids pAAS2 containing apple ACS (Dong et al., 1991) and pAE12 containing apple ACO (Dong et al., 1992) were kindly provided by Shang Fa Yang (U.C. Davis). The 1.6 kb cDNA encoding ACS was isolated from pAAS2 after digestion with *Bam*HI and was ligated into the binary vector pDU92.3103 (Tao et al., 1995) also digested with *Bam*HI creating two binary plasmids containing the ACS coding sequence in either a sense (pDU93.0114) or antisense (pDU93.0128) orientation with respect to the CaMV35S promoter in pDU92.3103 (Figure 1). The apple ACO cDNA was obtained from pAE12 after digestion with the restriction endonuclease *Xba*I and the ends were made flush with a fill-in reaction and then blunt-end ligated into the *Bam*HI site of pDU92.3103 also made flush with a fill-in reaction. Two binary plasmids containing the apple ACO cDNA were obtained in either a sense (pDU93.0412) or antisense (pDU93.0402) orientation with respect to the CaMV35S expression cassette in pDU92.3103 (Figure 1). These binary plasmids were then introduced into the disarmed Agrobacterium strain EHA101 via electroporation to create a functional Agrobacterium vector for plant transformation.

Plant transformation

Apple transformation was similar to that first reported by James et al. (1989) using the *Agrobacterium*-mediated transformation of leaf discs from the apple cultivar 'Greensleeves'. A detailed procedure for this transformation has been published (James & Dandekar, 1991) and although it is routine it takes 6–9 months to obtain transgenic plants. Selection was achieved with the antibiotic kanamycin and the scorable marker gene, GUS, greatly facilitated the rapid identification of transformants. GUS specific activity

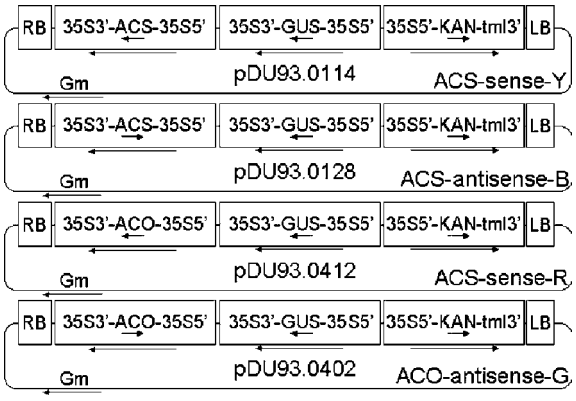

Figure 1. The *Agrobacterium* binary vectors pDU93.0114/pDU93.0128 for sense/antisense expression of apple ACC synthase (ACS) and pDU93.0412/pDU93.0402 for sense/antisense expression of apple ACC oxidase (ACO) respectively in apple. These vectors contain a kanamycin resistance (KAN) selectable marker gene driven by the CaMV35S promoter (35S5′), a β-glucuronidase scorable marker gene (*gusA*) driven by the CaMV35S promoter (35S5′), and the apple ethylene biosynthesis genes also driven by CaMV35S promoter. Arrows indicate the direction of transcription. LB and RB indicate the left and right T-DNA border sequences. The vector backbone contains a gene encoding resistance to the antibiotic gentamycin (Gm) for selection of the binary plasmid vector in *E.coli* and *Agrobacterium*.

was measured using fluorometry (Jefferson et al., 1987). Selected transformed shoots, based upon the level of GUS activity, were allowed to root in the presence of kanamycin. These plants were then acclimatized in the greenhouse and allowed to grow on their own roots. Buds from these plants were then grafted onto M9 and/or M26 rootstocks in the field at University of California, Davis (UCD) or to M9 rootstocks and grown in pots in an isolation greenhouse at Horticulture Research International (HRI), East Malling, UK. Transgenes were found to be stably incorporated and inherited in a simple Mendelian fashion (James et al., 1996).

Molecular characterization of transformants

Total cellular RNA was isolated by the hot borate method (Wan and Wilkins, 1994). Sixteen micrograms of RNA were resolved in formaldehyde agarose gel and transferred to nylon membrane. Transcripts were detected by hybridization with ^{32}P-labeled ACS and ACO DNA probes followed by analysis using a storage phosphor imager system (Typhoon, Amersham, Piscataway, NJ, USA).

Fruit collection and handling

Both transgenic and untransformed 'Greensleeves' apples were harvested at around 115 days after full bloom (DAFB) at the mature-green (preclimacteric) stage from the UCD research orchards. Selected lines have been analyzed for three consecutive growing seasons. The harvested apples were sorted to select those that were free from defects and to prepare matched samples of 1–5 apples per replicate and 3–5 replicates per line. At East Malling there were three trees per line, however, in California there were 3–6 trees per line. Two to five fruits per line were selected for ethylene measurement when untransformed fruit started to lose chlorophyll.

Maturity and quality parameters

Apples were evaluated for skin color, firmness, soluble solid content and titratable acidity at harvest and 12 days after storage at 20°C. Skin color was measured using a reflectance meter (Minolta Chromameter, Japan), at two different sites along the equatorial surface area. Firmness was measured at four peeled points along the equatorial region of each fruit using a penetrometer with an 11 mm probe. Cortical tissue was used to measure soluble solids content (°Brix) using a refractometer, and titratable acidity. Ethylene production and respiration rates were determined daily during storage for individual fruits at 20°C using a static system. Fruits were placed in 0.5 l jars and sealed for 30 min before measurements. Carbon dioxide and ethylene concentrations were determined by an infrared gas analyzer (Horiba, Irvine, CA) and a gas chromatograph (Hach Carle, Loveland, Co) equipped with a flame ionization detector (FID-GC), respectively. At East Malling fruits were stored individually at 4°C in sealed jars supplied with a continuous flow of air. Gas samples were taken at intervals for measurement of ethylene concentrations by FID-GC.

Analytical methods

Ethylene biosynthesis. ACC content and activities of ACS and ACO were determined as described by Gorny and Kader (1996). Briefly, for *in vitro* ACS activity apple cortical and skin tissues were homogenized in a chilled Waring Blender with

1 ml g^{-1} of 4°C extraction buffer (400 mmol potassium-phosphate [pH 8.5], 1 mmol EDTA, 5 ml l^{-1} 2-mercaptoethanol, 0.01 mmol pyridoxal phosphate). The homogenized tissue was filtered through four layers of cheesecloth and centrifuged at 28,000 × *g* for 30 min. The supernatant was discarded, and the pellet resuspended in extraction buffer and centrifuged for 20 min. The resulting pellet was solubilized and ACS activity assayed as described by Yip et al. (1991). For ACO, apple cortical and skin tissues were frozen in liquid nitrogen and homogenized with a mortar and pestle in 2 ml g^{-1} tissue of extraction solution containing 400 mmol 3-[*N*-Morpholino] propane–sulfonic acid (MOPS) buffer (pH 7.2), 100 ml l^{-1} glycerol, 30 mmol ascorbate, and 500 mg g^{-1} tissue polyvinyl polypyrrolidone (PVPP). Once the slurry melted, it was filtered through four layers of cheesecloth and centrifuged at 28,000 × *g* for 20 min. The supernatant was recovered and used for the enzyme assay. The standard reaction mixture contained 50 mmol MOPS (pH 7.2), 100 ml l^{-1} glycerol, 1 mmol ACC, 0.02 mmol $FeSO_4$, 5 mmol ascorbate, 1 mmol dithiothreitol, and 20 mmol sodium bicarbonate. The enzyme assay reaction was initiated by adding 0.2 ml of enzyme preparation to a 15 ml test tube containing the standard reaction mixture (1.8 ml). The test tube was then sealed, and after one hour of incubation with shaking at 30°C, a 1 ml gas sample was taken from the headspace for ethylene determination. Protein content of enzyme extracts used to determine *in vitro* ACS and ACO was determined by the Bradford method (Bradford, 1976) using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, California) and bovine serum albumin as a standard. For ACC content, 10 g of apple cortical and skin tissues were homogenized in a Polytron homogenizer in the presence of 20 ml of 9% trichloroacetic acid, and the mixture was maintained overnight at 5°C. Then the homogenated tissue was centrifuged at 27,000 × *g* for 20 min. The recovered supernatant was neutralized with 5N NaOH and ACC was determined according to Lizada and Yang (1979).

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 were homogenized in a Polytron homogenizer in the presence of 12 ml of water with 2 mM NaF (Tomas-Barberan et al., 2001). The homogenized tissue was filtered and centrifuged (20,000 × *g* for 20 min) at 4°C. Ten milliliters of the supernatant was placed into crim-seal 40 mL vials containing 2 g of NaCl. Prior to sealing the vials, 600 µl of the internal standard (IS) solution (1-octanol) was added to a final concentration of 500 nl l^{-1} IS. Headspace was sampled using a polydimethylsiloxane/divinylbenzene solid phase microextraction fiber (65 µm thickness). 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: 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}. Mass spectra were obtained by electron ionization at 70 eV. A spectra range from 40 to 250 *m*/*z* was used (Song & Bangerth, 1996). Identification of alcohols, aldehydes and esters was confirmed by comparison of collected mass spectra with those of authenticated reference standards and spectra in the National Institute for Standards and Technology mass spectra library.

Results

The cDNAs encoding ACS and ACO, respectively, from apple were used to construct binary vectors that expressed these cDNAs using the CaMV35S promoter in either a sense (pDU93.0114 and pDU93.0412, Figure 1) or antisense orientation (pDU93.0128 and pDU93.0402, Figure 1). These binary vectors were introduced into Agrobacterium and used to transform Greensleeves apple. Forty-two lines are currently maintained in a field planting in California. In addition, 18 lines were tested in the greenhouse at East Malling giving a total of 60 lines that were evaluated for this study (Table 1). Of these 60 lines, 52% were suppressed for ACS and 48% were suppressed for ACO (Table 1). Lines with the strongest suppression of ethylene biosynthesis in fruits appeared phenotypically uniform, and no significant differences

Table 1. Grouping of transgenic apple lines by their ethylene production rates (u L kg^{-1} h^{-1})

Vector		Group 1	Group 2	Group 3	Group 4	Group 5	All
		Very Low < 10	Low 10–40	Medium 41–70	High 71–100	Very High > 100	Total # lines
ACS sense	pDU93.0114	105Y***, 150Y***, 103Y***, *(130Y)*[a],***, (136Y)***	115Y***, 112Y***, *(133Y)****, (131Y)***, (*129Y*)[a]	107Y***, 113Y***, 119Y***, 125Y***, 114Y***, 140Y***, (134Y)*	129Y[a], 102Y**, 133Y***, (128Y), (137Y)	110Y***, (132Y)***	15, (6), (*3*)
ACS antisense	pDU93.0128		(187B)***		166B*, 190B[a],***	174B***, 175B***, (188B)***, (189B)	4, (3)
ACO sense	pDU93.0412	11R***, 21R***, (33R)[a],***, 9R***, 8R[a],***	49R***, 25R[a],***, (41R)***	(*40R*)***, 7R**, 35R***, (3R), (36R), (32R), (34R)*	13R***,	48R***, 20R[a],*, (38R)***	11, (7), (*1*)
ACO antisense	pDU93.0402	61G***, 67G[a],*** 68G***	54G***, 51G***, 55G***, 80G***, 75G[a],***, (76G)***		(78G)		8, (2)
Control	No vector				GS 1		1

Transgenic lines tested in the greenhouse are shown in brackets. Lines tested in green house and in the field are shown in brackets and in italic. Lines that contain a single copy of T-DNA are shown underlined.
[a]lines that contain two or more inserted T-DNA copies.
*, **, ***Values differ from control at $p < 0.05$, 0.01, 0.001, respectively.

were observed in tree size, leaf senescence or flowering.

Fruit obtained from transgenic apple trees grown in the field and greenhouse show a wide variation in ethylene production rates (Table 1). Almost half of the lines (47%; Groups 1 and 2; Table 1) are suppressed greater than 60% with about 22% being suppressed 90% and greater (Group 1). A quarter of the lines (23%) are suppressed between 30 and 60% (Group 3; Table 1) and about 15% (Group 4; Table 1) were indistinguishable from the control. There was also a group of clones (15%) that made more ethylene compared to the control (Group 5; Table 1). Specific activity measurements based on fluorometry (Jefferson et al., 1987) from several lines showed no correlation between the efficacy of the ethylene down-regulation and the level of expression of the co-transferred *gusA* gene (data not shown).

The peak of ethylene production as a percentage of non-transformed control fruit was, for almost all lines, consistent between years and across sites. Of the 42 California-grown lines that were used for ethylene measurements, 11 were used for more than 1 year. Of these, only one, 174B, behaved inconsistently, having a production rate greater than that of the control in 2001 and less than that of the control in 2000. Of the 22 lines examined at East Malling, ethylene measurements were made on 13 for more that 1 year. Of these 13, two were inconsistent. 132Y was a high producer in 2000 but not 2001, and 131Y had a low production in 2 years and a high production in 1 year. However, in California three clones consistently had a production rate less than 6% of the control, and at East Malling, three clones consistently had a production rate less than 20% of the control.

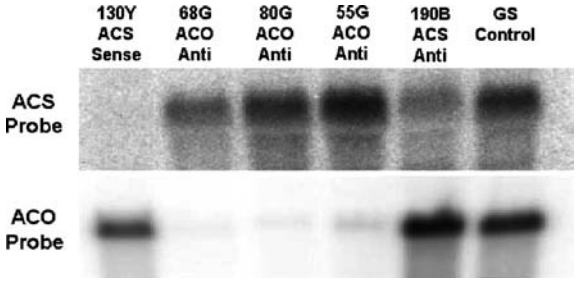

Figure 2. Comparison of ACS and ACO transcript abundance in wild-type and transgenic apple fruit using northern analysis. RNA was extracted from transgenic lines 130Y, 68G, 80G, 55G, 190B and from wild-type Greensleeves (GS) fruit and loaded in the lanes from left to right. The upper panel is a result of hybridization with apple ACS coding sequence and the lower panel is the result of hybridization with apple ACO coding sequence.

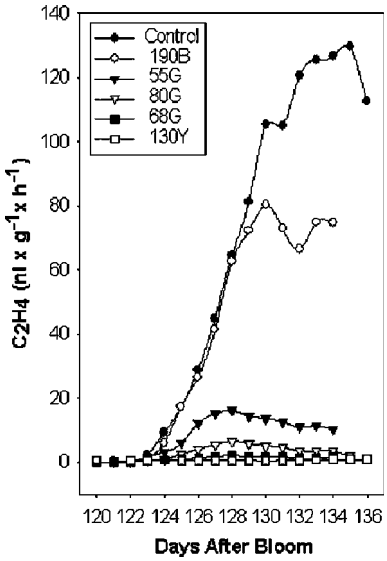

Figure 3. Ethylene biosynthesis in transgenic apple fruit suppressed for ACO (55G, 80G, 68G) or ACS (130Y, 190B) expression as compared to untransformed fruit. Ethylene production and respiration rates were determined every other day during storage for individual fruits at 20°C using a static system.

The regenerants were all initially screened for GUS activity. All lines selected for use in this study were transformed as they showed high GUS activity representing an unselected marker for the inserted T-DNA. Southern analysis showed no correlation between the number of T-DNAs incorporated and the level of suppression of ethylene biosynthesis (Table 1). Expression analysis was carried out with lines displaying different levels of suppression of ethylene biosynthesis and compared to the control (Table 1). In control fruit there was a strong signal for both ACS and ACO mRNA (Figure 2). However, in all lines that were suppressed for ethylene biosynthesis there was a reduction of steady state mRNA due to post-transcriptional gene silencing (PTGS), that was specific to the targeted gene. Apple fruit expressing ACO antisense (68G, 80G and 55G) show little mRNA for ACO but express levels of ACS comparable to the control. In the case of 68G, the level of ACS mRNA was lower than the control. ACS co-suppressed lines, such as 130Y, showed a marked signal for ACO (30–50% of the control) but not for ACS. Lines such as 190B (ACS antisense) that belong to Group 3 (Table 1) and show only a partial suppression of ethylene biosynthesis, do make ACS transcripts but less than that of the control. A dramatic effect on ethylene biosynthesis was observed when the progress of ethylene production was evaluated in harvested fruit. Autocatalytic ethylene biosynthesis was markedly suppressed in the silenced lines compared to the control fruit with the exception of line 190B, which showed a partial antisense suppression of ACS mRNA and displayed a distinct but reduced ethylene peak (Figure 3). An increase in respiration rate was observed in all clones (data not shown).

Further evidence for a distinct phenotype in these lines can be seen in the quality and biochemical analysis of fruit from the same lines shown in Table 1. Fruits deficient in ACO mRNA (Figure 2) have correspondingly less ACO activity (Table 2), and ACC accumulates to substantial quantities. Interestingly, the level of ACS activity is decreased in the ACO silenced lines because there is less ethylene to stimulate the expression of ACS (Table 2). Correspondingly, ACS deficient fruit do not accumulate ACC and display normal to slightly increased levels of ACO activity. Nevertheless, it was possible to obtain a dramatic reduction in the ethylene production rate in both the ACO and ACS silenced lines (Table 2).

Tables 3 and 4 show the quality attributes of some of these lines. When ethylene biosynthesis was suppressed or reduced, maturity parameters, including firmness, external color and aroma were also dramatically affected. The lines that made less ethylene were significantly firmer than

Table 2. ACS activity, ACC content, ACO activity and ethylene production rate of transgenic apples silenced for ethylene biosynthesis expressed as a percentage of that observed for the untransformed lines after storage for 12 days at 20°C

Line	ACS activity	ACC concentration	ACO activity	Ethylene production rate
GS	100 c[a]	100 b	100 b	100 c
190 B	35 ab	60 b	120 bc	40 b
55 G	68 b	nt	1.7 a	8 a
80 G	72 b	1200 c	0.8 a	6 a
68 G	62 b	3200 c	0.4 a	4 a
130 Y	10 a	12 a	158 c	6 a

Values of control: ACS activity = 2.5 nmol mg^{-1}(protein) h^{-1}, ACC concentration = 1.6 nmol ACC g^{-1} (tissue), ACO activity = 205 nl C_2H_4 mg^{-1}(protein) h^{-1}, ethylene production = 73 nl C_2H_4 $kg^{-1}h^{-1}$. nt – Not tested.
[a] Different letters indicate significant differences within a column at $p < 0.001$.

Table 3. Quality attributes at harvest and after storage for 12 days at 20°C

Line	At harvest				After 12 days at 20 °C			
	Firmness (Newton)	Soluble solids (°Brix)	Acidity[a] (%)	Color Hue angle	Firmness (Newton)	Soluble solids (°Brix)	Acidity (%)	Color Hue angle
GS	78 ± 3.91[b]a	15.7 ± 0.9 a	0.92 ± 0.2 a	104 ± 0.8 a	50 ± 2.6 a	16.8 ± 0.1 a	0.77 ± 0.3 a	97 ± 0.8 a
190 B	88 ± 7.0 ab	14.6 ± 0.9 ab	0.81 ± 0.1 a	110 ± 0.2 c	58 ± 3.9 a	17.0 ± 0.5 a	0.80 ± 0.2 a	101 ± 1.0 b
55 G	83 ± 5.8 a	15.2 ± 0.5 a	1.02 ± 0.2 a	106 ± 0.7 b	75 ± 4.2 b	16.7 ± 0.5 a	1.00 ± 0.2 a	102 ± 0.4 b
80 G	90 ± 5.0 b	15.2 ± 1.5 a	0.88 ± 0.2 a	110 ± 0.8 c	83 ± 3.5 b	17.7 ± 0.6 a	0.88 ± 0.1 a	107 ± 0.8 c
68 G	93 ± 3.2 b	13.7 ± 0.7 b	0.93 ± 0.3 a	108 ± 2.0 bc	82 ± 6.5 b	16.1 ± 1.6 a	0.86 ± 0.1 a	103 ± 1.0 b
130 Y	90 ± 8.2 b	13.9 ± 0.6 b	0.83 ± 0.1 a	107 ± 0.5 b	78 ± 5.5 b	17.2 ± 0.4 a	0.87 ± 0.2 a	105 ± 0.7 bc

[a] Titratable acidity as malic acid.
[b] Values are means ± SE of three replicates of five fruits each. Different letters indicate significant differences within a column at $p < 0.001$.

the untransformed control and appeared to have an increased shelf-life (Table 3; Figure 4). However, there were no significant differences in the content of soluble solids and no significant differences in the level of total acidity (Table 3). This suggests that climacteric ethylene does not control the sugar/acid balance in fruit and that this component of FFC is likely to be regulated by other factors.

We also examined the volatile component of FFC by examining the levels of some aldehydes, alcohols and esters in the mature fruit obtained from different transgenic lines (Table 4). Aroma production was characterized by the presence of more than 14 compounds that were identified and quantified in the 'headspace of apple tissue'. In all the transgenic lines analyzed there was a significant decrease in hexyl esters, especially hexyl butanoate, which is a major component of flavor in Greensleeves apples. No major changes were observed in the overall level of aldehyde compounds; however, there was a small but significant increase in the hexanal/(2E)-hexenal ratio in the ethylene suppressed lines compared to the control fruit. In addition, a reduction in alcohols was observed in the ACO suppressed line.

Levels of α-farnesene have been correlated with the development of superficial scald, a post harvest disorder. As shown in Table 4 the levels of α-farnesene are significantly decreased in the ACO and ACS suppressed lines, as has been shown by Fan and Mattheis (1999) by blocking ethylene action.

Discussion

Phenotypic and molecular analysis of ethylene silenced transgenic apple lines

A major objective of our research is to define the role of the gaseous hormone ethylene in the regulation of FFC in apple and therefore, we focused our efforts on those clones that were significantly

Table 4. Content of major volatile compounds in Greensleeves fruits derived from different lines

Compound	nl l^{-1}		
	GS	68G	130Y
Aldehydes			
Hexanal	273 ± 47 a	398 ± 23 b	320 ± 35 a
(2E)-Hexenal	420 ± 30 a	290 ± 40 b	300 ± 30 b
Ratio – Hexanal/(2E)-Hexenal	0.7 a	1.4 b	1.1 ab
Total	693 ± 77 a	688 ± 63 a	620 ± 65 a
Alcohols			
Butanol	12 ± 2 a	3 ± 1 b	8 ± 1 b
Methyl butanol	6 ± 2	ND	ND
Hexanol	74 ± 7 a	54 ± 7 b	73 ± 6 a
Total	92 ± 11 a	57 ± 8 b	81 ± 7 a
Esters			
Butyl butanoate	55 ± 5 a	45 ± 4 b	36 ± 5 b
Butyl 2-methylbutanoate	40 ± 7 a	8 ± 2 b	7 ± 2 b
Hexyl acetate	12 ± 2	ND	ND
Hexyl propanoate	41 ± 6 a	20 ± 4 b	ND
Hexyl butanoate	340 ± 20 a	100 ± 25 b	98 ± 9 b
Total	488 ± 40 a	173 ± 35 b	141 ± 16 b
Others			
α-farnesene	85 ± 17 a	34 ± 9 b	40 ± 13 b

Different letters indicate significant differences within a row at $p < 0.001$.
Fruits were evaluated after 12 days at 20°C.
ND = not detected.

suppressed in ethylene production (Table 1). Nonetheless, a range of ethylene suppression was observed among clones. Ethylene is an important plant growth regulator that affects diverse plant processes, including fruit and leaf abscission, senescence and response to biotic and abiotic stresses (Ecker & Davis, 1987; McKeon & Yang, 1987; Reid, 1987; Kieber and Ecker, 1993; Ecker, 1995). However, we did not observe any overt phenotypic differences among plants that developed fruits that were significantly suppressed in ethylene production. Ethylene production was measured on five clones that were grown at both sites. Of these, three had ethylene production rates less than 6% of the control at UCD and 10–34% of the control at East Malling. Line 129Y had a production rate the same as the control at UCD but was a lower producer at East Malling. It can be concluded that despite some variation within years and between sites, some lines could be identified as being consistently higher or lower producers of ethylene than the controls. Down regulation of ethylene production in apples has also been reported by Hradzina et al. (2000) when an antisense sequence of ACC synthase was used to transform a small number of clones of Gala and McIntosh. The resultant clones showed some down-regulation of ethylene in transgenic fruits, but there was no attempt to examine effects on flavor composition. Antisense apple ACO was also successfully used to reduce ethylene production in transgenic tomatoes, in one case to 95% of the control (Bolitho et al., 1997), suggesting a lack of species specificity for these enzymes. In a reverse situation, James et al. (unpublished data) showed that a tomato antisense ACO could also cause ethylene reduction in transgenic apple plants although only to a maximum of 60% in one clone. Other clones showed far less reduction, and in two of the six clones investigated, no reduction was observed at all. Measurement of steady state mRNA levels showed a reduction of mRNA corresponding to the gene silenced (ACO or ACS) (Figure 2), presumably due to PTGS. Since PTGS is highly sequence specific, one would expect normal levels

Figure 4. Transgenic apple tree and shelf life of fruit suppressed for ethylene biosynthesis. (a) three year old transgenic apple tree of the line 80G. (b) ACO silenced apple 1 month after storage at room temperature. (c) wild-type GS apple 1 month after storage at room temperature. (d) ACO silenced apple 3 months after storage at room temperature and (e) wild type 3 months after storage at room temperature.

of mRNA for ACO in ACS suppressed lines and ACS in ACO suppressed lines. This seems to be the case for some of the lines (80G and 55G). However, lines such as 68G and 130Y, which are significantly suppressed for ethylene biosynthesis, also accumulate lower levels of the non-suppressed mRNA, ACO in the case of 130Y and ACS in the case of 68G (Figure 2). Neither of these lines display autocatalytic ethylene production (see Figure 3) suggesting that ethylene is required for sustaining the steady state levels of ACO and ACS mRNA by either increased synthesis or decreased breakdown. It has been suggested that ethylene regulates many other processes in plants apart from ripening (Ecker & Davis, 1987; McKeon & Yang, 1987; Reid, 1987; Kieber & Ecker, 1993; Ecker, 1995). Therefore, it will be interesting to understand how the expression of silencing of ethylene biosynthesis using the CaMV35S promoter in our plants affects the fruits but not the rest of the plant. It is possible that the small amount of ethylene that is produced in our plants may be sufficient to regulate these other processes. However, it would be useful to examine the response of these plants to abiotic and biotic stress to further elucidate the role of ethylene level in regulating the vegetative growth of apple in response to different environmental stimuli. Finally, it is possible that higher levels of silencing affect vegetative growth and that these lines may have had problems growing in culture after transformation. Therefore, plants with higher levels of silencing may have been selected against in culture.

Texture and sugar regulation in ethylene silenced apple fruit

Texture, which is an important component of FFC as judged by softening, is clearly regulated by ethylene in apple. All ethylene suppressed fruit showed a significant increase in firmness with an extended shelf-life (Table 3). As shown in Figure 4 control apples were completely rotten during an extended storage at room temperature while the ethylene suppressed fruit still maintained their shape. All of the significantly suppressed lines showed a greatly reduced autocatalytic ethylene production (Figure 3). It is possible that the level of inhibition of ethylene biosynthesis in our most suppressed line is not enough to avoid the trigger action of ethylene, as observed for respiratory climacteric in melon by Ayub et al. (1996). In ACO antisense melon, suppression of ethylene production resulted in an almost complete inhibition of softening, indicating that cell wall disassembly is also triggered by ethylene in this fruit (Guis et al., 1997). In transgenic tomatoes producing low levels of ethylene, the rate of softening was similar to wild-type fruit during ripening but was retarded during the overripening phase (Klee, 1993). In apple lines that showed low level of ethylene production, firmness at harvest was higher than in control fruits (Table 3, Figure 4). The differences in firmness observed in apples after storage could be due to overripening processes that do not occur in clones with very low ethylene. The overripening process leads to apples that take on a soft, dry and mealy texture, which typically occurs when apples are allowed to remain on the tree after optimum physiological maturity or when apples are held under inappropriate conditions after harvest (Harker & Hallett, 1992). These silenced apples provide a unique opportunity to study the enzymes that regulate the texture of apple fruit as the process of overripening has been greatly slowed down.

Regulation of fruit flavor/aroma compounds in transgenic apples silenced for ethylene biosynthesis

One of the most significant components of FFC from the perspective of consumers is aroma created by the synthesis of volatile ester compounds in ripening fruit. As highlighted in Table 4, the ethylene silenced apples show a dramatic reduction in both the hexyl and butyl esters. In particular, a greater than 90% inhibition was observed for hexyl acetate, and a 60–80% inhibition was observed for butyl 2-methylbutanoate, hexyl propanoate and hexyl butanoate. Overall, the total ester production was inhibited by 65–70% in the transgenic fruit silenced for ethylene. In contrast, aldehyde and alcohol precursors were inhibited by 12–38% compared to the wild-type fruit. In the case of the aldehyde precursors, there was a significant change in the ratio of hexanal/(2E)-hexenal suggesting a possible regulation of either lipoxygenase or hydroperoxide lyase by ethylene. It has been shown by Sanz et al. (1997) that hexanal is synthesized from linoelic acid and (2E)-hexenal from linolenic acid via these two enzymes in two different pathways. Since there is a split in the pathway, there may be some differential regulation taking place. These pathways are also responsible for the synthesis of alcohols via alcohol dehydrogenase (ADH) and formation of esters via alcohol acyltransferase (AAT) (Sanz et al., 1997). Our results suggest that the enzymes responsible for ester production (AAT) in apple may clearly be under the control of ethylene. It has been previously shown that the production of volatile compounds is closely related to changes in respiration and ethylene production. For example, total aroma production increases with maturation and ripening (Song and Bangerth, 1996). Our results confirm the ethylene control of ester accumulation but not necessarily the upstream steps in the biosynthetic pathway (including alcohols and aldehydes) leading to ester biosynthesis as has been suggested earlier (Fellman et al., 2000; Lurie et al., 2002). This general behavior in aroma production is also in agreement with other work on apples where ethylene sensitivity had been reduced by using an ethylene action inhibitor (Fan & Mattheis, 1999) and in work with melons where ethylene production had been reduced by expression of ACO antisense mRNA (Bauchot et al., 1998).

Fruits that are silenced for ethylene biosynthesis thus show a unique phenotype that can be used to investigate FFC as they affect the volatile flavor component of FFC without having a significant effect on the sugar–acid component. These fruits show a distinct phenotype in their texture and shelf-life. In the future, apple fruit suppressed for ethylene can be used to study the channeling and regulation of metabolic pathways that lead to the manifestation of a complex trait such as fruit quality. This knowledge can lead to the development of more precise diagnostics for quality control and to a more consistent, high quality fruit for the consumer.

Conclusions

Despite some variation between sites and seasons, a sufficient number of transformed lines consistently had a higher or a lower ethylene production rate than the control, and the storage potential of the fruit was correspondingly altered. The availability of these lines suppressed in ethylene biosynthesis allowed the identification of events under ethylene control such as fruit softening, external color development and ester accumulation. The role of ethylene in total soluble solids and acidity is not clear, and further research needs to be performed to investigate the levels of other components affecting flavor including total phenolics and individual sugars. Ethylene supressed lines offer a unique opportunity to understand the ripening process by distinguishing ethylene dependent and independent mechanisms in tree fruits that regulate components of edible quality.

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