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VOLATILE BIOSYNTHESIS DURING RIPENING OF
'JONAGOLD' APPLE FRUIT: ASSOCIATION OF GENE
EXPRESSION WITH AROMA VOLATILES

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VOLATILE BIOSYNTHESIS DURING RIPENING OF 'JONAGOLD' APPLE FRUIT: ASSOCIATION OF GENE EXPRESSION WITH AROMA VOLATILES

By

Nobuko Sugimoto

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ABSTRACT

VOLATILE BIOSYNTHESIS DURING RIPENING OF 'JONAGOLD' APPLE FRUIT: ASSOCIATION OF GENE EXPRESSION WITH AROMA VOLATILES

By

Nobuko Sugimoto

Changes in the volatiles produced by 'Jonagold' apple (*Malus* x *domestica* Borkh) during ripening and senescence were related to changes in gene expression using a cDNA-based microarray containing over 10,000 gene fragments. Patterns for aroma biosynthesis, internal ethylene content, respiration, skin color, starch, and texture were typical for climacteric fruit. Volatile compounds and CO_2 increased after a rapid increase in ethylene production. Straight-chain esters hexyl acetate and butyl acetate and branched-chain esters 2-methylbutyl acetate and hexyl 2-methylbutanoate were found to be the major esters detected by GC/MS. Long chain esters predominated during the early stages of ripening and short chain esters increased later in proportion. Generally, the alcohols increased at an earlier development stage than the esters for which they acted as substrates. Esters are formed by combining alcohol with CoA derivative of fatty acid by the action of alcohol acyltransferase (AAT). Patterns in gene expression reflecting the rise and fall in ester formation were found in some putative genes for amino acid metabolism (branched-chain aminotransferase and branched-chain α-keto acid decarboxylase), fatty acid metabolism, and ester formation. The pathway for branched-chain ester biosynthesis is discussed.

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TABLE OF CONTENTS

LIST OF TABLES v

LIST OF FIGURES xiii

CHAPTER I
INTRODUCTION 1
    References 4

CHAPTER II
LITERATURE REVIEW 5
    Flavor 6
    Apple aroma 8
    Aroma physiology 10
        Cultural practices affecting aroma 11
        Timing of harvest 15
    Ester precursor formation 15
        Catabolic pathways 16
        Synthetic pathways 24
    Ester synthesis 27
    Hypothesis 30
    References 33

CHAPTER III
CHARACTERIZATION OF VOLATILE ESTER BIOSYNTHESIS BY 'JONAGOLD' APPLES DURING THE RIPENING PROCESS 54
    Introduction 55
    Materials and Methods 57
    Results 61
    Discussion 64
    Conclusion 70
    References 72

CHAPTER IV
GENE EXPRESSION ASSOCIATED WITH BRANCHED-CHAIN ESTER FORMATION IN 'JONAGOLD' APPLE FRUIT 114
    Introduction 115
    Materials and Methods 118
    Results 126
    Discussion 129
    Conclusion 136
    References 137

APPENDIX 154

LIST OF TABLES

CHAPTER 2

Table 1. Representative esters identified in apples with sensory description. Only esters that had an odor description are listed. Esters included acetates, propanoates, 2-methylpropanoates, butanoates, 2-methylbutanoates, pentanoates, and hexanoates. 47

CHAPTER 3

Table 2. Matrix of esters detected organized by acid and alcohol precursors for apple fruit. * indicates that the acid and alcohol combinations are reciprocal. Numbers indicate the maximum GC/MS response (TIC) detected during preclimacteric to postclimacteric stages of fruit ripening in millions. 77

CHAPTER 4

Table 3. Constraint table defining expression limits for microarray elements to identify candidate gene fragments for sequencing. Constraints were developed to identify changes in expression associated with eight distinct developmental stages (Days 0, 11, 25, 32, 39, 49, 60, and 70) during ripening for 'Jonagold' apple fruit. Expression levels are relative to Day 0 (in $\log_2$ scale). Categories are based on the pattern of expression and numbers denote the day following which, pattern changes were detected; 'low' indicates expression patterns that declined and 'high' indicates expression patterns that increased; 'middle' indicates no change in pattern from Day 0 through Day 70; 'peak' indicates a transient increase in gene expression on the indicated day; and 'double peak' indicates an expression pattern with peaks on Days 25 and 39. 142

Table 4. Gene with accession number and primer list for semi-quantitative RT-PCR. Apple cluster numbers are from Tree Fruit Technology genomic analysis tool apple database v.3.0 (http://genomics.msu.edu/fruitdb/analyses/apple.shtml). Accession and GI number indicates the longest EST from apple clusters. Genes include for which PCR was not successful. Single number in 'cycles needed' indicates that the same cycle was performed with both biological replications, rep. 1 and 2. Two numbers indicate that different cycles were performed between biological replications for optimum result. 143

APPENDIX

Table 5. Original data for Figure 10 (Chapter 3). Data are internal ethylene content, CO2 production, and the GC/MS response total ion count (TIC) for all aroma volatiles. Each value is the average of four replications. .. 155

Table 6. Original data for Figure 11 (Chapter 3). Force (N) for bending/tensile failure and compressive failure during ripening and senescence of 'Jonagold' apple fruit. 156

Table 7. Original data for Figure 12 (Chapter 3). Data are volatile compounds, GC retention time (second), and classes (esters, alcohols, and aldehydes) identified from 'Jonagold' apple fruit during ripening and senescence. . 157

Table 8. Original data for Figure 13A and 14A (Chapter 3). Data are the GC/MS response (total ion count, TIC) of alcohols produced by 'Jonagold' apple fruit during ripening and senescence. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications. 158

Table 9. Original data for Figure 13B and 14B (Chapter 3). Data are the percentages that each alcohol class comprises of all alcohols detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no alcohols of these classes were detected. 159

Table 10. Original data for Figure 14A (Chapter 3). Data are the GC/MS response (total ion count) of esters arranged by their alkyl (alcohol-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. 160

Table 11. Original data for Figure 14B (Chapter 3). Data are the percentages that each ester class comprises of all esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Esters are arranged by the source of the alkyl (alcohol-derived) moiety. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected. 161

Table 12. Original data for Figure 15C (Chapter 3). Data are the GC/MS response (total ion count, TIC) of ethanol esters arranged by their alkanoate (acid-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the

unique ion by the ion fraction. Each value is the average of four replications. 162

Table 13. Original data for Figure 15E (Chapter 3). Data are the percentages that each ester class comprises of all ethanol esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected. 163

Table 14. Original data for Figure 16C (Chapter 3). Data are the GC/MS response (total ion count, TIC) of propanol esters arranged by their alkanoate (acid-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications. 164

Table 15. Original data for Figure 16E (Chapter 3). Data are the percentages that each ester class comprises of all propanol esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected. 165

Table 16. Original data for Figure 17C (Chapter 3). Data are the GC/MS response (total ion count, TIC) of butanol esters arranged by their alkanoate (acid-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications. 166

Table 17. Original data for Figure 17E (Chapter 3). Data are the percentages that each ester class comprises of all butanol esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected. 168

Table 18. Original data for Figure 18B (Chapter 3). Data are the GC/MS response (total ion count, TIC) of pentanol esters arranged by their alkanoate (acid-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a

single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications. 169

Table 19. Original data for Figure 18C (Chapter 3). Data are the percentages that each ester class comprises of all pentanol esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected. 170

Table 20. Original data for Figure 19C (Chapter 3). Data are the GC/MS response (total ion count, TIC) of hexanol esters arranged by their alkanoate (acid-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications. 171

Table 21. Original data for Figure 19E (Chapter 3). Data are the percentages that each ester class comprises of all hexanol esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected. 172

Table 22. Original data for Figure 20C (Chapter 3). Data are the GC/MS response (total ion count, TIC) of 2-methylbutanol esters arranged by their alkanoate (acid-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications. 173

Table 23. Original data for Figure 20E (Chapter 3). Data are the percentages that each ester class comprises of all 2-methylbutanol esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected. 174

Table 24. Original data for Figure 21A (Chapter 3). Data are the GC/MS response (total ion count) of esters arranged by their alkanoate (acid-

derived) moiety during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. 175

Table 25. Original data for Figure 21B (Chapter 3). Data are the percentages that each ester class comprises of all esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Esters are arranged by the source of the alkanoate (acid-derived) moiety. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected. 176

Table 26. Original data for Figure 22B (Chapter 3). Data are the GC/MS response (total ion count, TIC) of acetate esters arranged by their alkyl (alcohol-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications. 177

Table 27. Original data for Figure 22C (Chapter 3). Data are the percentages that each ester class comprises of all acetate esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected. 178

Table 28. Original data for Figure 23B (Chapter 3). Data are the GC/MS response (total ion count, TIC) of propanoate esters arranged by their alkyl (alcohol-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications. 179

Table 29. Original data for Figure 23C (Chapter 3). Data are the percentages that each ester class comprises of all propanoate esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected. 180

Table 30. Original data for Figure 24B (Chapter 3). Data are the GC/MS response (total ion count, TIC) of butanoate esters arranged by their alkyl (alcohol-derived) moiety during ripening and senescence of 'Jonagold'

apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications. 181

Table 31. Original data for Figure 24C (Chapter 3). Data are the percentages that each ester class comprises of all butanoate esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected. 182

Table 32. Original data for Figure 25B (Chapter 3). Data are the GC/MS response (total ion count, TIC) of hexanoate esters arranged by their alkyl (alcohol-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications. 183

Table 33. Original data for Figure 25C (Chapter 3). Data are the percentages that each ester class comprises of all hexanoate esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected. 184

Table 34. Original data for Figure 26B (Chapter 3). Data are the GC/MS response (total ion count, TIC) of octanoate esters arranged by their alkyl (alcohol-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications. 185

Table 35. Original data for Figure 26C (Chapter 3). Data are the percentages that each ester class comprises of all octanoate esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected. 186

Table 36. Original data for Figure 27B (Chapter 3). Data are the GC/MS response (total ion count, TIC) of 2-methylbutanoate esters arranged by their alkyl (alcohol-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications. 187

Table 37. Original data for Figure 27C (Chapter 3). Data are the percentages that each ester class comprises of all 2-methylbutanoate esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected. 188

Table 38. Original data for Figure 31 (Chapter 4). Data are internal ethylene content, CO2 production, and the GC/MS response total ion count (TIC) for all aroma volatiles. Each value is the average of four replications. .. 189

Table 39. Original data for Figure 32A and 32C (Chapter 4). Data are the GC/MS response (total ion count, TIC) of 2-methylbutanol esters arranged by their alkanoate (acid-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications. 190

Table 40. Original data for Figure 32A and 32D (Chapter 4). Data are the GC/MS response (total ion count, TIC) of 2-methylbutanoate esters arranged by their alkyl (alcohol-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications. 191

Table 41. Original data for Figure 32B (Chapter 4). Data are the GC/MS response (total ion count, TIC) of 2-methylbutanol and 2-methylbutanal produced by 'Jonagold' apple fruit during ripening and senescence. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications. 192

Table 42. Original data for Figure 32E (Chapter 4). Data are the percentages that each ester class comprises of all 2-methylbutanoate esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected. 193

Table 43. Original data for Figure 33 (Chapter 4). Data are the relative luminosity (log2) of microarray elements compared to Day 0 for branched-chain aminotransferase (BCAT), pyruvate decarboxylase (PDC), and 2-isopropylmalate synthase during ripening and senescence of 'Jonagold' apple fruit. 194

Table 44. Original data for Figure 34 (Chapter 4). Data are luminosity of electrophoresis agarose gel spots relative to the maximum value for each gene from semi-quantitative RT-PCR products. The spot measurements are for branched-chain aminotransferase (BCAT) genes during ripening and senescence of 'Jonagold' apple fruit. All data were normalized relative to control gene 18s rRNA luminosity. Each value is the average of two replications. Figures below are original images from which luminosity data were extracted. 195

Table 45. Original data for Figure 35 (Chapter 4). Data are luminosity of electrophoresis agarose gel spots relative to the maximum value for each gene from semi-quantitative RT-PCR products. The spot measurements are for pyruvate decarboxylase (PDC) genes during ripening and senescence of 'Jonagold' apple fruit. All data were normalized relative to control gene 18s rRNA luminosity. Each value is the average of two replications. Figures below are original images from which luminosity data were extracted. 197

Table 46. Original data for Figure 36 (Chapter 4). Data are luminosity of electrophoresis agarose gel spots relative to the maximum value for each gene from semi-quantitative RT-PCR products. The spot measurements are for 2-isopropylmalate synthase gene during ripening and senescence of 'Jonagold' apple fruit. All data were normalized relative to control gene 18s rRNA luminosity. Each value is the average of two replications. Figures below are original images from which luminosity data were extracted. 198

LIST OF FIGURES

CHAPTER 2

Figure 1. Pathways having potential to supply alcohol and acyl-CoA substrates for ester formation. 48

Figure 2. Pathway for catabolism of fatty acids via β-oxidation. In plants, β-oxidation takes place in peroxisomes. Free fatty acid Cn acyl-CoA are reduced by two carbons during each cycle of β-oxidation by four steps. 1. Dehydrogenation by acyl-CoA oxidase, 2. addition of water by 2-trans-enoyl-CoA hydratase, 3. dehydrogenation by L-3-hydroxyacyl-CoA dehydrogenase, and 4. cleavage of acetyl-CoA by 3-ketoacyl-CoA thiolase to produce Cn-2 acyl-CoA. Stars indicate the carbon position during each reaction. Hydrogens in the carbon-hydrogen bonds are not shown. 49

Figure 3. Pathway for catabolism of lipids and fatty acids via lipoxygenase. In plants, lipoxygenase activity are found to be in chloroplasts (Hatanaka, 1993). Linoleic (18:2) or linolenic (18:3) acids are formed by lipase. Lipoxygenase peroxidizes linoleic and linolenic acid to 13-hydroperoxy linoleic acid (13-HPOD) and 13-hydroperoxy linolenic acid (13-HPOT), respectively. Hydroperoxide lyase cleaves 13-HPOD and 13-HPOT into hexanal and cis-3-hexenal, respectively. Hexanal and cis-3-hexenal is reduced by alcohol dehydrogenase to hexanol and cis-3-hexenol respectively. Hydrogens in the carbon-hydrogen bonds are not shown. . 50

Figure 4. Putative (dashed lines) and demonstrated (solid lines) pathways involved in branched-chain ester biosynthesis. * Indicates that gene is found in bacteria, but not in plants. Hydrogens in the carbon-hydrogen bonds are not shown. Stars and circles indicate the carbon position during each reaction. 51

Figure 5. Pathway for fatty acid biosynthesis through two-carbon chain elongation in plants. Acetyl-ACP (primer) and malonyl-ACP (chain extender) is condensed by 3-ketoacyl-ACP synthase III. The next 3 steps are reduction by 3-ketoacyl-ACP reductase, dehydration by 3-hydroxyacyl-ACP dehydrase, and reduction by enoyl-ACP reductase. The cycle repeats to condense with malonyl-ACP until the chain length is 16-18, in general. The final step is terminated by acyl-ACP thioesterase by hydrolysis. Stars indicate the carbon position during each reaction. Hydrogens in the carbon-hydrogen bonds are not shown. 52

Figure 6. Pathway for fatty acid biosynthesis through single-carbon chain elongation (α-keto acid elongation, αKAE) in plants. Pyruvate (primer) and acetyl-CoA (chain extender) is condensed by 2-isopropylmalate synthase.

The next 2 steps are isomerization and dehydration by isopropylmalate dehydratase, and decarboxylation by 3-isopropylmalate dehydrogenase. The cycle repeats to condense with acetyl-CoA or terminated to produce acyl-CoA. Stars indicate the carbon position during each reaction. Hydrogens in the carbon-hydrogen bonds are not shown. 53

CHAPTER 3

Figure 7. Pathways having potential to supply alcohol and acyl-CoA substrates for ester formation. 78

Figure 8. Tensile failure was measured on bars of cortex tissue using a 3-point bending rig. The tissue bars were square in cross-section (9 mm x 9 mm) and approximately 7cm in length and were cut parallel to the axis of the fruit. The force was applied perpendicular to the fruit axis and the maximum force encountered during the test was recorded. 79

Figure 9. Compressive failure was tested on cylinders of cortex tissue placed on the flat plate and compressed by a descending flat-faced probe. The cylinders were made with an internal diameter of 16.5 mm, trimmed to a length of 2 cm, and taken from tissue just beneath the skin. The cylinder was removed from the equator of the fruit, normal to the fruit axis. The force was applied perpendicular to the fruit axis and the maximum force encountered during the test was recorded. 80

Figure 10. Ontogeny of total volatiles, ethylene and respiration (CO_2 production) during ripening and senescence of 'Jonagold' apples. The apple fruit were examined from Sept 2, 2004 (Day 0) to Nov. 23, 2004 (Day 81). Fruits were harvested every 3-4 days from the field until Oct. 7, 2004 (Day 35), and thereafter maintained at room temperature (21±1°C). Each symbol represents the average of four replications for total volatiles and respiration, and ten replications for ethylene. Vertical bars represent mean ± SD. 81

Figure 11. Ontogeny of total volatiles and textural changes during ripening and senescence of 'Jonagold' apples. Tensile failure/bending force was to measure 'crispness' and compressive force for 'softening'. The apple fruit was examined from Sept 2, 2004 (Day 0) to Nov. 23, 2004 (Day 81). Fruits were harvested every 3-4 days from the field until Oct. 7, 2004 (Day 35), and thereafter maintained at room temperature (21±1°C). The samples were taken opposite sides of each fruit and each symbol represents four fruit replications. 82

Figure 12. Representative gas chromatograph of the headspace of 'Jonagold' apples at the respiratory climacteric on Day 42. Predominant esters were butyl acetate, 2-methylbutyl acetate, hexyl acetate, and hexyl 2-

methylbutanoate. A total of 31 esters, 3 aldehydes, and 5 alcohols were detectable at this point in development. 83

Figure 13. Patterns of alcohol emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of ethanol, propanol, butanol, hexanol, and 2-methylbutanol. B. Alcohol proportions (% of total alcohols). Each symbol represents the average of four replications. 85

Figure 14. Patterns of alcohol ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of propanol, propyl esters, butanol, butyl esters, hexanol, hexyl esters, 2-methylbutanol, and 2-methylbutyl esters. B. Alcohol and alcohol ester proportions (% of total alcohols or alcohol esters). Each symbol represents the average of four replications. 87

Figure 15. Patterns of ethanol ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total ethanol esters and ontogeny of ethylene. B. GC/MS response (TIC) of total ethanol. C. GC/MS response (TIC) of ethyl acetate, ethyl propanoate, ethyl butanoate, and ethyl 2-methylbutanoate. D. Ethanol proportion (% of total alcohols). E. Ethanol ester proportions (% of total ethanol esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD. 89

Figure 16. Patterns of propanol ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total propanol esters and ontogeny of ethylene. B. GC/MS response (TIC) of total propanol. C. GC/MS response (TIC) of propyl acetate, propyl propanoate, propyl butanoate, propyl 2-methylbutanoate, propyl hexanoate, and propyl octanoate. D. Propanol proportions (% of total alcohols). E. Propanol ester proportions (% of total propanol esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD. 91

Figure 17. Patterns of butanol ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total butanol esters and ontogeny of ethylene. B. GC/MS response (TIC) of total butanol. C. GC/MS response (TIC) of butyl acetate, butyl propanoate, butyl butanoate, butyl 2-methylbutanoate, butyl pentanoate, butyl hexanoate, butyl heptanoate, and butyl octanoate. D. Butanol proportions (% of total alcohols). E. Butanol ester proportions (% of total butanol esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD. 93

Figure 18. Patterns of pentanol ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total pentanol esters and ontogeny of ethylene. B. GC/MS response (TIC) of pentyl acetate, pentyl propanoate, pentyl 2-methylbutanoate, and pentyl hexanoate. C. Pentanol ester proportions (% of total pentanol esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD. 95

Figure 19. Patterns of hexanol ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total hexanol esters and ontogeny of ethylene. B. GC/MS response (TIC) of total hexanol. C. GC/MS response (TIC) of hexyl acetate, hexyl propanoate, hexyl butanoate, hexyl 2-methylbutanoate, hexyl hexanoate, and hexyl octanoate. D. Hexanol proportions (% of total alcohols). E. Hexanol ester proportions (% of total hexanol esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD. 97

Figure 20. Patterns of 2-methylbutanol ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total 2-methylbutanol esters and ontogeny of ethylene. B. GC/MS response (TIC) of total 2-methylbutanol. C. GC/MS response (TIC) of 2-methylbutyl acetate and 2-methylbutyl butanoate. D. 2-Methylbutanol

proportions (% of total alcohols). E. 2-Methylbutanol ester proportions (% of total 2-methylbutanol esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD. 99

Figure 21. Patterns of acid ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of acetates, propanoates, butanoates, 2-methylbutanoates, hexanoates, and octanoates. B. Acid ester proportions (% of total acid esters). Each symbol represents the average of four replications. 101

Figure 22. Patterns of acetate ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total acetate esters and ontogeny of ethylene. B. GC/MS response (TIC) of ethyl acetate, propyl acetate, butyl acetate, pentyl acetate, hexyl acetate, 2-methylpropyl acetate, and 2-methylbutyl acetate. C. Acetate ester proportions (% of total acetate esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD. 103

Figure 23. Patterns of propanoate ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total propanoate esters and ontogeny of ethylene. B. GC/MS response (TIC) of ethyl propanoate, propyl propanoate, butyl propanoate, pentyl propanoate, and hexyl propanoate. C. Propanoate ester proportions (% of total propanoate esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD. 105

Figure 24. Patterns of butanoate ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total butanoate esters and ontogeny of ethylene. B. GC/MS response (TIC) of ethyl butanoate, propyl butanoate, butyl butanoate, 2-methylbutyl butanoate, and hexyl butanoate. C. Butanoate ester proportions (% of total butanoate esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD. 107

Figure 25. Patterns of hexanoate ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total hexanoate esters and ontogeny of ethylene. B. GC/MS response (TIC) of propyl hexanoate, butyl hexanoate, pentyl hexanoate, and hexyl hexanoate. C. Hexanoate ester proportions (% of total hexanoate esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD. 109

Figure 26. Patterns of octanoate ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total octanoate esters and ontogeny of ethylene. B. GC/MS response (TIC) of propyl octanoate, butyl octanoate, and hexyl octanoate. C. Octanoate ester proportions (% of total octanoate esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD. 111

Figure 27. Patterns of 2-methylbutanoate ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total 2-methylbutanoate esters and ontogeny of ethylene. B. GC/MS response (TIC) of ethyl 2-methylbutanoate, propyl 2-methylbutanoate, butyl 2-methylbutanoate, pentyl 2-methylbutanoate, and hexyl 2-methylbutanoate. C. 2-Methylbutanoate ester proportions (% of total 2-methylbutanoate esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD. 113

CHAPTER 4

Figure 28. Pathways having potential to supply alcohol and acyl-CoA substrates for ester formation. 144

Figure 29. Putative (dashed lines) and demonstrated (solid lines) pathways involved in branched-chain ester biosynthesis. * Indicates that gene is found in bacteria, but not in plants. Hydrogens in the carbon-hydrogen bonds are not shown. Stars and circles indicate the carbon position during each reaction. 145

Figure 30. Experimental design for microarray analysis. Arrows indicate comparison made. Numbers indicate the days for the eight stages of development. Evaluated sixteen arrays were used with two biological samples and a dye swap to reduce variation. The tail of the arrow indicates RNA probe labeled with cyanine 3; the head of the arrow indicates RNA probe labeled with cyanine 5. 146

Figure 31. Internal ethylene concentration, total volatiles (TIC), and CO_2 production in pre-climacteric through post-climacteric 'Jonagold' apples. Eight stages (Days 0, 11, 25, 32, 39, 49, 60, and 70) were selected for genomic analysis based on physiological stages during ripening. Stage 1 (Day 0), early climacteric; stage 2 (Day 11), late preclimacteric and onset of trace ester biosynthesis; stage 3 (Day 25), onset of the autocatalytic ethylene and rapid increase of ester biosynthesis; stage 4 (Day 32), half-maximal ester biosynthesis and engagement of the respiratory climacteric; stage 5 (Day 39), near maximal ester biosynthesis, peak in respiratory activity, and onset of rapid tissue softening; stage 6 (Day 49), end of maximal biosynthesis, the conclusion of the respiratory climacteric, and completion of tissue softening; stage 7 (Day 60), midpoint in the decline in ester biosynthesis, maximal ethylene production, and onset of senescent; stage 8 (Day 70), postclimacteric minimum in ester production and high fruit senescent. 147

Figure 32. Patterns of 2-methylbutanol and 2-methylbutanoate ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total 2-methylbutanol and 2-methylbutanoate esters and ontogeny of ethylene. B. GC/MS response (TIC) of 2-methylbutanol and 2-methylbutanal. C. GC/MS response (TIC) of 2-methylbutyl acetate and 2-methylbutyl butanoate. D. GC/MS response (TIC) of ethyl 2-methylbutanoate, propyl 2-methylbutanoate, butyl 2-methylbutanoate, pentyl 2-methylbutanoate, and hexyl 2-methylbutanoate. E. 2-Methylbutanoate ester proportions (% of total 2-methylbutanoate esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD. 149

Figure 33. Gene expression based on microarray data (in log2 scale). For genes potentially involved in branched-chain ester formation including pyruvate decarboxylase (PDC), branched-chain aminotransferase (BCAT), and 2-isopropylmalate synthase gene expression relative to Day 0. 150

Figure 34. Gene expression of putative branched-chain aminotransferase (BCAT) for 'Jonagold' apple fruit ripened at room temperature performed by semi-quantitative RT-PCR. The value is based on spot density relative

to maximum value. 18s rRNA was used as a control. All data are normalized relative to control gene spot density. The control gene spot density ranged between 0.78-0.98. Each symbol represents the average of two replications. The average pooled standard deviation is 0.15. 151

Figure 35. Gene expression of putative pyruvate decarboxylase (PDC) for 'Jonagold' apple fruit ripened at room temperature performed by semi-quantitative RT-PCR. The value is based on spot density relative to maximum value. 18s rRNA was used as a control. All data are normalized relative to control gene spot density. The control gene spot density ranged between 0.78-0.98. Each symbol represents the average of two replications. The average pooled standard deviation is 0.10. 152

Figure 36. Gene expression of putative 2-isopropylmalate synthase for 'Jonagold' apple fruit ripened at room temperature performed by semi-quantitative RT-PCR. The value is based on spot density relative to maximum value. 18s rRNA was used as a control. All data are normalized relative to control gene spot density. The control gene spot density ranged between 0.78-0.98. Symbol represents the average of two replications. The pooled standard deviation is 0.06. 153

CHAPTER I

INTRODUCTION

Scent is one of the features of fruits that make them attractive to animals. Scent attraction to herbivores is a significant evolutionary response to a lack of mobility to employ animals for seed dispersal (Levey, 2004). Fruit maximize aroma production during ripening when seeds are mature. In fruits, the major compounds responsible for aroma are esters, aldehydes, acids, and terpenes. Most of the scents humans perceive as sweet are derived from esters. In apples and apple products, the volatile profile is known to contain over 200 compounds (Dimick and Hoskin, 1983).

During apple ripening, the volatile composition changes over time; quantitative and qualitative changes in volatiles stimulate the olfactory system of humans and mammals to indicate the presence of ripe fruit (Stoddart, 1980). In human olfaction, some compounds can be detected at parts-per-trillion level, but higher levels are needed for many compounds, depending upon their odor threshold. The degree to which a volatile impacts aroma is a function of the degree to which its concentration exceeds the odor threshold. In 'Delicious' apples, the concentration range for major esters is typically between 0.001-0.060 parts-per-million (ppm) (v/v) and the character impact compound ethyl 2-methylbutanoate can be detected as low as 0.0001 ppm (Flath et al., 1967).

Cultural practices (e.g. long-term controlled atmosphere (CA) storage) of preserving fruit quality significantly diminish aroma and the recovery is not reversible for most apple varieties (Ferenczi et al., 2006; Plotto et al., 1999). Despite consumer value placed in flavor, aroma research has been somewhat of a low priority. In other apple exporting countries, the focus on flavor is growing and is considered vital to the maintenance of strong marketing strategies.

The diverse aroma volatiles in apple are known to be produced by more than one biosynthetic pathway. But the actual pathways employed and their regulation are not well understood. Lack of progress in this area is in part a result of the difficulty associated with obtaining plant material, given the seasonality of the crop and the dynamic processes associated with ripening. Further, exploration of these pathways using forward or reverse genetic techniques is extremely difficult given the 5 to 10-year reproductive cycle of apple. Additionally, use of model organisms like *Arabidopsis thaliana* provides little insight, since *Arabidopsis* does not autonomously produce the same esters that are synthesized in apples.

The aim of this research is to: first, characterize aroma profiles and individual compounds during apple fruit ripening by establishing their temporal association with changes in fruit texture, skin color, starch content, sugar content, ethylene content, and respiratory activity; second, to relate changes in the expression of genes associated with putative ester biosynthetic pathways with patterns of ester production and other features of the ripening process. We expect that this information will help improve our understanding of the physiology and biochemistry of ester formation in apples.

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CHAPTER II

LITERATURE REVIEW

Flavor

Humans use five senses to enjoy food: sight, odor, taste, touch, and hearing. Using these senses, we classify food quality attributes into three sensory properties: appearance, flavor, and texture (Fisher and Scott, 1997). Consumers select fruits and vegetables based on these three properties and flavor is considered to be the most important factor (Péneau et al., 2006). Despite this preference for flavor, breeding of horticultural commodities has focused mostly on appearance and texture to aid in marketing and improve handling. The result has been that flavor is somewhat neglected.

Flavor consists of odor and taste. The taste component is due to the interaction of chemicals released from the food with the tongue. The odor component is due to the interaction of volatiles with the nose. The mixture of aroma notes and other taste factors determine characteristic flavors. For example, when Marsh et al. (2006) added sugar to kiwi fruit pulps, tasters perceived kiwi as more banana-like. When they added acid, kiwi was perceived less banana-like and had an increased lemon flavor.

Four categories of taste are prominent in fruits; sweetness, sourness, bitterness, and sometimes astringency. In fruits and vegetables, the perception of sweetness is primarily due to sugars and sugar alcohols, sourness due to organic acids, bitterness and astringency are due to alkaoloids and phenolics (Baldwin, 2004). Ripe fruit on average contain about 10-15% sugar by weight. The sugars are sometimes converted from stored starch as in apple, banana, and mango (McGee, 2004). Common sugars include sucrose, glucose and/or fructose, and sorbitol (Baldwin, 2002). Total acid content ranges from about 5% for the lemon to 0.1 % for the persimmon (Biale, 1964), and several forms of acids are found in fruits including ascorbic, citric, malic, quinic, tartaric, and oxalic. These organic acids are normally stored in the plant vacuoles. Ascorbic is described as soft, aspirin-like, astringent, and lemon-like; citric as sharp, fresh, and lemony; malic as lemony, tangy, bitter, sharp, and green apple-like; and quinic as chalky, aspirin-like, and fizzy (Marsh et al., 2006). Phenolic compounds range from low molecular weight, which tends to be bitter, to high molecular weight, which tends to be astringent (Drewnowski and Gomez-Carneros, 2000). One of the most important phenolic groups, flavonoids, includes flavonones, flavonols, flavones, isoflavones, flavans (catechins), and anthocyanins, and are found in many foods and beverages such as in green tea, red wine, soybean, grapefruit, and orange juice (Drewnowski and Gomez-Carneros, 2000). When phenolic compounds bind to proteins in saliva, it significantly reduces the lubricating qualities of saliva and decreases its viscosity, leading to an increase in friction, which we perceive as astringency (Prinz and Lucas, 2000).

There are three classes of aroma volatiles based on how the volatiles are generated. Thermally-generated aroma compounds require heating or cooking, disruption-dependent aroma requires the mixing of cellular contents normally held separate by various organelles, and autonomous aromas are produced without external intervention from ripening fruits and vegetables (Beaudry, 2000). Volatiles that add to flavor have various notes. For example, alcohols and aldehydes are perceived as green-grassy, esters as fruity and floral, phenolic compounds as spicy, furanones as nutty, pyrazines as earthy, lactones as creamy, and sulfur compounds as onion-like (McGee, 2004). Some odor compounds may add character-impact compounds, either desired or off-odor (Fan and Thayer, 2002), or may enhance the intensity of other flavors. Sweet flavor perception is enhanced in apple fruits by 2-methylbutyl acetate (Young et al., 1996).

Apple aroma

Apple is a climacteric fruit. As such it experiences a strong increase in ethylene production during ripening, which drives an increase in CO_2 production. Volatile compounds with odor-activity increase concurrently with or follow the ethylene increase (Ferenczi, 2003; Mattheis et al., 1991b) (Table 1). However, not all volatiles can be perceived as odorants and contribute to aroma. Further, while many of the volatiles are spontaneously produced from the intact fruit, several important volatiles are formed by oxidation, hydrolysis, or other enzymatic reactions as the fruit is crushed during mastication and exposed to oxygen (Drawert, 1975). When the apple is consumed, the mixture of spontaneous and induced compounds creates characteristic flavors.

In fresh and processed apples, more than 200 volatile compounds have been isolated; they consist mainly of alcohols, aldehydes, hydrocarbons, acids, and esters (Dimick and Hoskin, 1983). Of these volatiles, esters are the primary compounds that influence aroma and normally account for 80-95% of the total volatile emission (Paillard, 1990). Most esters contain two- to eight-carbon alkyl (alcohol-derived) or alkanoate (acid-derived) groups on either side of the oxygen atom. These alkyl or alkanoate groups can be straight or branched. For instance, hexyl acetate and butyl acetate are classified as straight-chain esters, and 2-methylbutyl acetate is a branched-chain ester. These three are the major esters considered to confer a characteristic apple aroma when the apple is ripening (Fellman et al., 2000). In general, they are perceived as fruity and floral (Plotto et al., 2000). The aldehydes, hexanal, cis-3-hexenal, and trans-2-hexenal are produced in abundance upon tissue disruption (as during mastication) by enzymatic oxidation and are responsible for green notes (Paillard, 1990) which are also important to the apple aroma. A low concentration of 4-methoxyallylbenzene (4-allylanisole) provides a ‘spicy’ or ‘anise-like’ aroma in some varieties and is thought to add a characteristic note (Williams et al., 1977). Other important aroma volatiles include 3-penten-2-ol (apple-like aroma) in ‘Starkspur Golden’ fruit (Vanoli et al., 1995) and β-damascenone (fruity odor) in ‘Cox Orange’ and ‘Elstar’ (Fuhrmann and Grosch, 2002). As fruit maturity shifts from preclimacteric to postclimacteric, the amount of individual compounds emitted by the fruit changes over time, altering the aroma profiles (Mattheis et al., 1991b). For example, in ‘Redchief Delicious’, longer carbon chains tend to predominate in esters produced at earlier ripening stages and shorter chain esters increase as fruit become overripe (Ferenczi, 2003). Unsaturated esters, which are normally in very low abundance in intact fruit, are formed when apple cells are crushed (Schreier et al, 1978). In processed apples, hexanal and trans-2-hexenal increase due to enzyme activation by heat and alter the aroma composition while others decrease by inactivation of enzymes (Su and Wiley, 1998).

Different cultivars emit different compounds (Kakiuchi et al., 1986). Cultivars can be classified by the type of esters they produce such as acetates in 'Calville blanc' and 'Golden Delicious', butanoates in 'Bell de Boskoop' and 'Canada blanc', propanoates in 'Richared' and 'Reinette du Mans', and ethyl esters in 'Starking' (Paillard, 1990).

Apple physiology

Ethylene is one of the plant hormones that regulate plant growth and development. From a postharvest perspective, it has a role in regulating fruit ripening. There are two systems to describe ethylene production (Biale, 1964; McMurchie et al., 1972; Pratt and Goeschl, 1969). System 1 is considered to be present in all fruit and plant tissues and is characterized as having feedback inhibition. System 2 is an autocatalytic system that is active in the climacteric fruit that stimulates ethylene production in response to ethylene presence. As climacteric fruits start to ripen, system 2 is activated, operating simultaneously with system 1.

The onset of ethylene production causes physiological changes in apples: respiration starts to increase, flesh softens, and volatile compounds are emitted. In some cultivars, chlorophyll is degraded and anthocyanins accumulate. The internal ethylene content that initiates the climacteric rise in mango can be as little as 0.04 $\mu L \cdot L^{-1}$, but most climacteric crops require 0.1 $\mu L \cdot L^{-1}$ or more (Biale, 1964; McGlasson, 1970). Suppression of the onset of system 2 ethylene is necessary to prolong storability substantially. Therefore, many studies have investigated inhibition of ethylene synthesis to delay ripening for long-term storage and export.

The ethylene pathway was established by Yang and Hoffman (1984). Ethylene is formed from s-adenosyl-methionine (SAM) by SAM synthetase which is derived from methionine. SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS), which is then oxidized by ACC oxidase (ACO), to ethylene. Differential expression of ACS and ACO gene family members result in the transition from auto-inhibitory to autocatalytic ethylene production (Barry et al., 2000; Leliévre et al, 1997). Inhibition of ethylene production can be accomplished by these two enzymes/genes. When ACS or ACO were silenced in fruit, firmness, sugar, and acid composition were maintained and shelf-life was increased; however, the total volatile esters were suppressed by 65-70% inhibition (Dandekar et al., 2004; Flores et al., 2002). In apple, *MdACO1* and *MdACS1* have been positioned on a molecular marker linkage map for breeders to develop new cultivars that maintain good shelf life (Costa et al., 2005). To an extent, continuous ethylene action and a high rate of ethylene production are required for full ester biosynthesis in apple (Fan et al., 1998).

Cultural practices affecting aroma

The impact of CA storage and the ethylene action inhibitor 1-methylcyclopropene (1-MCP, SmartFresh^{TM}) use on ripening and quality have been extensively studied. While these technologies permit preservation of fruit quality for a fairly long time, aroma production is diminished and the rate and extent of recovery is reduced upon transfer to air (Ferenczi et al., 2006; Plotto et al., 1999; Tough and Hewett, 2001; Yahia, 1991).

CA Storage. In 2005, about 84 % of the U.S. cold-stored apple crop was held in CA storage (USDA-NASS, 2005). CA storage has increased the marketing season of apples to year-round. It has an advantage in suppressing decay without necessitating the use of fungicides. It also delays ripening, maintains firmness, skin color, and acidity by inhibiting ethylene synthesis (Mattheis et al., 1998). However, improper management of storage condition can damage the crop significantly during storage. For example, low temperature, low O_2, and high CO_2 may cause fermentation (Mattheis et al., 1991a) or internal disorders (Argenta et al., 2002); low temperature, low O_2, and increased duration of storage greatly reduce aroma production (Brackmann et al., 1993; Echeverría et al., 2004a; Mattheis et al., 1991a; 1998; Plotto et al., 1999). On the other hand, Lavilla et al. (1999) reported that CA storage of 'Granny Smith' apple fruit did not diminish aroma production.

Under ultra-low oxygen (ULO) conditions, ethanol and aldehydes may accumulate to varying degrees depending on cultivar, causing off-flavors (Dixon and Hewett, 2000a). Plotto et al. (1999) reported that sourness and astringency were significantly higher in ULO-stored fruit than in apples held in air storage in which fruitiness and floral scent were maintained. Also, the accumulation of ethanol or methanol by ULO conditions may alter the ester composition. Because of the abundance of these alcohols in ULO-stored fruit, the methanol- or ethanol-derived esters increase while butyl and hexyl esters decrease (Argenta et al., 2004; Mattheis et al., 1991a). Moreover, ULO strongly suppresses straight-chain esters while branched-chain esters are not similarly suppressed by low O_2, but they are suppressed by high CO_2 (Brackmann et al., 1993). Similarly, Fellman et al. (1993) observed an increase in 2-methylbutyl acetate after 'Rome' apples were removed from low O_2 CA (1% CO_2) storage. Under the ULO condition, the suppression of ethylene action is probably the primary cause of reduced aroma volatile synthesis. Further, respiration may be suppressed, reducing carbon and energy available for ester biosynthesis (Rudell et al., 2002). It is also plausible that O_2-dependent processes in ester biosynthesis may be limited under these conditions.

When 'Gala' apple was stored in CA for 120 days and examined after 7 days at 20°C, ester emission was greater in fruits treated with ambient air 1-3 days per week for 8 hours and returned to 1 kPa O_2 compared to static 1 kPa O_2 (Mattheis et al., 1998). These experiments suggest a potential for hypoxia to assist in acclimation and recovery of aroma after a long period of storage.

Chemical treatments. Ethylene can be suppressed by chemical treatments in two ways: by inhibiting ethylene biosynthesis or ethylene action. One chemical tool is preharvest treatment with aminoethoxyvinylglycine (AVG), which inhibits ACS and can delay fruit maturity and ripening by reducing ethylene production. AVG application and additional CA storage benefit fruit quality (Brackmann and Waclawovsky, 2001).

Another important chemical treatment is 1-MCP. 1-MCP is approved in the US for commercial use on edible crops and is marketed as SmartFresh^{TM} (AgroFresh, Inc., Rohm and Haas). Food use registration for 1-MCP has been obtained in several countries. Use continues to expand, in terms of number of countries and variety of crops (Watkins and Miller, 2005).

Research suggests that 1-MCP is very effective in maintaining apple quality by blocking ethylene receptors and reducing physiological disorders during storage (Fan et al., 1999). However, the effectiveness of 1-MCP depends on cultivar and storage conditions (Watkins et al., 2000), concentration (Lurie et al., 2002), temperature and duration (DeEll et al., 2002), growing region, fruit maturity, and time from harvest to treatment (Blankenship and Dole, 2003). It also has a negative effect on disease control, thought to be imposed through an inhibition of the synthesis of phenolics in strawberry (Jiang et al., 2001). The significant decrease in volatile production is specific to climacteric fruit crops, dependent upon ethylene for normal ripening (Botondi et al., 2003; Defilippi et al., 2004; Ferenczi et al., 2006; Golding et al., 1999). Lurie et al. (2002) observed that 1-MCP-treated apples retained more alcohols, aldehydes, and β-damascenone. In bananas, application of 1-MCP after an initiation of ripening caused an increase in branched-chain alcohols, altering volatile composition (Golding et al., 1999). Botondi et al. (2003) reported that there is no 1-MCP effect on apricot color, but 1-MCP did cause a reduction in lactone synthesis and rise in terpenols. It is possible that when ethylene action is excessively limited, there can be a shortage of energy for maintenance reactions required for normal cellular activity, leading to undesirable reactions (Mir et al., 1999).

Timing of harvest

Timing of harvest can alter aroma production, composition, and recovery after storage (Echeverría et al., 2004a, 2004b). Early-picking of apples, harvested more than four weeks ahead of the optimal harvest date, delays the onset of volatile production and the amount is lower. If the fruit is harvested within two weeks of the optimal harvest date, the aroma production is normal after few days (Song and Bangerth, 1996). The fruits harvested at the climacteric stage produced more volatiles after removal of the fruit from storage than fruits harvested prior to the climacteric (Brackmann et al., 1993). Vanoli et al. (1995) found the change in volatile composition by harvest date that the optimum harvested apple had a best aroma composition, low content of butanoates and alcohols and high content of acetate esters.

Ester precursor formation

Apple skin is covered by a layer of wax and cutin (Ivanov and Dodova-Anghelova, 1974). When Dimick and Hoskin (1983) removed the oily wax coating on the skin, ester production was not limited and suggested that the ester source is from the skin or cortex. Guadagni et al. (1971) observed the greatest ester production was from peels of apples suggesting aroma biosynthesis mainly takes place in the skin rather than the flesh. Rudell et al. (2002) observed that the apple skin tissue displayed a greater capacity to synthesize pentanol esters than carpellary tissue (between the carpels and the core line) and hypanthial tissue (between the skin and the core line) when incubated with 1-pentanol.

Esters have an alcohol-derived (alkyl) group and an acid-derived (alkanoate) group. The alkyl and alkanoate groups can be straight-chain or branched-chain. The immediate precursors are an alcohol and a CoA thioester of a fatty acid (Figure 1). Alcohols are formed from aldehydes by alcohol dehydrogenase (ADH). Alkyl group precursors normally range from 1 to 6 carbons in length and the alkanoate group precursors range from 2 to 8 carbons (Paillard, 1990). Carbon entry into these pathways has been variously proposed to depend on catabolic and biosynthetic pathways (e.g. β-oxidation, lipoxygenase, amino acid metabolism, two- and single-carbon fatty acid synthesis). Although some experimental results support a role for catabolism (Sanz et al., 1997), sufficient evidence does not exist to disregard biosynthetic pathways. Once formed, the alcohols and acyl-CoAs are combined by alcohol acyltransferase (AAT) to form esters.

Catabolic pathways

According to one hypothesis, straight-chain ester precursors are proposed to be derived from catabolism of fatty acids via the β-oxidation (Figure 2) and lipoxygenase pathways (Figure 3) (Dixon and Hewett, 2000b; Fellman et al., 2000; Sanz et al., 1997; Yahia, 1994). Branched-chain ester precursors may be supplied from branched-chain amino acid (BCAA) metabolism (Figure 4) (Perez et al., 2002; Tressl and Drawert, 1973).

β-oxidation. In plants, β-oxidation takes place in peroxisomes rather than in mitochondria as in mammals. Peroxisomes are classified according to their physiological function: glyoxisomes, leaf peroxisomes, and unspecialized peroxisomes (Hayashi, 2000). Leaf peroxisomes, are specialized organelles for photorespiration, glyoxisomes are primarily dedicated to fatty acid breakdown (as in germinating seeds), and unspecialized peroxisomes remain undefined (Hayashi, 2000). The function of peroxisomes and glyoxisomes has some degree of plasticity; transformation from peroxisome to glyoxisome can be observed and vice versa during specific developmental stages (Hayashi and Nishimura, 2003). For example, the glyoxisomes first appear in a germinating seedling and, when the seedling begins photosynthesis, the glyoxisome is functionally transformed to a peroxisome (Hayashi and Nishimura, 2003). The major role of β-oxidation in the glyoxisome is to convert fatty acids into carbon skeletons that can be metabolized into sugars, amino acids, and nucleotides, and to provide energy (Graham and Eastmond, 2002).

The initial step of the β-oxidation pathway is the activation of free fatty acids with a high-energy thioester linkage with acyl-coenzyme A by acyl-CoA synthetase (EC 6.2.1.3). The activated acyl-CoAs are reduced by two carbons during each cycle of β-oxidation, which is comprised of four steps. 1) dehydrogenation, 2) addition of water, 3) dehydrogenation, and 4) cleavage of acetyl-CoA (Graham and Eastmond, 2002) (Figure 2). The first dehydrogenation step is catalyzed by acyl-CoA oxidase (ACX, EC 1.3.3.6). Acyl-CoA is converted to 2-*trans*-enoyl-CoA requiring FAD as a cofactor, passing electrons to O_2 and forming H_2O_2, which is further converted to H_2O and O_2 by catalase. There are multiple ACX isozymes with differences in substrate chain-length specificities (De Bellis et al., 1999, 2000; Hooks et al., 1996, 1999; Kirsch et al., 1986; Rylott et al., 2003). Chain-length specificities are categorized as: long- (>C14), medium- (C8-C14), and short-chain (C4-C8). Several peroxisomal ACX isozymes have been isolated and characterized in pumpkin (DeBellis et al., 1999, 2000) and cucumber (Kirsch et al., 1986). In *Arabidopsis*, several genes in the ACX family have been isolated and characterized (Hooks et al., 1999; Rylott et al., 2003). The second and third steps in β-oxidation are catalyzed by a multifunctional protein containing both enzyme activities: 2-*trans*-enoyl-CoA hydratase (EC 4.2.1.17), hydrating 2-*trans*-enoyl-CoA to 3-hydroxyacyl-CoA, and L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) requiring NAD as a cofactor and oxidizing 3-hydroxyacyl-CoA to 3-ketoacyl-CoA, respectively. The final step is catalyzed by 3-ketoacyl-CoA thiolase (EC 2.3.1.16), which uses CoASH to cleave two carbons from 3-ketoacyl-CoA to form acetyl-CoA and an acyl-CoA that is two carbons shorter than in the previous cycle. The shortened acyl-CoA product cycles again through β-oxidation until completely reduced to acetyl-CoA.

The major difference between mammalian mitochondrial and peroxisomal/glyoxisomal β-oxidation is that the first step in mammals is catalyzed by acyl-CoA dehydrogenase (ACDH, EC 1.3.99.3) instead of acyl-CoA oxidase (ACX) (Eaton et al., 1996; Graham and Eastmond, 2002). During the first step of dehydrogenation with ACDH, flavin adenine dinucleotide (FAD) passes electrons to the mitochondrial respiratory chain instead of to O_2 and forming H_2O_2. Similar to ACX, ACDH isozymes have different chain-length specificities of acyl-CoAs (Eaton et al., 1996). Chain-length specificities are categorized as: short- (C4-6), medium- (C4-12), long- (C8-12), and very long-chain (C12-24).

In plants, the complete degradation of fatty acids takes place in the peroxisome/glyoxisome and the function of mitochondrial β-oxidation is unknown (Hayashi, 2000). In contrast, mammals have a functional difference between two organelles in metabolizing fatty acids. Mammalian β-oxidation takes place primarily in mitochondria, but very long chain fatty acids such as hexacosanoic acid (C26) and less common fatty acids are oxidized in the peroxisome (Eaton et al., 1996). Interestingly, based on the phylogenetic tree of ACX, plant glyoxisomal short-chain ACX has a high similarity with mammalian mitochondrial ACDH, whereas plant glyoxisomal long-chain ACX has a high similarity with mammalian peroxisomal ACX (Hayashi, 2000).

Several 3-ketoacyl-CoA thiolase genes have been isolated and characterized in *Arabidopsis* (Germain et al., 2001). One of the 3-ketoacyl-CoA thiolases has substrate specificity based on chain-length. However, it is not clear whether chain-length specificity in *Arabidopsis* is imposed via ACX or the final step via 3-ketoacyl-CoA thiolase. No studies on the expression of β-oxidation genes have been reported for ester-producing plant organs.

Lipoxygenase pathway. The pathway of lipoxygenase involves several enzymes (Figure 3). These enzymes are bound to the thylakoid membrane of chloroplasts of green leaves (Hatanaka, 1993). Linoleic or linolenic acids are usually the most abundant fatty acids in plants. These fatty acids are released from phospholipids by lipase or hydrolase (Wang, 2001). The free fatty acids are oxidized by lipoxygenase (LOX, EC 1.13.11.12), requiring oxygen; LOX acts on C18 fatty acids to produce 9- or 13- hydroperoxy derivatives. Different forms of LOX have been analyzed in cotyledons and fruits, and in leaves of barley, spinach, and *Arabidopsis* (Feussner and Wasternack, 2002). The specificity of LOX determines the tendency toward C6 or C9 aldehyde synthesis. In apples, LOX is highly specific in peroxidizing linoleic acid to 13-hydroperoxy derivatives (Kim and Grosch, 1979), which would facilitate synthesis of hexanal. The second step involves the enzyme hydroperoxide lyase (HPL). HPL has been characterized as a special class of cytochrome P450 enzyme (Noordermeer et al., 2001) that cleaves the 13-hydroperoxy derivatives, 13-hydroperoxy linoleic acid (13-HPOD) and 13-hydroperoxy linolenic acid (13-HPOT) into hexanal and Z-3-hexenal, respectively. 13-HPOT can also be metabolized to C5 compounds such as 2-Z-pentenol or 1-penten-3-ol by a secondary activity of lipoxygenase under anaerobic conditions (Salch et al., 1995). The aldehyde products of HPL are reduced to corresponding alcohols (e.g. hexanol and Z-3-hexenol) by alcohol dehydrogenase (ADH). Salas et al. (2005 and 2006) studied silencing HPL and LOX genes in potato and *Arabidopsis* leaves. HPL knock-out plants had similar levels of C6 compounds but had 4-fold higher in C5 compounds, and LOX-silenced plants had severely decreased volatile production. The authors concluded that both LOX and HPL activities are required for volatile production. Finally, alcohols such as those produced by the LOX pathway can act as substrates for ester formation.

Branched-chain amino acid degradation. The pathways for branched-chain amino acid (BCAA) metabolism have been extensively studied in yeast and bacteria, and are an important in flavor development of microbial fermentations (Dickinson et al., 1997, 1998, 2000; Smit et al., 2005). The first step in BCAA catabolism is the removal of the amino group via branched-chain aminotransferase (BCAT, EC 2.6.1.42), which requires pyridoxal phosphate as a coenzyme (Figure 4). BCAT can transaminate all three BCAAs or may have a preference for a specific BCAA (Yvon et al., 2000). It is still not clear whether BCAT is regulated by an unique mechanism in plants, but in bacteria, nutritional factors such as carbohydrate and nitrogen source regulate aminotransferase activity (Chambellon and Yvon, 2003). In examples from non-ester producing plants, BCAA degradation is thought to occur in response to low carbohydrate availability (Graham and Eastmond, 2002). In a recent study with *Arabidopsis thaliana*, a total of six or possibly seven *BCAT* genes localized in mitochondria, plastids, and cytosol were cloned. The mitochondrial AtBCAT-1 is thought to contribute to the degradation of all three BCAA (Diebold et al., 2002; Schuster and Binder, 2005). Although BCAT enzymes catalyze a reversible reaction, the degradation is believed to contribute to aroma formation by bacteria since the mutant line of *BCAT* genes reduced the formation of branched-chain aldehydes and corresponding alcohols (Rijnen et al., 2003).

The branched-chain α-keto acid is metabolized via two pathways to supply immediate precursors to branched-chain esters, either to branched-chain acyl-CoAs (acid-forming pathway) or to branched-chain alcohols (alcohol-forming pathway). Mammals only have the capacity for catabolism in formation of branched-chain acyl-CoAs; a defect in branched-chain α-keto acid dehydrogenase in this pathway causes a disorder known as a maple syrup urine disease (Platell et al., 2000). In bacteria, metabolism of branched-chain α-keto acid differs among strains, favoring either the acid-forming or alcohol-forming pathway, or utilizing both at the same time (Helinck et al., 2004).

In the acid-forming pathway, a branched-chain α-keto acid is dehydrogenated to branched-chain acyl-CoAs by branched-chain α-keto acid dehydrogenase (BCKDH, EC 1.2.4.4), a multifunctional enzyme composed of three subunits (α-keto acid dehydrogenase, dihydrolipoyl acyltransferase, and dihydrolipoyl dehydrogenase) and structurally similar to pyruvate dehydrogenase (PDH) complex (Mooney et al., 2002). PDH catalyzes the oxidative decarboxylation of pyruvate to yield acetyl-CoA and NADH, and has two forms: plastidial and mitochondrial (Tovar-Méndez et al., 2003). In plants, BCKDH has been shown to take place both in peroxisomes (Gerblin and Gerhardt, 1988) and mitochondria (Taylor et al., 2004). Unlike BCAT, the activity of BCKDH is highly regulated through the mechanism of phosphorylation (inactivation) and dephosphorylation (activation) in mammals (Harper et al., 1984). However, regulation by phosphorylation has been difficult to prove in plants (Mooney et al., 2002). In mammals and bacteria, the branched-chain acyl-CoA (2-methylbutyl-CoA, for example, in figure 4) is further metabolized into acetyl-CoA and propionyl-CoA to supply for energy production (pathway not shown) (Harper et al., 1984; Massey et al., 1976). The same pathway is proposed in *Arabidopsis*, but, some of the enzymes are still waiting to be characterized to prove the pathway (Taylor et al., 2004). If the proposed pathway is active in plants, propionyl-CoA can serve as precursor of esters.

In the alcohol-forming pathway, the branched-chain α-keto acid is decarboxylated to branched-chain aldehyde by branched-chain α-keto acid decarboxylase (BCKDC, EC 4.1.1.72) with the release of CO_2. This product is further dehydrogenated to branched-chain alcohol by alcohol dehydrogenase (ADH, EC 1.1.1.1) (Wyllie et al., 1996). In bacteria, BCKDC activity is capable of forming an alcohol from branched-chain α-keto acid and is proposed to be the rate controlling step; however, it is present in only few strains of bacteria (Smit et al., 2004). Similarly, in yeast, pyruvate decarboxylase (PDC, EC 4.1.1.1), an important enzyme that cleaves pyruvate to acetaldehyde during alcoholic fermentation, has been reported to catalyze the decarboxylation of branched-chain α-keto acids (Yoshimoto et al., 2001), but may not be an essential step for forming branched-chain alcohol (ter Schure et al., 1998). Several genes (*PDC1*, *PDC5*, *PDC6*, *YDL080c*) have been reported to be responsible for decarboxylation of branched-chain α-keto acids to branched-chain aldehydes and branched-chain alcohols (Dickinson et al., 1997, 1998, 2000; Yoshimoto et al., 2001). Dickinson et al. (1997, 1998, 2000) suggests that the catabolic pathways of three BCAAs are accomplished in different ways, a single *YDL080c* gene (PDC-like gene) is likely responsible for leucine catabolism and any one of the isozymes of PDC is responsible for valine and isoleucine catabolism.

Yoshimoto et al. (2001) adds that a *PDC1* gene at least partially contributes to the formation of 3-methylbutanol in yeast.

In fruits, three PDC genes have been isolated from strawberries and one from grape berries (Moyano et al., 2004; Or et al., 2000). Also, PDC activities have been measured during maturation of 'Fuji' apples (Echeverría et al., 2004c). Since PDC plays a significant role in the conversion of pyruvate to acetaldehyde, which is further reduced to ethanol by ADH, the main purpose of these studies was to explore the mechanism of ethanol production under anaerobic conditions and the formation of ethanol-derived esters such as ethyl esters or acetate esters. However, they did not evaluate PDC potential for BCAA catabolism. Thus, PDC activity in BCAA catabolism in fruits is unknown. Tieman et al. (2006) recently found that 2-phenylethanol, an important flavor and insect attractant in tomato and rose, is synthesized from phenylalanine by participation of an aromatic amino acid decarboxylase. This report suggested that decarboxylase activity might contribute to aroma formation in ester-producing fruits. To date, the involvement of PDC in forming branched-chain esters has not been addressed in the literature, to our knowledge.

Synthetic pathways

There are two pathways for fatty acid biosynthesis, either by two-carbon (2-C FAB) or one-carbon (1-C FAB) elongation. For two-carbon elongation, fatty acid synthase (EC 2.3.1.85) plays a major role with an acyl carrier protein to synthesize long chain fatty acids for membranes, storage lipids, and waxes (Ohlrogge and Jaworski, 1997). For one-carbon elongation, an α-ketoacid elongation (αKAE) route is utilized, which may impact primary and secondary metabolic pathways including the tricarboxylic acid cycle, leucine biosynthesis, formation of sugar-ester acyl acids, and short-chain alcohols of yeast (Kroumova and Wagner, 2003). Two-carbon elongation has been extensively studied for its role in lipid biosynthesis, but, very limited information is available regarding one-carbon elongation. In certain strains of bacteria, isoleucine biosynthesis may utilize 1-C FAB pathway (Charon et al., 1974; Xu et al., 2004).

Branched-chain amino acid biosynthesis. Generally, isoleucine is synthesized from threonine in plants and bacteria. However, radioactive carbon-labeling studies indicate that most of the isoleucine is synthesized by a pathway independent of threonine in the *Leptospira* bacteria (Charon et al., 1974). Branched-chain amino acids are formed from branched-chain α-keto acids (Figure 4). These branched-chain α-keto acids, α-keto-β-methylvalerate, α-keto-isovalerate, and α-ketoisocaproate are substrates in the aminotransferase reaction synthesizing the BCAAs, isoleucine, valine, and leucine, respectively. The immediate precursor to isoleucine is the branched-chain α-keto acid α-keto-β-methylvalerate. This compound can potentially serve to synthesize 2-methylbutyl and 2-methylbutanoate esters without the formation of isoleucine.

Two-carbon elongation. 2-C FAB takes place in the plastids (Figure 5). Two enzyme activities are required, acetyl-CoA carboxylase (ACCase, EC 6.4.1.2) and fatty acid synthase (FAS, EC 2.3.1.85). ACCase provides the starting material of malonyl-CoA from acetyl-CoA, yielding CO_2. FAS refers to several enzymes required to form fatty-acids ranging in length from 12-22 carbons (Broun et al., 1999; Thelen and Ohlrogge, 2002). The predicted products found in most plants are 16- and 18-carbon fatty acids.

Fatty acid synthesis occurs in four steps (Ohlrogge and Jaworski, 1997) (Figure 5), 1) condensation, 2) reduction, 3) dehydration, and 4) reduction. First, malonyl-CoA is transacylated to malonyl-ACP (chain extender), requiring acyl-carrier protein (ACP) as a cofactor, and condenses with acetyl-CoA (primer) via the enzyme 3-ketoacyl-ACP synthase III (KAS III, EC 2.3.1.41). Steps 2-4 are catalyzed by 3-ketoacyl-ACP reductase (EC 1.1.1.100), 3-hydroxyacyl-ACP dehydrase (EC 4.2.1.17), and enoyl-ACP reductase (EC 1.3.1.9), respectively, forming butyryl-ACP after step 4. The cycle repeats to condense with malonyl-ACP with an enzyme 3-ketoacyl-ACP synthase I (KAS I) and KAS II.

KAS exhibits substrate specificity: KAS I prefers the shorter chain (C4-14)-ACP and KAS II for longer chain (>C14)-ACP (Shimakata and Stumpf, 1982). The fatty acid normally elongates to 16 to 18 carbons in length until acyl-ACP thioesterase (EC 3.1.2.14) terminates fatty acid synthesis by hydrolysis. The terminated fatty acid either leaves the plastid to the ER for further elongation or desaturation or for storage as a lipid. The acyl-ACP thioesterase has two types: one is relatively specific for 18:1-ACP (FatA) and the other for short-chain saturated acyl-ACP (FatB) (Hawkins and Kridl, 1998; Jones et al., 1995). When the 12:0-ACP thioesterase gene from seed of California bay trees was expressed in developing seed of *Arabidopsis*, the transgenic plant produced large amounts of laurate (12:0) (Voelker et al., 1992). If acyl-ACP thioesterase can cleave fatty acids to produce shorter length C4-C8 acids, they have a potential to produce precursors suitable for ester formation. However, to-date, no short- to medium-chain acyl-ACP thioesterase has been reported in fruit.

One-carbon elongation. The 1-C FAB pathway is rather specialized and is not found in all plants. This pathway consists of 3-4 steps, starting with condensation of acetyl-CoA (chain extender) and pyruvate (primer) by the enzyme 2-isopropylmalate synthase (EC. 4.1.3.12) (Figure 6). The next step involves isomerization, dehydration, and decarboxylation by isopropylmalate dehydratase (EC 4.2.1.33), and 3-isopropylmalate dehydrogenase (EC 1.1.1.85), to produce α-keto butyrate, extended by one-carbon relative to the primer, pyruvate (Kroumova and Wagner, 2003). Further elongation by one carbon is accomplished by successive rounds of acetyl-CoA addition and CO_2 elimination to make long odd- and even-numbered α-keto acids. Finally, α-keto acid dehydrogenase 2-oxovalerate dehydrogenase (often called 2-oxoacid dehydrogenase, EC 1.2.1.25) decarboxylates and acetylates to form an acyl-CoA with the same carbon number relative to the starting α-keto acid (Kroumova and Wagner, 2003). These acyl-CoAs can enter directly into the AAT reaction, forming straight-chain esters. Interestingly, α-keto butyrate produced in this pathway may also serve as a substrate for reactions leading to the formation of α-keto-β-methylvalerate and subsequent isoleucine biosynthesis (Figure 4). This possibility has not been demonstrated in plants to my knowledge.

Ester synthesis

The final step of ester formation is the combination of alcohol and acyl-CoA by alcohol acyltransferase (AAT, EC 2.3.1.84) (Figure 1). Of the various enzymes implicated in ester formation, only AAT has been characterized to any meaningful extent. The *AAT* gene has been identified in apple, strawberry, melon, and banana (Aharoni et al., 2000; Harada et al., 1985; Jayanty et al., 2002; Souleyre et al., 2005; Yahyaoui et al., 2002). Several isozymes of AAT have been studied and were determined to utilize a broad range of precursors although they do exhibit marked substrate preferences. The AAT reaction velocity is affected by carbon chain length, and architecture (e.g. straight- or branched-chain) of the acyl-CoAs, or alcohol substrates (Aharoni et al., 2000; Olías et al., 2002; Ueda et al., 1992; Yahyaoui et al., 2002). Substrate preference also differs by fruit species and, within a species, even between cultivars (Holland et al., 2005), and can not be predicted based on the sequence similarity among various members of the AAT family (Beekwilder et al., 2004). In apples, *MpAAT1* (Souleyre et al., 2005) and *MpAAT2* (Li et al., 2006) have been isolated. *MpAAT1* is characterized and found to be expressed in several different organs. MpAAT1 prefers to produce hexyl esters of C3, C6, and C8 acyl-CoAs, but with acetate esters substrate preference depends on precursor alcohol concentration. In strawberry, AAT has substrate preference in the order of hexyl>butyl>amyl>isoamyl when acetyl-CoA is the acyl donor, and acetate>butanoate>propanoate when butanol is the alcohol donor (Pérez et al., 1993). Four genes *CM-AAT1*, *CM-AAT2*, *CM-AAT3*, and *CM-AAT4* have been characterized in melon (El-Sharkawy et al., 2005; Yahyaoui et al., 2002). CM-AAT2 has no detectable activity; CM-AAT1 is capable of producing a wide range of esters but has a higher activity with hexanol relative to butanol in the production of hexyl and butyl esters (Yahyaoui et al., 2002). CM-AAT1 can also take branched-chain alcohols, but the activity rate depends on the position of the methyl group; it has higher activity for 2-methylbutanol than for 3-methylbutanol. CM-AAT3 accepts a wide range of substrates with strong preference in benzyl acetate and CM-AAT4 exclusively forms acetates with strong preference for cinnamoyl acetate (El-Sharkawy et al., 2005). Such substrate specificity is likely to have an effect on individual ester concentration and which types of esters are produced at various stages of ripening and senescence.

It is suggested that substrate supply is a major determinant rather than AAT enzyme activity for regulating the quantitative and qualitative composition of the aroma profile (Echeverría et al., 2004c; Wyllie and Fellman, 2000). In banana, Jayanty et al. (2002) observed AAT expression and the presence of AAT enzyme activity well before the onset of fruit ripening and ester biosynthesis, suggesting that the precursors are lacking, and therefore, the control must lie within the ester precursor biosynthetic pathway. However, Jayanty et al. (2002) also hypothesized that as aroma biosynthesis engages, the increase in AAT activity and gene expression exerts a major influence in overall ester formation. Also, since AAT cannot discriminate between 2-methylbutyl and 3-methylbutyl precursors (Wyllie et al., 1996), the fact that apple mostly produces 2-methylbutyl esters suggests control of biosynthesis must lie at the stage of ester precursor supply, rather than at the level of AAT (Wyllie and Fellman, 2000). An increase in AAT activity during ripening was observed for apple (Defilippi et al., 2005) and melon (Shalit et al., 2001) suggesting a significant, but not limiting role for AAT in ester production.

Hypothesis

The involvement of β-oxidation, the lipoxygenase pathway, and BCAA metabolism for ester formation is suggested by various substrate feeding studies. In 'Cox's Orange Pippin' apple, feeding of methyl hexanoate and methyl octanoate enhanced butanoate ester formation (Bartley et al., 1985) and feeding pentanoic acid increased propanoate esters in 'Golden Delicious' apples (De Pooter et al., 1983). In a deuterium-labeling study, feeding C18:0 and 18:1 fatty acids produced straight-chain C6-C8 alkanoate esters, hexanoic acids produced C4 alkyl and alkanoate esters, and linoleic acids only produced hexyl and hexanoate esters in apple (Rowan et al., 1999). On the other hand, labeled palmitic acid (C16) did not convert into volatile constituents in banana (Tressl and Drawert, 1973). These studies suggest that if LOX is active at the same time as β-oxidation, both pathways could act together to provide the large amount of C6 and the trace amount of unsaturated precursors found in esters.

Labeled-Leucine produced 3-methylbutyl and 3-methylbutanoate esters, valine produced 2-methylpropyl and 2-methylpropanoate esters, and isoleucine produced 2-methylbutyl and 2-methylbutanoate esters in strawberry, apple, and banana (Pérez et al., 2002; Rowan et al., 1996, 1998; Tressl and Drawert, 1973). In apples, esters are produced having branched-chain alkyl and branched-chain alkanoate groups that are likely from isoleucine and valine metabolism (Ferenczi, 2003). Additional feeding studies demonstrate that the catabolism of BCAAs can supply the needed products for branched-chain esters (Wyllie et al., 1996; 2000). Although leucine is abundantly found in ripening apple fruit (Burroughs, 1970; Hansen, 1970), there are little-to-no esters produced from leucine. Paradoxically during apple fruit ripening, isoleucine accumulates, but the other BCAAs do not (Nie et al., 2005). According to conclusions derived from feeding studies, the increase in 2-methylbutyl ester is a result of increased catabolism of isoleucine. However, this suggestion runs counter to the observation that isoleucine accumulates. Isoleucine accumulation data are more suggestive of enhanced isoleucine synthesis and/or a reduction in its catabolism. It is possible that increased isoleucine synthesis is accompanied by an increase in the pool of the isoleucine precursor α-keto-β-methylvalerate, α-keto-β-methylvalerate may directly feed into the synthesis of 2-methylbutanol and 2-methylbutanoate, without forming isoleucine.

1-C FAB can utilize several primers other than pyruvate to form various types of compounds such as glucosinolates and sugar esters (Kroumova and Wagner, 2003). Although there are some limitations by plant species, 1-C FAB is capable of making various chain length of fatty acids, generally 3-12 carbons; straight, branched, odd or even, short- or medium-chain length (up to C7 straight chain in petunia) (Kandra et al., 1990; Kroumova et al., 1994; Kroumova and Wagner, 2003; Oku and Kaneda, 1988). If 1-C FAB pathway is utilized in the fruit ester biosynthesis, it may give an explanation of forming esters with odd-number carbon chains (C3, C5, and C7) as well as even-number esters.

Extensive feeding studies demonstrate catabolic pathways have the potential to meet the needs for ester biosynthesis; however, genes/enzymes in these pathways are not well characterized in ester-forming fruits. In case of biosynthetic processes in ester formation, no studies have been evaluated the possible role in ester formation. Only the final step of ester formation, AAT gene expression and activity has been studied. Further investigation is needed at the gene/protein level to prove the theoretical pathways in ester-producing fruits.

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Esters	**Odor characters**	**Reference**
Ethyl acetate	pleasant, ethereal-fruity, brandy-like	Dimick and Hoskin, 1983
Ethyl acetate	Fruity, solvent like	Paillard, 1990
Butyl acetate	very diffusive, ethereal-fruity, pungent, pear odor	Dimick and Hoskin, 1983
Butyl acetate	slight wiskey	Advanced Biotech. Inc.
Butyl acetate	nail polish, gala	Plotto et al., 2000
Pentyl acetate	apple-like, sweet	Rizzolo et al., 1989
Pentyl acetate	banana oil, fruity pineapple	Paillard, 1990
hexyl acetate	Sweet fruity, slightly floral	Dimick and Hoskin, 1983
hexyl acetate	green, fruity	Salas et al., 2006
hexyl acetate	Gala, ripe, pear	Plotto et al., 2000
Heptyl acetate	fruity, fatty-green, slight floral odor	Dimick and Hoskin, 1983
2-methylbutyl acetate	solvent, gala	Plotto et al., 2000
2-methylbutyl acetate	apple, pear, banana	Advanced Biotech. Inc.
Z-3-hexenyl acetate	banana, green	Salas et al., 2006
Ethyl propanoate	ethereal, fruity-rum like odor	Dimick and Hoskin, 1983
Propyl propanoate	fruity	Plotto et al., 2000
Butyl propanoate	fruity, apple	Plotto et al., 2000
Hexyl propanoate	apple	Plotto et al., 2000
Ethyl 2-methylpropanoate	diffusive, sweet-ethereal, fruity odor	Dimick and Hoskin, 1983
Ethyl butanoate	fruity, ester-like, sweet	Ulrich et al., 1997
Ethyl butanoate	fruity, banana, pineapple	Paillard, 1990
Ethyl butanoate	powerful, ethereal-fruity odor, banana, pinapple	Dimick and Hoskin, 1983
Propyl butanoate	pineapple, apricot	Paillard, 1990
Butyl butanoate	rotten apple, cheesy	Plotto et al., 2000
Butyl butanoate	pear, pineapple	Paillard, 1990
Hexyl butanoate	green apple	Plotto et al., 2000
Methyl 2-methylbutanoate	sweet fruity	Plotto et al., 2000
Ethyl 2-methylbutanoate	powerful diffusive, green-fruity, pungent odor	Dimick and Hoskin, 1983
Ethyl 2-methylbutanoate	sweet strawberry	Plotto et al., 2000
Ethyl 2-methylbutanoate	apple-like, green , fruity	Paillard, 1990
Propyl 2-methylbutanoate	very sweet, strawberry	Plotto et al., 2000
Butyl 2-methylbutanoate	fruity, apple	Plotto et al., 2000
Hexyl 2-methylbutanoate	apple, grapefruit	Plotto et al., 2000
Hexyl 2-methylbutanoate	powerful, fresh-green fruity odor	Dimick and Hoskin, 1983
Butyl pentanoate	apple, raspberry	Paillard, 1990
Methyl hexanoate	fruity	Paillard, 1990
Ethyl hexanoate	fruity, fresh, sweet	Paillard, 1990
Butyl hexanoate	green apple	Plotto et al., 2000
Butyl hexanoate	pineapple	Paillard, 1990
Hexyl hexanoate	sweet floral	Advanced Biotech. Inc.

Table 1. Representative esters identified in apples with sensory description. Only esters that had an odor description are listed. Esters included acetates, propanoates, 2-methylpropanoates, butanoates, 2-methylbutanoates, pentanoates, and hexanoates.

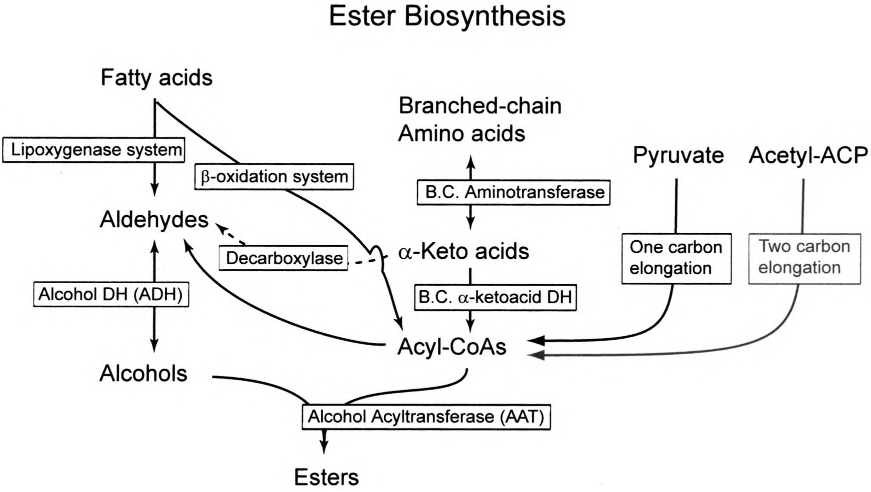


Figure 1. Pathways having potential to supply alcohol and acyl-CoA substrates for ester formation.

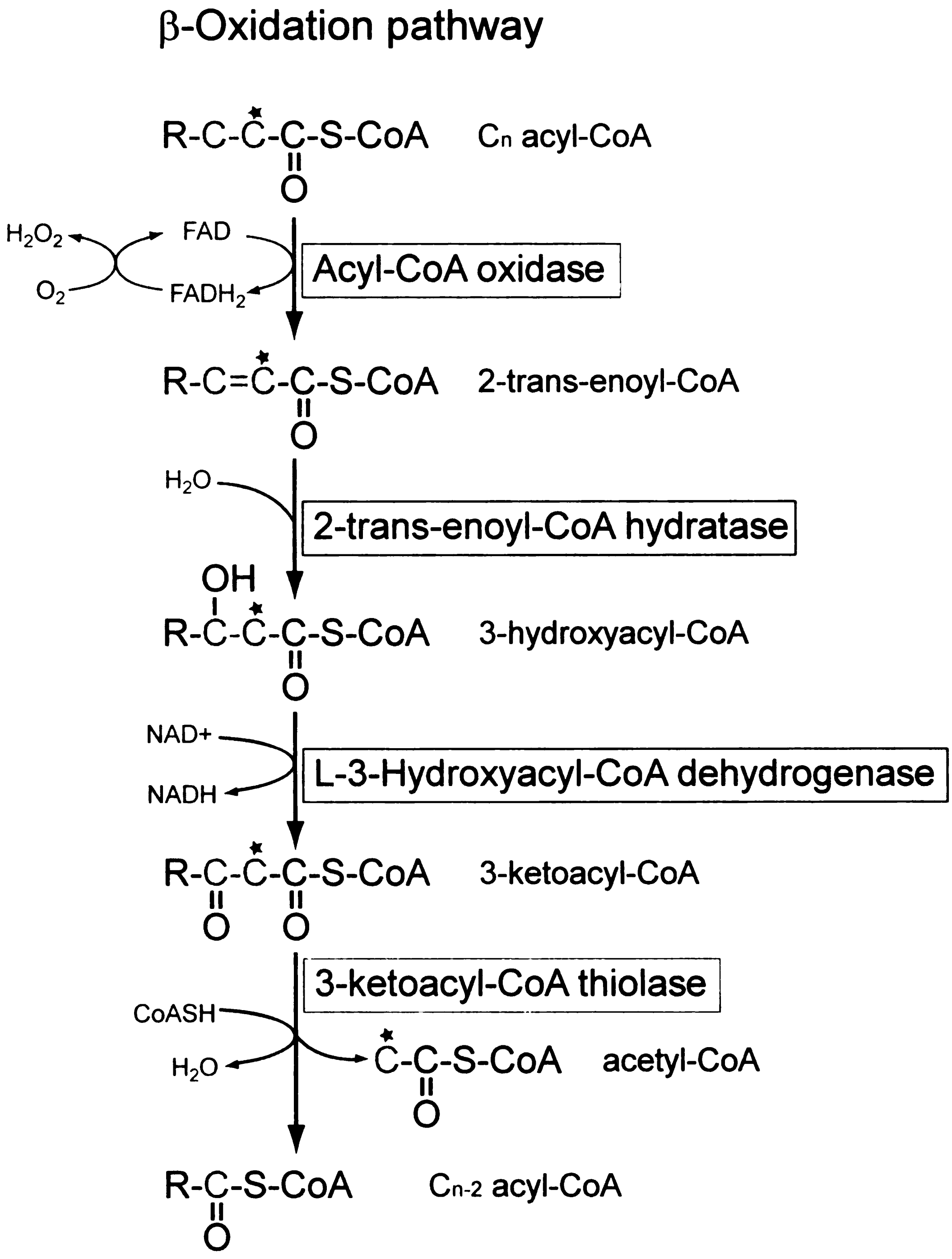


Figure 2. Pathway for catabolism of fatty acids via β-oxidation. In plants, β-oxidation takes place in peroxisomes. Free fatty acid Cn acyl-CoA are reduced by two carbons during each cycle of β-oxidation by four steps. 1. Dehydrogenation by acyl-CoA oxidase, 2. addition of water by 2-trans-enoyl-CoA hydratase, 3. dehydrogenation by L-3-hydroxyacyl-CoA dehydrogenase, and 4. cleavage of acetyl-CoA by 3-ketoacyl-CoA thiolase to produce Cn-2 acyl-CoA. Stars indicate the carbon position during each reaction. Hydrogens in the carbon-hydrogen bonds are not shown.

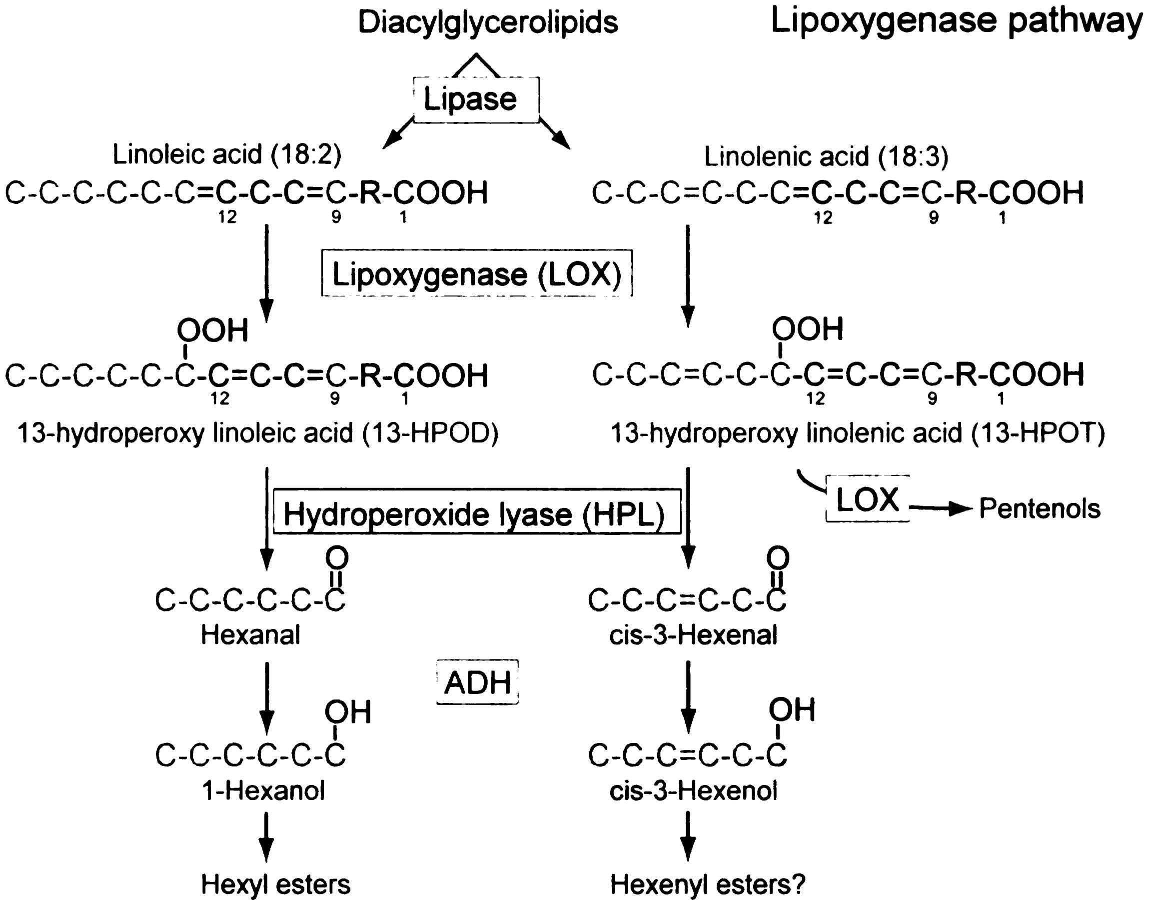


Figure 3. Pathway for catabolism of lipids and fatty acids via lipoxygenase. In plants, lipoxygenase activity are found to be in chloroplasts (Hatanaka, 1993). Linoleic (18:2) or linolenic (18:3) acids are formed by lipase. Lipoxygenase peroxidizes linoleic and linolenic acid to 13-hydroperoxy linoleic acid (13-HPOD) and 13-hydroperoxy linolenic acid (13-HPOT), respectively. Hydroperoxide lyase cleaves 13-HPOD and 13-HPOT into hexanal and cis-3-hexenal, respectively. Hexanal and cis-3-hexenal is reduced by alcohol dehydrogenase to hexanol and cis-3-hexenol respectively. Hydrogens in the carbon-hydrogen bonds are not shown.

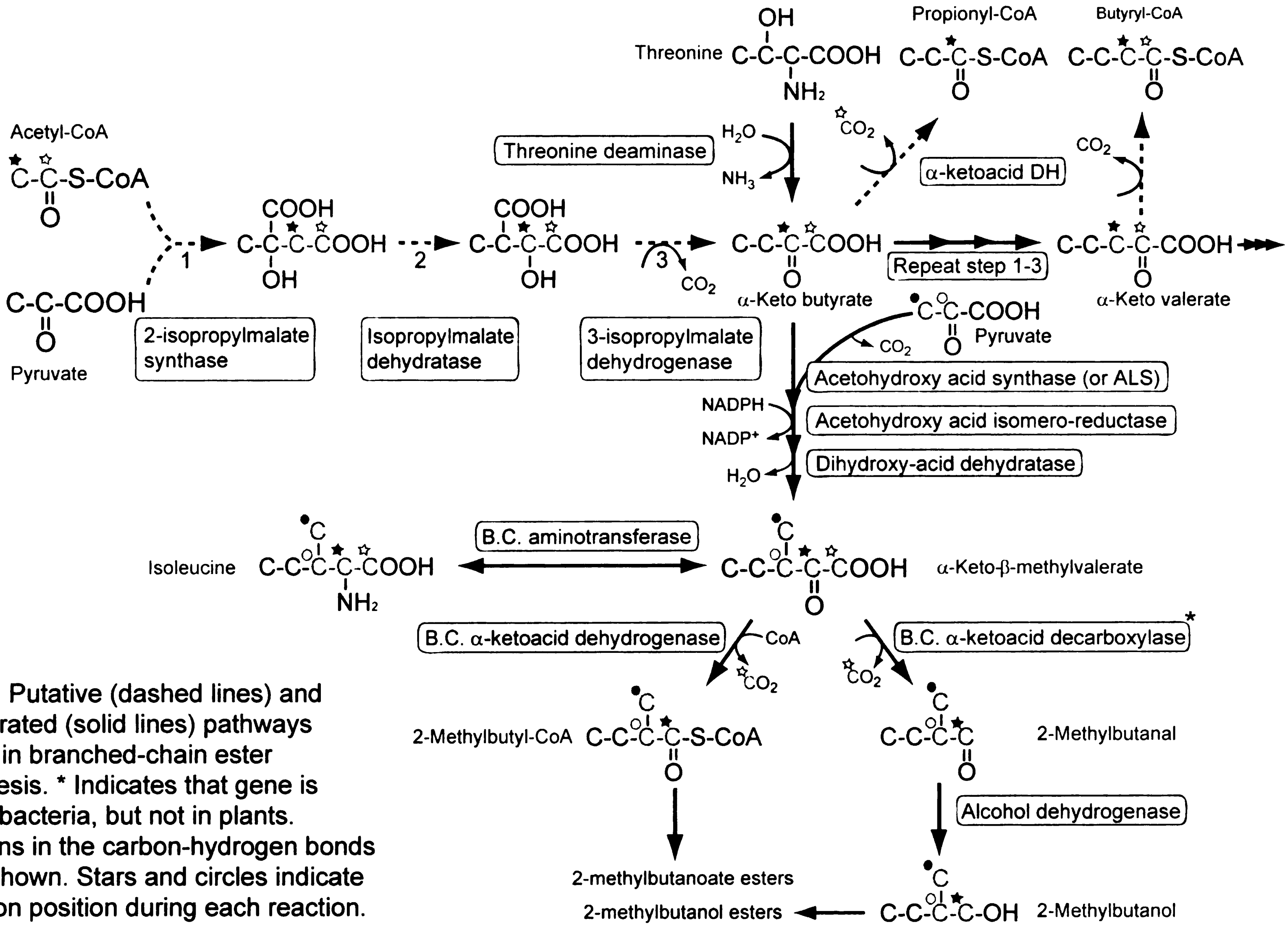
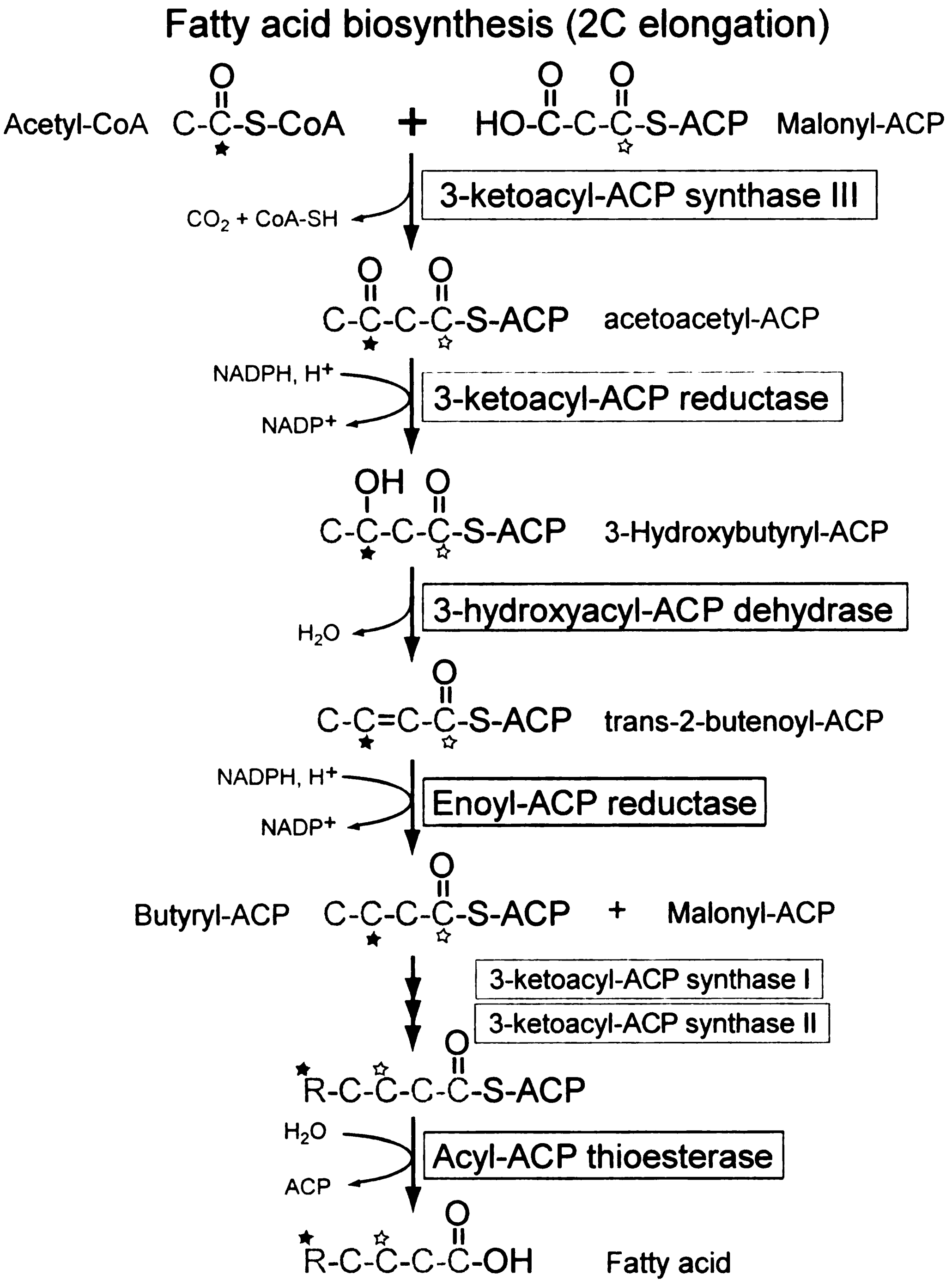
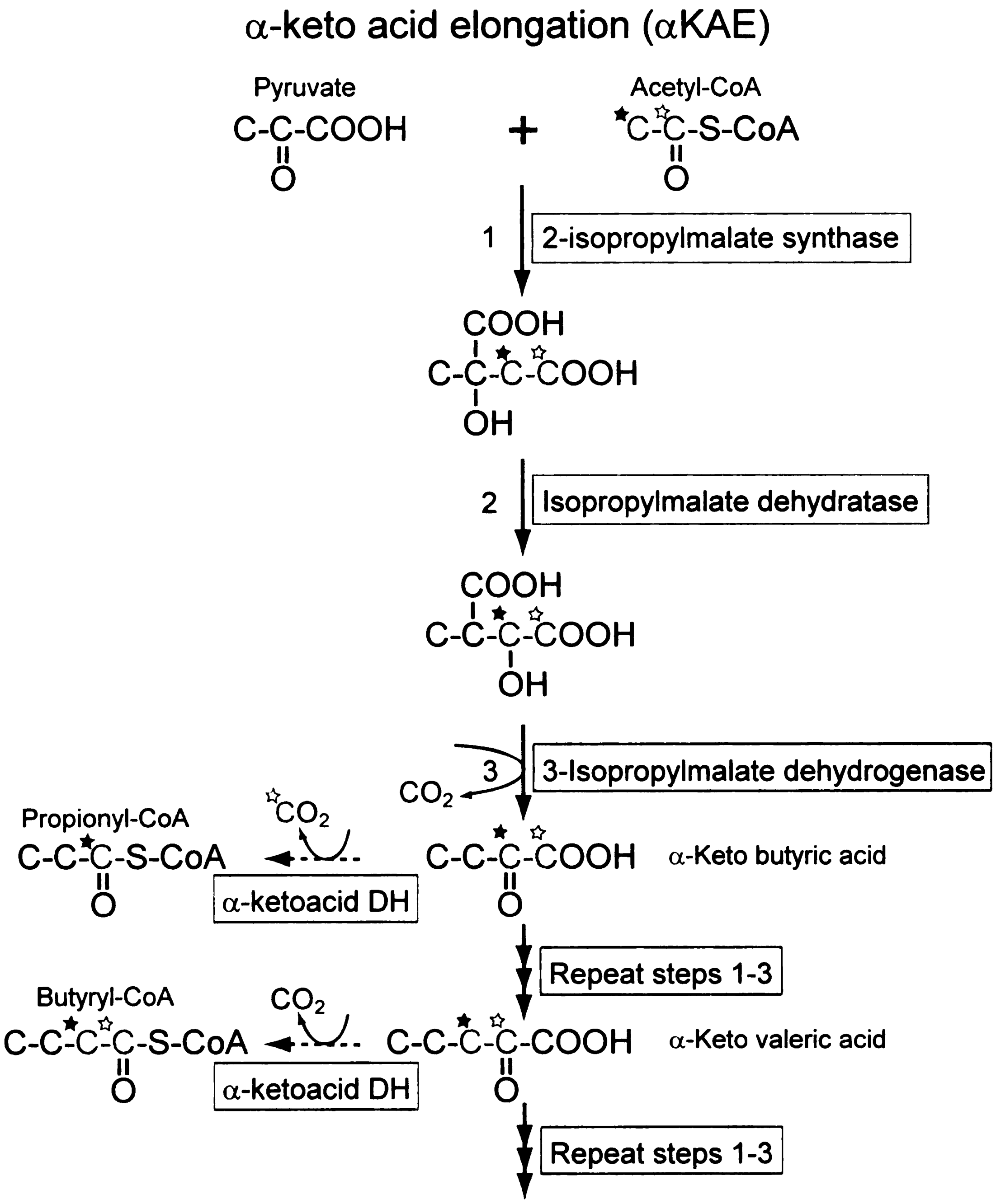


Figure 4. Putative (dashed lines) and demonstrated (solid lines) pathways involved in branched-chain ester biosynthesis. * Indicates that gene is found in bacteria, but not in plants. Hydrogens in the carbon-hydrogen bonds are not shown. Stars and circles indicate the carbon position during each reaction.



Fatty acid biosynthesis (2C elongation)

Figure 5. Pathway for fatty acid biosynthesis through two-carbon chain elongation in plants. Acetyl-ACP (primer) and malonyl-ACP (chain extender) is condensed by 3-ketoacyl-ACP synthase III. The next 3 steps are reduction by 3-ketoacyl-ACP reductase, dehydration by 3-hydroxyacyl-ACP dehydrase, and reduction by enoyl-ACP reductase. The cycle repeats to condense with malonyl-ACP until the chain length is 16-18, in general. The final step is terminated by acyl-ACP thioesterase by hydrolysis. Stars indicate the carbon position during each reaction. Hydrogens in the carbon-hydrogen bonds are not shown.



Kroumova and Wager, 2003

Figure 6. Pathway for fatty acid biosynthesis through single-carbon chain elongation (α-keto acid elongation, αKAE) in plants. Pyruvate (primer) and acetyl-CoA (chain extender) is condensed by 2-isopropylmalate synthase. The next 2 steps are isomerization and dehydration by isopropylmalate dehydratase, and decarboxylation by 3-isopropylmalate dehydrogenase. The cycle repeats to condense with acetyl-CoA or terminated to produce acyl-CoA. Stars indicate the carbon position during each reaction. Hydrogens in the carbon-hydrogen bonds are not shown.

CHAPTER III

CHARACTERIZATION OF VOLATILE ESTER BIOSYNTHESIS BY ‘JONAGOLD’ APPLES DURING THE RIPENING PROCESS

INTRODUCTION

Aroma is a flavor component with an important role in apple fruit quality. Of the aroma active compounds, esters are the predominant compounds produced by horticultural crops such as apple, banana, melon, and strawberry. The ratio and type of esters vary by fruit species and even among cultivars within species (Kakiuchi et al., 1986). Some aroma compounds increase intensity of 'sweet flavor' and can affect the perception of sweetness in fruits (Young et al., 1996) while others add additional flavor notes (Williams et al., 1977).

Characteristic fruit volatiles are produced via three routes: from autonomous production, from heat during cooking, and from tissue disruption (Beaudry, 2000). Some compounds are produced by more than one method, but to a significant extent these pathways provide distinct volatile aroma profiles. Fresh apples and prepared apple products (e.g. fresh apple slices, apple sauce, cooked apple products, apple cider) emit over 200 volatile compounds (Dimick and Hoskin, 1983). Fresh apples autonomously produce an abundance of hexyl acetate, butyl acetate, and 2-methylbutyl acetate during ripening. These esters are considered to confer typical apple aroma characteristics (Paillard, 1990). Despite the existence of 'typical' apple notes, apple cultivars have differing volatile profiles (Fellman et al., 1993; Guadagni et al., 1971; Kakiuchi et al., 1986; Mattheis et al., 1998; Song and Bangerth, 1996). For example, 'Golden Delicious' mostly produces acetate esters, 'Starking' produces a large amount of propanoate esters, and 'Richared' and 'Canada blanc' produce elevated levels of butanoate esters (Paillard, 1990). As the ripening stage changes from preclimacteric to postclimacteric, the amounts of individual compounds also change over time, altering the ester composition (Ferenczi, 2003; Mattheis et al., 1991b).

Esters in apples are comprised of an alkyl (alcohol-derived) group and an alkanoate (acid-derived) group. The alkanoate group is derived from a CoA thioester of a short- to medium-chain length fatty acid. The acyl-CoA and the alcohol are joined by an enzyme called alcohol acyltransferase (AAT) to form the ester (Figure 7). The carbon chain length of alcohol substrates for AAT normally ranges between 2-6 carbons whereas acyl-CoA substrates typically have 2-8 carbons (Ferenczi, 2003). Several hypotheses have been suggested to propose pathways by which ester precursors are formed. Straight-chain ester precursors may be formed from fatty acids by a combination of β-oxidation and lipoxygenase activities (Drawert, 1975; Sanz et al., 1997; Yahia, 1994). Branched-chain ester precursors may be formed from the metabolism of branched-chain amino acids (BCAA) (Pérez et al., 2002; Rowan et al., 1996, 1998; Tressl and Drawert, 1973). In apple, the primary branched-chain esters have 2-methylbutyl alkanoate and/or alkyl groups. These branched-chain groups are likely derived from isoleucine metabolism. A small amount of 2-methylpropyl esters are also found in apple, apparent products of valine metabolism. However, 3-methylbutyl esters which would be expected from leucine metabolism are typically absent. 3-Methylbutyl esters are abundantly produced in banana and provide this fruit its characteristic aroma (Jayanty et al., 2002).

Apple ripening is regulated by ethylene and ester biosynthesis begins as ethylene production commences (Biale, 1964; McGlasson, 1970). In addition to aroma volatile formation, ethylene induces several physiological changes including anthocyanin accumulation, chlorophyll loss, a rise in respiration, and cell wall degradation. These changes were used to measure the ripening stages of the fruit. The objective of this research was to investigate the pattern of ester biosynthesis as it relates temporally to physiological changes of ‘Jonagold’ apple during ripening and senescence. This study served to establish a physiological foundation for further genomic characterization.

MATERIALS AND METHODS

In order to temporally link the physiological changes of ‘Jonagold’ apple to the pattern of ester biosynthesis during ripening and senescence, ethylene, CO_2 production, texture, color, °Brix, firmness, and starch index were used to characterize the ripening stages of the fruit during an 81-day period. Data collection began well before the onset of ripening and continued until fruit were fully senescent.

Plant Material

‘Jonagold’ apples were harvested from the Michigan State University Clarksville Horticultural Experiment Station, Clarksville, MI. From September 2, 2004 (Day 0) until ripening was fully engaged on October 7, 2004 (Day 35), fruits were collected for examination every three to four days from the field. On each occasion, fruit were held overnight in the laboratory to equilibrate to laboratory temperature (21±1°C) and covered with ventilated, black, 4-mil thick plastic bags to avoid desiccation and responses to intermittent laboratory light before analysis. All fruits (approximately 200) remaining on the tree were harvested and transported to the laboratory at once on October 7, 2004 (harvest date, Day 35) after it was apparent that ripening was underway. This was done to avoid damage in the field due to freezing and fruit drop. Thereafter, fruit were maintained at room temperature (21±1°C) and covered with plastic bags as described previously. The fruit were examined every three to four days from harvest (October 7, 2004) until the conclusion of the study on November 23, 2004 (Day 81).

On each evaluation date, 20 apples were randomly chosen and the internal ethylene content of each was measured. Of these, the fourteen fruit having an internal ethylene content nearest the median were selected for further analysis. The four fruit having ethylene levels closest to the median were used for analysis of CO_2 production, ester emission, and textural properties. The remaining ten fruit were used to obtain skin color (percentage of redness), background, starch, °Brix measurements, and gene expression (Chapter IV).

Volatile analysis

To collect volatiles, apples were held at 20°C in a 1-L Teflon container (Savillex Corporation, Minnetonka, MN) fitted with two gas-sampling ports, each sealed with a Teflon-lined half-hole septum (Supelco Co., Bellefonte, PA). Headspace volatiles were sampled after 20 minutes using a 1-cm long solid-phase micro extraction (SPME) fiber (65 µm PDMS-DVB, Supelco Co., Bellefonte, PA). SPME sorption time was 3 min. The exposed fibers were immediately transferred to a gas chromatograph (GC) (HP-6890, Hewlett Packard Co., Wilmington, DE) injection port (230°C) and desorbed for 2 min. Desorbed volatiles were trapped on-column using a liquid nitrogen cryofocussing trap. Separation of volatiles was by capillary column (SupelcoWax-10, Supelco, Bellefonte, PA, 29 m x 0.2 mm i.d., 0.2 μm coating film). The temperature of the GC was programmed from 40 to 240°C at a rate of 50°C/min; the flow rate of the helium carrier gas was 1 mL/min and the GC was operated in splitless mode. Detection was by time-of-flight mass spectrometry (Pegasus II, LECO Corp., St. Joseph, MI) (GC/MS) according to the method of Song et al. (1997). Identification and quantification of compounds were by comparison of the mass spectrum and MS response (total ion count, TIC), respectively, with those of authenticated reference standards and spectra in the National Institute for Standard and Technology (NIST) mass spectra library (Search Version 1.5).

Measurement of respiration

To measure respiration, CO_2 accumulation in the 1-L Teflon container was measured on 0.1-mL gas samples withdrawn from the sampling port using an insulin-type plastic syringe. CO_2 was sampled at the same time volatiles were measured, following the 20-minute holding period. The gas sample was injected into an infrared gas analyzer (model 225-MK3; Analytical Development Co., Hoddesdon, England) operated in a flow-through mode with N_2 as the carrier gas and a flow rate of 100 $mL \cdot min^{-1}$. The CO_2 concentration was calculated relative to a certified standard (Matheson Gas Products Inc., Montgomeryville, PA) containing 0.979 $\mu L \cdot L^{-1}$ ethylene, 4.85% CO_2, and 1.95% O_2 balanced with N_2.

Measurement of ethylene production

The internal ethylene content of apple fruit was determined by withdrawing a 1-mL gas sample from the interior of the apples and subjecting the gas sample to gas chromatographic analysis (Carle Series 400 AGC; Hach Company, Loveland, CO) as previously described (Mir et al., 2001). The GC was fitted with a 6-m-long, 2-mm-i.d. stainless-steel column packed with activated alumina and was equipped with a flame ionization detector. The ethylene detection limit was approximately 0.005 $\mu L \cdot L^{-1}$. Ethylene concentrations were calculated relative to the certified standard noted previously.

Texture analysis

Texture was analyzed as the force (N) required to bring about tissue failure under both compressive and tensile strain using a texture analyzer (TA.XT2i, Texture Technologies, Scarsdale, NY). The signal was analyzed by commercial software (Texture Expert Exceed, version 2.60, Texture Technologies, Scarsdale, NY) (Figure 8). Tensile failure was measured on bars of cortex tissue cut from apple fruit using a 3-point bending rig (TA-92, 88.9 mm x 101.6 mm, the distance between the two supports was 28.57 mm). The tissue bars were square in cross-section (9 mm x 9 mm) and approximately 7cm in length and made using a French-fry maker (GPC-2549, Progressive International Corp., Kent, WA). To make the bars, apple fruit were trimmed to fit into the device and placed so that the tissue bars were cut parallel to the axis of the fruit.

The bars of tissue were placed on the bending rig such that the force applied was perpendicular to the fruit axis. The descending probe advanced at a rate of 2 mm/second and the maximum force encountered was recorded.

Compressive failure was tested on cylinders of cortex tissue taken from portions of the fruits not used for tensile failure samples. The cylinders were made using a cork borer with an internal diameter of 16.5 mm. The cylinder was removed from the equator of the fruit, normal to the fruit axis. The skin was removed and the core end was trimmed away to yield a cylinder 2 cm in length. Compressive failure of the tissue was tested by placing the cylinder between two flat plates on the texture analyzer (Figure 9). The probe speed was 2 mm/second. When the probe touched the fruit, force (N), distance (mm), and speed (s) were recorded. The instrument continued to apply force until the probe had traveled 8 mm from the point initial contact with the fruit. The maximum force encountered was recorded.

RESULTS

Skin color (percent of redness) increased from 22% on Day 0 to over 95 % by Day 39 (data not shown). Background color (green=5, yellow=1) had a reciprocal pattern to red color development, beginning at 5 (green) on Day 0 and gradually decreasing to 1 (yellow) on Day 81 (data not shown). Starch conversion to sugars, as measured by the starch index (1 - 8) started at 2 on Day 0 and continued to increase, reaching a maximum of 8 on Day 32 (data not shown). The pattern for soluble solids was similar to that of starch conversion;

the initial soluble solid was 12 °Brix on Day 0 and reached its maximum of 16 °Brix on Day 39 (data not shown).

Ethylene production remained low until Day 18 (Figure 10). Day 21 was considered to be the onset of the ethylene climacteric when the level rose above 0.2 $\mu L \cdot L^{-1}$. Esters were first detected at very low levels as early as Day 14, a week before the onset of a sustained increase in ethylene. Immediately after the ethylene increase, a rapid and large increase in ester biosynthesis began. On Day 32, ester biosynthesis was approximately half-maximal and the respiratory climacteric was engaged. On Day 39, ester biosynthesis approached its maximum, respiratory activity peaked, being twice as great as on Day 32, and rapid tissue softening began (Figure 11). The most abundant esters on Day 42, when the highest total ester response was recorded, were hexyl acetate, 2-methylbutyl acetate, butyl acetate, and hexyl 2-methylbutanoate; the complexity of the ester profile was at its maximum at this point (Figure 12). Near-maximal ester biosynthesis continued until Day 49 when the respiratory climacteric reached its conclusion and tissue softening was completed. On Day 60, the internal ethylene content reached its maximum (approximately 690 $\mu L \cdot L^{-1}$) and the decline in ester biosynthesis was approximately at its midpoint. By Day 70, fruit were highly senescent and esters reached a postclimacteric minimum. Data collection ceased on Day 81. A total of 39 volatile compounds, including 31 esters, 5 alcohols, and 3 aldehydes were detected and evaluated from early pre-climacteric through late post-climacteric stages of development (Table 2).

Alcohol patterns

Alcohols were detected only after the onset of the ethylene climacteric (Day 21). Alcohols detected included ethanol, propanol, butanol, hexanol, and 2-methylbutanol (Figure 13). 2-Methylpropanol, pentanol, heptanol, and octanol were not detected. Hexanol was at its maximum relative abundance in the early ripening stages and butanol, propanol, and ethanol increased in the later developmental stages. Ethanol did not appear until Day 53 and contributed only a small amount relative to other alcohols in total proportion. Butanol appeared on Day 25, and predominated in total proportion from Day 42 until senescence. 2-Methylbutanol was detected as early as Day 21 and had a high peak on Day 39, increasing 5- to 7-fold immediately after the final harvest date (October 7, 2004, Day 35), and declined sharply thereafter.

Ester patterns based on the alkyl moiety

Abundant esters included those with butyl, hexyl, 2-methylbutyl, and propyl alkyl groups; pentyl and ethyl esters were less abundant and heptyl and octyl esters were not detected (Table 2). The emission of esters of the above alcohols increased sharply after the onset of the ethylene climacteric (Figure 14). The patterns for the esters reflected the patterns of their corresponding alcohols (Figures 13-20); hexyl esters were most abundant in the early ripening stages and butyl, propyl, and ethyl esters rose in the later stages (Figures 14-19). Total ion count (TIC) response was greatest for acetate esters (Figures 15-20). Butyl esters were the most diverse with the butyl alkyl group being produced in combination with 2, 3, 4, 5, 6, 7, and 8 carbon straight-chain alkanoate groups and 2-methylbutanoate (Table 2). Several pentyl esters were detected but no pentanol was found.

Ester patterns based on the alkanoate moiety

Esters detected classed by the alkanoate moiety included: acetate, propanoate, butanoate, 2-methylbutanoate, hexanoate, and octanoate esters (Table 2). All esters rapidly increased after the onset of the ethylene climacteric (Figures 21-27). However, only acetate and propanoate esters were maintained at high levels during senescence, while butanoate, hexanoate, octanoate, and 2-methylbutanoate esters decreased after the climacteric peak on Day 42. Acetate esters had the greatest diversity of alkyl groups (Table 2). For each of the alkanoate ester classes, hexanol-derived alkyl groups predominated early in ripening and consistently declined in later development stages relative to other ester classes. Pentanoate and heptanoate esters were only produced with butanol-derived alkyl groups. No 2-methylpropanoate esters were detected.

DISCUSSION

The essentially concurrent events of autocatalytic ethylene production and ester synthesis in ‘Jonagold’ was similar to that previously described for ‘Bisbee Delicious’ (Mattheis et al., 1991b), ‘Golden Delicious’ (Song and Bangerth, 1996), and ‘Redchief Delicious’ apples (Ferenczi, 2003). This is consistent with previous studies demonstrating that ester production requires ethylene action (Defilippi et al., 2004; Ferenczi et al., 2006). Once climacteric ethylene production was engaged, the internal ethylene content did not decrease during senescence as previously observed in 'Redchief Delicious' apple (Ferenczi, 2003).

The predominant emission of acetate esters by 'Jonagold' fruit can permit this cultivar to be categorized as an 'acetate-producing' type (Paillard, 1990). The four major esters: hexyl acetate, 2-methylbutyl acetate, butyl acetate, and hexyl 2-methylbutanoate found in abundance at the climacteric peak (Day 42) in this study were similarly abundant at the climacteric peak for 'Redchief Delicious' apple (Ferenczi, 2003), although the proportion of these four major esters differed between the two cultivars. The high amount of butyl and hexyl esters are a characteristic of the 'Jonagold' aroma profile (Dixon and Hewett, 2000). Ethanol-derived esters such as ethyl acetate, ethyl butanoate, and ethyl 2-methylbutanoate, which contribute significantly to 'Delicious' or 'Bisbee Delicious' apple aroma (Guadagni et al., 1971; Mattheis et al., 1991a, 1991b), were only found during postclimacteric stage and the production was quite low relative to other esters. 2-Methylbutanoate esters, which have a fruity and sweet aroma (Plotto et al., 2000), increased during the climacteric peak. In addition to the esters described, 4-methoxyallylbenzene, which contributes a spicy aroma to apples (Williams et al., 1977) was also detected in the 'Jonagold' (data not shown). Other common apple odorants including 3-penten-2-ol (apple-like aroma) found in 'Starkspur Golden' fruit (Vanoli et al., 1995), or β-damascenone (fruity odor) found in 'Cox Orange' and 'Elstar' (Fuhrmann and Grosch, 2002), were not detected in 'Jonagold'. Despite the abundance of alcohol precursors, free acids were not detected or were tentatively detected below threshold levels, which is in contrast to data for 'Redchief Delicious' apple in which acetic acid, propanoic acid, 2-methylbutanoic acid, butanoic acid, and hexanoic acid were found (Ferenczi, 2003).

'Jonagold' apple is the result of a cross between 'Jonathan' and 'Golden Delicious' (Gianfranceschi et al., 1998). The ratios and abundance of butyl, 2-methylbutyl, and hexyl acetate esters in the 'Jonagold' may be a function of genetic descendance from the 'Jonathan' variety, which produced profiles similar to 'Jonagold' in butyl, 2-methylbutyl, and hexyl acetate esters (Kakiuchi et al., 1986).

As noted previously, the final step for ester formation is catalyzed by AAT. Substrate preferences for AAT isozymes have been characterized (Olías et al., 2002; Ueda et al., 1992). Souleyre et al. (2005) cloned MpAAT1 from 'Gala' apple and determined the substrate preference for the transcribed protein. The rate of acetate ester formation by MpAAT1 depended on substrate alcohol concentration; at low alcohol substrate concentrations, the preference order was 2-methylbutanol>hexanol>butanol. At high alcohol substrate concentrations, the preference order was hexanol>2-methylbutanol>butanol. In the current study, for the acetate esters in 'Jonagold', the abundance of the various alcohol classes seemed to reflect the abundance and availability of corresponding alcohols. As a result, it was not clear whether MpAAT1 exerted any influence on the profile due to its substrate preferences. Despite the abundance of 2-methylbutanol, only 2-methylbutyl acetate and 2-methylbutyl butanoate esters were produced. In contrast, 2-methylbutanoate esters were formed with five different alkyl groups from ethyl, propyl, butyl, pentyl, and hexyl alcohol. Interestingly, no 2-methylbutyl 2-methylbutanoate was detected, despite the high levels of 2-methylbutyl and 2-methylbutanoate esters. Furthermore, the production (TIC) of hexyl esters was greater than 2-methylbutyl esters even though the amount of hexanol detected was less than 2-methylbutanol. In apples, only *MpAAT1* (Souleyre et al., 2005) and *MpAAT2* (Li et al., 2006) have been isolated, but other AAT genes exist. In the Tree Fruit Technology genomic analysis tool apple database version 4.0 (http://genomics.msu.edu/fruitdb/analyses/apple.shtml), there are 13 clusters tentatively identified as AAT. The observed diversity of ester formation in the present study might be explained by the involvement of several AAT isozymes that differ in substrate preferences (Holland et al., 2005; Ueda et al., 1992).

The presence of butyl, hexyl, and 2-methylbutyl esters at low levels before the onset of ethylene production and the lack of detectable quantities of corresponding alcohols or acids suggest that isozymes of AAT were functional before the onset of the ethylene climacteric, but that precursors were low or limiting (Jayanty et al., 2002). This is in contrast to the suggestion that the AAT gene is fully under ethylene control (Defilippi et al., 2005a). Preclimacteric AAT activity has also been demonstrated for ‘Redchief Delicious’ apple (Ferenczi et al., 2006). Similarly in melon, the expression of *CM-AAT1* gene was not completely suppressed under the ethylene-suppressed antisense ACC oxidase melon fruit and fruit treated with 1-MCP (an inhibitor of ethylene action), although the expression was severely reduced in both lines (Yahyaoui et al., 2002).

Collectively, the data suggests that AAT activity may be highly regulated developmentally, having both constitutive and ethylene-driven components.

The rapid increase in branched-chain esters at the climacteric peak is indicative of elevated enzyme activity in the pathways for the formation of branched-chain ester precursors (Defilippi et al., 2005b; Nie et al., 2005). α-Keto-β-methylvalerate, which is a product of isoleucine degradation and a precursor in isoleucine formation, is believed to produce either 2-methylbuyl-CoA by branched-chain α-ketoacid dehydrogenase (BCKDH) or 2-methylbutanal by branched-chain α-ketoacid decarboxylase (BCKDC). 2-Methylbutanal is likely further converted to 2-methylbutanol by alcohol dehydrogenase (Wyllie et al., 1996). The fact that 'Jonagold' can produce both 2-methylbutanoate and 2-methylbutanol esters suggests that the apple utilizes BCKDH and BCKDC pathways where the branched-chain α-keto acid is either converted to acids or alcohols, respectively. This is in contrast to mammals, which can only catabolize branched-chain α-keto acid to form branched-chain acyl-CoAs (Platell et al., 2000).

Lipoxygenase activity may supply the saturated C6 alcohol, hexanol, for ester biosynthesis (Rowan et al., 1999). This supposition is supported by the finding that lipoxygenase activity in apple is highly specific in peroxidizing linoleic acid to 13-hydroperoxy derivatives (Kim and Grosch, 1979). β-Oxidation can theoretically form short- to medium-chain C4, C6, C8, and C10 fatty acids from long-chain fatty acids. However, the longest chain esters detected were octanoate esters. Feeding studies suggest that both β-oxidation and lipoxygenase pathway may play a major role in the abundance of even-chain number esters (Rowan et al., 1999). However, increase in the odd-number C3 alkyl and alkanoate groups in the later ripening stages is not supportive of the involvement of either lipoxygenase or β-oxidation, which do not easily allow an explanation for their biosynthesis. Further, the trends of decreasing C6 alkyl groups and increasing C3 alkyl and alkanoate groups, may be indicative of the involvement of an as-yet undescribed pathway. While C5 alcohols may be supplied from lipoxygenase, they have only been observed under anaerobic conditions (Salch et al., 1995). As for C7 esters, neither pathway explains their production easily without invoking α-oxidation. Since ester biosynthesis can not be readily explained by only fatty acid break down, it is possible that the biosynthetic pathways are involved.

Likely, the complex profile of esters produced by ripening apple fruit is the product of both synthetic and catabolic reactions. The recently characterized fatty acid synthetic pathway involving single carbon elongation (Kroumova and Wagner, 2003) may, for instance, be responsible for synthesizing odd-number carbon ester substrates and may also supplement even-number carbon substrates. This pathway may also impact the branched-chain esters, especially those thought to be derived from isoleucine. One of the products of the single-carbon fatty acid synthetic pathway is α-keto butyrate, a precursor in the formation of α-keto-β-methylvalerate and, ultimately, isoleucine.

The majority of the feeding studies suggest that ester biosynthesis is from fatty acid and amino acid catabolism pathways. Interestingly, however, no published studies have been found exploring the idea of synthetic or anabolic precursor formation. So far, few of the genes or proteins thought to supply the substrates of alcohols and acyl-CoAs in these pathways have been characterized relative to ester biosynthesis in fruit. Only the final step of ester formation involving AAT has been studied at protein and molecular levels in ester-producing fruits.

CONCLUSION

Physiological changes during fruit ripening were similar to those found for previous studies of apple. The abundance in butyl acetate, 2-methylbutyl acetate, hexyl acetate, and hexyl 2-methylbutanoate were characteristic for ‘Jonagold’ during the climacteric peak. Ethyl ester production did not significantly contribute to the volatile profile of this variety. Esters possessing long-chain alkyl and alkanoate moieties were most prevalent relative to those with shorter-chain groups at earlier ripening stages. Developmentally-dependent changes in alcohol abundance were similar to those of their corresponding esters suggesting that alcohol precursor availability exerts a major influence in ester biosynthesis. Presence of esters before the onset of ethylene suggests that the AAT is active before ripening begins, but precursors are limiting. Not all the reciprocal combinations of alcohols and acids were found in ‘Jonagold’, suggesting that several AAT isozymes with differing substrate preferences may be active.

The diversity of esters and changes in the ester profile during ripening suggested that, in addition to fatty acid catabolism, fatty acid biosynthesis pathways may contribute to ester precursor formation. Additional information of gene regulation and protein activity level will be useful in identifying controlling steps in ester formation pathways.

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Acid Alcohol	Acetic (2C)	Propanoic (3C)	2-Mpropanoic (branched) (4C)	Butanoic (4C)	2-Mbutanoic (branched) (5C)	Pentanoic (5C)	Hexanoic (6C)	Heptanoic (7C)	Octanoic (8C)
Ethanol (2C)	Ethyl* acetate 11	Ethyl* propanoate 3	---	Ethyl* butanoate 0.4	Ethyl* 2-Mbutanoate 1	---	---	---	---
Propanol (3C)	Propyl* acetate 188	Propyl* propanoate 81	---	Propyl* butanoate 29	Propyl 2-Mbutanoate 49	---	Propyl* hexanoate 23	---	Propyl octanoate 0.3
2-Mpropanol (branched) (4C)	2-Mpropyl acetate 35	---	---	---	---	---	---	---	---
Butanol (4C)	Butyl* acetate 369	Butyl* propanoate 166	---	Butyl* butanoate 115	Butyl* 2-Mbutanoate 148	Butyl pentanoate 3	Butyl* hexanoate 80	Butyl heptanoate 0.4	Butyl octanoate 4
2-Mbutanol (branched) (5C)	2-Mbutyl* acetate 403	---	---	2-Mbutyl* butanoate 13	---	---	---	---	---
Pentanol (5C)	Pentyl acetate 98	Pentyl propanoate 67	---	---	Pentyl 2-Mbutanoate 17	---	Pentyl hexanoate 10	---	---
Hexanol (6C)	Hexyl acetate 365	Hexyl* propanoate 110	---	Hexyl* butanoate 78	Hexyl 2-Mbutanoate 256	---	Hexyl* hexanoate 26	---	Hexyl octanoate 0.5
Heptanol (7C)	---	---	---	---	---	---	---	---	---
Octanol (8C)	---	---	---	---	---	---	---	---	---

Table 2. Matrix of esters detected organized by acid and alcohol precursors for apple fruit.
 * indicates that the acid and alcohol combinations are reciprocal.
Numbers indicate the maximum GC/MS response (TIC) detected during preclimacteric to postclimacteric stages of fruit ripening in millions.

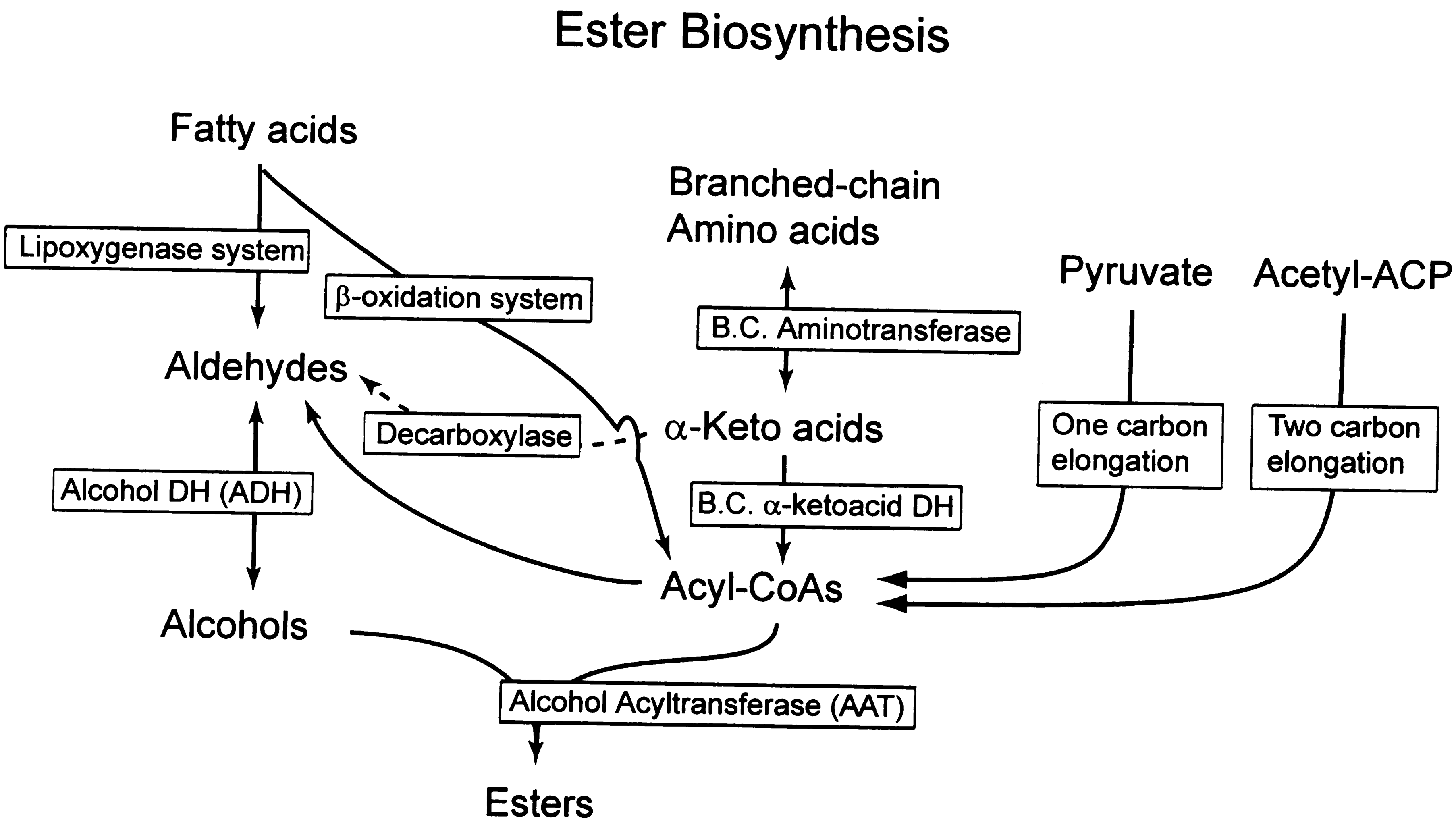


Figure 7. Pathways having potential to supply alcohol and acyl-CoA substrates for ester formation.

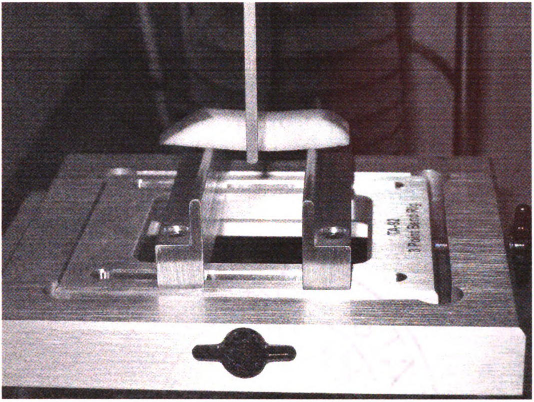


Figure 8. Tensile failure was measured on bars of cortex tissue using a 3-point bending rig. The tissue bars were square in cross-section (9 mm x 9 mm) and approximately 7cm in length and were cut parallel to the axis of the fruit. The force was applied perpendicular to the fruit axis and the maximum force encountered during the test was recorded.

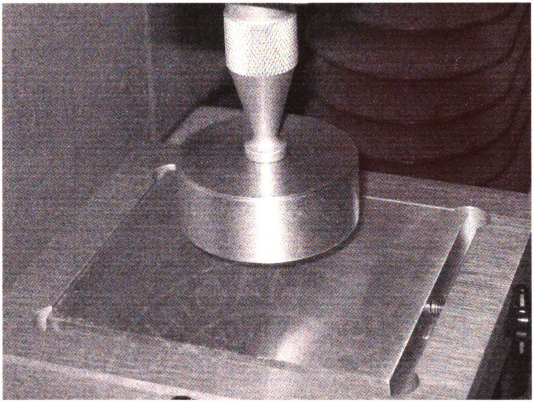


Figure 9. Compressive failure was tested on cylinders of cortex tissue placed on the flat plate and compressed by a descending flat-faced probe. The cylinders were made with an internal diameter of 16.5 mm, trimmed to a length of 2 cm, and taken from tissue just beneath the skin. The cylinder was removed from the equator of the fruit, normal to the fruit axis. The force was applied perpendicular to the fruit axis and the maximum force encountered during the test was recorded.

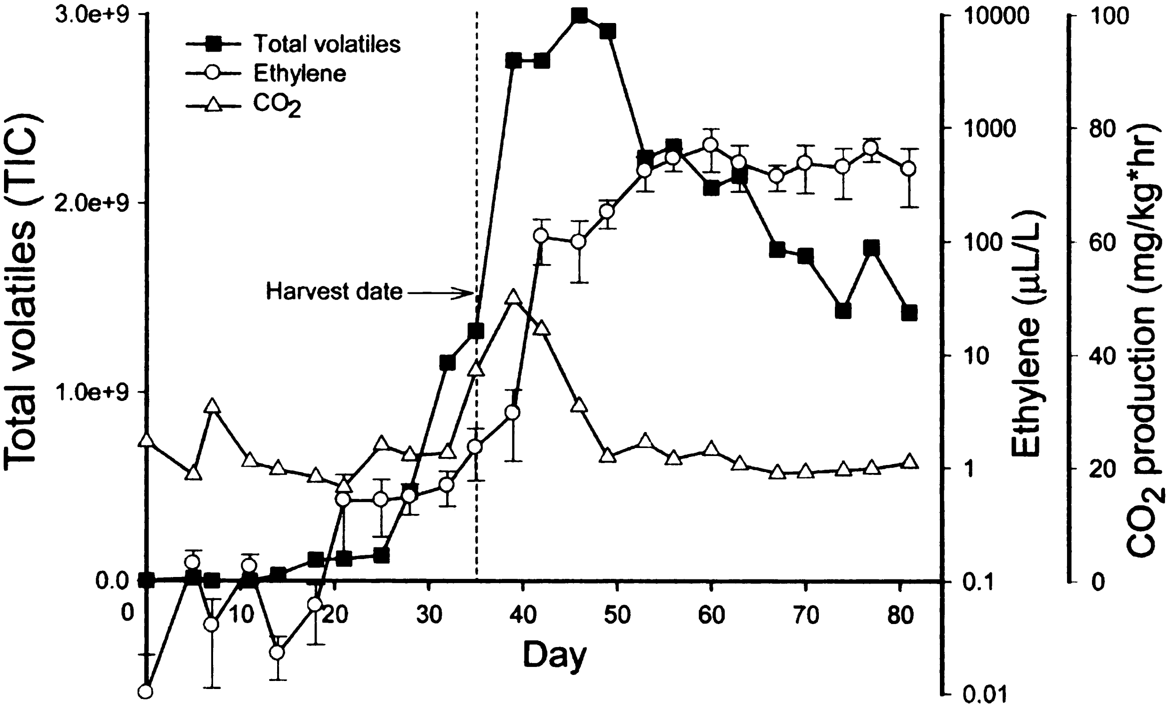


Figure 10. Ontogeny of total volatiles, ethylene and respiration (CO_2 production) during ripening and senescence of 'Jonagold' apples. The apple fruit were examined from Sept 2, 2004 (Day 0) to Nov. 23, 2004 (Day 81). Fruits were harvested every 3-4 days from the field until Oct. 7, 2004 (Day 35), and thereafter maintained at room temperature (21±1°C). Each symbol represents the average of four replications for total volatiles and respiration, and ten replications for ethylene. Vertical bars represent mean ± SD.

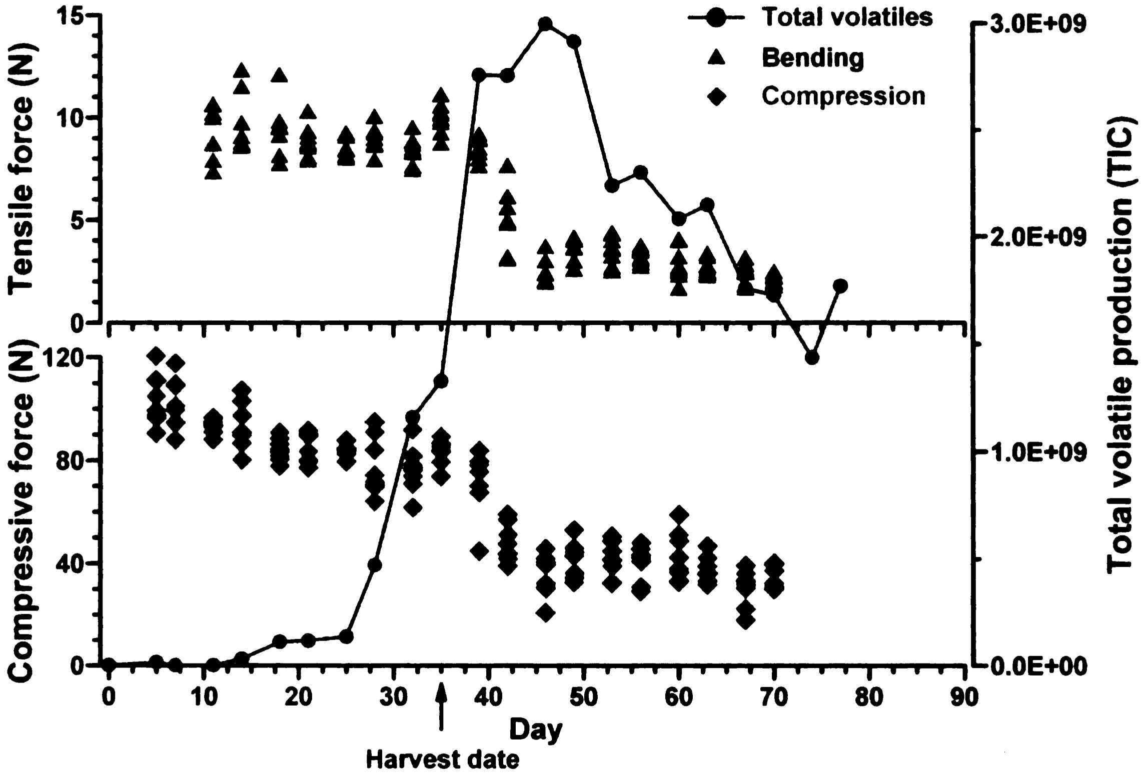


Figure 11. Ontogeny of total volatiles and textural changes during ripening and senescence of 'Jonagold' apples. Tensile failure/bending force was to measure 'crispness' and compressive force for 'softening'. The apple fruit was examined from Sept 2, 2004 (Day 0) to Nov. 23, 2004 (Day 81). Fruits were harvested every 3-4 days from the field until Oct. 7, 2004 (Day 35), and thereafter maintained at room temperature (21±1°C). The samples were taken opposite sides of each fruit and each symbol represents four fruit replications.

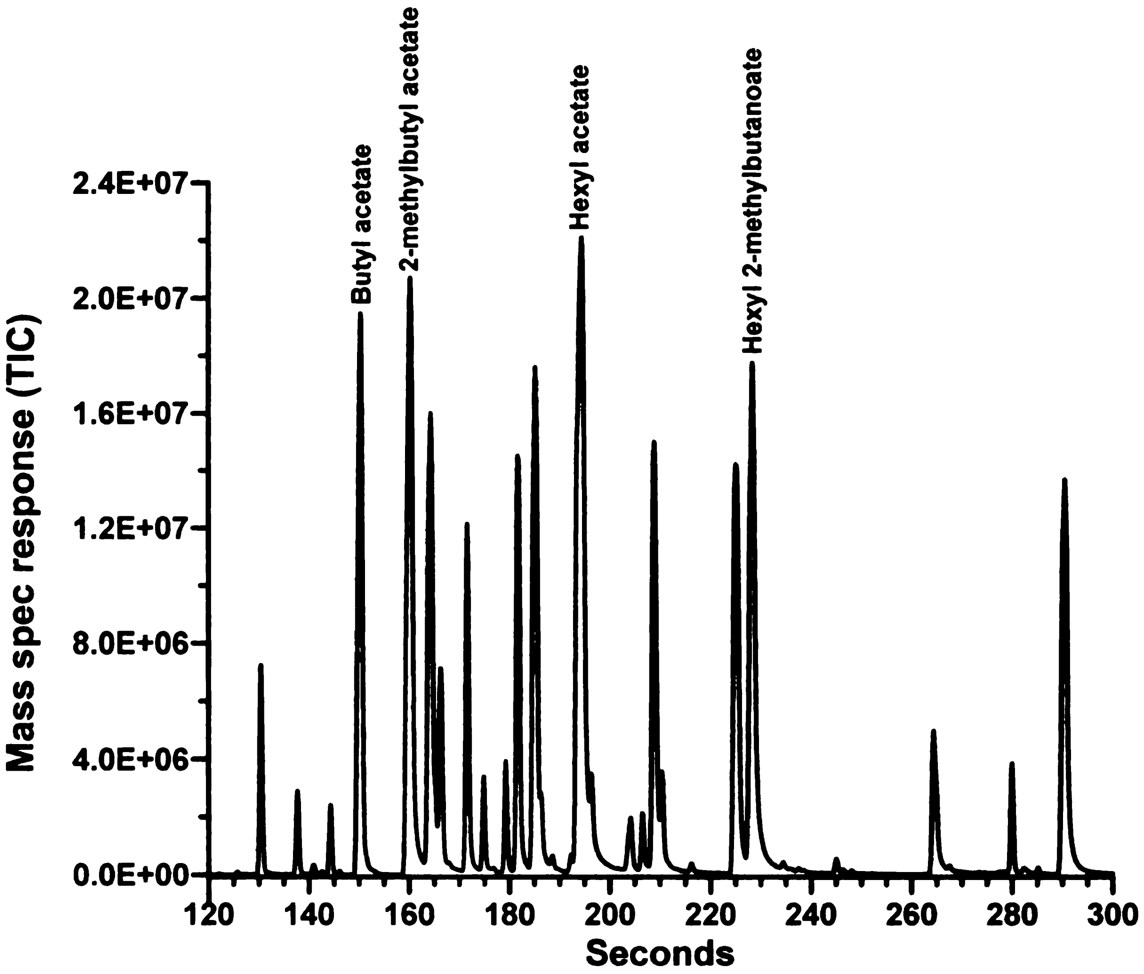


Figure 12. Representative gas chromatograph of the headspace of 'Jonagold' apples at the respiratory climacteric on Day 42. Predominant esters were butyl acetate, 2-methylbutyl acetate, hexyl acetate, and hexyl 2-methylbutanoate. A total of 31 esters, 3 aldehydes, and 5 alcohols were detectable at this point in development.

Figure 13. Patterns of alcohol emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of ethanol, propanol, butanol, hexanol, and 2-methylbutanol. B. Alcohol proportions (% of total alcohols). Each symbol represents the average of four replications.

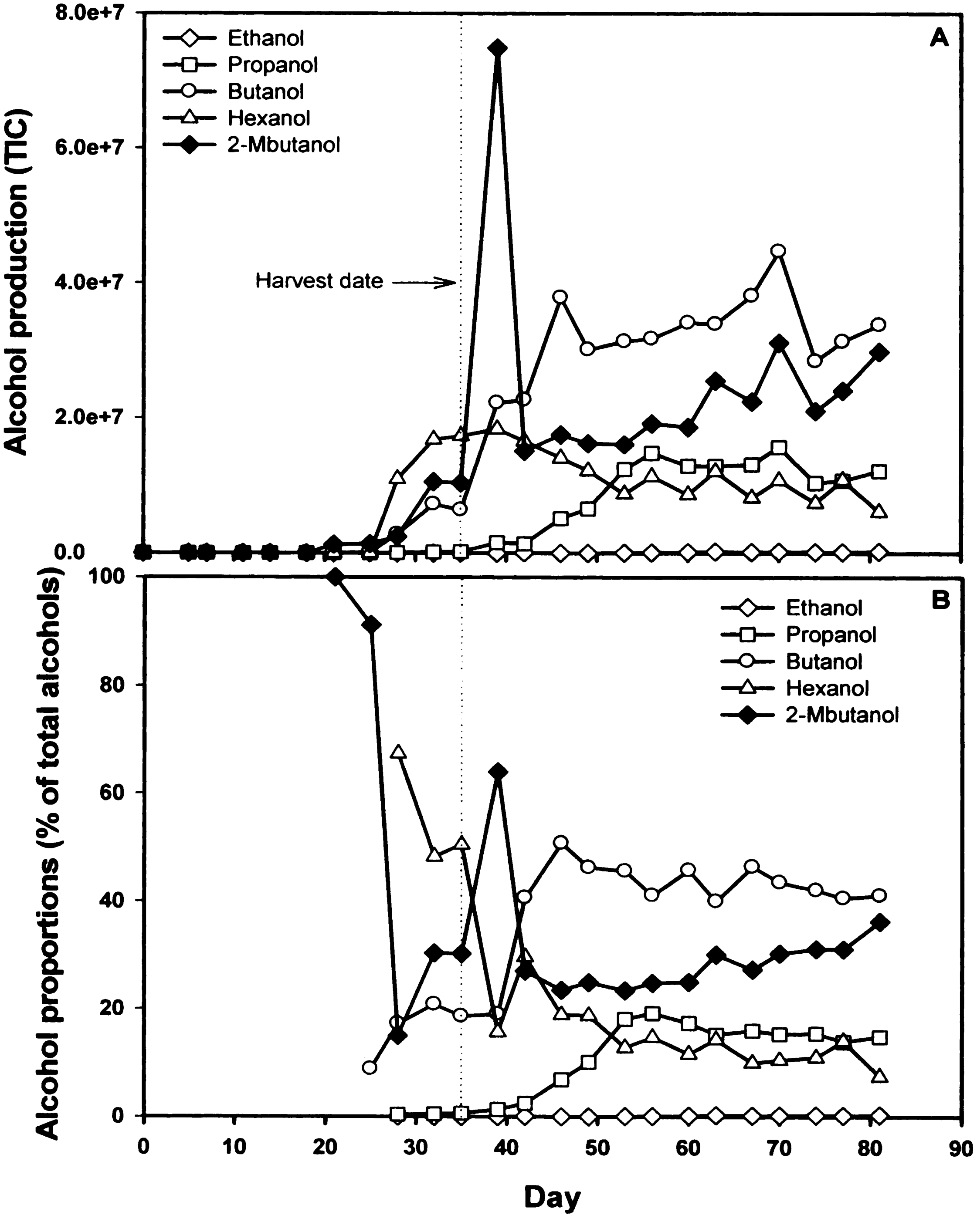

8.0e+7
6.0e+7
4.0e+7
2.0e+7
0.0
Alcohol production (TIC)
Ethanol
Propanol
Butanol
Hexanol
2-Mbutanol
A
Harvest date
100
80
60
40
20
0
Alcohol proportions (% of total alcohols)
B
0
10
20
30
40
50
60
70
80
90
Day

Figure 13.

Figure 14. Patterns of alcohol ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of propanol, propyl esters, butanol, butyl esters, hexanol, hexyl esters, 2-methylbutanol, and 2-methylbutyl esters. B. Alcohol and alcohol ester proportions (% of total alcohols or alcohol esters). Each symbol represents the average of four replications.

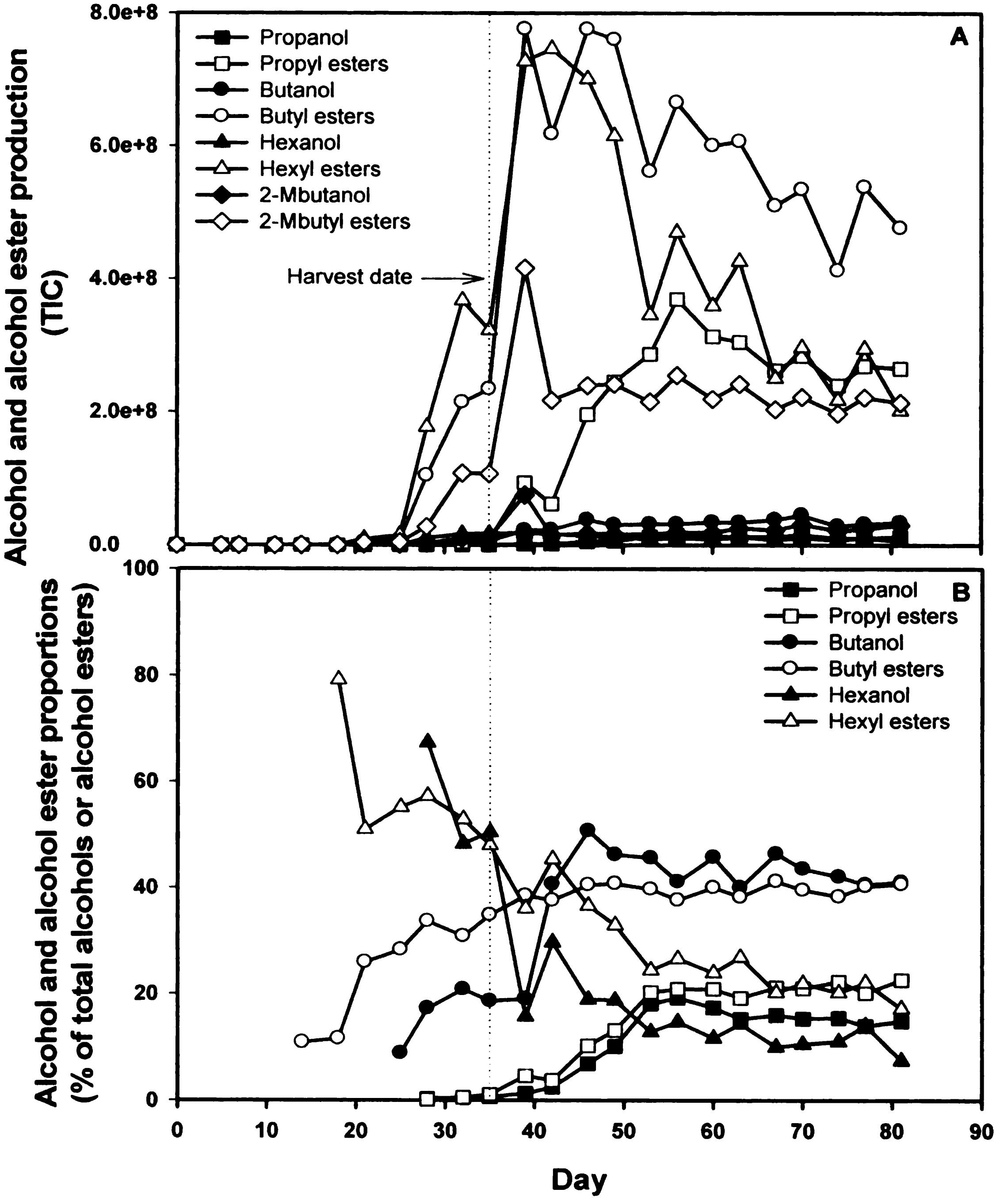

Alcohol and alcohol ester production (TIC)
8.0e+8
6.0e+8
4.0e+8
2.0e+8
0.0
A
Propanol
Propyl esters
Butanol
Butyl esters
Hexanol
Hexyl esters
2-Mbutanol
2-Mbutyl esters
Harvest date
Alcohol and alcohol ester proportions (% of total alcohols or alcohol esters)
100
80
60
40
20
0
B
Propanol
Propyl esters
Butanol
Butyl esters
Hexanol
Hexyl esters
0
10
20
30
40
50
60
70
80
90
Day

Figure 14.

Figure 15. Patterns of ethanol ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total ethanol esters and ontogeny of ethylene. B. GC/MS response (TIC) of total ethanol. C. GC/MS response (TIC) of ethyl acetate, ethyl propanoate, ethyl butanoate, and ethyl 2-methylbutanoate. D. Ethanol proportion (% of total alcohols). E. Ethanol ester proportions (% of total ethanol esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD.

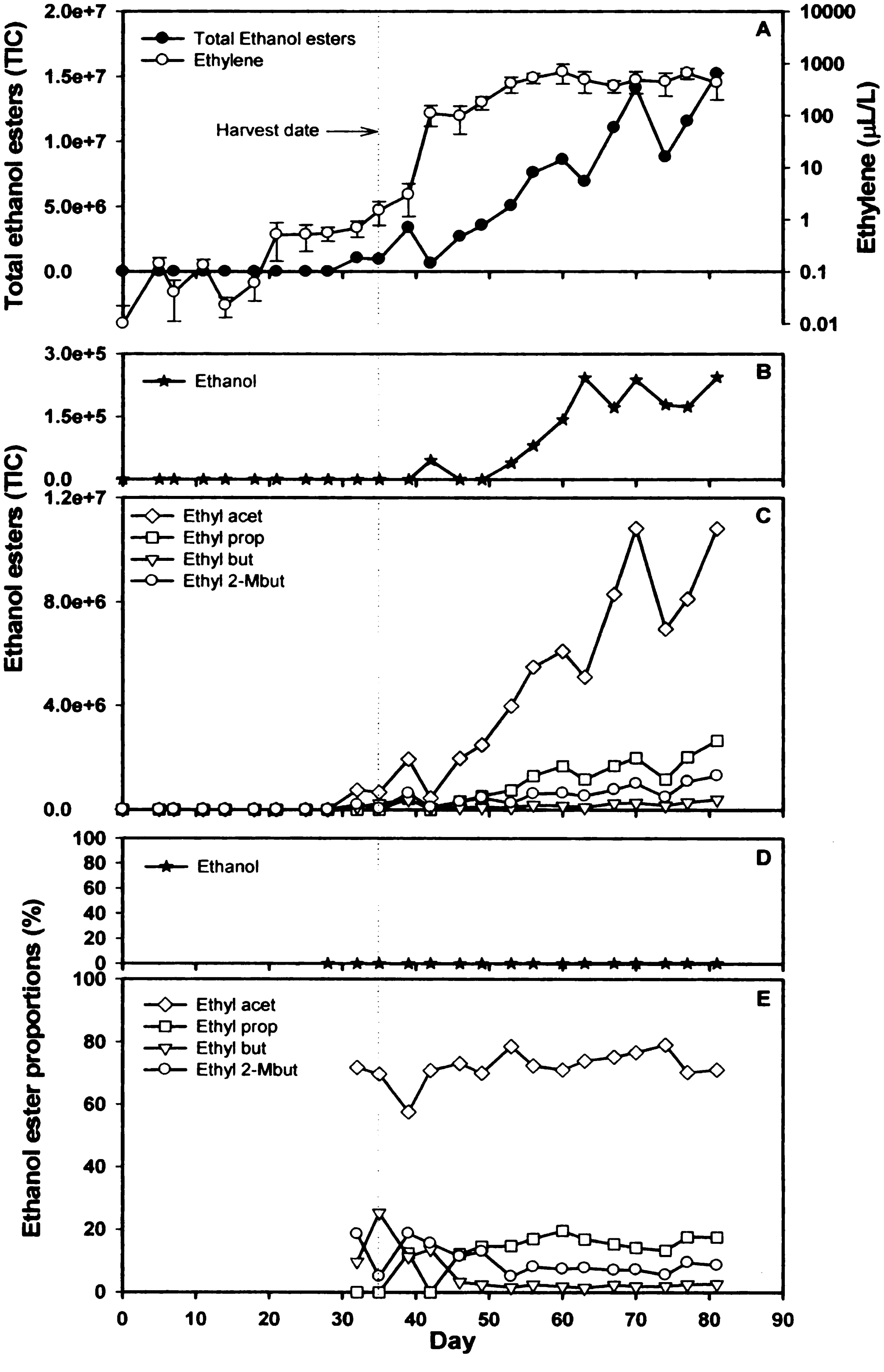

Total ethanol esters (TIC)
2.0e+7
1.5e+7
1.0e+7
5.0e+6
0.0
Total Ethanol esters
Ethylene
Harvest date
A
Ethylene (μL/L)
10000
1000
100
10
1
0.1
0.01
3.0e+5
1.5e+5
0.0
Ethanol
B
Ethanol esters (TIC)
1.2e+7
8.0e+6
4.0e+6
0.0
Ethyl acet
Ethyl prop
Ethyl but
Ethyl 2-Mbut
C
Ethanol ester proportions (%)
100
80
60
40
20
0
Ethanol
D
100
80
60
40
20
0
Ethyl acet
Ethyl prop
Ethyl but
Ethyl 2-Mbut
E
0
10
20
30
40
50
60
70
80
90
Day

Figure 15.

Figure 16. Patterns of propanol ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total propanol esters and ontogeny of ethylene. B. GC/MS response (TIC) of total propanol. C. GC/MS response (TIC) of propyl acetate, propyl propanoate, propyl butanoate, propyl 2-methylbutanoate, propyl hexanoate, and propyl octanoate. D. Propanol proportions (% of total alcohols). E. Propanol ester proportions (% of total propanol esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD.

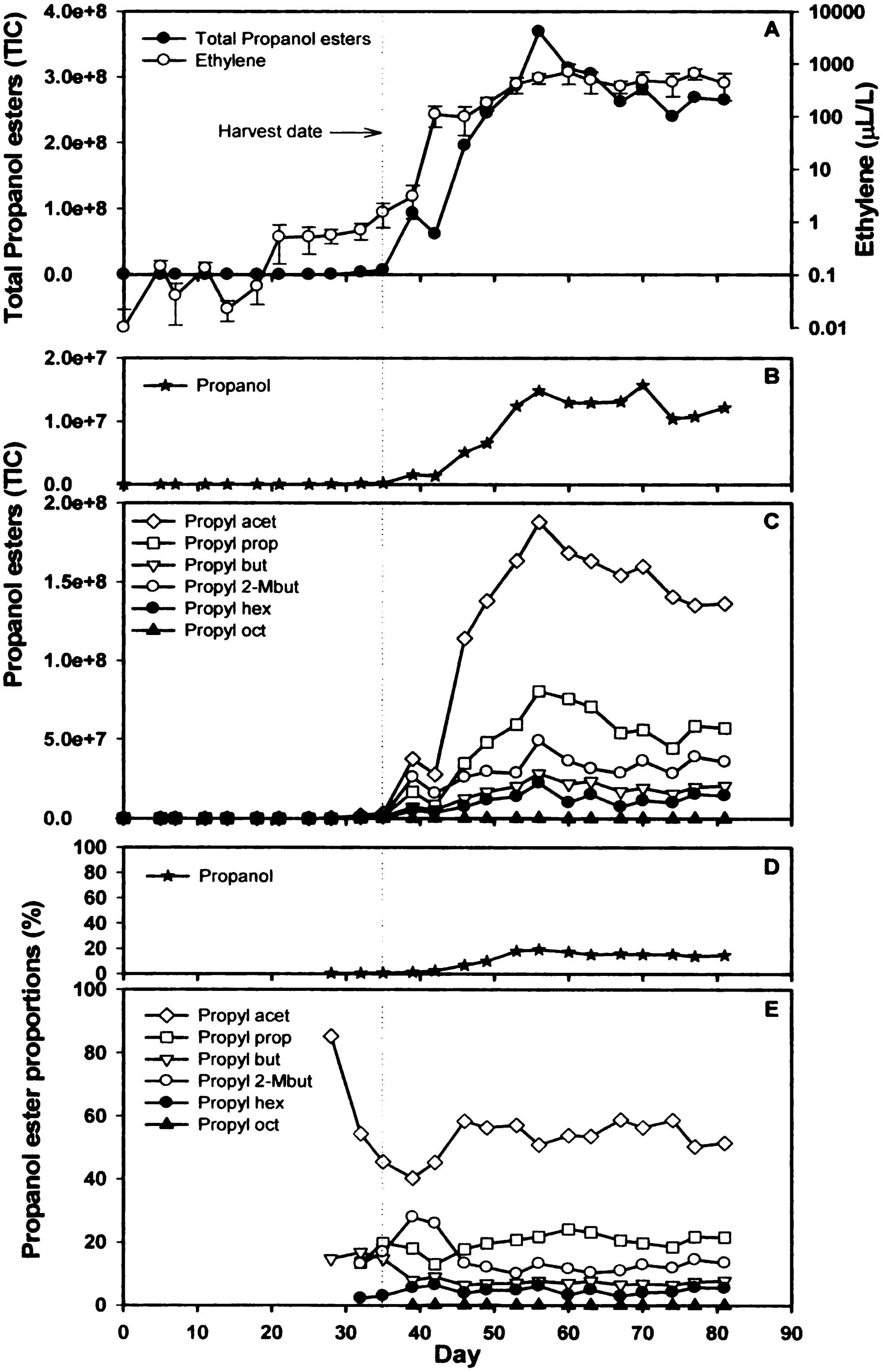
Total Propanol esters (TIC)
4.0e+8
3.0e+8
2.0e+8
1.0e+8
0.0
Total Propanol esters
Ethylene
Harvest date
A
Ethylene (µL/L)
10000
1000
100
10
1
0.1
0.01
Propanol esters (TIC)
2.0e+7
1.0e+7
0.0
Propanol
B
2.0e+8
1.5e+8
1.0e+8
5.0e+7
0.0
Propyl acet
Propyl prop
Propyl but
Propyl 2-Mbut
Propyl hex
Propyl oct
C
Propanol ester proportions (%)
100
80
60
40
20
0
Propanol
D
100
80
60
40
20
0
Propyl acet
Propyl prop
Propyl but
Propyl 2-Mbut
Propyl hex
Propyl oct
E
0
10
20
30
40
50
60
70
80
90
Day

Figure 16.

Figure 17. Patterns of butanol ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total butanol esters and ontogeny of ethylene. B. GC/MS response (TIC) of total butanol. C. GC/MS response (TIC) of butyl acetate, butyl propanoate, butyl butanoate, butyl 2-methylbutanoate, butyl pentanoate, butyl hexanoate, butyl heptanoate, and butyl octanoate. D. Butanol proportions (% of total alcohols). E. Butanol ester proportions (% of total butanol esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD.

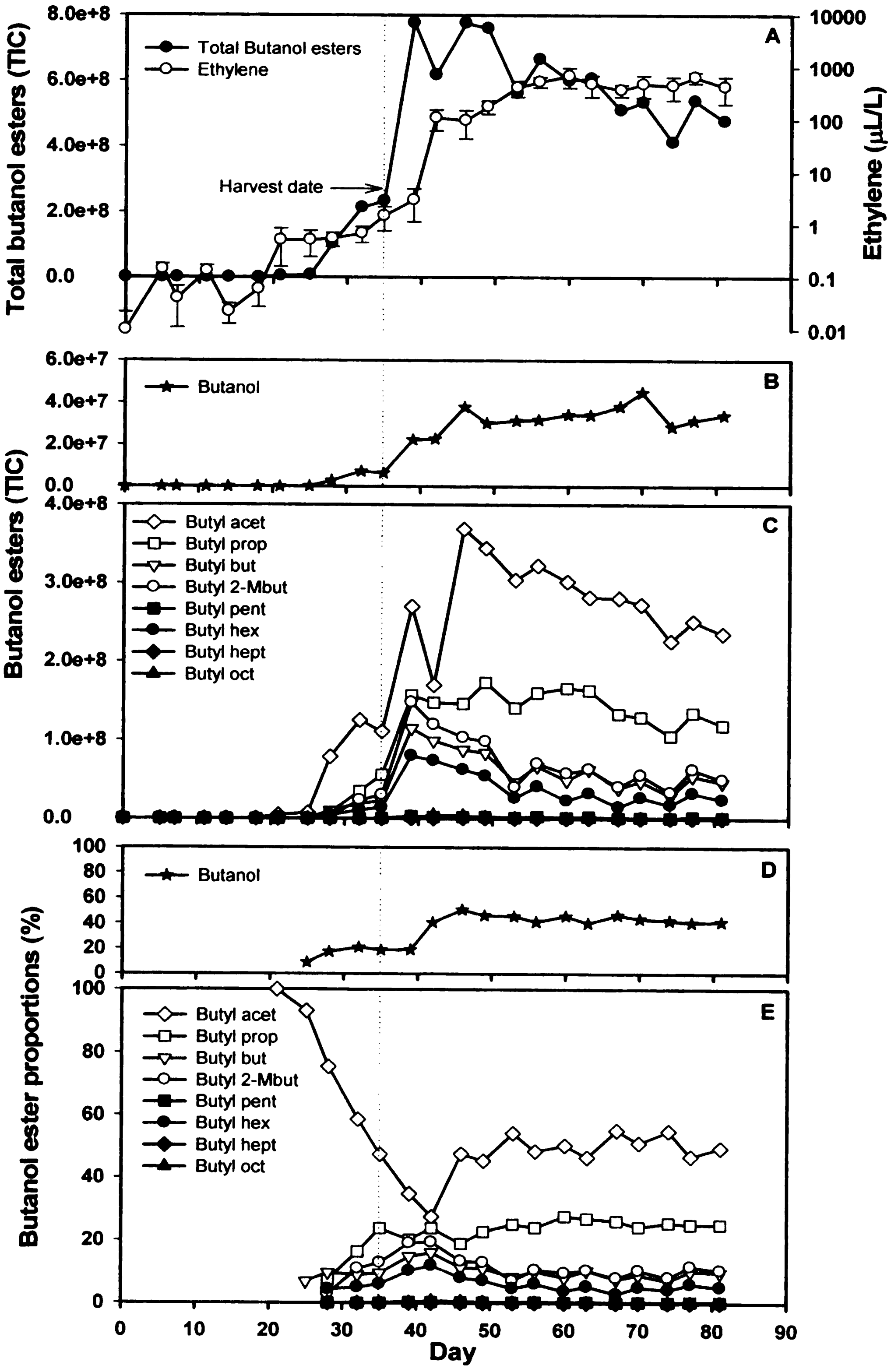
A
Total butanol esters (TIC)
8.0e+8
6.0e+8
4.0e+8
2.0e+8
0.0
Total Butanol esters
Ethylene
Harvest date
Ethylene (µL/L)
10000
1000
100
10
1
0.1
0.01
B
Butanol
6.0e+7
4.0e+7
2.0e+7
0.0
Butanol esters (TIC)
C
4.0e+8
3.0e+8
2.0e+8
1.0e+8
0.0
Butyl acet
Butyl prop
Butyl but
Butyl 2-Mbut
Butyl pent
Butyl hex
Butyl hept
Butyl oct
D
Butanol
100
80
60
40
20
0
Butanol ester proportions (%)
E
Butyl acet
Butyl prop
Butyl but
Butyl 2-Mbut
Butyl pent
Butyl hex
Butyl hept
Butyl oct
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
Day

Figure 17.

Figure 18. Patterns of pentanol ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total pentanol esters and ontogeny of ethylene. B. GC/MS response (TIC) of pentyl acetate, pentyl propanoate, pentyl 2-methylbutanoate, and pentyl hexanoate. C. Pentanol ester proportions (% of total pentanol esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD.

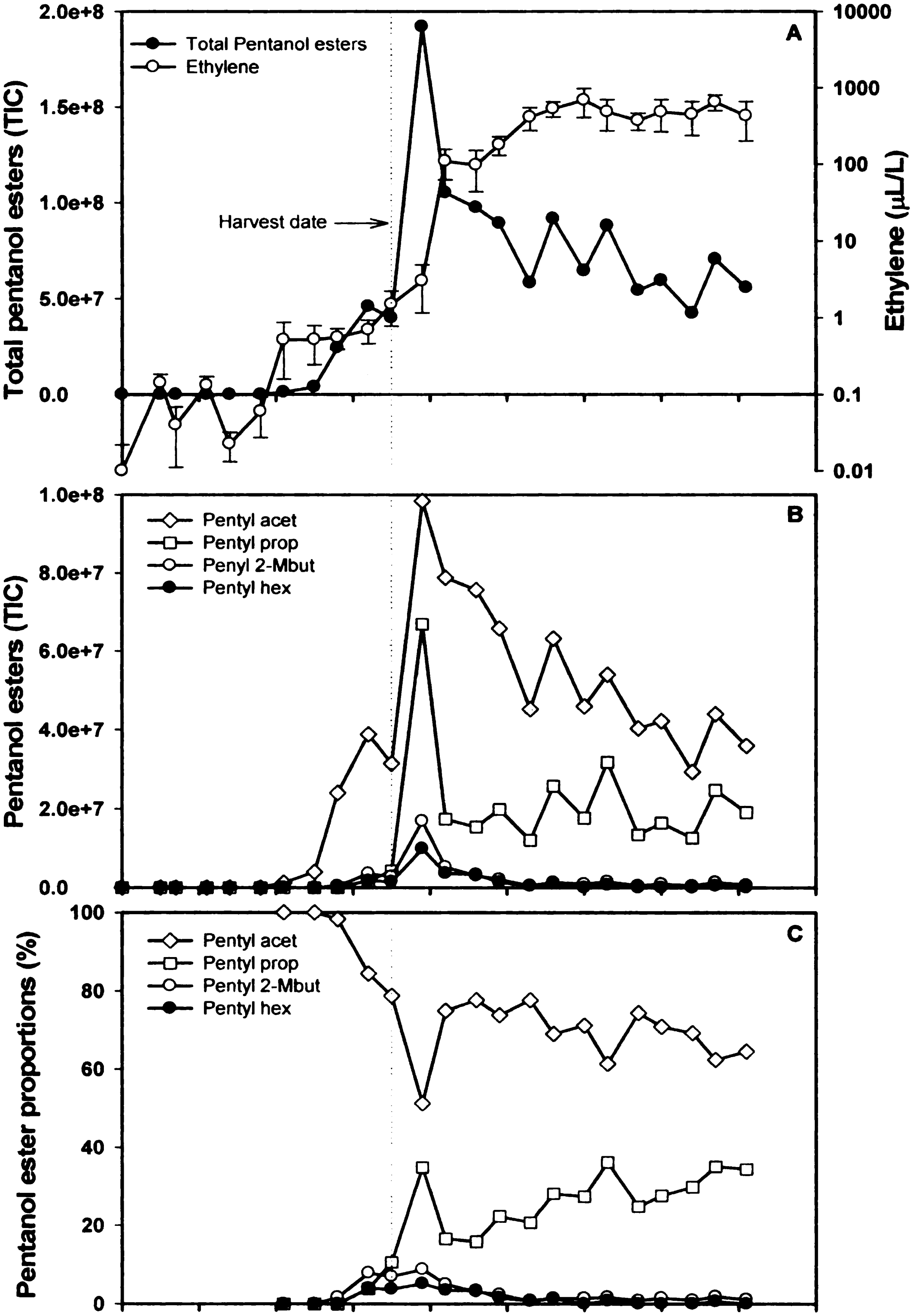

A
B
C
Total Pentanol esters
Ethylene
Harvest date
Total pentanol esters (TIC)
Ethylene (μL/L)
2.0e+8
1.5e+8
1.0e+8
5.0e+7
0.0
10000
1000
100
10
1
0.1
0.01
Pentyl acet
Pentyl prop
Penyl 2-Mbut
Pentyl hex
Pentanol esters (TIC)
1.0e+8
8.0e+7
6.0e+7
4.0e+7
2.0e+7
0.0
Pentyl acet
Pentyl prop
Pentyl 2-Mbut
Pentyl hex
Pentanol ester proportions (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
Day

Figure 18.

Figure 19. Patterns of hexanol ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total hexanol esters and ontogeny of ethylene. B. GC/MS response (TIC) of total hexanol. C. GC/MS response (TIC) of hexyl acetate, hexyl propanoate, hexyl butanoate, hexyl 2-methylbutanoate, hexyl hexanoate, and hexyl octanoate. D. Hexanol proportions (% of total alcohols). E. Hexanol ester proportions (% of total hexanol esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD.

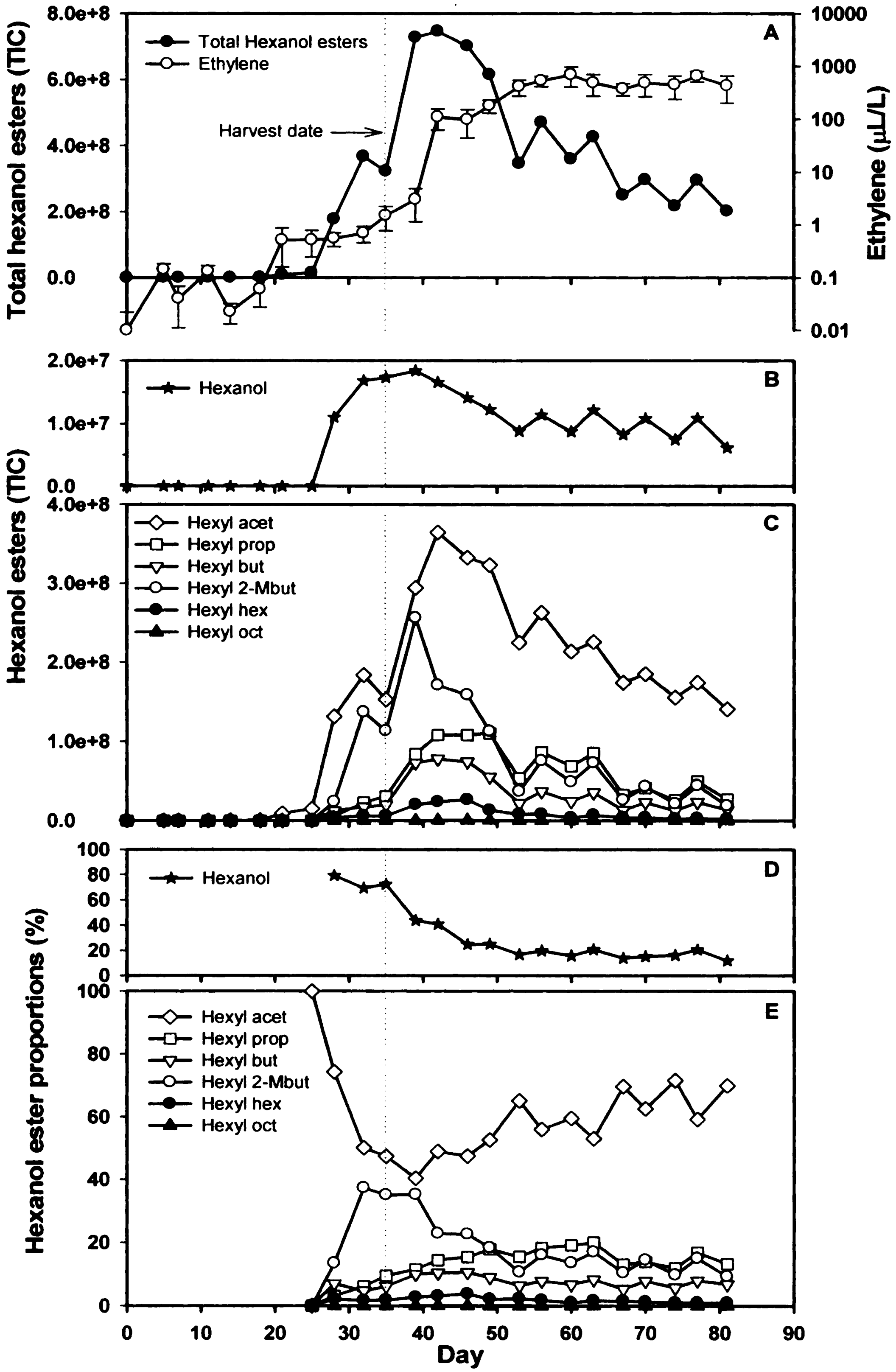

8.0e+8
6.0e+8
4.0e+8
2.0e+8
0.0
Total hexanol esters (TIC)
Total Hexanol esters
Ethylene
Harvest date
A
10000
1000
100
10
1
0.1
0.01
Ethylene (μL/L)
2.0e+7
1.0e+7
0.0
Hexanol
B
Hexanol esters (TIC)
4.0e+8
3.0e+8
2.0e+8
1.0e+8
0.0
Hexyl acet
Hexyl prop
Hexyl but
Hexyl 2-Mbut
Hexyl hex
Hexyl oct
C
100
80
60
40
20
0
Hexanol
D
Hexanol ester proportions (%)
100
80
60
40
20
0
Hexyl acet
Hexyl prop
Hexyl but
Hexyl 2-Mbut
Hexyl hex
Hexyl oct
E
0
10
20
30
40
50
60
70
80
90
Day

Figure 19.

Figure 20. Patterns of 2-methylbutanol ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total 2-methylbutanol esters and ontogeny of ethylene. B. GC/MS response (TIC) of total 2-methylbutanol. C. GC/MS response (TIC) of 2-methylbutyl acetate and 2-methylbutyl butanoate. D. 2-Methylbutanol proportions (% of total alcohols). E. 2-Methylbutanol ester proportions (% of total 2-methylbutanol esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD.

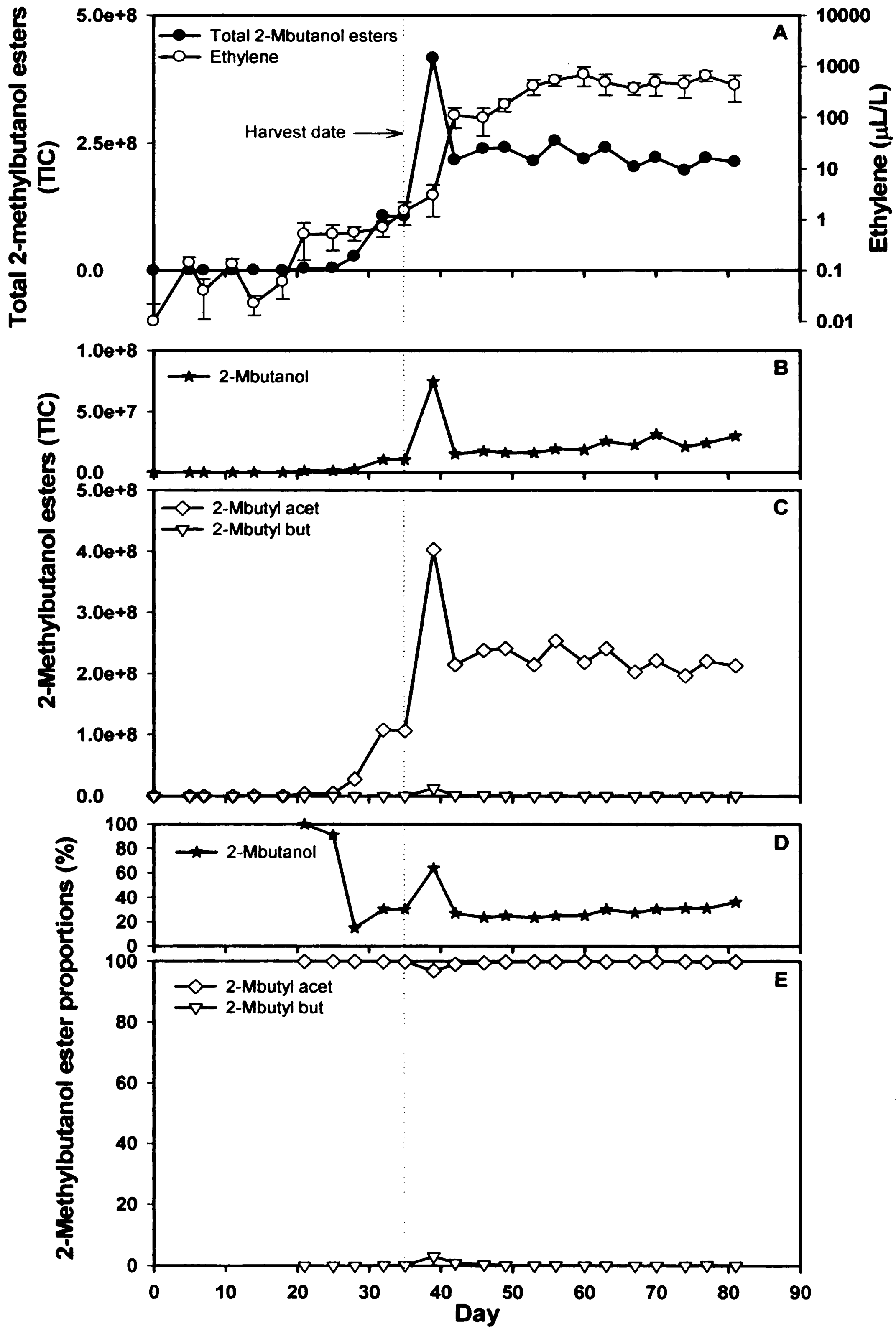
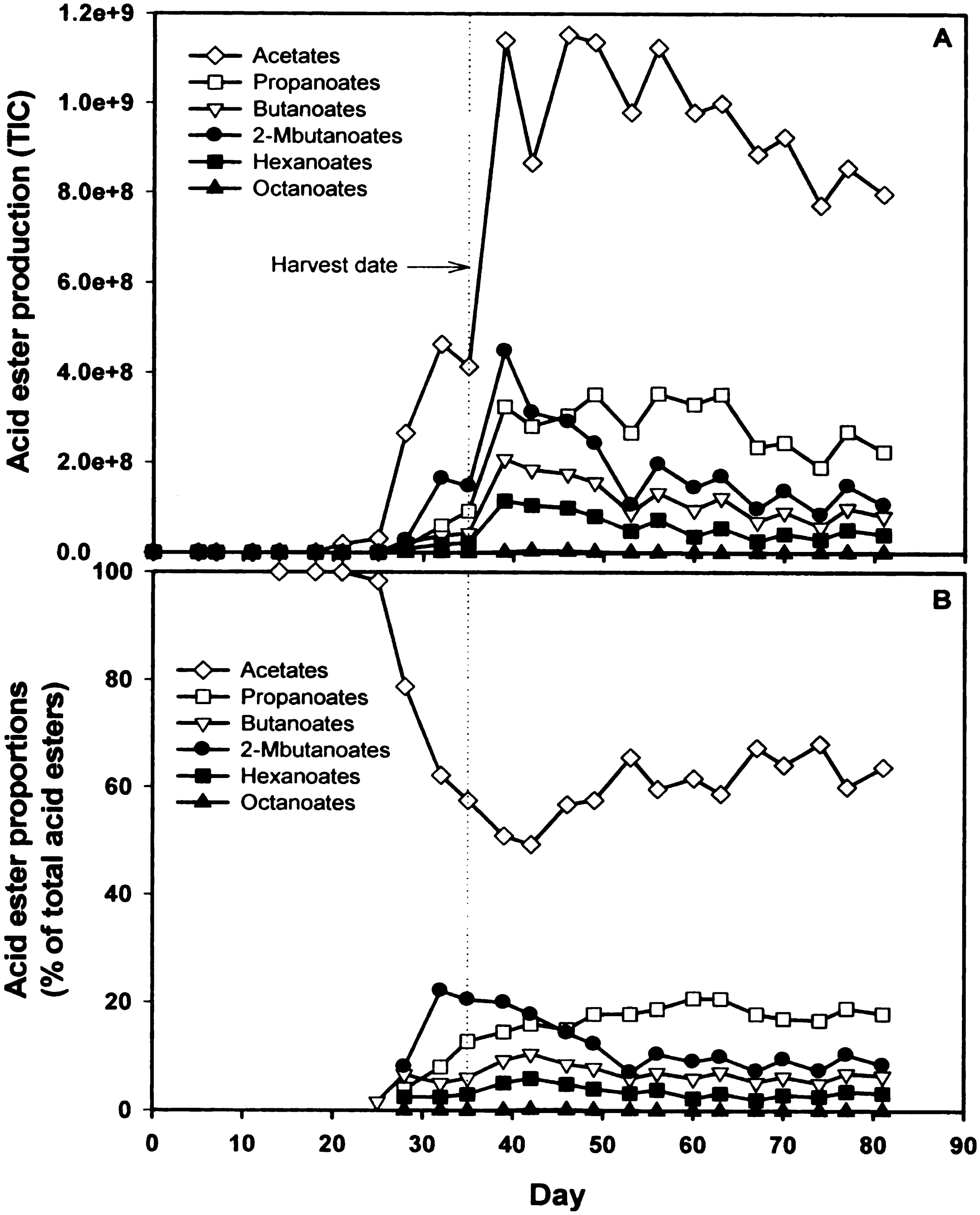

Total 2-methylbutanol esters (TIC)
5.0e+8
2.5e+8
0.0
Total 2-Mbutanol esters
Ethylene
A
Harvest date
Ethylene (µL/L)
10000
1000
100
10
1
0.1
0.01
1.0e+8
5.0e+7
0.0
2-Mbutanol
B
2-Methylbutanol esters (TIC)
5.0e+8
4.0e+8
3.0e+8
2.0e+8
1.0e+8
0.0
2-Mbutyl acet
2-Mbutyl but
C
2-Methylbutanol ester proportions (%)
100
80
60
40
20
0
2-Mbutanol
D
100
80
60
40
20
0
2-Mbutyl acet
2-Mbutyl but
E
0
10
20
30
40
50
60
70
80
90
Day

Figure 20.

Figure 21. Patterns of acid ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of acetates, propanoates, butanoates, 2-methylbutanoates, hexanoates, and octanoates. B. Acid ester proportions (% of total acid esters). Each symbol represents the average of four replications.



A
Acid ester production (TIC)
1.2e+9
1.0e+9
8.0e+8
6.0e+8
4.0e+8
2.0e+8
0.0
Acetates
Propanoates
Butanoates
2-Mbutanoates
Hexanoates
Octanoates
Harvest date
B
Acid ester proportions (% of total acid esters)
100
80
60
40
20
0
Acetates
Propanoates
Butanoates
2-Mbutanoates
Hexanoates
Octanoates
0
10
20
30
40
50
60
70
80
90
Day

Figure 21.

Figure 22. Patterns of acetate ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total acetate esters and ontogeny of ethylene. B. GC/MS response (TIC) of ethyl acetate, propyl acetate, butyl acetate, pentyl acetate, hexyl acetate, 2-methylpropyl acetate, and 2-methylbutyl acetate. C. Acetate ester proportions (% of total acetate esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD.

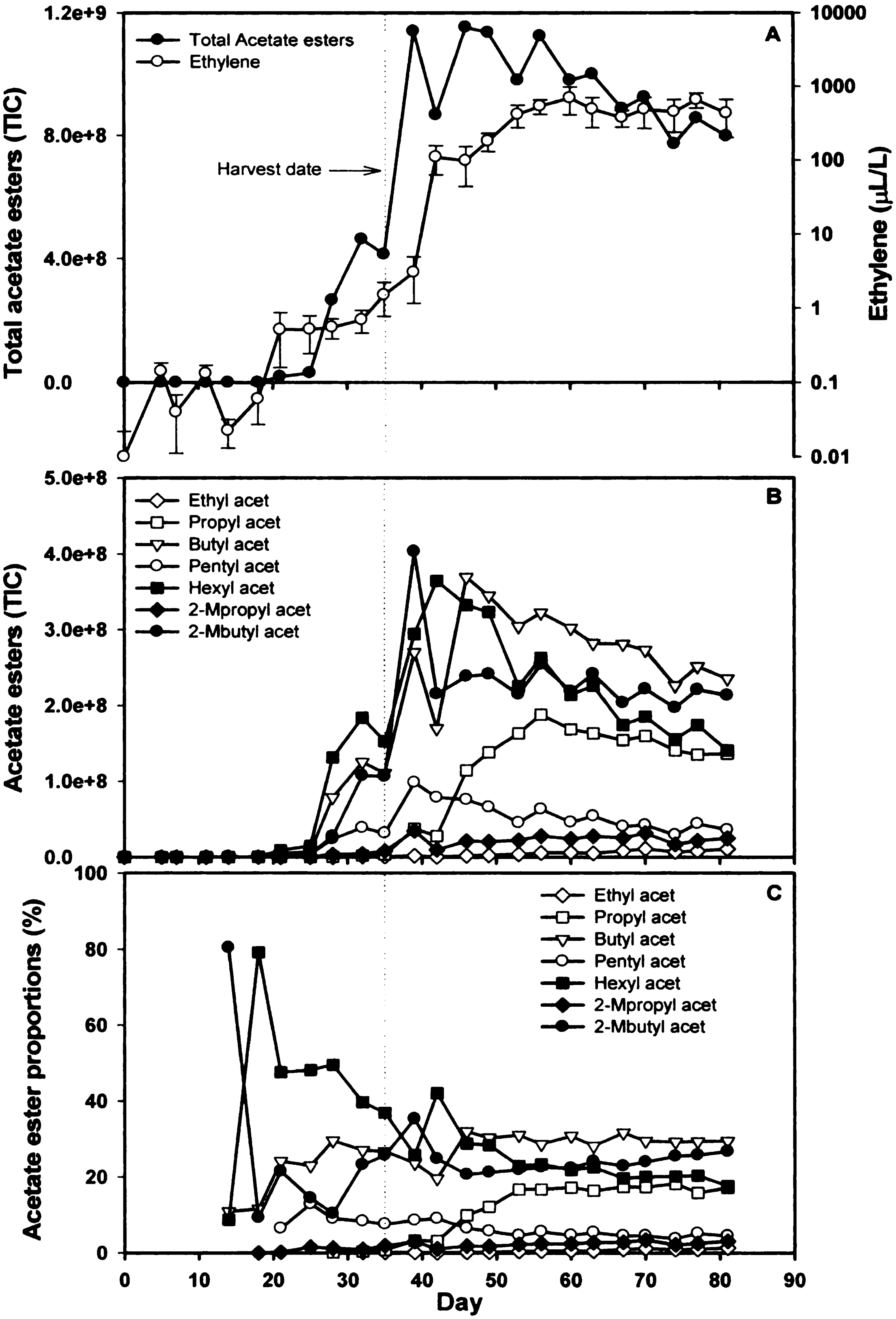

A
Total Acetate esters
Ethylene
Harvest date
Total acetate esters (TIC)
1.2e+9
8.0e+8
4.0e+8
0.0
Ethylene (µL/L)
10000
1000
100
10
1
0.1
0.01
B
Ethyl acet
Propyl acet
Butyl acet
Pentyl acet
Hexyl acet
2-Mpropyl acet
2-Mbutyl acet
Acetate esters (TIC)
5.0e+8
4.0e+8
3.0e+8
2.0e+8
1.0e+8
0.0
C
Ethyl acet
Propyl acet
Butyl acet
Pentyl acet
Hexyl acet
2-Mpropyl acet
2-Mbutyl acet
Acetate ester proportions (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
Day

Figure 22.

Figure 23. Patterns of propanoate ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total propanoate esters and ontogeny of ethylene. B. GC/MS response (TIC) of ethyl propanoate, propyl propanoate, butyl propanoate, pentyl propanoate, and hexyl propanoate. C. Propanoate ester proportions (% of total propanoate esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD.

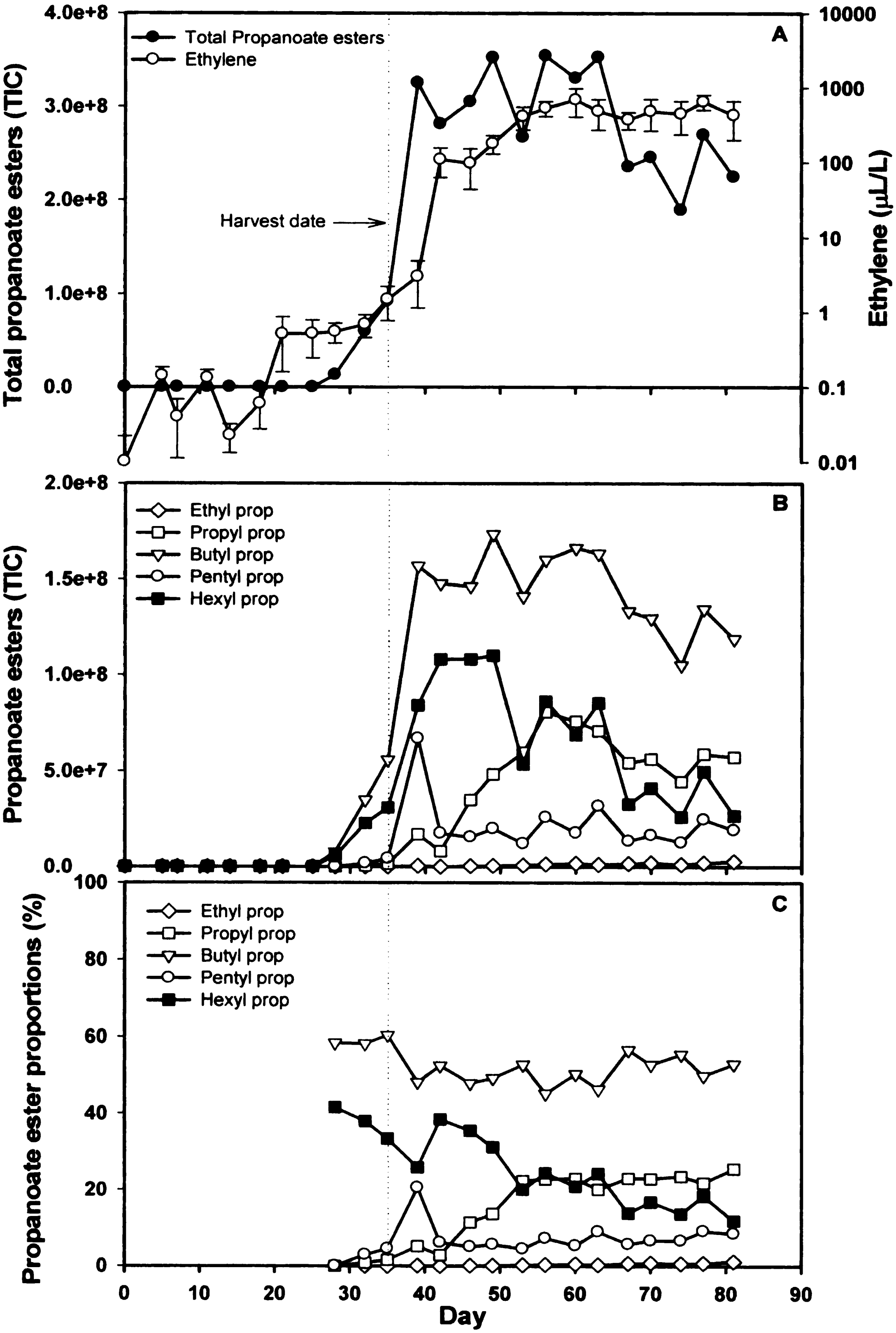

Total propanoate esters (TIC)
4.0e+8
3.0e+8
2.0e+8
1.0e+8
0.0
Total Propanoate esters
Ethylene
A
Harvest date
10000
1000
100
10
1
0.1
0.01
Ethylene (μL/L)
Propanoate esters (TIC)
2.0e+8
1.5e+8
1.0e+8
5.0e+7
0.0
Ethyl prop
Propyl prop
Butyl prop
Pentyl prop
Hexyl prop
B
Propanoate ester proportions (%)
100
80
60
40
20
0
Ethyl prop
Propyl prop
Butyl prop
Pentyl prop
Hexyl prop
C
0
10
20
30
40
50
60
70
80
90
Day

Figure 23.

Figure 24. Patterns of butanoate ester emissions during ripening and senescence of ‘Jonagold’ apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total butanoate esters and ontogeny of ethylene. B. GC/MS response (TIC) of ethyl butanoate, propyl butanoate, butyl butanoate, 2-methylbutyl butanoate, and hexyl butanoate. C. Butanoate ester proportions (% of total butanoate esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD.

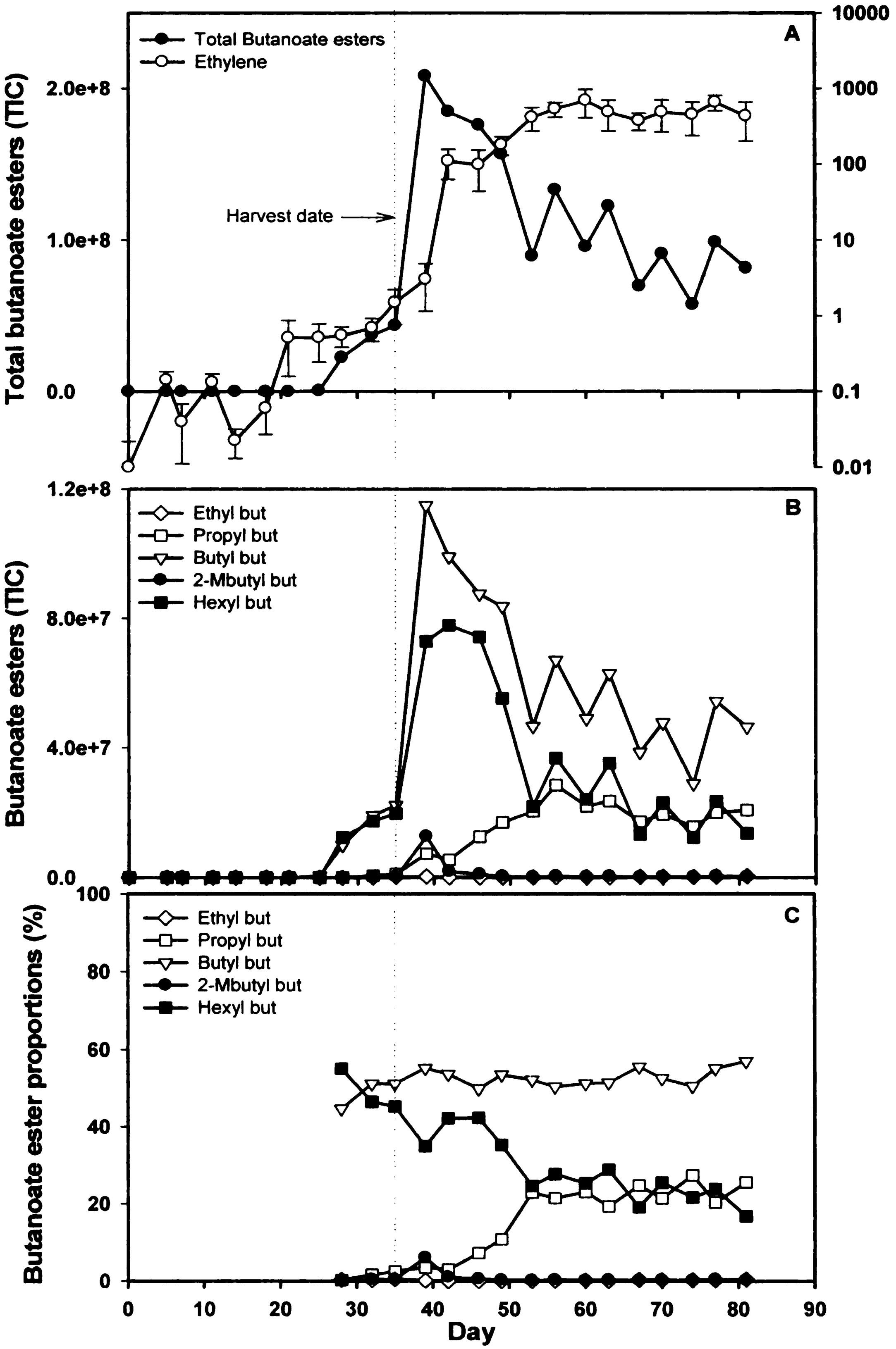

A
Total Butanoate esters
Ethylene
Harvest date
Total butanoate esters (TIC)
2.0e+8
1.0e+8
0.0
Ethylene (μL/L)
10000
1000
100
10
1
0.1
0.01
B
Ethyl but
Propyl but
Butyl but
2-Mbutyl but
Hexyl but
Butanoate esters (TIC)
1.2e+8
8.0e+7
4.0e+7
0.0
C
Ethyl but
Propyl but
Butyl but
2-Mbutyl but
Hexyl but
Butanoate ester proportions (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
Day

Figure 24.

Figure 25. Patterns of hexanoate ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total hexanoate esters and ontogeny of ethylene. B. GC/MS response (TIC) of propyl hexanoate, butyl hexanoate, pentyl hexanoate, and hexyl hexanoate. C. Hexanoate ester proportions (% of total hexanoate esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD.

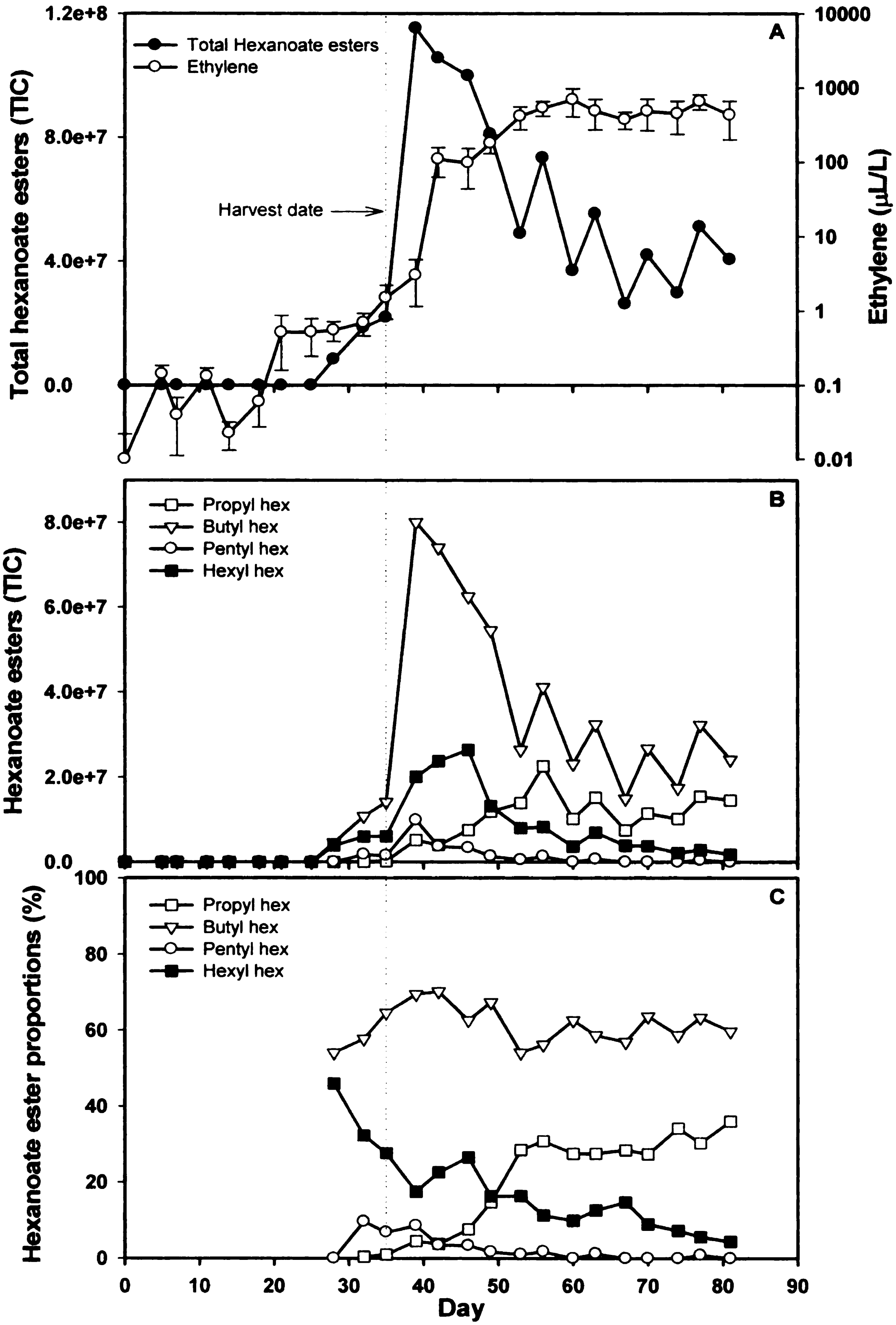
A
Total Hexanoate esters
Ethylene
Harvest date
Total hexanoate esters (TIC)
Ethylene (µL/L)
1.2e+8
8.0e+7
4.0e+7
0.0
10000
1000
100
10
1
0.1
0.01
B
Propyl hex
Butyl hex
Pentyl hex
Hexyl hex
Hexanoate esters (TIC)
8.0e+7
6.0e+7
4.0e+7
2.0e+7
0.0
C
Propyl hex
Butyl hex
Pentyl hex
Hexyl hex
Hexanoate ester proportions (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
Day

Figure 25.

Figure 26. Patterns of octanoate ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total octanoate esters and ontogeny of ethylene. B. GC/MS response (TIC) of propyl octanoate, butyl octanoate, and hexyl octanoate. C. Octanoate ester proportions (% of total octanoate esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD.

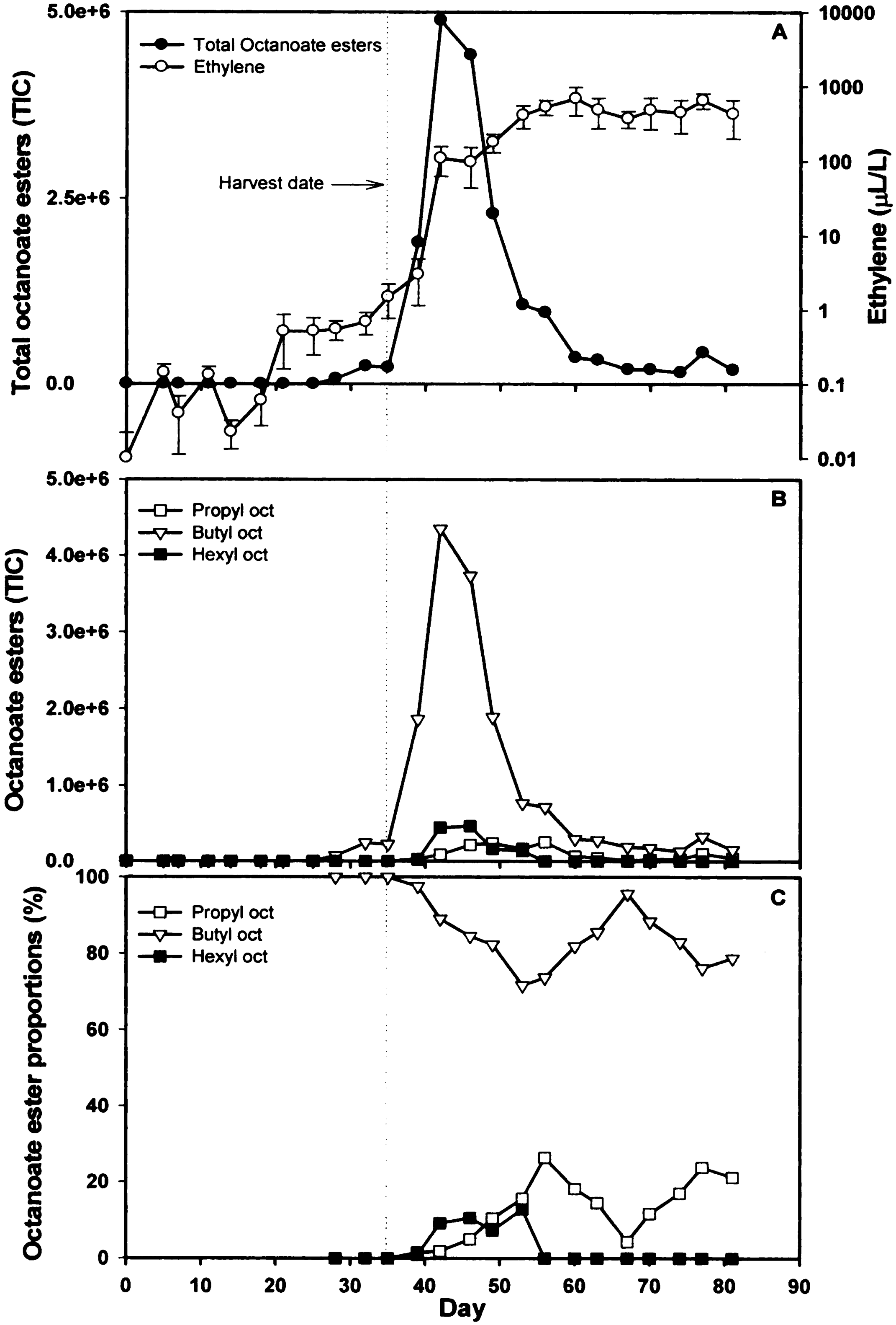

A
Total Octanoate esters
Ethylene
Harvest date
Total octanoate esters (TIC)
5.0e+6
2.5e+6
0.0
Ethylene (μL/L)
10000
1000
100
10
1
0.1
0.01
B
Propyl oct
Butyl oct
Hexyl oct
Octanoate esters (TIC)
5.0e+6
4.0e+6
3.0e+6
2.0e+6
1.0e+6
0.0
C
Propyl oct
Butyl oct
Hexyl oct
Octanoate ester proportions (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
Day

Figure 26.

Figure 27. Patterns of 2-methylbutanoate ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total 2-methylbutanoate esters and ontogeny of ethylene. B. GC/MS response (TIC) of ethyl 2-methylbutanoate, propyl 2-methylbutanoate, butyl 2-methylbutanoate, pentyl 2-methylbutanoate, and hexyl 2-methylbutanoate. C. 2-Methylbutanoate ester proportions (% of total 2-methylbutanoate esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD.

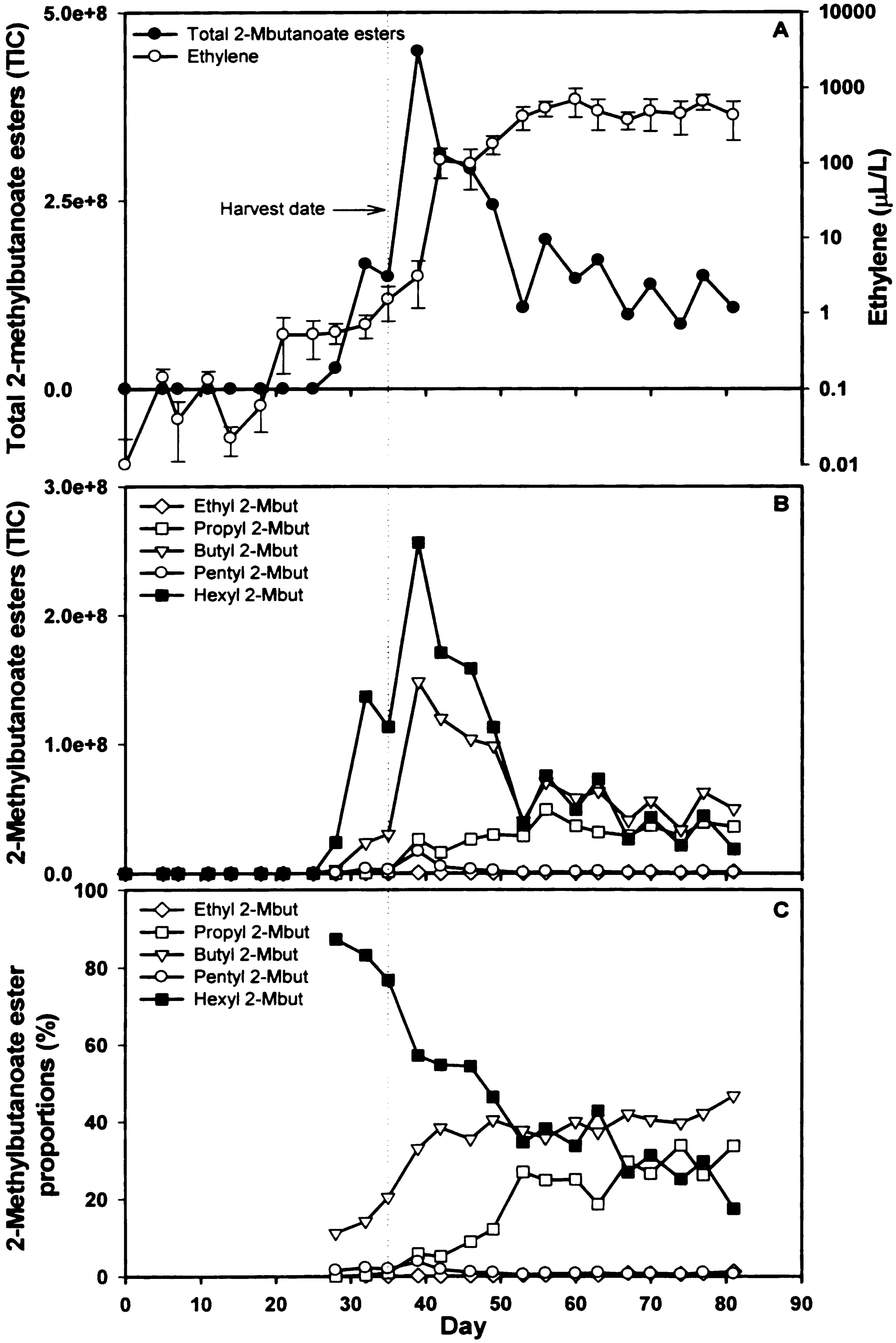

5.0e+8
2.5e+8
0.0
Total 2-methylbutanoate esters (TIC)
Total 2-Mbutanoate esters
Ethylene
A
Harvest date
10000
1000
100
10
1
0.1
0.01
Ethylene (µL/L)
3.0e+8
2.0e+8
1.0e+8
0.0
2-Methylbutanoate esters (TIC)
Ethyl 2-Mbut
Propyl 2-Mbut
Butyl 2-Mbut
Pentyl 2-Mbut
Hexyl 2-Mbut
B
100
80
60
40
20
0
2-Methylbutanoate ester proportions (%)
C
0
10
20
30
40
50
60
70
80
90
Day

Figure 27.

CHAPTER IV

GENE EXPRESSION ASSOCIATED WITH BRANCHED-CHAIN ESTER FORMATION IN 'JONAGOLD' APPLE FRUIT

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Steve van Nocker, Randolph Beaudry

INTRODUCTION

Esters, produced during ripening by many horticultural crops such as apple, banana, melon, and strawberry, contribute importantly to aroma. In apples, the esters hexyl acetate, butyl acetate, 2-methylbutyl acetate are abundantly produced and considered to confer typical apple aroma characteristics; however, the volatile profile is highly complex and differs with each cultivar (Kakiuchi et al., 1986).

Esters in apples are largely composed of either straight-chain or branched-chain alkyl (alcohol-derived) and alkanoate (acid-derived) groups (Figures 28 and 29). The alcohol and acid (actually acyl-CoA thioesters of fatty acids) precursors are joined by an enzyme called alcohol acyltransferase (AAT) to form the ester end product (Aharoni et al., 2000; Jayanty et al., 2002; Souleyre et al., 2005; Yahyaoui et al., 2002). Straight-chain ester precursors may be formed from fatty acid degradation via β-oxidation or lipoxygenase system (Sanz et al., 1997) or from fatty acid biosynthesis via single- or two-carbon fatty acid elongation synthetic pathways (Kroumova and Wagner, 2003; Thelen and Ohlrogge, 2002). Branched-chain ester precursors may be derived from branched-chain amino acid (BCAA) degradation (Rowan et al., 1996; Tressl and Drawert, 1973) or by diversion of branched-chain α-keto acid precursors to BCAAs. The single-carbon fatty acid synthesis pathway, if active in apple, may also produce α-keto butyrate which is necessary for the biosynthesis of α-keto-β-methylvalerate, the immediate precursor to isoleucine. While it can be demonstrated that apple fruit can make esters by metabolizing various exogenous substrates involving pathways such as lipoxygenase, β-oxidation, and amino acid degradation, isotopic evidence is not conclusive that these pathways operate in vivo in the same way.

2-Methylbutyl and 2-methylbutanoate esters are abundantly produced by 'Jonagold' apples (Mir et al., 1999; Chapter 3). The 2-methylbutyl alkyl and alkanoate moieties are likely derived from α-keto-β-methylvalerate. In isoleucine formation, α-keto-β-methylvalerate is transaminated by branched-chain aminotransferase (BCAT) to isoleucine (Figure 29). This reversible reaction is also the first step in isoleucine degradation. In apple, α-keto-β-methylvalerate can be formed from added isoleucine, suggesting that isoleucine degradation may be necessary for 2-methylbutyl and 2-methylbutanoate ester formation in apple (Rowan et al., 1996). Alternatively, α-keto-β-methylvalerate used in ester biosynthesis may be diverted from the pathway of isoleucine biosynthesis, which increases during apple ripening (Nie et al., 2005; Singh and Shaner, 1995).

In the biosynthesis of 2-methylbutyl and 2-methylbutanoate esters, α-keto-β-methylvalerate can be dehydrogenated by branched-chain α-ketoacid dehydrogenase (BCKDH) to 2-methylbutyl-CoA or decarboxylated by branched-chain α-ketoacid decarboxylase (a.k.a. branched-chain 2-ketoacid decarboxylase or pyruvate decarboxylase, PDC) to 2-methylbutanal (Wyllie et al., 1996). The dehydrogenase pathway is considered as a major route for BCAA catabolism in most organisms, whereas the PDC pathway has been extensively studied only in yeast and bacteria (Dickinson et al., 1997, 1998, 2000; Smit et al., 2004).

In the final enzymatic step, AAT can combine 2-methylbutyl-CoA and/or 2-methylbutanol with various alcohols and acyl-CoAs, respectively, to create a wide variety of esters. The substrate specificity of AAT is believed to markedly impact the ester profile (Aharoni et al., 2000; Olías et al., 2002; Souleyre et al., 2005; Ueda et al., 1992; Yahyaoui et al., 2002). Although AAT likely plays a major role in establishing the ester profile, control of ester synthesis probably lies at the level of ester precursor formation (Wyllie and Fellman, 2000). For branched-chain esters, control may lie in the BCAA catabolism pathway, either BCAT, BCKDH and/or PDC. However, little is known about activity and regulation of BCAT and PDC in higher plants. To our knowledge, no studies on ester synthesis in fruit have characterized the expression of genes associated with these enzymes.

Given that the biochemistry of ester formation is so poorly understood, a genomic analysis may aid in the identification of highly regulated genes in the ester formation pathways. The relationship between ester-production and gene expression has been studied in apple, strawberry, and pear fruit using expressed sequence tag (EST) analysis (Park et al., 2006) and microarrays made with ESTs (Aharoni et al., 2000; Fonseca et al., 2004). Fonseca et al. (2004) found that when the expression of the ethylene synthesis gene, ACC oxidase, increased, gene expression for a short-chain type alcohol dehydrogenase, pyruvate decarboxylase, and 3-ketoacyl-CoA thiolase (β-oxidation) increased at the same time in ripening pear fruit. Aharoni et al. (2000) found that *AAT* gene expression underwent a 16-fold increase in strawberry between the green and red fruit stages; they observed an increase in volatile esters as fruit turned from pink to red. Aharoni et al. (2000) also observed an increase in *PDC* gene expression during fruit development and explored the relationship between ethyl ester formation and *PDC* gene expression. Park et al., (2006) observed that expression of a lipoxygenase gene increased as total volatiles increased during apple ripening. However, the relationship between lipoxygenase gene expression and the production of C6 esters, which are believed to be derived from fatty acid metabolism via the lipoxygenase pathway, was not evaluated.

In order to improve our understanding of the pathways involved in ester formation, we developed an apple microarray for genomic analysis. The microarray was a composite of presumed ester formation-related ESTs, available at the time of printing and 10,000 unknown, unsequenced gene fragments obtained from a library representing genes expressed at all stages of apple fruit development. The objective of this experiment was to establish a correlative link between changes in gene expression and changes in branched-chain ester biosynthesis in ripening apple. We hypothesized that branched-chain ester formation was from BCAA catabolism and expected the expression of genes associated with BCAA catabolism such as *BCAT* and *PDC* to be upregulated during the increase in branched-chain ester production.

MATERIALS AND METHODS

Plant Material

'Jonagold' apples were harvested from the Michigan State University Clarksville Horticultural Experiment Station, Clarksville, MI. From September 2, 2004 (Day 0) until ripening was fully engaged on October 7, 2004 (Day 35), fruits were collected for examination every three to four days from the field. On each occasion, fruit were held overnight in the laboratory to equilibrate to laboratory temperature (21±1°C) and covered with ventilated, black, 4-mil thick plastic bags to avoid desiccation and responses to intermittent laboratory light before analysis. All fruits (approximately 200) remaining on the tree were harvested and transported to the laboratory at once on October 7, 2004 (harvest date, Day 35) after it was apparent that ripening was underway. This was done to avoid damage in the field due to freezing and fruit drop. Thereafter, fruit were maintained at room temperature (21±1°C) and covered with plastic bags as described previously. The fruit were examined every three to four days from harvest (October 7, 2004) until the conclusion of the study on November 23, 2004 (Day 81).

On each evaluation date, 20 apples were randomly chosen and the internal ethylene content of each was measured. Of these, the fourteen fruit having an internal ethylene content nearest the median were selected for further analysis. The four fruit having ethylene levels closest to the median were used for analysis of CO_2 production, ester emission, and textural properties. From the remaining ten fruit, the skin and a small amount of cortex were removed and immediately frozen in liquid nitrogen and stored at -80°C until extraction of RNA.

Two replicates were created from the ten apples; each replicate consisted of four to five apples.

Aroma Analysis

To collect volatiles, apples were held at 20°C in a 1-L Teflon container (Savillex Corporation, Minnetonka, MN) fitted with two gas-sampling ports, each sealed with a Teflon-lined half-hole septum (Supelco Co., Bellefonte, PA). Headspace volatiles were sampled after 20 minutes using a 1-cm long solid-phase micro extraction (SPME) fiber (65 μm PDMS-DVB, Supelco Co., Bellefonte, PA). SPME sorption time was 3 min. The exposed fibers were immediately transferred to a gas chromatograph (GC) (HP-6890, Hewlett Packard Co., Wilmington, DE) injection port (230°C) and desorbed for 2 min. Desorbed volatiles were trapped on-column using a liquid nitrogen cryofocussing trap. Separation of volatiles was by capillary column (SupelcoWax-10, Supelco, Bellefonte, PA, 29 m x 0.2 mm i.d., 0.2 μm coating film). The temperature of the GC was programmed from 40 to 240°C at a rate of 50°C/min; the flow rate of the helium carrier gas was 1 mL/min and the GC was operated in splitless mode. Detection was by time-of-flight mass spectrometry (Pegasus II, LECO Corp., St. Joseph, MI) (GC/MS) according to the method of Song et al. (1997). Identification and quantification of compounds were by comparison of the mass spectrum and MS response (total ion count, TIC), respectively, with those of authenticated reference standards and spectra in the National Institute for Standard and Technology (NIST) mass spectra library (Search Version 1.5).

Isolation of RNA

In brief, eight developmental stages were selected for analysis of expressed genes based on physiological changes during ripening. These stages are: stage 1 (Day 0), early climacteric; stage 2 (Day 11), late preclimacteric and onset of trace ester biosynthesis; stage 3 (Day 25), onset of the autocatalytic ethylene and rapid increase of ester biosynthesis; stage 4 (Day 32), half-maximal ester biosynthesis and engagement of the respiratory climacteric; stage 5 (Day 39), near maximal ester biosynthesis, peak in respiratory activity, and onset of rapid tissue softening (Chapter 3, Figure 11); stage 6 (Day 49), end of maximal ester biosynthesis, the conclusion of the respiratory climacteric, and completion of tissue softening; stage 7 (Day 60), midpoint in the decline in ester biosynthesis, maximal ethylene production, and onset of senescence; and stage 8 (Day 70), postclimacteric minimum in ester production and extensive fruit senescence.

Total RNA was isolated from the 'Jonagold' apple skin and 2-3 mm of underlying cortex tissue by hot borate/phenol extraction followed by LiCl precipitation (López-Gómez and Gómez-Lim, 1992).

Microarray Printing

Microarray slides were created by the Genomics Technology Support Facility (GTSF) of the Genomics Core in Michigan State University, East Lansing, MI. Approximately 10,000 unsequenced cDNA gene fragments were generated from the lambda phage cDNA library from 'Mutsu' apple fruit described by Gao et al. (2005) using a mass excision kit and protocols as described by the manufacturer (ZAP-cDNA synthesis kit, Stratagene, LaJolla, CA). In addition to the unsequenced cDNA fragments, an additional 116 nucleotide gene fragments were arrayed. These 116 genes were selected based on their putative identity using the Tree Fruit Technology genomic analysis tool apple database version 2.0 (http://genomics.msu.edu/fruitdb/analyses/apple.shtml). Putative gene identities included aminotransferase, alcohol acyltransferase, alcohol dehydrogenase, acyl-ACP thioesterase, fatty acid hydroperoxide lyase, lipoxygenase, 3-ketoacyl-CoA thiolase, acyl-CoA oxidase, enoyl-CoA hydratase, and omega-3 fatty acid desaturase, allene oxide synthase, mannitol dehydrogenase, mannitol transporter, NADP-dependent D-sorbitol-6-phosphate dehydrogenase, sorbitol dehydrogenase, and sorbitol transporter. Additional human and bacterial control genes were also placed on the array. These genes were used as controls for non-specific binding and to normalize for background signal noise when analyzing microarray data.

Microarray design and labeling

The experimental design was a one-way classification with a single multi-level treatment factor (stage of development) having eight levels. The comparison arrangement was mixed and consisted of loops and indirect comparisons (Figure 30) (Churchill, 2002; Yang and Speed, 2002). There were two biological and two technical replicates with dye swaps.

The microarray protocol used for labeling, hybridization, and washing has been described by Hegde et al. (2000). Reverse transcription to make cDNA was performed using total RNA (20 µg) and an oligo(dT) 20-mer primer (4 µg). Products were labeled with fluorescent dyes: cyanine 3 (cy3) and cyanine 5 (cy5). Labeled cDNA was mixed, vacuum dried, and re-suspended in 14 µL of hybridization buffer (50% formamide, 5x SSC, and 0.1% SDS) with COT1-DNA (20 μg) and Poly(A)-DNA (2 μg) to block nonspecific hybridization. The cy3 and cy5 probes were combined and placed on a microarray slide for hybridization and incubated for 16-20 hours at 42°C using a water bath. After washing and drying, the slides were scanned (Affymetrix 428 Array Scanner, Santa Clara, CA) at 635 nm for cy5 and 532 nm for cy3. Images were analyzed for feature and background intensities using GenePix Pro 3.0 (Molecular Devices, Union City, CA).

Statistical analysis

Raw data were submitted to linear models for microarrays Graphical User Interface (LimmaGUI) library modules for the R statistical package (http://bioinf.wehi.edu.au/limmaGUI/index.html) software (Wettenhall and Smyth, 2004). Data was analyzed with minimum background correction and normalized within array only using the global loess method. The least squares method was used for the linear model fit according to the Benjamini & Hochberg method to control the false discovery rate. The $\log_2$ differential expression ratio (M) value among the eight developmental stages was used to measure degree of expression changes relative to Day 0, which was considered as a reference. The probability of differences in expression was calculated. Gene fragments were selected for sequencing based on the statistical probability ($P<0.00025$) that expression changes were non-random. Selection was also accomplished using a constraint table developed to capture additional large expression changes associated with specific physiological events, but not statistically different from

Day 0 (greater than 60% of these had P<0.01) (Table 3). Sequencing was successful on approximately 85% of selected cDNA fragments. Gene identity was tentatively established by first screening for the presence of vector sequence. Following low quality sequence trimming, acceptable sequences were clustered and consensus sequences were obtained using stackPACK (http://genomics.msu.edu/stackpack/tool). Identity was tentatively assigned by BLAST analysis against the NCBI non-redundant protein and *Arabidopsis thaliana* protein database. Approximately 80% genes were identified for their identity from total of 758 genes BLASTed.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

PCR was used to verify microarray data and to compare expression of all available members for putative pyruvate decarboxylase (*PDC*), and branched-chain aminotransferase (*BCAT*) gene families and for the 2-isopropylmalate synthase gene. All genes evaluated using semi-quantitative RT-PCR, apple cluster number, primers, the expected size of the PCR-product, optimum cycle number, and optimum temperature for primer binding to gene are listed (Table 4). PCR for two *PDC* genes and one *BCAT* gene were unsuccessful. No products were evident for *PDC6* and *BCAT11* after two trials using different primers and *PDC7* was too short to design appropriate primers.

Two biological replicates were used for PCR analyses for each developmental stage used in the microarray analysis. cDNA synthesis and PCR reactions were performed according to the manufacturer (Invitrogen, Carlsbad, CA) directions. Before creating cDNA, total RNA was treated with DNase using RNase-free DNase set kit according to the manufacturer (Qiagen Inc, Valencia, CA). One µg of DNase-treated total RNA was reverse transcribed using oligo(dT)$_{12\text{-}18}$ primer and SuperScript II as described by the manufacturer (Invitrogen, Carlsbad, CA). The cDNA (1.0 µL) was used as a template in a 50 µL PCR reaction containing 10 µM of forward and reverse gene-specific primers designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The PCR reaction was performed as described in the following steps: 1) 5 min at 95°C, 2) 30 s at 95°C, 3) 30 s at 55-59°C, 4) 30 s at 72°C, repeating 18-35 cycles from steps 2-4, and final elongation 5 min at 72°C. The amplified PCR products were separated by electrophoresis on a 1.5% (w/v) agarose gel, visualized with ethidium bromide, and photographed. Relative light density of the bands was quantified by a digital imaging system (EagleEye II, Stratagene, La Jolla, CA). To identify the optimum cycle, the gene products amplified by PCR had to be visible on the gel electrophoresis and be able to quantifiable by light density measurement without saturation. The number of PCR cycles needed ranged from 26 to 35 (Table 4). A single number indicates that the same cycle was performed with both biological replications. Two numbers indicate that different number cycles were performed for each replicate.

PCR products were cleaned by QIAquick PCR purification kit (Qiagen inc., Valencia, CA) and sequenced at the GTSF facility to verify identity. All the PCR generated sequences had to be 97-100% identical to the reported apple sequence in the apple sequence database (version 3.0).

Semi-quantitative RT-PCR data analysis

A partial sequence of the 18S ribosomal RNA gene (gi85717895) was used as an internal control for PCR analyses. Expression data for all genes (PDC, BCAT, and 2-isopropylmalate synthase) were normalized based on the 18S spot density. The spot density for the 18S ribosomal RNA gene varied approximately ±10% across the eight developmental stages (data not shown). The values for PCR are calculated as the spot density relative to the maximum value obtained for each gene.

RESULTS

Branched-chain esters

Total ester production increased coincident with increased ethylene accumulation in the fruit and continued well beyond the end of the respiratory climacteric (Figure 31). The pattern for branched-chain esters was generally similar to that for total esters, but had a higher, sharper initial peak (Figure 32). The GC/MS response for all esters derived from 2-methylbutanoate (ethyl, propyl, butyl, pentyl, and hexyl 2-methylbutanoate) and from 2-methylbutanol (2-methylbutyl acetate and 2-methylbutyl butanoate) peaked on Day 39 and declined thereafter (Figure 32). 2-Methylbutanol esters had a lower diversity, but approximately the same abundance as 2-methylbutanoate esters based on total ion count (TIC). The most abundant 2-methylbutanoic acid-derived branched-chain esters were hexyl and butyl 2-methylbutanoate, and the most abundant alcohol-derived branched-chain ester was 2-methylbutyl acetate. 2-Methylbutanol and 2-methylbutanal production patterns were similar to those for 2-methylbutyl esters, rapidly increasing during the climacteric to a peak on Day 39 and declining rapidly thereafter, then undergoing a slow increase as ripening and senescence continued. Free 2-methylbutanoic acid was not detected.

Gene expression related to branched-chain amino acid metabolism

Of the 116 nucleotide gene fragments printed on the microarray slide, a branched-chain aminotransferase [BCAT, *MDC410280* (*BCAT10*)] was the only gene that was related with branched-chain amino acid (BCAA) metabolism. Based on the BLAST analysis of the 758 unknown microarray nucleotide fragments, only three genes were related to BCAA metabolism. Two pyruvate decarboxylase (PDC) clusters and one 2-isopropylmalate synthase singleton (gi7387848) were identified. The two PDC clusters were judged to be similar to a single apple gene (*MDC015210*, *PDC1*) found in the Tree Fruit Technology genomic analysis tool apple database (version 3.0, http://genomics.msu.edu/fruitdb/analyses/apple.shtml). There were no sequence similarities found in the apple sequence database for 2-isopropylmalate synthase. The apple sequence database contained a total of 11 putative *BCAT* and 7 putative *PDC* genes.

Microarray expression analysis

Relative expression of *BCAT10* changed relatively little, but peaked simultaneously with all branched-chain esters (Figure 33). The change in expression relative to Day 0 was not significant (P=0.36), but the overall range in expression between the peak and lowest expression levels was approximately a

12-fold change. Expression remained low until onset of ethylene accumulation on Day 25, maximized on Day 39 at 3.9x initial level, and decreased rapidly after Day 49 to a low of 0.3x on Day 70.

PDC1 relative expression remained very low until onset of ethylene accumulation on Day 25, but changed significantly thereafter ($P<0.005$). *PDC1* expression rapidly increased as volatiles began to increase, reached maximum of about 5.7x initial levels on Day 49, and decreased slightly during senescence.

2-Isopropylmalate synthase relative expression increased rapidly after Day 11 coincident with increasing ethylene and branched-chain ester production, reaching maximum 34-fold change on Day 39 and remained high during senescence.

Of the three genes characterized using the microarray, *BCAT10* had an expression pattern that was most similar to the pattern of branched-chain ester production. However, *PDC1* and 2-isopropylmalate synthase initial increases closely corresponded with the onset of branched-chain ester biosynthesis and, reflecting the pattern of production several of the branched-chain esters, continued to be highly expressed during the later stage of fruit development.

Semi-quantitative RT-PCR analysis

PCR product spot densities for *BCAT10*, *PDC1*, and 2-isopropylmalate synthase yielded patterns that were similar to the microarray data, validating the microarray analyses (Figures 34-36). Of the ten *BCAT* genes in addition to *BCAT10*, nine were found to be expressed in the fruit (Table 4). Four *BCAT* genes [*MDC021750* (*BCAT1*), *MDC028270* (*BCAT2*), *MDC238050* (*BCAT6*), *MDC405820* (*BCAT9*)] were expressed similarly to *BCAT10* during branched-chain ester production (Figure 34A), five genes [*MDC160840* (*BCAT3*), *MDC192460* (*BCAT4*), *MDC238040* (*BCAT5*), *MDC301230* (*BCAT7*), *MDC385660* (*BCAT8*)] had relatively stable expression until approximately Day 25, then declined slightly as ripening and senescence progressed (Figure 34B). Even though the expression of some *BCAT* genes followed the same pattern as branched-chain ester production, their relative expression change was not large.

Of the six *PDC* genes in addition to *PDC1*, five were detected as being expressed in the fruit (Table 4). Expression patterns for the five genes differed, however only one (*PDC1*) increased (Figure 35). *MDC206810* (*PDC4*) rapidly increased on Day 32 and declined thereafter. *MDC433930* (*PDC5*) was stable throughout ripening and slightly increased during senescence. *MDC133700* (*PDC2*) and *MDC206800* (*PDC3*) had the highest expression before the climacteric peak and gradually declined afterwards. With exception of *PDC5*, most of the *PDC* genes had a relatively high expression compared to the *BCAT* genes based on PCR cycle numbers (Table 4).

DISCUSSION

Ester synthesis

Autocatalytic ethylene production, which we considered to occur above an internal ethylene content of 0.2 $\mu L \cdot L^{-1}$, had a pattern relative to ester synthesis similar to that previously described for ‘Bisbee Delicious’ (Mattheis et al., 1991b), ‘Golden Delicious’ (Song and Bangerth, 1996), and ‘Redchief Delicious’ apples

(Ferenczi, 2003). This is consistent with previous studies demonstrating that the bulk of ester production requires ethylene action (Defilippi et al., 2004; Ferenczi et al., 2006).

The abundantly produced 2-methylbutyl acetate is also produced in large amounts by 'Bisbee Delicious' (Mattheis et al., 1991a, 1991b), 'Redchief Delicious' (Ferrenczi, 2003), 'Rome' (Fellman et al., 1993), and 'Golden Delicious' (Song and Bangerth, 1996) fruit. The 'Delicious' variety also produces ethyl 2-methylbutanoate in abundance, however, very little of this compound was produced by 'Jonagold', accounting for only 0.4% of total ion count (TIC) for all branched-chain esters on average. The high production of hexyl 2-methylbutanoate in 'Jonagold' is also found in 'Redchief Delicious' (Ferrenczi, 2003). On the other hand, 'Annurca' apple produces little or no branched-chain esters (Lo Scalzo et al., 2001). The maintenance of a high rate of production of 2-methylbutyl esters throughout ripening and senescence suggests a consistent production of 2-methylbutanol, which, in fact was detected. The sharp increase, followed by sustained production of 2-methylbutanol is suggestive of a sharp increase and subsequent sustained PDC activity.

Gene expression

The similarity in the expression pattern of putative *BCAT* genes with branched-chain ester production suggests expression activity may influence isoleucine metabolism and subsequent ester formation. Isoleucine metabolism is known to change during apple fruit ripening. A significant increase in isoleucine concentration takes place during apple ripening (Nie et al., 2005), indicating biosynthesis of isoleucine outpaces its catabolism. Deuterium-labeled feeding studies by Rowan et al. (1996) demonstrated that isoleucine catabolism can contribute to ester biosynthesis during apple fruit ripening. It is also possible, however, that increased synthesis of isoleucine is accompanied by increased availability of α-keto-β-methylvalerate, the precursor to isoleucine. α-Keto-β-methylvalerate rather than isoleucine may serve as the source of substrate for decarboxylation reactions leading to the formation of 2-methylbutanoate and 2-methylbutanol. In a recent study with *Arabidopsis thaliana*, a total of six or possibly seven *BCAT* genes have been cloned. BCAT proteins are found localized in different tissues and possess differing activities. AtBCAT-1 is localized in mitochondria, and has the capacity to initiate degradation of leucine, isoleucine, and valine in all tissues, AtBCAT-2, -3 and -5 are located in plastids, expressed at rather low levels, and, with exception of AtBCAT-3, transcribed in all tissues. AtBCAT-4 and -6 are cytoplasmically located, expressed in tissues associated with transport function and in meristematic tissues (Diebold et al., 2002; Schuster and Binder, 2005). Of these, mitochondrial AtBCAT-1 is suspected to be important in BCAA catabolism. None of the apple *BCAT* genes characterized in this study had a similarity with the mitochondrial *AtBCAT-1*, but were more similar to plastid-localized *AtBCAT-2* [*BCAT2* (*MDC028270*)], *AtBCAT-3* [*BCAT5* (*MDC238040*), *BCAT6* (*MDC238050*), *BCAT8* (*MDC385660*), and *BCAT10* (*MDC410280*)], and *AtBCAT-5* [*BCAT9* (*MDC405820*)]. The enzymatic functions of all BCAT proteins in *Malus* sp. are currently unknown.

In bacteria, nutritional factors such as carbohydrate and nitrogen source regulate aromatic aminotransferase and branched-chain aminotransferase gene expression (Chambellon and Yvon, 2003). In higher plants, BCAA degradation is thought to occur due to a limitation in carbon supply (Graham and Eastmond, 2002). It is possible that increase in *BCAT* gene expression changes in apple were in response to a limitation in the supply of carbon being delivered to the fruit after being detached from the tree. On the other hand, in apple, there is a plentiful supply of energy-rich carbohydrates stored in the fruit arguing against this possibility of starvation-induced *BCAT* expression.

Branched-chain α-ketoacid decarboxylases, or PDCs, are poorly studied in higher plants. Failure to detect *PDC6* gene expression in fruit suggests that it may lack a function in fruit and is therefore not expressed. Little information is found characterizing *PDC* genes relative to ester formation. In yeast, which produces important esters in some food products and beverages, five genes have been reported to be responsible for decarboxylation of branched-chain α-keto acids to branched-chain aldehydes (Dickinson et al., 1997, 1998, 2000; Yoshimoto et al., 2001). Dickinson et al. (1997, 1998, 2000) suggests that the catabolic pathways of three BCAAs are accomplished in different ways, a single *YDL080c* gene (PDC-like gene) is likely responsible for leucine catabolism and any one of the isozymes of PDC can enable valine and isoleucine degradation. It is possible that some apple PDCs may have a similar capacity to metabolize branched-chain α-keto acids. The predominant production of 2-methylbutyl and

2-methylbutanoate esters by 'Jonagold' apple fruit suggests a specificity of PDC activity for α-keto-β-methylvalerate, a component of isoleucine metabolism.

In fruits, three *PDC* genes were isolated from strawberries and one from grape berries (Moyano et al., 2004; Or et al., 2000). Also, PDC activities were measured during maturation of 'Fuji' apples (Echeverría et al., 2004). The main purpose of these studies was to relate PDC activity and expression to ethanol production under anaerobic conditions or to the formation of ethanol-derived esters such as ethyl esters, but not for BCAA metabolism. In 'Jonagold', the pattern of ethyl ester formation did not appear to reflect the pattern of expression of any of the *PDC* genes, they were found only at low levels and tended to increase only during later developmental stages. The TIC for total ethyl ester production only accounted for 1% of the TIC for total alcohol esters and the TIC for ethanol accounted for only 0.2% of the TIC for all alcohols. The lack of a correlation between ethanol ester production and expression for any of the five *PDC* genes argues against a causative relationship between the expression of these genes and ethanol metabolism.

In tomato fruits, Tieman et al. (2006) recently found that 2-phenylethanol, an important flavor and insect attractant in tomato and rose, is synthesized from phenylalanine by an aromatic amino acid decarboxylase. Tieman et al. (2006) also proposed the pathway that phenylalanine conversion to phenethylamine without producing phenylpyruvate as in yeast and then to 2-phenylacetaldehyde and to 2-phenylethanol. Theoretically, this report suggests that a decarboxylase may directly convert free branched-amino acid to 2-methylbutylamine for example, from isoleucine and then to 2-methylbutanal. The involvement of PDC in BCAA catabolism in ester synthesizing fruits awaits characterization.

2-Isopropylmalate synthase is normally not considered to be involved in isoleucine formation or degradation. It is engaged in the first step of leucine biosynthesis, transferring an acetyl group from acetyl-CoA to α-ketoisovalerate to form isopropylmalate. Given that Nie et al. (2005) observed high accumulation of isoleucine, but not leucine during ripening of apple, a rationale for the marked increase in 2-isopropylmalate synthase expression is not obvious. However, it is possible that the 2-isopropylmalate synthase protein may have a different activity; 2-isopropylmalate synthase may also be involved in fatty acid biosynthesis (Charon et al., 1974; Kroumova et al., 1994; Kroumova and Wagner, 2003).

The single-carbon fatty acid elongation pathway for fatty acid synthesis may contribute to α-keto-β-methylvalerate formation via α-keto butyrate and may also contribute to propionyl-CoA formation from this same metabolite (Figure 29). If so, 2-isopropylmalate synthase may be involved in supplying precursors of both straight- and branched-chain esters. The increase in branched-chain and propanol and propanoate esters at the climacteric peak (Chapter 3, Figures 14 and 21) and their maintenance at high levels during senesce supports a role for engagement of the single-carbon fatty acid elongation pathway in ester biosynthesis. Nevertheless, the single-carbon fatty acid elongation pathway needs to be more fully evaluated. Apart from the current study, we have not been able to find any studies linking this pathway to volatile ester biosynthesis.

However, there is solid data describing the involvement of the single-carbon fatty acid elongation pathway in sugar ester biosynthesis and excretion in several members of the *Solanaceae* family, including tobacco (*Nicotiana tabacum*), and petunia (Kroumova and Wagner, 2003). While no data are found verifying the function of the single-carbon fatty acid synthesis pathway in apple, the fact that 2-isopropylmalate synthase catalyzes the first step in this pathway, is suggestive that this gene plays a role in ester biosynthesis and/or other ripening processes not yet elucidated.

On average, the RT-PCR cycle number needed for *BCAT* family members tend to be larger than *PDC* or 2-isopropylmalate synthase genes. The lower number of PCR cycles required for quantification of *PDC* genes suggests transcript levels for these genes are several-fold higher than for *BCAT* genes. Further, the expression patterns for 2-isopropylmalate synthase and *PDC* exhibited a greater degree of change during ripening. The pattern of 2-methylbutanol, 2-methylbutanal, and 2-methylbutanol ester production remaining elevated or increasing slightly even in the senescence stage is similar to the expression pattern of *PDC1*. Since the expression patterns of *PDC2-5* declined sharply in the later ripening stage, we suggest that *PDC1*, detected on the microarray, may be involved in converting the isoleucine product/precursor α-keto-β-methylvalerate to 2-methylbutanal to form 2-methylbutyl esters in apples. Considering that 2-isopropylmalate synthase gene expression had a pattern similar to that for the production of branched-chain esters, we further suggest that this gene may have a role supplying α-keto butyrate for branched-chain α-keto acid (α-keto-β-methylvalerate) synthesis and ultimately contributing to branched-chain ester biosynthesis.

CONCLUSION

Several putative genes of branched-chain aminotransferase, pyruvate decarboxylase, and 2-isopropylmalate synthase were found to have an expression pattern that increase concurrently with branched-chain ester production. BCAA and 2-isopropylmalate synthase may be involved in supplying branched-chain α-keto acids. The high level of expression of 2-isopropylmalate synthase only during ester biosynthesis is suggestive of a role in the formation of straight-chain acyl-CoAs as well as branched-chain α-keto acids. The *PDC1* gene expression pattern reflected production patterns for 2-methylbutanal, 2-methylbutanol, and 2-methylbutyl esters. Considering the high level of *PDC* expression and its pattern, PDC may play an important role in regulating the formation of 2-methylbutyl esters.

Both BCAT and PDC activity needs to be further characterized at the protein level and the role of 2-isopropylmalate synthase in the single-carbon fatty acid biosynthesis pathway needs to be more fully evaluated.

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	Day						
Category	11	25	32	39	49	60	70
Low25	>-0.5	<-2					
Low32	>-0.5		<-2				
Low39	>-0.5	>-1		<-2			
Low49	>-0.5	>-0.5	>-1		<-2		
Low60	>-0.5	>-0.5	>-0.5	>-1		<-2	
Low70	>-0.5	>-0.5	>-0.5	>-0.5	>-1		<-2
High25	<0.5	>2	>3	>3	>3	>3	>3
High32	<1	<2	>3	>3	>3	>3	>3
High39	<0.5	<0.5		>2.5	>2.5	>2.5	>2.5
High49	<0.5	<0.5	<0.5		>2	>2	>2
HIgh60	<0.5	<0.5	<0.5	<0.5		>2	>2
High70	<0.5	<0.5		<0.5	<0.5		>2.5
Middle	>-0.2 and <0.2	>-0.2 and <0.2	>-0.2 and <0.2	>-0.2 and <0.2	>-0.2 and <0.2	>-0.2 and <0.2	>-0.2 and <0.2
Peak25	<2	>2		<2	<2	<2	<1
Peak32	<1		>2		<1	<1	<1
Peak39	<2			>2		<2	<1
Peak49	<1	<1	<1		>2		<2
Peak60	<1	<1	<1	<1		>2	<2
Double Peak	<2	>2	<2	>2		<2	<2

Table 3. Constraint table defining expression limits for microarray elements to identify candidate gene fragments for sequencing. Constraints were developed to identify changes in expression associated with eight distinct developmental stages (Days 0, 11, 25, 32, 39, 49, 60, and 70) during ripening for 'Jonagold' apple fruit. Expression levels are relative to Day 0 (in $\log_2$ scale). Categories are based on the pattern of expression and numbers denote the day following which, pattern changes were detected; 'low' indicates expression patterns that declined and 'high' indicates expression patterns that increased; 'middle' indicates no change in pattern from Day 0 through Day 70; 'peak' indicates a transient increase in gene expression on the indicated day; and 'double peak' indicates an expression pattern with peaks on Days 25 and 39.

Target	Apple cluster no.	Seq. length	Longest EST		Forward primer seq. (5'→ 3')	Reverse primer seq. (5'→ 3')	Expected size (bp)	Cycles needed	Annealing temp °C
			Accession no.	GI no.					
PDC 1*	MDC015210	1549	CK900568	GI:45438814	gcacaggattcttcaccaca	cagcctctaagcccaaatga	444	27	55
PDC 2	MDC133700	804	CX025334	GI:56435496	gatccctcgatgtctgcaag	gcccacccacagtgaaagta	336	27, 29	59
PDC 3	MDC206800	355	CO754399	GI:50889650	tggaccaccaaggttagcat	cattccagcagctctttgct	215	33	57
PDC 4	MDC206810	2097	DT002903	GI:71825511	gcggtgagattgtggagtct	accctcagaggctcatcctt	293	26, 27	57
PDC 5	MDC433930	600	CV567449	GI:54464858	tctgttccaccacatccaga	gtgtacccgccgttgtttat	307	28	57
PDC 6	MDC367810	473	CN491928	GI:46609400	ggatcgattggttggtctgt	tcttctcctccgttgctgtt	340	Failed	57
PDC 6	MDC367810	473	CN491928	GI:46609400	gacggcccttacaatgtgat	gggcggttattagcagttga	245	Failed	57
PDC 7	MDC341030	108	CN865916	GI:48122967	EST too short				
BCAT 1	MDC021750	685	DT003328	GI:71825936	caccgaagttggttgagcag	gagaacacgccttccttctg	290	35	57
BCAT 2	MDC028270	908	CV082955	GI:51561944	gagtacgatcgtgcctctcg	cacaggagcaacaccaacag	363	30, 32	57
BCAT 3	MDC160840	766	CO899974	GI:51239764	gggatttgcccatctgtacc	gggagaactcagtccggtta	300	31	57
BCAT 4	MDC192460	831	CX024412	GI:56433559	gtttgggagggtcttcgagt	attacgacgggtggtagctg	333	31	57
BCAT 5	MDC238040	572	CN581127	GI:46992677	tgcaactcctggtggtactg	ccttcactacgagcaacgtc	258	32	57
BCAT 6	MDC238050	1400	CO867683	GI:51097833	cctgctccggagtacacatt	ctggaatccttcgctacgag	369	29	57
BCAT 7	MDC301230	709	CN912350	GI:48384850	cgatcacatggttcatcgag	actgcatagaaagcgggtgt	279	33	57
BCAT 8	MDC385660	488	CV882612	GI:55857820	agagggagggttctggtgtt	gaggttgtcccattcgatgt	293	32	59
BCAT 9	MDC405820	636	CO903152	GI:51293455	cctgacacaacatgggacac	gttggtgcaggcatacacag	289	34	59
BCAT 10*	MDC410280	475	CO868030	GI:51098180	cccgagtccaaatcattcat	tcgaagggtaccaggttgag	352	28, 31	59
BCAT 11	MDC242390	724	DT003206	GI:71825814	ggtctcaaggcaatcaggac	gctgcatccagaaagaggac	326	Failed	57
BCAT 11	MDC242390	724	DT003206	GI:71825814	cttgcagccatgacttctga	tcctgattgccttgagacct	325	Failed	57
Control 18s rRNA		1063	DQ341382	GI:85717895	gagaaacggctaccacatcc	gagcgtaggcttgctttgag	390	18	59
2-isopropylmalate* synthase		701	AT5G23010.1	GI:7387848	caccgtgaaggcactatgaa	ggctcggagacaatccttct	456	27	57

Table 4. Gene with accession number and primer list for semi-quantitative RT-PCR. Apple cluster numbers are from Tree Fruit Technology genomic analysis tool apple database v.3.0 (http://genomics.msu.edu/fruitdb/analyses/apple.shtml). Accession and GI number indicates the longest EST from apple clusters. Genes include for which PCR was not successful. Single number in ‘cycles needed’ indicates that the same cycle was performed with both biological replications, rep. 1 and 2. Two numbers indicate that different cycles were performed between biological replications for optimum result.

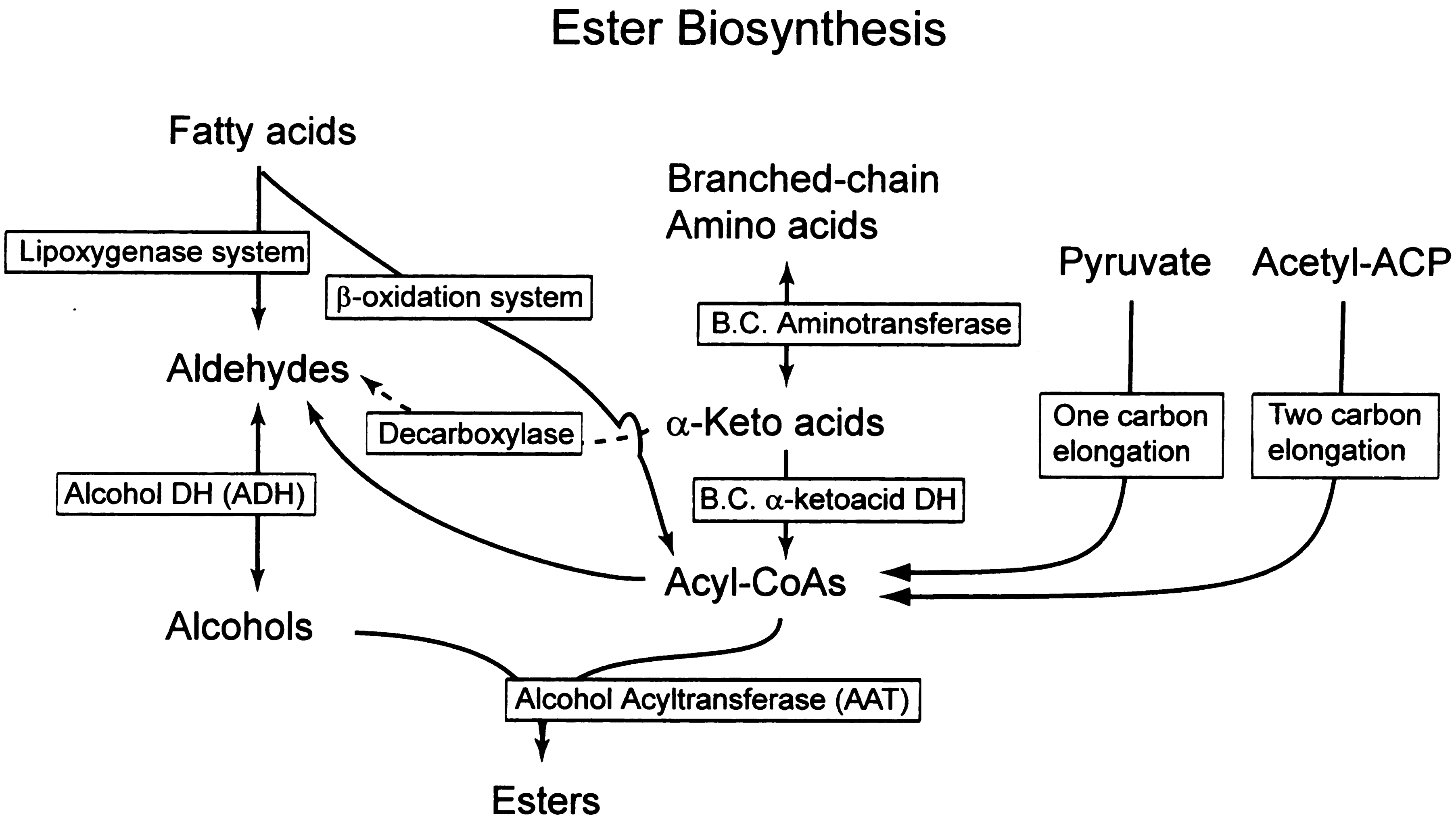

Figure 28. Pathways having potential to supply alcohol and acyl-CoA substrates for ester formation.

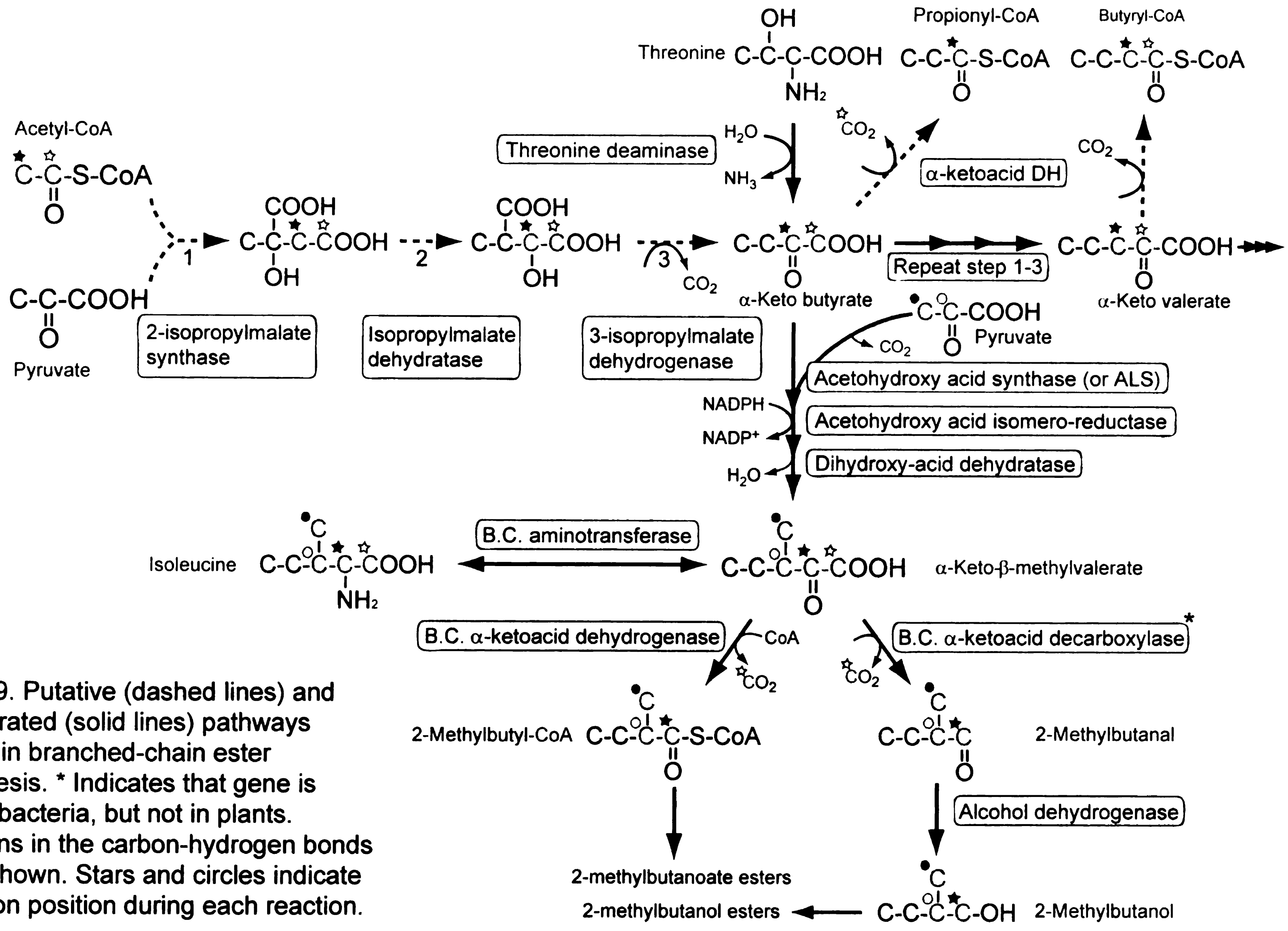

Figure 29. Putative (dashed lines) and demonstrated (solid lines) pathways involved in branched-chain ester biosynthesis. * Indicates that gene is found in bacteria, but not in plants. Hydrogens in the carbon-hydrogen bonds are not shown. Stars and circles indicate the carbon position during each reaction.

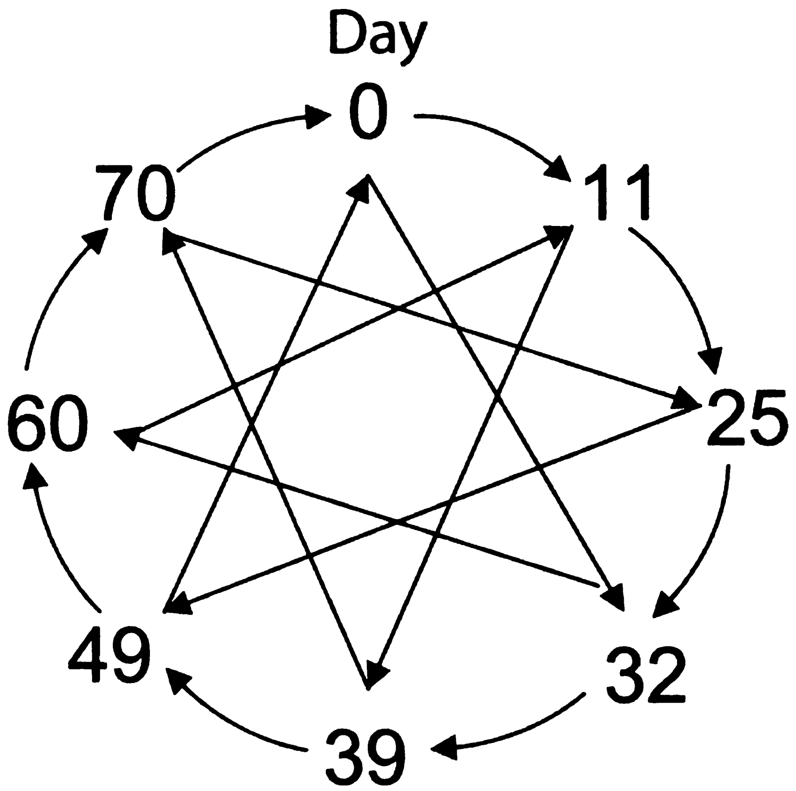

Figure 30. Experimental design for microarray analysis. Arrows indicate comparison made. Numbers indicate the days for the eight stages of development. Evaluated sixteen arrays were used with two biological samples and a dye swap to reduce variation. The tail of the arrow indicates RNA probe labeled with cyanine 3; the head of the arrow indicates RNA probe labeled with cyanine 5.

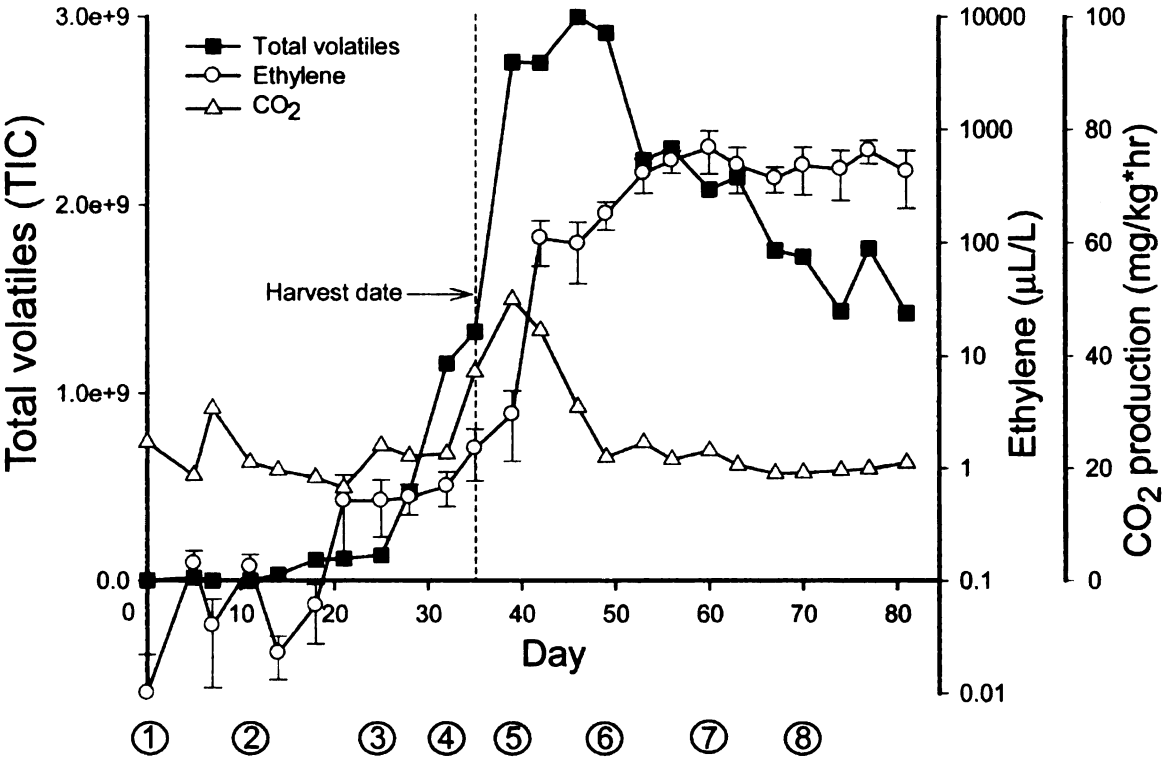

Figure 31. Internal ethylene concentration, total volatiles (TIC), and CO_2 production in pre-climacteric through post-climacteric ‘Jonagold’ apples. Eight stages (Days 0, 11, 25, 32, 39, 49, 60, and 70) were selected for genomic analysis based on physiological stages during ripening. Stage 1 (Day 0), early climacteric; stage 2 (Day 11), late preclimacteric and onset of trace ester biosynthesis; stage 3 (Day 25), onset of the autocatalytic ethylene and rapid increase of ester biosynthesis; stage 4 (Day 32), half-maximal ester biosynthesis and engagement of the respiratory climacteric; stage 5 (Day 39), near maximal ester biosynthesis, peak in respiratory activity, and onset of rapid tissue softening; stage 6 (Day 49), end of maximal biosynthesis, the conclusion of the respiratory climacteric, and completion of tissue softening; stage 7 (Day 60), midpoint in the decline in ester biosynthesis, maximal ethylene production, and onset of senescent; stage 8 (Day 70), postclimacteric minimum in ester production and high fruit senescent.

Figure 32. Patterns of 2-methylbutanol and 2-methylbutanoate ester emissions during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (Day 0) until late November (Day 81). The fruits were collected from the field until Oct. 7, 2004 (Day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. GC/MS response (TIC) of total 2-methylbutanol and 2-methylbutanoate esters and ontogeny of ethylene. B. GC/MS response (TIC) of 2-methylbutanol and 2-methylbutanal. C. GC/MS response (TIC) of 2-methylbutyl acetate and 2-methylbutyl butanoate. D. GC/MS response (TIC) of ethyl 2-methylbutanoate, propyl 2-methylbutanoate, butyl 2-methylbutanoate, pentyl 2-methylbutanoate, and hexyl 2-methylbutanoate. E. 2-Methylbutanoate ester proportions (% of total 2-methylbutanoate esters). Each symbol represents the average of four replications. Vertical bars represent mean ± SD.

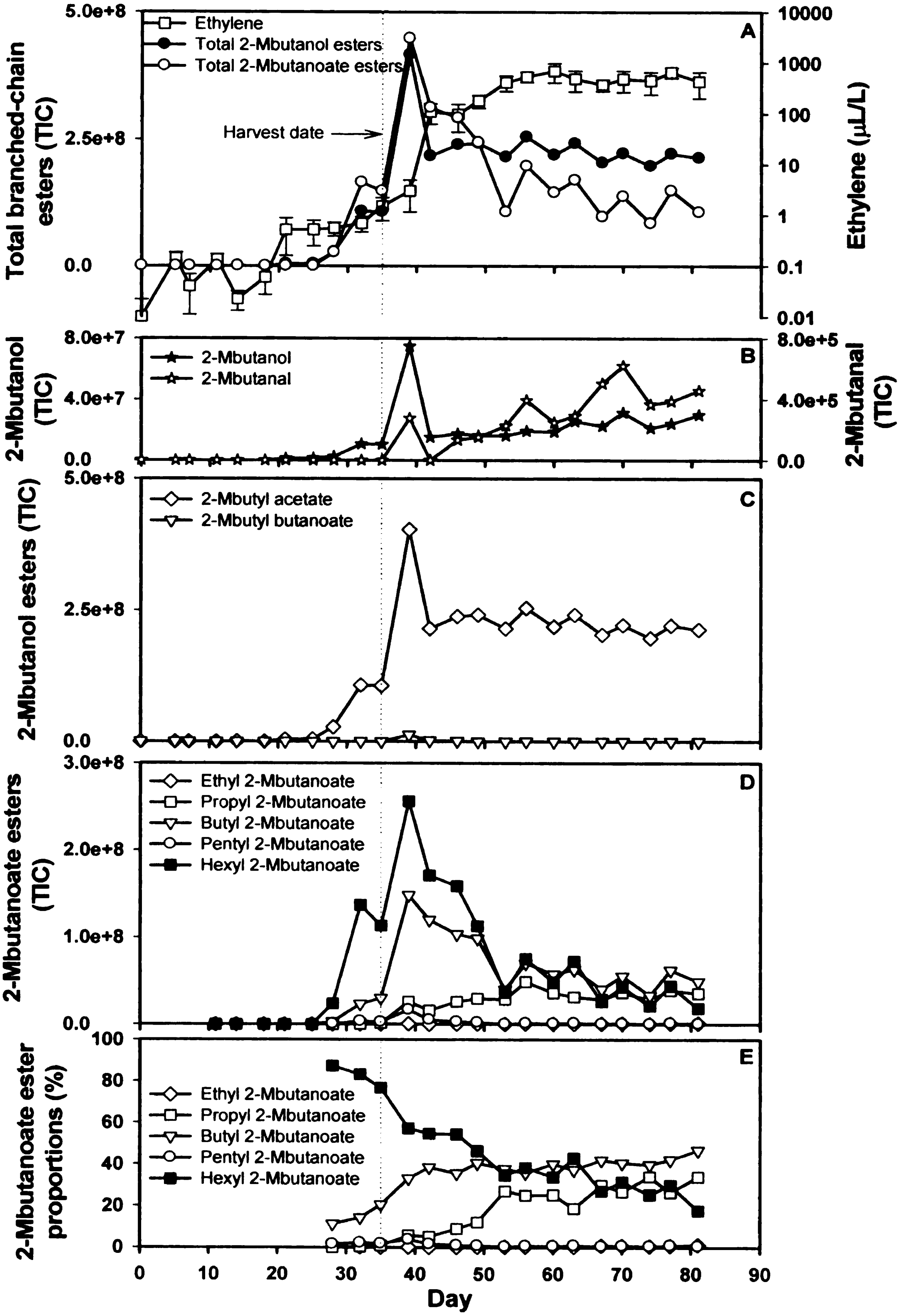
5.0e+8
2.5e+8
0.0
Total branched-chain esters (TIC)
Ethylene
Total 2-Mbutanol esters
Total 2-Mbutanoate esters
Harvest date
A
10000
1000
100
10
1
0.1
0.01
Ethylene (μL/L)
8.0e+7
4.0e+7
0.0
2-Mbutanol (TIC)
2-Mbutanol
2-Mbutanal
B
8.0e+5
4.0e+5
0.0
2-Mbutanal (TIC)
5.0e+8
2.5e+8
0.0
2-Mbutanol esters (TIC)
2-Mbutyl acetate
2-Mbutyl butanoate
C
3.0e+8
2.0e+8
1.0e+8
0.0
2-Mbutanoate esters (TIC)
Ethyl 2-Mbutanoate
Propyl 2-Mbutanoate
Butyl 2-Mbutanoate
Pentyl 2-Mbutanoate
Hexyl 2-Mbutanoate
D
100
80
60
40
20
0
2-Mbutanoate ester proportions (%)
Ethyl 2-Mbutanoate
Propyl 2-Mbutanoate
Butyl 2-Mbutanoate
Pentyl 2-Mbutanoate
Hexyl 2-Mbutanoate
E
0
10
20
30
40
50
60
70
80
90
Day

Figure 32.

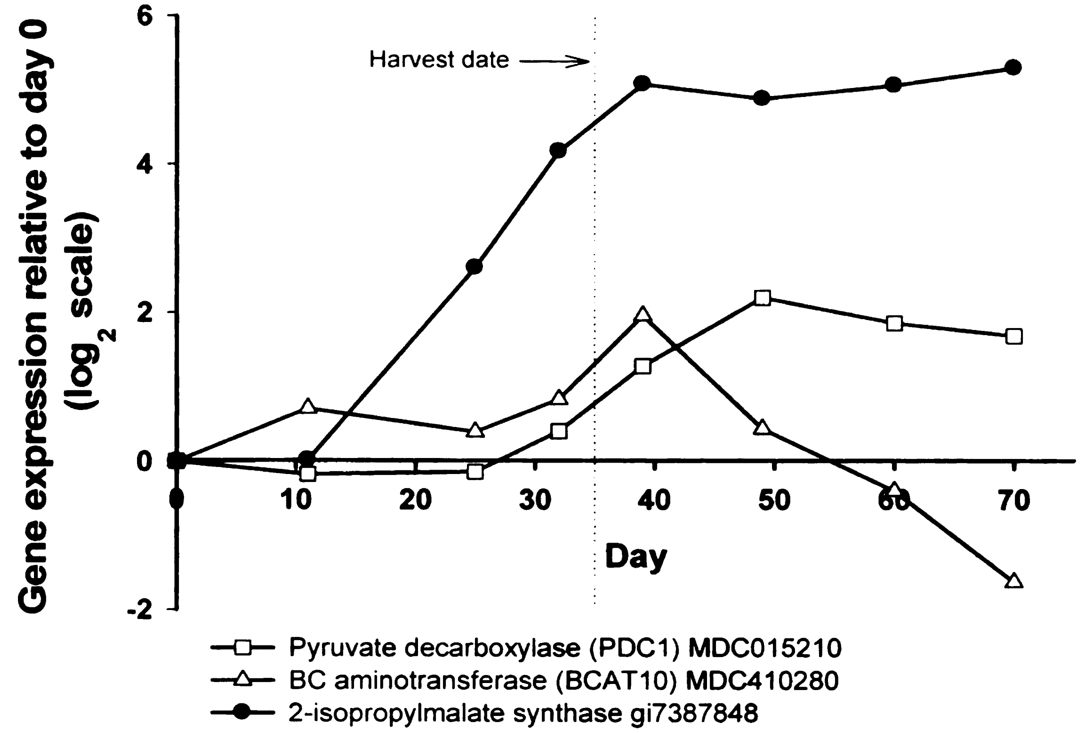

Figure 33. Gene expression based on microarray data (in $\log_2$ scale). For genes potentially involved in branched-chain ester formation including pyruvate decarboxylase (PDC), branched-chain aminotransferase (BCAT), and 2-isopropylmalate synthase gene expression relative to Day 0.

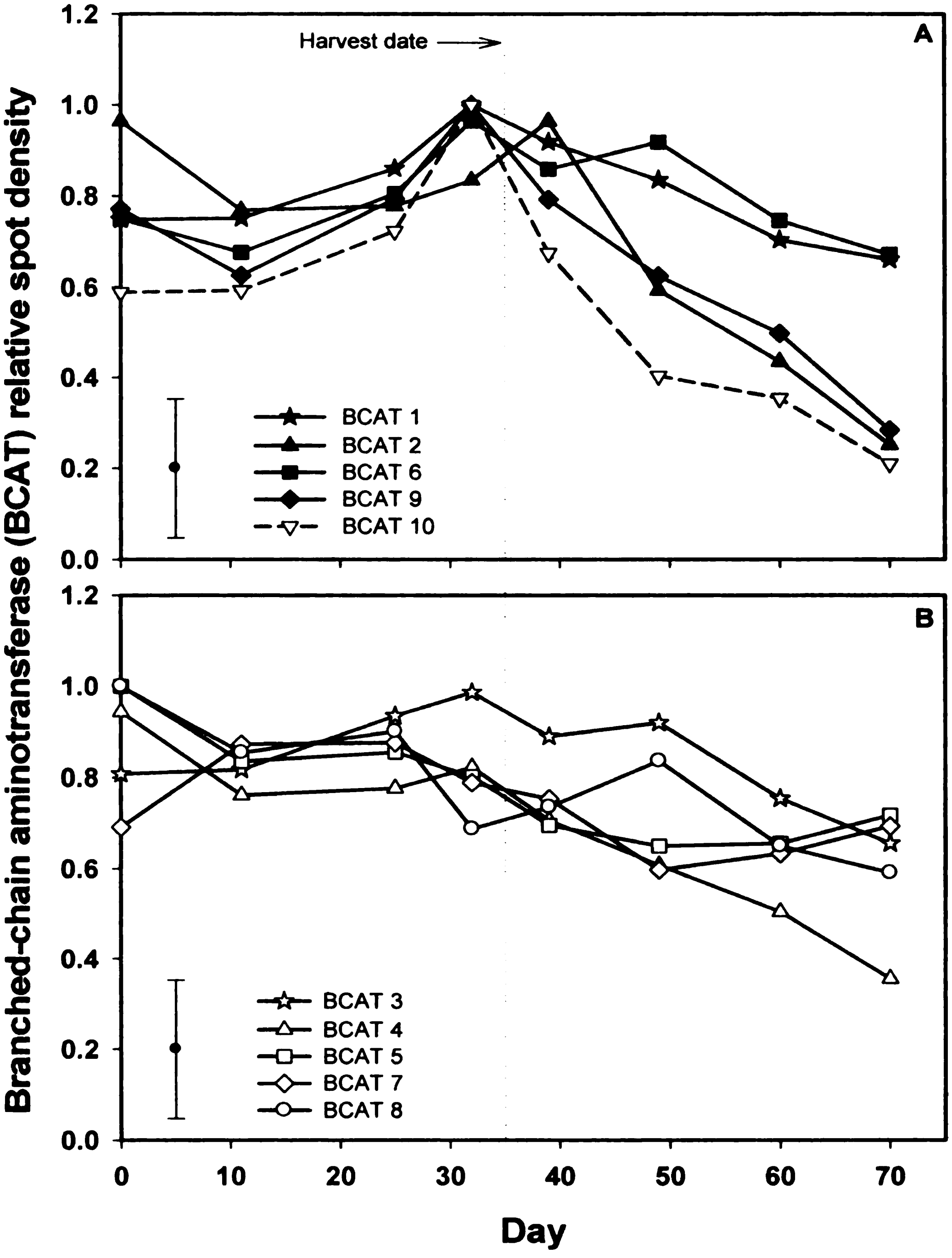

Figure 34. Gene expression of putative branched-chain aminotransferase (BCAT) for 'Jonagold' apple fruit ripened at room temperature performed by semi-quantitative RT-PCR. The value is based on spot density relative to maximum value. 18s rRNA was used as a control. All data are normalized relative to control gene spot density. The control gene spot density ranged between 0.78-0.98. Each symbol represents the average of two replications. The average pooled standard deviation is 0.15.

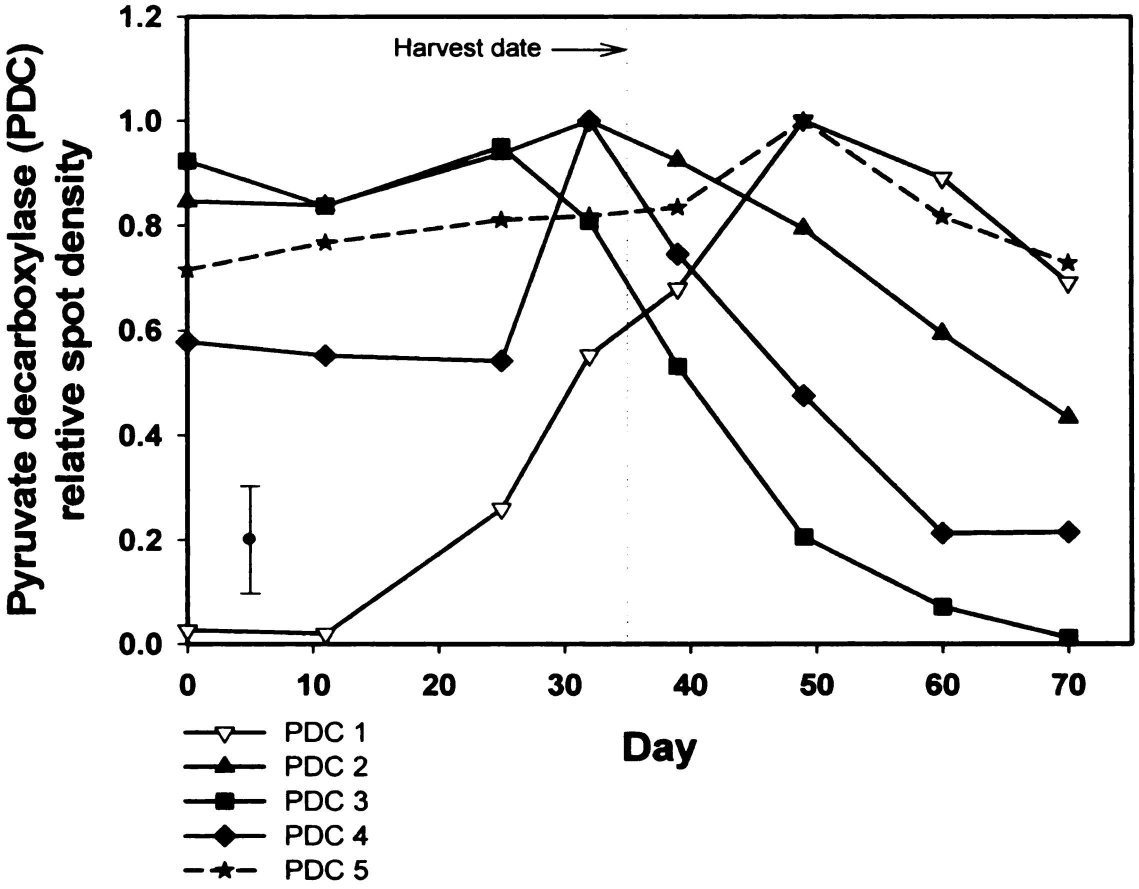

Figure 35. Gene expression of putative pyruvate decarboxylase (PDC) for 'Jonagold' apple fruit ripened at room temperature performed by semi-quantitative RT-PCR. The value is based on spot density relative to maximum value. 18s rRNA was used as a control. All data are normalized relative to control gene spot density. The control gene spot density ranged between 0.78-0.98. Each symbol represents the average of two replications. The average pooled standard deviation is 0.10.

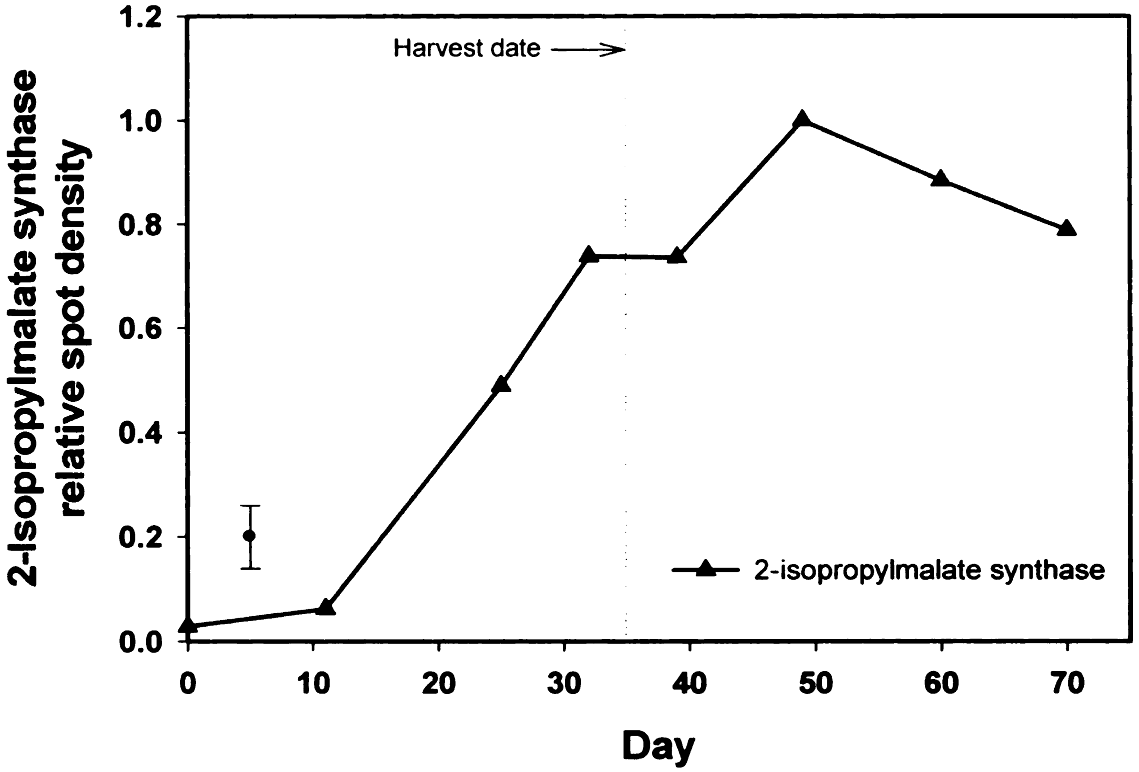

Figure 36. Gene expression of putative 2-isopropylmalate synthase for 'Jonagold' apple fruit ripened at room temperature performed by semi-quantitative RT-PCR. The value is based on spot density relative to maximum value. 18s rRNA was used as a control. All data are normalized relative to control gene spot density. The control gene spot density ranged between 0.78-0.98. Symbol represents the average of two replications. The pooled standard deviation is 0.06.

APPENDIX

Day	Ethylene (μL/L)	SD	CO_2 (mg/kg*hr)	Total Volatiles (TIC)
0	0.0074	0.0119	24.61	1509500
5	0.1423	0.0422	18.74	15432000
7	0.0399	0.0288	30.66	1162500
11	0.1326	0.0385	21.07	1057100
14	0.0225	0.0094	19.69	31861000
18	0.0602	0.0330	18.36	112090000
21	0.5149	0.3553	16.59	117370000
25	0.5182	0.2718	24.05	136640000
28	0.5538	0.1666	22.15	472390000
32	0.6956	0.2349	22.63	1160300000
35	1.5031	0.7281	37.21	1329000000
39	3.0271	1.8698	50.01	2758000000
42	109.536	46.980	44.50	2755600000
46	97.825	54.030	30.89	2997700000
49	179.507	49.798	21.99	2913700000
53	413.057	138.730	24.57	2239900000
56	533.818	119.016	21.55	2301200000
60	690.425	283.249	23.17	2083300000
63	485.390	212.733	20.59	2148300000
67	371.956	94.518	19.07	1759200000
70	482.844	217.468	19.22	1727100000
74	448.329	210.926	19.62	1438100000
77	653.707	150.345	19.88	1770300000
81	429.090	227.916	20.96	1427500000

Table 5. Original data for Figure 10 (Chapter 3). Data are internal ethylene content, CO_2 production, and the GC/MS response total ion count (TIC) for all aroma volatiles. Each value is the average of four replications.

Bending test (N)

	Day																	
Replication	11	14	18	21	25	28	32	35	39	42	46	49	53	56	60	63	67	70
Rep 1-1	8.61	8.97	9.40	8.69	9.00	8.88	8.36	8.64	8.88	4.85	2.31	2.88	3.54	3.52	3.89	2.17	2.33	1.84
Rep 1-2	7.23	9.62	9.47	8.60	9.14	9.95	7.51	9.83	8.18	4.73	2.27	2.49	3.88	3.49	3.94	2.55	3.03	1.69
Rep 2-1	10.54	8.53	8.03	7.95	8.37	8.55	9.41	9.14	8.39	3.00	1.85	4.03	3.45	3.07	3.09	2.42	2.35	1.72
Rep 2-2	10.10	8.49	7.64	8.94	7.91	8.65	8.24	10.16	7.55	3.09	1.96	3.89	4.22	2.62	2.47	2.67	2.52	1.91
Rep 3-1	7.79	11.39	9.03	9.21	8.00	7.82	7.34	10.45	9.08	5.50	1.96	3.92	4.26	3.36	1.57	3.11	1.73	2.10
Rep 3-2	8.64	12.20	7.64	10.20	8.36	8.63	8.59	11.01	8.92	6.03	2.27	3.52	3.13	3.63	2.21	3.24	1.59	1.91
Rep 4-1	10.49	8.65	11.98	7.81	8.33	9.15	8.74	9.66	8.81	7.55	2.90	3.57	2.42	2.96	2.39	2.30	2.71	2.37
Rep 4-2	9.89	8.53	9.70	8.47	8.07	9.25	8.17	10.05	7.89		3.58	2.88	2.58	2.79	2.59	2.20	2.41	2.19

Compression test (N)

	Day																			
Replication	5	7	11	14	18	21	25	28	32	35	39	42	46	49	53	56	60	63	67	70
Rep 1-1	120.39	101.09	93.15	89.89	83.29	77.13	87.71	91.15	78.24	83.32	83.76	44.73	39.46	32.60	44.64	40.81	58.82	35.57	39.05	40.00
Rep 1-2	110.81	117.58	88.07	97.40	90.82	89.49	84.39	74.07	76.20	86.52	70.04	41.85	40.46	44.23	32.40	45.50	50.98	31.77	32.86	29.85
Rep 2-1	111.12	88.02	94.87	89.59	84.29	91.55	83.53	70.38	61.48	89.14	67.58	43.73	32.10	45.87	50.43	47.99	48.66	42.15	31.60	37.09
Rep 2-2	99.21	99.68	96.48	102.98	80.40	79.38	83.50	71.70	73.71	79.37	78.06	39.12	20.66	52.92	48.60	42.11	42.30	36.32	35.95	32.27
Rep 3-1	104.91	108.98	93.59	107.06	88.50	80.01	84.62	63.98	81.65	85.21	79.77	51.14	30.55	42.98	41.22	29.09	32.73	35.94	17.91	29.81
Rep 3-2	97.62	94.55	94.99	86.60	81.64	83.54	79.43	94.75	76.94	86.63	78.26	58.97	30.17	36.15	38.84	30.79	37.88	46.62	22.25	30.79
Rep 4-1	90.60	94.62	91.04	90.88	86.26	90.44	87.53	84.02	70.74	73.66	75.36	47.54	45.65	34.36	41.54	47.95	33.30	38.83	30.33	39.55
Rep 4-2	96.38	109.41	96.31	80.11	77.65	91.72	82.46	69.68	91.87	83.85	44.73	56.93	41.84	36.10	32.21	43.14	36.50	33.13	33.35	30.75
Rep 4-3		94.82																		

Table 6. Original data for Figure 11 (Chapter 3). Force (N) for bending/tensile failure and compressive failure during ripening and senescence of 'Jonagold' apple fruit.

No.	Compound	RT (second)	Class
1	Butanal	114.31	Aldehyde
2	Ethyl acetate	114.86	Ester
3	2-Methyl butanal	120.13	Aldehyde
4	Ethanol	121.68	Alcohol
5	Ethyl propanoate	127.09	Ester
6	Propyl acetate	130.21	Ester
7	2-Methylpropyl acetate	137.68	Ester
8	Propanol	141.03	Alcohol
9	Ethyl butanoate	142.62	Ester
10	Propyl propanoate	143.89	Ester
11	Ethyl 2-methylbutanoate	145.97	Ester
12	Butyl acetate	149.89	Ester
13	Hexanal	153.21	Aldehyde
14	2-Methylbutyl acetate	160.37	Ester
15	Propyl butanoate	161.59	Ester
16	Butanol	163.64	Alcohol
17	Propyl 2-methylbutanoate	164.27	Ester
18	Butyl propanoate	165.29	Ester
19	Pentyl acetate	171.19	Ester
20	Pentyl propanoate	175.84	Ester
21	2-Methylbutanol	178.97	Alcohol
22	Butyl butanoate	182.70	Ester
23	Butyl 2-methylbutanoate	185.82	Ester
24	2-Methylbutyl butanoate	192.09	Ester
25	Hexyl acetate	194.95	Ester
26	Propyl hexanoate	203.79	Ester
27	Butyl pentanoate	203.84	Ester
28	Pentyl 2-methylbutanoate	207.07	Ester
29	Hexyl propanoate	209.40	Ester
30	Hexanol	210.10	Alcohol
31	Butyl hexanoate	225.22	Ester
32	Hexyl butanoate	225.50	Ester
33	Hexyl 2-methylbutanoate	227.82	Ester
34	Pentyl hexanoate	245.20	Ester
35	Butyl heptanoate	245.40	Ester
36	Propyl octanoate	245.98	Ester
37	Hexyl hexanoate	264.60	Ester
38	Butyl octanoate	264.96	Ester
39	Hexyl octanoate	300.86	Ester

Table 7. Original data for Figure 12 (Chapter 3). Data are volatile compounds, GC retention time (second), and classes (esters, alcohols, and aldehydes) identified from 'Jonagold' apple fruit during ripening and senescence.

Day	Ethanol	Propanol	Butanol	Hexanol	2-Mbutanol
0	0	0	0	0	0
5	0	0	0	0	0
7	0	0	0	0	0
11	0	0	0	0	0
14	0	0	0	0	0
18	0	0	0	0	0
21	0	0	0	0	1277184
25	0	0	130985	0	1356999
28	0	65117	2808173	10988762	2444501
32	0	172094	7243258	16803031	10570357
35	0	216622	6393902	17336530	10402166
39	0	1545900	22210397	18375822	74834387
42	45755	1377952	22670479	16568597	15101710
46	0	5126017	37799365	14080643	17507796
49	0	6574075	30097459	12208675	16221393
53	39537	12422605	31316951	8795541	16073867
56	80978	14816826	31705307	11363277	19166305
60	142928	12947110	34101107	8701354	18636585
63	242565	12920652	33910922	12122987	25520562
67	171391	13122409	38082128	8224758	22429805
70	237862	15718028	44701783	10800553	31140692
74	178244	10428265	28421878	7438556	21044632
77	172917	10763077	31327303	10844211	24048526
81	244142	12196363	33790393	6137536	29826410

Ion	45	31	33	31	41
Ion fraction	0.22358	0.55748	0.01690	0.09059	0.17844

Table 8. Original data for Figure 13A and 14A (Chapter 3). Data are the GC/MS response (total ion count, TIC) of alcohols produced by 'Jonagold' apple fruit during ripening and senescence. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications.

Day	Ethanol	Propanol	Butanol	Hexanol	2-Mbutanol
0	nd	nd	nd	nd	nd
5	nd	nd	nd	nd	nd
7	nd	nd	nd	nd	nd
11	nd	nd	nd	nd	nd
14	nd	nd	nd	nd	nd
18	nd	nd	nd	nd	nd
21	0.00	0.00	0.00	0.00	100.00
25	0.00	0.00	8.80	0.00	91.20
28	0.00	0.40	17.22	67.39	14.99
32	0.00	0.49	20.82	48.30	30.38
35	0.00	0.63	18.61	50.47	30.28
39	0.00	1.32	18.99	15.71	63.98
42	0.08	2.47	40.65	29.71	27.08
46	0.00	6.88	50.73	18.90	23.50
49	0.00	10.10	46.23	18.75	24.92
53	0.06	18.10	45.62	12.81	23.41
56	0.10	19.21	41.10	14.73	24.85
60	0.19	17.37	45.76	11.68	25.01
63	0.29	15.25	40.03	14.31	30.12
67	0.21	16.00	46.42	10.03	27.34
70	0.23	15.32	43.57	10.53	30.35
74	0.26	15.45	42.10	11.02	31.17
77	0.22	13.95	40.60	14.05	31.17
81	0.30	14.84	41.11	7.47	36.29

Table 9. Original data for Figure 13B and 14B (Chapter 3). Data are the percentages that each alcohol class comprises of all alcohols detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no alcohols of these classes were detected.

Day	Ethanol esters	Propanol esters	Butanol esters	2-Mbutanol esters	Pentanol esters	Hexanol esters
0	0	0	0	0	0	0
5	0	0	0	0	0	0
7	0	0	0	0	0	0
11	0	0	0	0	0	0
14	0	0	73330	544232	0	58889
18	0	0	83154	66706	0	570601
21	0	0	4650657	4135506	1248775	9151795
25	0	0	7648340	4476358	3988414	14924860
28	0	486171	104050337	27454993	24440953	177021079
32	1054211	3768227	214015744	107774833	45983035	367175606
35	957638	7505138	233873587	106544048	39889493	322774259
39	3383294	93199521	775917475	415832480	192039727	727724087
42	638924	61534735	617500508	217011577	105136342	745865025
46	2707736	195737296	775501480	239357798	97455215	700593932
49	3565715	245034720	759929280	241729378	89219198	614885143
53	5077692	286498097	561181076	215081835	58239977	345668171
56	7602839	369272427	664983847	254520293	91627767	469876634
60	8603966	313026664	600304346	219037073	64556391	359405892
63	6916506	304853959	606827901	241751786	87970809	426317622
67	11045524	262074575	509882747	203489888	54227242	250314174
70	14128221	283759336	534265021	221951898	59521053	296472154
74	8806151	239687287	412288858	197089486	42315368	217490846
77	11560499	268848735	537759061	221358650	70492799	294728574
81	15217937	264940302	476333456	213434085	55658328	201483545
Total	101266855	3200227190	8397070205	3352642903	1184010886	6542502888

Table 10. Original data for Figure 14A (Chapter 3). Data are the GC/MS response (total ion count) of esters arranged by their alkyl (alcohol-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications.

Day	Ethanol esters	Propanol esters	Butanol esters	2-Mbutanol esters	Pentanol esters	Hexanol esters
0	nd	nd	nd	nd	nd	nd
5	nd	nd	nd	nd	nd	nd
7	nd	nd	nd	nd	nd	nd
11	nd	nd	nd	nd	nd	nd
14	0.00	0.00	10.84	80.45	0.00	8.71
18	0.00	0.00	11.54	9.26	0.00	79.20
21	0.00	0.00	24.24	21.55	6.51	47.70
25	0.00	0.00	24.64	14.42	12.85	48.09
28	0.00	0.15	31.20	8.23	7.33	53.09
32	0.14	0.51	28.93	14.57	6.22	49.63
35	0.13	1.05	32.87	14.97	5.61	45.36
39	0.15	4.22	35.14	18.83	8.70	32.96
42	0.04	3.52	35.33	12.42	6.02	42.68
46	0.13	9.73	38.56	11.90	4.85	34.83
49	0.18	12.54	38.88	12.37	4.57	31.46
53	0.35	19.47	38.13	14.61	3.96	23.49
56	0.41	19.88	35.79	13.70	4.93	25.29
60	0.55	20.00	38.36	14.00	4.13	22.97
63	0.41	18.20	36.24	14.44	5.25	25.46
67	0.86	20.30	39.49	15.76	4.20	19.39
70	1.00	20.12	37.89	15.74	4.22	21.02
74	0.79	21.45	36.89	17.63	3.79	19.46
77	0.82	19.14	38.28	15.76	5.02	20.98
81	1.24	21.59	38.82	17.39	4.54	16.42

Table 11. Original data for Figure 14B (Chapter 3). Data are the percentages that each ester class comprises of all esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Esters are arranged by the source of the alkyl (alcohol-derived) moiety. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected.

Day	Ethyl acetate	Ethyl propanoate	Ethyl butanoate	Ethyl 2-Mbutanoate
0	0	0	0	0
5	0	0	0	0
7	0	0	0	0
11	0	0	0	0
14	0	0	0	0
18	0	0	0	0
21	0	0	0	0
25	0	0	0	0
28	0	0	0	0
32	757266	0	101763	195183
35	667936	0	241740	47962
39	1947659	420387	382040	633208
42	452915	0	86705	99303
46	1980734	329346	88585	309071
49	2496052	520588	87348	461727
53	3993076	749268	80848	254500
56	5506875	1296852	183483	615629
60	6117778	1685613	160476	640101
63	5117759	1169138	95136	534474
67	8312465	1695279	256405	781374
70	10836238	2004585	270795	1016602
74	6970138	1176384	171887	487742
77	8130761	2039725	293869	1096144
81	10825273	2672355	400953	1319356

Ion	43	57	88	102
Ion fraction	0.54264	0.26430	0.10491	0.14837

Table 12. Original data for Figure 15C (Chapter 3). Data are the GC/MS response (total ion count, TIC) of ethanol esters arranged by their alkanoate (acid-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications.

Day	Ethyl acetate	Ethyl propanoate	Ethyl butanoate	Ethyl 2-Mbutanoate
0	nd	nd	nd	nd
5	nd	nd	nd	nd
7	nd	nd	nd	nd
11	nd	nd	nd	nd
14	nd	nd	nd	nd
18	nd	nd	nd	nd
21	nd	nd	nd	nd
25	nd	nd	nd	nd
28	nd	nd	nd	nd
32	71.83	0.00	9.65	18.51
35	69.75	0.00	25.24	5.01
39	57.57	12.43	11.29	18.72
42	70.89	0.00	13.57	15.54
46	73.15	12.16	3.27	11.41
49	70.00	14.60	2.45	12.95
53	78.64	14.76	1.59	5.01
56	72.43	17.06	2.41	8.10
60	71.10	19.59	1.87	7.44
63	73.99	16.90	1.38	7.73
67	75.26	15.35	2.32	7.07
70	76.70	14.19	1.92	7.20
74	79.15	13.36	1.95	5.54
77	70.33	17.64	2.54	9.48
81	71.13	17.56	2.63	8.67

Table 13. Original data for Figure 15E (Chapter 3). Data are the percentages that each ester class comprises of all ethanol esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected.

Day	Propyl acetate	Propyl propanoate	Propyl butanoate	Propyl 2-Mbutanoate	Propyl hexanoate	Propyl octanoate
0	0	0	0	0	0	0
5	0	0	0	0	0	0
7	0	0	0	0	0	0
11	0	0	0	0	0	0
14	0	0	0	0	0	0
18	0	0	0	0	0	0
21	0	0	0	0	0	0
25	0	0	0	0	0	0
28	414457	0	71715	0	0	0
32	2048090	507334	633475	494961	84367	0
35	3413804	1485801	1113596	1268839	223098	0
39	37608468	16857424	7388455	26119739	5196125	29310
42	27873726	8012876	5581135	16001919	3975271	89808
46	114297757	34911242	12656492	26093516	7558456	219833
49	138006569	48247979	16984255	29639901	11916659	239356
53	163563464	59606493	20440790	28782475	13937652	167223
56	188014101	80665998	28531982	49177139	22626920	256286
60	168549933	75814458	22038081	36387441	10170995	65757
63	163483734	70828608	23633593	31639119	15221512	47393
67	154296474	54277559	17207960	28805571	7478334	8678
70	159947361	56294731	19451684	36552478	11489634	23448
74	140654281	44556421	15704732	28558255	10186096	27503
77	135337264	58784522	20012059	39151461	15461013	102416
81	136382064	57285036	20780573	35854075	14597304	41249

Ion	61	75	89	103	117	145
Ion fraction	0.15051	0.16752	0.11242	0.10392	0.12400	0.08257

Table 14. Original data for Figure 16C (Chapter 3). Data are the GC/MS response (total ion count, TIC) of propanol esters arranged by their alkanoate (acid-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications.

Day	Propyl acetate	Propyl propanoate	Propyl butanoate	Propyl 2-Mbutanoate	Propyl hexanoate	Propyl octanoate
0	nd	nd	nd	nd	nd	nd
5	nd	nd	nd	nd	nd	nd
7	nd	nd	nd	nd	nd	nd
11	nd	nd	nd	nd	nd	nd
14	nd	nd	nd	nd	nd	nd
18	nd	nd	nd	nd	nd	nd
21	nd	nd	nd	nd	nd	nd
25	nd	nd	nd	nd	nd	nd
28	85.25	0.00	14.75	0.00	0.00	0.00
32	54.35	13.46	16.81	13.14	2.24	0.00
35	45.49	19.80	14.84	16.91	2.97	0.00
39	40.35	18.09	7.93	28.03	5.58	0.03
42	45.30	13.02	9.07	26.00	6.46	0.15
46	58.39	17.84	6.47	13.33	3.86	0.11
49	56.32	19.69	6.93	12.10	4.86	0.10
53	57.09	20.81	7.13	10.05	4.86	0.06
56	50.91	21.84	7.73	13.32	6.13	0.07
60	53.85	24.22	7.04	11.62	3.25	0.02
63	53.63	23.23	7.75	10.38	4.99	0.02
67	58.88	20.71	6.57	10.99	2.85	0.00
70	56.37	19.84	6.85	12.88	4.05	0.01
74	58.68	18.59	6.55	11.91	4.25	0.01
77	50.34	21.87	7.44	14.56	5.75	0.04
81	51.48	21.62	7.84	13.53	5.51	0.02

Table 15. Original data for Figure 16E (Chapter 3). Data are the percentages that each ester class comprises of all propanol esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected.

Day	Butyl acetate	Butyl propanoate	Butyl butanoate	Butyl 2-Mbutanoate	Butyl pentanoate	Butyl hexanoate
0	0	0	0	0	0	0
5	0	0	0	0	0	0
7	0	0	0	0	0	0
11	0	0	0	0	0	0
14	73330	0	0	0	0	0
18	83154	0	0	0	0	0
21	4650657	0	0	0	0	0
25	7126722	0	521618	0	0	0
28	78576048	7754501	10032725	3038240	23596	4550864
32	125374274	34876400	19166243	23413937	155087	10785473
35	110905433	55736708	22333337	30182603	333355	14155968
39	270136771	156898323	114983654	148234603	3333613	80063605
42	169785919	147746452	99078766	119929683	2296889	74021672
46	369037955	146293135	87564772	103640952	2193415	62475437
49	344772295	173527613	83716422	98535206	2521761	54547534
53	304316462	141107484	46585937	40169381	1587307	26459589
56	322329500	160173677	67004923	70416587	2788663	41200400
60	302019260	166314968	48951982	57906444	1503062	23169541
63	281995191	163363226	62876066	63177714	2547400	32418850
67	280877951	133389806	38654196	40706810	1001717	14965410
70	272861267	129597161	47697412	55536206	1534070	26739338
74	226101851	105133987	28974460	33423413	955526	17495363
77	251296583	134593058	54297453	62561190	2155057	32326845
81	235478823	118927742	46367945	49531429	1602393	24188531

Ion	61	75	89	103	85	117
Ion fraction	0.06377	0.10197	0.12317	0.14318	0.12247	0.09374

Table 16. Original data for Figure 17C (Chapter 3). Data are the GC/MS response (total ion count, TIC) of butanol esters arranged by their alkanoate (acid-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications.

Day	Butyl heptanoate	Butyl octanoate
0	0	0
5	0	0
7	0	0
11	0	0
14	0	0
18	0	0
21	0	0
25	0	0
28	0	74365
32	0	244330
35	0	226181
39	402039	1864868
42	290288	4350839
46	562155	3733659
49	415686	1892763
53	186450	768466
56	355579	714518
60	142632	296456
63	168758	280696
67	94387	192470
70	121893	177673
74	70193	134066
77	201617	327258
81	83477	153117

Ion	113	145
Ion fraction	0.06613	0.08478

Table 16. Continued.

Day	Butyl acetate	Butyl propanoate	Butyl butanoate	Butyl 2-Mbut	Butyl pentanoate	Butyl hexanoate	Butyl heptanoate	Butyl octanoate
0	nd	nd	nd	nd	nd	nd	nd	nd
5	nd	nd	nd	nd	nd	nd	nd	nd
7	nd	nd	nd	nd	nd	nd	nd	nd
11	nd	nd	nd	nd	nd	nd	nd	nd
14	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
18	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
21	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
25	93.18	0.00	6.82	0.00	0.00	0.00	0.00	0.00
28	75.52	7.45	9.64	2.92	0.02	4.37	0.00	0.07
32	58.58	16.30	8.96	10.94	0.07	5.04	0.00	0.11
35	47.42	23.83	9.55	12.91	0.14	6.05	0.00	0.10
39	34.82	20.22	14.82	19.10	0.43	10.32	0.05	0.24
42	27.50	23.93	16.05	19.42	0.37	11.99	0.05	0.70
46	47.59	18.86	11.29	13.36	0.28	8.06	0.07	0.48
49	45.37	22.83	11.02	12.97	0.33	7.18	0.05	0.25
53	54.23	25.14	8.30	7.16	0.28	4.71	0.03	0.14
56	48.47	24.09	10.08	10.59	0.42	6.20	0.05	0.11
60	50.31	27.71	8.15	9.65	0.25	3.86	0.02	0.05
63	46.47	26.92	10.36	10.41	0.42	5.34	0.03	0.05
67	55.09	26.16	7.58	7.98	0.20	2.94	0.02	0.04
70	51.07	24.26	8.93	10.39	0.29	5.00	0.02	0.03
74	54.84	25.50	7.03	8.11	0.23	4.24	0.02	0.03
77	46.73	25.03	10.10	11.63	0.40	6.01	0.04	0.06
81	49.44	24.97	9.73	10.40	0.34	5.08	0.02	0.03

Table 17. Original data for Figure 17E (Chapter 3). Data are the percentages that each ester class comprises of all butanol esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of ‘Jonagold’ apple fruit. Each value is the average of four replications. The term ‘nd’ indicates percentages were not determined since no esters of these classes were detected.

Day	Pentyl acetate	Pentyl propanoate	Pentyl 2-Mbutanoate	Pentyl hexanoate
0	0	0	0	0
5	0	0	0	0
7	0	0	0	0
11	0	0	0	0
14	0	0	0	0
18	0	0	0	0
21	1248775	0	0	0
25	3988414	0	0	0
28	24028772	0	412181	0
32	38817751	1776737	3593381	1795166
35	31414858	4204037	2772000	1498598
39	98396608	66886681	16874175	9882263
42	78811290	17431405	5237831	3655815
46	75671392	15422169	3025049	3336606
49	65864975	19871052	2126883	1356288
53	45228066	12080687	412261	518962
56	63259797	25744242	1331161	1292567
60	45953173	17671340	931878	0
63	54003345	31785957	1503927	677580
67	40336013	13435316	455913	0
70	42191914	16425812	903327	0
74	29308441	12605036	401891	0
77	43996128	24717999	1337721	440952
81	35918068	19125035	615226	0

Ion	61	53	103	117
Ion fraction	0.06723	0.00346	0.14220	0.01772

Table 18. Original data for Figure 18B (Chapter 3). Data are the GC/MS response (total ion count, TIC) of pentanol esters arranged by their alkanoate (acid-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications.

Day	Pentyl acetate	Pentyl propanoate	Pentyl 2-Mbutanoate	Pentyl hexanoate
0	nd	nd	nd	nd
5	nd	nd	nd	nd
7	nd	nd	nd	nd
11	nd	nd	nd	nd
14	nd	nd	nd	nd
18	nd	nd	nd	nd
21	100.00	0.00	0.00	0.00
25	100.00	0.00	0.00	0.00
28	98.31	0.00	1.69	0.00
32	84.42	3.86	7.81	3.90
35	78.75	10.54	6.95	3.76
39	51.24	34.83	8.79	5.15
42	74.96	16.58	4.98	3.48
46	77.65	15.82	3.10	3.42
49	73.82	22.27	2.38	1.52
53	77.66	20.74	0.71	0.89
56	69.04	28.10	1.45	1.41
60	71.18	27.37	1.44	0.00
63	61.39	36.13	1.71	0.77
67	74.38	24.78	0.84	0.00
70	70.89	27.60	1.52	0.00
74	69.26	29.79	0.95	0.00
77	62.41	35.06	1.90	0.63
81	64.53	34.36	1.11	0.00

Table 19. Original data for Figure 18C (Chapter 3). Data are the percentages that each ester class comprises of all pentanol esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected.

Day	Hexyl acetate	Hexyl propanoate	Hexyl butanoate	Hexyl 2-Mbutanoate	Hexyl hexanoate	Hexyl octanoate
0	0	0	0	0	0	0
5	0	0	0	0	0	0
7	0	0	0	0	0	0
11	0	0	0	0	0	0
14	58889	0	0	0	0	0
18	570601	0	0	0	0	0
21	9151795	0	0	0	0	0
25	14924860	0	0	0	0	0
28	131498506	5522563	12387526	23743315	3869168	0
32	183912774	22733102	17414263	137088190	6027278	0
35	152882255	30808824	19759306	113268595	6055280	0
39	294259645	84247680	72800548	256254115	20144312	17787
42	364627548	108166313	77847525	170940079	23837029	446530
46	332492806	108249568	74261456	158676067	26447777	466259
49	323247968	110095687	55248853	112904713	13221424	166498
53	225050323	53572738	22004134	36898464	8005050	137463
56	262989871	86296100	36881668	75432768	8276227	0
60	213873194	68823064	24181018	48854871	3673745	0
63	225978774	85414278	35304101	72644413	6976055	0
67	174267987	32684977	13288105	26193534	3879571	0
70	185370126	41161098	23144300	43018220	3778410	0
74	155662452	25983911	12408862	21276635	2158986	0
77	174419158	49696094	23484719	44263571	2865032	0
81	140823494	26606633	13631588	18648496	1773334	0

Ion	61	75	89	103	117	145
Ion fraction	0.06301	0.09639	0.08893	0.13061	0.09131	0.03399

Table 20. Original data for Figure 19C (Chapter 3). Data are the GC/MS response (total ion count, TIC) of hexanol esters arranged by their alkanoate (acid-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications.

Day	Hexyl acetate	Hexyl propanoate	Hexyl butanoate	Hexyl 2-Mbutanoate	Hexyl hexanoate	Hexyl octanoate
0	nd	nd	nd	nd	nd	nd
5	nd	nd	nd	nd	nd	nd
7	nd	nd	nd	nd	nd	nd
11	nd	nd	nd	nd	nd	nd
14	nd	nd	nd	nd	nd	nd
18	100.00	0.00	0.00	0.00	0.00	0.00
21	100.00	0.00	0.00	0.00	0.00	0.00
25	100.00	0.00	0.00	0.00	0.00	0.00
28	74.28	3.12	7.00	13.41	2.19	0.00
32	50.09	6.19	4.74	37.34	1.64	0.00
35	47.37	9.55	6.12	35.09	1.88	0.00
39	40.44	11.58	10.00	35.21	2.77	0.00
42	48.89	14.50	10.44	22.92	3.20	0.06
46	47.46	15.45	10.60	22.65	3.78	0.07
49	52.57	17.91	8.99	18.36	2.15	0.03
53	65.11	15.50	6.37	10.67	2.32	0.04
56	55.97	18.37	7.85	16.05	1.76	0.00
60	59.51	19.15	6.73	13.59	1.02	0.00
63	53.01	20.04	8.28	17.04	1.64	0.00
67	69.62	13.06	5.31	10.46	1.55	0.00
70	62.53	13.88	7.81	14.51	1.27	0.00
74	71.57	11.95	5.71	9.78	0.99	0.00
77	59.18	16.86	7.97	15.02	0.97	0.00
81	69.89	13.21	6.77	9.26	0.88	0.00

Table 21. Original data for Figure 19E (Chapter 3). Data are the percentages that each ester class comprises of all hexanol esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected.

Day	2-Mbutyl acetate	2-Mbutyl butanoate
0	0	0
5	0	0
7	0	0
11	0	0
14	544232	0
18	66706	0
21	4135506	0
25	4476358	0
28	27454993	0
32	107605390	169444
35	106339430	204619
39	403136230	12696250
42	215097186	1914391
46	238299202	1058597
49	241304588	424791
53	214884501	197334
56	254053612	466681
60	218814342	222731
63	241371052	380734
67	203334247	155641
70	221685592	266306
74	196966986	122500
77	220904941	453709
81	213224107	209978

Ion	74	89
Ion fraction	0.02069	0.01143

Table 22. Original data for Figure 20C (Chapter 3). Data are the GC/MS response (total ion count, TIC) of 2-methylbutanol esters arranged by their alkanoate (acid-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications.

Day	2-Mbutyl acetate	2-Mbutyl butanoate
0	nd	nd
5	nd	nd
7	nd	nd
11	nd	nd
14	100.00	0.00
18	100.00	0.00
21	100.00	0.00
25	100.00	0.00
28	100.00	0.00
32	99.84	0.16
35	99.81	0.19
39	96.95	3.05
42	99.12	0.88
46	99.56	0.44
49	99.82	0.18
53	99.91	0.09
56	99.82	0.18
60	99.90	0.10
63	99.84	0.16
67	99.92	0.08
70	99.88	0.12
74	99.94	0.06
77	99.80	0.20
81	99.90	0.10

Table 23. Original data for Figure 20E (Chapter 3). Data are the percentages that each ester class comprises of all 2-methylbutanol esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected.

Day	Acetate esters	Propanoate esters	Butanoate esters	2-Mbutanoate esters	Hexanoate esters	Octanoate esters
0	0	0	0	0	0	0
5	0	0	0	0	0	0
7	0	0	0	0	0	0
11	0	0	0	0	0	0
14	676452	0	0	0	0	0
18	720461	0	0	0	0	0
21	19230579	0	0	0	0	0
25	31001745	0	521618	0	0	0
28	265402885	13277064	22491965	27193736	8420031	74365
32	462973702	59893573	37485187	164785652	18692283	244330
35	413714315	92235371	43652598	147539999	21932943	226181
39	1139999484	325310494	208250947	448115840	115286304	1911966
42	866341985	281357046	184508522	312208815	105489788	4887176
46	1152954103	305205461	175629902	291744655	99818276	4419750
49	1136140920	352262919	156461668	243668431	81041906	2298617
53	979235446	267116669	89309043	106517081	48921254	1073152
56	1124061950	354176869	133068737	196973284	73396115	970803
60	979347835	330309443	95554288	144720735	37014281	362214
63	999597405	352561208	122289631	169499647	55293997	328089
67	886777792	235482938	69562306	96943201	26323315	201148
70	924436609	245483388	90830496	137026833	42007382	201122
74	772449834	189455739	57382441	84147936	29840445	161568
77	855603196	269831398	98541809	148410087	51093842	429674
81	797389466	224616800	81391037	105968582	40559169	194366
Total	13808056163	3898576380	1666932196	2825464515	855131329	17984521

Table 24. Original data for Figure 21A (Chapter 3). Data are the GC/MS response (total ion count) of esters arranged by their alkanoate (acid-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications.

Day	Acetate esters	Propanoate esters	Butanoate esters	2-Mbutanoate esters	Hexanoate esters	Octanoate esters
0	nd	nd	nd	nd	nd	nd
5	nd	nd	nd	nd	nd	nd
7	nd	nd	nd	nd	nd	nd
11	nd	nd	nd	nd	nd	nd
14	100.00	0.00	0.00	0.00	0.00	0.00
18	100.00	0.00	0.00	0.00	0.00	0.00
21	100.00	0.00	0.00	0.00	0.00	0.00
25	98.35	0.00	1.65	0.00	0.00	0.00
28	78.79	3.94	6.68	8.07	2.50	0.02
32	62.22	8.05	5.04	22.15	2.51	0.03
35	57.52	12.82	6.07	20.51	3.05	0.03
39	50.92	14.53	9.30	20.02	5.15	0.09
42	49.37	16.03	10.51	17.79	6.01	0.28
46	56.80	15.04	8.65	14.37	4.92	0.22
49	57.62	17.86	7.93	12.36	4.11	0.12
53	65.62	17.90	5.99	7.14	3.28	0.07
56	59.71	18.81	7.07	10.46	3.90	0.05
60	61.70	20.81	6.02	9.12	2.33	0.02
63	58.81	20.74	7.20	9.97	3.25	0.02
67	67.42	17.90	5.29	7.37	2.00	0.02
70	64.20	17.05	6.31	9.52	2.92	0.01
74	68.15	16.72	5.06	7.42	2.63	0.01
77	60.09	18.95	6.92	10.42	3.59	0.03
81	63.79	17.97	6.51	8.48	3.24	0.02

Table 25. Original data for Figure 21B (Chapter 3). Data are the percentages that each ester class comprises of all esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Esters are arranged by the source of the alkanoate (acid-derived) moiety. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected.

Day	Ethyl acetate	Propyl acetate	Butyl acetate	Pentyl acetate	Hexyl acetate	2-Mpropyl acetate	2-Mbutyl acetate
0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0
14	0	0	73330	0	58889	0	544232
18	0	0	83154	0	570601	0	66706
21	0	0	4650657	1248775	9151795	43846	4135506
25	0	0	7126722	3988414	14924860	485391	4476358
28	0	414457	78576048	24028772	131498506	3430109	27454993
32	757266	2048090	125374274	38817751	183912774	4458158	107605390
35	667936	3413804	110905433	31414858	152882255	8090600	106339430
39	1947659	37608468	270136771	98396608	294259645	34514103	403136230
42	452915	27873726	169785919	78811290	364627548	9693400	215097186
46	1980734	114297757	369037955	75671392	332492806	21174258	238299202
49	2496052	138006569	344772295	65864975	323247968	20448473	241304588
53	3993076	163563464	304316462	45228066	225050323	22199554	214884501
56	5506875	188014101	322329500	63259797	262989871	27908195	254053612
60	6117778	168549933	302019260	45953173	213873194	24020155	218814342
63	5117759	163483734	281995191	54003345	225978774	27647550	241371052
67	8312465	154296474	280877951	40336013	174267987	25352654	203334247
70	10836238	159947361	272861267	42191914	185370126	31544110	221685592
74	6970138	140654281	226101851	29308441	155662452	16785685	196966986
77	8130761	135337264	251296583	43996128	174419158	21518361	220904941
81	10825273	136382064	235478823	35918068	140823494	24737638	213224107

Ion	43	61	61	61	61	56	74
Ion fraction	0.54264	0.15051	0.06377	0.06723	0.06301	0.11443	0.02069

Table 26. Original data for Figure 22B (Chapter 3). Data are the GC/MS response (total ion count, TIC) of acetate esters arranged by their alkyl (alcohol-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications.

Day	Ethyl acetate	Propyl acetate	Butyl acetate	Pentyl acetate	Hexyl acetate	2-Mpropyl acetate	2-Mbutyl acetate
0	nd	nd	nd	nd	nd	nd	nd
5	nd	nd	nd	nd	nd	nd	nd
7	nd	nd	nd	nd	nd	nd	nd
11	nd	nd	nd	nd	nd	nd	nd
14	0.00	0.00	10.84	0.00	8.71	0.00	80.45
18	0.00	0.00	11.54	0.00	79.20	0.00	9.26
21	0.00	0.00	24.18	6.49	47.59	0.23	21.50
25	0.00	0.00	22.99	12.87	48.14	1.57	14.44
28	0.00	0.16	29.61	9.05	49.55	1.29	10.34
32	0.16	0.44	27.08	8.38	39.72	0.96	23.24
35	0.16	0.83	26.81	7.59	36.95	1.96	25.70
39	0.17	3.30	23.70	8.63	25.81	3.03	35.36
42	0.05	3.22	19.60	9.10	42.09	1.12	24.83
46	0.17	9.91	32.01	6.56	28.84	1.84	20.67
49	0.22	12.15	30.35	5.80	28.45	1.80	21.24
53	0.41	16.70	31.08	4.62	22.98	2.27	21.94
56	0.49	16.73	28.68	5.63	23.40	2.48	22.60
60	0.62	17.21	30.84	4.69	21.84	2.45	22.34
63	0.51	16.35	28.21	5.40	22.61	2.77	24.15
67	0.94	17.40	31.67	4.55	19.65	2.86	22.93
70	1.17	17.30	29.52	4.56	20.05	3.41	23.98
74	0.90	18.21	29.27	3.79	20.15	2.17	25.50
77	0.95	15.82	29.37	5.14	20.39	2.51	25.82
81	1.36	17.10	29.53	4.50	17.66	3.10	26.74

Table 27. Original data for Figure 22C (Chapter 3). Data are the percentages that each ester class comprises of all acetate esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected.

Day	Ethyl propanoate	Propyl propanoate	Butyl propanoate	Pentyl propanoate	Hexyl propanoate
0	0	0	0	0	0
5	0	0	0	0	0
7	0	0	0	0	0
11	0	0	0	0	0
14	0	0	0	0	0
18	0	0	0	0	0
21	0	0	0	0	0
25	0	0	0	0	0
28	0	0	7754501	0	5522563
32	0	507334	34876400	1776737	22733102
35	0	1485801	55736708	4204037	30808824
39	420387	16857424	156898323	66886681	84247680
42	0	8012876	147746452	17431405	108166313
46	329346	34911242	146293135	15422169	108249568
49	520588	48247979	173527613	19871052	110095687
53	749268	59606493	141107484	12080687	53572738
56	1296852	80665998	160173677	25744242	86296100
60	1685613	75814458	166314968	17671340	68823064
63	1169138	70828608	163363226	31785957	85414278
67	1695279	54277559	133389806	13435316	32684977
70	2004585	56294731	129597161	16425812	41161098
74	1176384	44556421	105133987	12605036	25983911
77	2039725	58784522	134593058	24717999	49696094
81	2672355	57285036	118927742	19125035	26606633

Ion	57	75	75	53	75
Ion fraction	0.26430	0.16752	0.10197	0.00346	0.09639

Table 28. Original data for Figure 23B (Chapter 3). Data are the GC/MS response (total ion count, TIC) of propanoate esters arranged by their alkyl (alcohol-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications.

Day	Ethyl propanoate	Propyl propanoate	Butyl propanoate	Pentyl propanoate	Hexyl propanoate
0	nd	nd	nd	nd	nd
5	nd	nd	nd	nd	nd
7	nd	nd	nd	nd	nd
11	nd	nd	nd	nd	nd
14	nd	nd	nd	nd	nd
18	nd	nd	nd	nd	nd
21	nd	nd	nd	nd	nd
25	nd	nd	nd	nd	nd
28	0.00	0.00	58.41	0.00	41.59
32	0.00	0.85	58.23	2.97	37.96
35	0.00	1.61	60.43	4.56	33.40
39	0.13	5.18	48.23	20.56	25.90
42	0.00	2.85	52.51	6.20	38.44
46	0.11	11.44	47.93	5.05	35.47
49	0.15	13.70	49.26	5.64	31.25
53	0.28	22.31	52.83	4.52	20.06
56	0.37	22.78	45.22	7.27	24.37
60	0.51	22.95	50.35	5.35	20.84
63	0.33	20.09	46.34	9.02	24.23
67	0.72	23.05	56.65	5.71	13.88
70	0.82	22.93	52.79	6.69	16.77
74	0.62	23.52	55.49	6.65	13.72
77	0.76	21.79	49.88	9.16	18.42
81	1.19	25.50	52.95	8.51	11.85

Table 29. Original data for Figure 23C (Chapter 3). Data are the percentages that each ester class comprises of all propanoate esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected.

Day	Ethyl butanoate	Propyl butanoate	Butyl butanoate	2-Mbutyl butanoate	Hexyl butanoate
0	0	0	0	0	0
5	0	0	0	0	0
7	0	0	0	0	0
11	0	0	0	0	0
14	0	0	0	0	0
18	0	0	0	0	0
21	0	0	0	0	0
25	0	0	521618	0	0
28	0	71715	10032725	0	12387526
32	101763	633475	19166243	169444	17414263
35	241740	1113596	22333337	204619	19759306
39	382040	7388455	114983654	12696250	72800548
42	86705	5581135	99078766	1914391	77847525
46	88585	12656492	87564772	1058597	74261456
49	87348	16984255	83716422	424791	55248853
53	80848	20440790	46585937	197334	22004134
56	183483	28531982	67004923	466681	36881668
60	160476	22038081	48951982	222731	24181018
63	95136	23633593	62876066	380734	35304101
67	256405	17207960	38654196	155641	13288105
70	270795	19451684	47697412	266306	23144300
74	171887	15704732	28974460	122500	12408862
77	293869	20012059	54297453	453709	23484719
81	400953	20780573	46367945	209978	13631588

Ion	88	89	89	89	89
Ion fraction	0.10491	0.11242	0.12317	0.01143	0.08893

Table 30. Original data for Figure 24B (Chapter 3). Data are the GC/MS response (total ion count, TIC) of butanoate esters arranged by their alkyl (alcohol-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications.

Day	Ethyl butanoate	Propyl butanoate	Butyl butanoate	2-Mbutyl butanoate	Hexyl butanoate
0	nd	nd	nd	nd	nd
5	nd	nd	nd	nd	nd
7	nd	nd	nd	nd	nd
11	nd	nd	nd	nd	nd
14	nd	nd	nd	nd	nd
18	nd	nd	nd	nd	nd
21	nd	nd	nd	nd	nd
25	nd	nd	nd	nd	nd
28	0.00	0.32	44.61	0.00	55.08
32	0.27	1.69	51.13	0.45	46.46
35	0.55	2.55	51.16	0.47	45.26
39	0.18	3.55	55.21	6.10	34.96
42	0.05	3.02	53.70	1.04	42.19
46	0.05	7.21	49.86	0.60	42.28
49	0.06	10.86	53.51	0.27	35.31
53	0.09	22.89	52.16	0.22	24.64
56	0.14	21.44	50.35	0.35	27.72
60	0.17	23.06	51.23	0.23	25.31
63	0.08	19.33	51.42	0.31	28.87
67	0.37	24.74	55.57	0.22	19.10
70	0.30	21.42	52.51	0.29	25.48
74	0.30	27.37	50.49	0.21	21.62
77	0.30	20.31	55.10	0.46	23.83
81	0.49	25.53	56.97	0.26	16.75

Table 31. Original data for Figure 24C (Chapter 3). Data are the percentages that each ester class comprises of all butanoate esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of ‘Jonagold’ apple fruit. Each value is the average of four replications. The term ‘nd’ indicates percentages were not determined since no esters of these classes were detected.

Day	Propyl hexanoate	Butyl Hexanoate	Pentyl hexanoate	Hexyl hexanoate
0	0	0	0	0
5	0	0	0	0
7	0	0	0	0
11	0	0	0	0
14	0	0	0	0
18	0	0	0	0
21	0	0	0	0
25	0	0	0	0
28	0	4550864	0	3869168
32	84367	10785473	1795166	6027278
35	223098	14155968	1498598	6055280
39	5196125	80063605	9882263	20144312
42	3975271	74021672	3655815	23837029
46	7558456	62475437	3336606	26447777
49	11916659	54547534	1356288	13221424
53	13937652	26459589	518962	8005050
56	22626920	41200400	1292567	8276227
60	10170995	23169541	0	3673745
63	15221512	32418850	677580	6976055
67	7478334	14965410	0	3879571
70	11489634	26739338	0	3778410
74	10186096	17495363	0	2158986
77	15461013	32326845	440952	2865032
81	14597304	24188531	0	1773334

Ion	117	117	117	117
Ion fraction	0.12400	0.09374	0.01772	0.09131

Table 32. Original data for Figure 25B (Chapter 3). Data are the GC/MS response (total ion count, TIC) of hexanoate esters arranged by their alkyl (alcohol-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications.

Day	Propyl hexanoate	Butyl hexanoate	Pentyl hexanoate	Hexyl hexanoate
0	nd	nd	nd	nd
5	nd	nd	nd	nd
7	nd	nd	nd	nd
11	nd	nd	nd	nd
14	nd	nd	nd	nd
18	nd	nd	nd	nd
21	nd	nd	nd	nd
25	nd	nd	nd	nd
28	0.00	54.05	0.00	45.95
32	0.45	57.70	9.60	32.24
35	1.02	64.54	6.83	27.61
39	4.51	69.45	8.57	17.47
42	3.77	70.17	3.47	22.60
46	7.57	62.59	3.34	26.50
49	14.70	67.31	1.67	16.31
53	28.49	54.09	1.06	16.36
56	30.83	56.13	1.76	11.28
60	27.48	62.60	0.00	9.93
63	27.53	58.63	1.23	12.62
67	28.41	56.85	0.00	14.74
70	27.35	63.65	0.00	8.99
74	34.14	58.63	0.00	7.24
77	30.26	63.27	0.86	5.61
81	35.99	59.64	0.00	4.37

Table 33. Original data for Figure 25C (Chapter 3). Data are the percentages that each ester class comprises of all hexanoate esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected.

Day	Propyl octanoate	Butyl octanoate	Hexyl octanoate
0	0	0	0
5	0	0	0
7	0	0	0
11	0	0	0
14	0	0	0
18	0	0	0
21	0	0	0
25	0	0	0
28	0	74365	0
32	0	244330	0
35	0	226181	0
39	29310	1864868	17787
42	89808	4350839	446530
46	219833	3733659	466259
49	239356	1892763	166498
53	167223	768466	137463
56	256286	714518	0
60	65757	296456	0
63	47393	280696	0
67	8678	192470	0
70	23448	177673	0
74	27503	134066	0
77	102416	327258	0
81	41249	153117	0

Ion	145	145	145
Ion fraction	0.08257	0.08478	0.03399

Table 34. Original data for Figure 26B (Chapter 3). Data are the GC/MS response (total ion count, TIC) of octanoate esters arranged by their alkyl (alcohol-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications.

Day	Propyl octanoate	Butyl octanoate	Hexyl octanoate
0	nd	nd	nd
5	nd	nd	nd
7	nd	nd	nd
11	nd	nd	nd
14	nd	nd	nd
18	nd	nd	nd
21	nd	nd	nd
25	nd	nd	nd
28	0.00	100.00	0.00
32	0.00	100.00	0.00
35	0.00	100.00	0.00
39	1.53	97.54	0.93
42	1.84	89.03	9.14
46	4.97	84.48	10.55
49	10.41	82.34	7.24
53	15.58	71.61	12.81
56	26.40	73.60	0.00
60	18.15	81.85	0.00
63	14.45	85.55	0.00
67	4.31	95.69	0.00
70	11.66	88.34	0.00
74	17.02	82.98	0.00
77	23.84	76.16	0.00
81	21.22	78.78	0.00

Table 35. Original data for Figure 26C (Chapter 3). Data are the percentages that each ester class comprises of all octanoate esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of ‘Jonagold’ apple fruit. Each value is the average of four replications. The term ‘nd’ indicates percentages were not determined since no esters of these classes were detected.

Day	Ethyl 2-Mbut	Propyl 2-Mbut	Butyl 2-Mbut	Pentyl 2-Mbut	Hexyl 2-Mbut
0	0	0	0	0	0
5	0	0	0	0	0
7	0	0	0	0	0
11	0	0	0	0	0
14	0	0	0	0	0
18	0	0	0	0	0
21	0	0	0	0	0
25	0	0	0	0	0
28	0	0	3038240	412181	23743315
32	195183	494961	23413937	3593381	137088190
35	47962	1268839	30182603	2772000	113268595
39	633208	26119739	148234603	16874175	256254115
42	99303	16001919	119929683	5237831	170940079
46	309071	26093516	103640952	3025049	158676067
49	461727	29639901	98535206	2126883	112904713
53	254500	28782475	40169381	412261	36898464
56	615629	49177139	70416587	1331161	75432768
60	640101	36387441	57906444	931878	48854871
63	534474	31639119	63177714	1503927	72644413
67	781374	28805571	40706810	455913	26193534
70	1016602	36552478	55536206	903327	43018220
74	487742	28558255	33423413	401891	21276635
77	1096144	39151461	62561190	1337721	44263571
81	1319356	35854075	49531429	615226	18648496

Ion	102	103	103	103	103
Ion fraction	0.14837	0.10392	0.14318	0.14220	0.13061

Table 36. Original data for Figure 27B (Chapter 3). Data are the GC/MS response (total ion count, TIC) of 2-methylbutanoate esters arranged by their alkyl (alcohol-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications.

Day	Ethyl 2-Mbut	Propyl 2-Mbut	Butyl 2-Mbut	Pentyl 2-Mbut	Hexyl 2-Mbut
0	nd	nd	nd	nd	nd
5	nd	nd	nd	nd	nd
7	nd	nd	nd	nd	nd
11	nd	nd	nd	nd	nd
14	nd	nd	nd	nd	nd
18	nd	nd	nd	nd	nd
21	nd	nd	nd	nd	nd
25	nd	nd	nd	nd	nd
28	0.00	0.00	11.17	1.52	87.31
32	0.12	0.30	14.21	2.18	83.19
35	0.03	0.86	20.46	1.88	76.77
39	0.14	5.83	33.08	3.77	57.18
42	0.03	5.13	38.41	1.68	54.75
46	0.11	8.94	35.52	1.04	54.39
49	0.19	12.16	40.44	0.87	46.34
53	0.24	27.02	37.71	0.39	34.64
56	0.31	24.97	35.75	0.68	38.30
60	0.44	25.14	40.01	0.64	33.76
63	0.32	18.67	37.27	0.89	42.86
67	0.81	29.71	41.99	0.47	27.02
70	0.74	26.68	40.53	0.66	31.39
74	0.58	33.94	39.72	0.48	25.28
77	0.74	26.38	42.15	0.90	29.83
81	1.25	33.83	46.74	0.58	17.60

Table 37. Original data for Figure 27C (Chapter 3). Data are the percentages that each ester class comprises of all 2-methylbutanoate esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of 'Jonagold' apple fruit. Each value is the average of four replications. The term 'nd' indicates percentages were not determined since no esters of these classes were detected.

Day	Ethylene (μL/L)	SD	CO_2 (mg/kg*hr)	Total Volatiles (TIC)
0	0.0074	0.0119	24.61	1509500
5	0.1423	0.0422	18.74	15432000
7	0.0399	0.0288	30.66	1162500
11	0.1326	0.0385	21.07	1057100
14	0.0225	0.0094	19.69	31861000
18	0.0602	0.0330	18.36	112090000
21	0.5149	0.3553	16.59	117370000
25	0.5182	0.2718	24.05	136640000
28	0.5538	0.1666	22.15	472390000
32	0.6956	0.2349	22.63	1160300000
35	1.5031	0.7281	37.21	1329000000
39	3.0271	1.8698	50.01	2758000000
42	109.536	46.980	44.50	2755600000
46	97.825	54.030	30.89	2997700000
49	179.507	49.798	21.99	2913700000
53	413.057	138.730	24.57	2239900000
56	533.818	119.016	21.55	2301200000
60	690.425	283.249	23.17	2083300000
63	485.390	212.733	20.59	2148300000
67	371.956	94.518	19.07	1759200000
70	482.844	217.468	19.22	1727100000
74	448.329	210.926	19.62	1438100000
77	653.707	150.345	19.88	1770300000
81	429.090	227.916	20.96	1427500000

Table 38. Original data for Figure 31 (Chapter 4). Data are internal ethylene content, CO_2 production, and the GC/MS response total ion count (TIC) for all aroma volatiles. Each value is the average of four replications.

Day	2-Mbutyl acetate	2-Mbutyl butanoate	Total 2-Mbutanol esters
0	0	0	0
5	0	0	0
7	0	0	0
11	0	0	0
14	544232	0	544232
18	66706	0	66706
21	4135506	0	4135506
25	4476358	0	4476358
28	27454993	0	27454993
32	107605390	169444	107774833
35	106339430	204619	106544048
39	403136230	12696250	415832480
42	215097186	1914391	217011577
46	238299202	1058597	239357798
49	241304588	424791	241729378
53	214884501	197334	215081835
56	254053612	466681	254520293
60	218814342	222731	219037073
63	241371052	380734	241751786
67	203334247	155641	203489888
70	221685592	266306	221951898
74	196966986	122500	197089486
77	220904941	453709	221358650
81	213224107	209978	213434085

Ion	74	89
Ion fraction	0.02069	0.01143

Table 39. Original data for Figure 32A and 32C (Chapter 4). Data are the GC/MS response (total ion count, TIC) of 2-methylbutanol esters arranged by their alkanoate (acid-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications.

Day	Ethyl 2-Mbut	Propyl 2-Mbut	Butyl 2-Mbut	Pentyl 2-Mbut	Hexyl 2-Mbut	Total 2-Mbutanoate esters
0	0	0	0	0	0	0
5	0	0	0	0	0	0
7	0	0	0	0	0	0
11	0	0	0	0	0	0
14	0	0	0	0	0	0
18	0	0	0	0	0	0
21	0	0	0	0	0	0
25	0	0	0	0	0	0
28	0	0	3038240	412181	23743315	27193736
32	195183	494961	23413937	3593381	137088190	164785652
35	47962	1268839	30182603	2772000	113268595	147539999
39	633208	26119739	148234603	16874175	256254115	448115840
42	99303	16001919	119929683	5237831	170940079	312208815
46	309071	26093516	103640952	3025049	158676067	291744655
49	461727	29639901	98535206	2126883	112904713	243668431
53	254500	28782475	40169381	412261	36898464	106517081
56	615629	49177139	70416587	1331161	75432768	196973284
60	640101	36387441	57906444	931878	48854871	144720735
63	534474	31639119	63177714	1503927	72644413	169499647
67	781374	28805571	40706810	455913	26193534	96943201
70	1016602	36552478	55536206	903327	43018220	137026833
74	487742	28558255	33423413	401891	21276635	84147936
77	1096144	39151461	62561190	1337721	44263571	148410087
81	1319356	35854075	49531429	615226	18648496	105968582

Ion	102	103	103	103	103
Ion fraction	0.14837	0.10392	0.14318	0.14220	0.13061

Table 40. Original data for Figure 32A and 32D (Chapter 4). Data are the GC/MS response (total ion count, TIC) of 2-methylbutanoate esters arranged by their alkyl (alcohol-derived) moiety during ripening and senescence of 'Jonagold' apple fruit. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications.

Day	2-Methyl butanol	2-Methyl butanal
0	0	
5	0	
7	0	
11	0	0
14	0	0
18	0	0
21	1277184	0
25	1356999	0
28	2444501	0
32	10570357	0
35	10402166	0
39	74834387	275777
42	15101710	0
46	17507796	132737
49	16221393	151713
53	16073867	226604
56	19166305	393190
60	18636585	247492
63	25520562	291262
67	22429805	503137
70	31140692	618697
74	21044632	365645
77	24048526	387025
81	29826410	456629

Ion	41	41
Ion fraction	0.17844	0.21027

Table 41. Original data for Figure 32B (Chapter 4). Data are the GC/MS response (total ion count, TIC) of 2-methylbutanol and 2-methylbutanal produced by 'Jonagold' apple fruit during ripening and senescence. The GC/MS response is calculated from data for a single unique ion; the fraction that the ion comprises of the total ion count is provided. Each TIC value is the result of dividing the ion count for the unique ion by the ion fraction. Each value is the average of four replications.

Day	Ethyl 2-Mbut	Propyl 2-Mbut	Butyl 2-Mbut	Pentyl 2-Mbut	Hexyl 2-Mbut
0	nd	nd	nd	nd	nd
5	nd	nd	nd	nd	nd
7	nd	nd	nd	nd	nd
11	nd	nd	nd	nd	nd
14	nd	nd	nd	nd	nd
18	nd	nd	nd	nd	nd
21	nd	nd	nd	nd	nd
25	nd	nd	nd	nd	nd
28	0.00	0.00	11.17	1.52	87.31
32	0.12	0.30	14.21	2.18	83.19
35	0.03	0.86	20.46	1.88	76.77
39	0.14	5.83	33.08	3.77	57.18
42	0.03	5.13	38.41	1.68	54.75
46	0.11	8.94	35.52	1.04	54.39
49	0.19	12.16	40.44	0.87	46.34
53	0.24	27.02	37.71	0.39	34.64
56	0.31	24.97	35.75	0.68	38.30
60	0.44	25.14	40.01	0.64	33.76
63	0.32	18.67	37.27	0.89	42.86
67	0.81	29.71	41.99	0.47	27.02
70	0.74	26.68	40.53	0.66	31.39
74	0.58	33.94	39.72	0.48	25.28
77	0.74	26.38	42.15	0.90	29.83
81	1.25	33.83	46.74	0.58	17.60

Table 42. Original data for Figure 32E (Chapter 4). Data are the percentages that each ester class comprises of all 2-methylbutanoate esters detected on each date using the respective GC/MS response (total ion count) during ripening and senescence of ‘Jonagold’ apple fruit. Each value is the average of four replications. The term ‘nd’ indicates percentages were not determined since no esters of these classes were detected.

Day	BCAT10 (MDC410280)	PDC1 (MDC015210)	2-isopropylmalate synthase (gi:7387848)
0	0.000	0.000	0.000
11	0.711	-0.172	0.020
25	0.399	-0.137	2.605
32	0.835	0.408	4.172
39	1.961	1.282	5.078
49	0.440	2.205	4.880
60	-0.396	1.864	5.064
70	-1.623	1.693	5.300

Table 43. Original data for Figure 33 (Chapter 4). Data are the relative luminosity ($\log_2$) of microarray elements compared to Day 0 for branched-chain aminotransferase (BCAT), pyruvate decarboxylase (PDC), and 2-isopropylmalate synthase during ripening and senescence of 'Jonagold' apple fruit.

	BCAT1 MDC021750		BCAT2 MDC028270		BCAT3 MDC160840		BCAT4 MDC192460		BCAT5 MDC238040	
Day	Avg	STDEV	Avg	STDEV	Avg	STDEV	Avg	STDEV	Avg	STDEV
0	0.748	0.008	0.964	0.051	0.808	0.051	0.944	0.079	1.000	0.000
11	0.751	0.094	0.768	0.095	0.817	0.131	0.761	0.268	0.835	0.196
25	0.860	0.108	0.778	0.011	0.935	0.092	0.776	0.197	0.855	0.179
32	1.000	0.000	0.834	0.026	0.986	0.011	0.822	0.251	0.802	0.022
39	0.918	0.010	0.964	0.051	0.890	0.037	0.706	0.241	0.695	0.078
49	0.835	0.035	0.593	0.066	0.920	0.113	0.608	0.268	0.650	0.139
60	0.703	0.121	0.435	0.404	0.754	0.148	0.504	0.017	0.655	0.055
70	0.660	0.084	0.252	0.219	0.655	0.043	0.356	0.048	0.717	0.204

Avg SD	0.074	0.170	0.091	0.198	0.133

	BCAT6 MDC238050		BCAT7 MDC301230		BCAT8 MDC385660		BCAT9 MDC405820		BCAT10 MDC410280		18s rRNA gi:85717895
Day	Avg	STDEV	Avg	STDEV	Avg	STDEV	Avg	STDEV	Avg	STDEV	Avg
0	0.750	0.094	0.691	0.437	1.000	0.000	0.771	0.257	0.588	0.142	86790
11	0.676	0.045	0.873	0.180	0.854	0.100	0.626	0.129	0.593	0.036	91298
25	0.805	0.013	0.876	0.076	0.901	0.056	0.794	0.154	0.723	0.024	89126
32	0.965	0.050	0.789	0.140	0.687	0.220	1.000	0.000	1.000	0.000	83377
39	0.859	0.146	0.753	0.160	0.735	0.119	0.792	0.132	0.675	0.116	87071
49	0.918	0.115	0.597	0.032	0.836	0.050	0.624	0.026	0.404	0.363	72491
60	0.746	0.277	0.633	0.086	0.650	0.190	0.498	0.136	0.354	0.477	74058
70	0.671	0.261	0.694	0.164	0.591	0.054	0.284	0.128	0.210	0.297	73491

Avg SD	0.155	0.197	0.121	0.141	0.246

Pooled average SD	0.153

Table 44. Original data for Figure 34 (Chapter 4). Data are luminosity of electrophoresis agarose gel spots relative to the maximum value for each gene from semi-quantitative RT-PCR products. The spot measurements are for branched-chain aminotransferase (BCAT) genes during ripening and senescence of 'Jonagold' apple fruit. All data were normalized relative to control gene 18s rRNA luminosity. Each value is the average of two replications. Figures below are original images from which luminosity data were extracted.

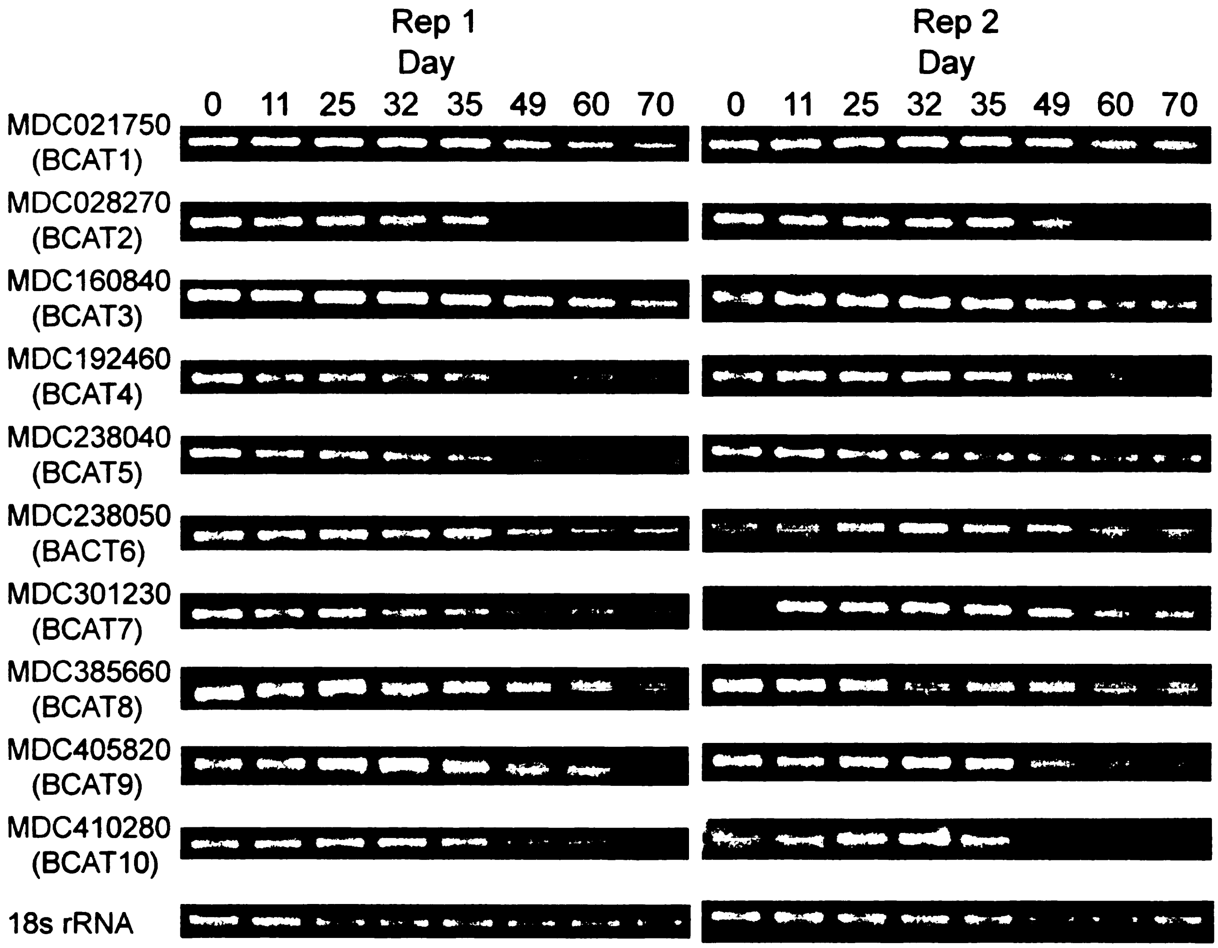

Table 44. Continued.

Day	PDC1 MDC015210		PDC2 MDC133700		PDC3 MDC206800		PDC4 MDC206810		PDC5 MDC433930		18s rRNA gi:85717895
	Avg	STDEV	Avg	STDEV	Avg	STDEV	Avg	STDEV	Avg	STDEV	Avg
0	0.026	0.037	0.846	0.137	0.923	0.108	0.578	0.087	0.716	0.070	86790
11	0.020	0.028	0.839	0.063	0.837	0.231	0.552	0.014	0.767	0.038	91298
25	0.260	0.005	0.937	0.070	0.951	0.065	0.542	0.125	0.811	0.038	89126
32	0.552	0.333	1.000	0.108	0.808	0.227	1.000	0.000	0.818	0.094	83377
39	0.679	0.086	0.924	0.079	0.531	0.083	0.745	0.040	0.834	0.037	87071
49	1.000	0.000	0.794	0.133	0.206	0.103	0.475	0.151	1.000	0.000	72491
60	0.890	0.040	0.593	0.181	0.071	0.049	0.213	0.097	0.817	0.012	74058
70	0.691	0.153	0.434	0.044	0.012	0.016	0.215	0.073	0.728	0.040	73491

Average SD	0.135	0.110	0.133	0.088	0.050

Pooled average SD	0.103

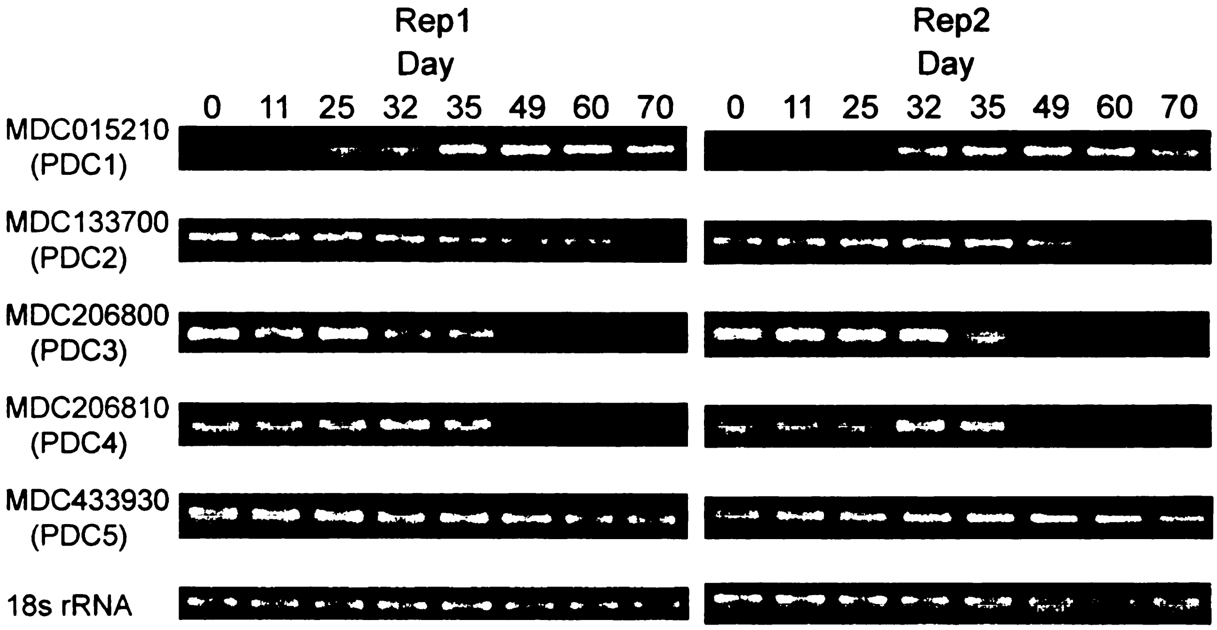

Table 45. Original data for Figure 35 (Chapter 4). Data are luminosity of electrophoresis agarose gel spots relative to the maximum value for each gene from semi-quantitative RT-PCR products. The spot measurements are for pyruvate decarboxylase (PDC) genes during ripening and senescence of ‘Jonagold’ apple fruit. All data were normalized relative to control gene 18s rRNA luminosity. Each value is the average of two replications. Figures below are original images from which luminosity data were extracted.

	2-isopropylmalate synthase gi:7387848		18s rRNA gi:85717895
Day	Avg	STDEV	Avg
0	0.029	0.017	86790
11	0.062	0.010	91298
25	0.489	0.013	89126
32	0.738	0.076	83377
39	0.736	0.070	87071
49	1.000	0.000	72491
60	0.884	0.109	74058
70	0.789	0.079	73491

Average SD	0.060

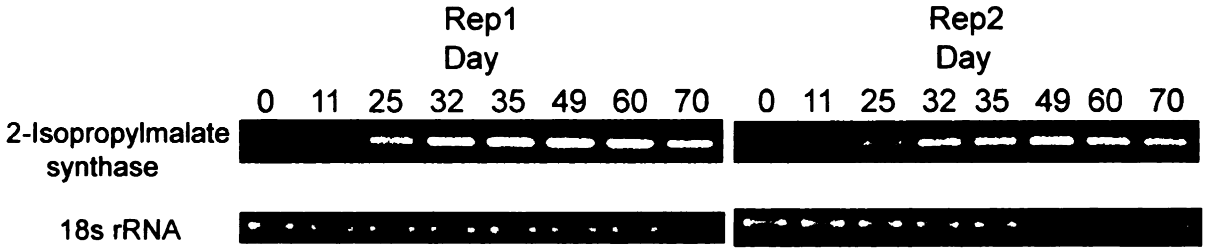

Table 46. Original data for Figure 36 (Chapter 4). Data are luminosity of electrophoresis agarose gel spots relative to the maximum value for each gene from semi-quantitative RT-PCR products. The spot measurements are for 2-isopropylmalate synthase gene during ripening and senescence of 'Jonagold' apple fruit. All data were normalized relative to control gene 18s rRNA luminosity. Each value is the average of two replications. Figures below are original images from which luminosity data were extracted.