FINAL PROJECT REPORT
WTFRC Project Number: PH-05-504

Project Title: Defining ethylene regulation of apple fruit quality traits

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Other funding Sources

Agency Name: N/A
Amount awarded:
Notes:

Total Project Funding: $128,295

Budget History:

Item	**Year 1:** 2005	**Year 2:** 2006	**Year 3:** 2007
Salaries	$19,326	$20,292	$21,307
Benefits	$10,109	$10,651	$11,146
Wages			
Benefits			
Equipment			
Supplies	$ 9,500	$12,000	$11,000
Travel	$ 1,000	$ 1,000	$ 1,000
Miscellaneous			
Total	**$39,935**	**$43,907**	**$44,453**

Objectives:
Our overall goal was to define the role of ethylene in the functional regulation of apple fruit quality. This was accomplished by using transgenic apple fruit modified in their capacity to synthesize endogenous ethylene or wild type fruit modified in their ethylene response via the application of 1-methylcyclopropene (1-MCP). Tissues were obtained from ripening wild type, transgenic and treated fruit (ethylene gas of 1-MCP) displaying significant differences in phenotypic traits responsive to ethylene. These differences in phenotypes were matched with changes in gene expression patterns which were used to identify ethylene regulated genes in apple fruit tissues. We created specialized resources and mining tools to utilize the information available in GeneBank to annotate the genes that we identified. Validation of the ethylene regulated genes was accomplished by quantitative real time PCR (RT-PCR) along with the analysis of metabolites from the same tissues to define the biochemical pathways by identifying the key metabolites, their precursors and the enzymes involved.

Objective 1. Identify specific transcripts that are differentially regulated in transgenic apple fruit silenced for ethylene synthesis or perception, and correlate them with flavor and texture development - The primary aim of this objective is to compile and annotate the most highly regulated transcripts expressed during postharvest apple fruit development. The transcriptome of developing apple fruit has been sampled over time, with particular emphasis on transcripts expressed in cortical and peel tissues. Our approach has been on two fronts one is to develop or deploy bioinformatic tools to do a digital analysis of expressed genes available in GenBank (NCBI) and to develop and deploy microarray technologies to investigate ethylene dependent pattern of expression in apple fruit. Microarray analysis revealed the expression pattern of ethylene regulated genes some of these were validated using real time PCR (RT-PCR). We have also developed the resource to visualize apple expression data as pathways to better understand the relationships between the expressed patterns of genes in tissues obtained from different treatments and their regulation by ethylene.

Objective 2. Functional validation of pathways *via* analysis of key metabolites and enzymes regulated by ethylene – We have focused on 2 transgenic lines that make very low ethylene these are 68G expressing antisense ACC oxidase (ACO) and 103Y a line expressing a sense version of the apple ACC synthase gene (ACS). We have refined our analysis by focusing on peel and cortical tissues obtained from samples harvested in 2005 and 2006. We have carried out phenotypic, biochemical, enzymatic and metabolic analysis focusing on the postharvest behavior of gene activity. We would like to discover the subset of genes that are regulated by 'system 2' ethylene regulation, i.e., those genes/traits that are specifically regulated by autocatalytic ethylene biosynthesis. The phenotypic, metabolic and biochemical data has been integrated using gene set enrichment analysis to visualize the functional caregories of genes regulated by ethylene.

Significant findings/accomplishments

1. Development of unique study design that involves 2 transgenic, 1 treated and 1 wild type fruit to create 9 phenotypically distinct treatments.
2. Validation of the study design demonstrated the ethylene is positively correlated with color, starch and weight and negatively correlated with firmness and acidity. Ethylene is not correlated with soluble solids.
3. Successful deployment of microarray resources and analysis tools to dissect the transcriptome of apple fruit.
4. Differential gene expression patterns obtained by microarrays identified 3029 genes significantly regulated in apple fruit with 658 in cortical, 2169 in peel tissue and 381 in both tissues respectively that are likely regulated by ethylene.

5. Expression of genes related with ethylene biosynthesis (ACO, ACS and ERF3), aroma volatiles-related biosynthesis (AAT, LOX, LOX1) and sorbitol biosynthesis (NAD-SDH5, NAD-SDH6, S6PDH, SDH4 and SDH5) down regulated in the GS (control) fruit treated with 1-MCP.
6. Sugar-acid balance is differentially regulated in cortical but not in peel.
7. Metabolite profiles showed that most of the statistically significant metabolites identified in cortical and peel are precursors of aroma-volatile compounds.
8. Ethylene is a modulator of most of the aroma related volatiles, especially the alcohol acyltransferase (AAT) enzyme activity.

Methods:

Plant material: The experiments utilized transgenic apple fruits suppressed in ethylene biosynthesis obtained from 2 different lines and one coltrol line grown in an experimental orchard.

Fruit collection and handling: Apples (Golden Delicious cv. 'Greensleeves', GS) were harvested from the research orchard when GS fruit was in a pre-climacteric stage (internal ethylene concentration lower than 0.3 μL L^{-1}) prior to the initiation of autocalylytic ethylene biosynthesis (Fig 1). Apples were transported to the Postharvest Pomology Research Laboratory at UC Davis and sorted to select those that were free from defects. Matched samples of 1 to 5 apples per replicate were prepared with 3 to 5 biological replicates per treatment.

Treatments: Fruits from selected 'Greensleeves' apples lines including transgenic 68G (ACO-antisense), 103Y (ACS-sense). Half of the fruit from each line was kept at 20ºC in an ethylene-free atmosphere and the other half was stored at 20ºC under the flow of air containing 80 μL L^{-1} ethylene during storage. Fruit were sampled at 1 day after 14 days of storage at 20ºC. The controls GS were also harvested at the same time as the transgenic lines and these were treated with 0 (control) or 1 □LL^{-1} 1-MCP in a 20L sealed glass jar for 20 h at 20ºC before storage at 20ºC for 14 days. Relative humidity was maintained close to 90-95%. The untransformed fruit (GS) and treated fruit were sampled at 1 day after 14 days of storage at 20ºC in air (ethylene-free atmosphere). After storage fruit tissues were dissected to obtain peel and cortical which were frozen in liquid N_2 and kept at -80 ºC until analysis. For all biochemical analysis, three replicates of five fruit each was used.

Ethylene and respiration rate measurements: Within each experiment ethylene production and respiration rates were determined at 1 and 14 days after storage for individual fruits using a static system. Exit air samples was collected from each jar and analyzed for CO_2 concentration (by an infrared gas analyzer) and ethylene concentration (by a flame ionization gas chromatograph) (Defillipi et al., 2004).

Maturity and quality parameters: An initial sample from each harvest was evaluated for skin color (by a Minolta Chromameter), starch pattern (by IKI staining), soluble solids content (by a refractometer), titratable acidity (by an automatic titration system), cortical firmness (by a Guss fruit texture analyzer and an Aweta Acoustic Firmness Sensor). All of these were used to document the mature apple phenotype and provide a "quality" reference for samples tested for comparison with ethylene-silenced fruit.

Microarray analysis of the transcriptome: A custom 12K oligonucleotide microarray was designed by CombiMatrix using the UniGene Build#14 (05 Apr 2006) their bioinformatics pipeline and design criteria and utilizing only the apple unigene entries that came from the 34

fruit libraries from GeneBank. Combimetrix synthesized the desired oligonucleotides on the chip surface and then sent us the chips. We isolated RNA from the two tissues (peel and cortex) from the 9 treatments (Fig 1), labeled RNA using the standard one color biotin labeling kit. The hybridization, imaging, stripping and re-hybridization was performed at the Microarray Core Facility at UC Davis as per the CombiMatrix protocols available on their website (http://www.combimatrix.com/docs/PTL006_00_12K_Hyb_Imaging.pdf). The chips were scanned at this facility using a GenePix scanner and the spots were manually aligned to the grid supplied by ComBimatrix to obtain a tiff image. The tiff image was preprocessed with global median scaling normalization, background (av of lowest 5% of signal of control probes) subtraction to obtain gene expression data. R package LMGene was used to perform one-way ANOVA to obtain p-value for each gene. R package multtest was used to BH adjust all p-values for multiple hypotheses. R package limma was used to obtain pair wise comparisons to identify tissue and treatment specific genes.

Determination of sugars, acids and related enzymes activities: Fruit cortical and peel tissues obtained from the various transgenic lines were analyzed for soluble sugars (sucrose, fructose, glucose, sorbitol), and acids (malic and citric acids) using a high resolution GC/MS equipment. Enzyme that regulate sugar-acid balance was assayed using methods described by Dey and Harborne (1990).

Additional Metabolite analysis: Metabolite analysis of samples obtained from all treatments was carried out at the Metabolomic facility of the Davis campus (http://fiehnlab.ucdavis.edu/) under the direction of Prof. Oliver Fiehn and the manager of the facility Dr. Valdimir Tolstikov. Cortical and peel tissues carefully separated and frozen in liquid N_2 and kept at -80 ºC, until sample preparation was carried out at the Metabolomic facility. Using their high resolution GC/MS equipment a relative abundance was obtained for 400 compounds that can be resolved using their protocol for each of our samples.

Results and Discussion

1. Development of unique study design that involves 2 transgenic, 1 treated and 1 wild type fruit to create 9 phenotypically distinct treatments.

Our overall goal was to define the role of ethylene in the functional regulation of apple fruit. The availability of transgenic apple fruit modified in their capacity to synthesize endogenous ethylene and the use of 1-methylcyclopropene (1-MCP), an ethylene action inhibitor, was used in our study design as they have distinct phenotypes demonstrated earlier by us (Dandekar et al., 2004). The various treatments used in our study are outlined below in Fig 1. Fruit harvested at a preclimacteric stage before the onset of system 2 ethylene formation and kept at 20°C for 14 days. Transgenic lines 68G and 103Y were incubated with and without ethylene whereas the controls were incubated with or without 1-MCP. This gave us the 9 treatments that we investigated in our study to define the ethylene responsive genes. The Fig 1 is adapted from our earlier publication that shows the

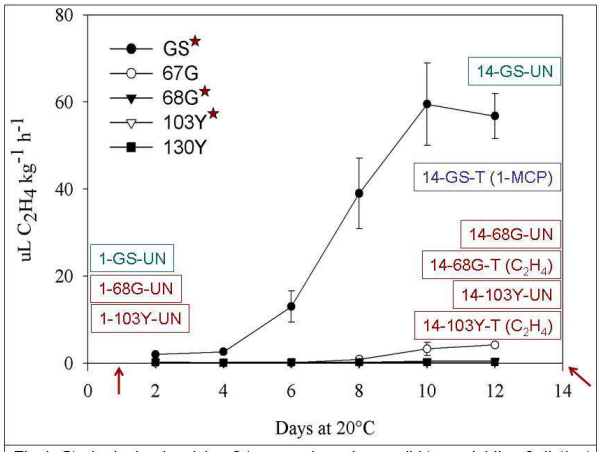

Fig 1: Study design involving 2 transgenic and one wild type yielding 9 distinct treatments modulated by or responsive to ethylene levels

autocatylytic ethylene biosynthesis in the wild type GS controls at day 14 but not in the transgenics.

2. Validation of the study design demonstrated the ethylene is positively correlated with color, starch and weight and negatively correlated with firmness and acidity. Ethylene is not correlated with soluble solids.

Application of 1-MCP to GS (control) apple fruit completely suppressed ethylene production. Application of exogenous ethylene to the 68G and 103Y transgenic lines did not produce increased ethylene biosynthesis. Weight, external color, firmness, starch index, and total acidity were regulated by ethylene in both 2005 and 2006, but total soluble solids were ethylene-independent (Fig 2). The apple fruit from the 2006 crop was affected by unseasonal and sustained heat at maturity and quality indicators showed ethylene dependence, but not color. Firmness, a primary measure of maturity and quality, is regulated by ethylene biosynthesis. Firmness was most affected in the 1-MCP treated fruit; the effect of ethylene suppression on firmness in transgenic lines was less obvious. Firmness after 1 day storage at 20 ºC was measured using a Guss fruit texture analyzer (destructive measurement method) and after 14 days firmness was measured first by an Aweta Acoustic Firmness sensor (AFS, non-destructive method) and then with the same fruits by the Guss Fruit Texture Analyzer. In 2005 the firmness value obtained using the the Guss fruit analyzer didn't showed good correlation with the firmness index obtained with the Aweta instrument. However in the second year of use the correlation was very high, a more extensive study including different varieties is needed to validate the Aweta non destructive method. This phenotypic analysis validated the phenotypic distinctions in the 9 treatments of our study design, the tissues (peel and cortex) form which were used to dissect the gene regulation.

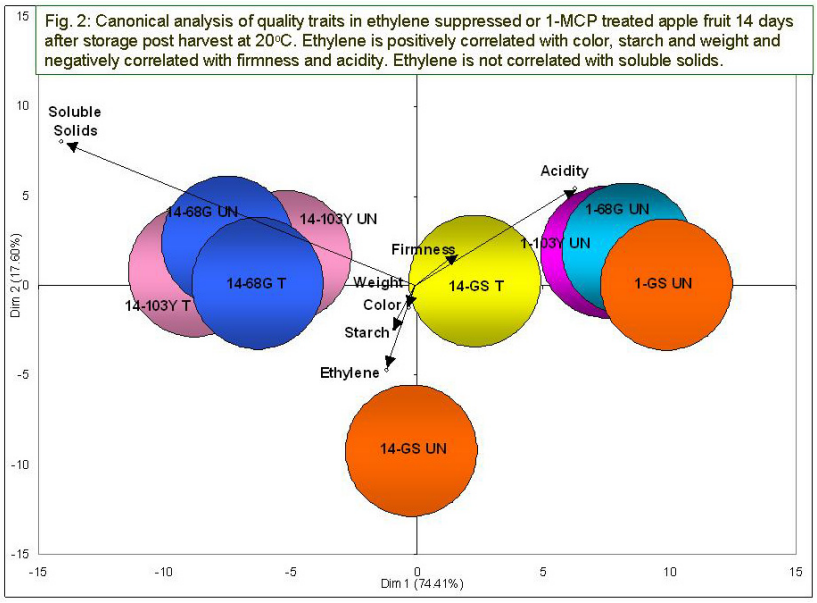

Fig. 2: Canonical analysis of quality traits in ethylene suppressed or 1-MCP treated apple fruit 14 days after storage post harvest at 20°C. Ethylene is positively correlated with color, starch and weight and negatively correlated with firmness and acidity. Ethylene is not correlated with soluble solids.

3. Successful deployment of microarray resources and analysis tools to dissect the transcriptome of apple fruit.

We successfully investigated the expressed genes in apple through the assembly of a cDNA anlaysis pipeline at the College of Agriculture and Environmental Science Core Genomics Facility (CA&ES CGF) at Davis. This pipeline is a series of programs that help examine all information stored in the GenBank public database, and it allows us to access raw sequence information for apple ESTs, which are terminal DNA sequences representing either the 5' of 3' end of an apple mRNA. These sequences were downloaded and stored in an Oracle database at Davis, then sorted to remove extraneous sequences that represent *E. coli*, chloroplast, or mitochondria DNA sequences. Only high quality sequence information was retained for further analysis. At the time we examined 160,620 of the current 256,249 entries in the public database

for Apple (GenBank, NCBI). Other available resources are the GDR which is the Rosaceae community wide resource and is more current and upto date in the analysis of all available apple EST sequences. Our resource and analysis is still web-accessible through the Core Genomics facility (CGF) website (http://cgf.ucdavis.edu/), available by clicking on the apple icon. Of the analyzed 160,620 ESTs, 45,414 (28.3% discovery rate) of the genes correspond to a unigene set, with a majority (25,232; 15.7%) being singletons (represented once in our database) and 20,182 (12.6%) being 'contigs' (represented more than once in our online database). This provided an estimated 45,414 unigenes. Since most of the apple ESTs represent 5' ends of mRNAs without any 3' anchor ESTs the unigene set we could derive was quite redundant. To avoid this problem we used the current 'unigene' (NCBI) assembly at the time which was build #14 (05 Apr 2006; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene). This particular build had about 17,180 unique genes that came from 106 different cDNA libraries. We divided these into two groups one vegetative and one fruit derived. We have utilized the fruit derived sequences to design oligonucleotides for microarray analysis.

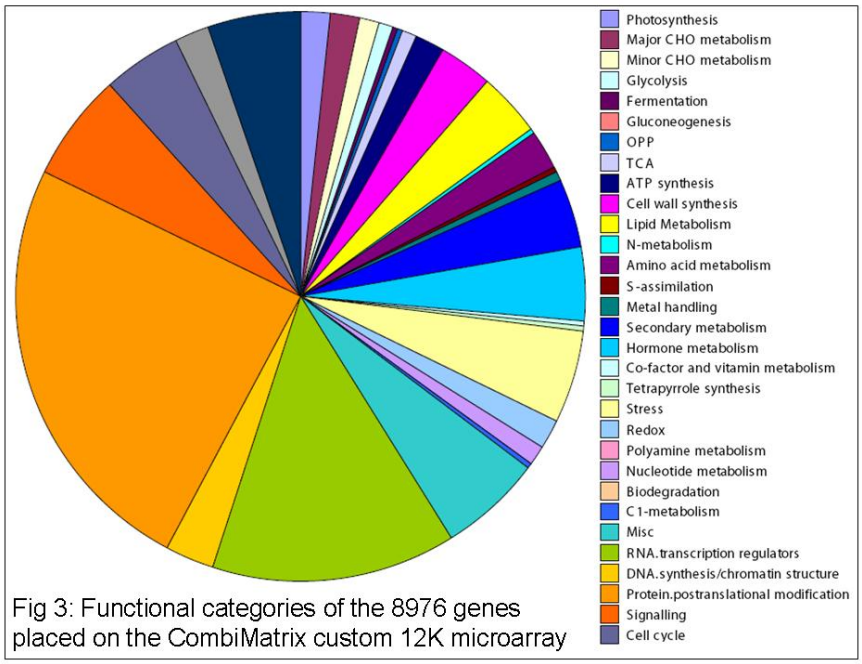

Fig 3: Functional categories of the 8976 genes placed on the CombiMatrix custom 12K microarray

A Combimatrix 12K custom microarray was designed by us using their design criteria and 8813 sequences from the UniGene Build#14 that came from the 34 fruit libraries and 163 genes of interest to us that we added for a total of 8,976 apple genes. The total number of probes synthesized were 12,395 of which 474 (4%) were control probes and 11,921 (96%) were apple probes. Oligo nucleotides were synthesized on the surface of the chip. Shown in Fig 3 are the functional categories of the genes that are represented on our chip. We used the MapMan tool developed by Mark Stitt et al. at the German Resource Center for Genome Research to develop the functional categories (http://gabi.rzpd.de/projects/MapMan/). MapMan project collaborators have developed an ontology which classifies Arabidopsis genes into 35 broad categories, and nearly 2000 sub-categories that correspond to all known functions in Arabidopsis.

4. Differential gene expression patterns obtained by microarrays identified 3029 genes significantly regulated in apple fruit with 658 in cortical, 2169 in peel tissue and 381 in both tissues respectively that are likely regulated by ethylene.

The microarray analysis involved 36 microarray hybridizations that included 6 treatments (14-GS-UN, 14-GS-T, 14-68G-UN, 14-68G-T, 14-103Y-UN and 14-103Y-T) 2 tissues (cortex and peel) and 3 replications. A one-way ANOVA model was first applied to each gene, respectively. Then the standard errors were modified using empirical Bayes methods. The resulting p-values were BH adjusted (Benjamini and Hochberg 2001) for multiple hypotheses testing. Genes with

adjusted p-values less than 0.05 were considered differentially expressed. Therefore, we report positive hits with high confidence. R package limma (Smyth, 2004) was used to perform the pair wise comparisons shown below in Table 1.

Table 1. Pairwise comparisons of differentially expressed genes in peel and cortical tissues in different treatments obtained from fruit after 14 days at 20°C

Comparisons	PEEL	CORTICAL
14-GS-T – 14-GS-UN	239	472
14-68G-UN – 14-GS-UN	389	626
14-68G-T – 14-GS-UN	1641	322
14-103Y-UN – 14-GS-UN	50	319
14-103Y-T – 14-GS-UN	680	507
14-68G-UN – 14-GS-T	388	20
14-68G-T – 14-GS-T	581	6
14-103Y-UN – 14-GS-T	70	3
14-103Y-T– 14-GS-T	1010	6
14-68G-T – 14-68G-UN	5	16
14-103Y-UN –14-68G-UN	4	67
14-103Y-T– 14-68G-UN	7	46
14-103Y-UN – 14-68G-T	13	14
14-103Y-T – 14-68G-T	32	14
14-103Y-T – 14-103Y-UN	2	28

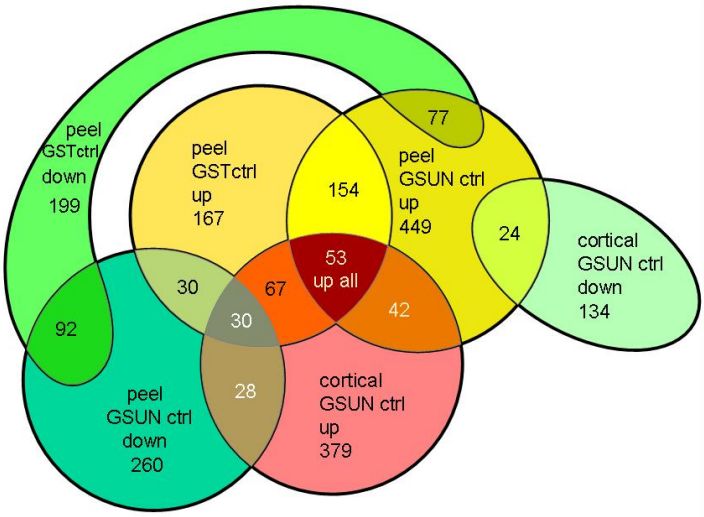

Fig 4: Expression patterns of 2339 genes up or down regulated in peel or cortex tissues among the treatments

The pair wise comparisons conducted for each of the 6 treatments (14-GS-UN, 14-GS-T, 14-68G-UN, 14-68G-T, 14-103Y-UN and 14-103Y-T) contain both up and down regulated genes. One can see that most number of different genes occurs when one compares the transgenics to either the control (14-GS-UN) or the control treated with 1-MCP (14-GS-T). Clearly the peel is much more transcriptionally active as compared to the cortex. This is a view of the complexity of gene expression patterns based on tissue type and treatment. The complex ven diagram presented in Fig 4 provides a view of expression patterns of 2339 genes in these various comparisons focusing on comparisons of the transgenics with either the control or the control treated with 1-MCP. The numbers indicate the number of different genes that are either up regulated or down regulated and the numbers within the intersections indicate genes that are ether up or down regulated in more than one tissue type or in more than one treatment. Fig 4 shows the complex expression pattern of ethylene regulated genes in peel and cortex tissues among the treatments. Since the comparisons involve tissues from our transgenics we can say that those particular genes are regulated by ethylene and in the case of the 1-MCP treatments are regulated by the ethylene response.

5. Expression of genes related with ethylene biosynthesis (ACO, ACS and ERF3), aroma volatiles-related biosynthesis (AAT, LOX, LOX1) and sorbitol biosynthesis (NAD-SDH5, NAD-SDH6, S6PDH, SDH4 and SDH5) down regulated in the GS (control) fruit treated with 1-MCP.

The expression of 47 target genes related mainly with ethylene biosynthesis and response, aroma biosynthesis, softening/texture, carbohydrate metabolism, amino acid metabolism and flavanoid biosynthesis were tested with cortical tissue using Real time (RT) PCR using a dual-labeled florogenic probe TaqMan Probe. The purpose of this analysis is the validation of data obtained from the microarray analysis. Expression of 12 out of 47 genes were shown to be significant at the threshold p-value<0.01, gene expression was represented as fold changes compared to the wild type GS control at harvest. Ethylene biosynthesis related genes, 1-aminocyclopropane-1-carboxylate oxidase (ACO), 1-aminocyclopropane-1- carboxylate synthase (ACS) and ethylene-response factor ERF3 showed a major reduction of ACO, ACS and ERF3 gene expression in GS treated with 1-MCP and stored for 14 days at 20ºC. 68G and 103Y samples treated with ethylene during storage showed an increase in ACS and AC0 gene expression, respectively (Table 2).

Table 2. ACO, ACS ERF3 and AAT genes expression[a]

	ACO		ACS		ERF3		AAT2	
Sample	**1 day**	**14 day**	**1 day**	**14 day**	**1 day**	**14 day**	**1 day**	**14 day**
GS	1.3544a	13.444b	1.3558a	58.21a	1.0264a	2.7990ab	1.0893a	3.1030ab
GS + 1MCP		0.1080b		0.120b		1.138b		0.0470b
68G	0.0142b	0.0290b	0.1010b	39.30a	1.1387a	2.6140ab	0.7307a	3.7720a
68G + C_2H_4		0.0790b		52.10a		1.9940ab		1.2450ab
103Y	0.3087b	7.6070b	0.2462b	1.630b	1.4774a	4.6310ab	0.6677a	0.3050b
103Y + C_2H_4		28.882a		1.120b		6.012a		2.5800ab

[a]Values represent fold changes compared to the mean of GS-1day samples. Different letters a or b indicate differences among sample at significance level p=0.05 within 1 day or 14 day. ACO = 1-aminocyclopropane-1-carboxylate oxidase, ACS = 1-aminocyclopropane-1- carboxylate synthase, EFR3 = ethylene-response factor and AAT = alcohol acyltransferase

Alcohol acyltranferase (AAT2), lipoxygenase (LOX), LOX1 gene expression, which are related with the biosynthesis of aroma-volatiles compounds showed a decrease in expression for GS control treated with 1-MCP and stored at 20ºC for 14 days. Whereas 14-103Y-T sample treated with ethylene showed a increase in AAT gene expression after harvest at 14 days. 68G and 103Y sample treated with ethylene showed an increase in LOX gene expression after the 14 day storage period at 20ºC. LOX2.3 gene expression showed and increase in GS control treated with 1-MCP, and in sample 103Y treated with ethylene after the the 14 day storage period at 20ºC (Tables 2 and 3).

Table 3. Lipoxygenases genes expression[a]

	LOX		LOX1		LOX2.3	
Sample	**1 day**	**14 day**	**1 day**	**14 day**	**1 day**	**14 day**
GS	1.2068a	14.2710ab	1.2963a	30.31a	1.5584a	2.0110b
GS + 1MCP		0.0670b		0.360b		7.7630b
68G	0.2103b	10.2460ab	0.7086a	22.930ab	1.4068a	1.6050b
68G + C_2H_4		21.264a		17.790ab		NA
103Y	0.0509b	9.9350ab	0.2719a	6.8700ab	1.0979a	7.6480b
103Y + C_2H_4		16.7750ab		1.360ab		20.894a

[a]Values represent fold changes compared to the mean of GSUN-1day samples. Different letters a or b indicate differences among sample at significance level p=0.05 within 1 day or 14 day. LOX = lipoxygenase.

Other significant genes related with sugar/acid balance are the genes associated with sorbitol metabolism. NAD-dependent sorbitol dehydrogenase 5 (NAD-SDH5), NAD-SDH6, sorbitol-6-phosphate dehydrogenase (S6PDH), sorbitol dehydrogenase 4 (SDH4) and SDH4 showed a

decrease in gene expression for the GS control treated with 1MCP after 14 day storage period at 20ºC when compared with GS control stored for 1 day (Table 4).

Table 4	NAD-SDH5		S6PDH		SDH4		SDH5	
Sample	**1 day**	**14 day**	**1 day**	**14 day**	**1 day**	**14 day**	**1 day**	**14 day**
GS	1.1587b	0.0706a	1.1149a	0.1881a	1.010b	0.28730ab	1.0080b	0.814b
GS + 1MCP		0.0309a		0.2221a		0.5901a		0.928b
68G	0.3948b	0.0032a	0.9062ab	0.5178a	1.118b	0.0333b	0.8791b	0.411b
68G + C_2H_4		0.0805a		0.2815a		0.3212ab		3.804a
103Y	2.1382a	NA	0.2881b	0.2158a	4.961a	0.0063b	1.6840a	0.510b
103Y + C_2H_4		0.0371a		0.2820a		0.0868ab		1.425b

[a]Values represent fold changes compared to the mean of GSUN-1day samples. Different letters a or b indicate differences among sample at significance level p=0.05 within 1 day or 14 day. NAD-SDH = NAD-dependent sorbitol dehydrogenase, S6PDH = sorbitol-6-phosphate dehydrogenase, SDH = sorbitol dehydrogenase.

6. Sugar-acid balance is differentially regulated in cortical but not in peel.

Flavor is one of the important non visual quality parameters that influences consumer acceptance. Flavor composition has been defined as a complex attribute of quality in which the mix of sugars, acids and volatiles play a primary role. The sugars sucrose, glucose and fructose are responsible for sweetness, with some contribution of sorbitol in apple fruit (Baldwin 2002). The behavior of individual sugars, malic and citric acid was analyzed in the various treatments in response to ethylene regulation in cortical and peel tissues using a high resolution GC/MS. The response of the sugars and acids in apple cortical and peel was different. Fig 5 is a canonical analysis of target sugar and acid compounds in both apple tissues. In the cortex ethylene is not correlated with citric acid, fructose, glucose and sucrose, but negatively correlated with malic acid and sorbitol. Fructose, glucose and sucrose are closely correlated with each other in a positive direction (Fig 5). In the peel, ethylene is slightly correlated with malic acid, fructose, glucose and sorbitol and sucrose are negatively correlated. Fructose, glucose and sucrose are closely related with each other in a positive direction and malic acid is strongly positively correlated with fructose (Fig 5).

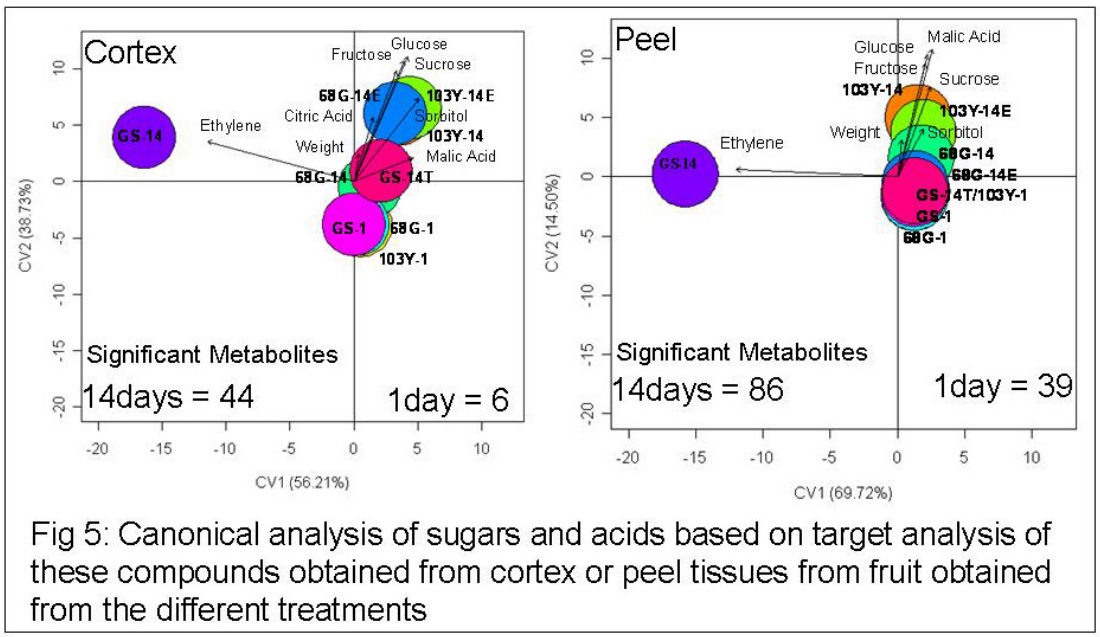

Fig 5: Canonical analysis of sugars and acids based on target analysis of these compounds obtained from cortex or peel tissues from fruit obtained from the different treatments

7. Metabolite profiles showed that most of the statistically significant metabolites identified in cortical and peel are precursors of aroma-volatile compounds.

The average number of metabolites detected in cortical tissue after 1 day and 14 days of storage at 20ºC were 155 and 136, respectively. The average number of metabolites detected in peel tissue after 1 day and 14 days of storage at 20ºC were 132 and 125, respectively. In both cases the metabolites observed included sucrose, glucose, fructose, sorbitol, and malic acid. After the statistical analysis we found that for cortical tissue 6 and 44 metabolites for 1 day and 14 days, respectively, are statistically different at the level of significance of $p<0.05$ (Fig 5). For peel tissue 39 and 86 metabolites for 1 and 14 days, respectively, are statistically different at the level of significance of $p<0.05$ (Fig 5). In cortical and peel tissue many of the significant metabolites identified are precursors of aroma volatile-related compounds, among these we found alcohols (arabitol and erythritol), fatty acids (palmitic acid, oleic acid and linoleic acid) and amino acids (valine, alanine, serine, glutamic acid and isoleucine). These metabolites can be used to evaluate differences among genotypes and treatments. Metabolite profiles can also be used to further define pathways that are operative in different tissues among treatments and can be used to validate the pathway data obtained from the microarray analysis.

8. Ethylene is a modulator of most of the aroma related volatiles, especially the alcohol acyltransferase (AAT) enzyme activity.

Fruit aroma is a complex trait, particularly in terms of the number of different biosynthetic pathways involved, accumulation of the final metabolites and their regulation. The aroma volatiles-related enzymes involved are alcohol acyltransferase (AAT), alcohol dehydrogenase (ADH) and lipoxygenase (LOX). And the main precursors of aroma volatiles are amino acids and fatty acids as mention above.

177 volatiles compounds were detected in cortical tissue by GC/MS using the method described by Defilippi et al (2004). 25 volatiles compounds that were present in 100% of the samples were statistically analyzed. The statistic results showed that the volatile profile of cortical tissue was dominated for aldehydes, alcohols and ketones at harvest. Alcohols and aldehydes showed an increase after 14 days of storage at 20ºC only for the control sample. Under ethylene suppression conditions, GS treated with 1-MCP, 68G and 103Y lines showed a major reduction of all groups of aroma volatiles. No recovery of volatiles compounds were observed when transgenic lines were exposed to ethylene. The reduction in aroma volatiles in 'Greensleves' cortical tissue treated with 1-MCP, an inhibitor of ethylene action, support the findings of early studies that ethylene is a modulator of volatiles responsible of aroma production (Defilippi et al 2004, 2006.; Lurie 2002). .

AAT the main enzyme in ester biosynthesis, showed and increase of enzyme activity of 40-60% between harvest and the end of storage in non transformed line. In transgenic lines lower levels of enzyme activity at harvest relative to the non-transformed lines were measured, and no significant changes in activity were observed until the end of the storage period. These results suggest that ethylene plays an important role in modulating AAT enzyme activity in GS apples. This observation is also supported by the use of 1-MCP, in which we observed an inhibition of enzyme activity relative to the non treated fruit. The AAT activity levels in peel was higher than in cortical for nontransformed and transformed lines at harvest and after 14 days.

ADH the enzyme responsible for the interconversion between aldehydes and alcohols, initially increase and then gradually declined in peel tissue or remain steady in all lines during the holding

period. Enzyme activity did not show any significant changes between the measurements done at harvest an after 14 days, under any conditions.

Lipoxygenase pathway plays an important role in the generation of aroma volatile-related during ripening. LOX enzyme activity levels showed a minor increase during the holding period, especially peel tissue obtained from GS line, application of ethylene only caused a minor increase in LOX activity in fruit from the 68G line.

Significance to the industry and potential economic benefits

Understanding the metabolic network and biosynthetic pathways active in apple fruit could facilitate extending postharvest life of flavor, aroma, and texture to match appearance. This, in turn, would promote consumption of fresh apple fruits and reduce losses during postharvest storage, stimulating demand while reducing costs to handlers and consumers. Identifying genes that determine and regulate fruit quality phenotypes can provide a new set of tools to improve management, breeding, and selection of existing and new cultivars.

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Executive Summary

Our overall goal of this project was to define the role of ethylene in the functional regulation of apple fruit quality. This was accomplished by using apple fruit obtained from 2 transgenic lines modified in their capacity to synthesize endogenous ethylene or wild type fruit modified in their ethylene response via the application of 1-methylcyclopropene (1-MCP). The study design used these unique resources to create 9 phenotypically distinct treatments. Validation of the study design demonstrated the ethylene is positively correlated with color, starch and weight and negatively correlated with firmness and acidity. These differences in phenotypes were matched with changes in gene expression patterns obtained by the successful deployment of microarray resources and analysis tools to dissect the transcriptome of apple fruit which were used to identify ethylene regulated genes in apple fruit tissues. Differential gene expression patterns obtained by microarrays identified 3029 genes significantly regulated in apple fruit with 658 in cortical, 2169 in peel tissue and 381 in both tissues respectively that are likely regulated by ethylene. These genes were functionally categorized to define their metabolic role in apple fruit. Expression of genes related with ethylene biosynthesis, aroma volatiles-related biosynthesis and sorbitol biosynthesis were down regulated in the control fruit treated with 1-MCP. Genes involved in sugar-acid balance were differentially regulated in cortical but not in peel. Metabolite profiles of the tissues validated the gene expression analysis and showed that most of the statistically significant metabolites identified in cortical and peel are precursors of aroma-volatile compounds. Thus ethylene is an important modulator of most of the aroma related volatiles, and a key gene that regulates this activity encodes the enzyme alcohol acyltransferase (AAT).