FINAL REPORT
WTFRC Project # __PH-02-241__

Project title: Regulation of flavor and texture in apple fruit genetically modified for ethylene biosynthesis

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Objectives:

Our overall goal is to understand the role of level of endogenous ethylene in the regulation of texture and flavor development in apple fruit. Our objectives to achieve this goal are to investigate the complex relationship between sugar, acid, texture and volatile components in transgenic fruit modified to various extents in their capacity to synthesize endogenous ethylene.

1 To outline the relationship between the level of endogenous ethylene, fruit softening and the metabolism of carbohydrates/acids in transgenic apple fruit suppressed for ethylene biosynthesis.
2 To determine the relationship between the level of endogenous ethylene and the pattern of volatile components in transgenic apple fruit suppressed for ethylene biosynthesis.

Significant findings:

1. Very low ethylene producing transgenic apple lines have been identified that are suppressed for either ACS (ACC Synthase) or ACO (ACC Oxidase) expression.
2. ACS suppressed lines show suppression of ACS mRNA accumulation, ACS enzymatic activity but display normal levels of ACO mRNA and enzyme.
3. ACO suppressed lines show suppression of ACO mRNA/enzyme, accumulate ACC but show normal levels of ACS mRNA and enzyme.
4. ACS/ACO suppressed lines have been identified that show no climacteric response (no autocatalytic ethylene production).
5. Firmness, external color and titratable acidity are highly regulated by ethylene.
6. ACS and ACO suppressed lines show that individual sugars are differentially regulated by suppression of ethylene biosynthesis, with sucrose and fructose showing an ethylene dependent behavior.
7. Down-regulation of ethylene biosynthesis significantly reduces loss of acids keeping the levels close to ones measured at harvest.

8. Accumulation of phenolics show a partial response to the suppression of ethylene biosynthesis, which suggest that part of this pathway may be under ethylene regulation.
9. ACS/ACO transgenic fruits that make very low ethylene are significantly suppressed in their capacity to make volatile esters, and this reduction is well correlated with a reduction in the activity of alcohol acyl transferase
10. Synthesis of volatile flavor precursors, aldehydes and alcohols is somewhat reduced.
11. Ethylene suppressed lines show a marked reduction in alfa-farnescene.

Methods:

Plant Transformation: Transgenic apple were obtained using a previously published procedure for the *Agrobacterium*-mediated transformation of leaf discs from the apple cultivar 'Greensleeves' (James and Dandekar, 1991).

Molecular Characterization of Transformants: Total cellular RNA was isolated by the hot borate method. Strandard methods were used to conduct northern analysis (Dandekar et al., 2004).

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. The harvested apples were sorted to select those that were free from defects and to prepare matched samples of 1 to 5 apples per replicate and 3 to 5 replicates per treatment. Two to five fruits per line were selected for ethylene measurement when untransformed fruit started to loose chlorophyll.

Analysis of fruit: An initial sample from each harvest was evaluated for skin color (by a Minolta Chromameter), flesh firmness (by a penetrometer), starch pattern (by IKI staining) soluble solids content (by a refractometer), and titratable acidity (by an automatic titration system). Ethylene production and respiration rates were determined every two days during storage of individual fruits at 20^{o}C using a static system. ACC content and activities of ACC synthase and ACC oxidase was determined as described by Gorny and Kader (1996).

Determination of metabolites: Sugars and organic acids were analyzed by HPLC according to Pérez *et al.* (1997). Individual phenolics were identified and quantified according to the procedure of Gil *et al.* (2000). Headspace sampling was done by using a polydimethylsiloxane/divinylbenzene (PDMS/DVB, 65 um thickness) SPME fiber. A GC-MS system equipped with a DB-Wax column (J&W Scientific, 30 m, 0.32 mm i.d., 0.25 um film thickness) was used for analysis. Mass spectra were obtained by electron ionization at 70 eV. A spectra range of 40 to 250 m/z was used (Song and Bangerth, 1996). 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:

We present and discuss our results in the context of the significant findings listed above.

1. Very low ethylene producing transgenic apple lines have been identified that are suppressed for either ACS (ACC Synthase) or ACO (ACC Oxidase) expression.

We currently maintain a field planting of 184 apple trees, corresponding to 52 independent transformed lines, for the 4 vectors. Fruits obtained from the individual lines were analyzed and they show a wide variation in the level of ethylene synthesis in fruit tissues with many showing very low ethylene production rates.

2. ACS suppressed lines show suppression of ACS mRNA accumulation, ACS enzymatic activity but display normal levels of ACO mRNA and enzyme.

Individual fruit obtained from either ACO/ACS suppressed lines were examined biochemically to confirm suppression of expression of these genes is related to the reduction in ethylene biosynthesis. Shown in Fig 1., is a northern analysis that examines the level of steady state mRNA, so the presence of a band indicates expression and the absence of a band would indicate suppression. Apple fruit expressing ACO antisense (55G, 68G and 80G) show no mRNA for ACO but express normal levels of ACS and conversely ACS suppressed lines make ACO but not ACS (130Y). This silencing of mRNA specific to the enzymes involved in ethylene biosynthesis causes a strong suppression of ethylene observed in the fruit obtained from these lines during the climacteric response as compared to the control (Fig 2). The line 190B that shows a partial sense suppression of ACS mRNA displays a distinct but reduced climacteric ethylene response (Fig. 2). Further evidence for a distinct phenotype in these lines can be seen in the biochemical analysis of fruit from the same lines shown below in Table 1.

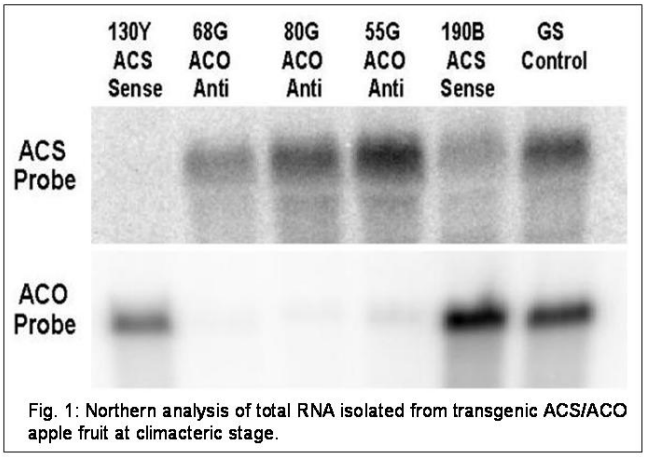

Fig. 1: Northern analysis of total RNA isolated from transgenic ACS/ACO apple fruit at climacteric stage.

Table 1. Analysis of transgenic apples silenced for ethylene biosynthesis relative to control apples

Apple lines	Genotype	Total Soluble Solids	Firmness	Ethylene level	ACO activity	ACC level
Control	WT	100	100	100	100	100
190B	ACS-Anti	106	110	78.4	270	47
55G	ACO-Anti	111	150	8.3	1.5	13,156
80G	ACO-Anti	100	140	3.1	1.3	19,969
68G	ACO-Anti	101	170	0.8	1.1	11,750
130Y	ACS-Sense	110	160	1.3	108	4

Values of Control Total soluble Solids = 17.0%, Firmness = 50 Newtons, Ethylene = 87.1 nl g^{-1} h^{-1}, ACC oxidase activity = 433.8 units, ACC level = 0.64 mM g^{-1}

3. ACO suppressed lines show suppression of ACO mRNA/enzyme, accumulate ACC but show normal levels of ACS mRNA and enzyme.

Here it can be seen that fruit deficient in ACO mRNA (Fig. 2) have correspondingly less ACO activity (Table 1). Interestingly in these very same fruit the precursor for ethylene biosynthesis namely ACC accumulates to massive quantities (Table 1). Correspondingly, ACS deficient fruit expectedly do not accumulate ACC and display normal levels of ACO activity. The suppression of the ethylene expression in fruit lines correspondingly displayed a greater degree of firmness as compared to an untransformed control and thus displays an increased shelf-life (Table 1). However and very interestingly there were no appreciable or significant differences in the content of soluble solids.

4. ACS/ACO suppressed lines have been identified that show no climacteric response (no autocatalytic ethylene production).

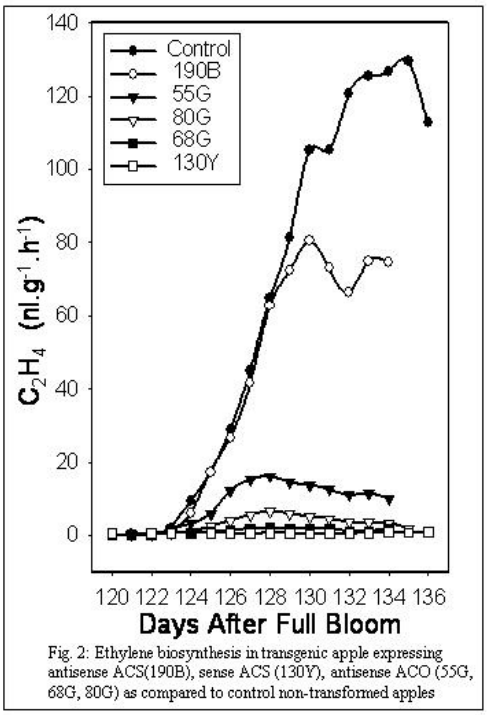

Fig. 2: Ethylene biosynthesis in transgenic apple expressing antisense ACS(190B), sense ACS (130Y), antisense ACO (55G, 68G, 80G) as compared to control non-transformed apples

Shown in Fig 2 is the typical rise in ethylene biosynthesis that can be seen in the sample obtained from control untransformed fruit. One can clearly see the steep rise in ethylene biosynthesis that is initiated at about 120 DAFB (Days after Full Bloom) and continues until about 135 DAFB. As can be seen in Fig. 2 this corresponds to expression of both ACS and ACO. In the case of line 190B these is a slight suppression of ACS (Fig 1) and this corresponds to a marked suppression of ethylene biosynthesis, however, the auto-catalytic ethylene production does still occur in this particular line. The rest of the lines 55G and 80G in particular show a very marked reduction in the ethylene response, which corresponds to the lack of ACO mRNA (Fig. 1) and a dramatic reduction in ACO enzymatic activity (Table 1). Finally, the lines 68G and 130Y show a complete elimination of the autocatalytic ethylene response in both cases this corresponds to the lack of ACO and ACS mRNA respectively. Shown in Table 1 is the ACO activity of the three clones 55G, 80G and 68G and the level of activity corresponds to the amount of ethylene made by these clones, with 68G making the least.

5. Firmness, external color and titratable acidity are highly regulated by ethylene.

Table 2. Quality indices of selected lines of Greensleeves apples stored at 20°C with or without exposure to 80 μL L^{-1} ethylene during storage.1

Line[2]	Firmness (Newton)		Total soluble solids (%)		Titratable acidity[3] (%)	
	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*
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
68G+ethylene		79 ± 7 b		16.8 ± 0.4 b*		0.69 ± 0.11 ab
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
103Y+ ethylene		60 ± 7 ab*		16.1 ± 0.4 ab*		0.70 ± 0.13 ab

[1]Values are means ± SD of 3 replicates of 5 fruits each. Means followed by different small letter within the same column are significantly different relative to the control treatment at *P=0.05*. Means follow by asterisk are significantly different relative to the evaluation at harvest within individual lines at *P=0.05*. [2]GS=non-transformed line, G=ACO antisense, Y=ACS sense. [3]Titratable acidity as malic acid.

When ethylene biosynthesis was suppressed or reduced, maturity and quality parameters, including firmness, external color and TA were affected, e.g. delay in softening, retention of green color (data not shown) and reduction in acid degradation rate (Tables 2), and they can be considered ethylene-dependent factors as previously demonstrated. On the other hand, another parameter like TSS, was slightly affected by a reduction in ethylene biosynthesis, and can be considered an ethylene-

independent parameter. The application of exogenous ethylene only produced a slight increase in ethylene biosynthesis, causing in the transgenic lines a differential enhancement of ethylene dependent processes, like change in color (data not shown) and loss of firmness, without affecting significantly loss of TA (Table 2). On the other hand, parameters that have been considered slightly dependent or independent of ethylene like TA and TSS, respectively, include a broad group of compounds like sugars (sucrose, glucose and fructose), phenolics and organic acids that can be differentially affected by ethylene and should be analyzed separately.

6. ACS and ACO suppressed lines show that individual sugars are differentially regulated by suppression of ethylene biosynthesis, with sucrose and fructose showing an ethylene dependent behavior.

As it was mentioned above, TSS has been considered an ethylene-independent process in apples; however, despite the importance of TSS as an indicator of sweetness of a fruit, the only metabolites determining its sweetness are the sugars glucose, fructose and sucrose. In the lines with down-regulation of ethylene biosynthesis total sugars did not accumulate at the levels of GS in which a 25% percent increase was observed between harvest and the end of storage (Defilippi et al. 2004).

Table 3. Changes in sugars (%) in fruit tissue of Greensleeves apples stored for 14 days at 20°C with or without exposure to 80 uL L^{-1} ethylene during storage.1

Lines[2]	Sucrose		Glucose		Fructose	
	At Harvest	After storage	At Harvest	After storage	At Harvest	After storage
GS	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	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		3.5 ± 0.38 a*		2.1 ± 0.15 a		5.5 ± 0.5 a*
103Y	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		3.0 ± 0.22 a*		2.6 ± 0.15 a		4.7 ± 0.5 a

[1]Values are means ± SD of 3 replicates of 5 fruits each. Means followed by different small letter are significantly different within the same row for an individual compound at *P=0.05*. Means follow by asterisk are significantly different relative to the evaluation at harvest within individual lines at *P=0.05*. [2]GS=non-transformed line, G=ACO antisense, Y=ACS sense.

However, when the lines were exposed to ethylene they reached control GS levels at the end of storage. In terms of individual sugars, sucrose and fructose showed the same behavior as total sugars, with glucose not being affected at all. It has been shown that hydrolysis of starch plays a role as a source of sugars in the last stages of fruit development, and prior research has concluded that loss of starch did not appear to be related with ethylene (Table 3). 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 observed changes in sugar levels.

7. Down-regulation of ethylene biosynthesis significantly reduces loss of acids keeping the levels close to ones measured at harvest.

A reduction in organic acids was observed between harvest and the end of storage, which was reflected in both the value of TA (Table 2), and in the concentrations of individual acids, i.e. malic and citric acids (Figure 3). Down-regulation of ethylene biosynthesis significantly reduced this loss of acids keeping the levels close to ones measured at harvest. The exposure of the fruit to ethylene only enhanced degradation of acids in the transgenic lines. It has been concluded that the main cause of

organic acids degradation in climacteric fruit is the large increase in respiration rate during ripening and with the suppression of ethylene biosynthesis and action we did notice a reduction of respiration rate. Exogenous ethylene in the transgenic lines increased the respiration rate close to the GS causing an increase in acid degradation relative to the non-treated fruit. Since changes of 0.08% in TA in apples are typically noticed by trained panelists, the effect of down regulation of ethylene on acid regulation will be reflected in the final overall flavor.

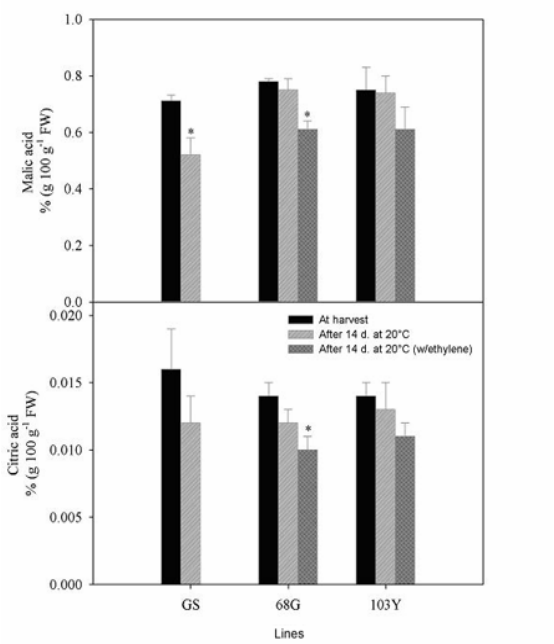

Figure 3. Acids content of Greensleeves apple lines stored at 20°C with or without exposure to 80 uL L-1 ethylene. Bars with (*) are significantly different relative to the evaluation at harvest within individual lines at P=0.05.

8. Accumulation of phenolics show a partial response to the suppression of ethylene biosynthesis, which suggests that part of this pathway, may be under ethylene regulation.

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 non-transformed line (Table 4). The main phenolic compounds identified were clorogenic acid, epicatechin and phloridzin. The transgenic lines showed a different pattern relative to GS with no significant changes between harvest and the end of storage at 20°C, in total or individual phenolics (Table 4). Moreover, applications of ethylene after harvest could not recover the levels of phenolics in the transgenic lines to those observed in the control indicating that the accumulation of phenolics during storage is an ethylene-independent process as has been suggested previously using controlled atmospheres (Awad and de Jager, 2000). However, these differences may also be due to a differential effect caused by the suppression at different stages of fruit development, i.e. in the transgenic lines ethylene biosynthesis is being affected from early stages of fruit development where most of the changes in phenolic concentration occurred.

Table 4. Changes in the phenolic content (μg g^{-1}) in fruit tissue of Greensleeves apples stored with or without exposure to 80 μL L^{-1} ethylene during 14 days at 20°C.[1]

Lines[2]	Total Phenolics		Chlorogenic acid		Epicatechin	
	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*	78 ± 15 a	123 ± 19 a*
68G	1100 ± 72 a	1150 ± 156 a	159 ± 12 a	153 ± 15 b	101 ± 16 a	100 ± 20 a
68 G plus ethylene		1180 ± 84 a		169 ± 9 ab		122 ± 6 a
103Y	1021 ± 57 a	1170 ± 150 a	137 ± 10 a	138 ± 17 b	43 ± 8 b	53 ± 10 b
103Y plus ethylene		1436 ± 231 a		155 ± 7 b*		60 ± 7 b*

[1]Values are means ± SD of 3 replicates of 5 fruits each. Means followed by different small letter are significantly different within the same column for an individual compound at *P=0.05*. Means follow by asterisk are significantly different relative to the evaluation at harvest within individual lines at *P=0.05*. [2]GS=non-transformed line, G=ACO antisense, Y=ACS sense.

9. ACS/ACO transgenic fruits that make very low ethylene are significantly suppressed in their capacity to make volatile esters, and this reduction is well correlated with a reduction in the activity of alcohol acyl transferase.

The effect of ethylene suppression was remarkable with a reduction or delay in the accumulation of ester compounds reaching levels of 12-15% in the transgenic lines relative to GS (Figure 4). These results confirm that ester production is under ethylene regulation. No major changes were observed in the levels of precursor aldehyde compounds. In addition a reduction/delay in the accumulation of alcohols was also observed in the transgenic lines, suggesting that not only are esters under the control ethylene, but also the steps upstream in the biosynthetic pathway of ester biosynthesis are under ethylene coordination. This statement can be supported by a recovery of alcohol and esters in the ethylene treated line, in which the levels of these compounds were significantly higher than the non-treated fruit. The behavior of ester production under theses conditions has been highly correlated with the behavior of alcohol acyl transferase activity (Figure 5).

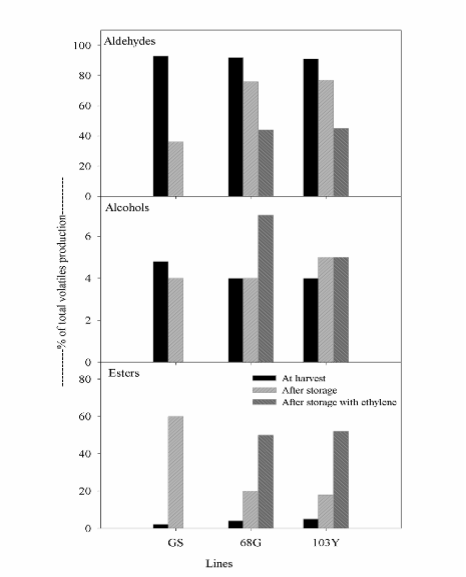

Figure 4. Relative content of aroma compounds of three Greensleeves apple lines stored at 20°C for 14 days with or without exposure to 80uL L^{-1} ethylene. Means of 3 replicates ± SD.

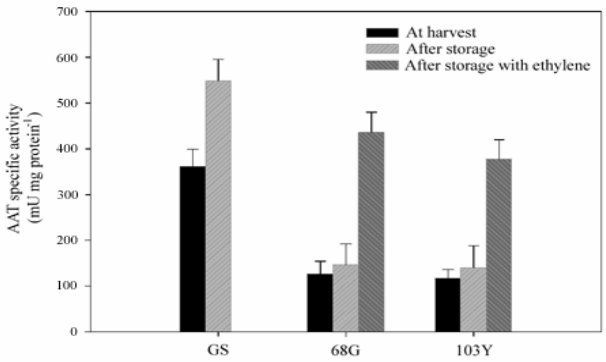

Figure 5. Alcohol acyl transferase (AAT) activity of three Greensleeves apple lines stored at 20°C for 14 days with or without exposure to 80uL L^{-1} ethylene. Means of 3 replicates ± SD.

The effect of ethylene suppression was dramatic, resulting in a remarkable reduction or delay in the accumulation of ester compounds reaching levels of 12-15% in the transgenic lines relative to GS (Figure 4). Therefore, these results confirm that ester production is under ethylene regulation in apples. No major effects were observed in the levels of aldehyde compounds. On the other hand, a reduction/delay in the accumulation alcohols was also measured in the transgenic lines, which suggests that not only esters are under ethylene control in Greensleeves fruit, but steps upstream in the biosynthetic pathway of ester biosynthesis are under ethylene coordination. This statement can be supported by a recovery of alcohol and esters in the ethylene treated line, in which the levels of these compounds were significantly higher than the non-treated fruit. The behavior of ester production under theses conditions has been highly correlated with the behavior of alcohol acyl transferase activity (Figure 5).

10. Synthesis of volatile flavor precursors, aldehydes and alcohols is somewhat reduced.

We also examined the major alcohol and aldehyde volatile flavor components that are precursors for the synthesis of esters but in many instances are significant flavor compounds themselves. Again we examined lines that made very little ethylene and these data are shown in Table 5. One of the major flavor precursors Hexanal was significantly increased, perhaps due to the lack of conversion to the ester (Table 5).

Table 5. Content of major volatile compounds in Greensleeves fruits derived from different lines. Fruits were evaluated after 12 days at 20°C

Compound (nL L^{-1})	GS	68G	130Y
Aldehydes			
Hexanal	273 ± 47	398 ± 23	320 ± 35
(E) 2-Hexenal	420 ± 30	290 ± 40	300 ± 30
Alcohols			
Butanol	12 ± 2	3 ± 1	8 ± 1
Methyl 2-butanol	6 ± 2	NP	NP
Hexanol	74 ± 7	54 ± 7	73 ± 6
Esters			
Butyl butanoate	55 ± 5	45 ± 4	36 ± 5
Butyl 2-methylbutanoate	40 ± 7	8 ± 2	7 ± 2
Hexyl acetate	12 ± 2	NP	NP
Hexyl propanoate	41 ± 6	20 ± 4	NP
Hexyl butanoate	340 ± 20	100 ± 25	98 ± 9
Alfa-farnescene	85 ± 17	34 ± 9	40 ± 13

11. Ethylene suppressed lines show a marked reduction in alfa-farnescene.
Finally, there are other metabolism that are related to fruit disorders like superficial scald where the levels of alfa-farnescene accumulation have been correlated to the development of this post harvest disorder. As shown in Table 5 the levels of alfa-farnescene appear to be regulated by ethylene with the ACO suppressed lines showing a dramatic reduction of alfa-farnescene.

In summary, fruit silenced for ethylene biosynthesis show a unique phenotype that can be used to investigate FFC in a meaningful way as they affect the volatile flavor component of FFC without having a significant effect on the sugar acid component. Also these fruit show a distinct phenotype in their texture and shelf-life. These data clearly show that antisense/ gene silencing technology is fully

functional and very specific in apple and we propose to use some of the specific lines (68G and 130Y) to identify ripening-related genes that determine FFC.

Significance to the industry and potential economic benefits:

-A greater understanding of the regulatory role of endogenous ethylene in flavor development in apple fruit.

-Enabling the development of new tools that will provide a better understanding of the role of endogenous ethylene in the regulation of texture and flavor development in apple fruit.

-Enabling the development of unique diagnostic tools to measure internal fruit quality in the field and during storage and distribution.

-Enabling the development of new tools for seedling selection in a breeding program to breed varieties with enhanced quality characteristics.

-Availability of unique tools to measure internal fruit quality development will lead to improved cultural practices and breeding strategies that will result in the production of higher quality fruit.

Budget

Project title: Regulation of flavor and texture in apple fruit genetically modified for ethylene biosynthesis.

PI: Abhaya M. Dandekar

Project duration: 2002-2005

Project total (3 years): $112,097

Year	Year 1 (2002)	Year 2 (2003)	Year 3(2004)
Total	$36,801	$37,081	$38,215

Year-by-year breakdown

Item	Year 1 (2002)	Year 2 (2003)	Year 3(2004)
Salaries PGRIV (50%)	$17,562	$18,440	$19,362
Student 20hr/wk for 6 months	$ 4,200	$ 4,200	$ 4,200
Benefits	$ 4,239	$ 4,441	$ 4,653
Equipment			
Supplies	$10,000	$ 9,000	$ 9,000
Travel	$ 800	$ 1,000	$ 1,000
Miscellaneous			
Total	$36,801	$37,081	$38,215

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