THE LIPOXYGENASE PATHWAY IN APPLE PEEL

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ABSTRACT

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Many aroma volatiles in fresh apple are produced autonomously, however, several important odor-active volatiles are also produced via processes engaged by cellular disruption brought about by cutting or mastication. Six-carbon (C_6-) volatiles, including the aldehydes *cis*-3-hexenal, *trans*-2-hexenal, and hexanal, as well as their corresponding alcohols, are produced from action of the lipoxygenase (LOX) pathway on substrates released by tissue disruption. This study provides evidence that disruption-dependent aldehyde biosynthesis and aroma perception are dependent upon changes or availability of lipid substrates and/or related to LOX gene expression. Three different approaches of the lipoxygenase pathway were investigated in 'Jonagold' apple: the products (volatiles), the substrates (lipid content) and the genes involved in the metabolic route. Sensory tests revealed that panelists were able to distinguish between non-ripening and normally ripening fruit more than two weeks before the onset of the ethylene and respiratory climacterics in control fruit, but their ability to discriminate was not related to changes in LOX-related volatiles. LOX-derived aldehydes *cis*-3-hexenal and hexanal were not detectable for whole fruit, consistent with its dependence on cellular disruption. For disrupted fruit, *cis*-3-hexenal declined markedly during ripening, coincident with autocatalytic ethylene formation, even as *trans*-2-hexenal and hexanal emissions increased. Changes in these aldehydes occurred two weeks after perceived differences in aroma. Analysis of free fatty acids and the polar lipids of ripening apple fruit revealed that, of the free fatty acids, 18:1 (oleic) and 18:2 (linoleic) increased several-fold during ripening, but 18:3 (linolenic) content was exceedingly

low and was unchanged during ripening. For polar lipids, the 18:1 and 18:2 fatty acid content increased modestly, while the 18:3 content declined sharply as ripening progressed. The increase in 18:2 free fatty acids is mirrored by the increase in emissions of hexanal and hexyl esters during ripening, however, there does not appear to be a similar relationship between 18:3 free fatty acids and *cis*-3-hexenal emissions. Rather, the decline in *cis*-3-hexenal more closely follows the pattern in the 18:3-content of polar lipids. Twenty-two LOX gene sequences were retrieved and assayed from the apple genome, and of these, only 6 LOXs were highly expressed in a ripening-dependent manner in tissue samples from apple peel. Expression analysis by qRT-PCR revealed that 4 genes were down-regulated and 2 were up-regulated as ripening progressed. Confocal microscopy analysis confirmed that 2 of the down-regulated genes were chloroplast-targeted and the other 4 were targeted to plasma membrane. The pattern of formation of cis-3-hexenal may be related to the decline in 18:3 polar lipids and gene expression. The pattern of formation of *trans*-2-hexenal and all other C6 esters cannot be explained by changes in LOX gene expression but may be related to increases in polar lipids and free fatty acids. The data suggest that much of the control of C6 ester formation is at the level of fatty acid biosynthesis, rather than the degradation of a pre-existing pool.

S.D.G. (Soli Deo Gloria)

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CHAPTER I

INTRODUCTION

Flavor has been defined as the impressions perceived via the chemical senses from a product in the mouth. Thus, flavor includes aroma, taste, and the chemical feeling factors that stimulate the nerve ends of the buccal and nasal cavities (astringency, spice, heat, metallic flavor, umami taste, etc.) (Meilgaard et al., 2007). Aroma is the odor of a food product and it is released in the mouth via the posterior nares (Meilgaard et al., 2007). Aroma is a complex trait to study due to the difficulty of describing sensations into identifiable terms, and also for the wide variety of volatiles that exist. More than 17,000 odorous compounds have been described, and a trained person can differentiate 150-200 odorous qualities (Meilgaard et al., 2007). For example a tomato produces more than 400 volatiles (Buttery, 1993), a strawberry around 360 volatiles (McFadden et al., 1965), an apple around 270 volatile compounds (Dimik y Hoskin, 1982). Apples produce a blend of volatiles during ripening and these compounds are classified in to several categories according to their chemical structure: terpenes, esters, aldehydes, alcohols and ketones. Esters, aldehydes, and alcohols are considered the most important for the contribution of aroma volatiles (Paliyath et al., 2008). And of these, esters are the primary aroma impact compounds produced in a ripening apple fruit, and normally account for 80% to 95% of the total volatiles emitted (Paillard, 1990).

Several metabolic routes synthesize aroma compounds from primary metabolites: 4-allyl anisole is a product of the phenylpropanoid pathway, which is derived from the amino acid phenylalanine (Gang et al., 2001); the mevalonic and non-mevalonic pathways contribute to the production of terpenoids (Ju and Curry, 2000; Eisenreich et al., 2004), branched-chain esters derived from isoleucine (Rowan et al., 1996, Sugimoto et al., 2011) and C-6 aldehydes and alcohols and related hexyl esters are possible products of the β-oxidation and lipoxygenase

(LOX) pathways (Rowan et al., 1999; Dixon and Hewett, 2000) derived mainly from linolenic and linoleic acids in addition to other fatty acids (Schaffer et al., 2007).

The LOX pathway consists of the sequential action of lipase, lipoxygenase (LOX), fatty acid hydroperoxide lyase (HPL) and alcohol dehydrogenase (ADH) activities (Matsui et al., 2000) (Fig. 1 and 2). It is believed that under normal intact cellular conditions, the release of the polyunsaturated fatty acids is initiated through the cleavage action of a lipase. The fatty acids released by the action of lipase become the substrates for the lipoxygenase enzymes, producing 9- and 13- hydroperoxides. The hydroperoxides are then oxidized by HPL to aldehydes (e.g., hexanal, and *cis*-3-hexenal) and the aldehydes can be converted to alcohols (1-hexanol and *cis*-3-hexenol, respectively) by ADH (Hatanaka, 1993). Additionally, *cis*-3-hexenal can undergo isomerization to *trans*-2-hexenal. Under disrupted cellular conditions, such as biting and/or mastication, the apple LOX pathway enzymes have free access to their substrates producing copious amounts of LOX-derived volatiles (Contreras and Beaudry, 2013).

Apart from aroma compounds, the LOX pathway via the 13-LOX branch is responsible for the synthesis of many metabolic end products via numerous secondary reactions. These metabolites of the LOX pathway have diverse biological roles including signaling (jasmonate pathway), peroxidation reactions, and mobilization of lipids through different developmental stages (Brash, 1999). On the other branch, 9-LOX functions as the main pathway for generating C-9 aldehydes via 9-hydroperoxides. Generally, 9-LOXs are involved in aroma and antifungal action for the cucurbit family (Matsui et al, 2006); conferring resistance against pathogens in species such as tobacco, potato and maize (Feussner and Wasternack, 2002); tuber growth regulation in potato (Kolomiets et al, 2001) and seed development and germination in rice (Mizuno et al, 2003). Only a few food plants (e.g., cucurbit family members), produce

predominantly (3*Z*)-nonenal, or (3*Z*, 6*Z*)-nonadienal from linoleic and linolenic acid, respectively (Blée, 1998). However C-9 aldehyde production is not common for other plant species, except for alfalfa and rice (Matsui, 2006).

Lipase. Although the lipases (EC 3.1.1.-) are widely considered to be the primary source of free fatty acids for the LOX pathway in intact tissues, these "free" fatty acids are thought to have little tendency to leave the membrane, and as a result, they may not be necessarily available to be acted upon by lipoxygenases (Brash, 1999). For disrupted tissue, fatty acids are probably also liberated by lipase action (Chapman, 1998; Matsui et al., 2000), or by a protease, as recently suggested by Liavonchanka and Feussner (2006). Interestingly, the evidence for the involvement of lipase, probably a phospholipase or galactolipase, following tissue disruption is limited (Matsui et al., 1998; Kondo et al., 1993; Matsui et al., 2000).

Lipoxygenase. Lipoxygenases (EC 1.13.11.12) are non-heme iron-containing dioxygenases that catalyze the addition of molecular oxygen at unsaturated positions on fatty acids. LOX enzymes are widely distributed among animals, fungi and plants (Siedow, 1991). Each species possess several different LOX enzymes that differ in substrate specificity, localization, and expression. Linoleic and linolenic acids, which contain two and three unsaturated carbon-to-carbon bonds respectively, are the most common fatty acid substrate for LOXs (Siedow, 1991). While LOX is often considered to act primarily on free fatty acids, there is also evidence that LOX is also active on other substrates such as phospholipid derived polyunsaturated fatty acids (PUFAs) (Brasch et al., 1987) and triglycerides (Feussner et al., 2001). Also Yamauchi et al. (1985) demonstrated that lipoxygenase can catalyze the oxygenation of mono and digalactolipids, such as monogalactosyldiacylglycerol (MGDG, 1 galactose residue) and digalactosyldiacylglycerol (DGDG, 2 galactose residues). Galactolipids are the predominant membrane lipids in plant cells,

they are located in the thylakoid membranes of chloroplasts, and consist of 1 or 2 galactose residues connected by a glycosidic linkage to C-3 of a 1,2-diacylglycerol (Nelson and Cox, 2008).

LOX enzymes exhibit considerable substrate specificity, acting at the C-9 (9-lipoxygenase or 9-LOX) or C-13 (13-lipoxygenase or 13-LOX) positions of the fatty acid substrate to generate 9- or 13-hydroperoxides, respectively (Feussner and Wasternack, 2002). These hydroperoxides are named HPOT (hydroperoxide linolenic acid) when derived from linolenic acid, and HPOD (hydroperoxide linoleic acid) if they derive from linoleic acid (Fig. 1 and 2).

Hydroperoxide lyase. Hydroperoxide lyases (HPL) (EC 4.2.1.92) are widespread in plant species (Stumpe and Feussner, 2006). HPL is a member of a novel subfamily of cytochrome P450 enzymes, which are chloroplast-localized, membrane-bound and catalyze the cleavage of fatty acid hydroperoxides to form short-chain aldehydes and oxo-acids (Matsui et al., 2000c). Based on substrate specificity, HPLs are divided into three classes: 9-, 13-, and non-specific hydroperoxide lyases with no hydroperoxide preference (Sanz et al., 1997).

HPL in tea leaves, bell pepper fruit, *Arabidopsis*, and tomato fruit, has shown high substrate specificity for the 13-hydroperoxides, and essentially no activity for the 9-hydroperoxides. However, in cucumber, HPL can act on both 9- and 13-hydroperoxides with preference to the former (Matsui et al., 2000b). Thus a 13-HPL will cleave a 13-HPO (hydroperoxide) to form C6-aldehydes (i) (*Z*)-3-hexenal if substrate is linolenic acid (Fig. 1), or *n*-hexanal if substrate is linoleic acid (Fig. 2), and (ii)12-oxo-(*Z*)-9-dodecenoic acid, 12C compound. Likewise, 9-HPL from 9-HPO of C18 fatty acids will form (i) C9-aldehydes (*Z,Z*)-

3,6-nonadienal if substrate is linolenic acid or (*Z*)-*3*-nonenal if substrate is linoleic acid, and (ii) 9-oxo-nonanoic acid (Matsui et al., 2006).

Alcohol dehydrogenase. Also known as ADH (EC 1.1.1.1), before or after isomerization of the aldehyde carbonyl compounds, alcohol dehydrogenase acts to produce corresponding alcohols in most plants (Sanz et al., 1997). Thoroughly studied in apple peel, ADH is located in the cytoplasm and uses $NADH^{+}$ as cofactor for reducing aldehydes (Bartley and Hindley, 1980). Apple ADH can reduce C2-C6 aldehydes at a decreasing rate with increasing carbon number. Interestingly, apple ADH can also reduce branched-chain aldehydes but at a lower rate than the straight chain isomers (Bartley and Hindley, 1980). Similar to apple LOX and HPL, apple ADH has also been demonstrated to be ethylene-regulated and highly ripening dependent (Dandekar et al., 2004, Harb et al., 2011).

LOX activity as a function of ripening. Lipoxygenase (or lipoxidase) activity and substrate specificity has been studied during ripening. Meigh and Hulme (1965) studied the "lipoxidase" activity of Cox's Orange Pippin apples "on" and "off" (stored at 12 °C) the tree and the relationship with lipids. In their study they found that "lipoxidase" activity followed the respiration pattern, "on" and "off" the tree, rising rapidly close to the climacteric peak, and then falling as the climacteric peak was passed. Also in both cases, the substrates, free and esterified fatty acids, were increasing before the climacteric and then their breakdown proceeded as rapidly as their previous synthesis. However, some inconsistencies were found, such as the degradation of other fatty acids while the "lipoxidase" continues firmly active suggesting other mechanisms for lipid degradation exist. Also, maybe "lipoxidase" can account for the rapid decrease of lipids, but to what extent β-oxidation is occurring is unknown (Meigh and Hulme, 1965). Wooltorton et al. (1965) studying the biogenesis of ethylene directed their attention to the lipid metabolism in

Cox's Orange Pippin apples. They found that, the form of the curves of ethylene production and "lipoxidase" activity were strikingly similar for apples stored at 12 °C; but for apples on the tree, "lipoxidase" activity preceeds the appearance of ethylene. Later, Meigh et al. (1967) in a more detailed study followed the climacteric pattern, ethylene, fatty acids and "lipoxidase" activity of apples. "Lipoxidase" activity was already rising some days before the climacteric peak and ethylene, and continued to rise in parallel to respiration for about 10 days, then lipoxidase and respiration start to decline. The authors concluded the sequence of events possibly begin with the activation or production of "lipoxidase". Later, in Fuji apples, LOX activity in the flesh was highly correlated to volatile ester production during ripening (Altisent et al., 2009). It is known that LOX activity is at least two-fold higher in the peel and core of apples than in the flesh (Wooltorton et al., 1965; Feys et al., 1980; Echeverria et al., 2004).

The profile of LOX-related aldehydes is not necessarily predictable based on enzymatic activity. In mature tomato fruits, even though the most abundant LOX is 9-LOX, it does not contribute to the typical volatile profile, which is primarily composed of hexanal, *cis*-3-hexenal, and *trans*-2-hexenal (Griffiths et al., 1999). The authors showed that an antisense suppression of the 9-LOX genes *TomLoxA* and *TomLoxB* in tomato fruits resulted in no significant impact in the flavor volatiles. Matsui et al. (2001) overexpressed 9-LOX in tomato fruits and surprisingly there was only a minor change in the composition of C-9 aldehydes and alcohols in the homogenate of the transgenic fruits. Also in tomato, Chen et al. (2004) demonstrated that *TomLoxA, TomLoxB* and *TomLoxE* (all of which are highly expressed in fruits), produced 9-hydroperoxides only, and are not directly involved in the generation of C-6 flavor volatile aldehydes and alcohols.

Finally, it is not known the extent to which ethylene influences the gene expression of lipoxygenases in apples. Schaffer et al. (2007) described from a microarray analysis using

exogenous ethylene treated apples that from the total of 11 lipoxygenases found, only two were up-regulated in response to ethylene. In tomato, of the five LOXs identified, 3 of them are ethylene-regulated; the expression of *TomloxC*, proven to be required for C-6 aldehyde formation, is enhanced in expression by ethylene (Chen et al., 2004). However, the orthologue from apple is not regulated by ethylene (Schaffer et al., 2007). Apparently, in all especies there are some members of the lipoxygenase family that are ripening-dependent or ethylene regulated.

The objective of this research was to understand how aroma volatiles are produced in apples and specifically through the study of the LOX pathway. A first approach was to understand the potential for the LOX pathway to contribute to aroma using sensory analysis, thus to determine when the perceived aroma of disrupted apple tissue begins to change as a function of ripening. Secondly, data of the lipid metabolism were collected to understand how they serve as substrates of the pathway. Finally, the study of changes in the expression of LOX genes relative to sensory changes, volatiles emissions, and lipid metabolism will help to understand the physiology of aroma biosynthesis.

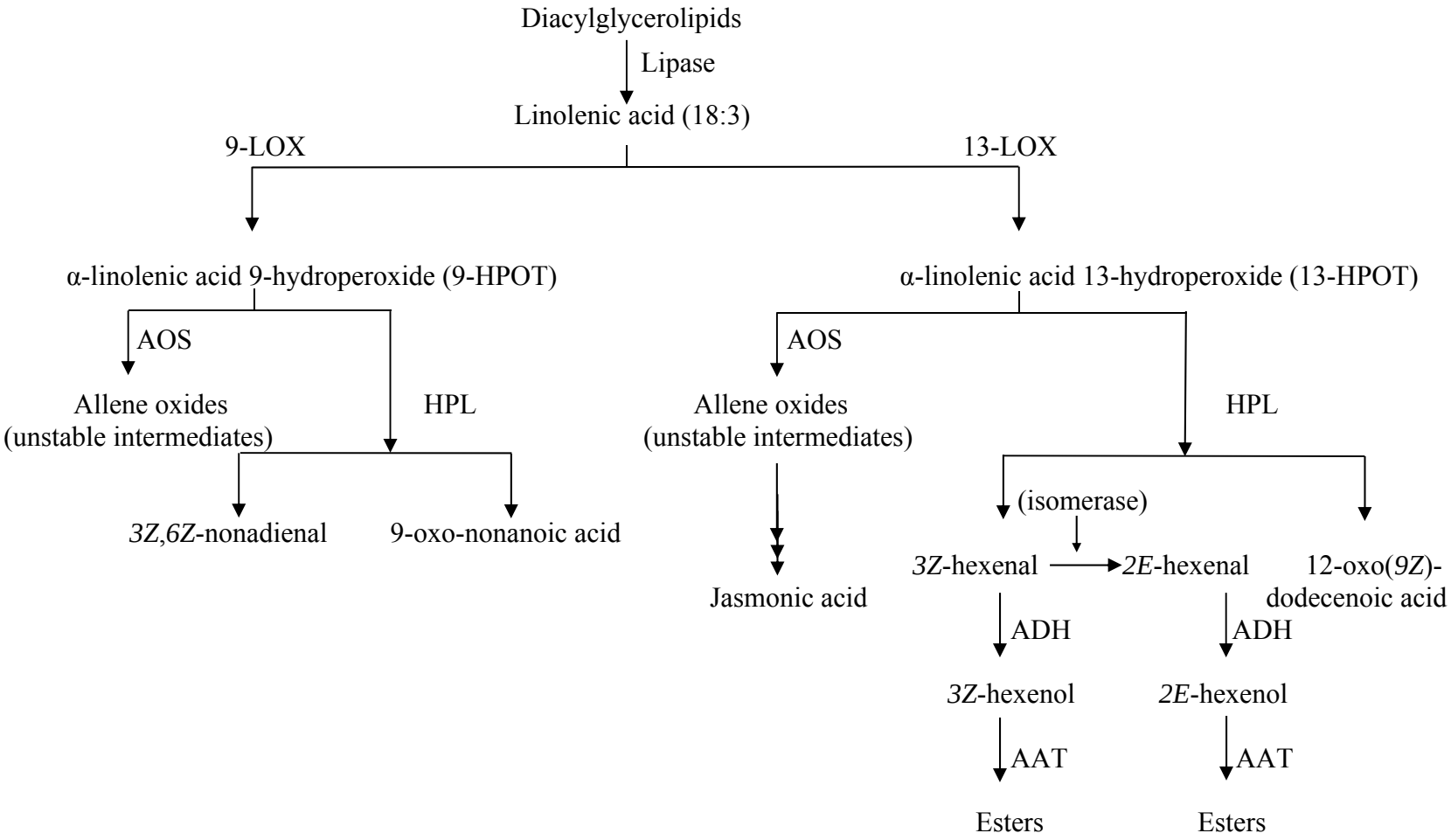

Figure 1. The LOX pathway using linolenic acid (18:3) as substrate. LOX (lipoxygenase), HPOT (hydroperoxide linolenic acid), AOS (allene oxide synthase), HPL (hydroperoxide lyase), ADH (alcohol dehydrogenase), and AAT (alcohol acetyl transferase).

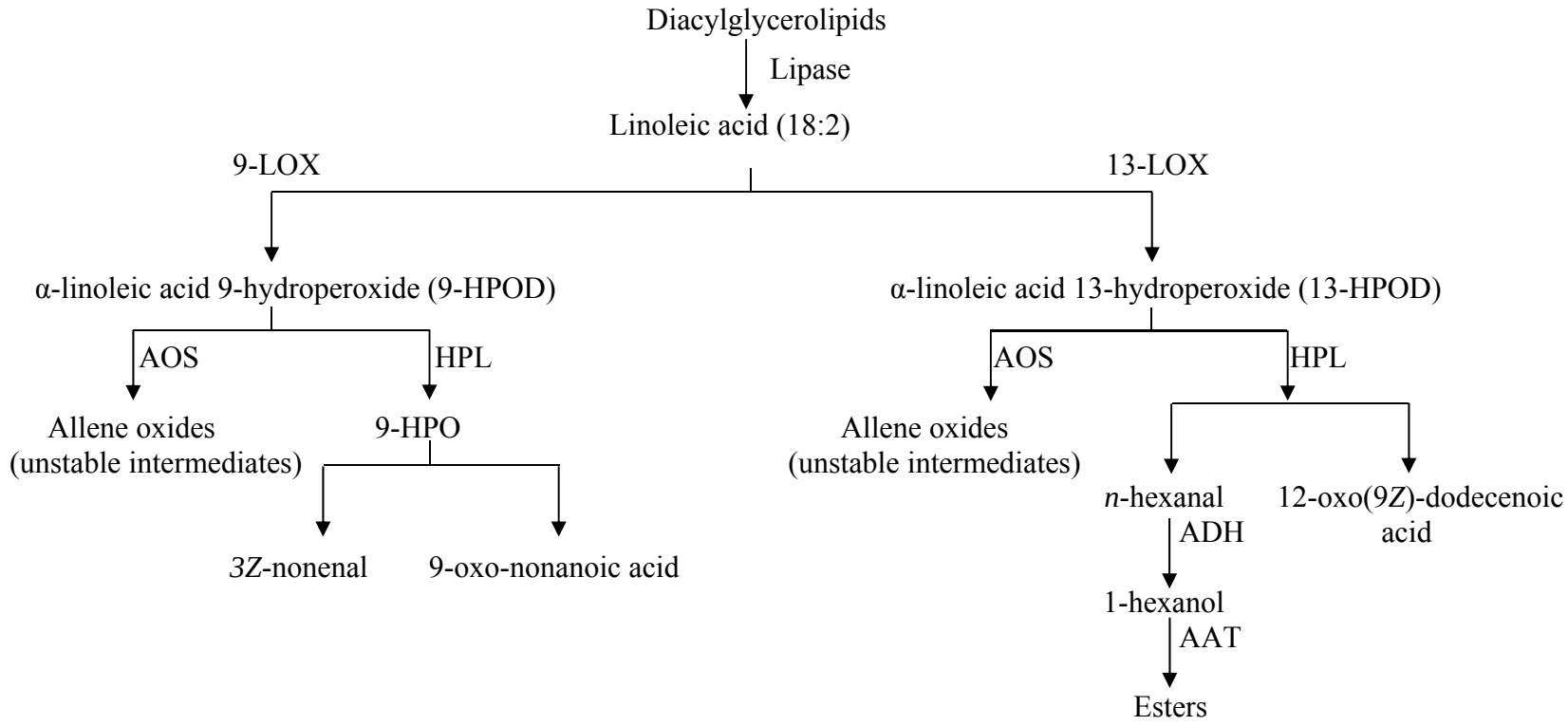

Figure 2. The LOX pathway using linoleic acid (18:2) as substrate. LOX (lipoxygenase), HPOD (hydroperoxide linoleic acid), AOS (allene oxide synthase), HPL (hydroperoxide lyase), ADH (alcohol dehydrogenase), and AAT (alcohol acetyl transferase).

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CHAPTER II

LIPOXYGENASE-ASSOCIATED APPLE VOLATILES AND THEIR RELATIONSHIP WITH AROMA PERCEPTION DURING RIPENING

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Introduction

When we consume apple fruit, we perceive aroma compounds synthesized by the intact organ prior to consumption as well as those synthesized in response to the cellular disruption caused by biting and mastication. Collectively these two routes of synthesis yield approximately 270 different volatile compounds (Dimick and Hoskin, 1982). Of these, 15 to 20 have been described as significant contributors to apple odor (Cunningham et al, 1986; Grosch, 1993; Komthong et al., 2006; Plotto et al., 2000). These volatiles belong to several chemical classes, including monoterpenes, esters, organic acids, aldehydes, alcohols, and ketones. Esters, aldehydes, and alcohols are considered the most important to aroma perception in apple (Plotto et al., 2000; Plotto and McDaniel, 2001).

An intact apple fruit develops its typical flavor profile during ripening with maximum volatile production near the peak of the respiratory climacteric and the rise in ethylene production (Mattheis et al., 1991; Dixon and Hewett, 2000). Most of the volatiles synthesized by intact fruit are under the control of ethylene (Defilippi et al., 2005; Ferenczi et al., 2006). The volatile profile of intact fruit is primarily composed of esters and can have significant content of various alcohols. Aldehydes, however, are produced at only very low amounts by intact fruits and it appears that their emissions decline as fruit maturity increases (De Pooter et al., 1987; Mattheis et al., 1991; Mehinagic et al., 2006)

In a cell-disrupted system, C6 aldehydes are produced in essentially all fresh produce as a result of the action of lipoxygenase (LOX) and other enzymes in the LOX pathway [e.g., lipase and fatty acid hydroperoxide lyase (HPL)] on substrates released by cell disruption (Galliard et al., 1977; Hatanaka et al., 1978). The synthesis of C6 aldehydes is essentially immediate following cell disruption (Bell et al., 1995; Chen et al., 2004; Hatanaka, 1993; Matsui et al.,

2000; Riley and Thompson, 1998; Wu and Liou, 1986). In ripe apple, the aroma of hexanal, generated during the maceration of apple tissue, while detectable, is partially masked by the large amount of volatile esters (Guadagni et al., 1971; Mattheis et al., 1991). Apple juice and apple products made from crushed apples have significant content of C6 aldehydes and alcohols (Dimick and Hoskin, 1982; Yajima et al. 1984; Su and Wiley, 1998), especially hexanal, which can sometimes correlate with sensory perception of off-flavors in certain commodities (Barrett et al., 2010). The content of hexanal and *trans*-2-hexenal continues to change well after the initial step of maceration (Su and Wiley, 1998). Llorente et al. (2011) found that alcohols (hexanol, *trans*-2-hexenol and pentanol) were the main aroma components in processed apple juice, followed by aldehydes (hexanal and *trans*-2-hexenal) and esters (butyl acetate). While the LOX pathway is active in the intact system prior to disruption, it is not known if it contributes in a meaningful way to the synthesis of aroma active compounds in non-disrupted tissue, although its involvement has been suggested (Dixon and Hewett, 2000; Rowan et al., 1999).

The core reactions of the LOX pathway consist of the sequential action of lipase, LOX, HPL and alcohol dehydrogenase (ADH) (Matsui et al., 2000). The release of the free fatty acids (FFAs) from membrane and storage lipids is thought to be by the cleaving action of a lipase (Chapman, 1998; Matsui et al., 2000). It has been commonly accepted that LOX enzymes then act on the FFA, however, there is accumulating evidence of LOXs attacking directly neutral lipids and phospholipids. Kondo et al. (1993) reported the production of phospholipid hydroperoxides by a LOX when a fungal elicitor was applied on soybean seedlings. Also, a cucumber root LOX capable of attacking phosphatidylcholine was reported by Matsui et al. (1998). In any case, the FFAs released by lipases become substrates for LOX enzymes, which oxidize the FFAs to 9 -and 13-hydroperoxides (HPODs). The HPODs then are oxidized by HPL

to aldehydes (e.g., hexanal, and *cis*-3-hexenal) and the aldehydes can be converted to alcohols (1-hexanol and *cis*-3-hexenol, respectively) by ADH (Hatanaka, 1993).

Hexanal and *cis*-3-hexenal are the primary volatile aldehydes produced by the LOX pathway (Hatanaka et al., 1978). These two volatiles have low odor thresholds and likely serve as odorants for most, if not all, fresh fruits and vegetables during mastication and consumption (Barrett et al., 2010). They are especially important in the aroma profile of some fresh products such as tomato, cucumber, peppers and apple, conferring significantly to the aroma character (Mehinagic et al., 2006; Baldwin, 2004).

It is not known the extent to which mastication-derived aldehydes explain apple aroma/flavor perception at the moment of consumption. Ozcan and Barringer (2011) reported in strawberry, that the concentration of *cis*-3-hexenal and *trans*-2-hexenal in the mouthspace of 25 individuals was two-fold higher than in the headspace of a whole strawberry. Also the persistence of LOX-derived compounds was higher than esters after 5 minutes of chewing in the mouthspace. Similar results were also reported for tomato (Xu and Barringer, 2010). Arvineset et al (2006) designed a model mouth system that reproduced mastication movements, hence the authors determined the importance of crushing apples to study the release of their volatile compounds, but the study did not include sensory tests. During ripening, the apple aroma profile has been demonstrated to change from aldehydes ("green-notes") to esters ("fruity-notes") (Mattheis et al., 1991; Plotto et al., 1999). Panasiuk et al. (1980) found a correlation between overall aroma intensity and the sum of *trans*-2-hexenal and hexanal for apple juice. Interestingly, the C6 aldehydes were positively correlated with 'ripe' aroma and were negatively correlated with 'unripe' aroma. 'Overripeness' was positively correlated with the rate of ester production.

The capacity for the disruption-dependent biosynthesis of C6 aldehydes and their corresponding alcohols and esters has not been explored in detail as a function of ripening for apple. Further, the relationship of this biosynthetic capacity to the perception of aroma is essentially unknown in apple, despite extensive work in tomato (Baldwin et al., 2004; Barrett et al., 2010; Buttery et al., 1987). We desired to learn the point in development when the aroma of disrupted apple tissue perceptibly changes and to determine if this perception can be correlated to changes in the biosynthesis of either disruption-dependent or autonomously-produced volatiles. To that end, we documented the relationship between volatile production by intact and disrupted apple tissues as affected by fruit maturation and ripening. The implications of changing trends in C6 aldehyde, alcohol, and ester formation are discussed in relation to expected changes in the capacities for LOX activity and ester forming capacity.

Materials and Methods

Plant material. 'Jonagold' (*Malus x domestica* Borkh.) apples from a commercial orchard in Sparta, MI were used to investigate the relationship between stage of development, the perception of aroma, and the volatile profile of tissue samples immediately after simulated mastication. The study was repeated in 2009 and 2010. The first harvest took place on 4 Sept. in 2009 and 8 Sept. in 2010. On the first harvest date, a lot of approximately 200 fruit was treated with 1 $\mu L\ L^{-1}$ of the ethylene action inhibitor 1-methylcyclopropene (1-MCP) for 24 h to suppress ripening and maintain fruit in a preclimacteric state ('non-ripening') throughout the study. For the remainder of the study, these fruit were held at 0 °C in 0.1 mm-thick black plastic bags to slow development, reduce moisture loss and avoid reactions to intermittent changes in lighting. After the initial harvest dates, 'normally-ripening' fruit were harvested twice per week,

every three to four days until ripening was imminent as judged by the average IEC being greater than 0.1 μL L^{-1} and by approximately 20% of 20-fruit maturity samples (see below) having ethylene levels greater than 10 μL L^{-1}. At that time, 9 Oct. in 2009 and 1 Oct. in 2010, approximately 200 additional fruit were harvested and thereafter allowed to ripen in a controlled environment chamber at 15 °C. This was done to avoid damage in the field due to freezing and fruit drop. These fruit were covered with plastic bags to minimize moisture loss as described previously and subsequently examined every three to four days until the conclusion of the study on 27 Oct. (day 53) in 2009 and 20 Oct. (day 42) in 2010. One day prior to each assay date, non-ripening and normally-ripening fruit were moved to the laboratory to equilibrate to room temperature (22 ± 1 °C). During this adjustment period, fruit were covered with black plastic bags to minimize moisture loss as described previously. On each assay date, normally-ripening apples were compared with non-ripening apples treated with 1-MCP.

On each evaluation date, 40 normally-ripening apples and 3 to 5 non-ripening apples were used for analysis. Maturity was assessed on 20 randomly selected, normally-ripening fruit by measuring internal ethylene (μL L^{-1}), as described below, and fruit weight (g), red skin coloration (%), background color (1-5 scale), firmness (N), soluble solids content (%), and starch index (1-8) according to Mir et al. (2001). These data were used to assess the readiness of the fruit to ripen for the final harvests noted previously and are summarized in Table 1. For the remaining 20 normally-ripening fruit, the internal ethylene content of each was measured and, of these, the 5 fruit having an internal ethylene content nearest the median were selected for analysis of CO_2 production as described below. These 5 normally-ripening fruit and non-ripening fruit were used for volatile analysis (whole fruit and crushed slices) and, within the next

4 h, were subjected to sensory analysis (crushed slices only). Slices were removed from the fruit and crushed no more than 2 minutes prior to analysis.

Ethylene, respiration rate and volatile analyses of whole fruit. The internal ethylene content of the fruit was determined by gas chromatographic (GC) analysis on a 1-mL gas sample withdrawn from the core of the apples as previously described (Mir et al., 2001). The GC (Carle Series 400 AGC; Hach Company, Loveland, CO) was equipped with an FID and a 2-m long × 6-mm i.d. stainless steel column packed with activated alumina. The column temperature was kept at 100 °C. The ethylene detection limit was approximately 0.005 μL L^{-1}. Ethylene concentrations were calculated relative to the certified standard (Matheson Gas Products Inc., Montgomeryville, PA) containing 0.979 μL L^{-1} ethylene, 4.85% CO_2, and 1.95% O_2 balanced with N_2.

Respiration was determined for whole fruit in a flow-through system at ambient temperature (22 ±1 °C). Fruit were sealed in 1-L Teflon containers (Savillex Corporation, Minnetonka, MN) flushed at approximately 40 mL min^{-1} with air. The atmosphere in the chambers was allowed sufficient time to achieve steady-state (i.e., when the volume inside the container was displaced 10 times) before measurements were made. For analysis, gas samples (100 μL) were withdrawn from the outlet port and immediately injected in an infrared gas analyzer (model 225-MK3; Hoddesdon, England) operated in a flow-through mode with N_2 as the carrier gas and a flow rate of 100 mL min^{-1}. CO_2 concentration was calculated relative to the certified gas standard noted earlier. CO_2 production was expressed as mg kg^{-1} h^{-1}.

Volatile analysis was performed on the headspace of the1-L containers used for respiratory measurements following a 20 min incubation period during which the flow of air through the container was stopped. Volatiles were collected using a solid phase micro extraction

(SPME) fiber (65 μm thickness PDMS-DVB, Supelco Inc., Bellefonte, PA). The fiber was exposed to the chamber headspace for 2 min adsorption, and immediately transferred to the GC (HP-6890, Hewlett Packard Co., Wilmington, DE) splitless injection port at 220 °C and desorbed for 2 min. Desorbed volatiles were trapped on-column using a liquid nitrogen cryofocusing trap. Separation of the volatiles was achieved using a 20 m long x 0.18 mm i.d. capillary column (SP-5, Supelco Inc., Bellefonte, PA) with a 0.18 μm thick stationary phase. The GC oven temperature was programmed to increase from 40 to 240 °C at a rate of 50 °C min^{-1}, holding at 240 °C for 1 min. The flow rate of the helium carrier gas was held constant at 0.8 mL min^{-1}. Volatile detection was by time-of-flight mass spectrometry (TOFMS) using electron impact ionization (Pegasus II, LECO Corp., St. Joseph, MI) according to Song et al. (1997). Identification of all quantified compounds was by comparison of the mass spectrum with authenticated reference standards and with spectra in the National Institute for Standard and Technology (NIST) mass spectral library (Search Version 1.5). Volatile compounds were quantified as previously described by calibrating with a standard mixture of 28 aroma compounds prepared with authenticated compounds (Sigma Co. and Fluka Chemika) of known purity (Bartelt, 1997; Song et al., 1997). The standard mixture contained equal volumes of each compound (ethanol, butanol, 1-hexanol, *cis*-3-hexenol, 2-methyl butanol, 3- methyl butanol, 2 methyl propanol, propanol, *trans*-2-hexen-1-ol, butyric acid, 2 methyl butyric, hexyl acetate, butyl acetate, butyl butanoate/butyrate, butyl hexanoate, ethyl acetate, ethyl 2-methylbutyrate, ethyl hexanoate, 2-methylbutyl acetate, propyl hexanoate, *cis*-3-hexenyl acetate, *trans*-2-hexenyl acetate, hexyl hexanoate, hexyl butanoate/butyrate, hexyl propanoate, hexanal, *trans*-2-hexenal and *cis*-3-hexenal). 1 μL of the standard mixture was applied to a small paper filter disk, which was dropped into a 4.4-L glass volumetric flask with a ground-glass top fitted with a Mininert

valve (Supelco Inc., Bellefonte, PA). The flask was sealed and the mixture was allowed to vaporize for at least 6 h. Volatilization of the compounds was found to be complete (i.e., the GC/MS response ceased increasing) after 4 h.

Sensory and volatile analysis of crushed fruit. To assess the difference in aroma between samples of normally-ripening and non-ripening fruit, a triangle difference test was performed (Meilgaard et al., 2007) in consecutive harvest seasons (2009 and 2010) on crushed tissue samples from the normally- and non-ripening fruit previously used for whole-fruit respiratory and volatile analysis. A panel composed of 22 experienced assessors was used each year. Sensory evaluations were conducted twice per week and in each session the panelists were presented with three coded samples, two of which were normally-ripening and the odd sample non-ripening or visa versa. Six different combinations/orientations were presented randomly to the panelists as, from left to right, ABB, BAA, AAB, BBA, ABA, and BAB. Each sample consisted of an 8-gram apple slice with the peel attached crushed in a ceramic mortar and pestle for 15 s to a uniform slurry and transferred to a 118-mL black polystyrene cup closed with a translucent lid (Fabri-Kal, Kalamazoo, MI). The lid had a large circular label that displayed the code for the sample and obscured the sample from view. Samples were presented to the assessors within two minutes of crushing. Panelists were instructed not view the samples and lift the lid to smell the headspace of each cup from left to right as presented, and select the odd sample.

For objective analysis of the volatiles of crushed samples, fruit were handled identically to those in the sensory study. Additional slices were removed from the same five fruit used in the sensory study immediately after the sensory session concluded, taking care not to include any previously cut tissue. Crushed tissue samples were placed into the same preparation procedure

and black polystyrene cups and lids as used in the sensory study. Approximately two minutes after crushing, aroma volatiles were collected from the plastic cup headspace by inserting a SPME fiber (65µm thickness PDMS-DVB) through a small hole in the center of the lid, taking care not to allow the fiber to contact the tissue within the cup. The absorption period was 2 min, after which the fiber was desorbed and the volatile profile analyzed as described previously.

*Experimental design and statistical analyses.*Volatile analysis experiments were performed with 5 replicates. The experimental unit was a single fruit. Analysis of variance for volatile data was performed in SAS (SAS 9.1, SAS Institute Inc., Cary, NC) using the PROC GLM procedure. A Tukey test with a 5% significance level was used for multiple comparison analysis. Triangle test significance was determined as indicated by Meilgaard et al. (2007). To explore the relationship between the sensory test results and volatile compounds across time points, principal component analysis (PCA) was performed using JMP® Version 9.0 (SAS Institute Inc., Cary, NC). Principal component analysis has two main functions: (1) to indicate relationships among groups of variables, and (2) relationships between objects. PCA proceeds by searching for linear combinations of variables that account for the maximum variation of the data and is used to facilitate interpretation of the data based on data visualization (Piggott and Sharman, 1986). The data for each year was independently studied. A PCA was carried out on thirteen different variable loadings composed of the concentration of 10 different volatile compounds (nL L^{-1}), correct sensory test answers (%), CO_2 production (mg kg^{-1} h^{-1}) and ethylene (log µL L^{-1}). Each of the 10 different volatiles was expressed in nL L^{-1}. The variable 'sensory test' reflects correct identification of normally-ripening and non-ripening apples.

Sample scores for the above loadings were from fifteen time points in 2009 and 13 time points in 2010. On each analysis date, normally-ripening apples were compared with non-ripening apples treated with 1-MCP. The resulting data matrix contained 30 samples (15 ripening and 15 non-ripening samples) in 2009, 26 samples (13 ripening and 13 non-ripening samples) in 2010, using the same 13 variables for each year. Principal components were obtained by computing the eigenvectors and eigenvalues of the correlation matrix. The eigenvectors correspond to principal components and the eigenvalues correspond to the variance explained by the principal components. Thus, variable loadings can be plotted on components as vectors in the dimensional component space. The maximum loading is 1, which means complete correlation and all variance explained. If two components provide a suitable representation of the data, the results are represented in biplots of the 2 principal components contain the greatest percentage of the information (Piggott and Sharman, 1986). In order to identify correlated or related pairs of variables, a scatterplot matrix is displayed with all pairwise plots of the data (JMP® Version 9.0). Pearson correlation coefficients were obtained with PROC CORR in SAS.

Results

Ethylene, respiration rate and fruit quality measurements. Data were collected for similar developmental stages each year, however, the 2010 spring and summer were warmer than in 2009, accelerating the fruit development. In 2009, the number of growing degree days (GDD) from April 1st with a base temperature of 4.4 °C (Edson, 1986; Valentini et al., 2001) until the onset of autocatalytic activity on 8 Oct. was 2138.6; in 2010, the GDD at the onset of autocatalytic ethylene on 23 Sept. was 2385.0. In both years, fruit underwent progressive changes in weight, color, red coloration, background color and °Brix, although, for brevity, these

data are only represented as ranges and averages (Table 1). In order to compare developmental stages for both years, the data were aligned on the date before the autocatalytic ethylene burst occurred (day 0) instead of chronological dates (Fig. 3). Autocatalytic ethylene production was fully engaged 12 Oct. 2009 and 30 Sept. 2010 as evidenced by a 5- to 10-fold increase in the three or four days from the previous evaluations, which were taken as day 0. The climacteric burst in respiration began on day 0 for both years, although the form of the climacteric differed somewhat between years, being more compressed in the warmer 2010 (Fig. 3). Even so, the rates of respiration before and after the climacteric and at the climacteric peak were similar between years.

Table 1. Quality parameters in Jonagold fruit for 57 and 43 days, during 2009 and 2010 years, respectively.

Quality Parameters	2009			2010		
	Min	Max	Avg	Min	Max	Avg
Weight (g)	159.8	257.0	212.7	226.0	298.7	249.0
Skin color or Redness (%)	19.0	98.0	70.5	29.0	90.5	69.6
Background color (*)	1.0	4.0	2.3	1.0	4.0	2.1
Firmness (N)	40	100	71	37	79	62
Soluble solid content (%)	10.8	13.8	12.5	12.3	15.2	13.9
Starch (*)	2.0	8.0	5.7	2.2	8.0	6.4

(*) Background color scale 1-5: 1= yellow and 5 = green. Starch scale 1-8: 1= 100% and 8= 0% starch staining in apple flesh tissue.

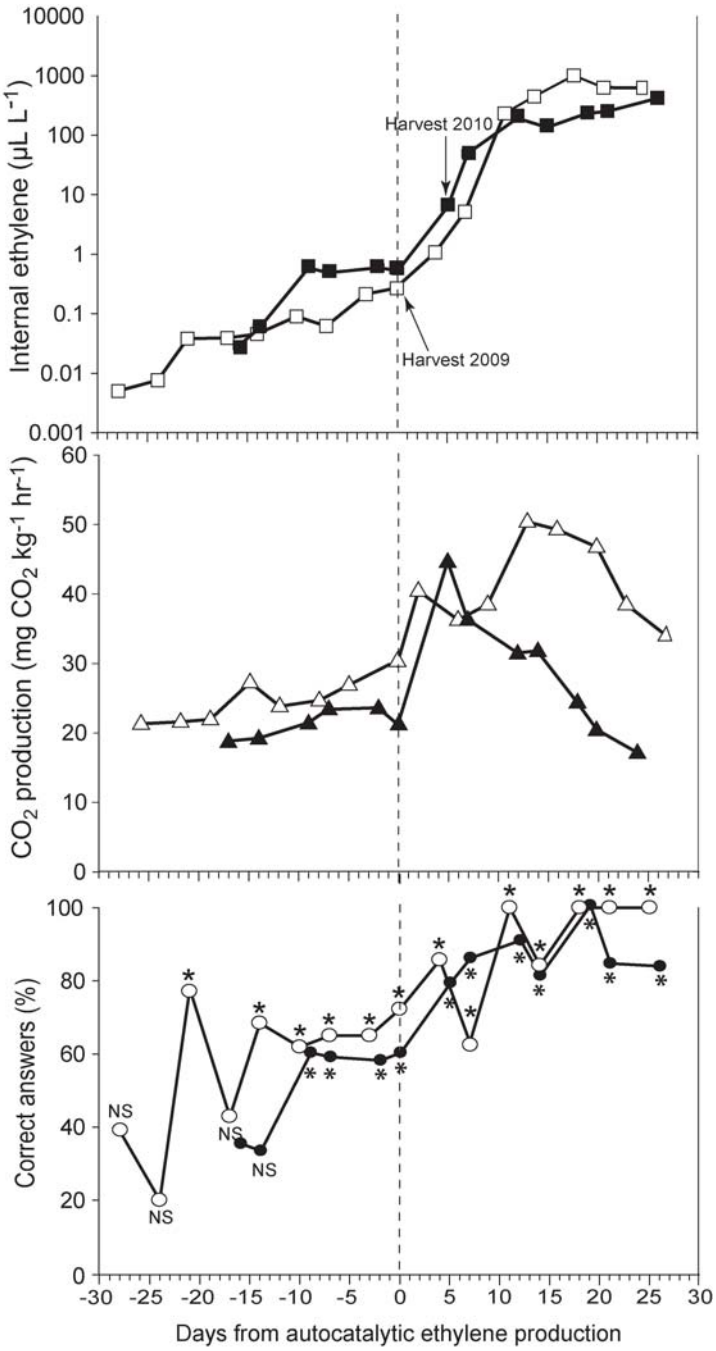

Figure 3. Internal ethylene concentration (top), CO_2 production rate (middle) and triangle sensory test (bottom) in Jonagold fruit. Percent correct answers for which the probability of is significantly different than chance are indicated with an asterisk. Data were collected from September 4 until October 27 and from September 8th to October 20th, during 2009 and 2010 years, respectively. The vertical dashed line represents time when autocatalytic ethylene commenced. Open and solid symbols represent 2009 and 2010 years, respectively.

Sensory analysis of crushed fruit slices. In 2009 and 2010, panelists were not able to distinguish between the scent of non-ripening and normally-ripening fruit in the early stages of maturation (Fig. 3). However, after the fourth triangle test (day -15) in 2009, panelists consistently discerned those fruit that were destined to ripen normally to those not able to ripen due to 1-MCP treatment, with 60% correct answers. Essentially 100% of the panelists could detect differences between ripening and non-ripening fruit by day +10. In 2010, 58% correct answers were obtained from the third evaluation date (day -10), reaching a high of 100% of correct answers by day +20.

Volatile profile. In intact apples, the production of hexanal was low and irregular. No clear pattern was found in 2009 whereas a slight increase was evident in the year 2010 (Fig. 4). However, two other hexyl volatiles, hexanol and hexyl acetate, had production patterns similar to one another, both increasing dramatically from the onset of autocatalytic ethylene production on day 0, rising to a peak in 7 to 12 days and declining thereafter (Fig. 4).

For crushed fruit tissue, the synthesis of LOX-derived C6 aldehydes *cis*-3-hexenal, *trans*-2-hexenal and hexanal was, at first, extremely high for non- and normally-ripening fruit such that the concentration of the aldehydes in the sample cup was several hundred times higher than their odor thresholds (Fig. 5). *Cis*-3-hexenal, while variable, remained constant throughout the experiment for non-ripening fruit, but declined markedly for normally-ripening fruit. Conversely, *trans*-2-hexenal and hexanal increased in macerated preparations of normally-ripening fruit as *cis*-3-hexenal declined, but not for non-ripening fruit. Changes in these aldehydes occurred two weeks after differences in aroma between 1-MCP treated and control fruit were first perceived. All volatiles, except for *cis*-3-hexenal and *cis*-3-hexenyl acetate, (i.e.,

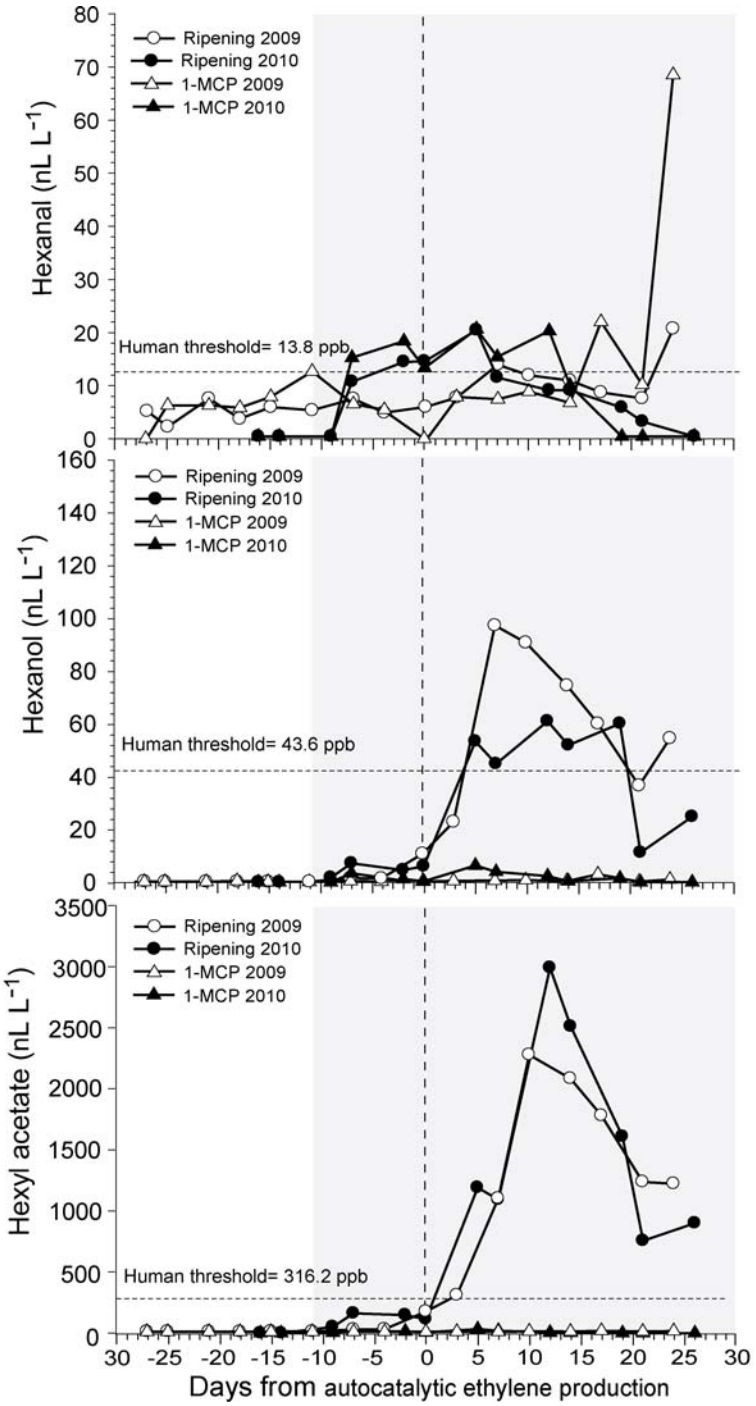

Figure 4. Volatile profiles of C6 volatiles, hexanal (top), hexanol (middle), and ester hexyl acetate (bottom) produced in intact Jonagold fruit throughout ripening. Vertical dashed line represents time when internal ethylene was considered to initiate its autocatalytic phase. The horizontal dashed line indicates the human threshold for detection (the human threshold for cis-3-hexenyl acetate is 316.2 nL L^{-1}). Grey area represents when panelists answered correctly the sensory analysis test for both years (-11 days).

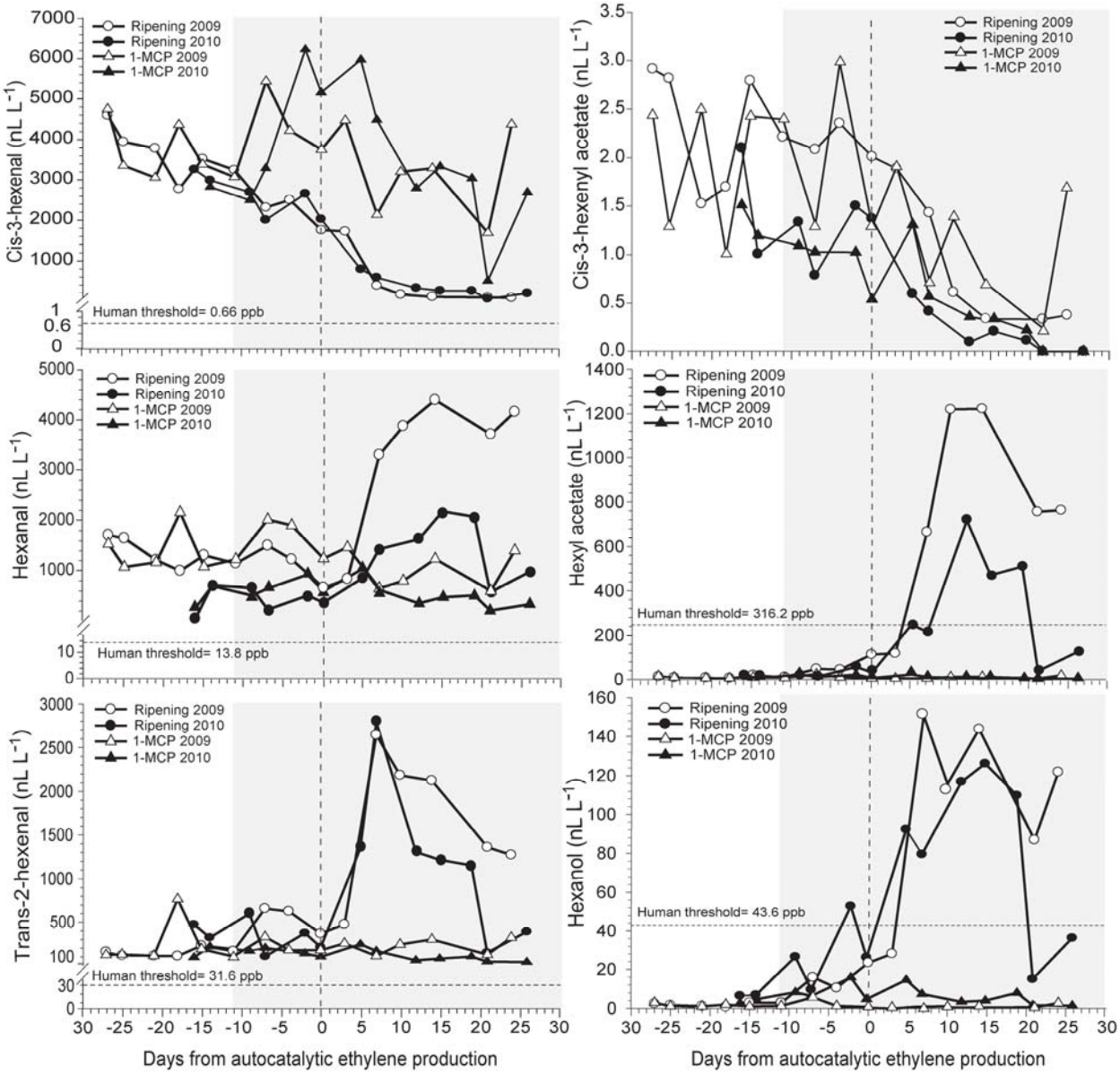

Figure 5. Volatile profiles of C6 aldehydes, cis-3-hexenal, trans-2-hexenal and hexanal (left), esters, *cis*-3-hexenyl acetate and hexyl acetate, and the alcohol hexanol (right) produced in crushed Jonagold fruit throughout ripening. Vertical dashed line represents time when internal ethylene exceeded 0.1 μL L^{-1}. The horizontal dashed line indicates the human threshold for detection (the human threshold for *cis*-3-hexenyl acetate is 316.2 nL L^{-1}). Grey area represents when panelists answered correctly the sensory analysis test for both years (-11 days).

trans-2-hexenal, hexanal, hexanol, and hexyl acetate) increased in concentration coincident with the highest percentage of correct identification of treatment differences by the panel (approximately 3-5 days after autocatalytic ethylene production). This was also the case for all other esters monitored including the predominant esters butyl acetate and 2-methylbutyl acetate (data not shown). 1-MCP effectively blocked production of all apple volatiles measured, except *cis*-3-hexenal and *cis*-3-hexenyl acetate.

The concentrations of volatiles in the container headspace for intact fruit and crushed slices differed markedly. *Cis*-3-hexenal and *trans*-2-hexenal were not detectable in the headspace of normally-ripening or non-ripening intact fruit, but were found at high levels in the headspace of crushed tissues. Hexanal emissions were detectable from both tissue types, but was many-fold higher for crushed apple tissue. The developmental patterns for hexanal differed between the two systems of production, however, with the post-climacteric rise being absent in the whole fruit system. The concentrations and patterns were similar in both systems for hexanol. Whole fruit yielded slightly higher headspace concentrations of hexyl acetate than crushed samples. *Cis*-3-hexenyl acetate was only detectable in the headspace of crushed fruit. *Trans*-2-hexenyl acetate was also only detectable in crushed fruit system and increased throughout ripening for normally-ripening fruit (data not shown), however its levels were too low for accurate quantification.

Principal Component Analysis and Pearson correlations. A PCA was used to determine the source of variation in the data throughout apple fruit development (Fig. 6). For 2009, PC1 contained 69% of the total variation whereas PC2 contained 13.9%. Similarly, for 2010 PC1 contained 63.4% of the total variation and PC2 contained 16.6%. With the exception of the

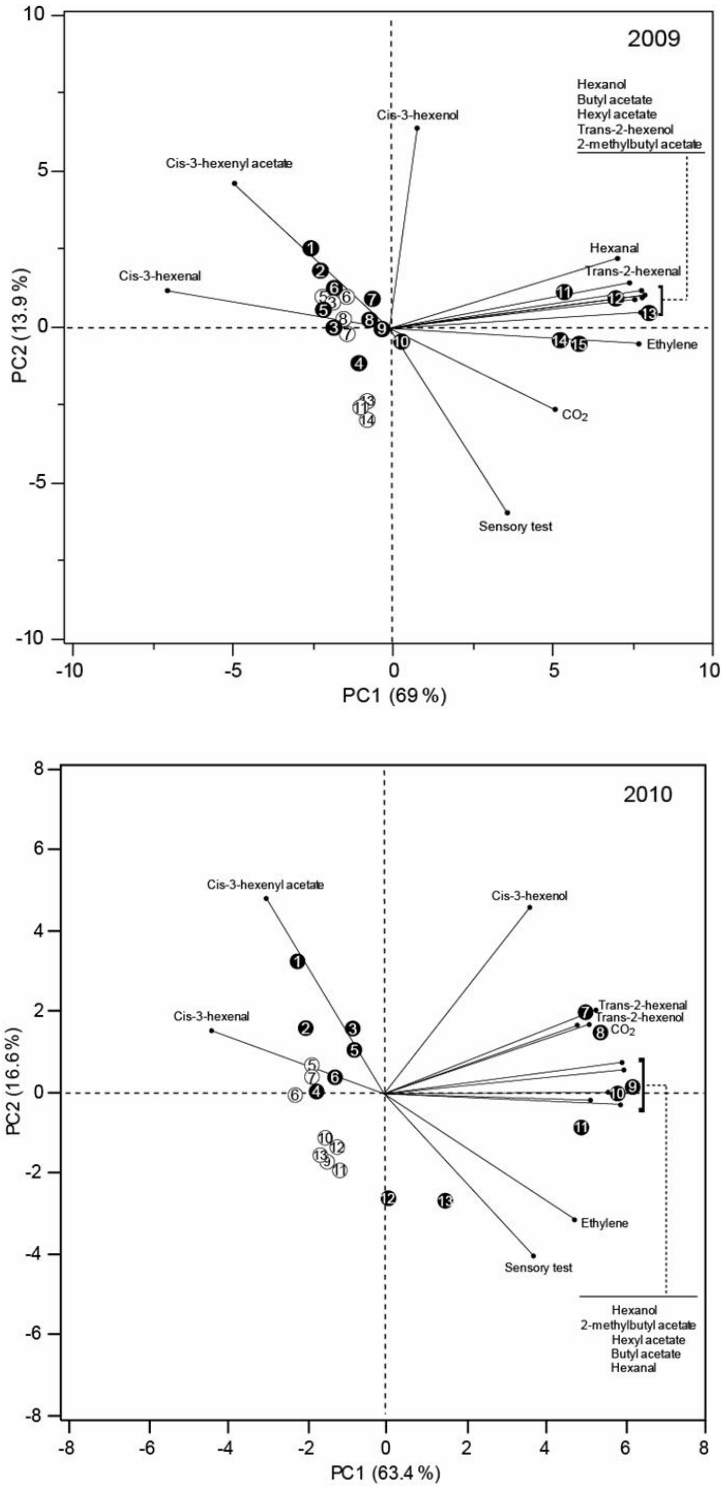

Figure 6. Principal component analysis (PCA) in Jonagold fruit. 2009 and 2010 biplots (loading variables and sample scores) of PC1 vs. PC2 are presented, top and bottom graphics, respectively. Open symbols represent non-ripening (1-MCP-treated) samples, and filled symbols represent normally-ripening samples. Numbers show the time points evaluated through ripening.

sensory test result, *cis*-3-hexenyl acetate and *cis*-3-hexenol, all variables were partially explained by PC1 in both years. Most of the volatiles clustered together and were heavily loaded on the positive side of component 1, whereas the C6 aldehyde *cis*-3-hexenal had a large negative loading on component 1 suggesting a negative correlation with the other volatiles.

Cis-3-hexenol had a large positive loading on component 2 in 2009 and smaller loading in 2010. *Cis*-3-hexenyl acetate had no clear pattern during 2009, being explained by both PCs (eigenvector -0.60 for PC1 and 0.57 for PC2), whereas in 2010, it was clearly loaded in PC2 (eigenvector 0.77). The loadings for variables such as CO_2 and ethylene, although with some variation between years, were significant on PC1. The sensory test variable had a large negative loading on PC2 (eigenvector -0.72 and -0.64 in 2009 and 2010, respectively).

The sample scores varied as a function of ripening in both years of study (Fig. 6). A group of early ripening fruits (filled symbols) and all non-ripening apple fruits (open symbols), clustered together with rather low and negative scores on component 1 (towards the center of the biplot) (Fig. 6). A second group composed of late ripening stages (filled symbols) with high positive scores were also found on component 1. During 2010, sample scores of early ripening and immature stages were more dispersed than in 2009, likely because in 2010, the warmer season resulted in highly senescent fruit with markedly reduced ester production by the end of the study. Two main clusters for the observations were found. Immature, normally-ripening fruit and non-ripening (1-MCP-treated) fruit clustered together close to *cis*-3-hexenal and its ester derivative, *cis*-3-hexenyl acetate, whereas ripe samples clustered separately along with the majority of the volatiles including most of the esters.

Cis-3-hexenyl acetate production, and to a lesser extent, that of *cis*-3-hexenal, were negatively related to the percentage of correct answers in the sensory test in both years. The

correlation between correct answers and these two compounds was negative and highly significant (Fig. 7). Interestingly, the correlations between the sensory test and volatiles associated with ripe fruit were somewhat lower, although they were all positive. The highest correlation found for the sensory test was with ethylene (r = 0.6). *Cis*-3-hexenal and *cis*-3-hexenyl acetate also had a negative correlation or no significant correlation with all variables apart from each other and the sensory test results. Very strong correlations were found between hexyl acetate and butyl acetate (r = 0.97); hexyl acetate and hexanol (r = 0.90); hexyl acetate and 2-methylbutyl acetate (r = 0.96); butyl acetate and hexanol (r = 0.95) and butyl acetate and 2-methylbutyl acetate (r = 0.92) (Fig. 7). Ethylene had strong correlations with most esters and hexanol. CO_2 had no strong correlations compared to ethylene or other variables.

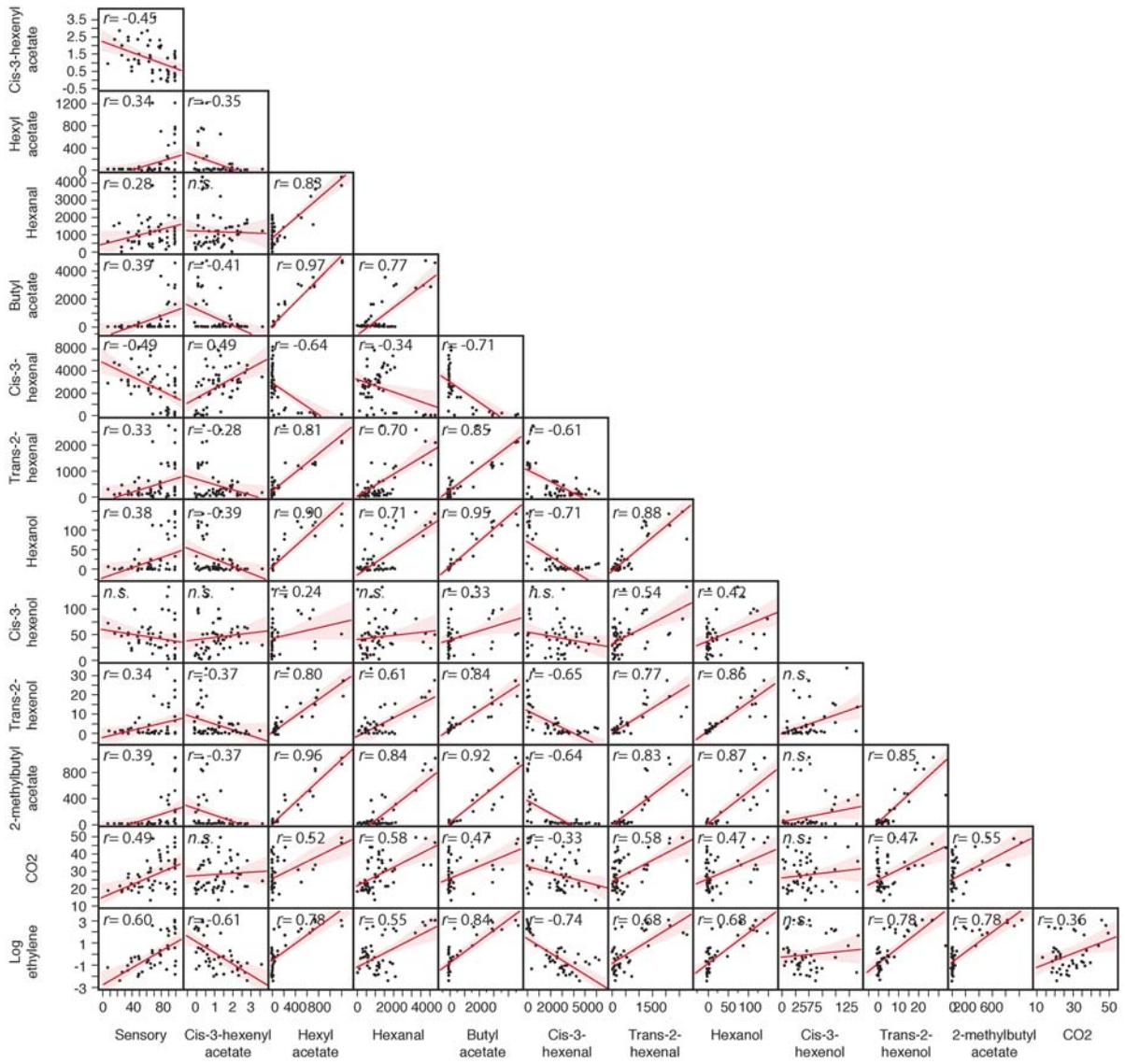

Figure 7. Scatter plot matrix of CO_2 production rate, internal ethylene concentration, triangle sensory test and aroma volatiles for Jonagold fruit. Pairwise plots represent data of 2009 and 2010 years.

Discussion

The progression of fruit maturity indices for 'Jonagold' is consistent with those previously published (Beaudry et al., 1993; Schwallier et al., 1995) (Fig. 3; Table 1). The onset of ester synthesis with the rise in ethylene is also consistent with the fact that ethylene action is required for the synthesis of ripening-related esters in apple fruit (Defilippi et al., 2005; Ferenczi

et al., 2006; Mir et al., 1999; Song and Bangerth, 1996), hence one would expect detection of typical apple aromas from this phenological stage (onset of the respiratory climacteric and autocatalytic ethylene production). However, panelists detected differences in aromas 1.5 to 2 weeks before this point as ethylene levels rose above 0.1 $\mu L\ L^{-1}$. During this period, low levels of some esters (e.g., hexyl acetate and butyl acetate) were detected, but their concentrations did not appear to rise above their odor threshold in the headspace of the cups used in the sensory analysis. It should be noted that the cups used for volatile analysis and sensory evaluations, while identical in sample mass, sample preparation, cup volume and incubation time, were derived a couple hours apart from one another and may have differed somewhat. It seems likely, however, that since the cups used by the panelists were opened and those for the volatile analysis were not, that the concentrations in the headspace of the later would have been slightly higher than the former. As such, there is less likelihood that those volatiles found at concentrations below the human threshold would have been above that threshold in the sensory analysis.

Upon tissue disruption, the LOX pathway contributes to apple aroma via the production of straight-chain C6 aldehydes and, possibly, alcohols and esters derived from these compounds (Guadagni et al., 1971, Mattheis et al., 1991; Dimick and Hoskin, 1982; Dixon and Hewett, 2000). However, it is not known if this pathway contributes significantly to volatile production in intact fruit. In this study, the accumulation of hexanal in the containers for intact ripening and non-ripening fruit, was quite low, remaining near the limit of human detection throughout development (Fig. 4). The data imply that hexanal synthesis by intact fruit is independent of ripening. Even so, emissions of hexanol and hexyl acetate were abundant after ripening began. Similarly, the data from Mattheis et al. (1991) show no similarity in the pattern of hexanal production by intact apple fruit with either hexanol or hexyl acetate production. This could be

interpreted to indicate that hexanal was rapidly converted to hexanol, thereby preventing the accumulation of hexanal. This possibility was also suggested by De Pooter et al. (1987). They found that C1 - C6 carboxylic acids yielded minimal accumulation of their respective aldehydes, but the production of esters derived from these aldehydes increased between 3- and 10-fold. These authors suggested that aldehyde formation may be rate-limiting such that aldehydes from exogenously applied carboxylic acid are transformed as quickly as it becomes available. However, the extractable activity of alcohol dehydrogenase (ADH), which is responsible for the conversion of aldehydes to alcohols (Bartley and Hindley, 1980), gradually declines or remains steady during ripening (Echeverria et al., 2004; Defilippi et al., 2005). Similarly, *ADH* expression decreases in apple after ethylene exposure (Defilippi et al., 2005; Schaffer et al., 2007). Steady or declining ADH activity is counterintuitive, given the relatively constant hexanal emissions under conditions of increased flux to hexanol. However, the capacity of ADH may be so much greater than the rate of formation of hexanal, that it is able to metabolize hexanal as fast as it is produced. An alternative explanation is that hexanol is derived from a metabolite other than hexanal (Mayorga, et al., 2001), although such a pathway has not been characterized in apple fruit. While the volatiles derived from the 18:3 fatty acids, *cis*-3-hexenal and *trans*-2-hexenal, were not detected in our study for intact apples (data not shown), other authors have detected low levels of "hexenal" in intact Golden Delicious apples (De Pooter et al., 1987), and *trans*-2-hexenal (Defilippi et al., 2005; Mattheis et al., 1991).

The high rate of *cis*-3-hexenal, hexanal and *trans*-2-hexenal production by ripening and non-ripening crushed apple tissue (Fig. 5) and the lack of *cis*-3-hexenal and *trans*-2-hexenal and their alcohol and ester products from intact fruit (Fig. 4) suggests that these volatiles are dependent upon tissue disruption and the action of LOX pathway enzymes. Further, the decline

in *cis*-3-hexenal production and increases in hexanal and *trans*-2-hexenal production suggests that changes in LOX pathway activity are part of the developmental ensemble of ripening processes. In tomato, Buttery (1993) found copious production of *cis*-3-hexenal after maceration. Similar results were found by Riley and Thompson (1998) in green and red tomato fruit homogenates. However, they also found that red ripe tomato had 4-5-fold greater capacity to generate *cis*-3-hexenal than green fruit when exogenous substrate was applied. This is contrary to our results where we found more *cis*-3-hexenal production in immature apples and very low levels in ripe apples. To our knowledge no other studies have shown the developmentally-dependent decline in *cis*-3-hexenal production by crushed tissue of normally-ripening apple fruit.

The burst in *trans*-2-hexenal formation from crushed apple fruit tissues has been previously detected by Paillard (1986) at the climacteric peak. Defilippi et al. (2005) also observed this increase in *trans*-2-hexenal apple ripening, even among slow to ripen fruit varieties intentionally modified to have suppressed ethylene biosynthesis. This burst in *trans*-2-hexenal is likely due to isomerization of *cis*-3-hexenal, but it is not clear if the process is enzymatically catalyzed. The existence of an isomerase that converts *cis*-3-hexenal to *trans*-2-hexenal has been proposed. Allmann and Baldwin (2010) reported, in mechanically wounded *Nicotiana attenuata* plants, large amounts of (*cis*) green leaf volatiles and smaller amounts of (*trans*) green leaf volatiles; these authors measured the conversion of *cis*-3-hexenal to *trans*-2-hexenal in an in vitro system of *Manduca sexta* oral secretions and found a much larger percentage (roughly 50%) of the added *cis*-3-hexenal was converted to *trans*-2-hexenal, implying the existence of an isomerase in the oral secretions. Buchhaupt et al. (2012) coexpressed LOX and HPL in a yeast strain in whole cell biotransformation experiments; they observed that the primary product was *cis*-3-hexenal, and only a small amount of it isomerized to *trans*-2-hexenal, suggesting the

chemical route of isomerization is of marginal importance. A (*3Z*):(*2E*)-enal isomerase, which would convert a (*Z*)-aldehyde to its (*E*) isomer has been proposed for several plant species. Partial purification and characterization of a (*3Z*):(*2E*)-enal isomerase from cucumber fruit showed *3Z* to *2E* isomerization (Phillips et al., 1979). Also, Takamura and Gardner (1996) and Noordermeer et al. (1999) demonstrated isomerase activity for soybean and alfalfa, respectively. However, Allmann and Baldwin (2010) in their studies of herbivore damage on *Nicotiana attenuata* plants, showed that a plant-derived isomerase was not responsible for the (*Z*) to (*E*) conversion of leaf aldehydes, but was due to an unknown and heat unstable oral compound secreted by the herbivore *Manduca sexta*. In any case, evidence of the involvement of plant isomerases in aldehyde conversion is still limited. The decline in *cis*-3-hexenal emanations by crushed, normally-ripening fruit may be partially related to an increase in its metabolism via in isomerase activity, which would also help explain the burst in *trans*-2-hexenal. Declining *cis*-3-hexenal production in normally-ripening fruit may also result from a decline in LOX pathway activity; either through changes in enzyme activity or changes in the availability of 18:3 fatty acid (linolenic acid). A ripening-related decline in LOX activity has been detected by Griffiths et al. (1999) for tomato fruit in which the expression of two out of three LOX enzymes declined during ripening. However, Defilippi et al. (2005) found no change in extractable LOX activity in apple and also found that exogenous ethylene did not impact the levels of aldehydes produced in homogenized preparations of apple. To our knowledge, there are no data on changes in lipase or HPL activity in apple during ripening that might help explain the decline in *cis*-3-hexenal formation.

The low level of *cis*-3-hexenyl acetate production from crushed fruit tissue is consistent with the findings of Yajima et al (1984), who also detected low amounts of this compound from

excised apple peel. The declining pattern of emission of *cis*-3-hexenyl acetate for ripening fruit parallels the pattern for *cis*-3-hexenal, which is the precursor for this ester (Fig. 5). The decline in emission of *cis*-3-hexenyl acetate for non-ripening fruit slices was less pronounced than for normally-ripening fruit, but similarly reflected the slight decline in *cis*-3-hexenal production in those fruit.

The climacteric-type rise in hexanal emissions from crushed tissue was only found for normally-ripening apple fruit after the onset of autocatalytic ethylene formation, consistent with the findings of Paillard (1986). The rise in hexanal emissions was opposite that for *cis*-3-hexenal, which declined approximately 90% during ripening. Presuming both aldehydes are the product of LOX activity, it is not clear how one would increase and the other decline. Three possibilities are proposed. (i) It is possible that the patterns in hexanal and *cis*-3-hexenal may reflect an increase in 18:2 fatty acids (linoleic acid) and a decline in 18:3 fatty acids (linolenic acid), respectively, which act as precursors in the LOX pathway. Galliard (1968) studied the composition of lipids from pulp of pre- and post-climacteric apples and found that the distribution of fatty acids from both, pre- and post-climacteric apples was similar except that 18:2 fatty acids were slightly higher in post-climacteric than in pre-climacteric fruit and 18:3 fatty acids in post-climacteric fruit were lower. Also, the author reported that 18:2 fatty acid was the major component of the total lipid and for many of the individual polar and non-polar lipids. Paillard (1986) reported similar results when investigated free fatty acids in epidermis and parenchyma of apple fruits throughout ripening. The 18:2 fatty acids increased after climacteric peak whereas 18:3 declined, although 18:3 fatty acids were 2-fold lower than 18:2. Likewise, Song and Bangerth (2003) determined that 18:2 fatty acids were low in pre-climacteric fruit, but rapidly increased 3- to 4-fold during climacteric peak. They also reported that 18:3 fatty acids

decreased in later ripening stages. (ii) An alternative explanation for this pattern in aldehyde emission is that the rate of metabolism of *cis*-3-hexenal is relatively greater than that of hexanal. In fact, the synthesis of *trans*-2-hexenal, presumably from *cis*-3-hexenal, did increase during ripening. On the other hand, the production of *cis*-3-hexenol was low and near the limits of detection of the instruments used (data not shown) and the synthesis of its ester product, *cis*-3-hexenal acetate, was only a fraction of that for hexyl acetate. These findings argue against more rapid metabolism of *cis*-3-hexenal than hexanal. (iii) Another alternative explanation for the observed patterns is that the hexanal released from crushed fruit during ripening is not derived via action of the LOX pathway. Mayorga, et al. (2001) found that significant quantities of hexanal as well as other volatiles were released from glycosides following tissue disruption. DePooter et al. (1987) also demonstrated that aldehydes could, to a limited extent, be produced from carboxylic acid reduction, although a mechanism was not suggested.

A burst in the production of hexanol and its ester product, hexyl acetate, was only detected in ripening (whole and crushed) fruit, implying hexanol formation was dependent on ethylene action. A simultaneous increase in hexanol and hexyl acetate has been previously reported (Mattheis et al., 1991; Song and Bangerth 1994; 1996; Dixon and Hewett, 2000). The patterns for the emanation of hexanol and hexyl acetate from crushed fruit were similar to respective patterns for hexanol and hexyl acetate from intact fruit, suggesting the route of synthesis is the largely the same for the two tissue systems.

Panelists began to correctly identify the odd sample at about the same time the declining pattern of *cis*-3-hexenal concentration could be discerned (Fig. 5). *Cis*-3-hexenal has been found to contribute strongly to the intensity of the aroma of apple and other fruits (Drawert et al., 1968, Buttery et al., 1987, Paillard, 1990, Baldwin et al., 1991, Matsui et al., 2000). Given that the

concentration of *cis*-3-hexenal in the cups was evidently many-fold higher than its detection threshold, it seems doubtful that the modest decline at this stage could be detected by the panelists, although this possibility should be further examined. The isomer *trans*-2-hexenal may contribute to aroma intensity based on its concentration relative to its odor threshold, which would be consistent with its linkage with apple-like aroma and its description as an odor-active compound in apple (Flath, 1967; Guadagni et al., 1971; Paillard, 1990: Panasiuk, 1980). The increase in *trans*-2-hexenal formation took place well after panelists could detect differences between non-ripening and ripening fruit, however.

Our findings that PCA discriminates volatiles as a function of ripening stages has been previously reported by Echeverria et al. (2004) and Reis et al. (2009), in which they showed volatiles from different harvest dates being grouped by maturity stages. However, both cited studies measured the volatiles in intact apples, and although Echeverria et al. (2004) considered disrupted tissue as juice extract they measured only acetaldehyde and ethanol with no comparable results to ours for C6 aldehydes and alcohols and their ester products. The high degree of association between aldehydes and unripe apples found in disrupted fruit tissue in our study by PCA is consistent with previous findings (Paillard, 1990) on whole fruit. The lower levels of hexanal and *trans*-2-hexenal produced in early stages of ripening are also consistent with previous results in crushed apples by Paillard (1986) and might explain why these aldehydes do not appear to be related with loadings of early time points. Both hexanal and *trans*-2-hexenal were present in quantities well above the human threshold such that panelists should have easily detected them, although this was not specifically tested. The high degree of association between esters and ripe apples in our study (i.e., later time points for normally-

ripening fruit preparations) is also consistent with previous findings on volatile emanations for whole fruit (Mattheis et al., 1991; Song and Bangerth 1994; Plotto et al., 2000).

The negative association between *cis*-3-hexenal and its ester *cis*-3-hexenyl acetate and ethylene in the scatter plot matrix (Fig. 7) suggests that these two volatiles may be negatively regulated by ethylene. Likely, the influence of ethylene would be primarily via the suppression of *cis*-3-hexenal, as *cis*-3-hexenyl acetate is derived from *cis*-3-hexenal. *Cis*-3-hexenal and *cis*-3-hexenol acetate were also the only two volatiles of those evaluated that were negatively correlated with correct sensory responses, suggesting that the decline of one or both may be related to the ability of the sensory panel to distinguish ripening from non-ripening fruit. Of the two, *cis*-3-hexenol acetate was detected at levels below the threshold of detection by humans and so seems less likely to have been important. It is also possible that *cis*-3-hexenal may mask other, ripening-related volatiles and its decline may unmask them. Conversely, the opposite may be true, as suggested by Plotto et al. (1999) for green-like attributes, which were perceived with higher intensities because the fruity notes (esters) were not as strong at the beginning of the ripening. It is also worth considering that volatiles we did not quantify would be more directly responsible for the shift in perception early in development. Further, while our results apply to olfactory perception, they may differ significantly from retronasal olfaction, which requires mastication and swallowing (Espinosa-Diaz, 2004), so results may have differed if the panelists had actually chewed the fruit.

The strong correlations between hexanal, hexanol, hexyl acetate, 2-methylbutyl acetate, and butyl acetate supports the supposition that each are somehow linked in the up-regulation of volatile biosynthesis associated with fruit ripening. Linkages may be at multiple levels in the biosynthetic pathways, but the increases in substrate (e.g. hexanal and hexanol) and product

(e.g., hexyl acetate) for some of these compounds suggests an upregulation in the biosynthetic pathways for ester precursors is important.

Conclusion

This study has shown an intriguing pattern of decline in *cis*-3-hexenal, which, to our knowledge, has not been reported in disrupted or macerated apples before. It is not disputed that the LOX pathway plays a major role in the production of this volatile following tissue disruption, but the increase in hexanal formation despite declining *cis*-3-hexenal formation offers an interesting paradox if the LOX pathway is considered to be the sole source of C6 aldehydes. It may well be that there is an alternate source of hexanal, leading to the formation of copious quantities of hexanol and hexyl esters during ripening. Characterization of genetic and enzymatic changes in the individual components of the LOX pathway is needed to help address this possibility. Another intriguing fact emerging from the current study is the high production of the isomer *trans*-2-hexenal, apparently from *cis*-3-hexenal, in ripening fruit. The data are consistent with an isomeration reaction driven by an as-yet undiscovered isomerase, which has important implications for changing flavor perceptions of masticated fruit during ripening. While our data here implicate unsaturated aldehydes in early changes in sensory perception of ripening fruit, more detailed sensory analysis is needed to test the validity of this proposition. Given that compounds may be present in sufficient amounts to be odor-active regardless of detection by the GC-MS (Plotto et al., 2000), additional GC olfaction investigations may be informative in this regard. Similarly, since odor-active compounds may interact with olfactory receptors to cause odor enhancement, suppression or addition (Plotto et al., 1998; Plotto and McDaniel, 2001) the use of flavor spiking (Baldwin et al., 2004) may be useful. Finally, it is incumbent upon us to

acknowledge that our sensory work is really quite limited in that it focused on the detection of initial changes in aroma and the underlying biology. Perhaps more important questions surround whether fruit have a desirable fruity or apple-like aroma and the biology controlling this feature, which would be of greater relevance to the consumer.

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CHAPTER III

RELATIONSHIP BETWEEN FREE AND ESTERIFIED FATTY ACIDS AND LOX-DERIVED VOLATILES DURING RIPENING IN APPLE

Introduction

In apple and other fruit, pathways for the synthesis of esters and other important aroma compounds are still being elucidated. Guadagni et al. (1971), in demonstrating that the major aroma compounds in apple fruit were esters, suggested that a "biochemical system" is involved and that the primary activity of this system is located in the peel rather than in the flesh of the fruit. Complicating our understanding of the pathways involved is the fact that some aroma compounds are produced autonomously, while others are induced, being synthesized in response to cellular disruption during mastication and cutting (Beaudry, 2000). Further, there is evidence that some of the volatiles are synthesized via anabolic pathways (Song and Bangerth, 2003; Sugimoto et al., 2011), while others are from catabolic pathways (Rowan et al., 1999).

In apple, it is known that the many autonomously produced esters increase with the ethylene burst during ripening (Guadagni et al., 1971; Mattheis et al., 1991; Fellman et al., 2000). However, volatiles induced by cellular disruption such as hexanal, *cis*-3-hexenal, and *trans*-2-hexenal, are produced before and after ripening, although their proportions vary as ripening progresses (Contreras and Beaudry, 2013). Thus, in the discussion of the formation of odor-active volatiles from fresh fruits and vegetables, it is important to distinguish between those processes engaged autonomously and those induced during food preparation and consumption. Curiously, fatty acid metabolism is thought to be involved in the biosynthesis of important aroma compounds in apple via both autonomous and induced routes.

Autonomously-produced aroma compounds for which fatty acid metabolism has been implicated include carboxylic acids, alcohols, aldehydes and, most importantly, esters, which are formed from the condensation of an alcohol and a Co-A derivative of a carboxylic acid by alcohol acyltransferase (AAT) (Ueda and Ogata, 1977). Both synthetic and catabolic processes

are capable of synthesizing the acids, alcohols and aldehydes that serve as ester precursors. Synthetic processes include the well-described 2-carbon elongation fatty acid synthetic pathway (Somerville et al., 2000), which is needed for the synthesis of the waxes and oils that accumulate in and on the cuticle of apple (Kolattukudy, 1966; Samuels et al., 2008). It has also been suggested that a one-carbon elongation fatty acid biosynthetic pathway may be active in apples during ripening (Sugimoto, et al., 2011). Catabolic processes include β-oxidation and the lipoxygenase (LOX) pathway (Sanz et al., 1997). In apple, β-oxidation produces acyl-CoAs that can be utilized to produce esters (Rowan et al., 1999); whereas the LOX pathway yields C-6 aldehydes, which are highly odor-active, but can also be converted to alcohols by alcohol dehydrogenase (ADH) and incorporated into esters (Tressl and Drawert, 1973; Fellman et al., 2000). Oxidation of fatty acids via the lipoxygenase (LOX) and β-oxidation pathways has been demonstrated in apple in various feeding studies (Paillard, 1979; Bartley et al., 1985; Rowan et al., 1997; Rowan et al., 1999) and has the potential to generate short to medium chain length aldehydes, alcohols and fatty acids for their incorporation into esters.

Volatiles induced by cellular disruption are overwhelmingly represented by C-6 aldehydes in apple and there is little disagreement that the LOX pathway is the primary route of their synthesis (Dixon and Hewett, 2000; Fellman et al., 2000; Contreras and Beaudry, 2013). Disruption of tissues permits the comingling of lipases, lipids and the enzymes of the LOX pathway, leading to the formation of aldehydes from free fatty acids (Siedow, 1991). Oxidation of linolenic acid (18:3) by LOX enzymes that form hydroperoxides at the 13-position (13-LOX) yields *cis*-3-hexenal, which in turn can undergo isomerization to *trans*-2-hexenal. Oxidation of linoleic acid (18:2) by 13-LOXs yields hexanal.

In apple, c*is*-3-hexenal production from disrupted tissue declines during ripening, even as the production of *trans*-2-hexenal and hexanal increases (Contreras and Beaudry, 2013). Given that *cis*-3-hexenal, *trans*-2-hexenal, and hexanal are all products of the LOX pathway and are likely liberated as a result of the activity of one or more of a family of 13-LOXs, their behavior is seemingly incongruous. How can *cis*-3-hexenal, the product of a 13-LOX reaction, decline while hexanal, also the product of 13-LOX, increase? This paradox has led to the hypothesis that the levels of the precursors upon which the 13-LOXs act, change in a ripening-dependent manner (Contreras and Beaudry, 2013). This is supported by previous analyses of fatty acids in apple (Galliard, 1968; Paillard, 1986; Song and Bangerth, 2003).

Whether the pathways involved are autonomous or induced, it can be expected that the composition of the fatty acids in the apple fruit will influence the profile of the acid, aldehyde, alcohol and ester emissions. In apple, most fatty acids are found esterified to polar lipids, a modest portion in the neutral lipids, and a very small portion comprises the free fatty acids (Meigh and Hulme, 1965; Christie, 2003). In the polar lipid fraction, the most abundant saturated and unsaturated fatty acids are palmitic (16:0) and linoleic acid (18:2), respectively (Paillard, 1990; Wang and Faust, 1992). Linolenic acid (18:3) is the predominant fatty acid in the monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), while linoleic acid is a common constituent in phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). Phosphatidylglycerol (PG) contains relatively more palmitic acid (16:0) (Wang and Faust, 1992). The content of phospholipids has been shown to increase slightly during ripening in apple; however, the rate of synthesis of phospholipids rises many-fold, suggesting synthesis is balanced to some degree by concomitant conversion of phospholipids to other cellular constituents (e.g., waxes and oils) (Galliard, 1968; Bartley, 1985).

LOX pathway activity has the potential to produce copious quantities of aldehydes in disrupted fruit tissues, but it is not clear whether the LOX pathway contributes significantly to autonomously-produced volatiles from intact fruit. In intact fruit, the LOX product *cis*-3-hexenal and its ester products are not reported (Contreras and Beaudry, 2013, Fellman et al., 2000). On the other hand, the production of hexanal, also a LOX product, increases with the onset of ripening, as does its product [via the alcohol dehydrogenase (ADH) reaction], hexanol and numerous hexyl esters. Thus, another seeming paradox exists. If LOX activity is responsible for the synthesis of C-6 precursors for esters in intact fruit, then how can hexanal, an 18:2 product, be produced without concomitant production of *cis*-3-hexenal from the abundant 18:3 fatty acids?

The answer may lie in the concentration and/or distribution of the fatty acids and 13-LOXs. Given that LOXs primarily act on free fatty acids rather than esterified fatty acids (i.e., mono-, di- and triacyl lipids) (Siedow, 1991), it seems likely that the selective production of hexanal can be explained by the selective production of 18:2 free fatty acids. This would infer the existence of a lipase with some specificity for hydrolysis of 18:2 relative to 18:3 from polar lipids or a decline in the rate of 18:3 formation. As yet, however, the link between the LOX pathway and autonomous ester formation has not been elucidated (Song and Bangerth, 2003).

The aim of this work is to further understand the temporal relationship between LOX substrates, their products, and the subsequent incorporation of these products into esters in both intact and disrupted tissues. We document changes in the pool of fatty acids found in the free (unesterified) and esterified (polar lipids only) fractions and relate these changes to the production of aldehydes, alcohols and esters expected to be derived from these fatty acids.

Materials and Methods

Plant material and developmental study. 'Jonagold' apples (*Malus x domestica* Borkh.) from a commercial orchard in Sparta, MI (43°06'13.9"N 85°41'56.0"W) were used to investigate the relationship between stage of development, volatile production, and the lipid profile and content of apple peels for intact fruit and for disrupted fruit tissue. The study was repeated in 2009, 2010, and 2012. The first harvest took place on 04 Sept. 2009, 08 Sept. in 2010 and 06 Sept. 2012. After the initial harvest, in 2009 and 2010, ripening fruit were harvested every three to four days, and in 2012 fruit were harvested on a weekly basis. Harvest continued until ripening was imminent as judged by two criteria: the average internal ethylene concentration (IEC) being greater than 0.1 μL L^{-1} and 3 to 4 fruit of 20-fruit sample having an IEC greater than 10 μL L^{-1}. At that time, (09 Oct. 2009, 01 Oct. 2010, and 17 Sept. 2012), approximately 400 additional fruit were harvested and thereafter allowed to ripen in a controlled environment chamber at 15 °C. This was done to avoid damage in the field due to freezing and fruit drop. These fruit were covered with plastic bags to minimize moisture loss and subsequently examined every three to four days in 2009 and 2010, and once per week in 2012 until the conclusion of the study on 27 Oct. (day 53) in 2009, 20 Oct. (day 42) in 2010 and 29 Oct. 2012 (day 53). One day prior to each assay date, fruit were moved to the laboratory to equilibrate to room temperature (22 ± 1 °C). During this adjustment period, fruit were covered with black plastic bags to exclude light and minimize moisture loss as described previously.

On each evaluation date, 40 apples were used for analysis. Maturity was assessed on 20 randomly selected fruit by measuring internal ethylene (μL L^{-1}) as described in Contreras and Beaudry (2013), and fruit weight (g), red skin coloration (%), background color (1-5 scale), firmness (N), soluble solids content (%), and starch index (1-8) according to Mir et al. (2001).

These data were used to assess the readiness of the fruit to ripen and accordingly schedule the final harvests as noted previously.

Ethylene, respiration rate and volatile analyses of whole and crushed apple tissue. For each assay date, the internal ethylene content of each of the remaining 20 fruit was measured and, of these, the 5 fruit having internal ethylene content nearest the median were selected for analysis of CO_2 production as described by Contreras and Beaudry (2013). After respiratory analysis, the inlet and outlet ports of the respirometer were sealed for 20 to 30 min and the accumulated volatiles were analyzed as described previously (Contreras and Beaudry, 2013). Following analysis of whole fruit volatiles, within the next 4 h, these same five fruit were subsequently used for volatile analysis of crushed slices (approx. 8 g each). Slices were removed from the fruit and crushed no more than 2 minutes prior to analysis as described by Contreras and Beaudry (2013). In 2012, no aroma volatiles were analyzed.

Lipid analysis. Free fatty acids and polar lipids were determined in apple peel tissue from ten representative fruit on each assay date in the 2009, 2010, and 2012 seasons. In 2009 and 2010, the ten fruit were those having internal ethylene nearest the ethylene median after those used for respiratory and volatile analysis were removed. In 2012, only five fruit were used, representing those closest to the median level of ethylene. From the fruit selected, the tissue was sampled by removing the epidermis and 2-3 mm of cortex with a manual rotary peeler (Model 8, Goodell, Antrim, NH), and immediately frozen in liquid nitrogen and stored at -80 °C. Tissue from the selected fruit was pooled and two biological replicates were created. Three technical replicates were analyzed per biological replicate.

Extraction. One gram per sample of frozen apple peel was ground to a powder in a mortar and pestle in liquid N_2, then the powder was added to 4 mL of boiling isopropanol and heated for 10 min at 75 °C. When samples were cooled, 20 µg of 17:0 free fatty acid (FFA), 250 µg of di 15:0 phosphatidylcholine and 50 µg of tri 13:0 triacylglycerol were added to each sample as internal standards. Lipids were extracted using hexane-isopropanol according to the method described in Hara and Radin (1978) and dried under nitrogen gas. Lipids were suspended in chloroform and stored at -20 °C.

Analysis. Polar and free fatty acids were separated by normal phase thin layer chromatography (TLC) using 200 mm by 200 mm TLC plates with 250 µm-thick silica coating (Particil K6, Whatman). Approximately 20% of each sample was loaded and developed in hexane:diethyl ether:acetic acid (70:30:1, v/v/v). TLC plates were sprayed with 0.01% primuline in 80% acetone and 20% water and visualized under UV-light. Lipids were identified by co-migration of commercial lipid standards. Polar lipids and free fatty acid were quantified as fatty acid methyl esters (FAMEs) as previously described via gas chromatography (Browse et al., 1986). Briefly, lipids were scraped from TLC plates into 7mL screw cap test tubes and 300 µL of toluene and 2 mL of 5% H_2SO_4 in methanol were added. Samples were incubated for 1.5 h at 75 °C. The resulting FAMEs were extracted using hexane containing 0.9% NaCl and dried down under nitrogen gas. FAMEs were resuspended in heptane and separated on a capillary column (DB-23, 30 m x 0.25 mm ID, 0.25µm film thickness, Agilent Technologies) using helium as carrier gas at a constant flow at 1.5 mL min^{-1}. The initial oven temperature was 140 °C for 3 min, followed by a 5 °C per minute increase until 230 °C, which was held for 3 min. The injector and detector were maintained at 250 °C throughout the analysis.

Experimental design and statistical analyses. For ethylene, respiratory and volatile analysis, the experimental unit was a single fruit. Means and standard deviations (error bars) from ethylene and CO_2 production were calculated over the 20 apples randomly selected as mentioned above. For the lipid analysis, means and standard deviations were calculated over the average of the two biological replicates mentioned above. In order to identify correlations or related pairs of free fatty acids and polar lipids against apple aromas, Pearson correlation coefficients were generated (JMP® Version 10.0, SAS, Cary, NC) for data from 2009 and 2010, with a level of significance of α=0.05.

Results and Discussion

Lipid analysis during apple ripening. Apple samples were collected at similar physiological stages each year of study, however, the 2010 and 2012 summer were warmer than in 2009, accelerating the fruit development. Internal ethylene concentration (IEC) steadily increased at the beginning of each season until the autocatalytic burst (Fig. 8). Autocatalytic ethylene production was fully engaged 12 Oct. 2009, 30 Sept. 2010 and 25 Sept. 2012 as evidenced by a 5- to 10-fold increase in the three or four days from the previous evaluations.

The climacteric peak for respiration was on 09 Oct. 2009, 29 Sept. 2010 and 25 Sept. 2012 with CO_2 production levels of 50.4, 44.6 and 37.2 mg kg^{-1} h^{-1} (Fig. 8), respectively, which are similar to those reported for other apples (Reid et al., 1973, Sharma et al., 2012). The form of the climacteric differed somewhat between years, being more compressed in the warmer 2010, but the rates of respiration before and after the climacteric were similar between years. The patterns of IEC, respiration and aroma production were similar to those reported for 'Golden Delicious' apples (Song and Bangerth, 2003).

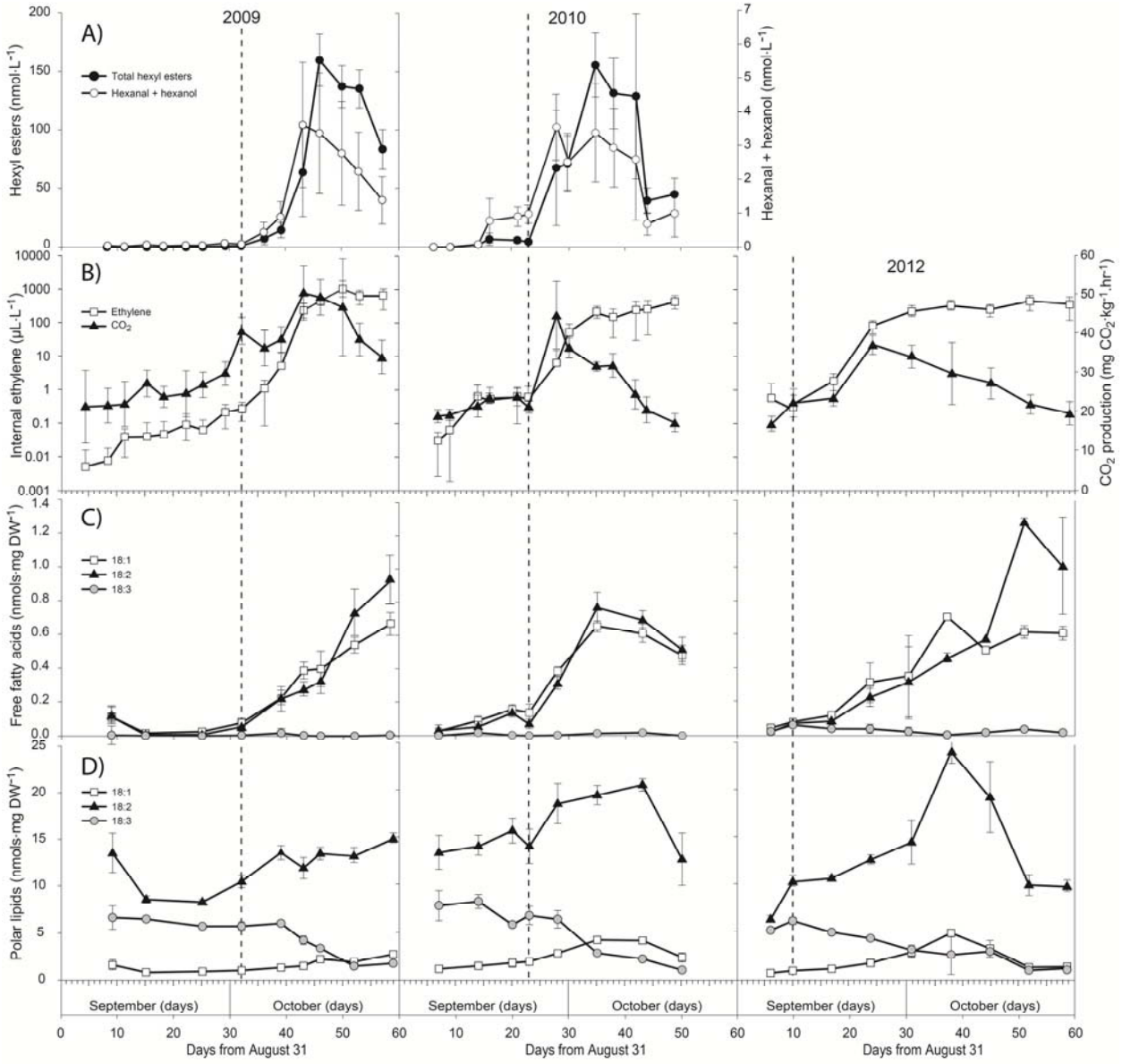

Figure 8. A) Emissions of hexyl esters and their precursors (the sum of hexanol and hexanal) from intact fruit; B) internal ethylene and CO_2 production rate; C) free fatty acids and D) polar lipids during ripening seasons of 2009, 2010 and 2012 in apple cv. 'Jonagold'. The vertical dashed line represents time when autocatalytic ethylene commenced or when internal ethylene exceeded 0.1 µL L-1. Vertical bars in the curves indicate the standard deviation.

Free fatty acids 18:1 (oleic) and 18:2 (linoleic acid) were initially very low (<0.15 nmol·mg^{-1} on a dry weight basis) in the preclimacteric fruit (mature, but non-ripening apple) in all three years (Fig. 8). Coincident with rising ethylene levels and about 7 to 10 d prior to the onset of the respiratory climacteric, the content of 18:1 and 18:2 in the free fatty acid fraction began to increase, reaching a maximum of 0.6 to 1.3 nmol mg^{-1}, an increase of approximately 9-fold for 18:1 and 14- to 20-fold for 18:2 from preclimacteric minimum levels. These data are consistent in their pattern relative to the respiratory climacteric compared to previously published analyses of free fatty acids in ripening 'Cox's Orange Pippin' (Meigh and Hulme, 1965) and 'Golden Delicious' (Song and Bangerth, 2003). Quantitatively, the data are similar with Meigh and Hulme (1965), but about 50% to 80% lower than levels reported by Song and Bangerth (2003). There was a slight change in the ratio of 18:1 to 18:2 throughout ripening in all studies. In the present study, 18:1 is in slight excess initially, and 18:2 is in slight excess late in ripening, which is similar to the study by Song and Bangerth (2003) and that of Mazliak (1960) for 'Calville blanc' fruit; Meigh and Hulme (1965) reported a 30% to 50% excess of 18:2 throughout ripening, which was similar to the ratio given by Davenport (1960) for 'Granny Smith' apples. These slight differences may result from the use of differing germplasm and/or the tissue types and methods used for extraction.

The level of 18:3 in the free fatty acid fraction was initially extremely low (0.005 to 0.02 nmol·mg^{-1}) and remained essentially unchanged throughout ripening, suggesting no link to ripening *per se* (Fig. 8). These results were similar to those for apple peel extractions from 'Cox's Orange Pippin' (Meigh and Hulme, 1965) and 'Golden Delicious' (Song and Bangerth, 2003) in which 18:3 was significantly lower than either 18:1 or 18:2 and underwent minor or no changes throughout ripening. These data contrast somewhat with early studies; Davenport

(1960) and Mazliak (1960) were unable to detect 18:3 in the free fatty acid fraction and Markley et al. (1935) found that 18:3 comprised a "very small amount" of the free fatty acids in pear (*Pyrus communis* L.).

Polar lipids were found to be in much greater abundance than free fatty acids present in apple peel. The 18:1 content of the polar lipids (0.7 to 5 nmol mg^{-1}) was roughly 10-fold greater than the 18:1 content of the free fatty acid fraction. The 18:2 content of the polar lipids (6 to 24 nmol mg^{-1}) exceeded that of the free fatty acid fraction 10- to 300-fold, with the ratio declining as ripening progressed. The 18:3 content of the polar lipids (0.8 to 8 nmol mg^{-1}) was typically 100- to 3000-fold higher than that of the 18:3 free fatty acid.

During ripening, the 18:1 and 18:2 contents of the polar lipids had similar patterns of either a continual increase (2009) or an increase, then decline (2010, 2012). Interestingly, the ratio of 18:2 to 18:1, which was initially 12, declined as ripening commenced, suggesting that there was preferential synthesis of 18:1 or mobilization of 18:2 or both. The ripening-related patterns for esterified 18:1 and 18:2 were similar to those in previous work (Meigh and Hulme, 1965, Song and Bangerth, 2003), with some differences in absolute concentrations. Generally, Meigh and Hulme (1965) had similar quantities of the fatty acids in the polar lipid fraction, but no change in the ratio of 18:2 to 18:1 during ripening and Song and Bangerth (2003) found much lower levels of both 18:2 and 18:1, but demonstrated a decline in the ratio of 18:2 to 18:1 similar to that reported here. Galliard (1968) found a three-fold increase in the 18:2 content of the monogalactosyl diglyceride (MGDG) fraction and a 50% increase if 18:2 in the digalactosyl diglyceride (DGDG) fraction in the pulp of ripe apples compared to preclimacteric fruit, which suggests that the rise in 18:2 content in polar lipids in our study was primarily due to shifts in the DGDG and MGDG fractions.

The 18:3 content of the polar lipid fraction declined 70% to 90% as ripening progressed, with the decline beginning coincident with autocatalytic ethylene production (Fig. 8) and coincident with a decline in the capacity of disrupted apple tissue to synthesize *cis*-3-hexenal (Contreras and Beaudry, 2013). The quantity of 18:3 in the polar lipid fraction was similar to that found by Meigh and Hulme (1965) in 'Cox's Orange Pippin', but much higher (3- to 10-fold) than that reported by Song and Bangerth (2003) for 'Golden Delicious'. No clear trend in ripening was evident in these studies. However, Galliard (1968) and Mazliak (1969) found a marked decline in the content of 18:3 in lipids of the pulp and skin of apple. Mazliak (1969) demonstrated this was primarily due to a reduction in the abundant DGDG, MGDG and phosphotidyl glycerol (PG) fractions, which underwent 80% to 90% reductions in their 18:3 content. Paillard (1986) also reported a decline in 18:3 fatty acids in epidermis and parenchyma with a slight peak at the onset of the climacteric. Mazliak (1969) noted that fatty acid biosynthesis was very active in ripening fruit, but that 18:3 synthesis seemed to be lower than that of other fatty acids.

In our study, the decline in the 18:3 content of the polar lipid fraction is likely due to a decrease in the 18:3 content in MGDG and DGDG, which are the two most abundant polar lipids in apple peel (Galliard, 1968; Mazliak, 1969). This shift suggests a loss in ω^3desaturase activity; however, Schaffer et al. (2007) found no decline in fatty acid desaturase genes linked with ethylene-induced ripening of apple. Galliard (1968) theorized that selective membrane degradation occurs during ripening, that is, plastid membranes disappear while mitochondria and the plasma membrane remain intact. Thus, the decrease of linolenic acid (18:3) would seem to be due to loss in structures rich in 18:3 containing polar lipids, rather than a decrease in rate of synthesis (Galliard, 1968).

The marked increase in the 18:1 and 18:2 content free fatty acids and polar fatty acids is suggestive of an enhancement of fatty acid synthesis during apple ripening. This interpretation is consistent with data demonstrating a sharply increased rate of fatty acid synthesis in ripening apple as evidenced by increased ^{14}C-acetate incorporation into lipids and waxes (Bartley, 1985; Galliard et al., 1968, Mazliak, 1969).

Intact fruit volatiles. The rise and fall in 18:1 and 18:2 free fatty acids parallels the rise and fall in the emission of esters from intact fruit containing hexyl moieties derived from hexanol and the rise and fall in hexanol and hexanal emissions (Fig. 8). In the current study, 18:1 and 18:2 free fatty acids were highly and positively correlated with hexanol and most of the esters quantified for intact fruit (Table 2). If free fatty acids serve as precursors for esters in intact fruit, it is anticipated that LOX pathway products would be hexanal and, via ADH, hexanol from 18:2 free fatty acids and *cis*-3-hexenal, *cis*-3-hexenol, *trans*-2-hexenal, and *trans*-2-hexenol from 18:3 free fatty acids. The noted strong correlations between 18:2 free fatty acids and hexanol and hexyl esters support this assertion, but are not conclusive. It may well be that there are multiple sources of hexanal and/or hexanol, including glycosidically-bound reservoirs, which have been found in ripe *Physalis peruviana* fruit (Mayorga et al., 2001).

Esters derived from hexanoic acid (only propyl-, butyl- and hexyl hexanoates were detected) also correlated well with 18:2 free fatty acids (Table 2). Hexanoate esters would ostensibly be derived from longer-chain free fatty acids via beta-oxidation (Paillard, 1979; Bartley et al., 1985; Rowan et al., 1997; Rowan et al., 1999). Synchrony in the induction of hexanol and hexanoate esters is consistent with the suggestion that these and other aroma-related pathways are engaged simultaneously by ethylene (Schaffer et al. 2007). Supporting this view,

the increase in free fatty acids in ripening apple can be suppressed by inhibitors of ethylene formation (Ju and Bramlage, 2001).

Table 2. Pearson correlation coefficients of free fatty acids and polar lipids with aroma volatiles for 2009 and 2010 years in intact apples cv. Jonagold. Only significant correlations are shown ($P<0.05$).

	Free Fatty acids			**Polar lipids**		
	18:1	**18:2**	**18:3**	**18:1**	**18:2**	**18:3**
2009						
Hexyl acetate	0.75	0.59	n.s.	0.78	0.56	-0.80
Hexyl propanoate	0.83	0.79	n.s.	0.91	0.61	-0.90
Hexyl 2-methylpropanoate	0.61	0.38	n.s.	0.59	0.44	-0.64
Hexyl butanoate	0.80	0.66	n.s.	0.83	0.57	-0.86
Hexyl 2-methylbutanoate	0.64	0.45	n.s.	0.67	0.45	-0.71
Hexyl pentanoate	0.89	0.80	n.s.	0.89	0.60	-0.95
Hexyl hexanoate	0.86	0.71	n.s	0.83	0.59	-0.89
Propyl hexanoate	0.82	0.94	n.s.	0.84	0.56	-0.84
Butyl hexanoate	0.87	0.81	n.s.	0.92	0.62	-0.93
2010						
Hexyl acetate	0.94	0.95	n.s.	0.97	0.82	-0.70
Hexyl propanoate	0.90	0.93	0.52	0.96	0.81	-0.71
Hexyl 2-methylpropanoate	0.51	0.45	n.s.	0.54	0.60	n.s.
Hexyl butanoate	0.93	0.93	0.43	0.98	0.89	-0.67
Hexyl 2-methylbutanoate	0.91	0.90	n.s.	0.96	0.89	-0.62
Hexyl pentanoate	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Hexyl hexanoate	0.94	0.95	0.45	0.98	0.85	-0.73
Propyl hexanoate	0.80	0.83	0.49	0.77	0.55	-0.83
Butyl hexanoate	0.93	0.94	0.47	0.93	0.75	-0.81
LOX products 2009						
Hexanal	0.93	0.90	n.s.	0.77	0.52	-0.91
Hexanol	0.75	0.53	n.s.	0.61	0.49	-0.73
LOX products 2010						
Hexanal	n.s.	n.s.	n.s.	n.s.	0.45	n.s.
Hexanol	0.91	0.86	n.s.	0.91	0.84	-0.61

Hexanal emissions were correlated with hexyl ester emissions in only one year. This may not be surprising since the capacity for hexanal conversion to hexanol by ADH is quite high

(DePooter et al., 1983; Drawert et al., 1973; Song et al., 1996) and the process may deplete the hexanal pool. Positive relationships between 18:2 linoleic acid and hexanal have been previously found by Paillard (1986), and these were highly associated with the respiratory climacteric.

The lack of an appreciable quantity of 18:3 in the free fatty acid fraction mirrors the absence of *cis*-3-hexenal, *cis*-3-hexenol, *trans*-2-hexenal, and *trans*-2-hexenol and their esters from the emissions of intact 'Jonagold' apple fruit (Contreras and Beaudry, 2013). In that 18:3 was abundant in the polar lipid fraction, the lack of these unsaturated volatiles in the emissions of intact apple fruit indirectly supports the contention that the free fatty acids, rather than esterified fatty acids, act as substrates for the generation of LOX products and the resulting hexanol and hexyl esters in intact fruit. Further, the negative correlations between the 18:3 polar lipids and ester emissions strongly suggests that esterified fatty acids do not contribute meaningfully to autonomous ester production in intact fruit. It is notable that *cis*-3-hexenol acetate, produced at low levels by crushed fruit, was not detected in emissions from intact fruit in this study. This is consistent with previous publications detailing volatiles detected from apples and apple products (Dimick and Hoskin, 1983; Yahia, 1994).

The mechanism allowing the accumulation of 18:1 and 18:2, but not 18:3, in the free fatty acid fraction is unclear. Our understanding of lipid metabolism suggests that these fatty acids would be released from glycerolipids by the action of a lipase, but no lipase specific for the hydrolysis of 18:1 and 18:2 relative to 18:3 has been reported. On the contrary, there are reports of lipases that favor the sn-1 or sn-2 positions (Mansfeld and Ulbrich-Hofmann, 2007; Bonaventure et al., 2011), rare cases of lipases partially specific for fatty acids with *cis*-9 configuration (Borgdorf and Warwel, 1999; Warwel and Borgdorf, 2000), lipases more active on

tri- than di- or monoacylglycerol species (Lin et.al., 1986; Sakaki et al., 2007), and the preference of lipase for galactolipids versus phospholipids (Mansfeld and Ulbrich-Hofmann, 2007, Sakaki et al., 2007).

Most assessments of available data have favored the hypothesis that esters in ripening fruit are synthesized from products of cellular degradation and disassembly (Paillard, 1986; Rowan et al., 1999; Dixon and Hewett, 2000). This reflects early thinking on ripening and senescence presented by Galliard (1968), who noted that early theories of ripening and senescence being linked to a loss in membrane integrity were being countered by mounting evidence to the contrary. Galliard (1968) concluded the new evidence indicated that the process of ripening required "an initial synthetic phase at the onset of ripening, during which specific enzymes are synthesized, cellular processes are under strict control and at least some cell organelles, e.g. mitochondria, retain their structure and activity." Similarly, and with specific reference to ester formation from lipids, Song and Bangerth (2003) posited that the synthesis of fatty acids is required for volatile ester production during ripening. These authors suggested a sequence of metabolic events consisting of changes in respiratory rate, leading to an increase in the availability of chemical energy in the form of ATP and requisite carbon skeletons, most importantly, acetyl Co-A, enabling and facilitating *de novo* fatty acid biosynthesis. The lack of unsaturated products of the LOX pathway produced by intact fruit in this study supports this view. Even hexanal, which we found at very low levels (Contreras and Beaudry, 2013), is often missing from the volatile profile of intact fruit (Fellman et al., 2000). Further, given the fact that biosynthesis of C-16 and C-18 fatty acids is known to increase during fruit ripening (Bartley, 1985; Galliard et al., 1968; Mazliak, 1969) and accumulate in the epicuticular waxes of ripening

apple fruit (Veraverbeke, 2004), the suggestion by Song and Bangerth (2003) would appear to have some merit.

Crushed fruit volatiles. The decline in 18:3 polar lipids (Fig. 8) is similar to the declining pattern of formation of *cis*-3-hexenal and *cis*-3-hexenyl acetate in crushed fruit reported by Contreras and Beaudry (2013). Given that the 18:3 content of the free fatty acid fraction is stable and near nil, and that the content of neutral lipids is low in apple (Mazliak, 1969), our data suggests that the synthesis of *cis*-3-hexenal is largely linked to the availability of 18:3 in the polar lipid fraction. Supporting this are the high correlations between 18:3 content of the polar lipids and emissions of *cis*-3-hexenal, *cis*-3-hexenol, and *cis*-3-hexenyl acetate (Table 3).

The contrasting correlations with ester emissions for *cis*-3-hexenal and *trans*-2-hexenal are interesting given that *cis*-3-hexenal is thought to spontaneously isomerize to *trans*-2-hexenal (Phillips et al., 1979; Takamura and Gardner, 1996; Noordermeer et al., 1999; Allmann and Baldwin, 2010). Conversely, it has been proposed that a ripening-induced isomerase may facilitate this interconversion (Contreras and Beaudry, 2013), which would help to explain the highly correlated relationship between *trans*-2-hexenal and other ripening induced volatiles. If the source of the *cis*-3-hexenal is largely from the polar lipids, previously published work allows us to speculate which subcellular organelles serve as the source of the 18:3 polar lipids that give rise to *cis*-3-hexenal. In most green plant tissues, the chloroplast is typically the richest source of lipids (Somerville et al., 2000). The plastids and microsomal membranes are enriched in MGDG and DGDG in bell pepper (*Capsicum annuum* L.) fruit pericarp (Whitaker, 1991). In spinach, 70% of the lipids in the chloroplast correspond to MGDG and DGDG (Block et al.,

Table 3. Pearson correlation coefficients of free fatty acids and polar lipids with aroma volatiles for 2009 and 2010 years in crushed apples cv. Jonagold. Only significant correlations are shown ($P<0.05$).

	Free Fatty acids			Polar lipids		
	18:1	**18:2**	**18:3**	**18:1**	**18:2**	**18:3**
2009						
Hexyl acetate	0.80	0.63	[z] n.s.	0.79	0.55	-0.85
Hexyl propanoate	0.79	0.69	n.s.	0.86	0.56	-0.87
Hexyl 2-methylpropanoate	0.27	0.10	n.s.	0.44	n.s.	n.s.
Hexyl butanoate	0.62	0.42	n.s.	0.66	0.43	-0.70
Hexyl 2-methylbutanoate	0.71	0.52	n.s.	0.71	0.48	-0.77
Hexyl pentanoate	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Hexyl hexanoate	0.69	0.56	n.s.	0.78	0.50	-0.78
Propyl hexanoate	0.76	0.91	0.26	0.74	0.50	-0.75
Butyl hexanoate	0.75	0.64	n.s.	0.83	0.54	-0.83
2-Methylbutyl acetate	0.89	0.78	n.s.	0.88	0.60	-0.93
2010						
Hexyl acetate	0.89	0.91	0.41	0.96	0.85	-0.63
Hexyl propanoate	0.84	0.88	0.58	0.93	0.82	-0.66
Hexyl 2-methylpropanoate	0.56	0.57	n.s.	0.41	n.s.	-0.79
Hexyl butanoate	0.79	0.71	n.s.	0.78	0.78	n.s.
Hexyl 2-methylbutanoate	0.85	0.80	n.s.	0.88	0.86	n.s.
Hexyl pentanoate	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Hexyl hexanoate	0.47	0.49	0.60	0.58	0.59	n.s.
Propyl hexanoate	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Butyl hexanoate	0.82	0.86	0.51	0.80	0.56	-0.85
2-methylbutyl acetate	0.81	0.76	n.s.	0.76	0.64	-0.52
LOX products 2009						
Cis-3-hexenyl acetate	-0.72	-0.68	n.s.	-0.63	n.s.	0.79
Cis-3-hexenal	-0.85	-0.71	n.s.	-0.69	-0.53	0.86
Hexanal	0.89	0.78	n.s.	0.86	0.59	-0.91
Trans-2-hexenal	0.68	0.44	-0.27	0.52	0.40	-0.69
Hexanol	0.87	0.69	n.s.	0.70	0.54	-0.85
Cis-3-hexanol	n.s.	-0.44	-0.27	n.s.	n.s.	0.25
Trans-2-hexanol	0.92	0.87	n.s.	0.92	0.67	-0.96
LOX products 2010						
Cis-3-hexenyl acetate	-0.69	-0.62	-0.26	-0.64	-0.48	0.62
Cis-3-hexenal	-0.95	-0.91	n.s	-0.86	-0.61	0.89
Hexanal	0.64	0.71	0.47	0.68	0.58	-0.51
Trans-2-hexenal	0.70	0.66	0.41	0.77	0.82	n.s.
Hexanol	0.88	0.85	0.42	0.95	0.89	-0.54

Table 3 (cont'd).

	Free Fatty acids			Polar lipids		
	18:1	**18:2**	**18:3**	**18:1**	**18:2**	**18:3**
LOX products 2010						
Cis-3-hexanol	n.s.	n.s.	0.29	n.s.	0.35	0.41
Trans-2-hexanol	0.46	0.34	n.s.	0.45	0.58	n.s.

1983; Somerville et al., 2000). Mitochondria, on the other hand, are much richer (approx. 80%) in the phosphoglycerides phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Bligny and Douce, 1980; Somerville et al., 2000). In barley (*Hordeum vulgare* L.), the lipid composition of the membrane fractions of the endoplasmic reticulum (ER), golgi and plasma membranes are rich in PC and PE (Brown and DuPont, 1989). PC and PE, while abundant in apple fruit, have relatively low levels of 18:3 (~12 to 15%) and the content of 18:3 in PC and PE does not change during ripening (Mazliak, 1969). On the other hand, MGDG and DGDG, are rich in 18:3, but undergo extensive depletion (50% to 70%) in amount while simultaneously undergoing a >20% loss in 18:3 content (Galliard, 1968; Mazliak, 1969). Paillard (1986) observed a correlation between total 18:3 content and degreening or chlorophyll loss, where linolenic acid decreased over a period of 50 days at 20 °C as chloroplast disassembly occurs during ripening. Collectively, these data are consistent with *cis*-3-hexenal generated following cellular disruption in apple being largely plastidic in origin. Interestingly, the bulk of the C-6 aldehydes produced during masceration in tomato are formed via the action of only one member of the small tomato LOX family, which, perhaps not coincidentally, is located in the plastid (Chen et al., 2004).

It is difficult to ascribe hexanal emissions from disrupted apple tissue to a particular organellar source. Hexanal emissions, as described by Contreras and Beaudry (2013), rise about 2- to 3-fold in concert with the rise in the 18:2 content of free fatty acids and polar lipids and the aldehyde is highly correlated with the 18:2 content of both lipid pools (Table 3). In that the polar lipids were far more abundant than the free fatty acids, they would seemingly be more likely to contribute significantly to hexanal emissions from crushed tissues. Both Mazliak (1969) and Galliard (1968) found that the 18:2 content of PC increased by 20% in apple. The PC fraction, which is largely non-plastidic in origin, accounts for approximately 25% of total lipids in apple and contains the greatest portion of 18:2 in the apple skin and flesh (Galliard, 1968; Mazliak, 1969). While the 18:2 content of MGDG and DGDG, which comprise the greatest portion of plastid lipids, increases by approximately 30% with ripening, total MGDG and DGDG levels decline sharply. Thus, the data do not suggest a particular organelle is the primary source of hexanal.

Conclusion

The work successfully demonstrates high correlations between polar lipid composition and the formation of *cis*-3-hexenal and hexanal in disrupted tissues and supports the contention that polar lipids are the predominant source of these highly odor-active aldehydes. The data also reveal a strong correlation between the free fatty acid composition and the formation of hexanal and its metabolites (hexanol and an array of hexyl esters) in intact fruit. The data suggest that ripening-dependent changes in the composition of free fatty acid and lipid pools are primarily responsible for changing profiles for several important odorants in both intact and disrupted tissue systems in apple fruit.

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CHAPTER IV

LIPOXYGENASE GENE EXPRESSION

Introduction

In the last decade, the lipoxygenase gene family has been thoroughly studied in a large number of plant species. Sequenced plant genomes have evidenced a large and expanded LOX family, for example cucumber has 23 LOX members, 15 in papaya, 21 in poplar, 18 in grapevine, 15 in rice and 18 in strawberry (Huang et al., 2009). The apple lipoxygenase family is not the exception with 23 putative LOXs found by Vogt et al. (2013). It has been suggested that the LOX family expansion may be the result of a genome-wide duplication (GWD) event, and this may be true for the domesticated apple *Malus* x *domestica* (Giovannoni et al., 2010). For cucumber and soybean, however, GWD is unlikely and other mechanisms such as tandem duplication might be playing a major role (Liu et al, 2011).

Although more than 20 putative apple LOX sequences have been identified in apple, little is known about the physiological importance, and the specific function of each isoform (Vogt et al., 2013). Park et al (2006), in their apple EST sequence analysis, were the first to report an apple LOX cluster (MD187410) overrepresented in apple cortex, with a LOX candidate gene possibly linked to volatile synthesis. Schaffer et al (2007) in a microarray in 'Royal Gala' apples found 11 LOXs, of these, 2 were ethylene-regulated. Sugimoto et al. (2008) in a microarray analysis in 'Jonagold' apples found a LOX gene (MD154490) which was highly upregulated and strongly linked to ester production during ripening. Dunemann et al (2009) mapped the LOX gene candidate, mentioned by Park et al (2006), which was genetically associated with a QTL cluster for ester-type volatiles. Later, Vogt et al. (2013) reported the QTL analysis linked to seven esters and the aldehyde hexanal. As a result, they found 23 putative functional LOXs, and 2 of these (*MdLox1a* and *MdLox5e*) were identified as gene candidates to be involved in fruit

aroma volatile production in apple. Together, these studies would suggest that 1 or 2 LOXs are strongly involved in apple aroma formation and they are ripening-dependent.

The LOX family is divided in 2 main groups based on the reactions they catalyze: the 9-LOX and the 13-LOX group. This classification operates according substrate specificity, that is, a LOX acting at the C-9 position of the fatty acid is termed a 9-lipoxygenase (9-LOX) and a LOX acting at the C-13 position is termed a 13-lipoxygenase (13-LOX). These reactions positions of the fatty acid substrate will generate 9- and 13-hydroperoxides (HPODs), respectively (Feussner and Wasternack, 2002). Generally, 13-LOXs exhibit a >95% preference for C-13 lipoxygenation, rather than lipoxygenation at C-9 position of a C-18 fatty acid, such as linolenic (18:3) or linoleic acid (18:2) (Feussner and Wasternack, 2002).

Feussner and Wasternack (2002) classified two subfamilies within the plant 13-LOX group: *type 1*-LOXs and *type 2*-LOXs. Those enzymes without a specific transit peptide and have a high sequence homology to each other (>75%) are called *type 1*-LOXs, whereas those enzymes possessing a putative chloroplast transit peptide sequence (cTP) and have a moderate homology to each other (~35%) are classified as *type 2*-LOXs. No similar cTP sequence is found in the 9-LOX genes (Royo et al., 1996, Feussner and Wasternack, 2002).

However, LOXs with probable dual reaction specificity (possessing both 9- and 13- LOX activity) have been previously reported (Porta and Rocha-Sosa, 2002; Palmieri-Thiers et al., 2009; Acosta et al. 2009). Acosta et al. (2009), in jasmonic acid biosynthesis related to sex determination in maize, reported that *TS1* lipoxygenase with 13-regiospecificity showed a production of 50:50 of 9- and 13-hydroperoxides. This dual role has not been previously described for a plastid-localized lipoxygenase, although the authors of this report do not overlook the possibility that this particular lipoxygenase probably acts with a separate 9-LOX

simultaneously. On the other hand, *PpLox3,* a 9-LOX identified in peach carries a plastid signal peptide in its N-terminal; however, the authors did not characterize the protein to determine regiospecificity (Han et al., 2011).

Back in the 1970s, it was unclear if the production of aldehydes was coming from linolenic acid and an 'enzyme system' was then proposed (Hatanaka and Harada, 1973). Hatanaka et al. (1978) provided evidence that the proposed 'enzymatic machinery' might be located in the chloroplast lamellae of green leaves. Nowadays, it is known that plant lipoxygenases are located in diverse compartments of the cell: in the cytosol, chloroplast, mitochondria and vacuoles (Baysal and Demirdoven, 2007), and also in peroxisomes, lipid bodies, plasma membrane and microsomal membranes (Liavonchanka and Feussner, 2006). The LOX enzymes in the chloroplast are thought to be exclusively 13-LOXs. In tomato, the chloroplastic *type 2* 13-LOX TomLoxC has been shown to be responsible for the synthesis of odor-active C-6 aldehydes in disrupted tomato fruit tissues (Chen et al., 2004). The majority of *type 2* LOXs and other enzymes that catalyze the synthesis of a variety of fatty acid derivatives are located in the stroma (Feussner and Wasternack, 2002). Conversely, Blee and Joyard (1996) reported that, in spinach, several lipoxygenases are located in the chloroplast envelope membranes.

It has been suggested that two important functions of 13-LOX (chloroplastic targeted) are (1) the conversion of lipid hydroperoxides to jasmonic acid cyclic precursors and derivatives such as traumatin and methyl jasmonate in intact tissues (Bell et al., 1995, Arimura et al., 2009), and (2) the synthesis of C-6 volatile compounds following cellular disruption (Chen et al., 2004). Hexanal and cis-3-hexenal are the primary aldehydes produced by 13-LOX following its action on linoleic and linolenic acid, respectively. The function of 9-LOX is less clear. The produced 9-

LOX hydroperoxides are converted to compounds whose physiological functions are not totally understood, although there is strong evidence of having an antimicrobial function (Siedow, 1991). This might explain that the major activity in many plant species is 9-LOX (Royo et al., 1996; Kolomiets et al., 2001; Chen et al., 2004); for example in tomato the majority of 9-HPOs (fatty acid hydroperoxides) formed by lipoxygenase activity are 9- isomers which are not directly involved in generation of C-6 flavor volatiles (Chen et al., 2004).

The identification, biochemical characterization and gene expression of many family LOXs have been well documented (Feussner and Wasternack, 2002), but validated protein function of LOXs and the physiological significance of LOX in plants remains to be determined for many species (Griffiths et al., 1999). In *Arabidopsis thaliana*, six LOX genes have been characterized: *AtLox1, 2, 3, 4, 5* and *6* (http://www.arabidopsis.org; Bannenberg et al., 2009). Of these, *AtLox2, 3, 4,* and *6* have a 13-LOX function, and they are required for the wound-induced synthesis of jasmonic acid (Chauvin et al., 2013). In tomato fruits, at least six lipoxygenases are present: *TomLoxA, B, C, D, E,* and *F* (Chen et al., 2004; Mariutto et al., 2011). Of these, *TomLoxC* and *D* are known to be chloroplast targeted and encode 13-LOX enzymes, and only *TomLoxC* has an impact on aroma profiles of the disrupted fruit tissue producing C-6 and C-5 volatile compounds when expression levels are reduced (Heitz et al., 1997; Griffiths et al., 1999; Chen et al., 2004; Shen et al., 2014). LOX genes characterization in other important plant species includes soybean (Hayashi et al., 2008; Lenis et al., 2010), rice (Acosta et al., 2009), potato (Royo et al., 1996), tobacco (Halitschke and Baldwin, 2003), barley (van Mechelen et al., 1999), eggplant (Pérez-Gilabert et al., 2001), pepper (Hwang and Hwang, 2010) among many others with substantial sequence information in updated databases. In regards to fruit crops, eleven putative LOX sequences were identified in apple in a microarray analysis by Schaffer et al.

(2007), and 23 putative LOXs were identified from the apple genome database using published ESTs for putative LOXs wich were used as query sequences by Vogt et al. (2013). To the best of our knowledge, none of the proteins have been characterized. In kiwifruit, 6 putative LOXs were identified and expressed during fruit ripening (*AdLox 1, 2, 3, 4, 5* and *6*) (Zhang et al., 2006); according to this study *AdLox1, AdLox3*, *AdLox4*, and *AdLox6* are grouped into the 13-LOX family. Mita et al. (2001) reported the first study in which a lipoxygenase has been characterized in the rosaceae family. *Lox1:Pd:1* a 9-LOX in almond, has activity associated with mitotic activity in young developing tissues and highly expressed in seeds.

As for protein characterization and functionality assay, several plant species with economical importance have been studied among them: rice (Ren et al., 2008), maize (Osipova, et. al. 2010), lentil (Hilbers et al., 1996), tomato (Hu et. al., 2011) and potato (Royo et al., 1996). However for fruit crops, much less information is available. From more to less information: olive (Palmieri-Thiers et al., 2009; Padilla et al., 2009), almond (Santino et al., 2005) and peach (Tian et al., 2011). Most of the LOXs characterized have been expressed in bacteria *E. coli* except for *TomloxD* (tomato lipoxygenase) which was expressed in yeast (Hu et al., 2011).

Materials and Methods

Phylogenetic tree and protein sequence aligment of the N-terminal. Twenty-two lipoxygenase gene full-length sequences were retrieved from the apple genome (Velasco et al., 2010), in the Genome Database for Rosaceae (GDR), to identify putative LOX gene candidates that might participate in the aroma production in apple. A Basic Local Alignment Search Tool (BLAST) search was performed using the 'Apple genome v1.0 predicted CDS' database. Alignment for the phylogenetic analysis was calculated with ClustalX2.1 (Larkin et al., 2007). The protein

alignments were then exported to MEGA5 (Kumar et al, 2008; Tamura et al., 2011), and evolutionary analyses were calculated with default parameters (Hall, 2011). The same software package was used to generate an unrooted phylogenetic tree based on the alignments obtained from the neighbour-joining method. The clustering included 137 aminoacid sequences from plant species (Table 4). Additionally, in order to compare the N-terminal of the lipoxygenase genes and to determine the presence of a signal peptide, the alignment of the N-terminal of the 22 lipoxygenases and other 26 protein sequences were drawn using Geneious version R7 (http://www.geneious.com/).

Table 4. List of sequences used for alignment and phylogenetic tree analysis. 137 aminoacid sequences are listed below.

Organism	Sequence ID	GenBank Accession	Accession N#	Source
1.Actinidia deliciosa (Kiwifruit)(1)	AdLox2	ABF60002	DQ497797	NCBI
2. Arabidopsis thaliana (6)	AtLox1	AT1G55020	AAA32827	NCBI
	AtLox2	AT3G45140	AAA32749	NCBI
	AtLox3	AT1G17420	AT1G17420	NCBI
	AtLox4	AT1G72520	AT1G72520	NCBI
	AtLox5	AT3G22400	AT3G22400	NCBI
	AtLox6	AT1G67560	AT1G67560	NCBI
3. Carica papaya (13)	-	-	Cpa evm.model.supercontig_8.58	Phytozome V5.0
	-	-	Cpa evm.model.supercontig_8.60	Phytozome V5.0
	-	-	Cpa evm.model.supercontig_17.119	Phytozome V5.0
	-	-	Cpa evm.model.supercontig_25.128	Phytozome V5.0
	-	-	Cpa evm.model.supercontig_32.35	Phytozome V5.0
	-	-	Cpa evm.model.supercontig_32.64	Phytozome V5.0

Table 4. (cont'd).

	-	-	Cpa evm.model.supercontig_43.30	Phytozome V5.0
	-	-	Cpa evm.model.supercontig_48.63	Phytozome V5.0
	-	-	Cpa evm.model.supercontig_58.126	Phytozome V5.0
	-	-	Cpa evm.model.supercontig_58.127	Phytozome V5.0
	-	-	Cpa evm.model.supercontig_458.2	Phytozome V5.0
	-	-	Cpa evm.model.supercontig_458.4	Phytozome V5.0
	-	AAR84664	Cpa emv. TU.supercontig_ 27.29	NCBI
4. Cucumber sativus (22)	-	-	Csa006732	Cucumber Genome Datab
	-	-	Csa007837	Cucumber Genome Datab
	-	-	Csa006736	Cucumber Genome Datab
	-	-	Csa006735	Cucumber Genome Datab
	-	-	Csa022478	Cucumber Genome Datab
	-	-	Csa013924	Cucumber Genome Datab
	-	-	Csa006734	Cucumber Genome Datab
	-	-	Csa006733	Cucumber Genome Datab
	-	-	Csa019335	Cucumber Genome Datab

Table 4. (cont'd).

	-	-	Csa009893	Cucumber Genome Datab
	-	-	Csa000832	Cucumber Genome Datab
	-	-	Cucsa.065240	Phytozome V5.0
	-	-	Cucsa.065250	Phytozome V5.0
	-	-	Cucsa.065260	Phytozome V5.0
	-	-	Cucsa.091390	Phytozome V5.0
	-	-	Cucsa.153610	Phytozome V5.0
	-	-	Cucsa.153620	Phytozome V5.0
	-	-	Cucsa.153630	Phytozome V5.0
	-	-	Cucsa.153640	Phytozome V5.0
	-	-	Cucsa.153650	Phytozome V5.0
	-	-	Cucsa.153660	Phytozome V5.0
	-	-	Cucsa.153670	Phytozome V5.0
5. Glycine max	GmLox1	AAA33986	P08170	NCBI
(Soybean)(7)	GmLox2	AAA33987	P09439	NCBI
	GmLox3	CAA31664	P09186	NCBI
	GmLox4	-	P38417	NCBI

Table 4. (cont'd)

	GmLox5	AAB67732	-	NCBI
	GmLox6	AAA96817	-	NCBI
	GmLox7	-	P24095	NCBI
6.Lycopersicon esculentum	TomLoxA	AAA53184	-	NCBI
(Tomato)(5)	TomLoxB	AAA53183	-	NCBI
	TomLoxC	AAB65766	-	NCBI
	TomLoxD	AAB65767	-	NCBI
	TomLoxE	AAG21691	-	NCBI
7. Malus domestica	Lox1	-	MDP0000874800	GDR
(Apple)(22)	Lox2	-	MDP0000154668	GDR
	Lox3	-	MDP0000215405	GDR
	Lox4	-	MDP0000224150	GDR
	Lox5	-	MDP0000300321	GDR
	Lox6	-	MDP0000277666	GDR
	Lox7	-	MDP0000211556	GDR
	Lox8	-	MDP0000174168	GDR
	Lox9	-	MDP0000281525	GDR

Table 4. (cont'd).

	Lox10	-	MDP0000753547	GDR
	Lox11	-	MDP0000864970	GDR
	Lox12	-	MDP0000272843	GDR
	Lox13	-	MDP0000755511	GDR
	Lox14	-	MDP0000257474	GDR
	Lox15	-	MDP0000264666	GDR
	Lox16	-	MDP0000923670	GDR
	Lox17	-	MDP0000172092	GDR
	Lox18	-	MDP0000423544	GDR
	Lox19	-	MDP0000312397	GDR
	Lox20	-	MDP0000450991	GDR
	Lox21	-	MDP0000146677	GDR
	Lox22	-	MDP0000135993	GDR
8. Nicotiana attenuata (3)	NaLox1	AAP83136	-	NCBI
	NaLox2	AAP83137	-	NCBI
	NaLox3	AAP83138	-	NCBI

Table 4. (cont'd).

9.Nicotiana tabacum (Tobacco)(1)	NtLox1	CAA58859	-	NCBI
10.Oriza sativa (Rice)(14)	OsLOX1	Q76I22	LOC_Os03g49260	NCBI & Phytozome
	OsLOX2	P29250	LOC_Os03g52860	NCBI & Phytozome
	OsLOX2.3	Q6H7Q6	LOC_Os02g10120	NCBI & Phytozome
	OsLOX3	Q7G794	LOC_Os03g49350	NCBI & Phytozome
	OsLOX4	Q53RBO	LOC_Os03g49380	NCBI & Phytozome
	OsLOX5	Q7XV13	LOC_Os04g37430	NCBI & Phytozome
	OsLOX6	Q8H016	LOC_Os03g08220	NCBI & Phytozome
	OsLOX7	P38419	LOC_Os08g39840	NCBI & Phytozome
	OsLOX8	Q84YK8	LOC_Os08g39850	NCBI & Phytozome
	OsLOX9	Q0IS17	LOC_Os011g36719	NCBI & Phytozome
	OsLOX10	Q0DJB6	LOC_Os05g23880	NCBI & Phytozome
	OsLOXRC11	Q9FSE5	LOC_Os012g37260	NCBI & Phytozome
	-	-	LOC_Os12g37350	Phytozome V5.0
11. Physcomitrella patens (Moss)(1)	Lox1F	F14C21.54	-	NCBI

Table 4. (cont'd).

12. Prunus armeniaca (Apricot)(1)	-	ABZ05753.1	EU439430	NCBI
13. Prunus dulcis (Almond)(2)	PdLox1	AJ404331	CAB94852	NCBI
	-	CAD10779.2	-	NCBI
14. Prunus persica (Peach)(16)	PpLox1	EU883638	ppa001082m	NCBI & GDR
	PpLox2	FJ029110	ppa001207m	NCBI & GDR
	PpLox3	FJ032015	ppa001016m	NCBI & GDR
	-	-	ppa000968m	GDR
	-	-	ppa001634m	GDR
	-	-	ppa001631m	GDR
	-	-	ppa001316m	GDR
	-	-	ppa001064m	GDR
	-	-	ppa001085m	GDR
	-	-	ppa001112m	GDR
	-	-	ppa001287m	GDR
	-	-	ppa001293m	GDR
	-	-	ppa001311m	GDR

Table 4. (cont'd).

	-	-	ppa001216m	GDR
	-	-	ppa026489m	GDR
	-	-	ppa002308m	GDR
15. *Solanum tuberosum*	StLox1	CAA64765	-	NCBI
(Potato)(5)	StLox2	AAD09202	-	NCBI
	StLox3	AAB67865	-	NCBI
	StLox4	CAA65268	-	NCBI
	StLox5	CAA65269	-	NCBI
16. *Vitis vinifera*	-	CAO17594	XP_002280651	NCBI & Phytozome
(Grape)(16)	-	-	XP_002284535	NCBI & Phytozome
	-	-	XP_002285574	NCBI & Phytozome
	-	-	XP_002278007	NCBI & Phytozome
	-	-	XP_002283135	NCBI & Phytozome
	-	-	XP_002273258	NCBI & Phytozome
	-	-	XP_002283123	NCBI & Phytozome
	-	-	XP_002283147	NCBI & Phytozome
	-	-	XP_002263854	NCBI & Phytozome

Table 4. (cont'd).

	-	-	XP_002283166	NCBI & Phytozome
	-	-	XP_002275268	NCBI & Phytozome
	-	-	XP_002265505	NCBI & Phytozome
	-	CAO21959	-	NCBI & Phytozome
	-	CAO40876	-	NCBI & Phytozome
	-	CAO15102	-	NCBI & Phytozome
	-	CAO21972	-	NCBI & Phytozome

RNA isolation and reverse transcription PCR (RT-PCR). Data for ethylene, respiration and volatile production was used to identify critical stages of development for *LOX* expression analysis (Fig. 9).

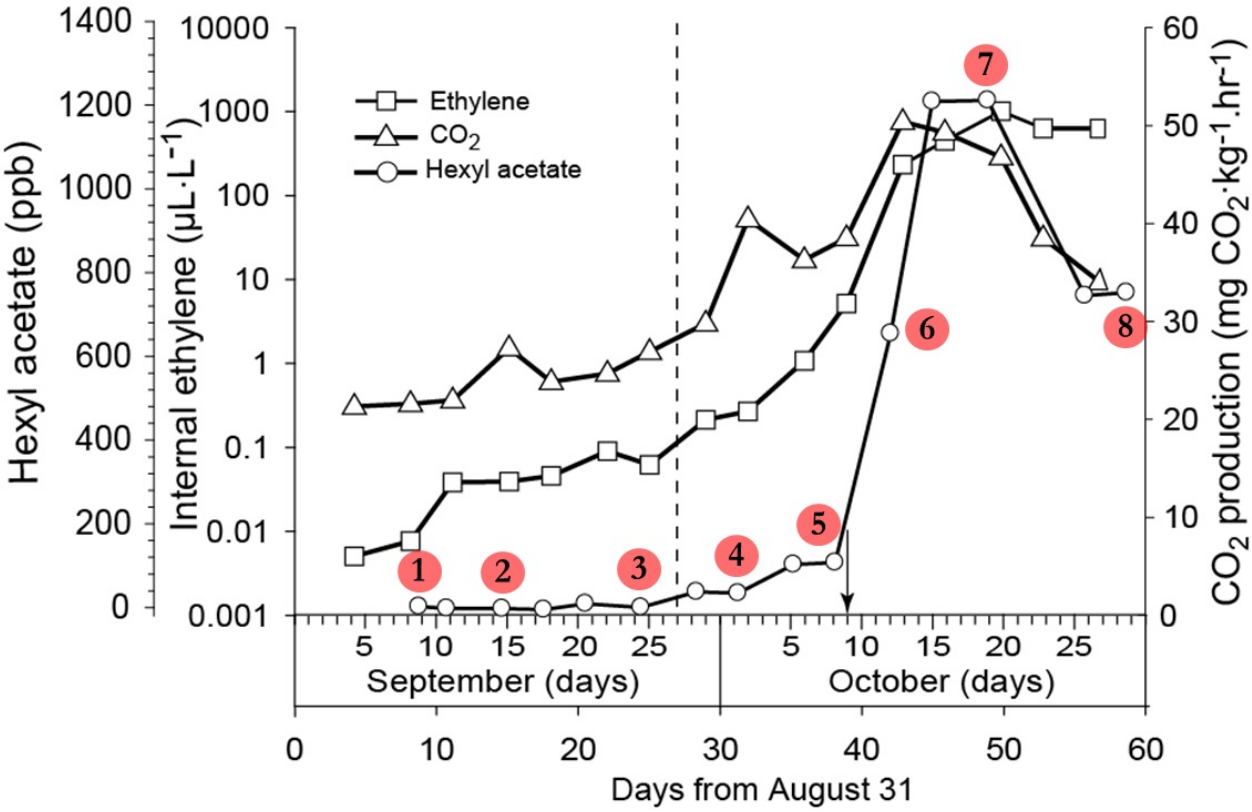


Figure 9. Hexyl acetate (ester), internal ethylene, and CO_2 production during fruit ripening in 'Jonagold' apples. Fruit were assessed from 4 Sept. 2009 (stage 1) to 27 Oct. 2009 (stage 8). Eight time points were selected for the gene expression analysis.

Eight stages were identified: immature apple (stage 1), mature with low levels of ethylene (stage 2), mature, just prior to the onset of autocatalytic ethylene production (stage 3), mature/ripening with autocatalytic ethylene and the respiratory climacteric engaged (stage 4), ripening, at the onset of rapid ester emissions (stage 5), ripening, at the peak of the respiratory climacteric (stage 6), ripe, at the peak of ester emissions and the onset of the decline in respiration (stage 7), and

overripe/senescent with declining ester synthesis and respiratory activity (stage 8). RNA was extracted from skin tissue at each stage using a hot borate extraction method procedure (Wang and Wilkins, 1994) with adaptations using RNeasy plant mini kit for RNA purification (Qiagen). Two micrograms of total RNA was pretreated with RNAse-free DNase set (Qiagen Inc. Valencia, CA) to remove contaminating genomic DNA. The concentration of total RNA was measured using a Nanodrop spectrophotometer. First-strand cDNA was synthesized from 2.0μg of treated total RNA, using SuperScript III kit as described by the manufacturer (Invitrogen, Carlsbad, CA) to a total volume of 20 μL. The relative expression of the 22 lipoxygenases and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were investigated using semi-quantitative PCR analysis. cDNAs (1μL) were used as templates in a 25-μL reaction containing 10μM of the forward and reverse gene-specific primer. Specific and non-conserved regions from each of the 22 lipogyxenases were amplified. Amplicon sizes ranged from 196 to 409bp. For each PCR, two biological and two technical replicates were analyzed. PCR primers were designed using Primer3 (Rozen and Skaletsky, 1999). Primer sequences, the expected size of the PCR product, optimum cycle number, and optimum temperature for primer binding are listed (Table 5). The PCR reaction was performed as follows: 1) 2 min at 94 °C, 2) 30 s at 94 °C, 3) 30 s at 55-61 °C, 4) 45 s at 72 °C, repeating 19 to 33 cycles from steps 2 to 4, and final elongation 5 min at 72 °C. Two genes, LOX16 and 19, were amplified using Touchdown (TD)-PCR with the following steps: 1) 5 min at 94 °C , 2) 30 s at 94 °C , 3) 30s at 72 °C (lowering 1 °C every cycle), 4) 45s at 72 °C, 5) repeating 9 cycles from steps 2 to 4, 6) 30s at 94 °C, 7) 30s at 62 °C, 8) 45s at 72 °C, 9) repeating 31 to 33 cycles from steps 6 to 8, and final 5 min elongation 72 °C. The amplified PCR products were separated by electrophoresis on a 1.2% (w/v) agarose gel, visualized with UV fluorescence of 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 quantifiable by light density measurement without saturation of pixels. The number of PCR cycles needed ranged from 20 to 34 (Table 5). PCR products were cleaned using ExoSAP-IT® for PCR product cleanup (Affymetrix) and sequenced at GTSF to verify identity. Expression data for all LOX genes were normalized based on the GAPDH control gene spot density. The spot density for the GAPDH varied approximately ±13% 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.

Table 5. Lipoxygenase gene ID, forward and reverse primer sequence (5→3'), expected PCR fragment size, optimum cycle number, and annealing temperature. n.e.: not expressed.

Gene	Apple Genome accession no.	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Amplicon size (bp)	Cycles	Annealing T° (°C)
LOX1	MDP0000874800	GTTTTCCCACCAGAAAGCAA	GCAGACATGTGCCTTAGCAA	372	27	56
LOX2	MDP0000154668	CTGGAAAGTACGCCATGGAT	AGCGACCCAAATTATCGTTG	386	22	56
LOX3	MDP0000215405	GCCATGGAGATTAGCTCTGC	AGCGACCCAAATAATCGTTG	375	23	56
LOX4	MDP0000224150	GAACGCCGTCCGATAGAATA	CGGAAGCTTTCGTTTCTTGT	389	34	56
LOX5	MDP0000300321	TGGGTAGCATCTGGTCATCA	TACAACCCCAGCTCCATTTC	399	26	55
LOX6	MDP0000277666	CACTCCTCCTCCTACCACCA	CCATCGACGACAATTTCCTT	390	24	56
LOX7	MDP0000211556				n.e.	
LOX8	MDP0000174168				n.e.	
LOX9	MDP0000281525	TCCCTGATCAACATCCTTCC	CAGCTCCAACCTCTCCAAAC	409	20	57
LOX10	MDP0000753547	ATGAAACAGTCGTACCTACC	AGTGCTGTCTCTAGCGAAGGG	386	21	57
LOX11	MDP0000864970				n.e.	
LOX12	MDP0000272843	CCTTATGCAACGGATGGACT	CCTGGCAGGGATGATAGAAA	395	28	57
LOX13	MDP0000755511	TCCTCCTCAAATTGGGTTTG	TCCGATCAAGAAATGGAAGG	384	24	55
LOX14	MDP0000257474	GTTTGGCCACGTGAAGTTTT	TGACTGGGGGAAACTCTTTG	377	28	56

Table 5. (cont'd).

Gene	Apple Genome accession no.	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Amplicon size (bp)	Cycles	Annealing T° (°C)
LOX14	MDP0000257474	GTTTGGCCACGTGAAGTTTT	TGACTGGGGGAAACTCTTTG	377	28	56
LOX15	MDP0000264666	TCATGGGTGTATCCTGCTGA	TATGGGTCCAAATCGTTCGT	384	28	56
LOX16	MDP0000923670	AGCAAAAACCACCAATCCAG	TCCAAATATGCTGCCTTTCC	390	34	54
LOX17	MDP0000172092	ATAGGCAGTTGAGCGTGCTT	GTTCCGAGTCCTTTTGGACA	390	24	57
LOX18	MDP0000423544	AGTGTTCTTGAAAACAATCACTGC	TGTAATCCTCTCCTCAATCTCCTC	196	32	57
LOX19	MDP0000312397	TATGCTGTCGATGGGATTGA	AGAACACTTTGTCGGGGTTG	376	32	61
LOX20	MDP0000450991	CCTCAACTTCGGACAGTTCTCTTA	CTCGGTTCTTCAGTTTCTCATCAT	350	26	57
LOX21	MDP0000146677				n.e.	
LOX22	MDP0000135993				n.e.	
GAPDH	MDP0000757565	TCGCTCTTCAGAGGGATGAT	CGTTCACACCAACAACGAAC	354	20	56

Real-time quantitative PCR (qPCR) assays.

Primer design. For the selected LOX genes, as well as for the housekeeping gene, PCR primers were designed using Primer Express Software for Real Time PCR version 3.0 (Applied Biosystems) according to the strategies set up by Yokoyama and Nishitani (2001). The high levels of sequence similarity between the apple LOX members were taken into account when designing quantitative real-time PCR (qPCR) primers to avoid cross-annealing of primers to untargeted LOX sequences. The gene-specificity of these primer sets was tested using the following procedures: (i) Genome Database for Rosaceae (GDR) BLAST searches of apple genome database were performed for each primer to confirm that no other sequence in the apple genome was similar to any primer; (ii) the specificity of PCR amplification was examined by monitoring the dissociation curves for qPCR reactions using the ABI PRISM 7900HT sequence detection system (Applied Biosystems). PCR amplification efficiencies for each primer were plotted in a semi-log regression plot, all primers efficient primers were in a range of -3.32 ±10% slope (-3.32 indicates a PCR reation with 100% efficiency). The length of all PCR products ranged from 50 to 70 bp. The eight primer sets used for qPCR are given in Table 6.

Table 6. Primer list for qPCR amplification of lipoxygenases genes and GAPDH control gene from 'Jonagold' apple.

Gene	Forward primer	Reverse primer
GAPDH	5'- CTGCCCCCAGCAAGGAT -3'	5'- TGGCTTGTATTCCTTCTCGTTCA -3'
LOX1	5'- GCCTACACTTGGCGGTAATAAAA -3'	5'- GGGCGACCGGTTCGA -3'
LOX4	5'- GGCATAAACCCTCTGAGCATAGA -3'	5'- TGGATGGATCAAGTTTGCTTACA -3'
LOX5	5'- TCTCTTGCTCGTGAATCACTTATCA-3'	5'- TTGGGCGAAAATGAGGTTTC -3'
LOX6	5'- GCCACCAATGGACGGAAA -3'	5'- CCACGCCGGTTGGAAA -3'
LOX15	5'- GAAAGCCAGCATACTTGGAGAAC -3'	5'- AGCCTCCCCTGCTGTTAAGG -3'
LOX17	5'- AAAGCCATCCCAGACCATCA -3'	5'- CTCTGAGTTTCTTCTCCCCAGTTT -3'
LOX20	5'- AAACTTCTGCAGCCTCATTTCC -3'	5'- AGGATTCCCCTGCCAATTG -3'

qPCR analysis. Serial dilutions of cDNA (10, 1, 0.1 and 0.01 ng/µL) were used with water for generating standard curves to test primer efficiency. PCR reactions were performed in a total volume of 10 µL: 3µL for each primer (1 µM), 5 µL of 2x SYBR Green PCR Master Mix (Applied Biosystems) and 2 µL of the cDNA template was used as a template for qPCR analysis on an ABI PRISM 7900HT sequence detection system (Applied Biosystems). The qPCR program included a preliminary step of 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. No-template controls for each primer pair were included in each run. Apple GAPDH gene was used as an internal control to normalize small differences in template amounts. The real time data generated was imported into Microsoft Excel and analyzed applying the delta-delta-Ct method (Livak and Schmittgen, 2001). At least two different RNA isolations and cDNA syntheses were used as biological replicates and three technical replicates

for the qPCR. Expression levels produced by qPCR were expressed as a ratio relative to the fruit time point number one, which was set to 1.

Constructions of Lipoxygenase-YFP fusions. Subcellular localization was predicted through the identification of signal peptide sequences using several prediction programs such as ProtComp (www.softberry.com/berry.phtml), PSORT (psort.nibb.ac.jp/) (Nakai and Horton, 1999), TargetP (www.cbs.dtu.dk/services/TargetP/), ChloroP (www.cbs.dtu.dk/services/ChloroP/), Signal-3L (http://www.csbio.sjtu.edu.cn/bioinf/Signal-3L/), and Predotar (http://urgi.versailles.inra.fr/predotar/predotar.html) (Small et al., 2004). Six constructs LOX-YFP were produced. All of them were designed to express a LOX protein sequence ranging from 166 to 300 amino acid residues which included the N-terminal of the protein carrying a chloroplast transit peptide (von Heijne and Nishikawa, 1991). All lipoxygenases were fused with a C-terminal yellow fluorescent protein (YFP). Phusion® High-Fidelity DNA polymerase (New England Biolabs) was used for PCR and cloning applications. Directional cloning was performed using the P-ENTR/D-TOPO entry vector kit (Life technologies, Carlsbad, CA). After sequencing, purified plasmids were digested with *Mlu*-I restriction enzyme (New England Biolabs) and a LR clonase recombination reaction between the entry clone and the pEarleyGate 101destination vector (35S-Gateway-YFP-HA tag-OCS 3', ABRC stock no. CD3-683 at Ohio State University) (Earley et al., 2006) was performed as described by the manufacturer (Life Technologies, Carlsbad, CA).

Transient expression of YFP fusions in tobacco plants. Five-week-old tobacco (*Nicotiana tabacum* cv Petit havana) greenhouse plants grown at 22°C were used for *Agrobacterium tumefaciens*-mediated transient expression. Briefly, each expression vector was introduced into

Agrobacterium tumefaciens strain GV3101 by heat shock. A single colony from the transformants was inoculated into 3mL of LB medium with 50μg/mL kanamycin, 50μg/mL gentamycin and 10μg/mL rifampicin. The bacterial culture was incubated at 28 °C with agitation for 16 h. 0.2 mL of bacterial culture then was pelleted in an Eppendorf tube by centrifugation at $4000g_n$ for 5min at room temperature. The pellet was resuspended in 0.5 mL of infiltration medium (50 mM MES pH=5.6, 2 mM NaH_2PO_4, 200 μM aceto-syringone, and 5mg/mL glucose). The bacterial suspension was diluted with infiltration buffer to adjust the inoculum concentration to the stated final OD_{600} value range of 0.05-0.1.The bacterial suspension was infiltrated using a 1-mL syringe without a needle by gentle pressure through the stomata on the abaxial surface. Transformed plants then were incubated under normal growth conditions for 72h (Brandizzi et al., 2002, Sparkes et al., 2006).

Sampling and confocal imaging. Confocal imaging was performed using an inverted Olympus IX81 laser scanning microscope model FV1000 fitted with 20- and 40-X air objectives (Melville, NY, USA). Excised plant tissues were mounted in water between a microscope slide and cover slip and examined. To detect EYFP fluorescence, an argon ion laser was used to produce excitation at 488 nm and fluorescence was detected in the range 505-530nm. Chlorophyll fluorescence was detected in the range 670–730 nm with a laser at 633nm. All images were acquired from single optical sections. Postacquisition image processing was done in Olympus Fluoview™ FV1000 software (Melville, NY, USA).

Lipoxygenase protein characterization.

cDNA cloning in expression vector. Three lipoxygenase genes were cloned for protein characterization: LOX1, LOX5, and LOX20. The same cDNAs mentioned above were used for protein characterization. Specifically for LOX1 and LOX5, cDNA from time point 2 was used, and for LOX20 time point 8. PCR was performed using forward and reverse primers for each LOX and Phusion® High-Fidelity DNA polymerase (New England Biolabs) was used for cloning applications. The PCR program used: 1) 30 s at 98 °C , 2) 10 s at 98 °C , 3) 30s at 60 °C, 4) 3 min at 72 °C, 5) repeating 34 cycles from steps 2 to 4, and a final 10 min elongation at 72 °C. The amplified PCR products were separated by electrophoresis on a 1.2% (w/v) agarose gel, visualized with UV fluorescence of ethidium bromide, and photographed. Bands were gel-excised with a razor blade and gel cleaned with QIAquick gel extraction kit (Qiagen). This cleaning procedure was unable to provide enough DNA for the cloning protocol. Instead, a PCR precipitation protocol was performed (Sambrook et al., 1989). The amount of DNA obtained after precipitation cleaning protocol was higher and cloning process was successful.

The pET 101/D-TOPO (Life technologies, Carlsbad, CA) expression vector containing C-terminal 6xHis tag was used. Full length proteins were expressed. Vectors were used to transform TOP10 *E. coli* competent cells, and the insert sequenced. The insertion of the full protein, correct frame and His-tag presence were analyzed with Geneious version R7 (http://www.geneious.com/). The screened constructs were used to transform to BL21(DE3) *E. coli* cells (Life technologies, Carlsbad, CA) for protein expression analysis. Colonies of the BL21 transformed cells were screened to check the presence of the insert.

Protein expression in E. coli. LOXs-pET 101/D-TOPO in BL21(DE3) cells were grown in 10 mL Luria-Bertani medium containing antibiotic ampicillin (100 $\mu g \cdot mL^{-1}$) at 37 °C for 2 h until the OD600 reached 0.6. The expression of the gene was induced by adding fresh isopropyl β-D-

1-thiogalactopyranoside (IPTG) optimized to a final concentration of 1 mM, and the incubation was continued for 10-16 hours at 15 °C. After incubation, the cells were harvested by centrifugation at 13,000 rpm for 10 min in 4 °C and the bacterial pellets were stored at –80 °C.

Expressed His-tag protein purification. Bacterial pellets that had been thawed on ice were homogenized with 400 µL lysis buffer (50 mM potassium phosphate buffer pH 7.8, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% TritonX-100, 10 mM imidazole). The cells were disrupted using a water bath sonicator (Cole-Parmer 8851, Vernon Hills, IL). Then, cells were lysed using pulsed sonication for 3 min followed by 1min with no sonication and the process was repeated 3-4 times. The water of the sonicator was kept at 10 °C. The disrupted cells were precipitated by centrifugation at 13,000 rpm for 5 min at 4 °C.

The protein concentration was determined by Bradford assay (Bradford, 1976) using bovine serum albumin as a standard and the protein concentration ranged from 0.2 to 0.4 $\mu g \cdot \mu L^{-1}$. The His-tagged protein was separated by electrophoresis on a 10% SDS-PAGE gel, stained using Coomassie Brilliant Blue R-250 and photographed.

Results and Discussion

Phylogenetic tree and protein sequence aligment of the N-terminal. Full sequences of the 22 LOX genes were aligned and used for the phylogenetic analysis (Fig. 10). An apple lipoxygenase contains 2,500 -2,800 base pairs and 930 amino acids. Phylogenetic analysis of translated amino acid sequences of different members of plant LOX families generated two large sub-trees and

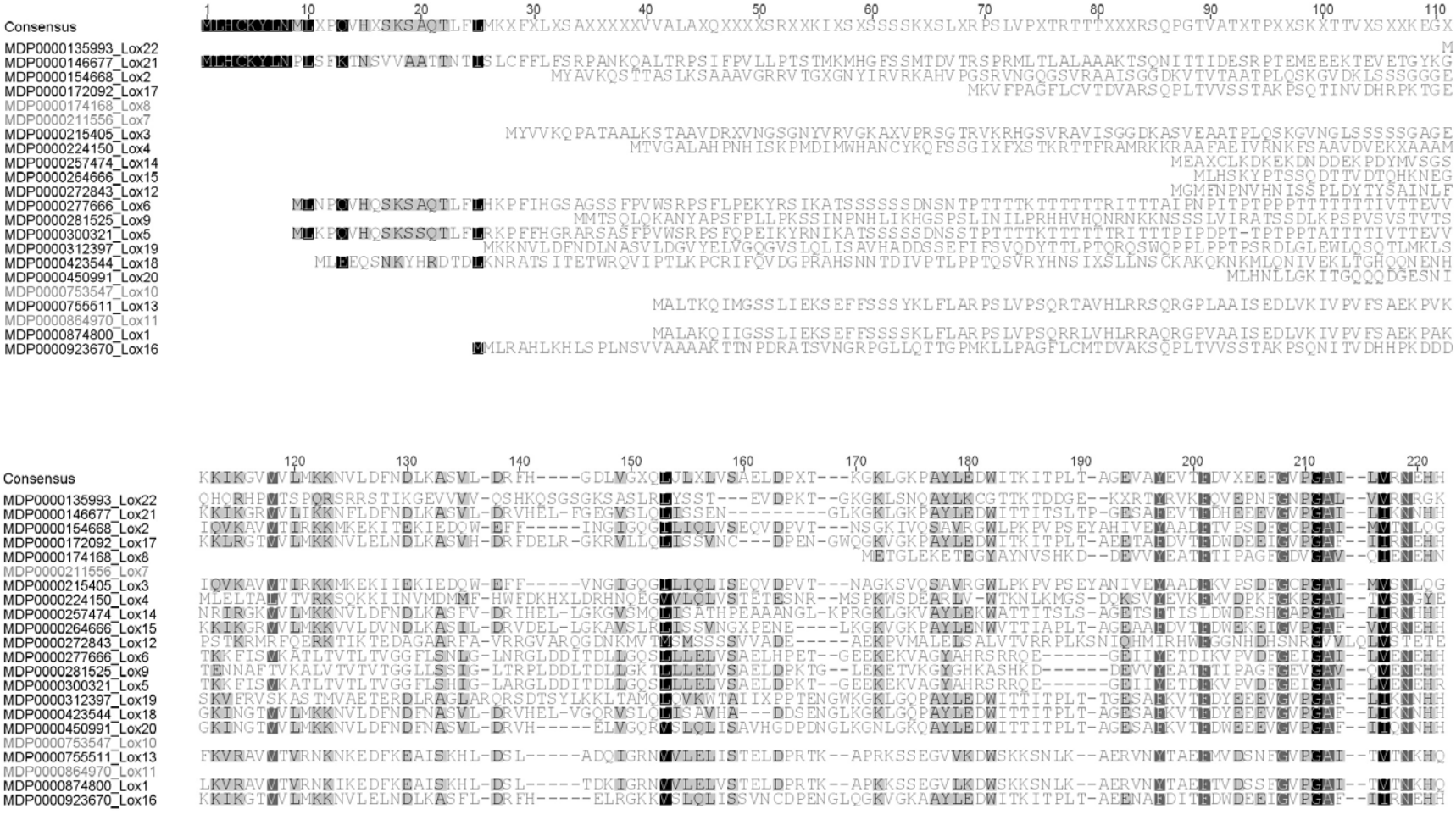
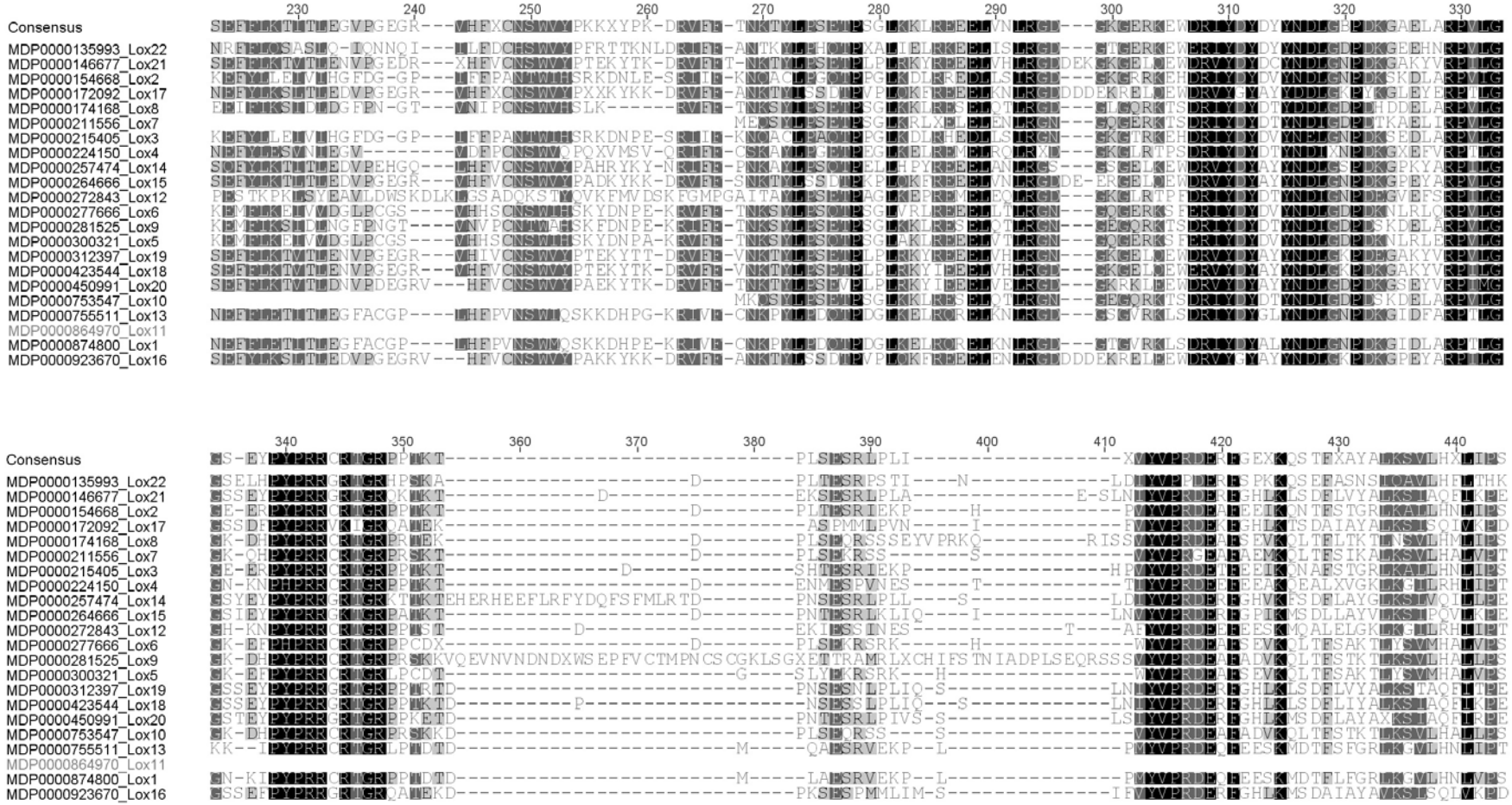


Figure 10. Aminoacid sequence alignment of the predicted lipoxygenases in apple (*Malus* x *domestica*) cv. Jonagold. The substrate binding site and the active site are enclosed in a rectangle (Vogt et al, 2013). Aminoacids being 70% identical or similar are shaded black or grey, respectively.

Figure 10. (cont´d).



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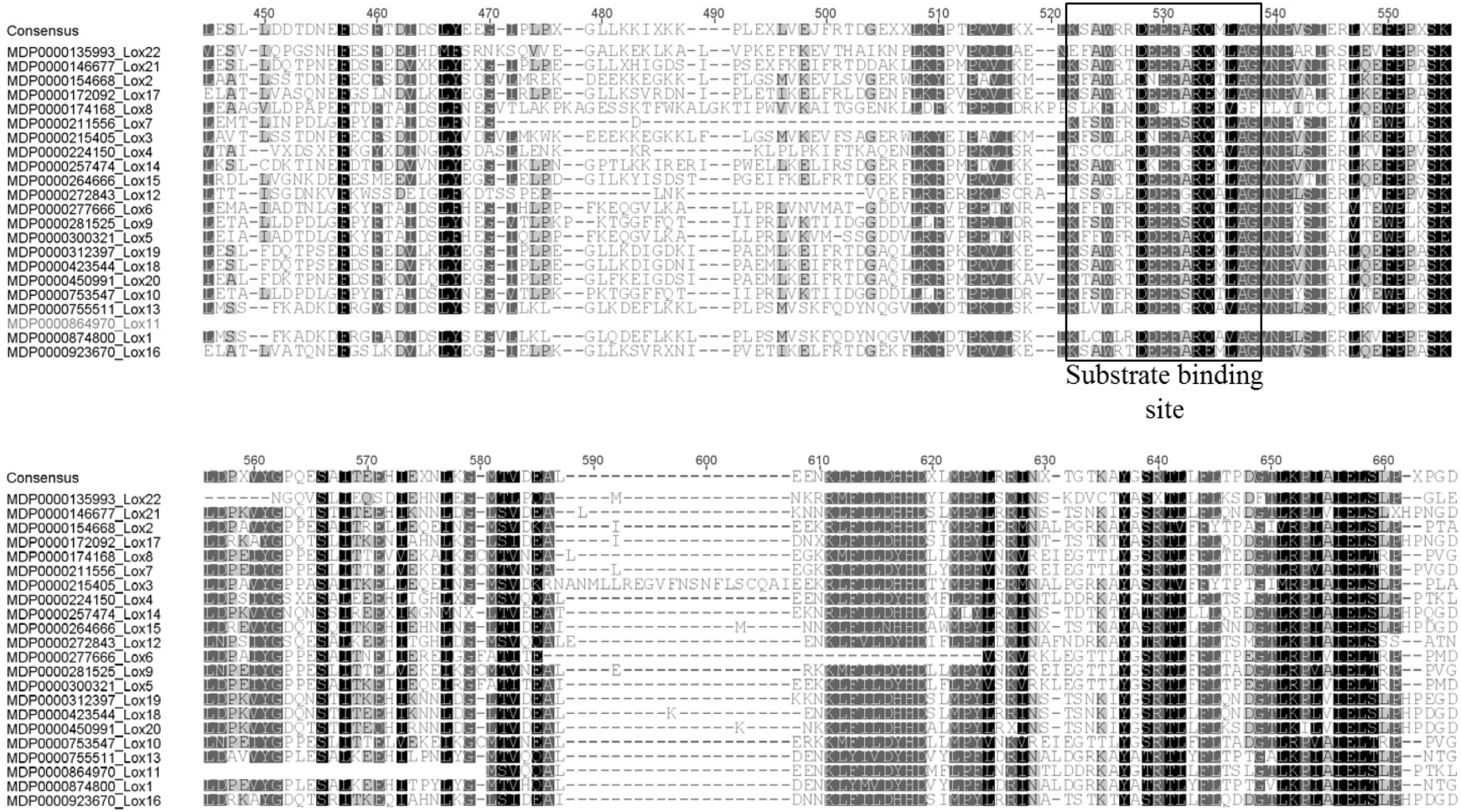

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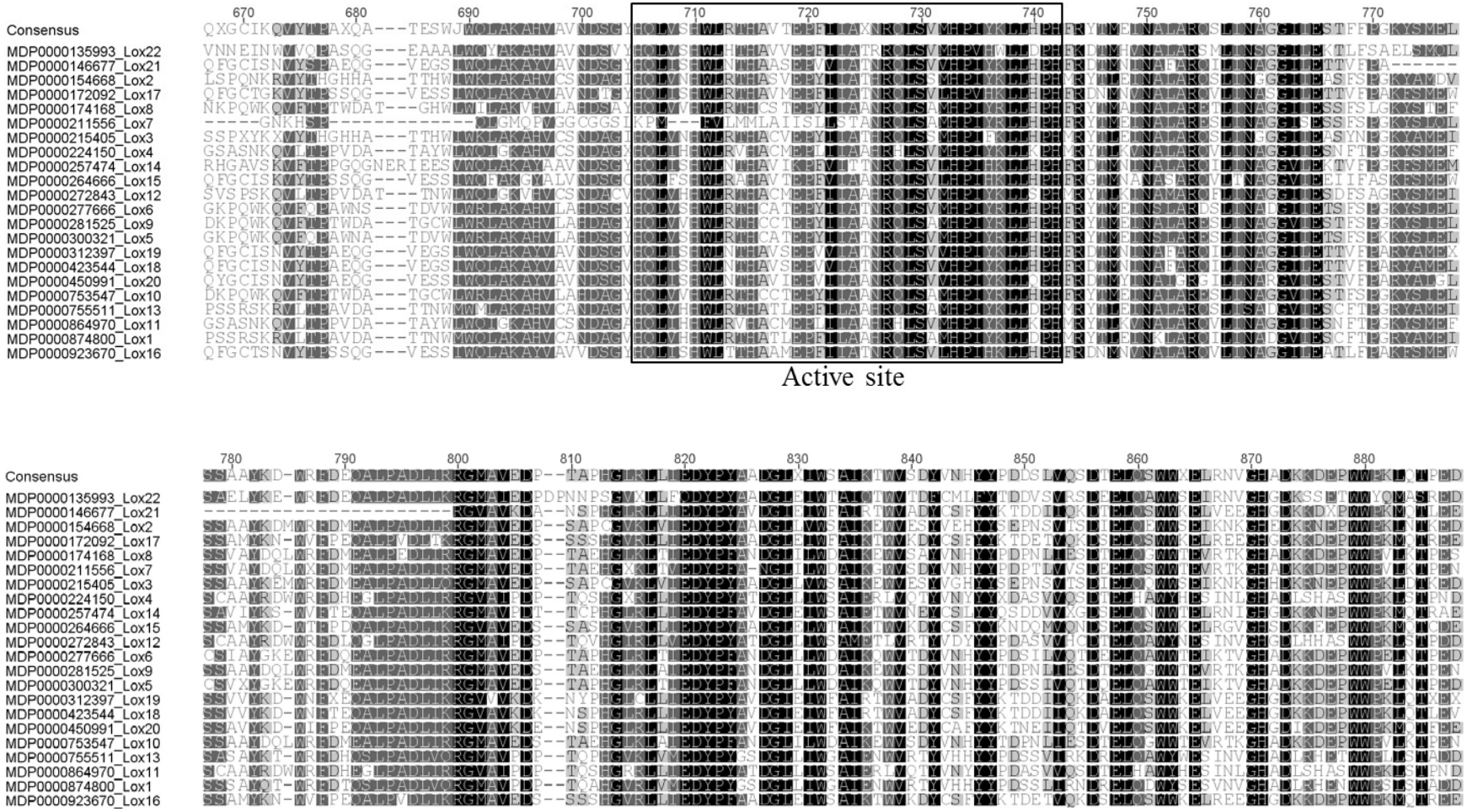

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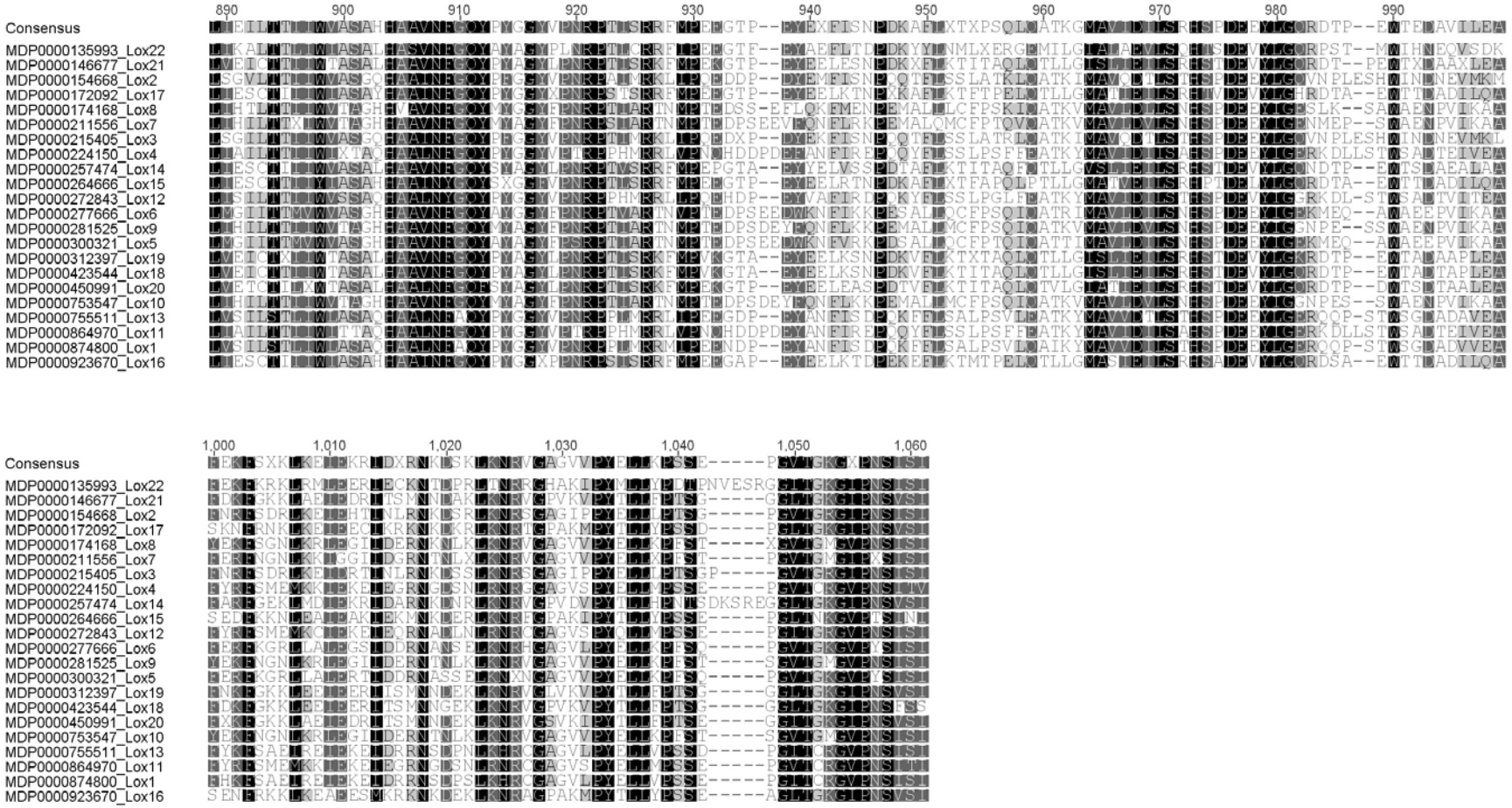


one small sub-tree: the 9-LOX group, the 13-LOX group and the smaller group with unknown function (Fig. 11), this was also reported by Vogt et al. (2013) in apple. Thirteen apple sequences were grouped in the 13-LOX group, whereas eight apple sequences were associated with the 9-LOX group. Only 4 sequences within the 13-LOX group had a putative chloroplast-transit peptide sequence (or *type 2* group).

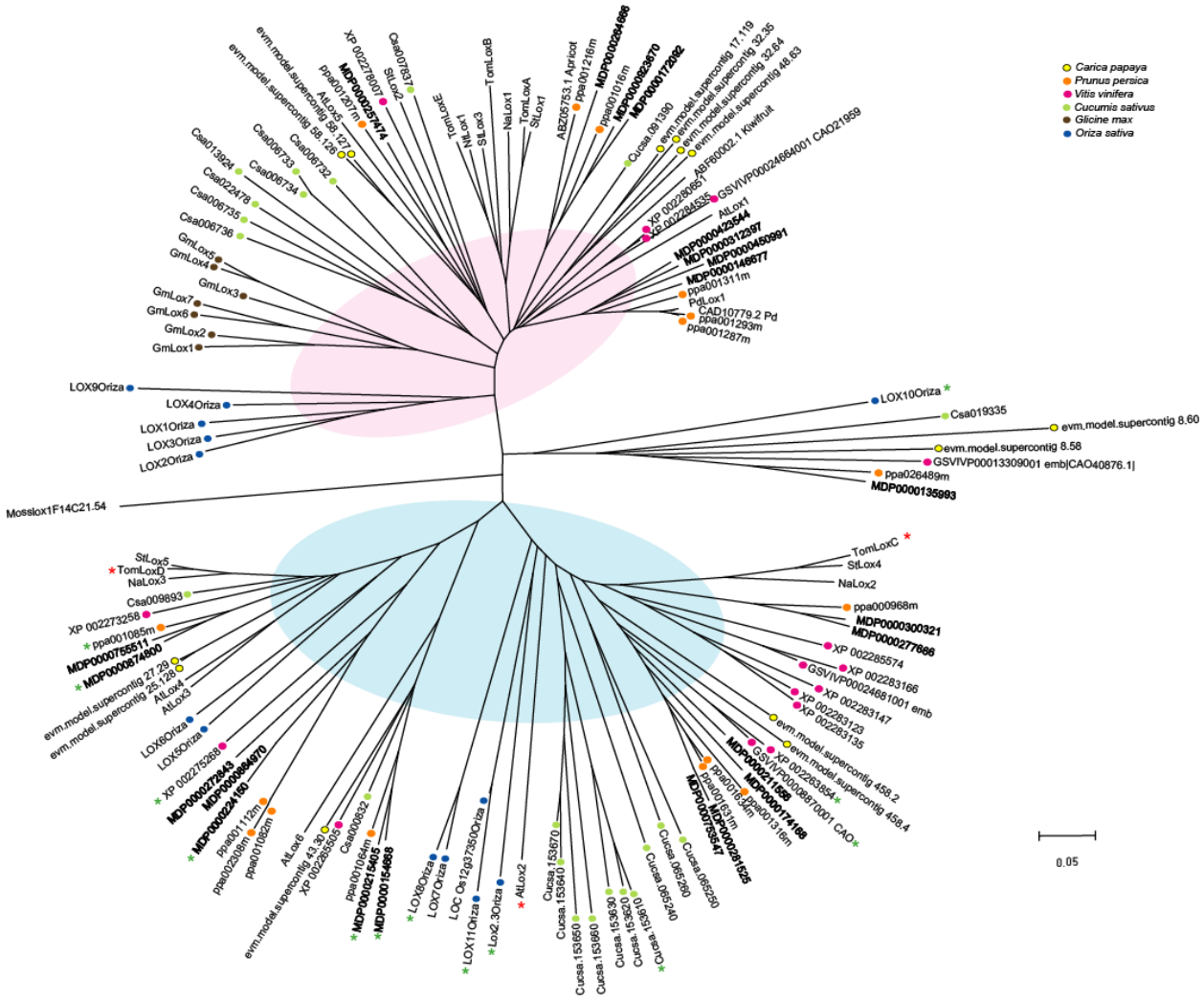


Figure 11. Phylogenetic tree of the LOX gene family using five sequenced dicot genomes and rice genome. The pink shaded area indicates the 9-LOX group and the blue shaded area indicates the 13-LOX group. Red marks represent chloroplastic targeted proteins, green marks represent predicted chloroplastic targeted genes, and names in bold correspond to apple LOX sequences.

The third and smaller group had one apple sequence. Interestingly, this uncharacterized group of sequences gathers at least one LOX member of each species. Podolyan et al. (2010) reported this unknown group in Sauvignon blanc grape berries where they found that one lipoxygenase gene (*VvLOXD*), was located in a different branch in the phylogenetic tree. *VvLOXD* was found to carry a leucine instead of a conserved phenylalanine at position 755, as well as a serine, rather than an alanine at the position determining LOX stereospecificity (position 730). Vogt et al. (2013) also reported a divergent apple sequence that apparently represents a unique gene that is phylogenetically distinct.

The present classification separates LOX proteins into groups based on their regiospecificity and the presence or absence of a plastid-targeting peptide. Both classification systems are not perfect, and actually lead to some confusion. For example, the soybean lipoxygenases have been classified as 13-LOXs *type 1* and as 9-LOXs by different authors (Podolyan et al., 2010; Acosta et al., 2009, Zhang et al., 2006). Both LOX groups lack a chloroplastic signal peptide, however, their regiospecificity differs. Another problem is that many LOXs possess dual regiospecificity (Porta and Rocha-Sosa 2002 ; Acosta et al., 2009; Palmieri-Thiers et al., 2009; Han et al., 2011) complicating their classification. Finally, LOXs are not only localized in the cytoplasm and plastids, but also in the vacuole, peroxisomes, lipid bodies, plasma membranes and microsomal membranes (Liavonchanka and Feussner 2006). Thus, the proposed classification is useful as a first step in predicting potential function and localization of individual members of large LOX families. However, functional characterization of LOX members is required to confirm sub-cellular location, regiospecificity and function (Podolyan et al., 2010).

As mentioned above, many plant species have a large LOX family; a possible physiological explanation for this expanded lipoxygenase pathway, is that it may be a complementary mechanism of defense against biotic stress (Huang et al., 2009). Cucurbitaceae family produces large amounts of (*E,Z*)-2,6-nonadienal with a fresh green flavor and confers resistance to bacteria and fungi (Blée, 1998; Matsui et al., 2000; Matsui et al, 2001). Likewise, apple fruit produce large amounts of hexanal, also with antimicrobial effects (Song et al., 1996), and apple aphid resistance factors have been found through QTLs studies suggesting that a lipoxygenase action might be involved in *Malus* aphid resistance reactions (Vogt et al., 2013).

Consequently with the phylogenetic study, the N-terminal alignment analysis of apple LOX sequences showed that the 13-LOX group aligns with the previously characterized N-termini of others species (Fig. 12). For the 9-LOX group, four apple sequences do show an N-terminal sequence, but according to the bioinformatic softwares none of these sequences carry a plastid transit peptide.

Figure 12. Terminal amino acids sequence comparison of plant LOX genes. The signal peptide sequence in *type 2* LOXs is indicated by the grey area. Accession numbers of sequences used to build the N-terminal alignment are as follows: *Arabidopsis thaliana*: *AtLox1* (AAA32827), *AtLox2* (AAA32749), *AtLox3* (AT1G17420), *AtLox4* (ATIG17420), *AtLox5* (ATIG72520); *Solanum tuberosum*: *StPotLox1* (AAB67858), *StLoxH2* (CAA65268), *StLoxH3* (CAA65269); *Lycopersicon esculentum*: *TomLoxA* (AAA53184), *TomLoxB* (AAA53183), *TomLoxC* (AAB65766), *TomLoxD* (AAB65767), *TomLoxE* (AAG21691); *Prunus persica*: *PpLox1* (EU883638),

Figure 12. (cont´d).

PpLox2 (FJ029110), *PpLox3* (FJ032015); *Oriza sativa*: *OsLox1* (CAA45738), *OsLox2* (A53054); *Glycine max*: *GmLox1* (AAA33986), *GmLox2* (AAA33987), *GmLox3* (CAA31664), *GmLox4* (P38417), *GmLox5* (AAB67732), *GmLox6* (AAA96817), *GmLox7* (AAC49159); *Actinidia deliciosa*: *AdLox2* (DQ497797); *Fragaria* x *ananassa*: *FaLox* (CAE17327); *Nicotiana tabacum*: *NtLox1* (AAP83134); *Vitis vinifera*: Vitis_XP002284535; *Prunus dulcis: PdLox1* (CAD10779). All apple sequences are the same as for the full alignment described above.

RT-PCR and real-time quantitative PCR (qPCR) assays. Semiquantitative RT-PCR of the 22 LOXs revealed that 17 were expressed in apple peel (Fig. 13). The expression of most genes exhibited no discernable pattern during ripening; however, at least 6 LOXs showed an expression pattern and were ripening-dependent. In the 13-LOX group, LOX4 showed a marked peak at stage 5 of development, which coincides with ethylene burst. LOX5 and 6 declined during ripening. In the 9-LOX group, LOX 14 also declined during ripening, while LOX 15 and 20 increased (Fig. 13). No visible PCR bands from LOX7, 8, 11, 21 and 22 were detected during fruit development.

qPCR was performed on 7 LOX genes to validate and monitor changes in transcript levels, obtaining essentially same results shown in the semiquantitative RT-PCR analysis. The qPCR assay included the 6 LOX genes that showed ripening-dependent patterns mentioned above, and additionally included LOX1 due to its little variation during ripening. In the 13-LOX group, LOX1 exhibited little change or a slight decrease in expression during ripening, LOX4 evidenced a sharp increase at stage 5, and LOX5 and 6 were down-regulated during ripening. As for the 9-LOX group, LOX14 was down-regulated, and LOX15 and 20 genes were up-regulated as ripening progressed (Fig. 14). The variation of LOX gene expression in fruit peel during ripening has been shown previously in apple (Sugimoto et al., 2008) and several other species, such as tomato (Ferrie et al., 1994; Chen et al., 2004), grape (Podolyan et al., 2010), peach (Han et al., 2011), kiwifruit (Zhang et al., 2006; 2009) among others. In apple, and particularly for the 13-LOX group genes, Vogt et al. (2013) reported basically the same results we obtained for the gene LOX1, with stable expression and transcripts detected in all samples of ripening tissue for ‘Golden Delicious’ and ‘McIntosh’ apples. As for the 9-LOX group, Sugimoto et al. (2008) in a microarray analysis found several LOXs with small variation during ripening, but one

lipoxygenase in particular increased as hexyl ester increased, and continued high even in senescence stage. BLAST searches, revealed that this LOX corresponded to LOX20 in our study, the LOX gene with the highest level of expression, being a hundred-fold higher than any other LOX member (Fig. 14). Also in our study, the dramatic increase of LOX20 gene expression coincides with the increase in hexyl esters during ripening (Fig. 9). Vogt et al. (2013) also identified this same gene, LOX20, to be involved in fruit aroma production through QTL analysis. In their gene expression studies LOX20 showed high expression in young leaves and fruit ripening after harvest. Another 9-LOX gene with an interesting pattern is LOX15. This gene has been suggested to be involved in flower development processes, particularly by the contribution of the formation of 9,10-ketol-octadecanoid acid (KODA), a 9-LOX product (Kittikorn et al., 2010). This gene may be involved in flower bud formation in apple by providing precursors for KODA production (Vogt et al., 2013). In apple fruit peel for 'Golden Delicious' and 'McIntosh', this gene had low expression (Vogt et al., 2013), whereas in our study LOX15 was highly expressed specifically at the end of ripening. Likewise, Schaffer et al. (2007) in a microarray analysis based on antisense ACC oxidase transgenic 'Royal Gala' apples, found that two genes were up-regulated when ethylene-induced, these two genes corresponded to LOX20 and LOX15 of our study, which coincides with our conclusion where these genes increase their expression along with ripening when ethylene autocatalytic burst starts (Fig. 14).

In tomato for example, *TomLoxB* and *E*, appear to play a role in ripening and senescence because they are up-regulated, with high expression in late stages of ripening and they are essentially fruit-specific (Griffiths et al., 1999; Chen et al., 2004). It is suggested then, that the increase on transcript levels of LOX20 is directly related with the aroma production in apple.

Zhang et al. (2009) showed in kiwifruit a strong relationship between the down-regulated genes and C6-aldehydes, while the up-regulated genes were strongly related to esters associated with kiwifruit ripening and senescence. However, this association in apple fruit may not occur. In the mentioned study, the C6-aldehydes such as *trans*-2-hexenal and hexanal, decreased as ripening progressed, while Contreras and Beaudry (2013) found the opposite trend in apple peel. There are many other examples in the literature demonstrating that plant LOXs gene expression is ripening-dependent and ultimately ethylene regulated (Defilippi et al., 2005; Schaffer et al., 2007). The most extensive data available exist for tomato, which for many years as served as a model fruit for investigation for genetic and biochemical regulation of ripening (Baldwin et al., 2000).

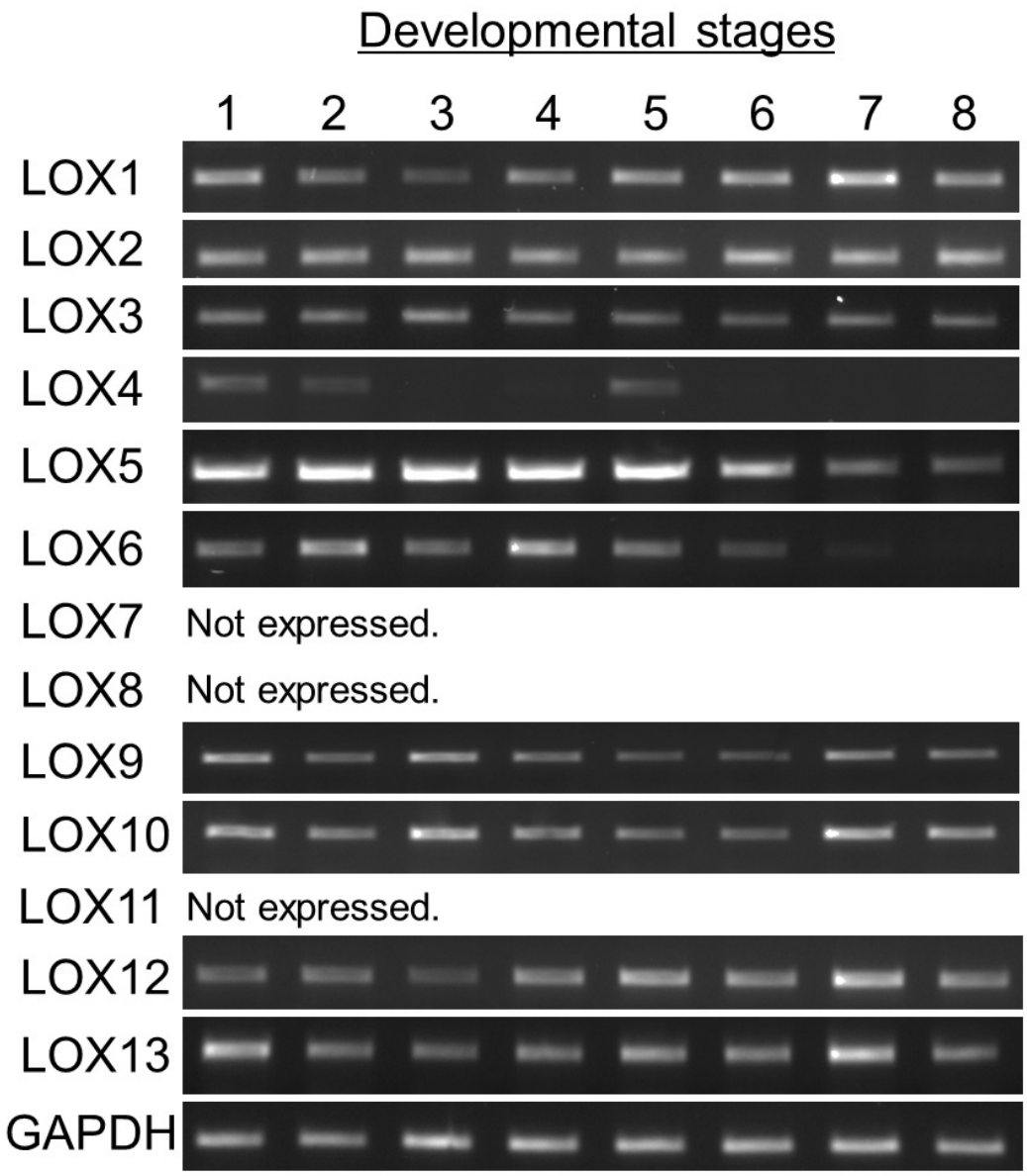
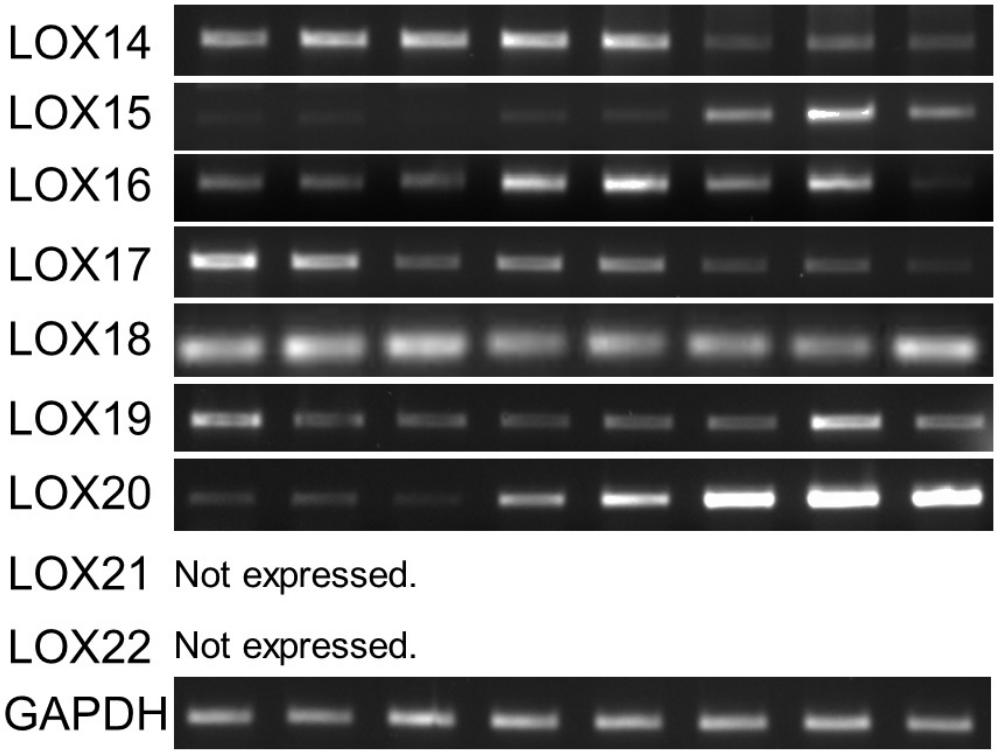


Figure 13. RT-PCR analysis of lipoxygenase expression for 'Jonagold' apple fruit peel during ripening. Eight time points were selected based on distinct physiological stages during which fruit were collected from 4 Sept. 2009 (stage 1) to 27 Oct. 2009 (stage 8). Total RNA was isolated from fruit at each time point and GAPDH gene was used as a control.

Figure 13. (cont´d).



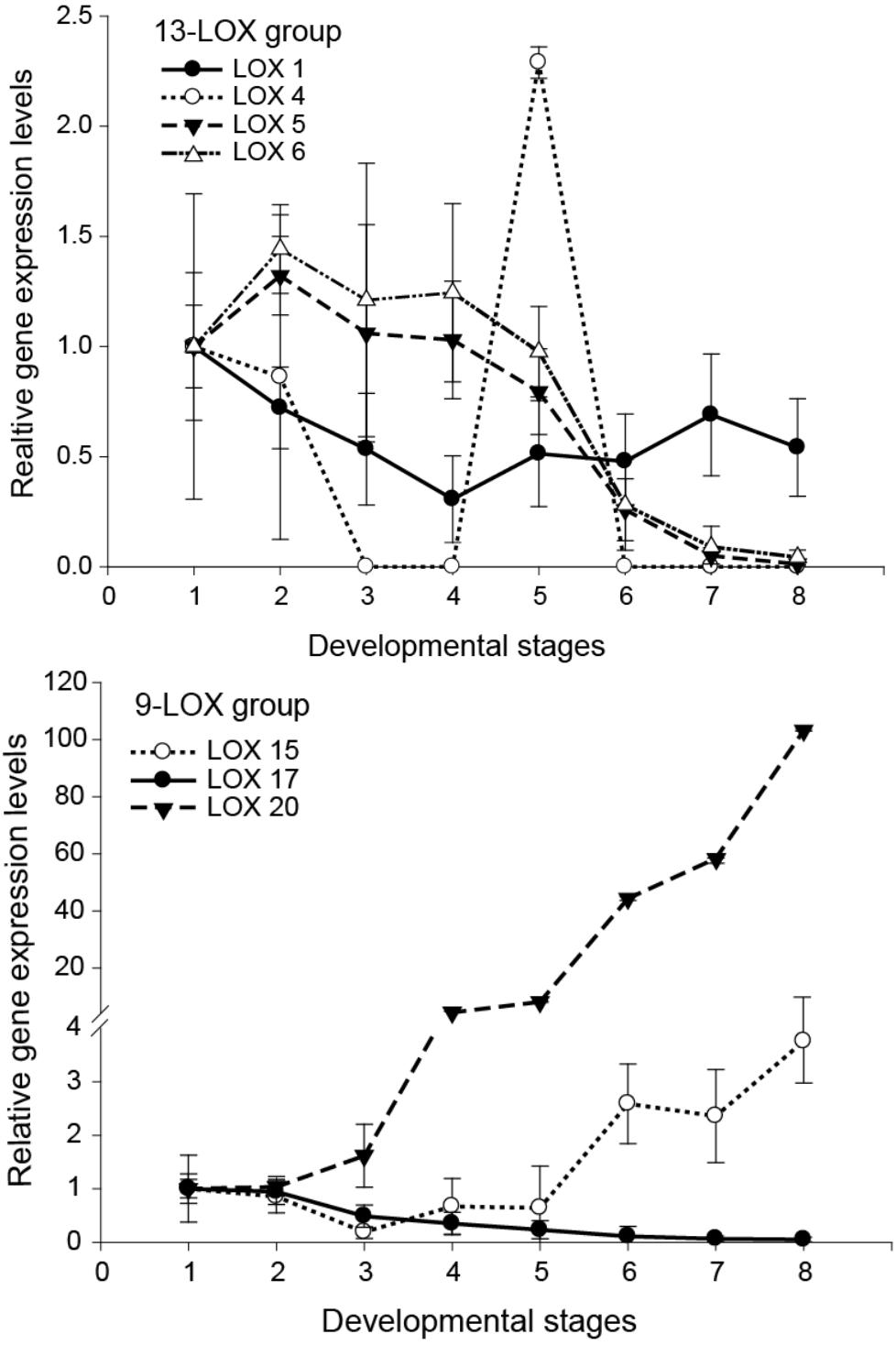


Figure 14. Levels of LOX transcripts (qPCR) in 'Jonagold' apple fruit peel during ripening. Time points selected are the same chosen for the RT-PCR analysis.

Subcellular localization and protein characterization. All six gene products were localized in cell structures. Clear differences between the patterns of protein accumulation were observed among the 6 lipoxygenases studied (Fig.15). For the 13-LOX group, LOX1 was not targeted to the chloroplast as was predicted by various subcellular prediction tools. This gene presented a weaker fluorescence pattern compared to all other LOXs. LOX4 and LOX5 carry a transit peptide sequence for chloroplast, according to the bioinformatic software used and their localization in chloroplasts is consistent with this prediction. As for the 9-LOX group genes, LOX15, LOX17 and LOX20, none of the three members had putative chloroplast targeting peptides and, consistent with this, none were found to co-locate with chloroplasts in the tobacco system (Fig. 15). Not many confocal studies are available for lipoxygenase cellular localization, and in only one case an aroma-related lipoxygenase was investigated, *TomLoxC*, a tomato lipoxygenase, which was expressed in the chloroplasts (Chen et al., 2004). Unlike *TomLoxC* where the pattern of expression was a diffused and evenly distributed fluorescence within the chloroplasts, LOX4 and LOX5, presented green dots within the chloroplasts (Fig. 15). This dotted pattern has been seen before not only in LOXs but also in genes that participate downstream of the LOX pathway (HPL, AOS, AOC) (Farmaki et al, 2007). These authors studied the distribution of the LOX pathway in potato chloroplasts, and found that the number of fluorescence spots detected in the chloroplasts coincides with the average number of grana present in the chloroplast, which might suggest an association with thylakoid membranes. Each chloroplast contained 1-4 grana maximum, although the size and shape depended on the type of tissue studied (i.e. leaves, fruit, etc.) (Farmaki et al, 2007). On the other hand, diffuse fluorescence patterns within the chloroplasts may indicate abundance of the LOX protein in the stroma, but it does not exclude associations with thylakoid membranes (Farmaki et al, 2007).

The spatial distribution of the LOXs in our study may suggest that at least one LOX would participate in aroma volatile production, since LOX enzymes associated with disruption-dependent aroma compounds are known to be localized in the chloroplasts (Chen et al, 2004, Arimura et al., 2009). In this case, LOX5 and LOX6 would be good candidates due to their expression pattern mimicking the decline in *cis*-3-hexenal emissions from disrupted tissues during ripening. It is more difficult to ascribe a particular LOX to the formation of autonomous C-6 volatiles and their ester products. The only LOX undergoing a substantial increase in expression that would parallel the emissions of hexanal, hexanol and hexyl esters, is LOX20. However, this LOX has been annotated as a 9-LOX and would be expected to yield 9-carbon aldehydes and alcohols. Nevertheless, if this LOX has a dual role (i.e., possesses both 9- and /13-LOX function) it is possible that it may be contributing to autonomous aroma biosynthesis.

Protein expression. Attempts to express LOX1, LOX5, and LOX20 proteins in *E.coli* were unsuccessful despite several changes made to the expression protocol. Several induction temperatures were tested: 25 °C, 17 °C, 10 °C and 4 °C, as well as different induction times: 4 h, 6 h, 10 h, 16 h, 20 h, 24 h. Ampicillin was changed to carbenicillin due to its higher stability during induction time. Other authors have reported failing LOX expression in bacteria system. Podolyan et al. (2010) in wine grape failed on the expression of some full-length proteins; they argued most probably due to the presence of targeting peptides that may have inappropriately targeted the proteins to *E. coli* membrane fractions or, more likely, directed the recombinant protein into *E.coli* inclusion bodies. In contrast, the constructs encoding proteins, in which the authors removed the signal peptide, successfully produced soluble proteins that exhibited LOX activity.

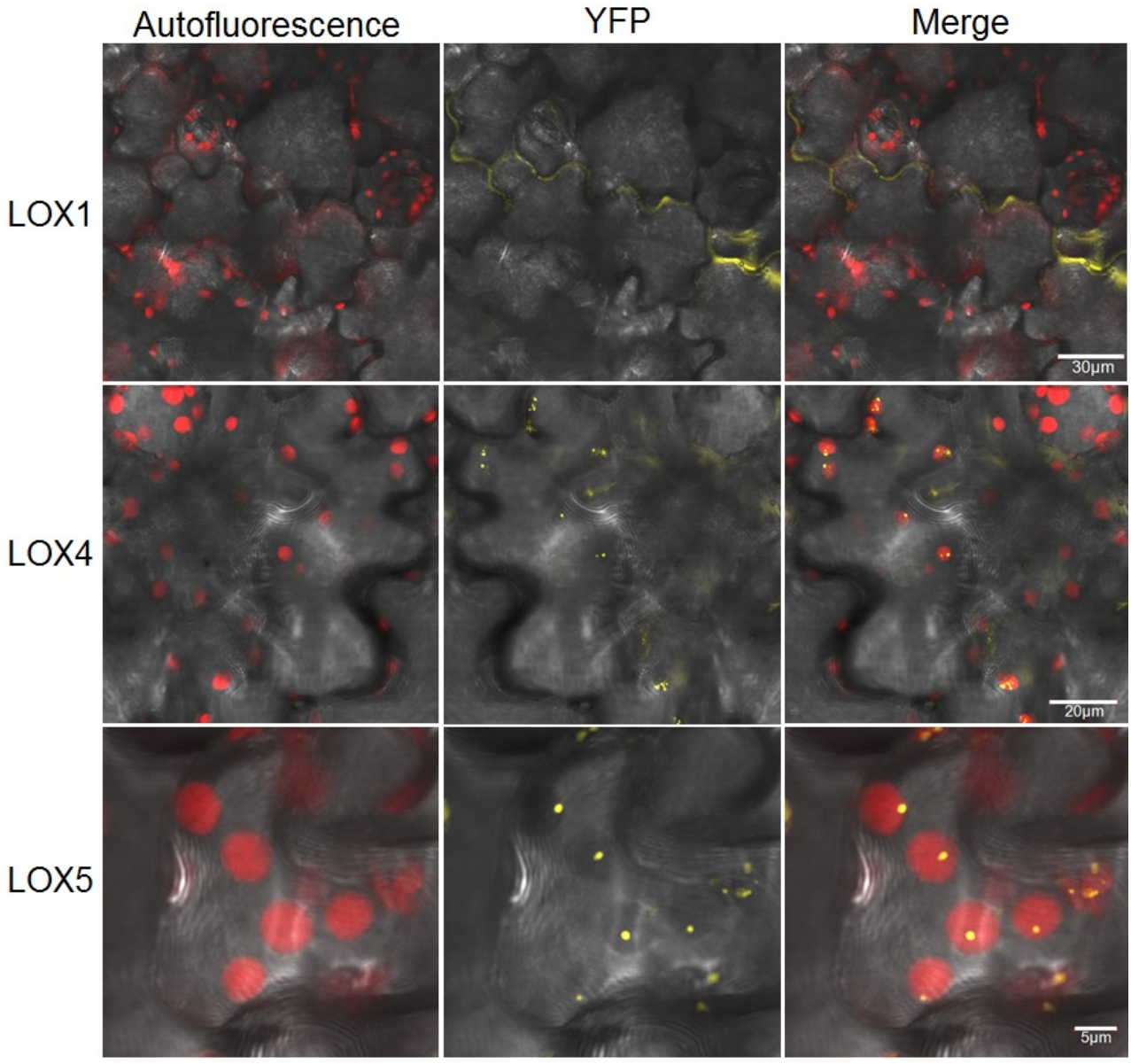
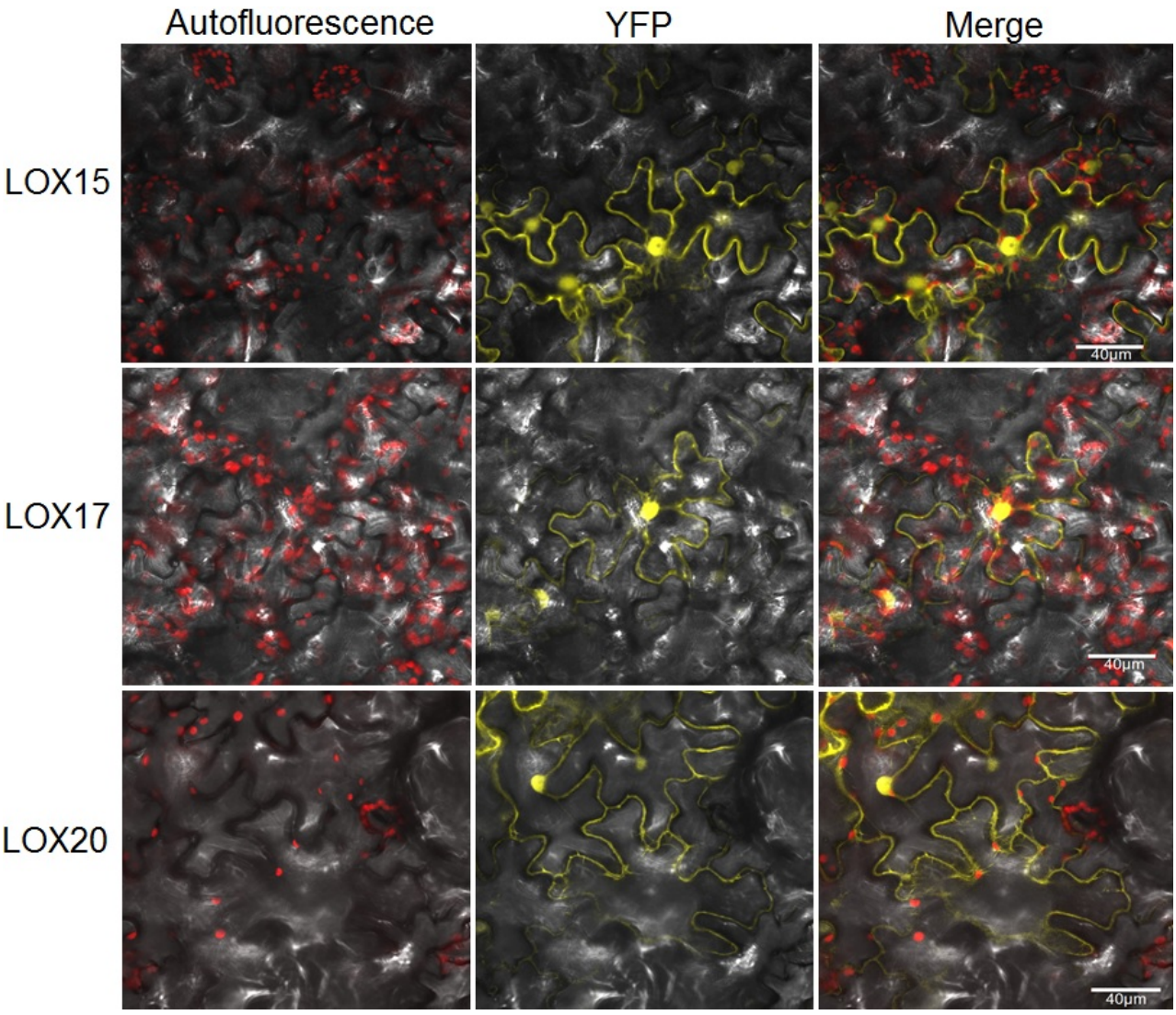


Figure 15. Transient expression of LOX1, LOX4, LOX5 (13-LOX group, 30, 20, and 5 µm ruler, respectively), and LOX15, LOX17 and LOX20 (9-LOX group, 40µm ruler) in tobacco (*Nicotiana tabacum* cv. Petit havana). N-terminal of LOX genes fused with YFP was infiltrated into tobacco leaves and the infiltrated areas were analyzed after three days by confocal microscopy as described by Brandizzi et al. (2002). Left column shows chloroplast autofluorescence, middle column shows the protein fused with YFP, and the right column shows the overlay image.

Figure 15. (cont´d).



Conclusion

Collectevely, the data support same findings as in other species: the majority of the LOX genes correspond to 9-LOXs. This fact might indicate that the main function of the LOX pathway in apple has an antimicrobial function and defense mechanisms. Our research provides evidence for gene expression profiles and cellular localization that narrows the possibilities

among the apple LOX family members to find the LOX-aroma related candidate. As well as in other species where only one lipoxygenase is implicated in aroma production, in apple there are at least two lipoxygenase candidates that may be involved in aroma biosynthesis. Importantly, this study also support the idea of other researchers, that a better classification system is needed for lipoxygenases, since based on latest evidence of dual role LOXs, the existence or absence of a cTP does not inform about the regiospecifcity of a determined LOX. Since, it was not possible to express these LOXs in a bacteria system, a protein expression in yeast might be successful. Thus, would be possible to finally elucidate which of these LOXs is participating in aroma biosynthesis in apple.

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CONCLUSION

Aroma volatiles, low-molecular-weight substances, derived from the metabolism of fatty acids, amino acids, and carbohydrate pools are commercially important for food, pharmaceutical, agricultural and chemical industries. Food flavor and fragrances are among the most profitable businesses in the aroma field, giving rise to the recent interest in understanding the formation of these compounds and engineering their biosynthesis (Schwab et al., 2008). As part of the current trend in flavor modification, our work provides some insight in the lipoxygenase pathway in apple fruit, and its relationship to aroma and provides the groundwork for future technical innovations in the field of aroma compound synthesis.

Research contribution to the field

Probably, one of the biggest contributions of this research is the understanding of the biology behind the aroma production in an intact tissue. Previously, we did not know if the LOX pathway contributed to the formation of autonomously-generated aroma compounds; we now understand that lipoxygenases are likely important contributors to the aroma profile of an intact apple. We also know that the substrates, free fatty acids, are being synthesized at precisely the right time for their catabolism by lipoxygenases. Simultaneously, other pathways are also likely contributing to the formation of aroma compounds and their precursors, e.g. β-oxidation and in order to develop a systems level understanding of fruit aroma it is important to consider the contribution and interactions between these additional pathways.

Another contribution to our understanding of aroma perception, is that the 'machinery' for synthesizing aroma is preformed at an early stage of fruit development and is not necessarily linked to the ethylene burst or the respiratory climacteric. This may represent an evolutionary strategy in which the plant attempts to guarantee seed dispersal by preparing the requisite

enzymatic systems, leaving only the formation of select precursors to the latter stages of fruit development. This hypothesis is supported by data obtained from our own studies which suggest that panelists started to differentiate immature apples from ripening apples up to two weeks prior to the autocatalytic burst. However, the impact of a continual low level of ethylene production, prior to the climacteric phase of ripening, on aroma formation cannot be entirely discounted.

Another interesting discovery presented in this research relates to linolenic acid (18:3) metabolism, which, to the best of our knowledge, has not been previously described in apple fruit. *Cis*-3-hexenal, which is produced from linolenic acid, was found to decrease throughout ripening, leading us to study linolenic acid in apple peel. The finding that there is a decline of linolenic acid in polar lipids during ripening, suggested that *cis*-3-hexenal is being synthesized from this source as opposed to free fatty acids.

Finally, the paradigm of aroma being derived primarily from degradative processes, a hypothesis favored by most other authors, is not supported by our data. The evidence provided here shows a pool of fatty acids being synthesized for the production of hexanal, and later, hexyl esters.

Research drawbacks

One of the limitations for this research, in a quantitative sense, was the sensory analysis. Sensory studies are limited by the sensations of the judge, which then have to be sorted into identifiable terms (Meilgaard et al., 2007). This subjectivity, in addition to other sources of variability such as the intensity of the odor sample and capacity of the judge to sense the aroma compounds, brought extra challenges to the experiments. In both years of our study, sensory test results were not able to link an increase in a specific odorant to the early detection of fruit destined

to ripen versus those whose ripening has been impaired. The opposite may be true: we hypothesize that the decline of *cis*-3-hexenal could provide a partial explanation of the fact that panelists detected differences two weeks before ethylene burst. One way to elucidate this possibility, could have been by determining the impact of *cis*-3-hexenal decline against a background of increasing production of another compound such as the isomer *trans*-2-hexenal or related esters. Thus, it would have been possible to find out if *cis*-3-hexenal had a masking effect over other incipient volatiles produced during the early stages of ripening, such that when *cis*-3-hexenal started to decrease, the impact of other volatiles, now unmasked, would rise.

An additional problem in directly comparing aroma production from whole fruit with sensory analysis relates to the the fact that for the latter analysis we utilized an 8-g-slice. Basically, our methodology allowed us to elucidate the biology involved in the production of autonomously-produced aroma volatiles and the production of volatiles by disrupted tissue, but we could not compare both systems directly.

Even though the lipid analysis presented in this research utilized a novel methodology that permitted the determination of free and esterified fatty acids from the same extraction, it still had limitations. For example, polar lipids were the major lipids represented, but it was not possible to determine the polar lipid class. We did not know, for instance, if the largest lipid contribution was coming from the glycolipids or the phospholipids. It is known that phospholipids are abundant in apple flesh, particularly phosphatidylcholine, but it is not known where in apple skin that aroma is formed. An interesting approach to accurately locate the substrates of the LOX pathway could have been the use of a fatty acid marker coupled with confocal microscopy. For example, previous experience on neutral fatty acids colocalization and quantification exist in mammalian cells (Malhi et al., 2006; Kuerschner et al., 2008). We also made many attempts to separate polar lipids using

thin layer chromatography (TLC) but the problem was the background resulting in strong variation between the replicates. In order to clean the samples we ran them through small columns in a gas chromatograph (GC), but we still had issues with reproducibility.

One of the biggest challenges in this work was the subcellular localization using confocal microscopy. *LOXs* are usually large genes (~2.5-2.8 kb), and are therefore difficult to clone. Only when LOX genes where cut, conserving their N-terminal portion, were they amenable to cloning and therefore permitted the infiltration in tobacco leaves. Unfortunately, the use of partial *LOX* sequences, rather than whole sequences, may have altered the organelle in which fluorescence was detected.

Future challenges

Aroma in apple has been negatively influenced by breeding efforts for long-storing varieties and the development of new technologies to extend storage. Research tools in fruit breeding have been focused mainly on crop production, decay resistance, chilling injury disorder resistance, and nutrition and pharmaceutical value such as antioxidants, rather than being focused on flavor retention (Shulaev et al., 2008). The result has been the generation of relatively odorless (though highly successful) long-storing varieties, including Red Delicious, Rome Beauty and Granny Smith. In addition, many technical approaches have been implemented in order to impact ripening physiology and extend the time of storage and commercialization, often having, unfortunately, a negative consequence for fruit quality and flavor. Some examples of these approaches are: modified and controlled atmospheres, ethylene blockers (1-MCP and AVG), and preconditioning treatments among others (Ferenczi et al., 2006). There is major concern today about consumer preference and fruit quality characteristics to enhance consumption and promote

healthy eating habits (Blanck et al., 2008). Fruit quality deserves more emphasis in the motivation for cultivar improvement and determination of research priorities (Shulaev et al., 2008).

Although many of the volatile constituents of aromas have been identified, many of the enzymes and genes involved in their biosynthesis are still unknown. However, modification of flavor by genetic engineering is dependent on the knowledge of genes that encode enzymes of key reactions that influence the biosynthetic pathways of plant-derived volatiles. Here we report a major progress resulted from the use of molecular techniques, in apple aroma biology. Our data suggests the lipoxygenase pathway contributes to aroma formation in intact apples; however, when apple tissue has been disrupted, the LOX pathway likely accounts for the majority of the volatiles compounds produced. Currently, by volume, short-chain acids and alcohols (C5 and C6) are the most prominent aroma-active compounds produced by biotechnology (Schwab et al., 2008). These compounds are widely used as food additives because of their 'fresh green' odor, specifically for their intense smell or sour taste, in the case of the acids, or as substrates for enzymatic synthesis of flavor esters (Schwab et al., 2008). Considering this fact, the LOX pathway may represent a potential route for aroma modification in fresh fruit, and an unprecedented path for the apple industry.

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APPENDIX

PRESTORAGE CONDITIONING AND DIPHENYLAMINE IMPROVE RESISTANCE TO CONTROLLED-ATMOSPHERE-RELATED INJURY IN 'HONEYCRISP' APPLES

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Introduction

The Minnesota Agricultural Experimental Station released 'Honeycrisp' in 1991 (Luby and Bedford, 1992). Since then, its unusual texture attributes and flavor has made 'Honeycrisp' very popular among consumers (The Packer, 2013; Yue and Tong, 2011). 'Honeycrisp' has been described as a cultivar with excellent storage characteristics, with the potential for low temperature storage of six months without atmosphere modification (Luby and Bedford, 1992; Tong et al., 1999). Significant production can now be found in Michigan, New York, and Washington (NASS 2011a, 2011b, 2011c).

As the growing area dedicated to 'Honeycrisp' has grown, the need for continued improvement of storage performance has taken on a greater significance. Unfortunately, long-term storage has been a challenge for 'Honeycrisp', which has proven to be very sensitive to low temperatures (Watkins and Rosenberger, 2000; Watkins et al., 2004, 2005) and can be damaged by controlled atmosphere (CA) conditions (Beaudry and Contreras, 2009). The low temperature disorders (i.e., chilling injuries) described for 'Honeycrisp' have been diagnosed as soggy breakdown and soft scald (aka. ribbon scald or deep scald) as described by Ramsey et al. (1917) and Plagge (1925, 1929). Tong et al. (2003) reported 'Honeycrisp' susceptibility to soft scald is orchard-specific. Sensitivity to this disorder is enhanced by greater fruit maturity at harvest (Brooks and Harley, 1934; Watkins et al., 2004), but is not necessarily related to ethylene production rate (Tong et al., 2003). In some apple cultivars, soft scald and soggy breakdown may or may not appear simultaneously (Plagge, 1929; Watkins et al., 2005). Delayed cooling or prestorage conditioning of 'Honeycrisp' appears to be effective in controlling soft scald and soggy breakdown (Watkins and Rosenberger, 2000; DeLong et al., 2004; Watkins et al., 2004, 2005). Successful prestorage conditioning temperatures range from 10-20 °C and 4-7 d in duration.

Many fruit cultivars develop physiological disorders in response to exposure to the low O_2 and elevated CO_2 partial pressures of CA storage. CA storage injury (CA injury) can be caused by low O_2, elevated CO_2 or a combination of both (Pierson et al., 1971). Injury can be manifested as large or small brown lesions, the largest of which are frequently surrounded by a narrow band of healthy tissue at the periphery of the fruit skin, and resemble soggy breakdown (Pierson et al., 1971). CO_2 can cause an injury described by Snowdon (1990) as 'brown heart', which is exacerbated by low O_2 (Plagge, 1929). Affected fruit are described as possessing small lesions of brown flesh distributed randomly between the skin and the core (Snowdon, 1990). Initially, the injured tissue is firm and moist, but after prolonged storage they become spongy and dry developing cavities, or lens-shaped voids (Plagge 1929; Snowdon, 1990). 'Empire' apple develops both internal and external injuries and is known to be susceptible to CA storage atmospheres containing 3-5 kPa CO_2, and the damage is exacerbated by levels of O_2 of 1.5 kPa or below (Burmeister and Dilley, 1995). For 'Empire', the external CO_2 injury occurs early during storage (Burmeister and Dilley, 1995). Strategies and/or recommendations to control damage in 'Empire' include the use of diphenylamine (DPA) (Burmeister and Dilley, 1995; Fawbush et al., 2008; Wang et al., 2000; Watkins et al., 1997), use of CA storage with CO_2 levels below 2 kPa, and preferably nearer to 1 kPa (Watkins and Liu, 2010), and delaying CA in combination with 1-MCP to retard fruit softening (DeEll and Ehsani-Moghaddam, 2012a; Watkins and Nock, 2012b). CO_2 injury, expressed as internal browning, is also found in 'Braeburn' apples and is known as "Braeburn browning disorder" (BBD) per Elgar et al. (1998). BBD is triggered by elevated CO_2 and reduced O_2 during CA storage; hence the recommendations are to store 'Braeburn' apples at <1 kPa CO_2 and 3 kPa O_2 (Elgar et al., 1998). The use of delayed CA is additionally recommended (Saquet et al., 2003).

Prestorage conditioning treatments have been used for the alleviation of storage disorders for nearly a century (Brooks et al., 1920; Harley and Fisher, 1930; Plagge, 1925, 1929). Prestorage conditioning treatments typically involve exposing fruit to elevated temperatures (i.e., elevated relative to storage temperatures) prior to storage and/or by delaying the application of refrigeration. Reports on prestorage conditioning effectiveness have been inconsistent, with incidence of the disorder increasing, decreasing or both, depending on cultivar (Brooks et al., 1920; Brooks and Harley, 1934; Harley and Fisher, 1930; Plagge and Maney, 1937). However, to our knowledge, no studies have shown that prestorage conditioning suppresses CA injury in 'Honeycrisp'.

The primary aim of this study was to determine the extent to which CA injury in 'Honeycrisp' was due to low O_2 and/or elevated CO_2 and to evaluate the influence of prestorage conditioning and DPA treatment on the severity of CA injury of Honeycrisp. A secondary objective was to evaluate whether the use of 1-methylcyclopropene (1-MCP) in air storage, as an alternative to CA storage, would compromise quality. The work was conducted in two phases. The first phase (experiment 1) was conducted in 2008 and designed primarily to determine whether 'Honeycrisp' apples were susceptible to CA injury, to determine the relative influence of O_2 and CO_2, and to identify a treatment combination that would reliably generate symptoms so that control measures could be subsequently evaluated. The second phase (experiment 2) was designed to evaluate whether prestorage conditioning treatments or the DPA could suppress CA injury and whether 1-methylcyclopropene could be used as an alternative to CA storage, thereby avoiding CA injury. The latter experiment was conducted over three years (2009 - 2011). An attempt was made to link accumulated heat units and rainfall to disorder incidence over the three years of the study.

Materials and Methods

Experiment 1.

Plant material. Fruit were harvested from four grower cooperators at different locations in Michigan in the fall of 2008. Harvest dates ranged from 18 Sept. to 1 Oct., at the same time as the main commercial 'Honeycrisp' harvest at each farm. Fruit from each location were separated into 27 lots of 50 apples each for storage experiments. An additional 10 fruit were used for maturity analysis [fruit weight, red skin coloration, background color, internal ethylene concentration (IEC), fruit firmness, starch pattern index, and soluble solids concentration (SSC)] the day after harvest.

Maturity analysis. Fruit weight (g) was determined using a calibrated balance (Toledo Scale). Percent red coloration was estimated on individual fruit by trained assessors. IEC (μL·L-1) was determined by withdrawing a 1-mL gas sample from the interior of the apples using a disposable plastic syringe and subjecting the gas sample to gas chromatographic (Carle Series 400 AGC; Hach Company, Loveland, CO) analysis as previously described by Mir et al. (2001). Fruit firmness (N) was measured on opposite, paired sides of each fruit using a drill stand-mounted penetrometer (Effegi FT-327, McCormick Fruit Tree Inc., Yakima, WA) fitted with an 11.1 mm-diameter probe. SSC (°Brix) was measured using juice expressed from the penetrometer wound using a hand-held refractometer (Atago N1, Atago Co. Ltd., Tokyo, Japan). Background color (1-4) was determined by comparison to a McIntosh background color index chart (Cornell University, 1948). The starch index (1-8) was determined by comparison of the iodine staining pattern of a transverse section of the fruit cut through the seed cavity according to the method of Blanpied and Silsby (1992).

Treatments. Of the 27 fruit lots from each of the four orchards, nine were stored for 1 month, nine were stored for 3 months, and the remaining nine held for 6 months. All fruit were stored at 3 °C, rather than 0 °C, to minimize chilling injury and avoid confusion of soggy breakdown symptoms with CA-related injury symptoms. For each storage duration, each of the lots was given one of nine different storage treatments, which are described as the following O_2/CO_2 concentration (kPa) combinations: 1/0; 3/0; 1/3; 3/3; 21/3; 21/0 (air); 21/0 with 1-methylcyclopropene (1-MCP, AgroFresh Inc., Spring House, PA); 21/0 conditioned 3 d at 10 °C, and 21/0 conditioned 5 d at 10 °C. At the altitude of the research site, roughly 270 m above sea level, 1% of atmospheric pressure equals 0.98 kPa. Fruit were placed into specially-built 0.93 m3 aluminum chambers (Storage Control Systems, Sparta, Mich.) and atmospheres were regulated with an automated atmosphere control system (ICA 61 Lab System, International Controlled Atmosphere Ltd., Paddock Wood, UK). All the CA treatments were imposed on the day of harvest; fruit were not cooled prior to placement in CA. The storage temperature was maintained by placing the CA chambers in controlled environment chambers held approximately 0.5 °C cooler than the target temperature of the CA chamber. Chamber temperatures were monitored continuously. The ethylene action inhibitor, 1-MCP, was applied at an initial concentration of 1 $\mu L \cdot L^{-1}$ in a sealed CA chamber and held for 24 h. 1-MCP application took place one to three days after harvest while fruit were being held at 3 °C. Fruit given prestorage conditioning treatments were placed at 10 °C on the day of harvest and held for 3 or 5 d before the long-term storage at 3 °C in air. The intent was to determine if prestorage conditioning would lead to any problems with quality apart from any problems caused by CA. During prestorage conditioning, the fruit were covered with 3-mil (76.2 μm) thick low-density polyethylene (LDPE) bags to reduce moisture loss.

Post-storage evaluations. Fruit removed after each storage period were sorted to eliminate fruit with excessive decay, which might obscure disorder detection. Ten fruit from each lot were assessed for IEC, firmness, and SSC. The remainder were covered in LDPE bags as previously described and held for 7 d at 20 °C and afterwards assessed for external and internal disorder incidence. External physiological disorders such as soft scald, lenticel breakdown, and bitter pit were evaluated visually and the incidence of the damage determined. CA-related injury to the cortex (brown lesions and lens-shaped cavities, the latter typically associated with smaller brown lesions) and senescence breakdown were evaluated by cutting apples transverse to the longitudinal axis at two points. The fruit section with the most extreme symptoms was used to assess the type, severity and incidence of the disorder. Disorders were assessed after 1, 3, and 6 months of storage at 3 °C plus 7 d at 20ºC.

Experiment 2.

Plant material. During 2009, 2010, and 2011 seasons, 'Honeycrisp' apples were harvested from 7, 8, and 6 locations, respectively; across the state of Michigan and transported to the Post-harvest Physiology Laboratory at Michigan State University on the morning of harvest. Harvests were between 10 Sept. and 1 Oct. in 2009, 6 and 28 Sept. in 2010, and 13 and 28 Sept. for 2011. Every year for each harvest, fruit were distributed into 30 lots of 50 to 70 fruit and randomly assigned to the various prestorage conditioning treatment/storage condition combinations. Maturity at harvest and after 7 d at 20 °C was assessed on 20 fruit as described previously. Eleven different orchards were used in this study.

Treatments. From the 30 fruit lots, 15 were stored for 3 months and the remaining 15 were stored for 6 months. For each storage duration, 5 of the lots were conditioned at 3 °C, 5 lots were conditioned at 10 °C, and the remaining 5 lots were conditioned at 20 °C. Prestorage conditioning treatments were imposed the day of harvest and the duration was 5 d. During prestorage conditioning, fruit were covered with LDPE bags to reduce moisture loss as described. Lots from the prestorage conditioning treatment/storage duration combinations was subjected to one of the following five O_2/CO_2 partial pressure (kPa) combinations: 3/0, 3/3, 21/0, 3/3 with DPA, and 21/0 with 1-MCP. After prestorage conditioning, the fruit were placed into custom-made CA chambers previously described and atmospheric conditions imposed. DPA treatments were applied the day of harvest. DPA-treated fruit were dipped in a DPA solution (No Scald, 31% a.i., Elf Atochem) containing 1 $g \cdot L^{-1}$ a.i. for 1 min, allowed to drain and dry for 1 hour, and then given one of the three prestorage conditioning treatments. 1-MCP was applied as described previously.

Post-storage evaluations. The evaluations performed for physiological disorders were carried out as for Experiment 1. Additionally, for 2011, the lens-shaped cavities and smaller lesion injuries were tracked independently. The purpose of this was to determine if these are different disorders and how they develop during storage.

Statistical Analysis. For experiment 1, the experimental design was completely randomized with a factorial combination of orchard (4), storage duration (3), and postharvest treatments/regimens (9). For experiment 2, the experimental design was completely randomized with a factorial combination of orchard (6-8), storage duration (2), prestorage conditioning treatment (3), and postharvest storage regimens (5). Analysis of variance for both years was performed using SAS

(Version 9.1; SAS Institute Inc., Cary, NC) software using PROC GLIMMIX. Tukey's test was used for multiple comparison analysis. An arcsine square root transformation was performed on percentage data before statistical analysis. Non-transformed data are presented. We explored whether relationships existed between the incidence of CA-induced lesions and growing degree days (GDDs), accumulated precipitation, log IEC, and starch index using a stepwise multiple linear regression. All data from 2009, 2010, and 2011 was used for the regression.

Daily GDD and accumulated precipitation data were collected from Michigan Enviro-weather Automated Weather Station Network (MAWN) from nine stations across Michigan located near orchards used in the study. The weather stations corresponded to: Romeo, Benton Harbor/SWMREC, Sparta, Fremont, Ludington, Hart, Hartford, East Leland and Berrien Springs, Mich. The accumulated GDDs were calculated relative to base temperatures of 4, 10, 15, 21, and 26 °C. The number of GDDs between 4 and 10 °C, 10 and 15°C, 15 and 21°C, and 21 and 26°C were also calculated. GDDs and rainfall data were collected from full bloom date until harvest date for each orchard. The PROC REG and STEPWISE selection method were performed in SAS (Version 9.1; SAS Institute Inc., Cary, NC) using a P value of 0.10. Variables in the model were log transformed, tested for normality and residual against predicted values plots were examined.

Results and Discussion

Experiment 1.

'Honeycrisp' apple fruit exhibited a high sensitivity to both low oxygen and elevated CO_2 levels (Fig. 16). We found that the CA atmospheres used induced injuries typical of those associated with CO_2 (i.e., small brown lesions and associated lens-shaped cavities) and also, larger dark-brown lesions with often irregular margins (Fig. 17). The extent of the injury was higher for

those fruit in an atmosphere with elevated CO_2 for each level of O_2. Watkins and Nock (2012b) also studied 'Honeycrisp' under CA conditions and reported this cultivar as highly susceptible to physiological disorders, especially internal CO_2 injuries, which were exacerbated with high levels of CO_2 during storage. After one, three, and six months of storage, the treatments 1/3 and 3/3 yielded the highest incidence of internal browning, coinciding with the findings of Watkins and Nock (2012b). In the current study, up to 70% of the fruit from individual lots suffered mild to extreme internal browning (data not shown) under low O_2 (both 1 and 3 kPa O_2) when the carbon dioxide level was at 3 kPa. Without CO_2, the internal browning severity was markedly reduced, but still significant as long as the O_2 level was low. When the O_2 level was increased to 21 kPa, internal browning only occurred when CO_2 was present, although the degree of damage was relatively minor. 1-MCP and prestorage conditioning at 5 or 10 °C yielded fruit with a very low incidence of internal browning disorders. 1-MCP in air did not influence total disorders when compared to air storage alone. However, disorder incidence declined in air-stored fruit when prestorage conditioning was applied (Fig. 16).

Interestingly, only individual effects of O_2, CO_2, and orchard were significant for the development of disorders (Table 7). The interaction of oxygen and carbon dioxide was not significant, suggesting that these two gases do not have a synergistic effect, but rather are additive on the appearance of the disorders. Importantly, there was great variability between orchards, with some orchards producing susceptible fruit (up to 73% damaged fruit in the treatment 1/3), and others producing fruit with little propensity for disorder development (as low as 5% incidence for the same treatment) (Table 8).

The extent of the CA injury seemed to be near its maximum after only one month in CA (Fig. 18). Total damage did not increase as the storage duration increased, but the distribution of

the categories damage did change slightly (e.g., brown lesion injury decreased over time whereas cavities increased).

Experiment 2.

The CA injury seen in the preliminary study (2008) was reproduced the subsequent three years (2009, 2010 and 2011), but with varied intensity. High variability between orchards and years was observed (Table 9).

Brown lesions and cavities in the cortex were suppressed by prestorage conditioning at 20 ºC only in 2009, when incidence was highest (Table 9). Unlike Watkins and Nock (2012b) who found high incidence of senescent breakdown during 2009 and 2010, we had a low incidence all three years and in all treatments and orchards. Prange et al. (2011) showed that senescent breakdown in 'Honeycrisp' increases with later harvest, especially in fruit heavier than 250 g. High temperatures (10 and 20 °C) during the prestorage conditioning period did not increase bitter pit incidence relative to 3 °C in any of the years. However, orchard affected bitter pit incidence markedly. Soft scald appeared only rarely in our 4 years of study (data not shown).

DPA effectively eliminated the CA injury each year of study (Table 9). DPA reduces chilling injuries (soggy breakdown and soft scald) in 'Honeycrisp' only slightly (Watkins et al., 2004). However, CO_2-related injuries are known to be prevented by DPA (Argenta et al., 2002; de Castro et al., 2008; Mattheis and Rudell, 2008; Meheriuk et al, 1984; Wang et al., 2000; Watkins et al., 1997). In the case of 'Honeycrisp', we found that the brown lesions in the cortex were completely suppressed by DPA application, even when the prestorage conditioning temperature was 3 °C. The incidence of cavities ranged from 0.1% to 0.3% under the same DPA treatment. On the other hand, the most affected treatment was 3/3, followed by 3/0, 21/0, and 21/0 plus 1-MCP.

Although the effects of DPA in preventing disorders are well known, it is unclear how DPA prevents or reduces the disorders. Whitaker (2004) reported that its probable action is as a free radical scavenger, suggesting that oxidative reactions are involved in CO_2 injuries. Toivonen and Brummell (2008) suggested the antioxidative properties of DPA might be involved in protecting against membrane lipid degradation and inhibiting the release of phenylpropanoid substrates into the cytosol, where polyphenyloxidase (PPO) catalyzes browning and polymerization of phenol substrates. Lee et al. (2012) showed in 'Braeburn' apples, an accumulation of amino acids, acetaldehydes, and ethyl ester compounds linked to appearance of internal browning, suggesting that DPA decreases the amino acid production or increases the protein degradation.

As pointed out above, there was variation between orchards for brown lesions, cavities with associated small brown lesions, and bitter pit (Table 9). Incidence of damage for brown lesions in the orchards (averaged across all treatment combinations) varied from 0 % to 14%, from 0% to 3%, and from 0.1% to 4% for 2009, 2010, and 2011, respectively. Cavity incidence ranged from 0.8 to 6%, 0% to 3% and 0.4 to 2% in 2009, 2010 and 2011, respectively. In 2011, the lens-shaped injury increased during storage up to 2%, whereas smaller brown lesions plateaued after 3 months with a low incidence of 0.3% damage (data not shown). The data may indicate that the smaller brown lesions convert to the lens-shaped cavities over time.

Weather data were extracted from weather stations near the orchards to explore whether CA-injury susceptibility may have been linked to environmental influences. For instance, rainfall was found to influence firmness (Lachappelle et al., 2013) and susceptibility to soft scald (Moran et al., 2009). In 2009, the level of CA-injury was highest yet accumulated growing degree days were lowest (1891 GDDs, based on a 10 °C threshold) compared to the 2010 and 2011 growing seasons, which had 2378 and 2192 GDDs, respectively. However, stepwise regression resulted in

GDDs, in addition to rainfall and starch index, being dropped from the model as they were not significant. Only one variable, ethylene, emerged to explain a significant portion of the model, revealing that disorder incidence declined as IEC at harvest increased. This was reminiscent of the findings of Ehsani-Moghaddam and DeEll (2013) where a strong negative correlation was found between soft scald and IEC at harvest. DeEll and Ehsani-Moghaddam (2012b) found ethylene to be implicated in the sensitivity of 'Empire' apple fruit to CO_2-related storage injury. In 'Honeycrisp', we found the relationship between CA-injury incidence and IEC was described by the following equation:

Disorder incidence = 37.71 - 8.27 log(IEC), R^2= 0.35, P value= 0.0043

The significant linear regression with the log of the IEC suggests that immature apples were more susceptible to CA-related physiological disorders (Fig. 19). According to our results, apples harvested with an IEC less than approximately 20 μL·L-1 have a greater potential to develop CA-related injuries. This result suggests that a later harvest would help to minimize the risk of disorders. However, it is also known that soft scald increases with later harvest (Tong et al., 2003; Watkins et al., 2004), which would complicate 'Honeycrisp' handling for the fruit industry.

Harvest maturity is considered the most important factor affecting sensitivity of apples to physiological disorders during ripening and storage (Ferguson et al., 1999). Predictive equations have been used to estimate the incidence of disorders (Autio et al., 1986; Moran et al., 2009). In addition to the influence of maturity, many studies have also demonstrated a strong relationship between disorder susceptibility and orchard practices and/or environmental conditions (Ferguson et al., 1999). Watkins and Liu (2010) suggested mineral nutrition could be used to predict

susceptibility to CO_2 injury, but Watkins and Nock (2012a) found no correlations between mineral concentrations and incidence of CO_2 injury in 'Honeycrisp' apples. A better understanding of the factors involved in fruit storage disorders and how they relate to each other will allow optimization of storage quality, and development of methods for predicting disorder risk.

Although it can take several years to establish a storage protocol due to validation for fruit harvested in different years, optimizing handling protocols for the new cultivars have been successful in the past in order to reduce the incidence of physiological disorders to a manageable level (Johnston and Brookfield, 2012). Development of such protocols has allowed, for example, reducing the incidence of soft scald in 'Honeycrisp' by managing appropriate storage temperatures (Watkins and Rosenberger 2000; Watkins et al., 2004).

The development of recommendations for the storage of this cultivar in CA will be subject to a number of considerations. For instance, while the 7-day prestorage conditioning treatments provided some protection against the development of CA injury, shorter durations should be investigated to prevent quality loss due to excessive ripening, which could cause increased skin greasiness and undesirable flavor profile (Delong et al., 2009). Given the rapid rate of formation of CA injury, it seems advisable to minimize the exposure to elevated CO_2 during room loading and prestorage conditioning in addition to the first month or so of CA storage. If 'Honeycrisp' behave like 'Empire' apple fruit with regard to CA injury development, it may be possible to increase CO_2 after first 4 to 6 weeks of CA storage. Use of DPA can be used to avoid CA injury and with concentrations of CO_2 as high as 3%, but fruit may still have to be conditioned and would need to be held at 3 ºC to minimize the risk of chilling injury. Finally, in air storage has merit in extending the storage duration of 'Honeycrisp' in that its use avoids the issue of CA injury altogether.

Extended storage of 'Honeycrisp' in CA or with 1-MCP application in air storage requires additional considerations not directly related to the storage atmosphere. Given the propensity of 'Honeycrisp' to develop bitter pit (Rosenberger et al., 2004; Watkins et al., 2005), storage and handling recommendations should probably include preharvest management of bitter pit (e.g., through Ca^{+2} applications and crop thinning). While the current study suggests that prestorage conditioning does not exacerbate this disorder, previous work does (Watkins et al., 2004) and therefore vigilance is probably justified. While decay was not specifically evaluated in the current study, the decay rate was approximately 5% to 10% in 3- and 6-month stored fruit. There is some concern, therefore, that prestorage conditioning and elevated storage temperatures may promote decay, so a good preharvest decay control strategy would be advisable for this decay-susceptible cultivar (Rosenberger, 2004; Watkins et al., 2005). While starch index was not linked to CA injury, low ethylene production was. Watkins et al. (2005), found a starch index above 5 was associated with increasing ethylene levels, suggesting this might be an appropriate target for harvest maturity for fruit destined for CA storage.

Table 7. Individual effects and interactions of controlled atmosphere (CA) gases (1.5, 3, and 21 kPa O_2 with and without 3 kPa CO_2), orchard (four orchards) and storage time (1, 3 and 6 months) for CA injury incidence of 'Honeycrisp' apples stored at 3 °C in Expt. 1.

Source of variation	F Value	Pr > F
Oxygen	20.38	<.0001
CO_2	13.70	0.0005
Orchard	30.79	<.0001
Duration of Storage	0.67	0.5182
Oxygen *CO_2	1.54	0.2231
Oxygen*duration	1.58	0.1950
CO_2*duration	0.35	0.7042
Oxygen *CO_2*duration	0.10	0.9807

Table 8. Controlled atmosphere injury incidence of 'Honeycrisp' apples for four Michigan orchards stored 6 months at 3 °C under different combinations of O_2 and CO_2 with additional treatments of prestorage conditioning (2 and 5 d at 10 °C) and 1-methylcyclopropene (1-MCP; 1µL·L-1) for fruit held in air. Treatment atmospheres are given in kPa. Tukey's test was used for multiple comparison analysis of averages. Numbers in the column followed by differing letters were different.

Treatment (O_2/CO_2)	Prestorage treatment	Total disorder incidence (%)				Average (%)
		Orchard 1	Orchard 2	Orchard 3	Orchard 4	
1/0	none	39.33	15.67	24.66	1.66	20.33ab
1/3	none	73.33	20.67	35.66	5.33	33.75a
3/0	none	34.66	9.0	5.33	2.66	12.91bc
3/3	none	71.33	28.0	21.66	4.33	31.33a
21/0	none	7.33	7.67	2.66	1.0	4.66c
21/3	none	15.33	11.67	4.33	0.0	7.83c
21/0 1-MCP	1-MCP	9.0	5.67	4.0	0.0	4.66c
21/0 3d at 10°C	3d at 10°C	1.0	10.5	3.33	0.0	3.70c
21/0 5d at 10°C	5d at 10°C	2.0	5.0	1.33	0.0	2.10c

Table 9. Total CA-injury incidence of 'Honeycrisp' apples with or without prestorage conditioning treatments and stored 3 months at 3 °C under different controlled atmosphere treatments in 2009, 2010 and 2011. Treatment atmospheres are in kPa. Tukey's test was used for multiple comparison analysis. DPA= diphenylamine; 1-MCP = 1-methylcyclopropene.

	Internal Disorders									External Disorders		
	Controlled atmosphere-related injury						Senescent breakdown (%)			Bitter pit (%)		
	Brown lesions (%)			Cavities (%)								
Effects	2009	2010	2011	2009	2010	2011	2009	2010	2011	2009	2010	2011
Prestorage conditioning temperature												
3 °C	10.3a	1.3a	1.9a	4.1a	1.1a	1.4a	0.0a	0.8a	0.3a	5.9a	13.6a	8.4a
10 °C	6.5a	1.5a	1.2a	2.2a	1.0a	0.9a	0.0a	0.7a	0.4a	7.6a	14.6a	10.9a
20 °C	0.6b	0.5a	0.6a	0.7a	0.3a	0.6a	0.0a	0.7a	0.2a	6.2a	18.8a	6.7a
Storage treatment (O_2/CO_2)												
3/0	7.7b	0.8b	0.3b	2.3b	0.3b	0.9b	0.0a	0.4a	0.0a	7.3a	16.7a	5.0a
3/3	13.6a	3.3a	5.8a	6.3a	3.0a	3.7a	0.0a	0.6a	0.4a	4.1a	14.5a	10.3a
3/3+DPA	0.0c	0.0b	0.0b	0.3b	0.3b	0.1b	0.0a	0.3a	0.1a	6.6a	13.8a	7.8a
Air	3.4b	1.0b	0.0b	1.4b	0.2b	0.1b	0.0a	1.5a	0.5a	6.3a	20.7a	10.8a
1-MCP	4.4b	0.3b	0.2b	1.4b	0.1b	0.1b	0.0a	0.8a	0.5a	8.4a	12.5a	9.5a
Orchard												
1	0.0b	0.0a	4.1a	0.8a	0.0c	0.7a	0.0a	0.0b	0.0b	15.3a	31.1b	25.3a
2	0.0b	1.1a	2.2a	5.7a	0.3bc	0.4a	0.0a	0.5b	0.1b	5.0b	0.8ef	3.2d
3	9.7a	1.9a	0.1a	0.8a	3.2a	1.2a	0.0a	0.0b	0.0b	4.2b	22.6b	0.3d
4	3.0b	3.1a	0.7a	1.5a	0.0c	1.8a	0.0a	0.1b	0.6ab	15.2a	13.5c	14.5b
5	11.6a	0.4a	0.3a	4.0a	0.0c	0.8a	0.0a	0.0b	0.0b	0.6c	0.0f	7.8c

Table 9. (cont'd).

Effects	Internal Disorders									External Disorders		
	Controlled atmosphere-related injury						Senescent breakdown (%)			Bitter pit (%)		
	Brown lesions (%)			Cavities (%)								
	2009	2010	2011	2009	2010	2011	2009	2010	2011	2009	2010	2011
Orchard												
6	14.3a	0.0a	0.1a	2.5a	0.2bc	0.9a	0.0a	1.4b	1.2a	0.3c	47.0a	1.1d
7	2.2b	0.0a	-	0.9a	0.3bc	-	0.0a	0.0b	-	5.1b	5.9dc	-
8	-	2.2a	-	-	2.2ab	-	-	3.7a	-	-	4.3de	-

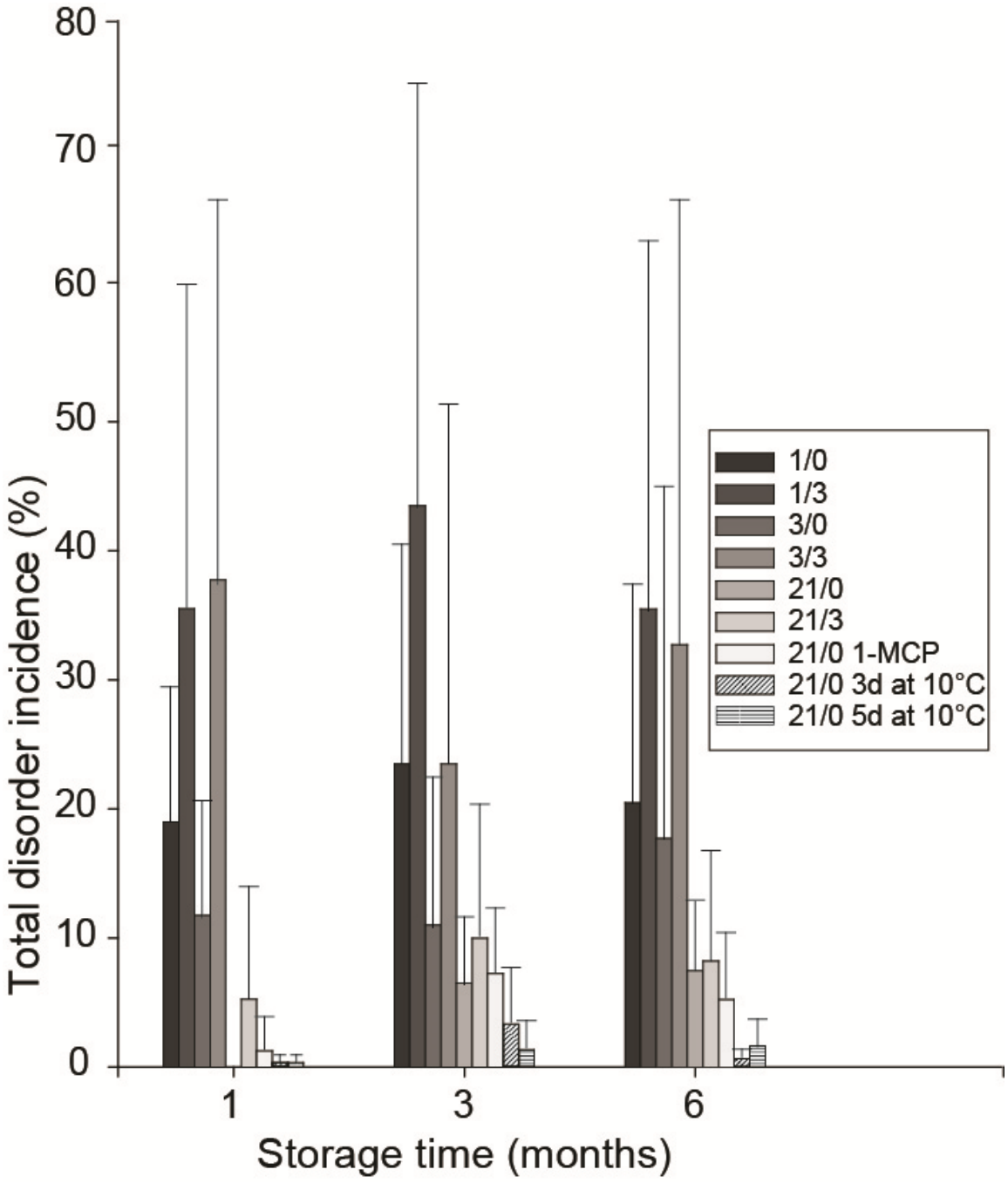

Figure 16. Effect of controlled atmosphere (CA) storage and prestorage conditioning on CA injury incidence of 'Honeycrisp' apples after 1, 3, and 6 months of storage at 3 °C. For each storage duration, each of the lots was given one of nine different storage treatments, which are described as the following O_2/CO_2 concentration (kPa) combinations: 1/0; 3/0; 1/3; 3/3; 21/3; 21/0 (air); 21/0 with 1-methylcyclopropene (1-MCP), 21/0 conditioned 3 d at 10 °C, and 21/0 conditioned 5 d at 10 °C. Each data point represents the average of 50 fruit from each of four orchards (a total of 1800 fruit). Bars represent 1 SD.

Figure 17. Transverse cross-sections of ‘Honeycrisp’ fruit depicting a range of severity of controlled atmosphere (CA)-related injury after 1 month of storage under 3 kPa O_2 and 3 kPa CO_2 at 3 °C.

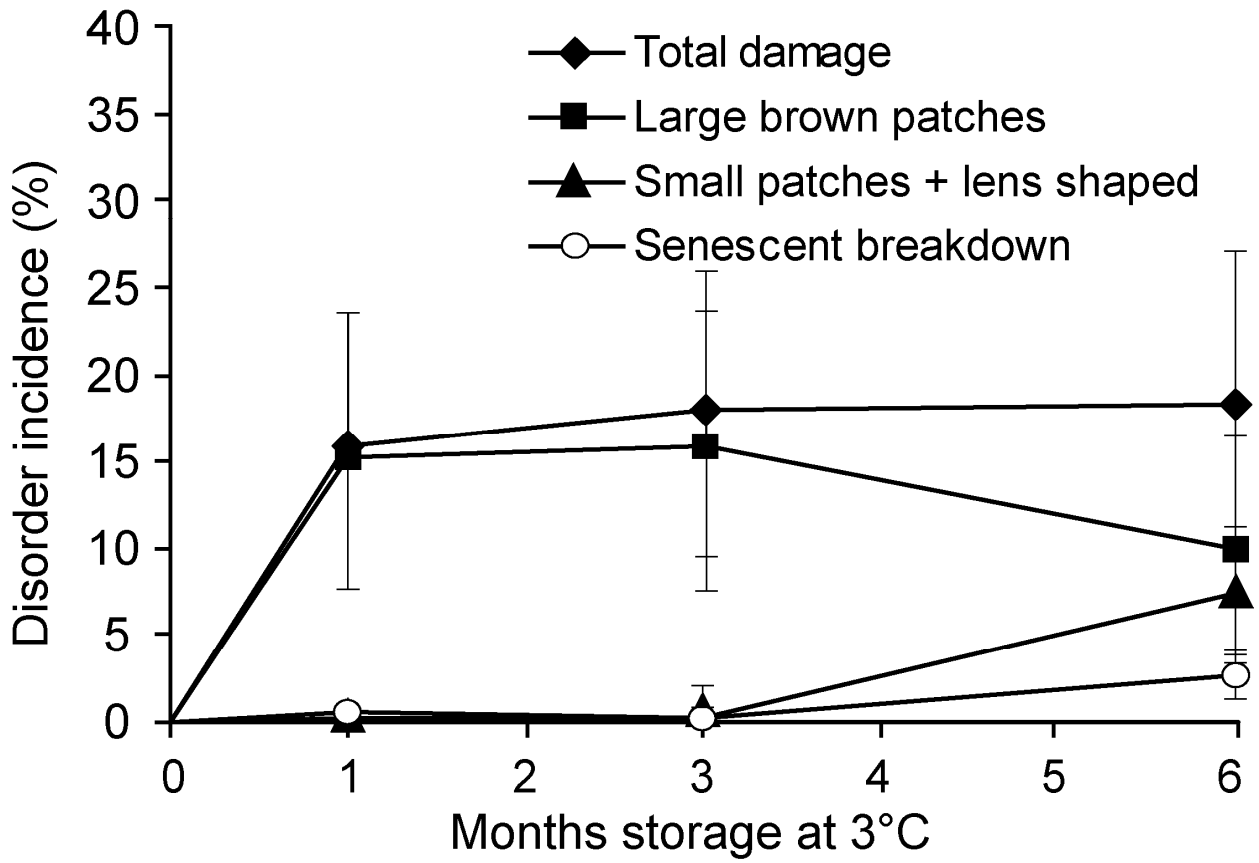

Figure 18. Incidence of controlled atmosphere (CA)-related injury in 'Honeycrisp' apples after 1, 3 and 6 months of CA storage under 3 kPa O_2 and 3 kPa CO_2 at 3 °C. Each data point represents the average for fruit from all nine treatments noted in the caption for Fig. 16 and all four orchards (a total of 1800 fruit). Bars represent 1 SD.

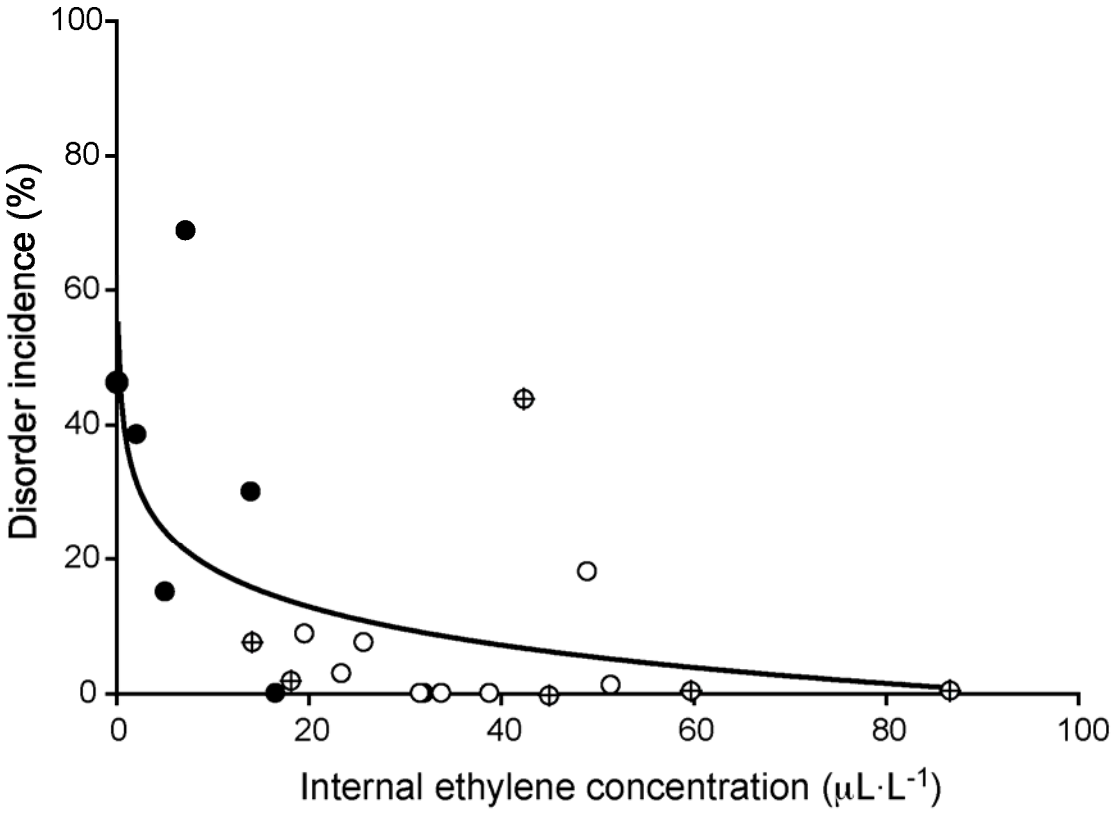

Figure 19. Relationship between controlled atmosphere (CA)-related injuries (brown flesh lesions only) and internal ethylene concentration (IEC) at harvest for 'Honeycrisp' apples after 3 months of CA storage under conditions of 3 kPa O_2 and 3 kPa CO_2 at 3 °C. Each symbol corresponds to the average of 50 fruit from one orchard. Open symbols correspond to year 2009; closed symbols to 2010 and open cross symbols to 2011.

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