FATTY ACID DERIVED ESTER RELATED GENE EXPRESSION, ACTIVITIES, AND VOLATILES FOR ON-TREE RIPENING AND STORAGE OF APPLES

By

CHRISTOPHER DANIEL STARR

A thesis submitted in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE IN MOLECULAR PLANT SCIENCES

WASHINGTON STATE UNIVERSITY
Program in Molecular Plant Sciences

DECEMBER 2009

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of CHRISTOPHER DANIEL STARR find it satisfactory and recommend that it be accepted

John K. Fellman, PhD, Chair

N. Richard Knowles, PhD

B. Marcus Lange, PhD

ACKNOWLEDGEMENTS

I would like to express gratitude to the following persons who in some way helped me to complete this work. I would like to thank my parents, Robert and Carol Starr, for their unwavering love and constant encouragement throughout a sometimes difficult experience. I would like to thank my advisor, Dr. John Fellman, for his patience and helpful suggestions, and for prodding me when I needed it. I would like to thank Dr. Michael Finnegan, who allowed me to work for him under sometimes difficult circumstances and encouraged me to try new things. I would like to thank Dr. Cameron Peace, who provided financial support in exchange for work directly related to this thesis. I would like to thank Scott Mattinson, who performed the volatile analyses with great skill and who constantly provided advice throughout every step of my project. I would like to thank Michelle Kruger for her diligent, skillful, and cheerful assistance with collecting samples and performing apple quality analyses, and for her constantly positive attitude. I would like to thank Dr. Suresh Iyer, who provided advice on the gene expression experiments and helped me interpret the results of them. I would like to thank Dr. Richard Knowles for allowing me to use equipment in his lab and for valuable advice. I would like to thank Dr. Mark Lange, who provided sound advice with abundant patience and good humor. Finally, I would like to thank my friends Dan Cuthbertson, Jeremy Dahmen, and Max Feldman, who made graduate school an interesting experience and who provided advice and encouragement when I needed it (and sometimes when I didn't).

FATTY ACID DERIVED ESTER RELATED GENE EXPRESSION, ACTIVITIES, AND VOLATILES FOR ON-TREE RIPENING AND STORAGE OF APPLES

Abstract

By Christopher Daniel Starr, M.S.
Washington State University
December 2009

Chair: John K. Fellman

Although apple fruit ripening has been the focus of many studies, many questions about the regulation of the ripening process remain unresolved. Most studies do not focus on freshly harvested fruit during the transition to autocatalytic ethylene production, which marks the beginning of many ripening-related processes, including the increased production of volatiles. To provide insight into the regulation of apple fruit volatile production during ripening, apple samples were taken off the tree every 3-4 days during and just after the transition to autocatalytic ethylene production. The lipoxygenase (LOX) and alcohol acyltransferase (AAT) enzymes, which are important to the production of straight-chain ester volatiles and the focus of much attention in apple flavor studies, were examined at both the enzyme activity and gene expression levels. Aldehydes and esters, which are products of LOX and AAT, respectively, and alcohols, which are substrates for AAT, were also examined in both headspace emissions and juice content. Headspace emissions were found to be a more reliable method of measuring physiological changes. While the LOX isoform examined in this work increased in early ripening, LOX activity decreased, along with headspace aldehydes. AAT gene expression and activity increased in late ripening, after the increase in headspace esters, indicating a role for

AAT activity in regulating later ripening. Acetyl-coA carboxylase (ACCase) gene expression was also measured to try to determine any involvement in the regulation of flavor volatiles. Results showed changes in gene expression during ripening. Attempts were made to measure ACCase activity, but none was detected.

Apple fruit were placed in 1.5% O_2 controlled atmosphere (CA) storage, and the measurements taken in freshly harvested fruit were also taken just after removal from storage and during ripening afterwards. Esters and alcohols increased during CA storage, while aldehydes decreased. During ripening after CA storage, AAT activity does not reach the level it reaches in ripe fruit, while LOX activity does. Esters, including butyl acetate and hexyl acetate, make a strong recovery after CA storage, indicating a strong recovery of the straight chain ester synthesis pathway.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

IEC	Internal Ethylene Content
CA	Controlled Atmosphere
AAT	Alcohol Acyltransferase
LOX	Lipoxygenase
HPL	Hydroperoxide Lyase
1-MCP	1-Methylcyclopropene
ACCase	Acetyl-CoA Carboxylase
DAFB	Days After Full Bloom
SPME	Solid Phase Microextraction
GC	Gas Chromatograph
PVPP	Polyvinylpolypyrrolidone
DEPC	Diethyl Pyrocarbonate
2-BE	2-butoxyethanol
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
SE	Standard Error
Ppm	parts per million
BLAST	Basic Local Alignment Search Tool
DPM	Disintegrations Per Minute

CHAPTER 1

INTRODUCTION

Apple ripening and storage

Apple ripening is a complex process involving physical and biochemical changes within the fruit. These changes include fruit softening, loss of green color, and production of flavor molecules characteristic to each variety (Dixon and Hewett 2000). As apple is a climacteric fruit (Kidd and West 1945), it is also characterized by an increase in respiration and ethylene production during ripening (Lelièvre et al 1997). Specifically, there is a rapid increase in ethylene production and respiration, followed by a decline late in ripening (Dixon and Hewett 2000).

Ethylene is the principal hormone involved in climacteric fruit ripening (Burg and Burg 1965). Ethylene production in climacteric fruits, including apple, can be divided into two "systems" – system I and system II. System I represents the ethylene produced at very low levels before the onset of ripening. System II is responsible for the dramatic increase in ethylene production during ripening of climacteric fruit (Lelièvre et al 1997). Ethylene has been shown to act as a modulator of ripening in apples, constantly controlling the ripening process. Early ripening events, like conversion of starch into sugar, are not as dependent on ethylene but are sensitive to very low ethylene concentrations. In contrast, later ripening events, like volatile production, are highly dependent on ethylene and require higher ethylene concentrations to take place (Johnston et al 2009).

Because of the importance of ethylene in apple ripening, the best way to track the maturity of apples is to measure ethylene. There are two general approaches to measuring ethylene: one can measure ethylene evolved from the fruit, or one can measure the ethylene

taken from the fruit core, which is called internal ethylene content (IEC). IEC is a well-established and reliable way of measuring physiological maturity in many apple cultivars (Chu 1984). The pattern of IEC during ripening has been studied in detail in 'Red Delicious' apples. These results suggest that an increase in IEC indicates the onset of climacteric ripening, while evolved ethylene levels rise later. Specifically, 'Red Delicious' fruit enter climacteric ripening when IEC rises above 1 μl/l (Fellman et al 2003).

Although IEC is the best way of measuring physiological maturity, it is not a good indicator of harvest maturity for apples. Optimum harvest maturity is when picked apples can be stored with a minimum of disorders and maximum dessert quality (Haller and Smith 1950). Less mature fruit develop poor color and flavor and are susceptible to some physiological disorders, while fruit harvested too late are too soft and susceptible to other physiological disorders. Because optimum harvest maturity can have varying relationships with ethylene production, and because the timing of climacteric ethylene production depends on cultivar, strain, orchard, growing conditions, and nutrition, IEC does not always work as a harvest indicator. Therefore, in practice harvest maturity is determined through the collection of a wide variety of maturity indices and the judgment of industry personnel (Watkins et al 2004). Other maturity indices with similar or better reliability, across-the-board consistency, and predictive power as IEC would be welcome to the industry.

Controlled atmosphere (CA) storage is used extensively by apple growers to extend the life of fruit so that apples can be sold year-round. A CA storage facility is a room where carbon dioxide levels, oxygen levels, and temperature are regulated. CA storage mainly serves to prevent softening and delay storage disorders (Smock 1979). Oxygen and carbon dioxide levels that can be effectively used for a particular variety run within a narrow range. Within this range,

CA conditions extend fruit life by lowering respiration and ethylene production rates. However, anaerobic respiration, lower pH, and lowered ATP levels occur in CA stored fruit, leading to changes in enzyme activities and a detrimental production of acetaldehyde, ethanol, ethyl acetate, and/or lactate (Kader 2004).

Apple volatiles and their synthesis

Among all plant organs, flowers and fruits produce the largest amounts of volatiles (Dudareva et al 2006). In apples, over 300 compounds are produced (Cunningham et al 1986). Apple flavor consists of taste and odor; odor, which determines the character of apple fruits, comes from volatiles released when one bites into an apple (Yahia 1994, Fellman et al. 2000). About 20-40 compounds are responsible for apple aroma (Cunningham et al 1986). Volatile esters from apple, which produce a characteristic sweet and fruity "apple peel" smell, are responsible for the majority of the aroma profile in apples (Cunningham et al 1986, Yahia 1994, Fellman et al. 2000). Specifically, butyl acetate, 2-methylbutyl acetate, and hexyl acetate are primary contributors to apple-like aroma in most cultivars (Fellman et al 2000). However, all volatiles are important to produce the complete aromatic character of the apple (Paillard 1990). Aldehydes, which are mainly produced when fruit tissue is crushed during chewing (Flath et al 1967, Paillard 1986), can produce green, aromatic, and/or fruity notes (Panasiuk et al 1980, Fellman et al 2000). Specifically, hexanal and 2-hexenal were identified early as important constituents of apple-like aromas when fruit is crushed (Flath et al. 1967). In a study of the sensory quality of commercial apple essences, cis-3-hexenal and trans-2-hexenal contributed strongly to aroma intensity (Dürr and Schobinger 1981). Studies on commercial apple odor have correlated bad essences with high levels of alcohols, especially ethanol and hexanol, and good

essences with high levels of some aldehydes and esters, including hexanal, 2-hexenal, butyl acetate, and hexyl acetate (Petró-Turza et al 1986, Dürr and Schobinger 1981)

Volatile esters are synthesized in apple through at least two different pathways: one that originates from amino acids and produces branched chain esters and one that originates from fatty acids and produces straight chain esters. Little work has been done on the synthesis of branched chain esters. Strong evidence from feeding of deuterium-labeled substrates established that 2-methylbutyl and 2-methylbutanoate esters are produced from isoleucine (Rowan et al 1996). Analogies to banana fruit also indicate that valine and leucine are precursors for other apple volatiles (Paillard 1990, Fellman et al 2000).

By contrast, the straight chain ester biosynthesis pathway, which will be the main focus of the present work, has been well established. Straight chain esters, which are produced from the metabolism of fatty acids, are mostly produced in peel tissue, likely because peel tissue is rich in fatty acids (Yahia 1994, Fellman et al 2000, Guadagni et al 1971). A large amount of early evidence from feeding experiments provided an initial view of the synthesis pathway. Feeding of alcohols to apple fruit produced increases in esters, showing that esters are formed from alcohols (Knee and Hatfield 1981, Berger and Drawert 1984, Bartley et al 1985). To make an ester an alcohol combines with an acyl-CoA; this acyl-CoA can be produced from a carboxylic acid (De Pooter et al 1983, De Pooter et al 1987) or produced through other metabolic processes (Fellman et al 2000). The enzyme that catalyzes this step is alcohol acyltransferase (AAT) (Fellman et al 2000). Esterases, which can hydrolyze esters back to their components, have also been shown to exist in apple (Knee and Hatfield 1981, Goodenough and Entwistle 1982). Feeding of alcohols also produced aldehydes (Knee and Hatfield 1981, Berger and Drawert 1984), a step catalyzed by alcohol dehydrogenase (ADH) (Bartley and Hindley 1980).

Feeding of aldehydes produced alcohols through the action of ADH, which are incorporated into esters (De Pooter et al 1983). Besides forming an acyl-CoA, a given carboxylic acid can form an alcohol, likely through the reduction to an aldehyde (De Pooter et al 1983). β-oxidation, which takes two carbons away from a compound, acted on added alcohols, aldehydes, and carboxylic acids (De Pooter et al 1983, Bartley et al 1985, De Pooter et al 1987). Feeding of unlabeled precursors is not always an accurate way of determining synthesis pathways, so the results mentioned above were tested by feeding apples with deuterium-labeled precursors. Figure 1 shows the synthesis pathway discovered by this experiment, which is similar to the one implied by feeding of unlabeled precursors (Rowan et al 1999).

Precursors for straight chain volatile synthesis come from two sources. Saturated carboxylic acids mostly come from β-oxidation of saturated and monounsaturated fatty acids (Bartley et al 1985, Paillard 1979, Rowan et al 1999, Fellman et al 2000). Unsaturated aldehydes, along with some hexanal and hexanoic acid, come from linoleic acid (18:2, $\Delta^{9,12}$) and linolenic acid (18:3, $\Delta^{9,12,15}$) (Rowan et al 1999, De Pooter and Schamp 1989). Lipoxygenase (LOX) acts on linoleic or linolenic acid to form 13- or 9- hydroperoxides. In the creation of hexanal and hexenal, first linoleic acid is oxidized to 13(S)-hydroperoxy-cis-9,trans-11-octadecadienoic acid, and linolenic acid is oxidized to 13(S)-hydroperoxy-cis-9-trans-11-cis-15-octadecatrienoic acid. These fatty acid hydroperoxides are then cleaved by hydroperoxide lyase (HPL) between the hydroperoxide-bearing carbon and the double bond to create an aldehyde and an oxo-acid. Specifically, 13(S) specific HPL creates hexanal from the hydroperoxide of linoleic acid and cis-3-hexenal from the hydroperoxide of linolenic acid. In a similar fashion, nonenals can be created from 9-hydroperoxides (Gardner 1995).

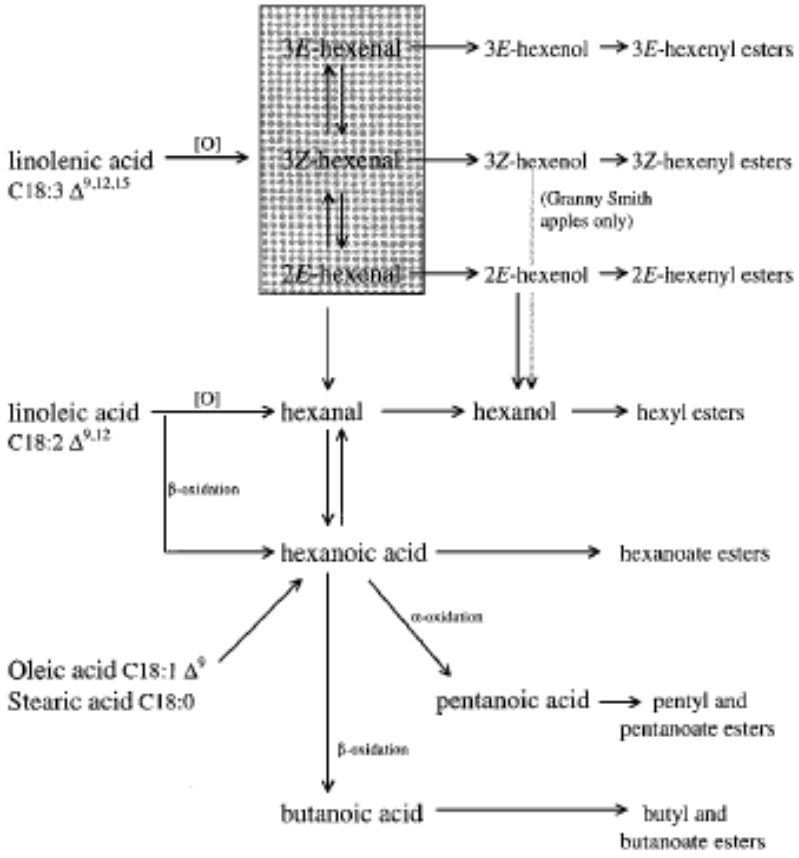


Fig. 1. Synthesis of straight-chain volatile esters. Taken from Rowan et al 1999.

Volatile levels throughout ripening

When flowers are ready for pollination and fruits are fully ripe, they produce the highest levels of volatiles (Dudareva et al 2006). Apple fruit is no exception to this rule. Apples produce low concentrations of aroma volatiles when they are immature. During climacteric ripening, levels of volatiles rise until the fruit is fully ripe. After this, levels of volatiles decline (Yahia 1994, Dixon and Hewett 2000). The specific pattern of volatile emissions also varies during ripening, forming the basis of a potential maturity indicator that has been measured with an "electronic nose" and used with some success to measure apple maturity (Young et al 1999).

Esters usually are sporadically detected before the onset of climacteric ripening, and then increase during climacteric ripening with increasing ethylene production. This general pattern held true in volatile emission from whole fruit of 'Red Delicious' apples (Mattheis et al 1991), in volatile content of juice of 'Red Delicious' apples (Fellman et al 2003), in volatile emissions from 'Pink Lady' apples (Villatoro et al 2008), in volatile emissions from 'Fuji' apples (Echeverrìa et al 2004), and in volatile emissions from 'Golden Orange' apples (Rizzolo et al 2006). Two major esters that will be analyzed in this work are hexyl acetate and butyl acetate. They followed the general pattern for esters of a major increase during climacteric in all of the experiments listed above. Butyl acetate content in juice increased with respiration (Paillard 1986). However, in experiments with 'McIntosh' and 'Cortland' apples, hexyl acetate content was high in immature apples and decreased during ripening (Yahia et al 1990).

Alcohols show cultivar-dependent patterns of changes throughout ripening. Two major alcohols that will be analyzed in these experiments are 1-hexanol and 1-butanol. A 'Red Delicious' volatile emissions study showed individual alcohols to have different patterns; 1-butanol decreased before climacteric, then increased with climacteric, while 1-hexanol stayed constant throughout ripening (Mattheis et al 1991). In volatile content of 'Red Delicious' juice 1-butanol also increased over climacteric (Fellman et al 2003). In another experiment measuring volatile content butanol rose with respiration (Paillard 1986). In volatile emissions from 'Pink Lady' 1-hexanol and 1-butanol were only detectable in late stages of ripening (Villatoro et al 2008). Volatile emissions from 'Fuji' apples showed that 1-hexanol and 1-butanol increased during ripening (Echeverrìa et al 2004).

Aldehydes show varying patterns depending on the individual aldehyde and whether volatile content in juice or emissions from whole fruit are being measured. In contrast with

alcohols or esters, aldehyde levels are mainly determined by measuring aldehyde content in juice. In one experiment measuring volatile content, hexanal rose at the beginning and 2-hexenal showed a distinct peak early in ripening (Paillard 1986). However, in 'Red Delicious' hexanal and 2-hexenal increased in late ripening (Fellman et al 2003). In volatile content of 'Greensleeves' hexanal decreased slightly and 2-hexenal increased (Defillippi et al 2005a). For volatile contents in juice of 'Mcintosh' and 'Cortland' apples, 2-hexenal continuously increased during ripening and hexanal increased with climacteric ethylene production (Yahia et al 1990). In volatile emissions from 'Red Delicious', aldehydes increased until a couple of weeks before the start of climacteric ripening and declined slowly thereafter (Mattheis et al 1991). However, in the headspace of 'Golden Orange' apples 2-hexenal was associated with unripe fruit (Rizzolo et al 2006). Aldehyde content in juice and whole fruit does not depend on ethylene production (Defilippi et al 2004, Defilippi et al 2005a, Lurie et al 2002).

Volatiles after controlled atmosphere storage

During CA storage, a slow decline in the ability of apples to produce volatiles takes place. However, just after removal from CA storage, temperature-induced 'outgassing' takes place, which causes an initial rise in volatiles (Fellman et al 2003). Also, many volatiles have different trends during ripening after CA storage compared to ripening on the tree (Yahia et al 1990b, Patterson et al 1974). CA stored apples are generally characterized by increased production of acetaldehyde, ethanol, and ethyl acetate (Kader 2004)

Straight chain esters, including the major esters butyl acetate and hexyl acetate, were reduced by CA storage in the headspace of 'Golden Delicious' (Harb et al 2008), 'Fuji' (Lara et al 2006), and 'Red Delicious' (Mattheis et al 1995) apples. All these measurements were taken

after apples were acclimated to air. CA storage reduced butyl acetate emission from 'Gala' apples (Lara et al 2007). During ripening after removal from CA, hexyl acetate and butyl acetate increased in the headspace and juice of 'Red Delicious' apples (Mattheis et al 1995, Fellman et al 2003), and butyl acetate emission increased during ripening after CA storage in 'Gala' (Lara et al 2007). In contrast, hexyl acetate decreased in measurements of volatile content of 'Macintosh' and 'Cortland' during ripening after CA storage (Yahia et al 1990b).

The important straight chain alcohols butanol and hexanol were reduced by CA storage in emissions from 'Golden Delicious' (Harb et al 2008), 'Fuji' (Lara et al 2006), and 'Red Delicious' (Mattheis et al 1995) apples. Butanol emissions were reduced by CA storage in 'Gala' apples (Lara et al 2007). Straight chain alcohols usually increased along with their corresponding esters during ripening after CA storage (Lara et al 2007, Lara et al 2006, Fellman et al 2003). However, emissions patterns of 'Red Delicious' apples showed an increase in both butanol and its corresponding ester butyl acetate, while the pattern for hexanol depended on harvest date and storage conditions (Mattheis et al 1995).

The influence of CA storage on aldehyde production remains relatively obscure. After CA storage, hexanal and 2-hexenal developed in lower amounts in the volatile content of juice (Yahia et al 1990b). Also for volatile content, hexanal increased during ripening after CA storage and 2-hexenal spiked temporarily before decreasing (Fellman et al 2003).

Regulation of straight chain volatile ester synthesis

Many authors have used results from precursor feeding studies or analysis of volatiles and/or enzyme activities during ripening to speculate about regulation of straight chain volatile ester synthesis. The generally accepted conclusion is that regulation of the final enzyme,

AAT (alcohol acyltransferase), is not as important as availability of precursors in determining the level of ester production. A timeline for ester biosynthesis was proposed by Paillard (1986), who concluded from tracking of volatile synthesis and enzyme activity over time that aldehyde production takes place first. When climacteric was reached, aldehydes decreased and alcohol increased, followed by ester biosynthesis. Feeding of alcohols to fruit showed that the concentrations of esters are governed by alcohol concentration (Knee and Hatfield 1981). Feeding of carboxylic acids show that concentrations of these precursors can also limit concentrations of esters (De Pooter et al 1987). One experiment used principal component analysis on enzyme activity and volatile concentrations, and a regression model showed that 93% of total variability in production of volatile esters could be explained by availability of precursors (Villatoro et al 2008).

Ethylene is important for regulating the synthesis of many volatiles, continuously controlling their production throughout ripening (Mattheis et al 2005, Defilippi et al 2004, Fan et al 1998). Specifically, the ethylene signaling pathway is involved in controlling volatile synthesis (Defilippi et al 2005b, Ferenczi et al 2006). Ester production is highly dependent on ethylene, as shown by experiments on transgenic apples and 1-MCP (1-Methylcyclopropene) treated fruit (Fan et al 1998, Defilippi et al 2004, Defilippi et al 2005a, Mattheis et al 2005). Straight chain esters are particularly affected by ethylene action suppression with 1-MCP (Mattheis et al 2005). Alcohol production also seems to be dependent on ethylene (Defilippi et al 2004, Mattheis et al 2005). Aldehyde content and emissions do not depend on ethylene production (Defilippi et al 2004, Defilippi et al 2005a, Lurie et al 2002). A significant change in the ratio of hexanal/(2E)-hexenal in transgenic fruit suppressed for ethylene production takes place, suggesting a possible regulation of either specific isoforms of lipoxygenase, or the regulation of HPL, by ethylene

(Dandekar et al 2004, Dandekar et al 2005a). A comprehensive overview of gene expression during ripening was completed, and showed that not all genes are regulated by ethylene, but the genes that represent the first and last steps of many pathways are regulated by ethylene (Schaffer et al 2007).

Regulation of straight chain ester volatile development after CA storage principally depends on biosynthetic regulation of ester precursors, specifically, production of alcohols seems to be an important regulatory step. An early precursor study found that CA stored fruit built up aldehydes when they were fed carboxylic acids, showing that formation of alcohols was inhibited (De Pooter et al 1987). Mathematical modeling after measuring many variables showed the importance of alcohols in limiting production of esters in CA stored 'Gala' and 'Fuji' apples (Lara et al 2006, Lara et al 2007). Strikingly, in a regression model the correlation coefficients between emission of butyl and hexyl acetates and their alcohol precursors 1-butanol and 1-hexanol were 0.93 and 0.85, respectively. It has been suggested that high levels of acetaldehyde may out-compete butanal and hexanal for reduction, leading to a shortage of alcohols used in ester synthesis (Rudell et al 2002). A decrease in LOX activity, leading to a shortage of aldehydes, has also been implicated in the shortage of precursors for ester synthesis (Lara et al 2006, Lara et al 2007). After ultra low oxygen storage, LOX activity in the flesh but not the skin was highly correlated with ester regeneration (Altisent et al 2009). Generally, two modeling studies showed that ester synthesis could be explained by precursor availability 71% (Lara et al 2007) and 76% (Villatoro et al 2008) of the time.

Fatty acid precursors to straight chain volatile esters

There are two contrasting views about the origins of the fatty acid precursors to straight chain ester volatiles. One view says that the most important source of linoleic and linolenic acids used by LOX is breakdown of chloroplasts. During ripening of apples, it is clear that chloroplasts break down (Rhodes and Wooltorton 1967). Further evidence for this viewpoint comes from studies that reveal decreases in free fatty acids, and specifically linolenic acid, during ripening, which is evidence that fatty acids from the breakdown of chloroplasts are being used up to make straight chain esters (Meigh and Hulme 1965, Galliard 1968). More recently, another view has developed which proposes that synthesis of fatty acids is the most important source of fatty acids used for straight chain ester synthesis. Evidence for this view comes from experiments which show a constant increase in fatty acid levels with climacteric ripening, particularly, an increase in levels of linoleic acid seems to take place (Song and Bangerth 1996, Song and Bangerth 2003, Defilippi et al 2005a).

Acetyl-coA carboxylase

One of the enzymes studied in this work is acetyl-CoA carboxylase (ACCase). This enzyme is one of two involved in synthesis of fatty acids, along with fatty acid synthase. It catalyzes the first committed step, carboxylation of acetyl-CoA to malonyl-CoA. It is a multisubunit protein with a biotin carboxyl carrier protein, biotin carboxylase and carboxyltransferase. Two forms of ACCase exist; the one in the plastids is the one that produces the fatty acids that are part of volatile ester synthesis (Sasaki and Nagano 2004). Being the first committed step, this enzyme is the most likely target for regulation, although all enzymes in fatty acid synthesis could also be coordinately regulated (Ohlrogge and Jaworski 1997). Evidence

from EST abundance suggests coordinated gene expression of ACCase subunits in Arabidopsis (Mekhedev et al 2000). Protein activity has not been measured in apple. However, ACCase protein has been detected in strawberry fruit (Bianco et al 2009), and measurements of gene expression and ribosome loading indicate that ACCase is actively being transcribed and translated in ripening tomatoes (Kahlau and Bock 2008). However, one large-scale gene expression study in apple did look at a few potential isoforms of ACCase and did not find regulation by ethylene (Schaffer et al 2007). In developing seeds, both increases and decreases in ACCase gene expression have been noted, and at least one ACCase gene was able to be regulated by ethylene (Nakkaew et al 2008, Zuther et al 2004, Figueroa-Balderas et al 2006).

Lipoxygenase

Because it plays an important part in straight chain volatile synthesis and has been the focus of previous work in apple, one of the enzymes studied in this work is LOX. The role of lipoxygenase in straight chain volatile synthesis was discussed earlier. Generally, lipoxygenases are fatty acid dioxygenases, which can catalyze the oxygenation of polyenoic fatty acids at the C9 or C13 positions (Dudareva et al 2006). Apple lipoxygenase activity is mainly on linoleic acid, and acts on the C13 position (Kim and Grosch 1979). Some large-scale gene expression studies including potential LOX genes have been done in apple. In one study, a lipoxygenase from apples was upregulated from unripe fruit to fruit ready for harvest, then downregulated (Goulao and Oliveira 2007). In another study, two genes were found to be induced by ethylene (Schaffer et al 2007). One of these genes was examined in another study, and was found to increase during ripening (Park et al 2006). LOX enzyme activity in apple has been examined during ripening in multiple studies. Two early studies showed generally that LOX activity

increases during ripening (Meigh and Hulme 1965, Meigh and Hulme 1967). A later study showed that LOX specific activity in peel decreases dramatically around commercial harvest, then slowly increases. However, LOX activity in the pulp follows an opposing trend, which could negate the changes in peel activity (Echeverrìa et al 2004). LOX activity seems to be independent of ethylene (Defilippi et al 2005a). In one study done on apples taken out of long-term CA storage, LOX activity declined from where it was before CA storage, although samples were allowed to acclimate to air (Lara et al 2007).

Alcohol acyltransferase

AAT is responsible for the final step in ester synthesis and is therefore of great interest in studies of volatiles. Generally, it is part of the BAHD acyltransferase family, in clade III. The members of this clade are found in flowers and fruits, and usually use acetyl CoA and a range of alcohol substrates to produce volatile esters (d'Auria 2006). Apple AAT has been proven to mainly use acetyl CoA, and accepts a range of alcohol substrates (Holland et al 2005). Two AAT genes, MpAAT1 and MdAAT2, have been officially named, although other putative AAT genes have been studied in apples. Both MpAAT1 and MdAAT2 increased during ripening (Zhu et al 2008, Li et al 2006). Also, both genes are induced by ethylene (Defilippi et al 2005b, Schaffer et al 2007, Li et al 2006). Inconsistent results have been reported on studies of AAT enzyme activity during ripening, it has been reported to both increase (Fellman et al 2000, Li et al 2006) and decrease (Echeverrìa et al 2004). AAT activity seems to be modulated by ethylene. Transgenic apples suppressed for ethylene had lower AAT activity, which recovered when high levels of ethylene were applied but not when lower levels were applied (Defilippi et al 2005b). AAT activity has been reported to be strongly inhibited by long-term CA storage (Fellman et al

2000, Lara et al 2007). AAT activity can recover after CA storage in some cases (Villatoro et al 2008).

Hypothesis and Objectives

Evidence supporting or contradicting the following hypotheses was collected in this study:

1. Straight chain ester production during on-tree ripening of apple is regulated by biosynthesis of precursors, and steps before alcohol synthesis play the primary role.
2. Straight chain ester production during ripening after CA storage of apple is regulated by biosynthesis of precursors, and alcohol synthesis plays the primary role.
3. Lipoxygenase protein levels could be used as a tool to help determine harvest maturity for apple.
4. Changes in acetyl-CoA carboxylase levels are involved in regulating biosynthesis of straight-chain esters, indicating that fatty acid synthesis is important in producing straight-chain esters.
5. Acetyl-CoA carboxylase protein levels could be used as a tool to help determine harvest maturity for apple.

One objective of this study was to examine regulation of the straight-chain ester synthesis pathway during ripening in apple. To achieve this, gene expression, activity, and associated volatiles for important steps in straight-chain ester synthesis were followed over the period just before and after the transition to autocatalytic ethylene synthesis for

apples harvested fresh off the tree. The steps catalyzed by lipoxygenase and alcohol acyltransferase were chosen for examination. Another objective was to examine the effects of CA storage on the straight-chain ester synthesis pathway. To achieve this, the genes, activities, and volatiles measured during on-tree ripening were examined during and after CA storage. Another objective of this study was to test for the first time if acetyl-CoA carboxylase, the first step in fatty acid synthesis, is regulated during apple ripening.

CHAPTER 2

MATERIALS AND METHODS

Plant material

Apples (*Malus x domestica* Borkh. cv. 'Red Chief Delicious') were harvested in 2007 from the Tukey Orchard on the Washington State University campus. For each sampling date, fruit were harvested from 16 random trees (chosen utilizing www.random.org). Apples were of similar size and shape, and to ensure a uniform level of ripeness within each sample, fruit were taken from inside the canopy near the bottom of each tree, with similar numbers of apples taken from each cardinal direction (see Farhoomand et al 1977, Miller et al 1998). Starting at 116 days after full bloom (DAFB), samples were taken once per week. At 144 DAFB, when previous experience indicates that apples may start to ripen, samples were taken every three or four days until internal ethylene concentration (IEC) exceeded 10 ppm, specifically at 147 DAFB, 151 DAFB, 154 DAFB, 158 DAFB, and 161 DAFB. A final sample was taken at 165 DAFB. Separately, just before IEC reached 1 ppm, specifically at 154 DAFB, fruit were harvested from 40 random trees (chosen utilizing www.random.org) and placed into low oxygen storage. Storage treatment was 0.5 °C at 1.5 kPa (1.5 %) oxygen and 0.2 kPa carbon dioxide controlled atmosphere storage (CA). Apples were removed from storage after four months and an initial sample, referred to as Day 0, was processed within five hours. Apples were subsequently placed at room temperature for one week, and samples were taken after four days (Day 4 sample) and after seven days (Day 7 sample). Samples of skin along with a thin layer of cortex were taken from 10 apples for each sampling date after low oxygen storage, and from 6 apples for each off-tree sampling date starting at 144 DAFB. Skin + cortex samples were chopped into small pieces and flash frozen in liquid nitrogen, then stored at –80 ºC for later use.

Internal ethylene concentration

Internal ethylene concentration was measured for all sampling dates as the sole physiological measurement of maturity. After fruit were allowed to warm to room temperature, a 0.5 cm^3 gas sample was withdrawn with a 1 cm^3 syringe using a 50-gauge hypodermic needle from the core of 20-25 fruit per assessment day. The gas sample was injected into a gas chromatograph (GC) (HP 5830A, Hewlett Packard, Palo Alto, CA) equipped with a PLOT-Q column (Agilent, Avondale, PA.) (15 m x 0.53 mm I.D.). The injector was a packed type with a flame ionization detector (FID). The pre-purified nitrogen carrier gas flow rate was 8 ml· min^{-1}. Injector and detector temperatures were 200 °C and the oven temperature 100 °C.

Volatile analysis from juice

Three replications of a 10 apple juice composite were sampled on each date and were stored at –20 °C until analysis. Solid phase microextraction (SPME) was employed to determine the concentration of major volatile compounds in apple flesh. One sample consisted of 2.0 ml of apple juice with 0.65 g NaCl in a 4.0 ml vial with a Teflon lined cap. The SPME device (Supelco, Co., Bellefonte, PA, USA) consisted of a fused silica fiber coated with 65 μm poly (dimethylsiloxane)/ divinylbenzene phase. The SPME fiber was exposed to the headspace of the sample for exactly one hour before GC injection. SPME injection was achieved by splitless injection for 2 min at 200 °C into a Hewlett-Packard 5890II/5970 GC/MSD equipped with a DB-1 column (60m x 0.32 mm, 0.25 μm film). Chromatographic conditions were as described by Mattheis et al. (1991) except transfer line temperature and ion source was held at 250 °C. The GC inlet contained a 0.75 mm SPME injection sleeve which assures peak sharpness, especially

for early eluting peaks (Yang and Peppard, 1994). The compound identification was made by comparison of spectra from sample compounds with those contained in the Wiley-NIST library and by comparing retention indices of sample compounds and authentic standards. Quantification was accomplished by using selected ion monitoring for base peaks. Values were calculated using response factors generated from injection of authentic standard compounds.

Volatile analysis from headspace of whole fruit

Headspace volatile analyses were measured by placing three or four fruit in a polytetrafluoroethylene jar (Berghof/America, Coral Springs, FL), and by passing purified air through the jar at 100 $mL \cdot min^{-1}$ for 60 min, to collect 6 L of headspace atmosphere onto a 65 μm PDMS/DVB SPME fiber. The SPME was injected into the same gas chromatography/mass spectrometry system described above. The column and GC oven conditions were the same. The compound identification was the same as listed and the quantification was accomplished by generating response factors from authentic standard compounds over a one hour sample time. Each sample consisted of three replications utilizing up to nine apples per treatment. The data are reported similar to Rudell et al. (2002) as $\mu g \cdot kg^{-1} \cdot hr^{-1}$.

Alcohol acyltransferase activity

At least four fresh apples from each sampling date were stored overnight at 4 °C and used the next day for alcohol acyltransferase (AAT) activity measurements. For each sampling date, 100 g of sample, consisting of skin and the thin layer of cortex tissue immediately underneath it, was taken and flash frozen in liquid nitrogen. This tissue was homogenized in a Waring blender with 160 ml 0.1 M potassium phosphate buffer (pH 7.0) containing 7.5 % (w/v) PVPP

(polyvinylpolypyrrolidone). The homogenate was centrifuged at 20,000 x g for 20 min at 4 °C, and the supernatant was filtered with a Whatman 4 filter paper. The filtrate was then slowly brought up to 40 % saturation with solid ammonium sulfate and stirred for a total of 30 min. This solution was centrifuged at 20,000 x g for 20 min at 4 °C, and the supernatant was reserved. This was slowly brought up to 80 % saturation with solid ammonium sulfate and stirred for a total of 30 min. This solution was centrifuged at 20,000 x g for 20 min at 4 °C, and the supernatant was discarded. The pellet was resuspended in 3 ml 0.1 M potassium phosphate buffer (pH 7.0). This mixture was put into a dialysis bag with pore size 6-8 K and dialyzed overnight at 4 °C in 500 ml 0.1 M potassium phosphate buffer (pH 7.0). Two hundred microliters of protein extract was assayed with a method previously developed in our lab (Fellman et al. 2000). Error bars indicate three to four assays.

RNA isolation

For each sampling date, three extractions were done with four apples used per extraction, with a total of at least 6 apples used per sampling date. The mortar and pestle were baked at 200 °C overnight. All glassware was either treated with RNaseZap (Ambion) or baked at 200 °C overnight. The Oak Ridge centrifuge tubes were treated with RNaseZap. All aqueous solutions were made with diethyl pyrocarbonate (DEPC) -treated water. RNA was extracted according to Manning (1991) with some modifications. Using a mortar and pestle, 1.2g frozen tissue was ground to a powder in liquid nitrogen, transferred to a 40 ml Oak Ridge centrifuge tube, and homogenized by shaking for 10 minutes in 6 ml pre-cooled extraction buffer. Extraction buffer contained 0.2 M boric acid and 10 mM Na_2EDTA, and was adjusted to pH 7.6 with Tris base. Both β-mercaptoethanol and SDS (sodium dodecyl sulfate) were added to the extraction buffer

in 1/50 (v:v) of final volume. The extract was centrifuged at 20,000 x g for five minutes at room temperature. The supernatant was decanted into a fresh 15 ml polypropylene tube, and two phenol: chloroform: isoamyl alcohol (25:24:1) extractions were done, after each extraction samples were centrifuged at 3,000 x g for 10 minutes at room temperature. Next, the upper phase containing total nucleic acids was decanted into a 40 ml Oak Ridge centrifuge tube, diluted 2.5 times with DEPC-treated water, and adjusted to a final sodium concentration of 80 mM using 1 M sodium acetate. To this solution, 0.4 volume of 2-butoxyethanol (2-BE) was added, and the solution was placed on ice for 45 minutes prior to centrifugation at 20,000 x g for 15 minutes at 0°C. The supernatant was collected without touching the gel-like pellet containing polysaccharides and other carbohydrates. The total nucleic acids were precipitated by adding 1 volume of 2-BE and by placing the solution on ice for 45 minutes prior to centrifugation at 20,000 x g for 10 minutes at 0°C. The pellet was washed three times with 70 % (v/v) ethanol; after each wash step, the solution was centrifuged at 20,000 x g for 10 minutes at 0°C. Residual ethanol was evaporated at room temperature in a fume hood for fifteen minutes and the pellet dissolved in 1 ml DEPC-treated water. 500 µl of 12 M LiCl was added (4 M LiCl final), the solution was transferred to a fresh 1.5 ml eppendorf tube, and the RNA was precipitated overnight at 0˚C. The solution was centrifuged at 11,600 g for 15 minutes. The pellet was rinsed once with 70 % (v/v) ethanol and the residual ethanol was evaporated for five minutes in a fume hood at room temperature. Finally, the pellet was dissolved in 30 µl of DEPC-treated water. Following DNase treatment (DNA-free kit, Ambion, Inc.), RNA was quantified at 260 nm using a Cary 100 Bio UV-visible spectrophotometer (Varian, Walnut Creek, CA). RNA quality was checked by agarose gel electrophoresis.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

First-strand cDNA synthesis was done with oligo $(dT)_{18}$ primer using 0.2 µg of total RNA with the Fermentas First Strand cDNA Synthesis Kit. Primer design was done using Vector NTI (ver. 10). Primer sequences, along with accession numbers and expected fragment sizes, are listed in Table 1. Each set of primers was verified by sequencing the fragment produced and checking this sequence against the published sequence. Sequencing was performed at the Laboratory for Bioanalysis and Biotechnology at WSU. PCR reactions were done using GoTaq® Green Master Mix following manufacturer's instruction (Promega Corporation). PCR cycling conditions for all genes were: denature at 95˚C for 2 min, 30 cycles at 95˚C for 30 sec each; 50 ˚C for 30 sec, 72 ˚C for 60 sec followed by a 5 min extension. PCR products (10 µl from 25 µl total PCR reactions) were resolved using a 1.5 % agarose gel at 95 V for 30 min and checked against the O'GeneRuler 100 bp DNA ladder.

Lipoxygenase activity

Extractions for lipoxygenase assays were performed according to Feys et al (1980) with some modifications. All steps were performed at 4 °C. Frozen tissue from five apples (5 g total) for each sampling date was homogenized with an Ultra-Turrax homogenizer for 2 min in 5 ml of 0.25 M sodium phosphate buffer (pH 7) containing 1 % (v/v) Triton X-100 and 0.01 M sodium metabisulfite (added fresh daily) to prevent rapid darkening of the homogenates. The pH of the homogenates was adjusted to pH 7 with a few drops of concentrated NaOH. The homogenates were centrifuged at 8000 g for 20 min, and the supernatant was retained and filtered through four layers of cheesecloth. This was used immediately as the source of the enzyme.

The substrate for the assay was prepared according to Axelrod et al. (1981). Specifically, .026 g sodium linoleate and 25 µl Tween-20 were dissolved in about 5 ml degassed 0.1 M potassium phosphate buffer (pH 8). Forty microliters of 5 M NaOH was added, and the solution was made up to 10 ml with degassed buffer. After mixing thoroughly, the solution was distributed into 4 ml test tubes stored on ice. The tubes were capped with sleeve stoppers, flushed with nitrogen gas, and stored at –20 °C for not more than 10 days.

Lipoxygenase was assayed by measuring the increase in absorbance at 234 nm. Hydroperoxide formation was calculated using a molar extinction coefficient of 25000 M^{-1} cm^{-1} (Axelrod et al. 1981). The assay mixture consisted of 885 µl 0.25 M sodium phosphate buffer (pH 7), 15 µl enzyme extract, and 100 µl substrate solution in a quartz cuvette. The blank consisted of 900 µl 0.25 M sodium phosphate buffer (pH 7) and 100 µl substrate solution. The buffer was added first, followed by the extract, followed by the substrate solution (removed from the stoppered vial using a syringe). Two assay mixtures along with a blank were mixed for 45 sec each by pipetting up and down, and measured for 5 min in an HP 8453 UV/Vis spectrophotometer. Three independent extractions were performed for each sampling date, and at least two assays were performed per extraction. Results for each extraction were averaged; error bars represent the three extractions.

Statistical analysis

When it seemed necessary to confirm or deny the significance of particular changes between sampling dates, *t*-tests were done to compare up to two pairs of sampling dates for a figure. Differences were considered significant at $P < 0.05$.

Name	Forward Primer (5' -> 3')	Reverse Primer (5' -> 3')	Genbank Accession	Size (bp)
ACC3	GGTGGGAAATAAAGTCAAC	GATGGCAGCAGATAAAAC	ES790098	310
LOX1	TGAGAAACTGAAGAACCGA	ATCCCATACAATACAATACATA	ES790104	180
MpAAT1	ATACTATGGCAATGCATTTG	GCGGCAACTGAAATTTAATTT	AY707098	624

Table 1: Primer information for PCR amplification of flavor-related genes

CHAPTER 3

RESULTS

Maturity through the ripening phase

A detailed analysis of the changes in gene expression, enzyme activities, and volatile levels was performed during apple fruit ripening. Internal ethylene concentration (IEC) data was chosen as a reliable, precise measurement of physiological maturity (Fellman et al 2003, Chu 1984). Data were collected weekly for fruit samples starting at 116 days after full bloom (DAFB), when apples are pre-climacteric. At 144 DAFB, when prior experience has indicated apples may start to ripen, samples were collected every three or four days in order to obtain information at the onset of apple ripening. Ethylene was first detected at 0.17 ppm at 151 DAFB (Fig. 2A). Levels were elevated to 0.88 ppm at 154 DAFB and were higher afterwards, reaching a peak at 161 DAFB. System II climacteric ethylene production started after 1 ppm (Fellman et al 2003), which was slightly after 154 DAFB for this experiment. A dramatic increase in percent standard error (%SE) has been reported to take place at the climacteric break, at times, this provides a better indication of when apples enter climacteric ripening (Fellman et al 2003, Lin and Walsh 2008). In this experiment, %SE was at 100% at 151 and 154 DAFB and was lower after ethylene production reached 1 ppm (parts per million) (Fig. 2B).

Gene expression during ripening

Three genes along the straight-chain ester synthesis pathway were chosen for analysis during on-tree ripening and ripening after storage. Accession numbers are listed in Table 1. An alcohol acyltransferase (or AAT), MpAAT1, was chosen, as its corresponding protein has been well studied (Souleyre et al 2005), and as the expression of this gene has been shown to increase

with the addition of ethylene (Shaffer et al 2007) and during ripening (Zhu et al 2008). A lipoxygenase (LOX) gene was chosen as the enzyme activity of LOX has been well studied in apples, but gene expression has not been studied as much (see introduction). However, two LOX contigs have been examined as part of large scale gene expression studies; one of these is nearly identical to a portion of the other (Park et al 2006, Schaffer et al 2007). A BLAST (Basic Local Alignment Search Tool) search confirmed that the larger contig, called LOX1 (Schaffer et al 2007), has a 69% identity with a potato LOX (X95513) and a 69% identity with LOX1 from Arabidopsis (NM_104376). This contig has been shown to be upregulated during ripening (Park et al 2006) and is induced by ethylene (Schaffer et al 2007), so it was chosen for further study. An acetyl-CoA carboxylase (ACCase) gene was chosen to try to determine if fatty acid synthesis is regulated during ripening. ACCase genes have received very little study in apple, however, a contig named ACC3 has been studied (Schaffer et al 2007). A BLAST search confirmed an 89 % identity to the ACCase biotin carboxylase subunit from tobacco, so ACC3 was chosen for further study.

Figure 3A shows gene expression data, measured by RT-PCR (Reverse Transcriptase Polymerase Chain Reaction), for apples freshly harvested throughout ripening and for apples collected after removal from CA storage. ACC3 expression may show a slight increase from 144 to 147 DAFB, but after this it does not change. Both of these harvest dates were early in ripening, before ethylene was detected. LOX1 expression also changes early in ripening, but the changes are more marked. It clearly increases from 144 to 151 DAFB before leveling off. At 151 DAFB apples are still early in the ripening process and producing system I ethylene. In contrast, MpAAT1 expression stays constant from 144 to 158 DAFB, then increases at 165 DAFB. At 165 DAFB apples are well into system II ethylene production and far into the ripening process.

Enzyme activity during ripening

LOX and AAT activities were studied in parallel with gene expression. Figure 4 compares enzyme activity and gene expression for LOX and AAT. LOX activity showed a complex pattern of change during ripening. It decreased between 144 and 154 DAFB (Fig. 4B), following the opposite trend as gene expression (Fig. 4A). 154 DAFB is where IEC is approximately 1 ppm, near climacteric onset. Between 154 and 158 DAFB LOX activity showed a small and statistically insignificant ($P>0.05$) increase that was not reflected in RT-PCR data, then LOX activity stayed constant to 165 DAFB (Fig. 4B). AAT activity also decreased between 144 and 151 DAFB, with no activity found at 151 DAFB (Fig. 4B). When IEC reached 1 ppm at 154 DAFB, AAT activity spiked. At 158 DAFB AAT activity was not found. After this, late in ripening, AAT activity rose. RT-PCR data showed an increase in AAT only at 165 DAFB (Fig. 4A), after AAT activity had started to rise.

Volatile levels during ripening

Volatiles important for flavor and related to LOX and AAT were measured in parallel with activity and enzyme data already discussed. Figure 5 compares summed ester and alcohol levels for juice volatile content and emissions in headspace to AAT activity. As volatile content was measured at more time points than volatile emission, the time scale on the x axis of fig. 5A is wider than that of fig. 5B, and the 165 DAFB value for AAT activity is left off of fig. 5B. Juice volatile content measurements indicate that ester levels were very low, but nonzero, before 144 DAFB, when they jumped before decreasing on 147 DAFB (fig. 5A). Starting at 151 DAFB, when ethylene levels began to rise, esters also began to rise and continued to rise until the end of the experiment (fig. 5A). Total juice alcohol content was higher than ester content before 144

DAFB, and from 144 to 154 DAFB, which is just before climacteric system II ethylene production, it parallels total juice esters (fig. 5A). Content of alcohols then rises at 158 DAFB before leveling off (fig. 5A). Headspace volatile content behaved somewhat differently (fig. 5B). There was a continuous rise in headspace esters that became dramatic starting at 151 DAFB, while alcohol levels did not change (fig. 5B). At 151 DAFB ethylene levels had just begun to rise. Comparing these results to AAT activity, both juice and ester volatiles rise before the major, sustained rise in AAT activity that takes place at 161 DAFB (fig. 5).

Figure 6 compares selected straight-chain esters and alcohols to AAT activity. As volatile content was measured at more time points than volatile emission, the time scale on the x axis of fig. 6A is wider than that of fig. 6B, and the 165 DAFB value for AAT activity is left off of fig. 6B. For volatile content, hexanol and hexyl acetate are much lower than butanol and butyl acetate (fig. 6A). The only notable trend in hexanol and hexyl acetate levels is the rise of both at 158 DAFB, after the beginning of system II ethylene production (fig. 6A). Higher hexyl acetate levels were present after this until the end of the experiment. Butanol and butyl acetate in juice parallel each other; both increased at 144 DAFB, decreased at 147 DAFB, and starting at 151 DAFB, when ethylene levels began to rise, these volatiles began to rise and generally continued to rise until the end of the experiment (fig. 6A). Butyl acetate levels were consistently higher than hexyl acetate levels (fig. 6A). Somewhat different patterns were evident for volatile emissions (fig. 6B). Surprisingly, headspace butanol was undetectable (fig. 6B). Hexanol levels were low and flat (fig. 6B). Both esters began to increase in headspace measurements starting at 151 DAFB, when ethylene levels rose above zero (fig. 6B). As in juice, butyl acetate levels were consistently higher than hexyl acetate levels.

Since hexanal and various hexenals are produced by LOX, LOX activity was compared to hexanal, 2-hexenal, and cis-3-hexenal (Gardner 1995). Results for freshly harvested fruit are shown in figure 7. As juice volatiles were measured at more time points than headspace volatiles, the time scale on the x axes of Fig. 7A and Fig. 7B is wider than the time scale on Fig. 7C and Fig. 7D, and the 165 DAFB value for LOX activity is left off of Fig. 7C and Fig. 7D. In juice, a decrease was evident in all three volatiles until 148 DAFB, followed by an increase (fig. 7A). Ethylene begins to rise at 151 DAFB. Hexanal and 2-hexenal then were increased to 154 DAFB and were approximately constant afterwards, while cis-3-hexenal was decreased until 158 DAFB, when it remained constant (fig. 7A). In headspace, all three volatiles showed a small and statistically insignificant ($P>0.05$) increase from 144 to 147 DAFB (Fig. 7C). When ethylene began to increase at 151 DAFB, all three volatiles began to decrease (Fig. 7C). Hexanal also makes a striking decrease from 154 to 158 DAFB, after the start of system II ethylene production (Fig. 7C). Notably, LOX activity was independent of aldehyde production in all cases.

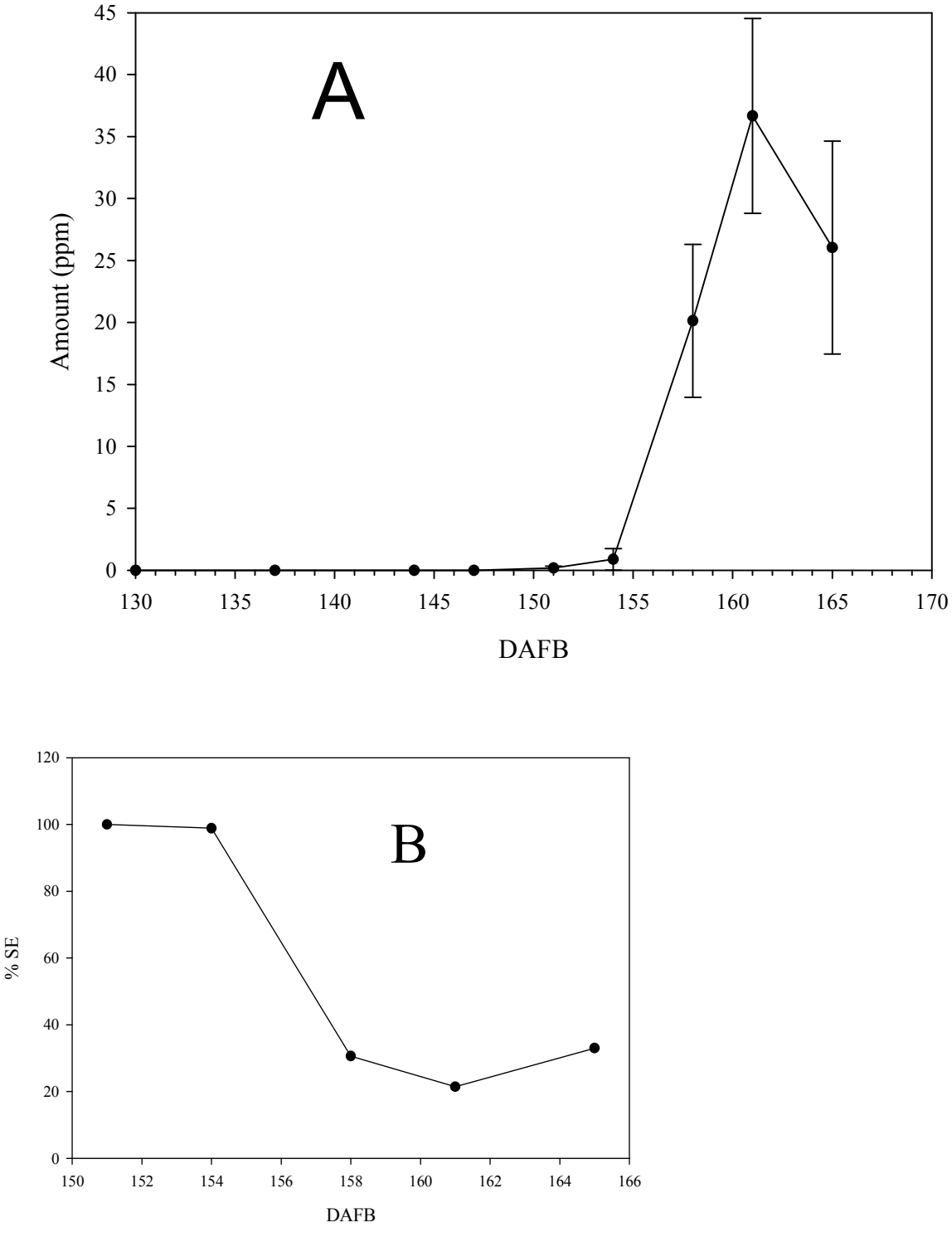


Fig. 2. At harvest IEC (A), % S.E. IEC (SE/mean) (B). Error bars represent S.E., *n* = 20-25

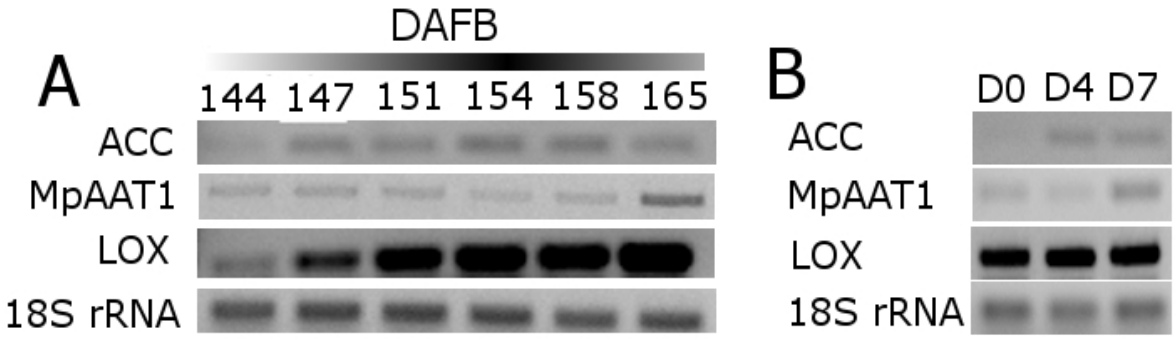
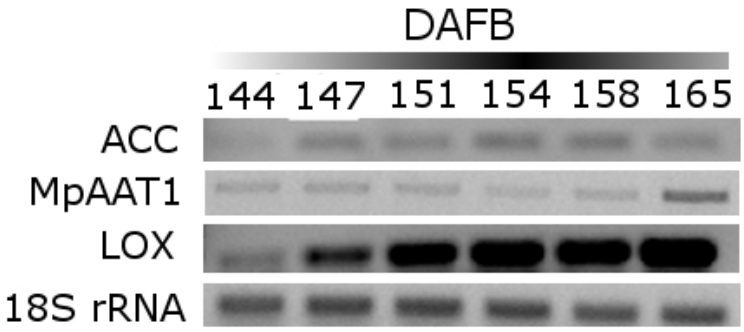


Fig. 3. Semi-quantitative RT-PCR expression analysis for the MpAAT1 gene and likely ACC3 and LOX1 genes. 18S rRNA was used as a loading control. PCR was done on skin tissue picked during ripening on the tree from 144-165 DAFB (A). Apples from the 154 DAFB harvest were placed in CA storage for 4 months, and PCR was done on skin tissue from CA stored apples collected less than 5 hours (D0), 4 days (D4) and 7 days (D7) after removal from CA storage (B). Typical results for three extractions are shown.

A



B

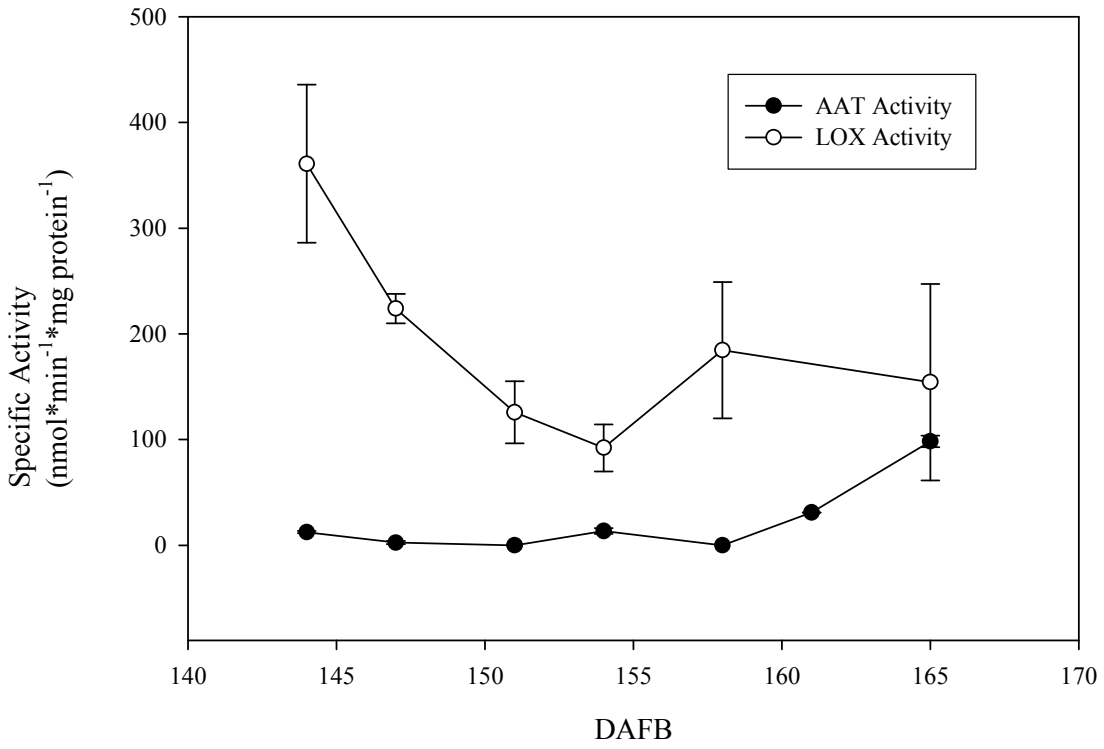
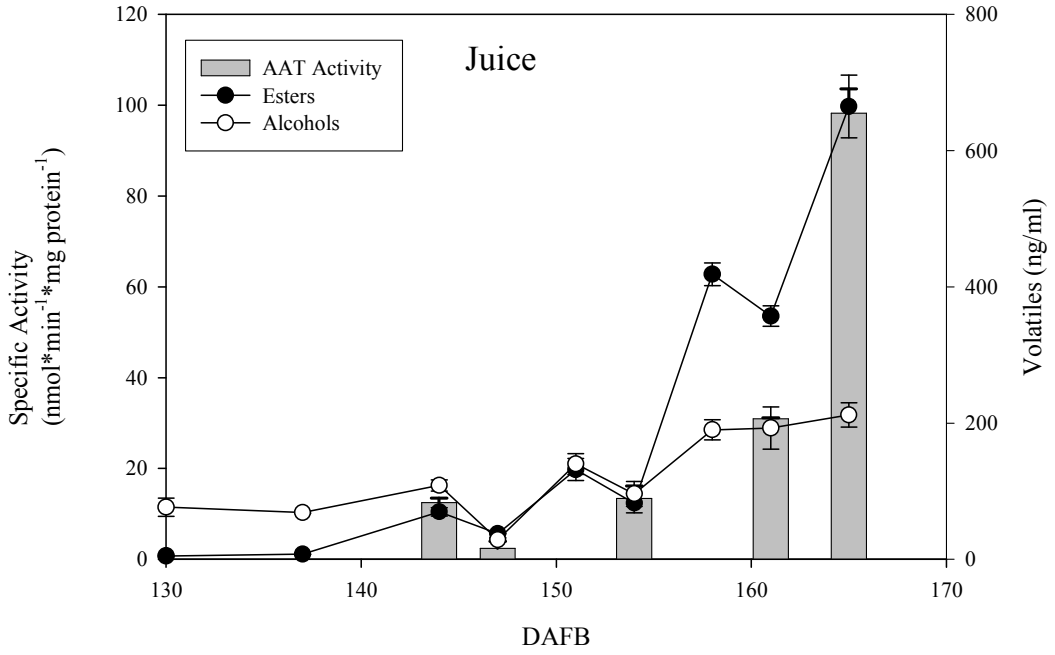


Fig. 4. Semi-quantitative RT-PCR compared to enzyme activities for on-tree ripening. (A) PCR conditions are identical to those described in fig. 3A. (B) Enzyme activities (mean of 3 repetitions +/- S.E.).

A



B

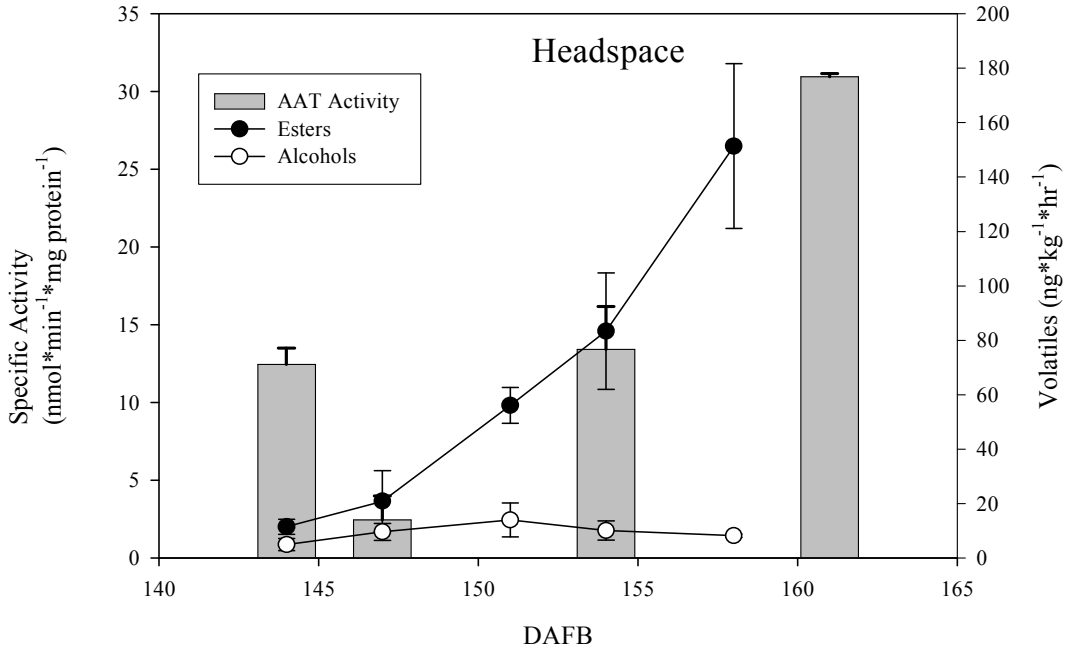
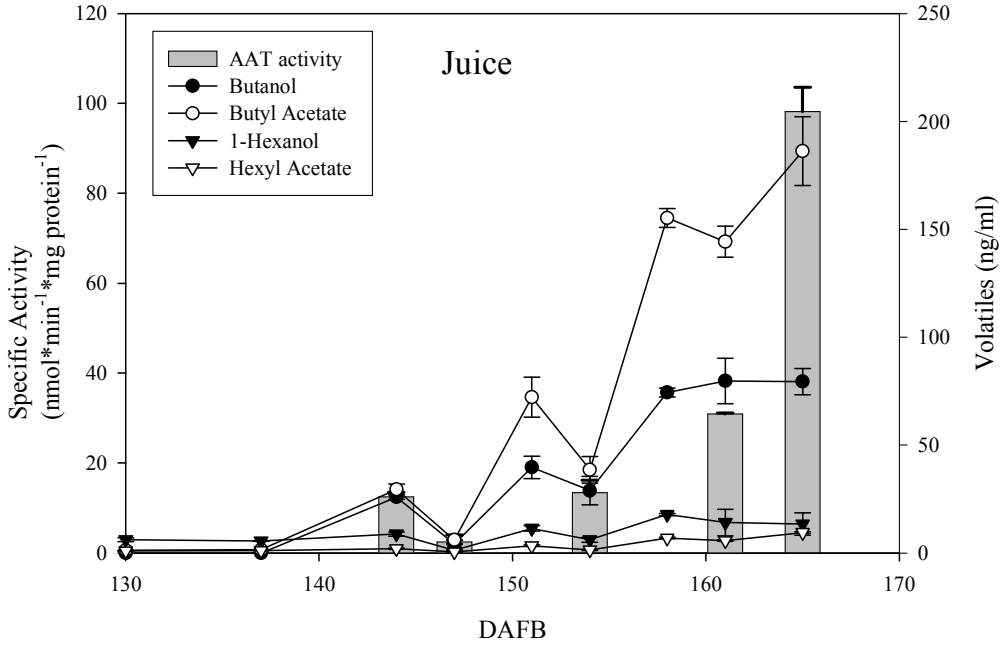


Fig. 5. AAT activity and volatiles for on-tree ripening. (A) AAT activity compared to total esters and alcohols in juice. (B) AAT activity compared to total esters and alcohols in the headspace of whole fruit. Activity and volatile data are means of 3 repetitions +/- S.E.

A



B

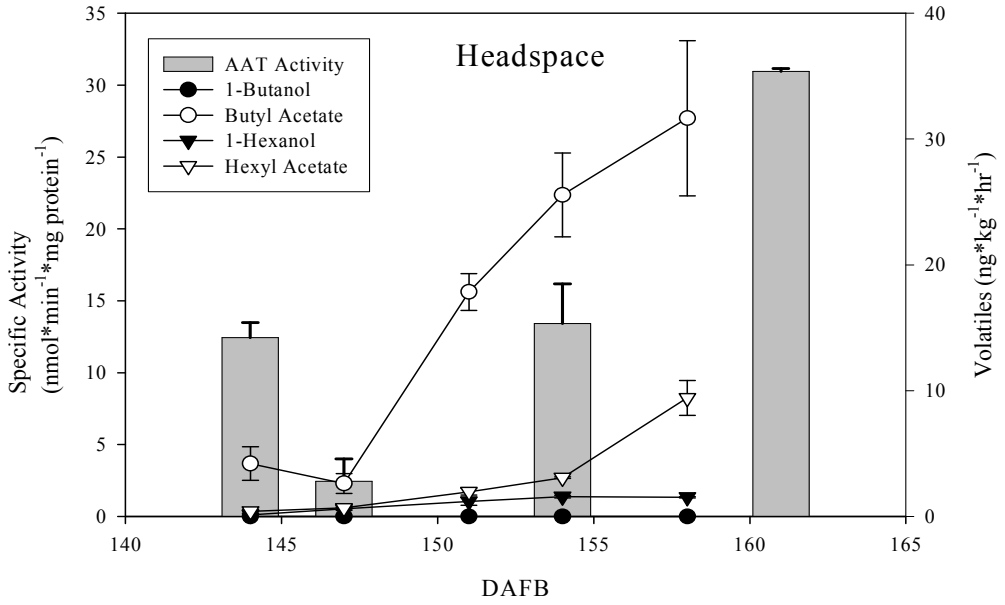


Fig. 6. AAT activity compared to important alcohols and esters for on-tree ripening. (A) Juice volatiles. (B) Headspace volatiles from whole fruit. Activity and volatile data are means of 3 repetitions +/- S.E.

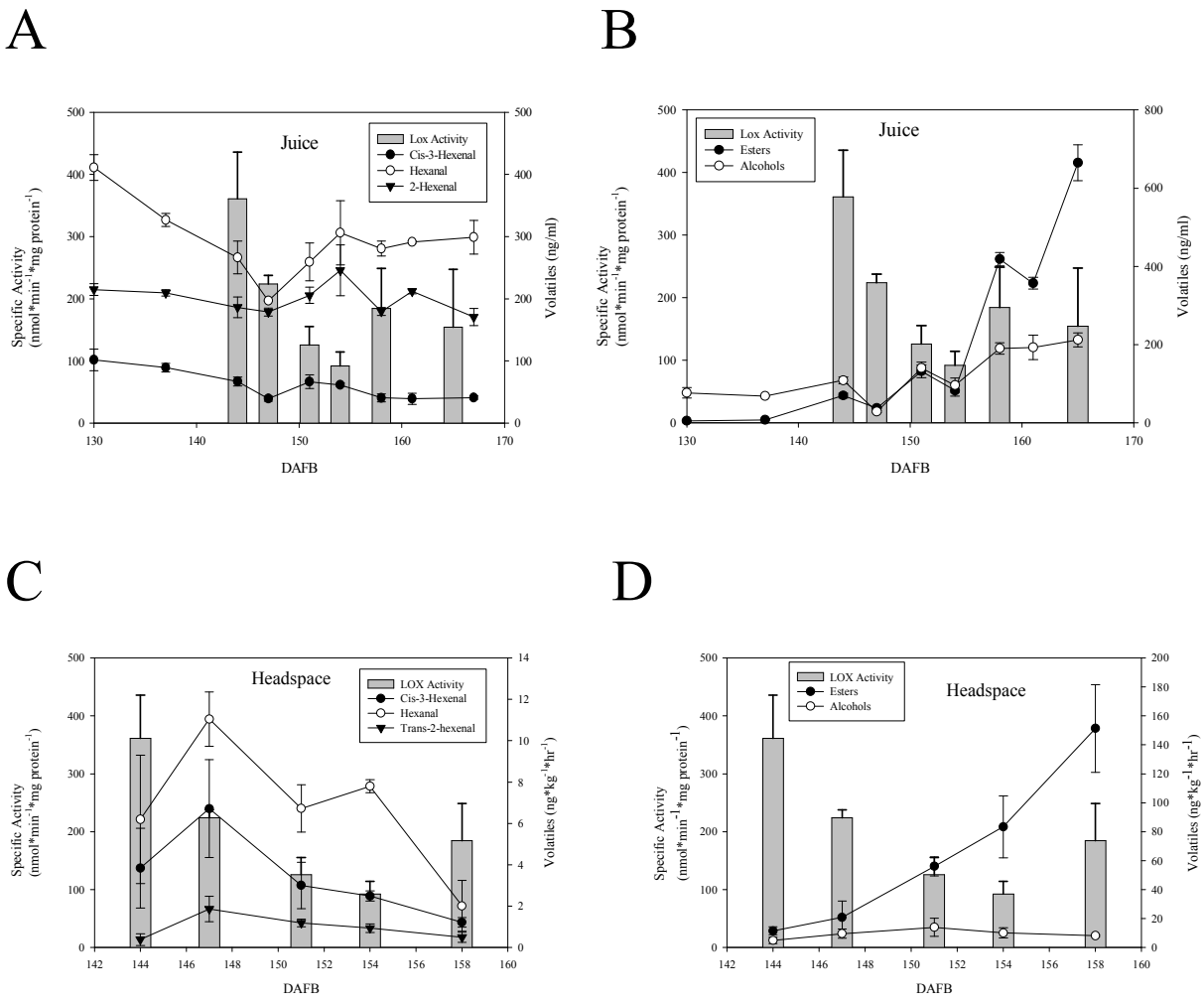


Fig. 7. LOX activity compared to volatiles for fresh harvest dates. (A) and (B) juice volatiles. (C) and (D) headspace volatiles from whole fruit. Activity and volatile data are means of 3 repetitions +/- S.E.

Changes during controlled atmosphere storage

To examine gene expression and enzyme activity changes during controlled atmosphere (CA) storage, gene expression and enzyme activity were measured on a sample of fruit on the day fruit were placed in CA storage (154 DAFB) and within a few hours after fruit were removed from CA storage. While MpAAT1 and LOX1 gene expression levels stayed approximately the same during CA storage, ACC3 gene expression seemed to decrease slightly (Fig. 3). Comparisons of activity before and after CA storage did not reveal statistically significant changes, although LOX activity showed an increase and AAT activity showed a decrease (Table 2). A statistical analysis also revealed that the concentration of all juice volatiles significantly changed during the 4 months of CA storage. Esters and alcohols, including the straight chain volatiles butyl acetate, hexyl acetate, butanol, and hexanol all significantly increased (Table 2). In contrast, the three lipoxygenase-associated aldehydes that were measured all significantly decreased (Table 2).

Ripening after CA storage

IEC was near zero just after CA storage (Fig. 8). IEC then rapidly increased until 4 days after CA storage. From 4 to 7 days after CA storage an increase was evident in IEC, but the rate of increase was slower.

Gene expression and enzyme activity after CA storage

In order to determine how gene expression and enzyme activity were related to straight chain ester synthesis development after CA storage, measurements were taken a few hours, 4 days, and 7 days after fruit were removed from CA storage (Fig. 9A). Gene expression of ACC3

may have increased slightly between a few hours and 4 days after CA storage, but stayed constant between day 4 and day 7. Gene expression of MpAAT1 was constant between a few hours and 4 days after CA storage and increased at day 7. LOX gene expression did not change after CA storage. AAT activity shows an increasing trend during ripening after CA storage, but a comparison between a few hours after CA storage and 7 days after showed no significant increase ($P > 0.05$, Fig. 9B, 10B). LOX activity shows no significant changes after CA storage ($P > 0.05$, Fig. 9B).

Volatile levels after CA storage compared to activities of biosynthetic enzymes

To determine how volatile development changes after CA storage and to determine how volatiles recover after CA storage, the same volatiles were measured after CA storage as during on-tree ripening. Total esters and alcohols for emissions in the headspace and content in the juice are shown in Figure 10. Juice alcohols show a slow increase after CA storage, while ester levels from juice are high a few hours after CA storage, much lower after 4 days, and increase until 7 days after storage (Fig. 10B). Volatile levels for headspace were not measured a few hours after removal from CA storage. From day 4 to day 7 after storage, ester emissions increased while alcohols decreased (Fig. 10C).

The same selected volatiles that were measured during on-tree ripening were also measured after CA storage. Hexyl acetate content stayed at consistently low levels after CA storage in juice, while its corresponding alcohol hexanol also remained at consistently low levels (Fig. 11A). In contrast, emissions of hexyl acetate increased from 4 days to 7 days after CA storage, although hexanol stayed consistently low (Fig. 11B). For measurements of volatile content, both butanol and butyl acetate started high and showed a decrease from a few hours to 4

days after CA storage, then showed an increase from 4 to 7 days after CA (Fig. 11A). In headspace, butyl acetate was increased from day 4 to day 7, while butanol was not detectable (Fig. 11B).

Changes in LOX activity did not relate to changes in aldehydes produced by LOX (Fig. 12). The y-axis in Figure 12C should be noted, as it shows that headspace aldehydes are very low compared to other measured volatiles. Cis-3-hexenal and trans-2-hexenal did not show striking changes over time in either volatile content or emissions, and cis-3-hexenal was never detected in headspace (Fig. 12). Hexanal had different patterns for juice content and headspace emissions. In juice there was a decrease to zero on day 4 from a positive value just after removal from CA, then an increase until day 7, while in headspace, hexanal values remained constant from day 4 to day 7 (Fig. 12).

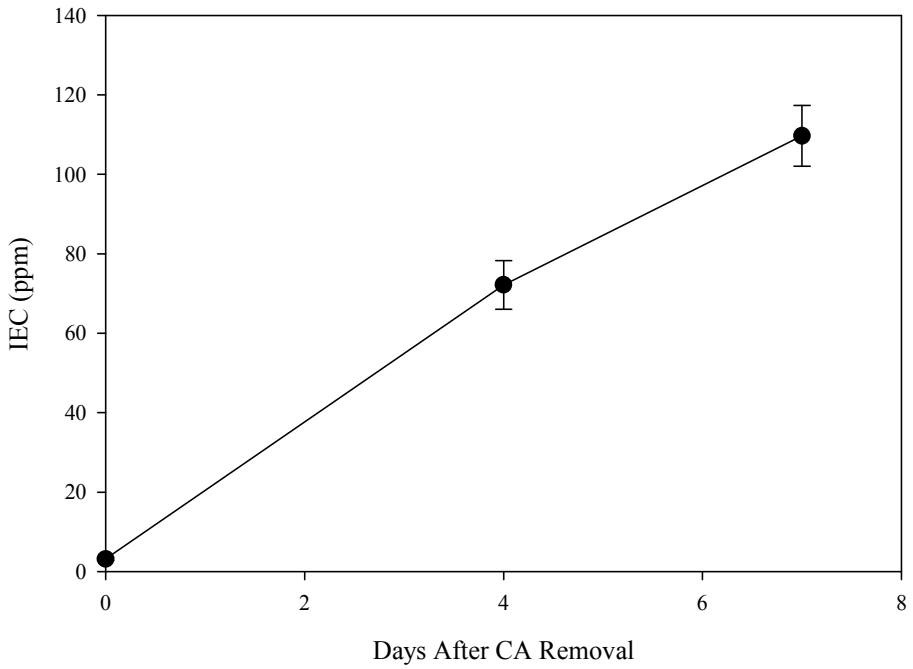
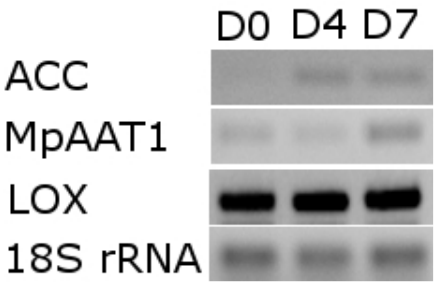


Fig. 8. Internal ethylene concentration in apples after they were removed from 4 months of CA storage. Zero days after CA removal indicates less than 5 hours. Error bars represent S.E., *n* = 20.

	Before CA	**After CA**
LOX Activity (nmol*min^{-1}*mg protein^{-1})	92.1	342.7 n.s.
***cis*-3-Hexenal (ng/ml)**	61.6	22.5
Hexanal (ng/ml)	306.1	74.5
2-Hexenal (ng/ml)	245.6	64.4
AAT Activity (nmol*min^{-1}*mg protein^{-1})	13.4	6.7 n.s.
Esters (ng/ml)	82.5	579.0
Alcohols (ng/ml)	96.1	169.5
Butyl Acetate (ng/ml)	38.6	100.3
Hexyl Acetate (ng/ml)	1.4	4.8
Butanol (ng/ml)	28.9	69.1
Hexanol (ng/ml)	6.1	16.4

Table 2. Activities and volatile levels before and after 4 months of CA storage. Fruit were harvested at 154 DAFB, just before ethylene reach 1 ppm, and placed into CA storage for 4 months (Before CA). A sample was removed and tissue was used or frozen for analysis within 5 hours (After CA). LOX and AAT activity values are specific activity (nmol*min^{-1}*mg protein^{-1}). Volatiles are from juice (ng/ml) and are means of three repetitions. Significance determined by Student's t-test, $P < 0.05$; n.s., non significant.

A



B

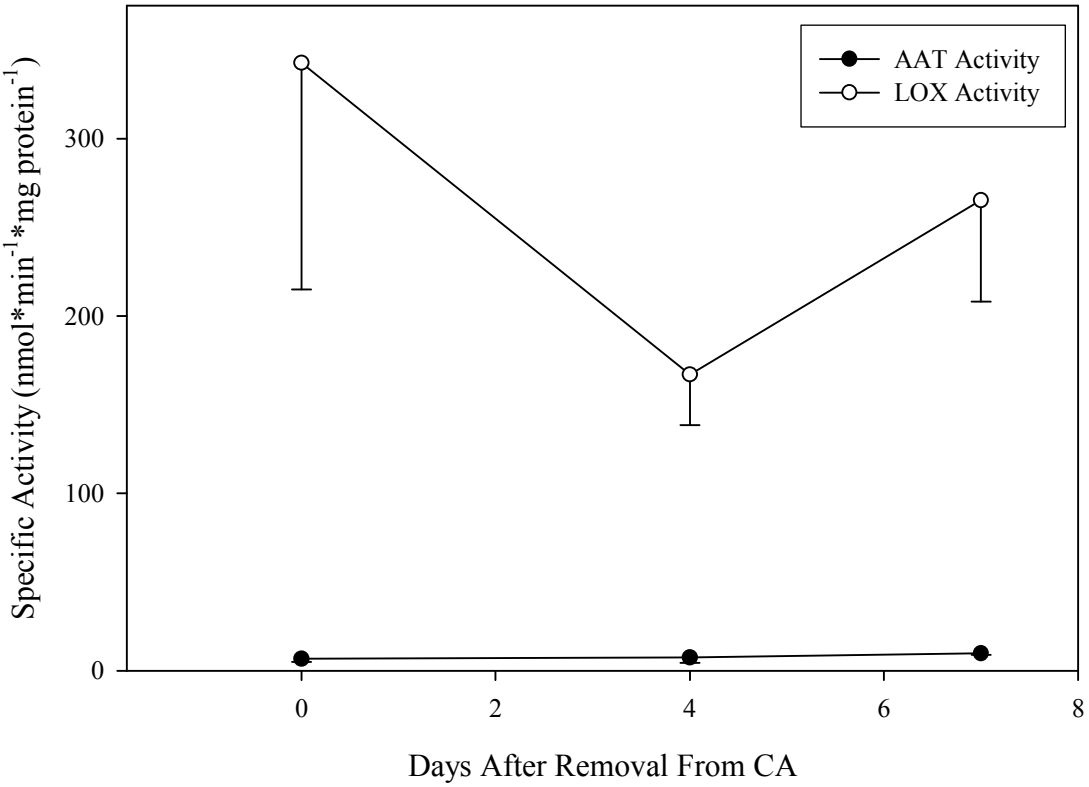
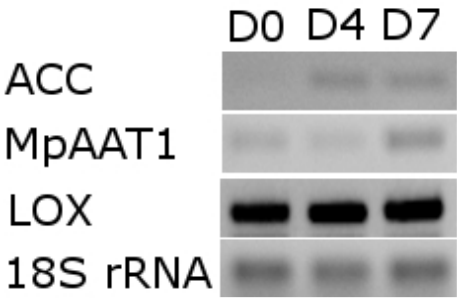
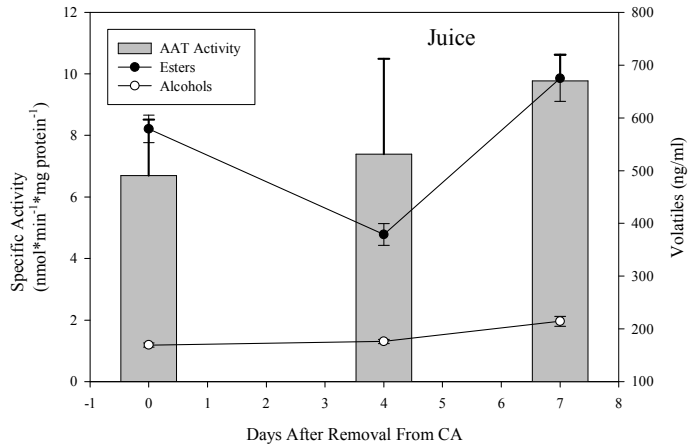


Fig. 9. Semi-quantitative PCR compared to enzyme activities after CA storage. (A) PCR conditions are identical to those described in fig. 3B. (B) Activity data are means of 3 repetitions +/- S.E, 0 days after removal from CA indicates less than 5 hours.

A



B



C

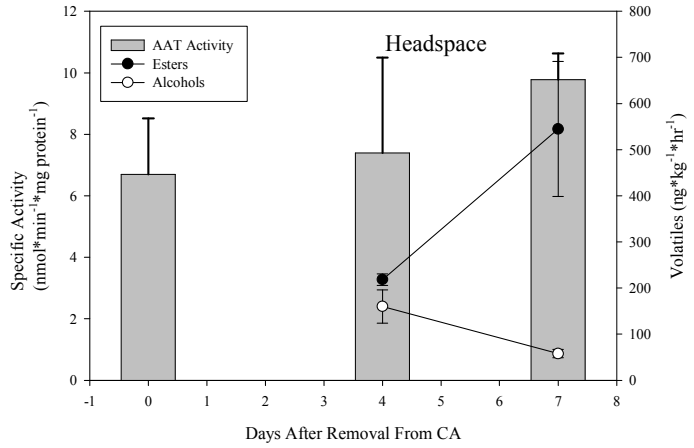
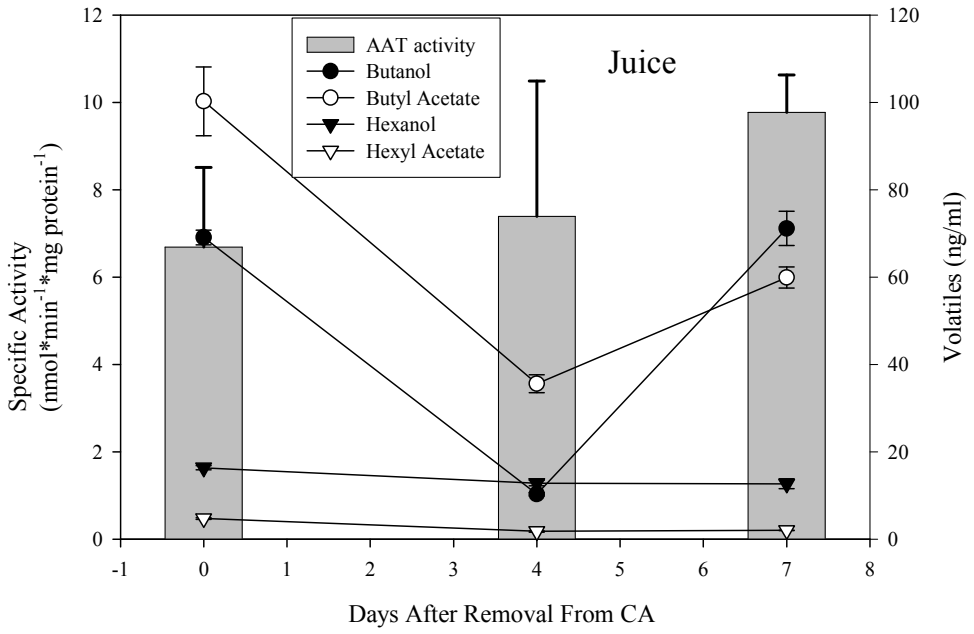


Fig. 10. Semi-quantitative PCR, AAT activity, and volatiles after CA storage. (A) PCR conditions are identical to those described in fig. 3B. (B) AAT activity vs. juice volatiles. (C) AAT activity vs. headspace volatiles. Activity and volatile data are means of 3 repetitions +/- S.E, 0 days after CA removal indicates less than 5 hours.

A



B

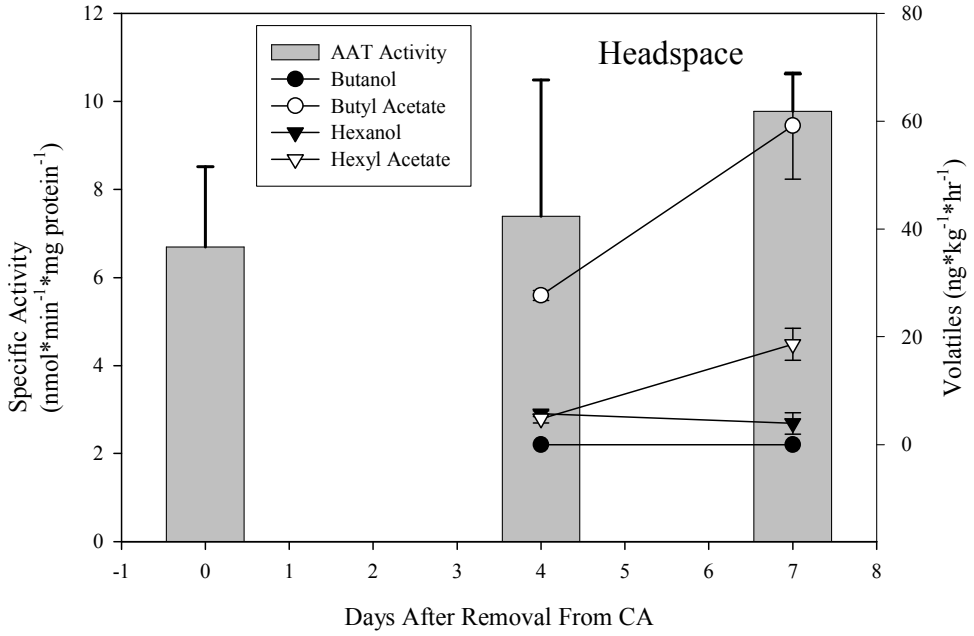


Fig. 11. AAT activity compared to important alcohols and esters. (A) Juice volatiles. (B) Headspace volatiles. Activity and volatile data are means of 3 repetitions +/- S.E, 0 days after CA removal indicates less than 5 hours.

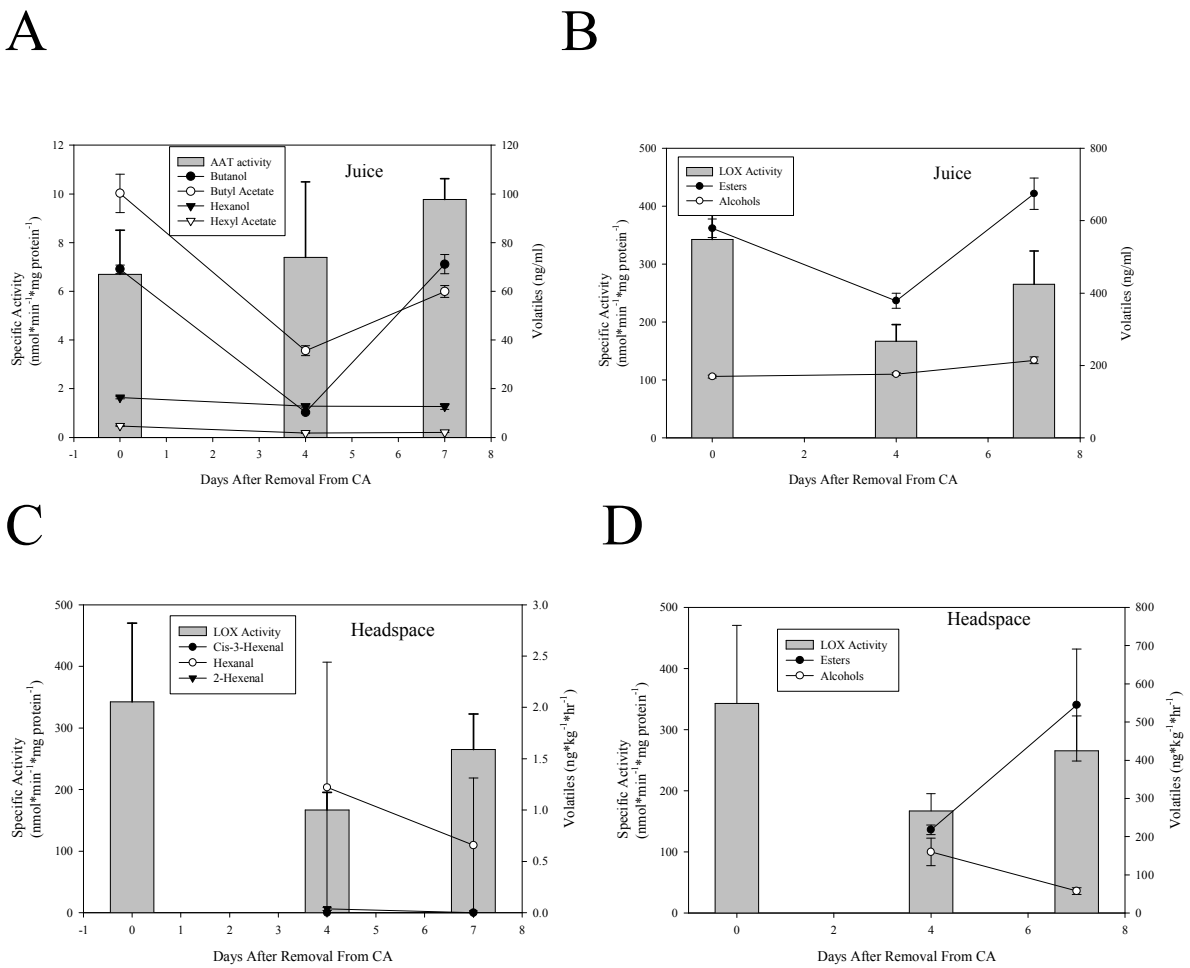


Fig. 12. LOX activity compared to volatiles after CA storage. (A) and (B) juice volatiles. (C) and (D) headspace volatiles. Activity and volatile data are means of 3 repetitions +/- S.E, 0 days after CA removal indicates less than 5 hours.

CHAPTER 4

DISCUSSION

Development of volatile esters is an important process in defining the flavor of apples and is therefore of interest to the fresh apple industry. Apples contain both straight-chain and branched-chain esters, but only straight-chain esters have a well-established synthesis pathway in apples (see Fig. 1). Two well-defined, interesting points along this pathway were chosen and gene expression, enzyme activity, and associated volatiles were followed through ripening on the tree, controlled atmosphere (CA) storage, and for a few days after CA storage. These points were the last step of ester synthesis, which is alcohol acyltransferase (AAT), and lipoxygenase (LOX), which splits the fatty acid originators of straight-chain esters to create aldehyde precursors. The objective was to find out whether and how these points are regulated, and the importance of potential regulation to straight-chain ester levels in ripening fruit. Acetyl-CoA carboxylase (ACCase) was also chosen for study to determine if synthesis of fatty acids is regulated during ripening, although only gene expression was detectable. An attempt was also made to measure enzyme activity of ACCase. To the author's knowledge, no results have been reported for ACCase enzyme activity in apple. Attempts to measure activity with two methods gave inconclusive results. See appendix A for details.

Freshly harvested fruit

Apple, as a climacteric crop, has a quick, well-defined transition to physiological maturity, marked by a rapid increase in internal ethylene content (IEC) as ethylene transitions from system I production in unripe fruit that mainly governs stress response to the positive feedback loop of system II production that stimulates ripening (Kidd and West 1945, Lelièvre et

al 1997). Studying the narrow window of time slightly before, during, and after the rise in IEC, when ethylene transitions from system I production to system II production, could give useful insights into processes that take place during ripening. Although apple ripening is a frequent target of study, most studies do not specifically target this relatively narrow time window. When this narrow time window is studied, sometimes fruit are harvested when unripe and allowed to ripen off the tree. In order to obtain the most accurate picture of the regulation of important ripening-related processes, samples were harvested during this narrow window of time.

AAT is commonly studied because it combines alcohols and acyl CoAs in the final step of ester synthesis (Fellman et al 2000). Multiple studies that examined AAT gene expression and activity revealed increases in both during development, so it was not surprising that both MpAAT1 gene expression and AAT activity increased during ripening in this experiment. It is difficult to interpret the AAT activity data before 161 days after full bloom (DAFB) in this experiment, but AAT activity did clearly increase starting at 161 DAFB, which is about a week after the beginning of system II ethylene production (Fig. 2A, 5A). AAT gene expression was not measured at 161 DAFB, but the measurement at 165 DAFB did show an increase from the previous measurement at 158 DAFB (Fig. 3A). Although gene expression data showing an increase at 161 DAFB would have confirmed this, it seems like AAT gene expression is stimulated by high ethylene levels, which leads to an increase in activity. This is supported by a publication showing that high levels of ethylene, but not low levels, can produce an increase in AAT activity in apples (Defilippi et al 2005b). MpAAT1 gene expression has been measured during on-tree ripening, showing an increase as ripening progressed, but these measurements were taken every two weeks, giving them limited value (Zhu et al 2008). A previous study in our lab on AAT specific activity over apple ripening revealed four different periods of change during

ripening. There was a slow decrease before climacteric, then a spike just before climacteric, then a slow increase as ethylene increased, then finally a decrease late in ripening (Rudell 2000). My data before 161 DAFB is difficult to interpret, but it could possibly support a decrease before the beginning of IEC production, followed by a slow increase thereafter, if the lack of activity at 158 DAFB is discounted as a statistical aberration (Fig. 5). This is possible, as 4 apples total at minimum were used for activity measurements, and as the experience in our lab with apple ripening has revealed a wide range in ripeness of individual apples taken at a specific time point. This opens up the possibility that by chance, the four apples chosen at 158 DAFB had undetectable AAT activity, while the overall population of apples at 158 DAFB had higher AAT activity. In this case, the increase in gene expression would take place after the increase in activity. This could be possible if AAT activity is regulated by both gene expression and a post-transcriptional or post-translational process which provides an initial increase in AAT activity.

Total substrates (alcohols) and products (esters) of AAT were measured to further define the role that regulation of AAT plays in regulating ester production. Measurements of alcohol and ester emissions in the headspace show that esters rise starting at 151 DAFB, while alcohols stay constant (Fig. 6B). In contrast, AAT activity is still low at 147 and 151 DAFB (Fig. 6B). This implies that increases in AAT activity do not provide the initial increase in ester synthesis, in other words, that precursor levels help determine ester levels, which is generally accepted as being the case (Villatoro et al 2008, Lara et al 2007). As ethylene starts to increase at 151 DAFB, it also confirms the strong control of ethylene over ester production (Fan et al 1998, Defilippi et al 2004, Defilippi et al 2005a, Mattheis et al 2005). However, alcohol levels stay steady, giving evidence that the production of alcohols is also not the important step regulating ester synthesis. Headspace measurements of butyl acetate and its corresponding alcohol butanol, along with

hexyl acetate and its corresponding alcohol hexanol, show that straight chain esters follow the same pattern as total esters (Fig. 6B). This is not surprising, as straight chain esters are under particularly tight control by ethylene (Mattheis et al 2005). As 151 DAFB is the beginning of autocatalytic ethylene synthesis, ethylene could regulate an earlier step in the straight chain ester synthesis pathway. The same alcohols and esters were also measured in juice volatiles, with different results (Fig. 5A and 6A). In volatile content of juice, changes in alcohol levels roughly parallel changes in ester levels. After CA storage, alcohols in juice and in headspace from whole fruit also contrast; alcohols in juice increase while alcohols in headspace decrease (Fig. 10B and 10C). This seeming discrepancy could be explained as an artifact of processing. Apple juice is an acidic solution (Brown and Harvey 1971), and studies of the enzyme produced by MpAAT1 show that it works best in a neutral or slightly basic solution (Souleyre et al 2005). This indicates that measurements of juice volatile content can be problematic. Although the cuticular and wax layers of the skin could create a barrier for the escape of flavor volatiles, lenticels and cracks in the skin surface of apples likely allow enough escape of flavor volatiles into the headspace to make emissions measurements a reasonably accurate reflection of processes occurring inside the fruit (Watkins et al 2004).

Contrasting patterns were shown between the gene expression of the LOX isoform that was studied and LOX enzyme activity. Gene expression of LOX1 increased dramatically before ethylene production started and stayed constant after this (Fig. 3A). LOX1 gene expression has not been studied in detail during ripening, but a study of LOX1 expression done at widely spaced intervals during ripening showed an increase during development (Park et al 2006). In contrast, LOX activity decreases in early ripening and increases slightly, but not significantly afterwards (Fig. 4B). This supports an earlier observation, where a decrease in LOX specific activity in the

skin was also found near commercial harvest, and where a slow increase subsequently occurred (Echeverrìa et al 2004). The slight increases observed during the final phase of this experiment may correspond to the onset of the slow increase measured by Echeverrìa et al (2004), who took samples weekly as opposed to the narrower time frame in this study. The discrepancy between LOX1 gene expression and LOX activity could be explained in two ways. First, many isoforms of LOX have been found in apple, so LOX1 could make only a minor contribution to LOX activity (Schaffer et al 2007). Because only linoleic acid was used in measuring activity, a more intriguing possibility is that the protein encoded by LOX1 preferentially accepts linolenic acid as a substrate. LOX isoforms specific for linoleic and linolenic acid have been found in *Arabidopsis*, making this a strong possibility (Bannenberg et al 2009). Further evidence that specific isoforms of lipoxygenase play a role in ripening is provided by a significant change in the ratio of hexanal/(2E)-hexenal in transgenic apple fruit suppressed for ethylene production, suggesting regulation of specific isoforms of lipoxygenase by ethylene (Dandekar et al 2004, Dandekar et al 2005a).

Since hexanal and various hexenals are produced by LOX, LOX activity was compared to hexanal, 2-hexenal, and cis-3-hexenal (Gardner 1995). Aldehyde emissions have not been studied as much as emissions of other volatiles. One study during ripening showed that hexanal and 2-hexenal increased about two weeks before climacteric, then sharply decreased (Mattheis et al 1991). In this study, headspace volatiles were not followed over such a long time period. All three aldehydes showed a slight non-significant increase before the start of ethylene production, and then decreased during ripening (Fig. 7C). This indicates that aldehydes are being consumed during ripening. Since no pre-ripening increase in aldehyde production was observed, no conclusion regarding aldehydes as the precursors that determine the increase in straight-chain

ester production can be reached. Again, the pattern of juice volatiles differs from the pattern of headspace volatiles, but the pattern over time of juice volatiles is not as interesting because it is less physiologically relevant. This is especially important because aldehyde levels are higher in crushed apple tissue (Dixon and Hewett 2000). Interestingly, LOX activity over time does not correlate with levels of headspace alcohols or esters (Fig. 7D).

Development during and after CA storage

To examine changes that take place during CA storage, we measured gene expression, activities, and volatiles the day fruit were put in storage and the day fruit were removed from storage. To the author's knowledge, gene expression of AAT, LOX, or ACCase has not been examined after CA storage in any member of the Rosaceae family. MpAAT1 and LOX1 gene expression stayed constant while ACCase gene expression decreased (Fig. 3). This regulation of ACCase supports the hypothesis that changes in ACCase levels are involved in regulation of straight-chain ester biosynthesis. Changes in AAT and LOX activity, although present, were not statistically significant. Both LOX and AAT activity have been reported to decline after CA storage (Fellman et al 2000, Lara et al 2007). These studies used apples that had been allowed to acclimate to air for at least 24 hours, which likely explains the difference. When changes in volatiles during CA storage were examined, esters and alcohols, including straight-chain esters and alcohols specifically examined, increased significantly during storage, while aldehydes decreased significantly (Table 2). This indicates that AAT and alcohol dehydrogenase (ADH) are working during low oxygen CA storage, while LOX is not. The LOX result is easily explained, as oxygen is a substrate for LOX.

To study the recovery of the straight chain ester pathway after CA storage, the same gene expressions, activities, and volatile levels were also measured 4 and 7 days after removal from CA storage. Ester emissions, including production of butyl acetate and hexyl acetate, recover after CA storage (Fig. 10C, 11B), as shown previously (Mattheis et al 1995). The recovery of butyl acetate and hexyl acetate was robust (Compare Fig. 11B to Fig. 6B), which indicates a strong recovery of the straight chain ester pathway. However, straight chain alcohol and aldehyde concentrations remain very low and do not change between 4 and 7 days after CA storage (Fig. 11B and 12C). Compared to normal ripening, headspace emissions of all three measured aldehydes were suppressed (compare Fig. 6C and Fig. 12C). Likely, they are being used by AAT and ADH immediately after they are produced. Interestingly, the ACC3 gene is regulated, showing an increase at 4 and 7 days after CA storage compared to the day after removal from CA (fig. 9A). This probably reflects the general restarting of metabolism after CA storage, although it could also play a role in the recovery of straight chain esters. LOX1 gene expression did not change, and LOX activity did not change at statistically significant levels after CA storage. In fact, LOX activity stays within approximately the same range of values that it had during on-tree ripening, showing that LOX activity fully recovers after CA storage. MpAAT1 gene expression seems to start to recover from its very low levels 7 days after CA storage. However, AAT activity is very low after CA storage (compare Fig. 10 with Fig. 5) and the slight increases during ripening after CA storage are not significant. The results of these experiments highlight the fact that some volatiles are unaffected by CA storage, some are suppressed, and some are stimulated (Yahia 1994). In whole fruit in these experiments, emissions of the important flavor volatiles hexyl acetate and butyl acetate are unaffected or even stimulated by

CA storage, while emissions of the important flavor volatiles hexanal, cis-3-hexenal, and 2-hexenal are suppressed by CA storage.

Regulation of straight chain ester synthesis

Based on many previous experiments, it was hypothesized that synthesis of precursors was of primary importance in determining levels of straight-chain ester volatiles. Evidence collected in this experiment did not confirm this. Specifically, measurements of whole-fruit volatile production showed that aldehyde precursors were available before parallel increases in ester synthesis and autocatalytic ethylene production. It would be expected that if aldehyde precursors were of primary importance to determining levels of straight-chain ester volatiles, aldehyde levels would stay steady or increase during ripening, reflecting increased production of aldehydes. In these experiments, a decrease in aldehyde levels took place during ripening, showing that aldehydes start at a high level before ripening and are likely being used up by other enzymes in the straight-chain ester pathway. Also, in these experiments lipoxygenase, which is involved in production of straight-chain aldehydes, decreases just before ester production increases, which likely is partially responsible for the decrease in aldehydes.

In contrast, in these experiments AAT production increases at 154 DAFB when ethylene production increases, and also increases at 161 and 165 DAFB. This could explain rises in straight-chain ester production that take place after 151 DAFB. However, ester levels increase starting at 151 DAFB. LOX was not measured in flesh tissue in this work, but an increase in LOX activity in apple flesh was found during early ripening by Echeverría et al (2004). This could explain the increase in ester levels. The increase could also be explained by an increase in carboxylic acid production. However, to the authors knowledge, no studies have been published

which track carboxylic acids involved in ripening over development. The increase in gene expression of ACC3 between 144 and 147 could be involved in the increase in esters at 151 DAFB. If this was true, the delay would be explained by the fact that the ACCase enzyme is the first step in the pathway leading to carboxylic acid synthesis, and an increase may take time to develop. As ethylene is undetectable before 151 DAFB, the increase in ACCase is likely not ethylene-dependent. It could be part of a general increase in metabolic activity that takes place during apple fruit ripening as reflected by the increase in respiration.

The hypothesis examined here was based on earlier studies involving either feeding experiments or mathematical modeling (see introduction). Feeding experiments showed that AAT activity exists in unripe apples but did not track AAT activity closely over development. These experiments indicate that a rise from low but measurable AAT activities may be partly responsible for increased ester production during ripening. Mathematical modeling highlighted interesting correlations but these may not always have been relevant to the physiology of the organ under study.

Origin of fatty acid precursors to straight chain volatile esters

The origin of fatty acid precursors to straight chain volatile esters has not been resolved. It was hoped that measurements of ACCase activity along with gene expression would help to determine if fatty acid synthesis plays an important role in production of fatty acids in ripening fruit. Some recent evidence indicates that fatty acid synthesis plays a role in ripening, including an experiment showing that ACCase is actively being transcribed and translated in ripening tomatoes (Kahlau and Bock 2008) and measurements that indicate some fatty acids increase during ripening (Song and Bangerth 1996, Song and Bangerth 2003, Defilippi et al 2005a).

Traditionally, fatty acids in ripening fruit have been thought to come from the breakdown of chloroplasts during ripening (Meigh and Hulme 1965, Galliard 1968). This work is inconclusive and could be used to support both hypotheses. The increases in ACC3 gene expression with ripening indicate that ACCase and thus fatty acid synthesis may be important in fruit ripening. However, the lack of demonstrable ACCase activity in this work (see Appendix A) indicates that ACCase activity is very low in apples, which suggests that breakdown of chloroplast lipids is a more likely source of fatty acids for volatile synthesis.

Maturity index

The dramatic change in LOX1 gene expression over early ripening may reveal a potential indicator for apple maturity, as the increases take place near an optimum maturity for harvest (see Fellman et al 2003). If LOX1 protein levels reflect LOX1 gene expression levels, an enzyme-linked immunosorbent assay (ELISA) indicator specific for LOX1 may be useful as a maturity indicator. Much more work, including measurements of LOX1 protein levels over maturity in many varieties of apples, would be needed to confirm or deny this.

Conclusions

1. Increases in AAT gene expression and activity after climacteric ethylene production starts may lead to increases in esters in freshly harvested fruit.

2. Higher ester production at the start of climacteric ripening in freshly harvested fruits is likely not caused by increases in LOX activity and aldehyde levels in the skin, although other published work indicates LOX activity in the flesh may be important.

3. The straight chain ester synthesis pathway makes a strong recovery after CA storage.

4. Trends during ripening in volatile content measured in juice do not match trends in volatile emissions found in headspace. Processing samples to measure volatile content disrupts metabolic processes taking place in whole fruit. Volatile content may be more important in studies of apple flavor than in studies of apple physiology.

5. Gene expression levels of at least one isoform of the acetyl-coA carboxylase gene are regulated during ripening, during CA storage, and after CA storage.

Fig. 13 shows a visual summary of the results of this work. At 144 DAFB, ethylene levels are very low, LOX activity and aldehyde levels are high, and AAT activity and associated straight-chain metabolite levels are low. At 151 DAFB, LOX activity and aldehydes are lower, while straight-chain ester levels are higher, although alcohol levels and AAT activity stays the same. At 161 DAFB, AAT activity increases. LOX activity is estimated to be similar to that at 151 DAFB (Fig. 4). Combining the results from headspace and juice volatile studies, it seems that straight-chain ester levels are dramatically higher at 161 DAFB while straight-chain alcohol levels are still low (Fig. 6A and 6B). As discussed earlier, during CA storage ADH and AAT activity are present, producing alcohols and esters, while LOX activity is absent. Seven days after CA storage, LOX activity has recovered, AAT activity has recovered to a lower level, and ester levels have recovered, although alcohols and aldehydes are low.

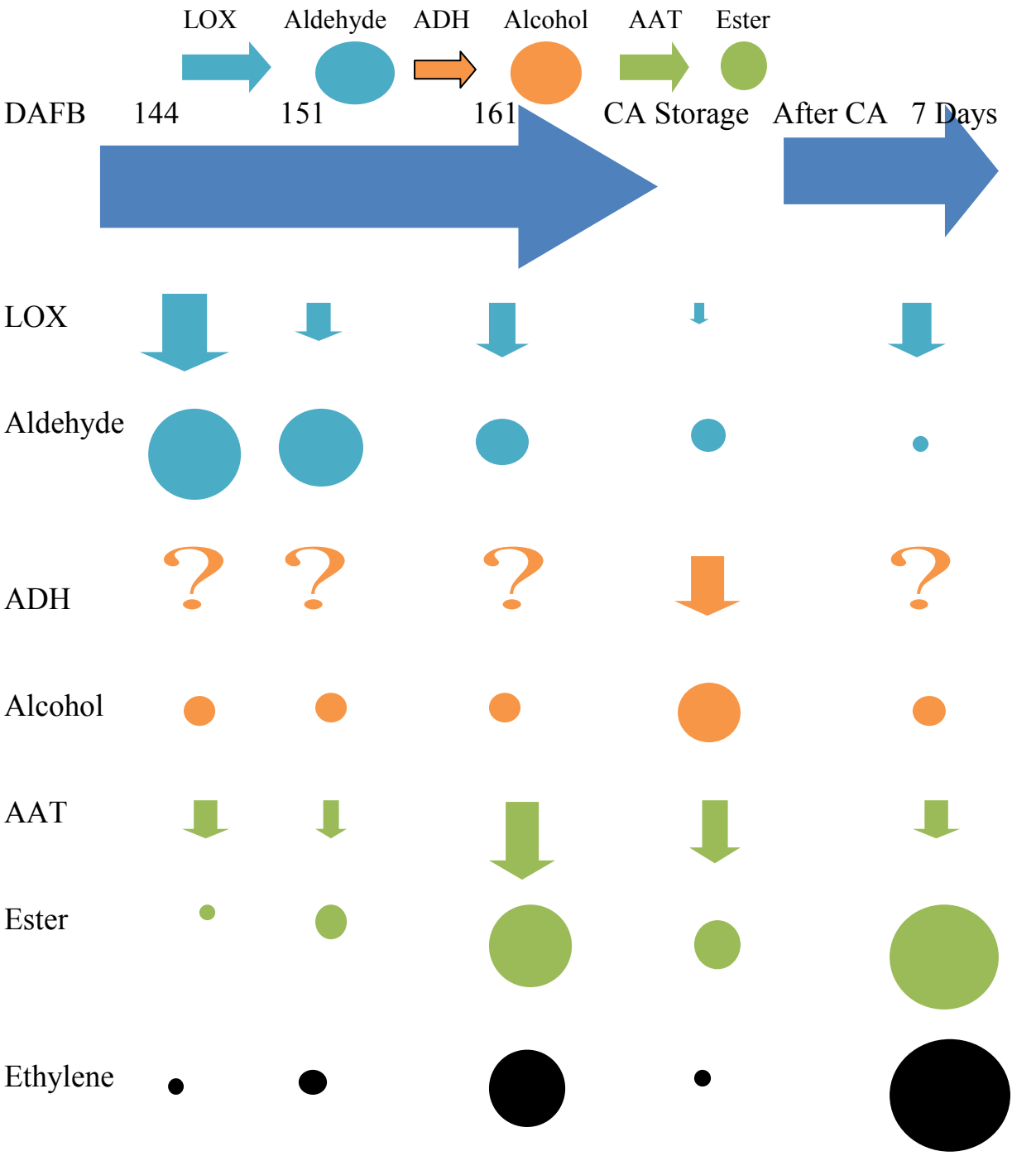


Fig. 13. Graphical representation of straight-chain ester biosynthesis pathway changes occurring during on-tree ripening and after storage of apples as described in this study. The straight-chain ester biosynthesis pathway along with associated symbols used in the figure is at the top. The dates of important changes in the pathway that were noted in this work are at the top of the figure, while the levels of enzymes or metabolites associated with these dates are represented below. A larger symbol represents a higher enzyme activity or greater amount of metabolite. Question marks for ADH indicate that ADH activity was not measured in this experiment. Activities during CA storage were estimated from changes in metabolite levels during CA storage, see discussion for details.

CHAPTER 5

SUGGESTIONS FOR FUTURE RESEARCH

Steps needed to improve the results contained in this work

1. The AAT activity measurements need to be improved. Instead of one extraction using 4 apples for each extraction date, it would be feasible to do two parallel extractions using samples from greater than 10 apples total on fresh tissue.
2. After removal from CA storage, the same data that were collected after 4 and 7 days need to be collected on both the day of removal (Day 0) and the day after removal (Day 1).
3. LOX1 is interesting enough to deserve a full-length sequence and an official name, and ACC3 should probably be sequenced too. RACE (Rapid Amplification of cDNA Ends) could be used to generate a full-length cDNA for both genes, which could then be sequenced.
4. The gene expression data in these experiments should also be collected using realtime RT-PCR.

Steps to further advance knowledge on apple flavor regulation

1. The expression of more isoforms of LOX genes could be examined during ripening and after CA storage.
2. LOX isoforms could be cloned and expressed in an *in vitro* system, and their specificity for either linoleic or linolenic acid could be determined.
3. The LOX activity measurements done in these experiments on skin tissue could be repeated on flesh tissue.

4. MdAAT2 gene expression data could be measured once every few days during ripening just like MdAAT1 was measured in this experiment.
5. Carboxylic acid levels could be followed throughout ripening and after storage.
6. The activity of hydroperoxide lyase could be followed throughout ripening and after storage.
7. Based on the work of Schaffer et al (2007), a potentially important carboxylesterase could be fully sequenced and followed throughout ripening and after storage. The activity of carboxylesterase could also be followed throughout ripening and after storage.
8. To determine if carboxylic acids or carboxylesterases are regulated by ethylene, fruits could be treated with 1-MCP. A comparison of carboxylic acid levels and carboxylesterase activity of 1-MCP treated fruits and control fruits over the same time period could then be made.

APPENDIX

APPENDIX A

Acetyl-coA carboxylase measurement

In a first attempt to measure activity, a phosphate sensor (Invitrogen) was used to measure phosphate produced during the course of the ACCase reaction. Extraction procedures were modified from Luo and Matsumoto (2002), including the addition of PVPP to remove polyphenols, and the reaction mixture was similar to Liu et al (2007). A phosphate mop (Brune et al 1994, Nixon et al 1998) was added to remove phosphate contamination competitively with the reaction (Martinez-Senac and Webb 2005). Although a variety of extraction procedures and concentrations of the phosphate mop were used, we did not successfully measure activity with this method. Since this method is not commonly used, and since, to the author's knowledge, it has not been tested in plant systems, it was not optimized extensively.

In a second attempt to measure ACCase activity, the commonly used radioactive bicarbonate assay was tried. The extraction procedure was modified from Luo and Matsumoto (2002), including the addition of PVPP to the extraction buffer to remove polyphenols. Reaction procedures were modified from Burke et al (2006), with the addition of $MgCl_2$ to the reaction buffer. Modifications were made to the dry down step, and varying amounts of radioactive bicarbonate, protein extract, and acetyl CoA were tried in the reaction. Although successful positive controls were done in extracts from wheat, we were unable to get conclusively positive readings from apple extracts. Nearly all corrected disintegrations per minute (DPM) readings (test – control) for apple were between positive and negative 300, and readings higher than this could not be replicated. Most corrected DPM readings were less than 200. The readings for apple were low enough that they could be interpreted as machine noise, especially considering the large number of negative corrected DPM readings. Although the reaction procedure was

optimized extensively, the extraction procedure was not. Specifically, raw protein extract for apples contains higher levels of protein, so the use of raw extract, while not ideal, may be necessary.

LITERATURE CITED

Altisent R., Echeverría G., Graell J., López L., and I. Lara. 2009. Lipoxygenase Activity Is Involved in the Regeneration of Volatile Ester-Synthesizing Capacity after Ultra-Low Oxygen Storage of 'Fuji' Apple. *J. Ag. Food Chem.* 57:4305–4312.

Axelrod B., Cheesbrough T.M, and S. Laakso. 1981. Lipoxygenase from soybeans. *Methods in Enzymology* 71:441-451.

Bannenberg G., Martínez M., Hamberg M., and C. Castresana. 2009. Diversity in the enzymatic activity of the lipoxygenase gene family in *Arabidopsis thaliana. Lipids* 44:85–95.

Bartley I.M. and S.J. Hindley. 1980. Alcohol dehydrogenase of apple. *J. Exp. Bot.* 121:449-459.

Bartley I.M., Stoker P.G., Martin A.D.E., Hatfield S.G.S. and M Knee. 1985. Synthesis of aroma compounds by apples supplied with alcohols and methyl esters of fatty acids. *J. Sci. Food Ag*. 36:567-574.

Berger R.G. and F. Drawert. 1984. Changes in the composition of volatiles by post-harvest application of alcohols to 'Red Delicious' apples. *J. Sci. Food Ag*. 35:1318-1325.

Bianco L., Lopez L., Scalone A.G., Carli M., di Desiderio A., Benvenuto E. and G. Perrotta. 2009. Strawberry proteome characterization and its regulation during fruit ripening and in different genotypes. *J. Proteomics* 72: 586-607.

Brown A.G. and D.M. Harvey. 1971. The nature and inheritance of sweetness and acidity in the cultivated apple. *Euphytica* 20:68-80.

Brune M., Hunter J.L., Corrie J.E.T., and M.R. Webb. 1994. Direct, real-time measurement of rapid inorganic phosphate release using a novel fluorescent probe and its application to actomyosin subfragment 1 ATPase. *Biochemistry* 33:8262-8271.

Burg S.P. and E.A. Burg. 1965. Ethylene action and the ripening of fruits: ethylene influences the growth and development of plants and is the hormone which initiates fruit ripening. *Science* 148:1190-1196.

Burke I.C., Burton J.D., York A.C., Cranmer J., and J.W. Wilcut. 2006. Mechanism of resistance to clethodim in a johnsongrass (Sorghum halepense) biotype. *Weed Science* 54:401-406.

Chu C.L. 1984. Use of Internal Ethylene Concentration as a maturity index of eleven apple cultivars. *Acta Hort*. 157:129-134.

Cunnigham D.G., Acree T.E., Barnard J., Butts R., and P. Braell. 1986. Charm analysis of apple volatiles. *Food Chem.* 19:137-147.

Dandekar A.M., Teo G., Defilippi B.G., Uratsu S.L., Passey A.J., Kader A.A., Stow J.R., Colgan R.J., and D.J. James. 2004. Effect of down-regulation of ethylene biosynthesis on fruit flavor complex in apple fruit. *Transgenic Res.* 13:373-384.

d'Auria J.C. 2006. Acyltransferases in plants: a good time to be BAHD. *Cur. Op. Plant Biol*. 9:331 -340.

Defilippi B.G., Dandekar A.M., and A.A. Kader. 2004. Impact of Suppression of Ethylene Action or Biosynthesis on Flavor Metabolites in Apple (*Malus domestica* Borkh) Fruits. *J. Ag. Food Chem.52:* 5694-5701.

Defilippi B.G., Dandekar A.M., and A.A. Kader. 2005a. Relationship of ethylene biosynthesis to volatile production, related enzymes, and precursor availability in apple peel and flesh tissues. *J. Ag. Food Chem.* 53: 3133-3141.

Defilippi B.G., Kader A.A., and A.M. Dandekar. 2005b. Apple aroma: alcohol acyltransferase, a rate limiting step for ester biosynthesis, is regulated by ethylene. *Plant Sci.* 168:1199-1210.

De Pooter H.L., Montens J.P., Willaert G.A., Dirinck P.J., and N.M. Schamp. 1983. Treatment of golden delicious apples with aldehydes and carboxylic acids: effect on the headspace composition. *J. Ag. Food Chem.* 31:813-818.

De Pooter, H.L., Van Acker, M.R. and N.M. Schamp. 1987. Aldehyde metabolism and the aroma quality of stored Golden Delicious apples. *Phytochemistry* 26:89-92.

De Pooter, H.L. and N.M. Schamp. 1989. The study of aroma formation and ripening of apples cv. Golden Delicious by headspace analysis. *J. Essent. Oil Res.* 2:47-56.

Dixon J. and E. Hewett. 2000. Factors affecting apple aroma/flavour volatile concentration: a review. *NZ J. Crop Hort. Sci*. 28:155-173.

Dudareva N., Negre F., Nagegowda D.A., and I. Orlova. 2006. Plant volatiles: recent advances and future perspectives. *Crit. Rev. Plant Sci.* 25:417-440.

Dürr P. and U. Schobinger. 1981. The contribution of some volatiles to the sensory quality of apple and orange juice odor, p. 179-193. *In*: P. Schreier (Ed.). Flavour '81. Walter de Gruyter, Berlin.

Echeverría G., Graell J., López M.L., and I. Lara. 2004. Volatile production, quality and aroma-related enzyme activities during maturation of 'Fuji' apples. *Postharvest Biol. Technol.* 31:217-227.

Fan X., Mattheis, J.P. and D. Buchanan. 1998. Continuous Requirement of Ethylene for Apple Fruit Volatile Synthesis. *J. Ag. Food Chem.* 46:1959-1963.

Farhoomand, M.B., Patterson, M.E., and C.L. Chu. 1977. The Ripening Pattern of 'Delicious' Apples in Relation to Position on the Tree. *J. Am. Soc. Hort. Sci.* 102:771-774.

Fellman J.K., Miller T.W., and D.S. Mattinson. 2000. Factors that influence biosynthesis of volatile flavor compounds in apple fruits. *HortScience* 35:1026-1033.

Fellman J.K., Rudell D.R., Mattinson D.S., and J.P. Mattheis. 2003. Relationship of harvest maturity to flavor regeneration after CA storage of 'Delicious' apples. *Postharvest Biol. Technol*. 27:39-51.

Ferenczi A., Song J., Tian M., Vlachonasios K., Dilley D., and R. Beaudry. 2006. Volatile Ester Suppression and Recovery following 1-Methylcyclopropene Application to Apple Fruit. *J. Am. Soc. Hort. Sci.* 131:691-701.

Feys M., Naesens W., Tobback P. and M Edward. 1980. Lipoxygenase Activity in Apples in Relation to Storage and Physiological Disorders. *Phytochemistry* 19:1009-1011.

Figueroa-Balderas, R.E. Garcia-Ponce, B. and M. Rocha-Sosa. 2006. Hormonal and stress induction of the gene encoding common bean acetyl-coenzyme A carboxylase. *Plant Phys.* 142: 609-619.

Flath R., Black D.R., Guadagni D.G., McFadden W.H., and T.H. Schultz. 1967. Identification and organoleptic evaluation of compounds in Delicious apple essence. *J. Ag. Food Chem.* 15:29-35.

Galliard, T. 1968. Aspects of lipid metabolism in higher plants-II. The identification and quantitative analysis of lipids from the pulp of pre- and postclimacteric apples. *Phytochemistry 7:* 1915-1922.

Gardner H.W. 1995. Biological Roles and Biochemistry of the Lipoxygenase Pathway. *HortScience* 30:197-205.

Goodenough P.W. and T.G. Entwistle. 1982. The hydrodynamic properties and kinetic constants with natural substrates of the esterase from *Malus Pumila. Eur. J. Biochem*. 127:145-149.

Goulao L.F. and C.M. Oliveira. 2007. Molecular identification of novel differentially expressed mRNAs up-regulated during ripening of apples. *Plant Sci.* 172:306-318.

Guadagni D.G., Bomben J.L., and J.S. Hudson. 1971. Factors influencing the development of aroma in apple peels. *J. Sci. Food Ag.* 22:110-115.

Haller, M.H. and E. Smith. 1950. Evaluation of indexes of maturity for apples. USDA Technical Bulletin No. 1003.

Harb J., Streif J., and F. Bangerth. 2008. Aroma volatiles of apples as influenced by ripening and storage procedures. *Acta Hort.* 796:93-103.

Holland D., Larkov O., Ya'akov I.B., Bar E., Zax A., Brandeis E., Ravid U., and E. Lewinsohn. 2005. Developmental and varietal differences in volatile ester formation and acetyl-CoA:alcohol acetyl transferase activities in apple (Malus domestica Borkh.) fruit. *J. Ag. Food Chem.* 53:7198-7203.

Johnston J.W., Gunaseelan K., Pidakala P., Wang M. and R.J. Schaffer. 2009. Co-ordination of early and late ripening events in apples is regulated through differential sensitivities to ethylene. *J. Exp. Bot.* 60:2689–2699.

Kader A.A. 2004. Controlled Atmosphere storage. *In* Agriculture Handbook Number 66: The Commercial Storage of Fruits, Vegetables, and Florist and Nursery Stocks. USDA, ARS.

Kahlau S., and R. Bock. 2008. Plastid transcriptomics and translatomics of tomato fruit development and chloroplast-to-chromoplast differentiation: chromoplast gene expression largely serves the production of a single protein. *Plant Cell* 20: 856–874.

Kidd, F. and C. West. 1945. Respiratory activity and duration of life of apples gathered at different stages of development and subsequently maintained at a constant temperature. *Plant Phys.* 20:467-504.

Kim I.S. and W. Grosch. 1979. Partial purification of a lipoxygenase from apples, *J. Ag. Food Chem*. 27:243-246.

Knee M. and S.G.S. Hatfield. 1981. The metabolism of alcohols by apple fruit tissue. *J. Sci. Food Ag.* 32:593-600.

Lara I., Graell J., Lòpez M.L., and G. Echeverrìa. 2006. Multivariate analysis of modifications in biosynthesis of volatile compounds after CA storage of ‘Fuji’ apples. *Postharvest Biol. Technol.* 39:19-28.

Lara I., Echeverrìa G., Graell J., and M.L. Lòpez. 2007. Volatile Emission after Controlled Atmosphere Storage of Mondial Gala Apples (Malus domestica): Relationship to Some Involved Enzyme Activities. *J. Ag. Food Chem.* 55: 6087-6095.

Lelièvre J.M., Latché A., Jones, B., Bouzayen M and J-C Pech. 1997. Ethylene and fruit ripening. *Phys. Plantarum* 101:727-739.

Li, D.P., Xu, Y.F., Sun. L.P., Liu, L.X., Hu, X.L., Li, D.Q. and H.R. Shu. 2006. Salicylic acid, ethephon, and methyl jasmonate enhance ester regeneration in 1-MCP-treated apple fruit after long-term cold storage. *J. Ag. Food Chem.* 54:3887-3895.

Lin S-F and C.S. Walsh. 2008. Studies of the tree factor and its role in the maturation and ripening of 'Gala' and 'Fuji' apples. *Postharvest Biol. Technol.* 48:99-106.

Liu Y., Zalameda L., Kim K.W., Wang M., and J.D. McCarter. 2007. Discovery of Acetyl-Coenzyme A Carboxylase 2 Inhibitors: Comparison of a Fluorescence Intensity-Based Phosphate Assay and a Fluorescence Polarization-Based ADP Assay for High-Throughput Screening. *ASSAY and Drug Dev. Technol.* 5:225-234.

Luo X-Y and H. Matsumoto. 2002. Susceptibility of a broad-leaved weed, *Acanthospermum hispidum*, to the grass herbicide fluazifop-butyl. *Weed Biol. Management* 2:98-103.

Lurie S., Pre-Aymard C., Ravid U., Larkov O., and E. Fallik. 2002. Effect of 1-methylcyclopropene on volatile emission and aroma in cv. Anna apples. *J. Ag. Food Chem. 50*: 4251-4256.

Manning K. 1991. Isolation of nucleic acids from plants by differential solvent precipitation. *Anal. Biochem.* 195:45-50.

Martinez-Senac M.M. and M.R. Webb. 2005. Mechanism of Translocation and Kinetics of DNA Unwinding by the Helicase RecG. *Biochemistry* 44:16967-16976.

Mattheis J.P., Fellman J.K., Chen P.M., and M.E. Patterson. 1991. Changes in Headspace Volatiles during Physiological Development of Bisbee Delicious Apple Fruit. *J. Ag. Food Chem.* 39:1902-1906.

Mattheis J.P., Buchanan D.A., and J.K. Fellman. 1995. Volatile compound production by Bisbee Delicious apples after sequential atmosphere storage. *J. Ag. Food Chem.* 43:194-199.

Mattheis J.P., Fan X. and L.C. Argenta. 2005. Interactive Responses of Gala Apple Fruit Volatile Production to Controlled Atmosphere Storage and Chemical Inhibition of Ethylene Action *J. Ag. Food Chem*. 53:4510-4516.

Meigh D.F., and A.C. Hulme. 1965. Fatty Acid Metabolism in the Apple Fruit during the respiration climacteric. *Phytochemistry* 4: 863-871.

Meigh D.F.**,** Jones J.D., and A.C. Hulme. 1967. The respiration climacteric in the apple: production of ethylene and fatty acids in fruit attached to and detached from the tree. *Phytochemistry* 6: 1507-1515.

Mekhedov S., Martínez de Ilárduya O., and J. Ohlrogge. 2000. Toward a functional catalog of the plant genome: a survey of genes for lipid biosynthesis. *Plant Phys*. 122:389-401.

Miller T.W., Fellman J.K., Mattheis J.P., Mattinson D.S. 1998. Factors that influence volatile ester biosynthesis in 'Delicious' apples. *Acta Hort* 464:195-200.

Nakkaew A., Chotigeat W., Eksomtramage T., and A. Phongdara. 2008. Cloning and expression of a plastid-encoded subunit, beta-carboxyltransferase gene (accD) and a nuclear-encoded subunit, biotin carboxylase of acetyl-CoA carboxylase from oil palm (Elaeis guineensis Jacq.). *Plant Sci.* 175:497-504.

Nixon A.E., Hunter J.L., Howell S.A., Bonifacio G., Eccleston J.F., and M.R. Webb. 1998. Purine nucleoside phosphorylase: its use in a spectroscopic assay for inorganic phosphate with the aid of phosphodeoxyribomutase. *Anal. Biochem.* 265:299-307.

Ohlrogge J.B. and J.G. Jaworski. 1997. Regulation of fatty acid synthesis..*Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:109-136.

Paillard, N.M.M. 1979. Biosynthesis of apple volatiles: formation of alcohols and esters from fatty acids. *Phytochemistry* 18:1165-1171.

Paillard N.M.M. 1986. Evolution of the capacity of aldehyde production by crushed apple tissues, during an extended storage of fruits, p. 369-378. *In*: G. Charalambous (ed.). The shelf life of foods and beverages. Elsevier Science, Amsterdam.

Paillard N.M.M. 1990. The flavour of apples, pears, and quinces, p. 1-41. *In*: I. Marton and A.J. McLeod (eds.). Food Flavours Part C: The Flavour of Fruits. Elsevier, Amsterdam.

Panasiuk O., Talley F.B., and G.M. Sapers. 1980. Correlation between aroma and volatile composition of McIntosh apples. *J. Food Sci.* 45:989-991.

Park S., Sugimoto N., Larson M.D., Beaudry R., and S. van Nocker. 2006. Identification of genes with Potential Roles in Apple Fruit Development and Biochemistry through Large-Scale Statistical Analysis of Expressed Sequence Tags. *Plant Phys.* 141:811-824.

Patterson B.D., Hatfield S.G.S., and M. Knee. 1974. Residual effects of controlled atmosphere storage on the production of volatile compounds by two varieties of apples. *J. Sci. Food Ag.* 25:843-849.

Petró-Turza M., Szarföldi-Szalma I., Madarassy-Mersich E., Teleky-Vamossy, G. and K. Füzesi-Kardos. 1986. Correlation between chemical composition and sensory quality of natural apple aroma condensates. *Nahrung* 30:765-774.

Rhodes M.J.C. and L.S.C. Wooltorton. 1967. The respiration climacteric in apple fruits. : The action of hydrolytic enzymes in peel tissue during the climacteric period in fruit detached from the tree. *Phytochemistry* 6:1-12.

Rizzolo A., Grassi M., and P. Eccher-Zerbini. 2006. Influence of harvest date on ripening and volatile compounds in the scab-resistant apple cultivar 'Golden Orange' *J. Hort. Sci. Biotech.* 81:681-690.

Rowan D.D., Lane H.P., Allen J.M., Fielder S. and M.B. Hunt. 1996. Biosynthesis of 2-Methylbutyl, 2-Methyl-2-butenyl, and 2-Methylbutanoate Esters in Red Delicious and Granny Smith Apples Using Deuterium-Labeled Substrates. *J. Ag. Food Chem.* 44:3276-3285.

Rowan D.D., Allen J.M., Fielder S., and M.B. Hunt. 1999. Biosynthesis of Straight-Chain Ester Volatiles in Red Delicious and Granny Smith Apples Using Deuterium-Labeled Precursors. *J. Ag. Food Chem.* 47:2553-2562.

Rudell, D.R. 2000. Studies of apple fruit ripening and aroma volatile biosynthesis for improving harvest timing and post-storage quality. Master's Thesis, Washington State University, Pullman, WA.

Rudell, D. R., Mattinson, D. S., Mattheis, J. P., Wyllie, S. G., and J.K. Fellman. 2002. Investigations of aroma volatile biosynthesis under anoxic conditions and in different tissues of 'Redchief Delicious' apple fruit (*Malus* × *domestica* Borkh.). *J. Ag. Food Chem*. 50:2627-2632.

Sasaki Y. and Y. Nagano. 2004. Plant acetyl-coA carboxylase: structure, biosynthesis, regulation, and gene manipulation for plant breeding. *Biosci. Biotechnol. Biochem.* 68:1175-1184.

Schaffer R.J., Friel E.N., Souleyre E.J.F., Bolitho K., Thodey K., Ledger S., Bowen J.H., Ma J-H., Nain B., Cohen D., Gleave A.P., Crowhurst R.N., Janssen B.J., Yao J-L., and R.D. Newcomb. 2007. A Genomics Approach Reveals That Aroma Production in Apple Is Controlled by Ethylene Predominantly at the Final Step in Each Biosynthetic Pathway. *Plant Physiol.* 144:1899-1912.

Smock R.M. 1979. Controlled Atmosphere storage of fruits. *Hort. Rev.* 1:301-336.

Song J. and F. Bangerth. 1996. The effect of harvest date on aroma compound production from 'Golden Delicious' apple fruit and relationship to respiration and ethylene production. *Postharvest Biol. Technol.* 8:259-269.

Song J, and F. Bangerth. 2003. Fatty acids as precursors for aroma volatile biosynthesis in pre-climacteric and climacteric apple fruit. *Postharvest Biol. Technol*. 30:113-121.

Souleyre E.J.F., Greenwood D.R., Friel E.N., Karunairetnam S. and R.D. Newcomb. 2005. An alcohol acyl transferase from apple (cv. Royal Gala), MpAAT1, produces esters involved in apple fruit flavor. *FEBS Journal* 272: 3132–3144.

Villatoro C., Altisent R., Echeverrìa G., Graell J., López M.L., and I. Lara. 2008. Changes in biosynthesis of aroma volatile compounds during on-tree maturation of 'Pink Lady' apples. *Postharvest Biol. Technol.* 47: 286-295.

Watkins, C., Kupferman, E., and D. Rosenberger. 2004. "Apple". *In* Agriculture Handbook Number 66: The Commercial Storage of Fruits, Vegetables, and Florist and Nursery Stocks. USDA, ARS.

Yahia E.M., Acree T.E., and F.W. Liu. 1990. The evolution of some odour-active volatiles during the maturation and ripening of apples on the tree. *Lebensm.-Wiss. Technol.* 23:488-493.

Yahia E. M., Liu F.W., and T.E. Acree. 1990b. Changes of some odor-active volatiles in controlled atmosphere- stored apples. *J. Food Quality* 13:185-202.

Yahia E.M. 1994. Apple flavor. *Hort. Rev.* 16:197-234.

Yang, X. and T. Peppard. 1994. Solid-Phase micorextraction for flavor analysis. *J. Ag. Food Chem*. 42: 1925-1930.

Young H., Rossiter K., Wang M., and M. Miller. 1999. Characterization of Royal Gala Apple Aroma Using Electronic Nose Technology ─ Potential Maturity Indicator. *J. Ag. Food Chem.*47: 5173-5177.

Zhu Y., Rudell D.R., and J.P. Mattheis. 2008. Characterization of cultivar differences in alcohol acyltransferase and 1-aminocyclopropane-1-carboxylate synthase gene expression and volatile ester emission during apple fruit maturation and ripening. *Postharvest Biol. Technol*. 49:330-339.

Zuther E., Huang S., Jelenska J., Eilenberg H., Arnold E.M., Su X., Sirikhachornkit A., Podkowinski J., Zilberstein A., Haselkorn R., and P. Gornicki. 2004. Complex nested promoters control tissue-specific expression of acetyl-CoA carboxylase genes in wheat. *PNAS* 101:1403-1408.