BRANCHED-CHAIN ESTER BIOSYNTHESIS IN RIPENING APPLE FRUIT

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ABSTRACT

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In apple fruit, aroma is an essential element of organoleptic quality and it can suffer in response to a number of pre- and post-harvest cultural treatments. Of the several classes of odor-active compounds, esters are the most important, but little is known regarding pathways of biosynthesis. This research presents evidence for a 'new' pathway for ester biosynthesis in apple that uses the starting products pyruvate and acetyl-CoA for the synthesis of precursors to branched-chain (BC) and certain short, straight-chain (SC) esters. The initial step in the pathway involves the formation of citramalic acid from pyruvate and acetyl-CoA by citramalate synthase (CIM). Citramalic acid then provides for the formation of α-keto-β-methylvalerate and its tramsaminated product isoleucine via α-ketobutyrate, and also for the BC ester precursors 2-methylbutanol or 2-methylbutanoate. The hypothesized pathway also provides for the formation of 3-, 4-, and 5-carbon fatty acids via the process of single-carbon elongation of α-keto acids, which are metabolized to short-chain fatty acids. These short-chain fatty acids are proposed to contribute to SC ester formation. Analysis of ripening fruit revealed that citramalic acid increased about 120-fold as ester production increased during ripening. At the same time, the content of isoleucine increased more than 20-fold, while other amino acids remained steady or declined. Apple disc feeding studies documented the incorporation of ^{13}C-labeled acetate into citramalic acid and isoleucine and into esters derived from 2-methylbutanoate, 2-methylbutanol, propanoate, and butanoate, supporting the hypothesized pathway. Furthermore, two novel genes were identified from apple, the sequence of which suggests that they are members of the 2-isopropylmalate

synthase (*IPMS*) gene family. Purified His-tag protein from these genes was found to form citramalate and 2-ethylmalate from the α-keto acids pyruvate and α-ketobutyrate, respectively, when acetyl-CoA was added. Substrate specificity for α-keto acids in decreasing order was α-ketobutyrate, pyruvate, and α-ketovalerate and is characteristic of CIM. Therefore, the two genes (*MdCIM*1 and *MdCIM*2) are apparently alleles coding for CIM, which initiates carbon flux into esters via the 'citramalate pathway'. The sequence of MdCIM1 differed from that of MdCIM2 by two amino acids, yet MdCIM2 was essentially non-functional, possessing only a fraction of the activity of MdCIM1. The hypothesized pathway constitutes a conceptual shift in the regulation of ester biosynthesis in that it implies synthetic, rather than catabolic pathways are responsible for ester precursor supply.

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CHAPTER I

INTRODUCTION

The characteristic aroma in essentially all horticultural fruits is derived from volatile compounds produced during ripening. Aroma is one of the components of flavor along with taste and is considered to be the most important factor in consumer's fruit choice (Péneau et al., 2006) and, therefore, is of significant value.

There are two physical paths for aroma compounds to be to perceived by the olfactory epithelium, one is from the front of the nose by sniffing and the other from a retronasal path via the back of the throat (Mayr et al., 2003). The latter is primarily a product of mastication, involving shearing, salivation, and temperature and pH changes to affect the release of aroma compounds during consumption.

Despite the importance of flavor, breeding of horticultural commodities has focused mostly on appearance, texture, and disease resistance to aid in marketing and improve handling. One of the reasons for a lack of focus on aroma may be that apple grading criteria include maturity, size, color, shape, cleanliness, and freedom from injury, damage, disease, insects, and internal disorder incidence, but not flavor (AMS-USDA, 2002). For apples to be marketable, emphasis on breeding for disease resistance started as early as 1945, in which Purdue University, Rutgers University, and University of Illinois collaborated in the PRI apple scab resistance breeding program (Crosby et al., 1992; Janick, 2006). A number of resultant apple varieties (e.g. 'GoldRush', 'Prima', 'Enterprise') have been released. Others have evaluated the resistance of germplasm lines to blue mold (Janisiewicz et al., 2008) and pest (plum curculio) pressure (Myers et al., 2007) for potential breeding programs. The result has been that flavor has been somewhat neglected in various breeding programs despite the consumer's demand for better flavor (Baldwin, 2002).

Aroma is negatively affected by current cultural practices such as controlled atmosphere (CA) storage and use of the ethylene action inhibitor 1-methylcyclopropene (1-MCP, SmartFresh^{TM}) (Marin et al., 2009). The impact of CA storage and 1-MCP use on ripening and quality have been extensively studied and have increased the marketing season of apples to year-round. In 2010, most of the apples were stored in refrigerated storages and, of these, 81 % were stored in CA for release to the market at a later time in the season (USDA-NASS, 2010). These practices have an advantage in suppressing decay without necessitating the use of fungicides and reducing physiological disorders during storage (Fan et al., 1999). While these technologies permit preservation of fruit quality for a fairly long time, volatile composition is altered, in some cases by the induction of fermentation (Mattheis et al., 1991). For some cultivars, off-flavors are produced by an accumulation of ethanol and aldehydes (Dixon and Hewett, 2000; Ke et al., 1991). Further, aroma production is diminished, and the rate and extent of recovery is reduced upon transfer to air (Ferenczi et al., 2006; Plotto et al., 1999; Tough and Hewett, 2001; Yahia, 1991).

In ripening apple fruit, volatile compounds are spontaneously produced from the peel rather than the flesh (Guadagni et al., 1971) and more than 200 compounds have been isolated (Dimick and Hoskin, 1983). These volatiles consist mainly of esters, alcohols, aldehydes, hydrocarbons, and acids. Of these volatiles, esters are the primary compounds that influence aroma and normally account for 80% to 95 % of the total volatile emission (Paillard, 1990). In ripening apples, the esters hexyl acetate, butyl acetate, and 2-methylbutyl acetate are autonomously and abundantly produced and considered to confer typical apple aroma characteristics (Dimick and Hoskin, 1983; Fellman et al., 2000). In general, they are perceived as fruity and floral (Plotto et al., 2000). Different cultivars emit different compounds (Kakiuchi et

al., 1986) or blends of compounds that determine their aroma characteristics. Secondary aroma volatiles come from the pulp when the tissue is disrupted during consumption. The aldehydes, hexanal, cis-3-hexenal, and trans-2-hexenal are produced in abundance upon tissue disruption (as during mastication) and all responsible for green notes. When the apple is consumed, internal tissue compounds and aroma from the skin mix to create characteristic flavors.

The diversity of the esters produced by apple is largely known. However, the biochemistry of formation of precursors of esters (e.g., the Co-A derivative of a carboxylic acid and an alcohol) is poorly understood. Only the final enzymatic step of ester formation, alcohol acyltransferase, has been thoroughly studied (Aharoni et al., 2000; Harada et al., 1985; Souleyre et al., 2005; Yahyaoui et al., 2002). Extensive feeding studies such as application of exogenous fatty acids or branched-chain amino acids (BACC) demonstrated catabolic pathways involving lipoxygenase, β-oxidation, and amino acid degradation can contribute to ester biosynthesis; however, these pathways are not well characterized in ester-forming fruits. While it can be demonstrated that apple fruit can make esters by metabolizing various exogenous substrates, feeding study evidence is not conclusive that these pathways operate in vivo in the same way. The current dogma of ester biosynthesis is that they are formed primarily via degradative pathways, but we propose synthetic processes may also be involved, perhaps to a significant extent.

The objective of this research was to understand the biology of apple aroma biosynthesis, emphasizing the pathways for the formation of precursors of branched-chain (BC) esters. A thorough investigation of BCAA metabolism leading to BC ester formation led subsequently to the development of a proposal for a ‘new’ pathway for ester biosynthesis. Finally, data were collected to prove the existence of the biosynthetic pathway and to isolate and characterize the

gene(s)/protein(s) involved in the pathway. It is thought that characterization of the gene and the mechanism of ester biosynthesis will help in the development of new highly flavored varieties in the market.

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CHAPTER II

LITERATURE REVIEW

Aroma diversity

Esters are the primary aroma impact compounds and contribute importantly to the quality of ripening apple (*Malus ×domestica*) fruit. The esters are largely composed of either straight-chain (SC) or branched-chain (BC) groups. Major esters in ripening apples are hexyl acetate (fruity, floral), butyl acetate (pear like), and 2-methylbutyl acetate (fruity, banana-like) collectively, and are considered to confer typical apple aroma characteristics (Dimick and Hoskin, 1983; Fellman et al., 2000; Holland et al., 2005). The profile of individual esters changes over time during ripening, altering the aroma (Ferenczi, 2003; Mattheis et al., 1991a). For example, in 'Bisbee Delicious', the ester ethyl acetate is produced predominately during early stage of ripening but decreases as ripening progresses, whereas 2-methylbutyl acetate is produced little during the early stage of ripening but significantly increases in the later stages of ripening (Mattheis et al., 1991a). Each ester has its odor threshold in human olfactory system and some can be detected at parts-per-billion to parts-per-trillion level. For example, ethyl 2-methylbutanoate which is perceived as an apple-like fruity note (Paillard, 1990) can be detected as low as 0.0001 parts-per-million (ppm) (v/v) (Flath et al., 1967) and contributes to overall apple aroma. Non-ester volatiles also contribute to fruit aroma. 4-Methoxyallylbenzene (4-allylanisole) provides a 'spicy' or 'anise-like' aroma (Williams et al., 1977), 3-penten-2-ol provides apple-like aroma (Vanoli et al,. 1995), and β-damascenone, its detection threshold only 2 pg/g in water, provides fruity odor (Fuhrmann and Grosch, 2002; Roberts and Acree, 1995). An Italian cultivar 'Annurca' produced δ-octalactone which has never been reported in apples (Lo Scalzo et al., 2001), but is an odor perceived as creamy and coconut (Perfumer & Flavorist magazine, IL). Terpenes such as ocymene (floral, rose, spicy), cymene (citric), α-bergamotene

(fruity, bergamote), nerolidol (citric, green, apple) were identified for the first time in a Portugese cultivar 'Bravo de Esmolfe' (Reis et al., 2009).

The type and amount of esters produced by fruit are dependent on cultivar (Kakiuchi et al., 1986). For example, acetates are predominately produced in 'Calville Blanc' and 'Golden Delicous', butanoates in 'Bell de Boskoop' and 'Canada Blanc', propanoates in 'Richard' and 'Reinette du Mans', and ethyl esters in 'Starking' (Paillard, 1990). 'Annurca' produced primarily SC esters (96.8%) (Lo Scalzo et al., 2001). Aroma profiles are influenced by a number of cultural factors. 'Delicious' stored for 30 days in 0.05% O_2 and 0.2% CO_2 at 1 °C increased in the concentration of ethyl acetate which was derived from the accumulation of ethanol and acetaldehyde as a result of anaerobic metabolism (Mattheis et al., 1991b). When 'Golden Delicious' was stored up to 8 months, the production of 2-methylbutyl acetate and 2-methylbutanol was suppressed by high CO_2 (3%) but not by low O_2 (1%) (Brackmann et al., 1993). Aroma profiles are also affected by rootstock type (Lo Bianco et al., 2008), harvest season (Echeverria et al., 2004a), maturity stage (Echeverria et al., 2004b), and ripening stage (Brackmann et al., 1993). Optimally-harvested apple has its best aroma composition, whereas early-harvested or overripe fruit can have a low level of volatile production (Song and Bangerth, 1996; Vanoli et al., 1995).

Ester formation

Esters are synthesized by the condensation of an alcohol and a Co-A derivative of a carboxylic acid by alcohol acyltransferase (AAT, EC2.3.1.84) (Ueda and Ogata, 1977). The AAT gene was identified in several horticultural crops such as apple, strawberry (*Fragaria virginiana*), melon (*Cucumis melo*), banana (*Musa* sp.), kiwifruit (*Actinidia* spp.), and mountain

papaya (*Vasconcellea pubescens*) (Aharoni et al., 2000; Balbontín et al., 2010; Günther et al., 2011; Harada et al., 1985; Jayanty et al., 2002; Souleyre et al., 2005; Yahyaoui et al., 2002). AAT has features that have the potential to influence the ester profile: it can utilize a broad range of alcohol and acyl-CoA precursors and exhibits a marked substrate preference (El-Sharkawy et al., 2005; Günther et al., 2011; Yahyaoui et al., 2002). The reaction velocity is affected by carbon chain length and architecture (e.g., straight- or branched-chain) of acyl-CoA, or alcohol substrates (Aharoni et al., 2000; Olias et al., 2002; Ueda et al., 1992; Yahyaoui et al., 2002). The substrate preference differs by fruit species and, within a species, between cultivars (Holland et al. 2005), and cannot be predicted based on the sequence similarity among various members of the AAT family (Beekwilder et al., 2004). However, substrate preference can be predicted based on structural modeling (Morales-Quintana et al., 2011). In apples, four AAT genes are reported, but so far only MdAAT1 was characterized for its enzyme activity. *MdAAT1* from 'Royal Gala' was isolated and is expressed in leaves, flowers, and fruit (Souleyre et al., 2005). The expression of *MdAAT3* and *MdAAT4* decreased as ripening progressed in 'Golden Delicious' (Zhu et al., 2008). An accumulation of MdAAT2 protein was observed during fruit ripening and was found exclusively in the fruit peel by immunolocalization analysis in 'Golden Delicious' (Li et al., 2006). For enzymatic activity, MdAAT1 utilizes a broad range of alcohol substrates ranging from C3-C10 SC, BC, aromatic, and terpene alcohols, and acid substrates of short-medium-chain CoAs (Souleyre et al., 2005). While MdAAT1 produces a wide range of esters, its preference depends on substrate concentration when synthesizing acetate esters. It is generally accepted that AAT will influence the ester profile by its substrate specificity and availability, but control of ester synthesis probably lies at the level of ester precursor formation (Ferenczi et al., 2006; Wyllie and Fellman, 2000).

Esters have an alcohol-derived (alkyl) group and an acid-derived (alkanoate) group. Alkyl groups normally range from 1-6 carbons in length and alkanoate groups range from 2-8 carbons (Paillard, 1990). Ester precursors are proposed to form from both degradative and synthetic pathways; however, the current concept of ester biosynthesis is mainly supported via degradation pathways. SC ester precursors have been proposed to be from fatty acid degradation via β-oxidation or the lipoxygenase system (Sanz et al., 1997) and BC ester precursors have been proposed to be derived from branched-chain amino acid (BCAA) degradation (Gonda et al., 2010; Rowan et al., 1996; Tressl and Drawert, 1973; Wyllie and Fellman, 2000). The proposed degradative pathways are supported by exogenous feeding studies. For example, feeding methyl hexanoate and methyl octanoate enhanced butanoate ester formation in 'Cox's Orange Pippin' (Bartley et al., 1985). Application of deuterated C18:0 and C18:1 fatty acids produced C6-C8 alkanoate esters and linoleic acid produced only hexyl and hexanoate esters in 'Granny Smith' (Rowan et al., 1999). Feeding isoleucine increased 2-methylbutyl and 2-methylbutanoate ester content in 'Granny Smith' and strawberry (*Fragaria ×ananassa*) (Pérez et al., 2002; Rowan et al., 1996). ^{14}C-labeled leucine yielded 3-methylbutanol and 3-methylbutanoate and their respective esters, and ^{14}C-labeled valine metabolism produced 2-methylpropanol and 2-methylpropanoate and their respective esters in banana (Tressl and Drawert, 1973). On the other hand, synthetic pathways such as one- (1-C FAB) and two-carbon fatty acid biosynthesis (2-C FAB) may not be disregarded. 2-C FAB pathway provides for membrane fatty acids, storage lipids, and waxes (Ohlrogge and Jaworski, 1997), and 1-C FAB pathway is involved in sugar-ester acyl-acids (Kroumova and Wagner, 2003), short-chain alcohols of yeast (Vollbrecht, 1974) and bacteria (Atsumi and Liao, 2008), and isoleucine biosynthesis in bacteria (Howell et al., 1999; Xu et al., 2004). These synthetic routes may play an important role in providing ester

precursors. Carbon entry into these degradative and synthetic pathways now will be discussed further.

Proposed catabolic pathways

β-oxidation pathway. Evidence for the involvement of β-oxidation in ester synthesis is based on feeding studies using isotopically labeled substrates (Rowan et al., 1999). β-Oxidation takes place in plant peroxisomes/glyoxisomes rather than mitochondria as in mammals (Charlton et al., 2005; Eaton et al., 1996). Four enzymes act to remove acetyl-CoA from the fatty acid, which are, in order of action, acyl-CoA oxidase (ACX, EC 1.3.3.6), 2E-enoyl-CoA hydratase (EC 4.2.1.17), 3S-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), and 3-ketoacyl-CoA thiolase (KAT, EC 2.3.1.16) (a.k.a. thiolase). In *Arabidopsis*, four out of six ACX isozymes are characterized and have a substrate preference for fatty acid chain-length: either long- (C14-20), medium-long- (C12-C16), medium- (C8-C14), and short- (C4-C8) chain (De Bellis et al., 1999, 2000; Graham, 2008; Hooks et al., 1996, 1999; Kirsch et al., 1986, Rylott et al., 2003). Three KAT genes are found in Arabidopsis and *KAT2* is strongly expressed during seed germination. The *Kat2* mutant lacks long-chain (C16-C20) fatty acyl-CoAs and C4 acetoacetyl-CoA thiolase activity suggesting that the KAT protein has broad substrate specificity (Graham, 2008).

The gene expression involved in β-oxidation have been studied in ester-forming fruit species in few publications (Schaffer et al., 2007; Sugimoto et al., 2008) and protein characterization is completely lacking. Sugimoto et al. (2008) found that the expression of two of the four genes (acyl-CoA oxidase and putative 2E-enoyl-CoA hydratase) in the pathway increased to a maximum at the peak in ester synthesis, declining thereafter. The β-oxidation pathway may contribute the synthesis of fatty acid ester precursors such as C4-C8 fatty acid ester

precursors; however, β-oxidation results in even numbered carbon fatty acids, leaving in question the formation of the odd numbered carbon fatty acid ester precursors. In apple fruit the formation of C5 compounds such as pentanol was suggested to occur through α-oxidation of C6 compounds (Rudell et al., 2002), but this assumption was not verified. Also, the formation of the C3 alcohol propanol by decarboxylation of α-ketobutyrate, an intermediate in isoleucine synthesis, has been demonstrated to occur in yeast *(Saccharomyces cerevisiae*) (Guymon et al., 1961). However, there is no study presented in plants, thus in vivo synthesis of 3- to 8-carbon fatty acids is still unclear in ester forming fruit.

Lipoxygenase pathway. The lipoxygenase pathway may supply saturated and unsaturated C6 and C9 aldehydes that could potentially be converted to alcohols, which act as precursors for ester formation. C6 and C9 aldehydes are generated by the action of lipoxygenase pathway enzymes on linoleic (C18:2) or linolenic acids (C18:3). Meigh and Hulme (1965) observed a significant accumulation of both saturated and unsaturated free fatty acids and esterified fatty acids, especially in the unsaturated C18 acids, in 'Cox's Orange Pippin' peel during ripening. When palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid, linolenic acid were quantified during unripe to ripe stage in 'Golden Delicious' peel, oleic and linoleic acid rapidly increased as fruit ripened (Song and Bangerth, 2003). Linoleic acid was one of the most abundant free fatty acids (Meigh and Hulme, 1965; Meigh et al., 1967) and the ratio of oleic to linoleic to linolenic acid was approximately 3:4:1 during the later stages of ripening (Song and Bangerth, 2003).

The first step in the lipoxygenase pathway is the release of linoleic and linolenic acid from phospholipids by lipase or hydrolase (Wang, 2001). These fatty acids are then oxidized by

lipoxygenase (LOX, EC 1.13.11.12). LOX can be classified into two groups: 9-LOX mainly produces 9-carbon-hydroperoxides (9-HPO) and 13-LOX produces 13-carbon-hydroperoxides (13-HPO). In strawberry fruit, LOX activity preferred linolenic acid to produce 13- and 9-HPOs in a 7:3 ratio (Pérez et al., 1999). However, in apples, LOX is highly specific in peroxidizing linoleic acid to 13-HPO (Kim and Grosch, 1979), while in bananas, LOX produced nearly equal amounts of 13- and 9-HPO (Tressl and Drawert, 1973). When Salas et al. (2005) silenced LOX genes in potato and studied linoleic acid metabolism in leaf tissue, the affected plants had severely decreased C6 volatile production from leaf disks. Salch et al. (1995) reported that C5 compounds such as 2-Z-pentenol or 1-penten-3-ol can be produced from 13-HPO under anaerobic conditions in soybean seed. In kiwifruit, different LOX genes are differentially regulated during fruit ripening and six LOX genes have been identified (Zhang et al., 2006). Some are active in ripening and are responsive to ethylene, while others are constitutively expressed. Application of linoleic and linolenic acid to kiwifruit promoted LOX activity and produced n-hexanal and E-2-hexenal, respectively (Zhang et al., 2009). In tomato fruit, TomloxB is likely related with fruit ripening and senescence (Griffiths et al., 1999), TomloxC is involved in the production of C6 aldehydes associated with characteristic tomato aromas (Chen et al., 2004), and TomloxD is suggested to be involved in defense signaling pathway (Heitz et al., 1997).

The next step in the lipoxygenase pathway involves the enzyme hydroperoxide lyase (HPL, EC 4.1.2.) in which 9-HPL cleaves 9-HPO to form C9 oxoacids and C9 aldehydes and 13-HPL cleaves 13-HPO to form C12 oxoacids and C6 aldehydes. There are three types of HPL activity based on the substrate preference: 9-HPL has a strong preference for 9-HPO, 9/13-HPL can accept either 9- or 13-HPO, and 13-HPL prefers 13-HPO. The activity of 9-HPL is found in

pear (*Pyrus communis*) fruit (Kim and Grosch, 1981) , 9/13-HPL is in cucumber (*Cucumis sativus*) seedlings (Matsui et al., 2000) and melon (*Cucumis melo*) fruit (Tijet et al., 2001), and 13-HPL is in tomato (*Solanum lycopersicon*) fruit (Matsui et al., 2007), guava (*Psidium guajava*) fruit (Tijet et al., 2000), and olive (*Olea europaea* cv. Picual) fruit (Padilla et al., 2010). When HPL gene was silenced in Arabidopsis, C6 compounds from linoleic acid accumulated and the total amount of C5 compounds was 4-fold higher in mutant lines (Salas et al., 2006). Currently, there is little information about the relationship of HPL activity with aroma biosynthesis in fruit. In apple, autonomously produced unsaturated esters are rare but are abundantly produced upon tissue disruption (De Pooter et al., 1987). The two aldehydes hexanal and trans-2-hexenal are formed from the crushing of apples (Feys et al., 1980), suggesting the lipoxygenase pathway may be important for volatile production during chewing of apples (Yahia, 1994).

Branched-chain amino acids (BCAA) pathway. The first step of the reaction produces α-keto acids from three BCAAs in a proposed degradative pathway for BC ester biosynthesis,. The three BCAAs, isoleucine, valine, and leucine are transaminated to produce their respective α-keto acids, α-keto-β-methylvalerate, α-keto-isovalerate, and α-ketoisocaproate, respectively, by branched-chain aminotransferase (BCAT, EC 2.6.1.42). These α-keto acids are the most immediately related product to ester precursors. In apple, isoleucine is proposed to rapidly degrade into α-keto-β-methylvalerate to form 2-methylbutanol and 2-methylbutanoate esters via BCAT. To date, none of the apple BCAT genes have been characterized and the enzymatic function of protein is unknown, but only the EST expression is reported as an ester biosynthesis-related gene (Newcomb et al., 2006; Schaffer et al., 2007). In melon, *CmBCAT1* gene was isolated and is highly expressed in ripe fruit (Gonda et al., 2010). The enzyme converted

isoleucine and leucine to α-keto-β-methylvalerate and a-ketoisovalerate, respectively, and suggested the BCAT gene is the initial step in the formation of BC esters.

Feeding studies also demonstrated that BC esters can be produced directly from exogenously supplied BC α-keto acids rather than BCAAs (Gonda et al., 2010). It is important to note that the α-keto acids and not the BCAAs serve as substrates for the formation of alkyl and alkanoate BC precursors to BC esters. Since BC α-keto acids are most immediately related to ester precursor formation, 2-methylbutyl- and 2-methylbutanoate esters can be expected to result from enhanced catabolism of α-keto-β-methylvalerate, rather than from isoleucine per se in apple fruit. Moreover, the BCAT reaction is readily reversible so that it can be stated the BC α-keto acids are in approximate equilibrium with their respective BCAAs, and it would be expected that the pools of BCAAs roughly mirror the pools of their respective BC α-keto acids (Tewari et al., 2000). Furthermore, the predominant direction of carbon flux can not be determined by the gene expression pattern, but rather by the concentration of the reactants and products.

Synthetic pathways

The formation of C3-C8 ester precursors may also be the product of synthetic processes. 2-C FAB will provide even-number carbon ester precursors and 1-C FAB will provide odd- and even-number carbon ester precursors. As well, 2-methylbutanol and 2-methylbutanoate ester precursors may be formed as the result of isoleucine synthesis.

Two-carbon fatty acid biosynthesis. The proportion of saturated free fatty acids is not significantly large compared to unsaturated free fatty acids, but palmitic acid (C16:0) and stearic acid (C18:0) in apple accumulate during ripening and senescence (Defilippi et al., 2005; Meigh

and Hulme, 1965), which implies that the biosynthesis of fatty acids is quite active throughout the ripening process.

Fatty acid biosynthesis in plants is localized in plastids (Ohlrogge and Jaworski, 1997) and the first committed step is the formation of malonyl-CoA from acetyl-CoA by acetyl-CoA carboxylase (EC 6.4.1.2). The malonyl-CoA is transacylated to malonyl-ACP by malonyl-CoA:ACP transacylase requiring acyl-carrier protein as a cofactor. The EST expression of acyl-carrier protein from strawberry is associated with fruit ripening (Manning, 1998) and both acyl-carrier protein and malonyl-CoA:ACP transacylase from ripening apple skin is strongly expressed (Park et al., 2006). Malonyl-ACP next condenses with acetyl-CoA by an enzyme 3-ketoacyl-ACP synthase lll (KAS lll, EC 2.3.1.41) producing 3-ketobutyryl-ACP. From this, butyryl-ACP is produced by the action of three additional enzymes: 3-ketoacyl-ACP reductase (EC 1.1.1.100), 3-hydroxyacyl-ACP dehydratase (EC 4.2.1.17), and enoyl-ACP reductase (EC 1.3.1.9) in that sequence. ESTs of KAS lll and acyl-carrier protein are expressed in ripening papaya (*Carica papaya*) fruit (Devitt et al., 2006), which produces C4, C6, and C8 alkanoate esters (Fuggate et al., 2010). Butyryl-ACP then condenses with malonyl-ACP by the enzyme 3-ketoacyl-ACP synthase l (KAS l) and KAS ll, repeating the cycle to produce even numbered carbon fatty acids until acyl-ACP thioesterase (EC 3.1.2.14) terminates fatty acid synthesis. KAS has a substrate preference: KAS l prefers the shorter chain (C4-14)-ACP and KAS ll prefers the longer chain (>C14)-ACP (Shimakata and Stumpf, 1982). Acyl-ACP thioesterase isozymes also have a substrate preference either in cleaving 18:1-ACP (FatA) or saturated 8:0- to 18:0-acyl-ACP (FatB) which has a broader specificity (Hawkins and Kridl, 1998; Jones et al., 1995). Enzyme activity of FatA from mangosteen (*Garcinia mangostana*) seed had a substrate preference mostly for 18:1-ACP, whereas FatB preferred 16:0-ACP (Hawkins and Kridl, 1998).

California bay (*Umbellularia californica*) cotyledons contained two activities either with preference for 12:0-ACP or 18:1-ACP (Pollard et al., 1991), and when the 12:0-ACP thioesterase gene was expressed in developing seed of Arabidopsis, the transgenic plant produced large amounts of laurate (12:0) (Voelker et al., 1992). It is possible that the increase in the C4-C8 esters during ripening is related to fatty acid synthesis, especially when both KAS l and acyl-ACP thioesterase are active to produce shorter chain length of C4-C8 acid precursors of esters. However, to my knowledge, no data exists to support this possibility.

Branched-chain amino acid biosynthesis. In apples, BC esters 2-methylbutanol and 2-methylbutanoate esters predominate and 2-methylpropanol and 2-methylpropanoate can be detected occasionally at low levels (Ferenczi, 2003; Mattheis et al., 1998; Ortiz et al., 2010; Plotto et al., 2000; Sugimoto, 2007). Although a small amount of leucine is found in ripening apple fruit (Burroughs, 1970; Hansen, 1970), there are few 3-methylbutanol and 3-methylbutanoate esters produced. During apple fruit ripening, isoleucine has been reported to accumulate, but not the other BCAAs (Defilippi et al., 2005; Nie et al., 2005). An increase in isoleucine is indicative that its synthesis is active during` fruit ripening.

In general, isoleucine is thought to be derived from the threonine pathway, but a non-threonine, 'citramalate pathway' (a part of 1-C FAB) for the formation of isoleucine has been demonstrated in certain strains of bacteria and in yeast (Howell et al., 1999; Vollbrecht, 1974; Xu et al., 2004).

Threonine pathway. Isoleucine is synthesized from threonine via aspartate in plants. Aspartate originates from the TCA cycle and is used to synthesize several amino acids including lysine,

methionine, and threonine (Azevedo et al., 1997). To synthesize isoleucine, threonine is deaminated to α-ketobutyrate by threonine deaminase (TD, EC 4.2.1.16) (Binder, 2010) (Figure 1). The isoleucine precursor, α-keto-β-methylvalerate, is synthesized from α-ketobutyrate by three enzymes: acetohydroxy acid synthase [AHAS, a.k.a. acetolactate synthase, (ALS), EC 4.1.3.18], acetohydroxy acid isomero-reductase (EC 1.1.1.86), and dihydroxy-acid dehydratase (EC 4.2.1.9) acting in succession. These enzymes also catalyze the formation of α-ketoisovalerate from pyruvate to synthesize valine. Leucine synthesis, however, begins with the valine precursor, α-ketoisovalerate, which is acted upon by three enzymes: 2-isopropylmalate synthase (IPMS, EC 2.3.3.13), 2-isopropylmalate isomerase (EC 4.2.1.33), and 3-isopropylmalate dehydrogenase (EC 1.1.1.85), to form α-ketoisocaproate, the immediate precursor to leucine.

Biosynthesis of all three BCAAs is responsive to feedback regulation. TD is inhibited by isoleucine, but stimulated by valine, ALS is inhibited by valine and leucine, and IPMS is inhibited by leucine (Eisenstein, 1991; Mourad and King, 1995; Singh and Shaner, 1995; Wessel et al., 2000). An accumulation of isoleucine during apple ripening is counter intuitive with the feedback mechanism, the increase in isoleucine during ripening suggests that either TD activity becomes insensitive to feedback regulation or an alternative pathway exists to synthesize α-ketobutyrate via isoleucine that bypasses threonine. In plants, isoleucine biosynthesis is thought to be exclusively via the threonine pathway. TD activity is required for autotrophy in *Nicotiana plumbaginifolia* (Sidorov et al., 1981). An isoleucine-requiring Nicotiana TD mutant (ILE401) was able to restore its autotrophy with a yeast TD (ILV1) gene in a complementation study (Colau et al., 1987), demonstrating that threonine degradation is the sole pathway for isoleucine biosynthesis. In most plant species, TD is considered to have only one gene whereas tomato has

two forms (Gonzales-Vigil et al., 2011). In tomato, one of the TD genes is present in the younger leaves and is responsive to isoleucine regulation, whereas the other gene is present in older leaves but insensitive to isoleucine and it is suggested that the role of the isoleucine insensitive TD is in the nitrogen remobilization in senescing leaves (Szamosi et al., 1993). To date, no TD activity has been characterized in apple.

Non-threonine pathway. There are three pathways reported to synthesize α-keto-β-methylvalerate and its product, isoleucine, via alternative routes that do not involve threonine deamination. In Arabidopsis, methionine-γ-lyase (MGL, EC 4.4.1.11) activity may produce α-ketobutyrate from methionine (Joshi and Jander, 2009; Rebéillé et al., 2006). A second pathway has been found in yeast in which cystathionine-γ-lyase (Cys3, EC 4.4.1.1) produces α-ketobutyrate from cystathionine via cysteine biosynthesis (Ono et al., 1999). The third pathway for isoleucine biosynthesis has been described only in several strains of bacteria and is via a 'citramalate' pathway established using isotope feeding studies (Table 1) (Gray and Kornberg, 1960; Hochuli et al., 1999; Risso et al., 2008; Westfall et al., 1983). In this pathway, acetyl-CoA and pyruvate are substrates for the formation of citramalic acid by citramalate synthase (CIM, EC 2.3.1.182). Citramalate, in turn, is converted to α-ketobutyrate by 2-isopropylmalate isomerase and 3-isopropylmalate dehydrogenase, which are the same enzymes involved in leucine biosynthesis. The citramalate pathway is suggested to be present in yeast (*Saccharomyces cerevisiae*) (Vollbrecht, 1974) and CIM activity is reported (Losada et al., 1964; Sai et al., 1969) but no nucleotide or amino acid sequence was reported nor documented in the yeast genome database. In plants, Kroumova and Wagner (2003) reported the involvement of 1-C FAB in sugar ester biosynthesis and excretion in members of the Solanaceae [e.g., tobacco

(*Nicotiana tabacum*) and petunia (*Petunia ×hybrida*)] suggesting that the 'citramalate pathway' is the entry point for the 1-C FAB pathway. It is interesting that methylthioalkylmalate synthase 3 (MAM3, EC 2.3.3), which is involved in glucosinolate biosynthesis in Arabidopsis, was capable of synthesizing citramalic acid from pyruvate and acetyl-CoA (Textor et al., 2007). However, its primary function was ultimately in glucosinolate formation.

The citramalate pathway has been suggested to confer the capacity for chain elongation by adding one carbon to the initial starting product α-keto acid. The condensation of α-ketobutyrate and acetyl-CoA is catalyzed by an enzyme tentatively named 2-ethylmalate synthase (EC 2.3.3.6) in yeast (Canovas et al., 1965), which is likely a member of the IPMS family such as IPMS or CIM. The production of 2-ethylmalate and 2-propylmalate, which are products of chain elongation of α-ketobutyrate, has been demonstrated in yeast (Strassman and Ceci, 1967). The reactions of the citramalate pathway have the potential to synthesize various chain length fatty acids. It has been proposed to be employed in the formation of fatty acids of 3-12 carbons of straight-, branched-, odd- or even-, short- or medium-chain length (up to C7 straight-chain in petunia) (Kandra et al., 1990; Kroumova et al., 1994; Kroumova and Wagner, 2003; Oku and Kaneda, 1988). A similar chain elongation cycle is also reported for AtMAMs. AtMAM1 and AtMAM3 condense 4-methylthio-2-oxobutanoate with acetyl-CoA with two steps to generate the elongated product 5-methylthio-2-oxopentanoate and repeats the cycle to produce 6-methylthio-2-oxohexanoate in glucosinolate synthesis (Textor et al., 2004; 2007). If the citramalate pathway is utilized in fruit ester biosynthesis, it may explain the accumulation of isoleucine during apple ripening and the formation of esters with odd as well as even numbered carbon chains. Further, if the production of α-keto-β-methylvalerate and its product, isoleucine, occurs via the citramalate synthase pathway or one of the other alternative pathways mentioned,

it would explain the unique and unexpected accumulation of isoleucine during apple ripening. Isoleucine normally inhibits its own accumulation by feedback inhibition of TD (Mourad and King, 1995) and alternative routes of synthesis may not be similarly regulated. Despite the existence of the citramalate pathway in bacteria, no conclusive data for this pathway (e.g., isolate the citramalate synthase gene) has been demonstrated in plants or in yeast.

Citramalic acid has been detected in apple, pear, banana, citrus, and tomato (Degu et al., 2011; Fraser et al., 2007; Rudell et al., 2008; Ulrich, 1970). Hulme (1954) isolated citramalic acid from the peel of mature 'Edward VII' apple fruit and observed its absence in pulp tissue. Hulme and Wooltorton (1958) studied the changes of citramalic acid content in 'Bramley's Seedling' apple during 100 days of storage at 15 °C and reported that no citramalic acid was detected at the time of harvest, but increased to 0.1 $mg \cdot g^{-1}$ after 25 days, and maximized at 0.25 $mg \cdot g^{-1}$ after 100 days. Hulme and Wooltorton (1957) also found that citramalic acid was absent at all stages of immature apple fruit, taking a measurement from the date of petal fall on May 10, 1952 to fruit maturity. Although citramalic acid has been detected in several fruits, there is no data supporting any particular source or pathway of biosynthesis. Hulme (1954) proposed citramalate participates in the TCA cycle but its function has been the subject of speculation.

Both CIM and IPMS belong to an acyltransferase family (EC 2.3.3) sharing a common reaction between α-keto acids and acetyl-CoA. Other relevant enzymes that belong to the acyltransferase family include citrate synthase (EC 2.3.3.1), homocitrate synthase (EC 2.3.3.14), malate synthase (EC 2.3.3.9), and MAM, but each differs in substrate preference, preferring oxaloacetate, α-ketoglutarate, glyoxylate, and various ω-methylthio-α-ketoalkanoates, respectively (Textor et al., 2004).

Despite the existence of citramalte pathway in several strains of bacteria, only CIM gene from *Methanococcus jannaschii* (gi: 15669582) and *Leptospira interrogans* (*LiCMS*) (gi: 24215050) has been isolated and characterized for its activity (Howell et al., 1999; Xu et al., 2004). Recently, the structure analysis was reported in *Leptospira interrogans* (Ma et al., 2008; Zhang et al., 2009). The size of *LiCMS* is 56 kDa as a mononer, functions as a dimer, requires Mn^{2+} as a divalent cation, and the monovalent cation K^+ or NH_4^+ act as a co-activator. The enzyme contains R-region and is negatively regulated by isoleucine.

IPMS is the first enzymatic step for leucine biosynthesis. The enzyme activity is characterized in several strains of bacteria including *Salmonella typhimurium* (Kohlhaw et al., 1969; Leary and Gunter, 1972), *Mycobacterium tuberculosis* LeuA (Koon et al., 2004; de Carvalho and Blanchard, 2006a, 2006b; Singh and Bhakuni, 2007), *Neurospora* (Cassady et al., 1972; Webster and Gross, 1965), yeast *Saccharomyces cerevisiae* Leu4 and Leu9 (Casalone et al., 2000; Hampsey and Khlhaw, 1981; Kohlhaw, 1988; Ulm et al., 1972), and plant *Arabidopsis thaliana IPMS*1 (At1g18500) and *IPMS*2 (At1g74040) (de Kraker et al., 2007) and *Spinacia oleracea* (Hagelstein and Schultz, 1993).

The structure of IPMS is either in dimer (*Mycobacterium tuberculosis* and yeast) or a tetramer (*Salmonella typhimurium* and Arabidopsis) and the molecular weight ranges from 63 to 70 kDa as a monomer depending on species. The optimum pH is around 7.0-9.0, requires Mg^{2+}, and is activated or stimulated by K^+, but inhibited by Zn^{2+}. IPMS activity is inhibited by leucine which binds to the R-region located in the C-terminal region (Cavalieri et al., 1999). Bacteria have only one IPMS gene but yeast and Arabidopsis have two genes. In bacteria, IPMS is localized in mitochondria and in mitochondria and cytosol for yeast, whereas IPMS is localized in chloroplast in spinach.

My overall objective was to explore the biology associated with the synthesis of BC esters in apple culminating in an evaluation of the potential for the citramalate pathway to contribute to the synthesis of several BC ester precursors. It should be noted that there are linkages between the putative citramalate pathway and the capacity to form short SC esters. Evidence for an active citramalate pathway, with its attendant capacity for one-carbon chain elongation, will demonstrate that aroma formation is at least, in part, the product of an active synthesis, rather than pathways of catabolism and degradation.

Table 1. Reported existence of citramalate pathway in several strains of bacteria supported by feeding studies and with characterized citramalate synthase gene name. (n.d. indicates not determined; n.a. indicates not available).

Species	Type	Feeding	Gene	Reference
Chlorobaculum tepidum	anaerobic, thermophilic, photosynthetic green sulfur bacteria	^{13}C-acetate, ^{13}C-pyruvate	n.a.	Feng et al. (2010)
Chlorobium vibrioforme	anaerobic, phototrophic, green sulfur bacteria	^{13}C-acetate	n.a.	Nesbakken et al. (1988)
Cyanothece sp. ATCC 51142	aerobic	^{13}C-glycerol	n.a.	Wu et al. (2010)
Dehalococcoides ethenogenes	anaerobic	^{13}C-sodium acetate, ^{13}C-sodium bicarbonate	n.a.	Tang et al. (2009)
Geobacter sulfurreducens	anaerobic	^{13}C-acetate, ^{13}C-fumarate	GSU1798	Risso et al. (2008)
Haloarcula hispanica	aerobic chemo-organotrophs, halophilic archaea	^{13}C-glycerol	n.a.	Hochuli et al. (1999)

Table 1 (cont'd).

Species	Type	Feeding	Gene	Reference
Leptospira interrogans	aerobic	^{14}C-CO_2		Charon et al. (1974)
Serotype lai		^{14}C-CO_2, ^{14}C-pyruvate		Westfall et al. (1983)
Strain 56601		n.d.	LA2350	Xu et al. (2004)
Methanococcus	aerobic	n.d.	MJ1392	Howell et al. (1999)
Jannaschii				
Methanogenic	archaebacteria	^{13}C-acetate	n.a.	Ekiel et al. (1984)
Bacteria (6 different species)				
Pseudomonas ovalis	aerobic	^{14}C-acetate,	n.a.	Gray and Kornberg, (1960)
Chester		^{14}C-pyruvate		
Serratia marcescens	anaerobic	n.d.	n.a.	Kisumi et al. (1977)
Thermoanaerobacter	anaerobic, thermophilic	^{13}C-glucose,	n.a.	Feng et al. (2009)
sp. Strain X514		^{13}C-pyruvate		
Thermoproteus	anaerobic, thermophilic	^{13}C-acetate	n.a.	Schäfer et al. (1989)
neutrophilus				

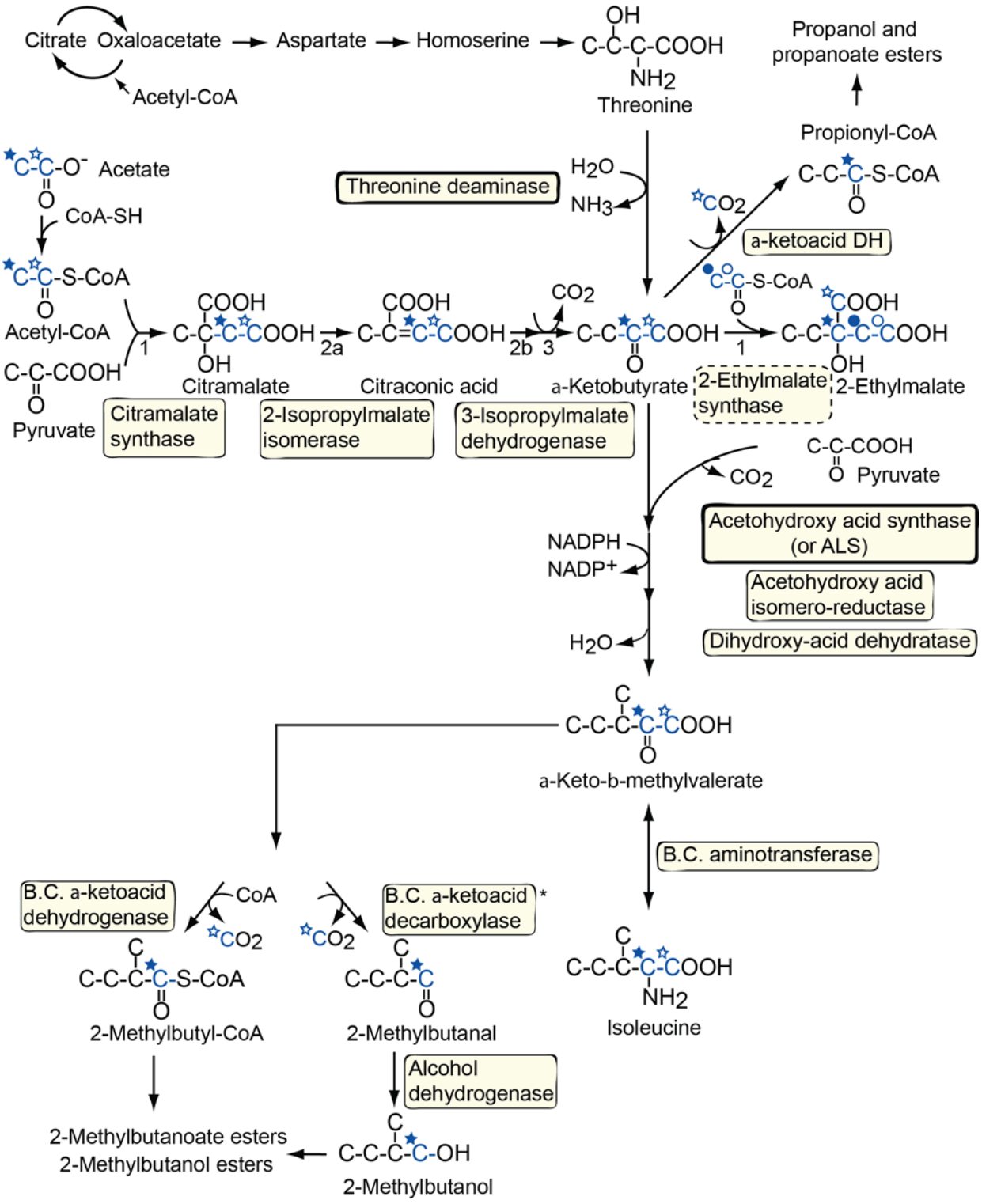
Citrate
Oxaloacetate
Aspartate
Homoserine
Threonine
Acetyl-CoA
Propanol and propanoate esters
Propionyl-CoA
Acetate
CoA-SH
Threonine deaminase
a-ketoacid DH
Acetyl-CoA
Pyruvate
Citramalate
Citraconic acid
a-Ketobutyrate
2-Ethylmalate
Citramalate synthase
2-Isopropylmalate isomerase
3-Isopropylmalate dehydrogenase
2-Ethylmalate synthase
Acetohydroxy acid synthase (or ALS)
Acetohydroxy acid isomero-reductase
Dihydroxy-acid dehydratase
a-Keto-b-methylvalerate
B.C. aminotransferase
B.C. a-ketoacid dehydrogenase
B.C. a-ketoacid decarboxylase
2-Methylbutyl-CoA
2-Methylbutanal
Isoleucine
Alcohol dehydrogenase
2-Methylbutanoate esters
2-Methylbutanol esters
2-Methylbutanol

Figure 1.

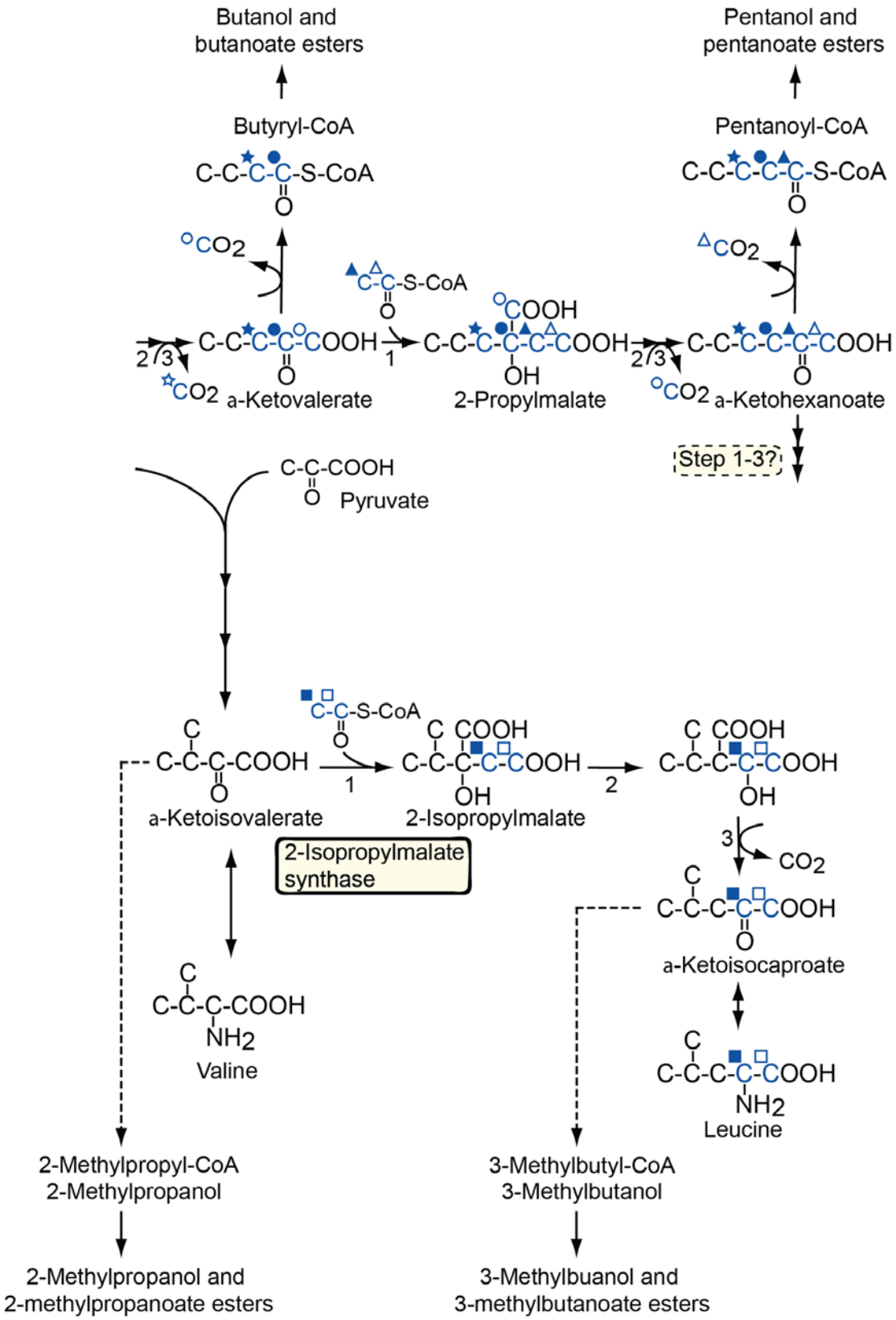

Figure 1 (cont'd).

Figure 1. Pathways involved in branched-chain ester biosynthesis in apple. Genes in bolded boxes are regulated by feedback inhibition. Of these, threonine deaminase is inhibited by isoleucine, but stimulated by valine, acetohydroxy acid synthase [a.k.a. acetolactate synthase, (ALS)] is inhibited by valine and leucine, and 2-isopropylmalate synthase is inhibited by leucine. The trivial name of 2-ethylmalate synthase, in dashed box, is tentatively given in yeast (Canovas et al., 1965). An asterisk indicates the gene is found in bacteria, but not in plants. Hydrogens in carbon-hydrogen bonds are not shown. Enzymatic steps numbered 2 and 3 in the citramalate pathway are considered to be the same enzymes used in leucine biosynthesis, namely 2-isopropylmalate isomerase and 3-isopropylmalate dehydrogenase, respectively. Carbons derived from the C-1 and C-2 positions of acetyl-CoA are indicated with open and solid symbols adjacent to the carbon atoms, respectively. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

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CHAPTER III

CHANGES IN FREE AMINO ACID CONTENT IN 'JONAGOLD' APPLE FRUIT AS RELATED TO BRANCHED-CHAIN ESTER PRODUCTION, RIPENING, AND SENESCENCE

Introduction

Esters are the primary aroma impact compounds produced in ripening apple (*Malus ×domestica*) fruit and normally account for 80% to 95% of the total volatiles emitted (Paillard, 1990). Fresh apples autonomously produce an abundance of hexyl acetate, butyl acetate, and 2-methylbutyl acetate, which confer typical apple aroma characteristics (Paillard, 1990). The esters are largely composed of either straight- or branched-chain alkyl (alcohol-derived) and alkanoate (acid-derived) groups. The ester product is formed from the condensation of an alcohol and a Co-A derivative of a carboxylic acid by alcohol acyltransferase (AAT) (Ueda and Ogata, 1977). Ester precursors are suggested to be produced primarily by degradative processes. Straight-chain ester precursors have been proposed to form from fatty acid degradation via β-oxidation or the lipoxygenase system (Sanz et al., 1997) and BC ester precursors have been proposed to be derived from branched-chain amino acid (BCAA) degradation (Gonda et al., 2010; Rowan et al., 1996; Tressl and Drawert, 1973; Wyllie and Fellman, 2000).

The BC α-keto acids, α-keto-β-methylvalerate, α-keto-isovalerate, and α-ketoisocaproate are substrates for the synthesis of the three BCAAs, isoleucine, valine, and leucine, respectively. The reaction is catalyzed by one or more BCAT proteins (Figure 2). This reversible reaction is also the first step in BCAA degradation. Feeding studies have demonstrated that labeled leucine yields 3-methylbutanol and 3-methylbutanoate and their respective esters, valine metabolism produces 2-methylpropanol and 2-methylpropanoate and their respective esters, and isoleucine yields 2-methylbutanol and 2-methylbutanoate and their respective esters (Pérez et al., 2002; Rowan et al., 1996, 1998; Tressl and Drawert, 1973; Wyllie et al., 1996; Wyllie and Fellman, 2000). The BC α-keto acids are in approximate equilibrium with their respective BCAAs (Tewari et al., 2000), so it is expected that the pools of BCAAs roughly mirror the pools of their

respective BC α-keto acids. Since the BC α-keto acids, not the BCAAs, serve as direct substrates for the reactions leading to the formation of alkyl and alkanoate BC precursors to BC esters, it might be more correctly stated that BC α-keto acids, rather than BCAAs, are most immediately related to ester precursor formation. In fact, BC esters can be produced directly from exogenously supplied BC α-keto acids (Gonda et al., 2010), which suggests the BC esters can be formed without participation of the BCAAs. To some extent, therefore, in apple fruit, 2-methylbutyl- and 2-methylbutanoate esters can be thought to result from enhanced catabolism of α-keto-β-methylvalerate, the precursor to isoleucine, rather than isoleucine per se.

In apples, esters originating from α-keto-β-methylvalerate (isoleucine precursor) predominate and those from α-keto-isovalerate (valine precursor) can be detected occasionally at low levels (Ferenczi, 2003; Mattheis et al., 1998; Ortiz et al., 2010; Plotto et al., 2000; Sugimoto, 2007). Although a small amount of leucine is found in ripening apple fruit (Burroughs, 1970; Hansen, 1970), there are few esters produced from α-ketoisocaproate (leucine precursor). During apple fruit ripening, isoleucine has been reported to accumulate, but not the other BCAAs (Defilippi et al., 2005; Nie et al., 2005). The increase in isoleucine is accompanied by an increase in its respective BC esters, BC alcohols, and BC aldehydes (Nie et al., 2005; Pérez et al., 2002; Tressl and Drawert, 1973).

The interconversion of α-keto-β-methylvalerate to the alcohol required for 2-methylbutyl esters is via two enzymatic steps. The first involves its decarboxylation by BC α-ketoacid decarboxylase (a.k.a. BC 2-ketoacid decarboxylase or pyruvate decarboxylase, PDC) to 2-methylbutanal. In the second step, 2-methylbutanal is acted on by alcohol dehydrogenase to form 2-methylbutanol (Wyllie et al., 1996). The conversion of α-keto-β-methylvalerate to acids required for 2-methylbutanoate esters is via dehydrogenation by branched-chain α-ketoacid

dehydrogenase (BCKDH) to 2-methylbutyl-CoA. The dehydrogenase pathway is considered as a major route for BCAA catabolism in most organisms, whereas the PDC pathway has been extensively studied only in yeast and bacteria (Dickinson et al., 1997, 1998, 2000; Smit et al., 2004).

In the final enzymatic step of the BC ester biosynthetic pathway, AAT combines 2-methylbutyl-CoA and/or 2-methylbutanol with various alcohols and acyl-CoAs, respectively, to create a wide variety of esters. The substrate specificity of AAT is believed to markedly impact the ester profile (Aharoni et al., 2000; Olías et al., 2002; Souleyre et al., 2005; Ueda et al., 1992; Yahyaoui et al., 2002). However, the AAT of apple cannot discriminate between 2-methylbutyl and 3-methylbutyl precursors (Wyllie et al., 1996). The predominance of 2-methylbutyl and lack of 3-methylbutyl esters in apple therefore suggests that precursor formation and availability is critical for BC ester biosynthesis. It is generally accepted that AAT will influence the ester profile, but control of ester synthesis probably lies at the level of ester precursor formation (Ferenczi et al., 2006; Wyllie and Fellman, 2000).

Isoleucine is synthesized from threonine via aspartate in plants. Aspartate originates from the TCA cycle and is used to synthesize several amino acids including lysine, methionine, and threonine (Azevedo et al., 1997). To synthesize isoleucine, threonine is deaminated to α-ketobutyrate by threonine deaminase (TD) (Binder, 2010) (Figure 2). The isoleucine precursor, α-keto-β-methylvalerate, is synthesized from α-ketobutyrate by three enzymes: acetohydroxy acid synthase [AHAS, a.k.a. acetolactate synthase, (ALS)], acetohydroxy acid isomero-reductase, and dihydroxy-acid dehydratase acting in succession. These enzymes also catalyze the formation of α-ketoisovalerate from pyruvate to synthesize valine. Leucine synthesis, however, begins with the valine precursor, α-ketoisovalerate, which is acted upon by three enzymes 2-isopropylmalate

synthase (IPMS), isopropylmalate isomerase, and 3-isopropylmalate dehydrogenase, to form α-ketoisocaproate.

Biosynthesis of all three BCAAs is responsive to feedback regulation. TD is inhibited by isoleucine, but stimulated by valine, ALS is inhibited by valine and leucine, and IPMS is inhibited by leucine (Eisenstein, 1991; Singh and Shaner, 1995; Wessel et al., 2000). The accumulation of isoleucine in some ripening apple fruit (Nie at al., 2005) is not consistent with its known regulation by feedback inhibition.

To our knowledge, detailed information about the concentration of the free amino acids in apple throughout ripening and senescence is not available. The objective of this work was to examine the relationship between the free amino acid content, the expression of genes related to BCAA metabolism, and the production of BC esters during apple fruit ripening.

Materials and Methods

Plant material. 'Jonagold' apples were harvested for examination every three to four days from research plots at the Michigan State University Clarksville Horticultural Experiment Station in Clarksville, Mich. from 2 Sept. 2004 (day 0) until ripening was fully engaged on 7 Oct. 2004 (day 35). On each occasion, fruit were held overnight in the laboratory to equilibrate to laboratory temperature (20±1 °C) and covered with ventilated, black, 0.1-mm-thick plastic bags to avoid desiccation and responses to intermittent laboratory light before analysis. All fruit (approximately 200) remaining on the trees were harvested and transported to the laboratory on 7 Oct. 2004 (day 35) after it was apparent that ripening was underway. This was done to avoid damage in the field due to freezing and fruit drop. Thereafter, these fruit were maintained at room temperature (20±1 °C), 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 23 Nov. 2004 (day 81).

On each evaluation date, 20 apples were randomly chosen and the internal ethylene content of each was measured as described below. Of these, 14 fruit having an internal ethylene content nearest the median were selected for further analysis. The four fruit having ethylene levels closest to the median were used for analysis of CO_2 production and ester emission. Maturity analysis (percent red coloration, background color, soluble solids concentration (%), and starch index) was performed on the remaining 10 fruit on each date as described by Mir et al. (2001). From these fruit, the skin and 2 to 3 mm of underlying cortex tissue were removed and immediately frozen in liquid nitrogen and stored at –80 °C. Two replicates were created, each consisting of pooled tissue samples from five fruit. Pooled tissue samples were used for amino acid and gene expression studies.

Measurement of internal ethylene. The internal ethylene content of apple fruit was determined by withdrawing a 1-mL gas sample from the interior of the apples and subjecting the gas sample to gas chromatography (GC) analysis (Carle Series 400 AGC; Hach Company, Loveland, CO) as previously described (Mir et al., 2001). The GC was fitted with a 6-m-long, 2-mm-i.d. stainless-steel column packed with activated alumina and was equipped with a flame ionization detector. The ethylene detection limit was approximately 0.005 $\mu L \cdot L^{-1}$. Ethylene concentrations were calculated relative to the certified standard (Matheson Gas Products Inc., Montgomeryville, PA) containing 0.979 $\mu L \cdot L^{-1}$ ethylene, 4.85% CO_2, and 1.95% O_2 balanced with N_2.

Measurement of respiration. Respiration was sampled at the same time volatiles were measured. Apples were each placed into 1-L Teflon chambers (Savillex Corporation, Minnetonka, MN) and held for 20 min at 20 °C. CO_2 accumulation was measured by withdrawing 0.1-mL gas samples from a sampling port in the lid of the chamber, which was sealed with a Teflon-lined half-hole septum (Supelco Co., Bellefonte, PA) using an insulin-type plastic syringe. The gas sample was injected into an infrared gas analyzer (model 225-MK3; Analytical Development Co., Hoddesdon, England) operated in a flow-through mode with N_2 as the carrier gas and a flow rate of 100 $mL \cdot min^{-1}$. The CO_2 concentration was calculated relative to the certified standard noted previously.

Volatile analysis. Ester, alcohol and aldehyde measurement was by gas chromatography (GC) coupled with time-of-flight mass spectrometry (TOFMS). Headspace volatiles were sampled using a 1-cm long, solid-phase micro extraction (SPME) fiber (65 μm PDMS-DVB, Supelco). Following a 3 min sorption time, the SPME fiber was immediately transferred to a GC (HP-6890, Hewlett Packard Co., Wilmington, DE) splitless injection port at 230 °C and desorbed for 2 min. The GC was equipped with a mass selective detector (Pegasus II, LECO Corp., St. Joseph, MI). Conditions of GC separation and TOFMS analysis were as previously described (Song et al., 1997). Identification of compounds was by comparison of the mass spectrum with authenticated reference standards and/or with spectra in the National Institute for Standard and Technology (NIST) mass spectrum library (Version 05). Volatile compounds were quantified by calibrating with a known amount of an authenticated, high purity standard mixture of 20 volatilized alcohols, aldehydes, and esters as previously described (Song et al., 1997). Where no standard was available, volatiles were quantified by estimation of the instrument response factor based on the

Kovats index for the compound of interest (Bartelt, 1997). There were four replicate samples on each analysis date.

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

Approximately 5 g of 'Jonagold' apple skin and 2 to 3 mm of underlying cortex tissue was used to isolate total RNA by hot borate/phenol extraction followed by LiCl precipitation (López-Gómez and Gómez-Lim, 1992).

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. The expression of BCAT, PDC, TD genes and the 18S ribosomal RNA (18s rRNA) gene was measured using semi-quantitative PCR analysis. Contigs of BCAT, PDC, and TD were assembled using all available ESTs in the non-redundant nucleotide database in NCBI. The methods used to identify ESTs for the creation of contigs and contig assembly and annotation were as described in Park et al. (2006). With the exception of BCAT3, PCR primers were

contained within a single EST used for assembling contigs. For BCAT3, the forward primer spans two ESTs, CO541320 and CO899974. The BCAT10 clone (CO868030) was donated by Dr. Korban (University of Illinois, Urabama-Champaign) and was re-sequenced from 3' end at the Genomics Technology Support Facility (GTSF) of the Genomics Core in Michigan State University, East Lansing, Mich. The PDC1 clone (CK900568) was re-sequenced from 3'end at GTSF. The resulting sequences were used to generate primers for PCR analysis.

For each PCR analysis, two biological replicates were used with the exception of *TD* for which only one replicate was analyzed. cDNA synthesis and PCR reactions were performed using commercially available kits according to manufacturer (Invitrogen, Carlsbad, CA) directions. Before creating cDNA, total RNA was treated with DNase using an RNase-free DNase kit according to the manufacturer (Qiagen Inc, Valencia, CA). One microgram of DNase-treated total RNA was reverse transcribed using oligo(dT)12-18 primer or random hexamer and SuperScript II as described by the manufacturer (Invitrogen, Carlsbad, CA). cDNA created with oligo (dT)12-18 primer was used for 18s rRNA, *BCAT* and *PDC* expression and cDNA created with random hexamer was used for *TD* expression analysis. cDNAs (1.0 μL) were used as templates in a 50-μL PCR reaction containing 10 μM of the forward and reverse gene-specific primers. 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 2). The PCR reaction was performed as follows: 1) 5 min at 95 °C, 2) 30 s at 95 °C, 3) 30 s at 55-59 °C, 4) 30 s at 72 °C, repeating 18 to 35 cycles from steps 2 to 4, and final elongation 5 min at 72 °C. The amplified PCR products were separated by electrophoresis on a 1.5% (w/v) agarose gel, visualized with 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 26 to 35 (Table 2). A single number indicates that the same cycle was performed with both biological replications. Two numbers indicate that different number cycles were performed for each replicate. PCR reactions for two PDC genes and one BCAT gene were unsuccessful. No products were evident for PDC6 and BCAT11 after two trials using different primers and PDC7 was too short to design appropriate primers.

PCR products were cleaned using a PCR purification kit (QIAquick, Qiagen inc., Valencia, CA) and sequenced at GTSF to verify identity. All the PCR generated sequences were 98% to 100% identical to apple fruit ESTs as reported in NCBI.

A partial sequence of the 18s rRNA gene (gi:85717895) was used as an internal control for PCR analyses. Expression data for all genes (PDC, BCAT, and TD) were normalized based on the 18s rRNA spot density. The spot density for the 18s rRNA varied approximately ±10% across the eight developmental stages (data not shown). The values for PCR are calculated as the spot density relative to the maximum value obtained for each gene.

Amino acid extraction. The same eight developmental stages that were selected for gene expression analysis as described previously were used for amino acid analysis (Figure 3). Two biological replicates were created within the same stage as previously described.

Approximately 5 g of the frozen apple tissue were ground into fine powder using a liquid N_2-cooled mortal and pestle. The ground tissue was further divided into three technical replicates, each containing 0.5 g of tissue. The powdered tissue was transferred into 1 mL of preheated

water containing 10 μM deuterated methionine (Met-d_3) (Cambridge Isotope Laboratories Inc., Andover, MA) as an internal standard, mixed vigorously, and incubated at 90 to 95 °C for 10 min. After centrifugation at 5000 g_n for 5 min, the supernatant was filtered through a 0.45-μm filter (Millex HA, Millipore, Billerica, MA). The cleared filtrate was diluted into one fifth of its original concentration with water containing the internal standard and used for analysis.

Amino acid analysis. The 20 amino acids (Sigma-Aldrich Corporation, St. Louis, MS) listed in Table 3 were dissolved in water containing 10 μM Met-d_3 to make individual stock solutions of 1 mM. A master mixture was created by mixing all 20 amino acids to a final concentration of 50 μM each. A series of six working standards ranging from 0.5 to 40 μM, each containing 10 μM Met- d_3, was prepared by serial dilutions from the 50 μM master mixture using water containing the internal standard. Amino acid samples were quantified by calibration curves obtained from six working standards using linear regression, plotting amino acid concentration as a function of ratio of the amino acid peak area to the Met-d_3 peak area

Amino acids were analyzed using a tandem mass spectrometer (Waters Quattro micro, Waters Inc., Milford, MA) coupled to a high pressure liquid chromatograph (LC-20AD HPLC, Shimadzu, Columbia, MD) equipped with an autosampler (SIL-5000, Shimadzu, Columbia, MD). The 2.1 x 100 mm column was packed with a 3.5-μm-diameter C18 stationary phase (Symmetry, Waters, Milford, MA) and held isothermally at 30 °C. Injection volume was 10 μL and solvents used were 1 mM perfluoroheptanoic acid (mobile phase A) and acetonitrile (mobile phase B); the flow rate was held constant at 0.3 mL·min^{-1}. The gradient program was as follows: 98% mobile phase A and 2% mobile phase B at start, 20% mobile phase B after 0.1 min, increasing to 40% mobile phase B at 2.3 min, decreasing to 2% mobile phase B at 4.1 min for re-equilibration of

the column. The total run time was 6 min. Mass spectra were acquired using electrospray ionization in positive ion mode (ESI+). The capillary voltage was 3.17 kV, the extractor voltage was 4 V, the rf lens was held at 0.3 V, the cone gas flow rate was 20 $L \cdot h^{-1}$, the desolvation gas flow rate was 400 $L \cdot h^{-1}$, the source temperature was 110 °C, and the desolvation temperature was 350 °C as described in Gu et al. (2007). The data acquisition method was split into two functions, the first from 0 to 1.8 min and the second from 1.8 to 6.0 min. Ten multiple reaction monitoring (MRM) transitions were included in function 1 and 11 in function 2. Function number, collision energies, and masses of observed ions for each amino acid are listed in Table 3. Data were collected and quantified with proprietary software (MassLynx 4.0 and QuanLynx; Waters).

Results

Fruit maturation. Skin color (percent of redness) increased from 22% on day 0 to over 95% by day 39 (data not shown). Background color (green=5, yellow=1) had a reciprocal pattern relative to red color development, beginning at 5 (green) on day 0 and gradually decreasing to 1 (yellow) on day 81 (data not shown). Starch conversion to sugars, as measured by the starch index (1 to 8) started at 2 on day 0 and increased linearly with time, reaching a maximum of 8 on day 32 (data not shown). The pattern for soluble solids was similar to that of starch conversion; the initial soluble solid was 12% on day 0 and reached its maximum of 16% on day 39 (data not shown). Fruit internal ethylene concentration (IEC) remained low until day 18 (≤ 0.1 $\mu L \cdot L^{-1}$) (Figure 3), after which time IEC rose above 0.2 $\mu L \cdot L^{-1}$ by day 21. The autocatalytic increase in ethylene did not occur until after day 32, with IEC increasing to over 500 $\mu L \cdot L^{-1}$ by day 60. The respiratory climacteric occurred after day 32 and appeared to be complete by day 49.

BC esters. Esters were first detected at very low levels as early as day 14, a week before ethylene levels greater than 0.2 $\mu L \cdot L^{-1}$ were sustained. As the ethylene content exceeded 0.2 $\mu L \cdot L^{-1}$, a rapid and large increase in ester biosynthesis began. Total ester production increased coincident with increased ethylene accumulation in the fruit (Figure 3). The pattern for BC esters was generally similar to that for total esters, but had a higher, sharper initial peak for 2-methylbutanol esters (Figure 4A and B). 2-Methylbutanol esters (2-methylbutyl acetate and 2-methylbutyl butanoate) had a lower diversity, but were approximately twice as abundant as 2-methylbutanoate esters (ethyl-, propyl-, butyl-, pentyl-, and hexyl 2-methylbutanoate). The most abundant 2-methylbutanoic acid-derived BC esters were hexyl and butyl 2-methylbutanoate, and the most abundant 2-methylbutanol-derived BC ester was 2-methylbutyl acetate. 2-Methylbutanol and 2-methylbutanal production patterns were similar to those for 2-methylbutanol-derived esters; both peaked on day 39 and declined rapidly thereafter, then undergoing a slow increase as ripening and senescence continued (Figure 4C). Free 2-methylbutanoic acid was not detected.

Amino acids. Two amino acids, glycine and cysteine, were not quantifiable because of limitations with the methodology used, but the remaining 18 amino acids were quantified readily. Patterns of change in amino acid content during fruit development were diverse and could be classed either as declining; moderately increasing; increasing markedly; declining and then increasing; or increasing, then declining (Figure 5). Six amino acids, alanine, arginine, aspartate, glutamate, serine, and threonine, decreased relatively steadily throughout ripening, decreasing to 15% to 20% of preclimacteric levels. Three amino acids, leucine, tryptophan, and valine increased moderately (2 to 3-fold). Asparagine, glutamine, phenylalanine and methionine

contents decreased as fruit began to ripen then gradually increased during the latter phase of senescence. The concentration of histidine, lysine, proline, tyrosine, and tryptophan peaked in concert with the respiratory climacteric. Isoleucine was the only amino acid to increase continuously during ripening, with the onset of the increase coincident with the increase in internal ethylene content. The final concentration of isoleucine was more than 20-fold higher than preclimacteric levels. The compositional change in isoleucine (isoleucine as a percent of total amino acids) shifted almost 85-fold from 0.13% to 11% of total amino acids in unripe to ripe fruit. Isolecuine levels at their peak were 10- and 20-fold higher than valine and leucine, respectively.

Gene expression related to BCAA metabolism. Of the eleven putative BCAT genes, ten were expressed in the fruit (Table 2). Of these, four (*BCAT1*, *BCAT6*, *BCAT9*, and *BCAT10*) had expression patterns peaking on day 32 then declining thereafter, and one (*BCAT2*) had an expression pattern that peaked on day 39 then declined thereafter (Figure 6A). The remaining five putative BCAT genes (*BCAT3*, *BCAT4*, *BCAT5*, *BCAT7*, and *BCAT8*) had relatively stable expression until approximately day 25, then declined slightly as ripening and senescence progressed (Figure 6B). The change in expression of the BCAT genes was not great; increases (relative to initial) were no greater than 40% and decreases (relative to maximum) were no greater than 80%.

Of the seven putative PDC genes, five were detected as being expressed in the fruit. Expression patterns for the five genes differed, but only one, (*PDC1*) underwent a sustained increase during ripening and senescence (Figure 7). Of the remaining four, one (*PDC4*) rapidly increased on day 32 and declined thereafter, one (*PDC5*) was stable throughout ripening and

slightly increased during senescence, and two (*PDC2* and *PDC3*) had their highest expression before the climacteric peak and gradually declined afterwards. With the exception of *PDC5*, most of the PDC genes had a relatively high expression compared to the BCAT genes based on PCR cycle numbers (Table 2).

The two putative TD genes (*TD1* and *TD2*) were expressed in the fruit (Table 2). Unlike BCAT and PDC genes, expression for *TD1* and *TD2* remained constant during ripening (Figure 8). Based on the PCR cycles required to obtain an adequate signal, the abundance of *TD1* and *TD2* transcription products was similar to that for the bulk of the putative BCAT genes.

Discussion

Maturation. The progression of fruit maturity indices for 'Jonagold' is consistent with those previously published (Beaudry et al., 1993; Schwallier et al., 1995). 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 (Defilippe et al., 2005; Ferenczi et al., 2006; Mir et al., 1999; Song and Bangerth, 1996).

Ester synthesis. The diversity and quantity of esters that apple fruit produce are cultivar-specific (Dixon and Hewett, 2000; Kakiuchi et al., 1986; Paillard, 1990). With regard to the synthesis of BC esters, some varieties produce much greater quantities than others. Based on its copious production of 2-methylbutyl and 2-methylbutanoate esters, 'Jonagold' can be classified as a BC ester producing cultivar, as are 'Bisbee Delicious' (Mattheis et al., 1991a, 1991b), 'Redchief Delicious' (Ferrenczi, 2003), 'Rome' (Fellman et al., 1993), and 'Golden Delicious' (Song and Bangerth, 1996). There are cultivars that could be classified as low BC producers, such as

'Annurca' (Lo Scalzo et al., 2001). The maintenance of a high rate of production of 2-methylbutyl esters throughout ripening and senescence suggests a consistent production of 2-methylbutanol, which had a pattern of production that reflected that of 2-methylbutyl esters. It may be that high BC ester producing lines also produce elevated levels of α-keto-β-methylvalerate, the isoleucine precursor. However, to our knowledge, no analysis has been performed to test this relationship.

Patterns of amino acid content and gene expression. Total amino acid content in unripe fruit was similar to that quantified in wild type 'Columbia' arabidopsis (*Arabidopsis thaliana*) seeds (Lu et al., 2008). While the free amino acid content in apple fruit was not directly comparable to previously studies on amino acids in apples (Ackermann et al., 1992; Burroughs, 1957; Defilippi et al., 2005; Nie et al., 2005) due to methodological differences, comparison of the fraction of each amino acid was possible. Averaging across developmental stages, asparagine (45%), aspartate (16%), glutamate (23%), and serine (5%) were the major amino acids in 'Jonagold' fruit and accounted for more than 80% of the quantified 18 amino acids. These four amino acids were also found to predominate in 'Court Royal', 'Morgan Sweet', 'Reine des Pommes', 'Kingston Black', 'Yarlington Milll', and 'Dabinett' (Burroughs, 1957), in 'Collaos', 'Meana', 'Picona Rayada', and 'Raxao' (Blanco Gomis et al., 1992), in 'Granny Smith' (Magné et al., 1997), and in 'Glockenapfel' (Ackermann et al., 1992) cultivars. However, our numbers did not agree with the data from Wu et al. (2007) for a 'Jonagold' juice extract, in which alanine (25%) had the highest proportion, followed by serine (6%), glutamine (5%), and asparagine (5%). Aspartate and glutamate levels were not reported in this study, however. Differences in our results may be related to either the stage of development when the fruit was examined, the

extraction method for free amino acids, or the fact that the data for aspartate and glutamate were not reported in the cited study.

Five amino acids, asparagine, lysine, threonine, methionine, and isoleucine (through threonine), are synthesized from aspartate, which originates from oxaloacetate from the TCA cycle in the mitochondria (Azevedo et al., 1997; Bartlem et al., 2000; Coruzzi and Last, 2000). With the exception of isoleucine, the concomitant decline in aspartate and its products asparagine, lysine, threonine, and methionine, is consistent with their linkages with aspartate metabolism. Even so, the lysine pattern was somewhat different from that of the other aspartate-derived amino acids, perhaps because lysine biosynthesis is considered to be strongly regulated by itself without affecting the flux of the aspartate pathway (Azevedo et al., 1997). The increase in isoleucine, despite a decrease in threonine, is difficult to explain. Increasing isoleucine and depletion of threonine could result from an increase in the activity of TD. However, the lack of an increase in *TD* expression would suggest that simple genetic regulation at this step is not responsible. Protein synthesis remains highly active in ripening fruit (Frenkel et al., 1968), so it seems unlikely that isoleucine would accumulate preferentially due to a decline in its incorporation in proteins or enhanced proteolysis. Given that isoleucine inhibits the activity of TD in plants (Mourad and King, 1995), it is not clear how isoleucine was able to accumulate and threonine to decline. Generally, suppression of a step in a metabolic pathway leads to the accumulation of intermediates in earlier steps. For instance, Bartlem et al. (2000) observed that the inhibiting threonine synthesis caused the methionine accumulation in arabidopsis.

Isoleucine and the other BCAAs are products of an aminotransferase reaction in which glutamate acts as a donor to transfer its amino group to BC α-keto acids to form the BCAAs and α-ketoglutarate (Goto et al., 2003). In this way, glutamate contributes to the synthesis of valine,

isoleucine, and leucine from α-ketoisovalerate, α-keto-β-methylvalerate, and α-ketoisocaproate, respectively. Our data shows that glutamate decreases as 'Jonagold' fruit ripen. However, the decrease in glutamate was not reflected by declines in any of the three BCAAs, suggesting that glutamate was likely not limiting to the aminotransferase reactions. That being said, it can be deduced that glutamate levels did not promote transferase reactions. In fact, according to Tewari et al. (2000), the formation of the BC α-keto acid is energetically favored over the BCAA in the BCAT reaction. Similarly, Gonda et al. (2010) found that CmBCAT1 from melon (*Cucumis melo*) converted isoleucine and leucine to α-keto-β-methylvalerate and a-ketoisovalerate, respectively, and vice versa, indicating that the enzyme carries out a freely reversible reaction. Therefore, to explain the accumulation of isoleucine, the decline in glutamate would have to be balanced by a significant increase in the content of α-keto-β-methylvalerate, or a marked decline in α-ketoglutarate. To our knowledge, changes in the levels of these metabolites during apple fruit ripening have not been determined. However, Aharoni et al. (2002) determined that α-ketoglutarate declined several-fold during fruit ripening in strawberry (*Fragaria ×ananassa*).

The greater than 20-fold increase in isoleucine content found in this study was similar to that found in 'Greensleeves' apples, which experience a more than 10-fold increase in isoleucine during ripening (Defilippi et al., 2005). Nie et al. (2005) also observed the increase in isoleucine during apple ripening in 'Starkrimson'. The data from our study and these two previous studies indicate that biosynthesis of isoleucine outpaces its catabolism.

Since 2-methylbutyl BC esters in apple appear to be formed from the dehydrogenation and decarboxylation of α-keto-β-methylvalerate (Gonda et al., 2010), 2-methylbutyl BC esters may be considered a by-product of α-keto-β-methylvalerate metabolism. Viewing BC ester formation in apple as a function of BC α-keto acid synthesis, rather than BCAA degradation,

runs counter to the way BC ester formation is often described. Investigators have suggested that isoleucine is converted into α-keto-β-methylvalerate via BCAT, thereby facilitating BC ester formation (Gonda et al., 2010; Pérez et al., 2002; Rowan et al., 1996). However, as noted previously, isoleucine levels increase, rather than decrease, reflecting net synthesis rather than net degradation or depletion.

There are three pathways reported to synthesize α-keto-β-methylvalerate and its product, isoleucine, via alternative routes of α-ketobutyrate synthesis that do not involve threonine deamination. In arabidopsis, methionine-γ-lyase (MGL) activity may produce α-ketobutyrate from methionine (Joshi and Jander, 2009; Rebéillé et al., 2006). A second pathway has been found in yeast in which cystathionine-γ-lyase (Cys3) produces α-ketobutyrate from cystathionine via cysteine biosynthesis (Ono et al., 1999). The third pathway for isoleucine biosynthesis has been described in *Methanococcus jannaschii* and *Leptospira interrogans* bacteria (Drevland et al., 2007; Howell et al., 1999; Westfall et al., 1983; Xu et al., 2004). In this pathway, acetyl-CoA and pyruvate are substrates for the formation of citramalic acid by citramalate synthase (CIM), which is a member of the IPMS family (Figure 2). Citramalate, in turn, is then converted to α-ketobutyrate. This 'citramalate pathway' has not been conclusively demonstrated in plants and yeast, but data exist to support this possibility (Textor et al., 2007; Vollbrecht, 1974). Further, detailed isotope labeling studies point to the possible existence of a novel pathway of fatty acid biosynthesis for sugar esters in members of the Solanaceae [e.g., tobacco (*Nicotiana tabacum*) and petunia (*Petunia ×hybrida*) led to the suggestion that some plants may possess CIM activity in specialized tissues (Kroumova and Wagner, 2003)]. Interestingly, Hulme (1954) isolated citramalic acid from the peel of the mature apple fruit. It was postulated that citramalic acid participated in the TCA cycle, although no data supported this suggestion. If the production of

α-keto-β-methylvalerate and its product, isoleucine, occurs via the citramalate synthase pathway or one of the other alternative pathways mentioned, it would explain the unique and unexpected accumulation of isoleucine during 'Jonagold' ripening. Isoleucine normally inhibits its own accumulation by feedback inhibition of TD (Mourad and King, 1995) and alternative routes of synthesis may not be similarly regulated.

In plants, TD activity is required for autotrophy in *Nicotiana plumbaginifolia* (Sidorov et al., 1981) and autotrophy was restored in an isoleucine-requiring Nicotiana *TD* mutant (ILE401) with a yeast TD gene (*ILV1*) in a complementation study (Colau et al., 1987). These data suggest that threonine degradation is the sole pathway for isoleucine biosynthesis in some plant tissues. Thus, Schaffer et al. (2007) suggested that TD contributes to the regulation of BC ester production by controlling threonine degradation and entry of carbon into the isoleucine synthetic pathway based on the up regulation of one of three *TD* ESTs (CN878598) in ripening apple. Our *TD* expression data are not in accord with the findings by Schaffer et al. (2007), however, which would imply the converse of their interpretation, although realistically, little can be concluded regarding TD activity in vivo. The only related data from the current study is the difficult to reconcile increasing isoleucine content with the known feedback inhibition of TD by isoleucine. Inhibition of TD by isoleucine should yield accumulation of threonine (Mourad and King, 1995).

PDC1 gene expression was low in preclimacteric fruit, increased during ripening, and remained elevated or increased slightly even during the latter stages of senescence. *PDC1* was the only one of the five PDC genes that had an expression pattern that, by virtue of its timing and degree, may be induced by the ripening process and associated with ester formation. However, the function of apple PDCs have not been characterized and a conclusive relationship to ester synthesis awaits further study. It is possible that one or more PDC may metabolize BC α-keto

acids. The idea that one or two PDC isozymes could have sufficient specificity to be primarily responsible for decarboxylation of BC α-keto acids is supported by findings in yeast (*Saccharomyces cerevisiae*) (Dickinson et al., 1997, 1998, 2000; Yoshimoto et al., 2001). Dickinson et al. (1997, 1998, 2000) concluded that a single PDC-like enzyme (a product of the gene *YDL080c*) is likely responsible for leucine catabolism and two other isozymes of PDC are used for valine and isoleucine degradation.

Unfortunately, branched-chain α-ketoacid decarboxylases (PDCs) are poorly studied in higher plants and little information is found characterizing PDC genes relative to ester formation. In fruit, PDC gene expression pattern was studied in developing grape berries (Or et al., 2000) and the crude extract of PDC activity was measured during maturation of strawberry and 'Fuji' apple (Echeverría et al., 2004; Moyano et al., 2004). The main purpose of these studies was to relate PDC activity and expression to ethanol production under anaerobic conditions or to the formation of ethanol-derived esters such as ethyl esters, not for BCAA metabolism. In the present study, the pattern of ethyl ester formation in 'Jonagold' appeared to reflect the pattern of only *PDC1* expression, however, despite ethyl esters being found only at low levels (data not shown). The relatively high *PDC1* expression in 'Jonagold' and the low ethyl ester production argue against a causative relationship between the expression of the gene and ethanol metabolism per se. The relevance of the changes in *PDC* expression relative to the production of 2-methylbutyl and 2-methylbutanoate esters by 'Jonagold' apple fruit cannot be elaborated at this time and clarification of its role awaits characterization of the various apple PDC enzymes.

Conclusion

Collectively, the data support reassessing the perspective that the precursors for ester biosynthesis result from primarily degradative processes. In particular, the data surrounding isoleucine metabolism provide indications of enhanced synthetic activity. Importantly, the findings here may point to the existence of an alternative route of synthesis of α-keto-β-methylvalerate and its product, isoleucine. This possibility is supported by the large and sustained increase in isoleucine content despite declines in the concentrations of its amino acid precursors, the apparent lack of feedback inhibition to control the accumulation of isoleucine, and the lack of patterns in the expression of BCAT, and TD genes that parallel isoleucine accumulation and BC ester formation. Further, the presence of citramalate in apple and the potential for its contribution to the synthesis of α-ketobutyrate formation provides a rationale supporting the existence of a citramalate-based pathway for the formation of α-keto-β-methylvalerate and its product, isoleucine, that is not feedback regulated similarly to that in bacteria. We propose that an alternative 'citramalate pathway' exists in apple that by-passes threonine and permits the rapid and extensive accumulation of isoleucine through enhanced formation of α-keto-β-methylvalerate. Given the coincidental timing of increased isoleucine formation with changes in internal ethylene and respiratory activity, it seems likely that if the citramalate pathway is active in apple fruit, it may be influenced by ethylene or is dependent upon other developmental cues associated with ripening.

Table 2. Putative branched-chain aminotransferase (*BCAT*), pyruvate decarboxylase (*PDC*), threonine deaminase (*TD*) genes and the 18S ribosomal RNA (18s rRNA) gene with accession number, GenBank number, forward and reverse primer sequence (5'→3'), and expected PCR gene fragment size (bp) for semi-quantitative RT-PCR. With the exception of BCAT3, accession numbers represent one of the ESTs used in the assembly of contigs that contained the region between the forward and reverse primers. Genes are included for which PCR was not successful. A single number in 'cycles' indicates that the same cycle was performed with biological replications 1 and 2. Two numbers indicate that different cycles were performed between biological replications for optimum result. 'Temp.' indicates annealing temperature.

Target	Accession no.	GenBank gi no.	Forward primer sequence	Reverse primer sequence	Size (bp)	Cycles	Temp. (°C)
PDC 1	JK045128	336041422	gcacaggattcttcaccaca	cagcctctaagcccaaatga	275	27	55
PDC 2	CX025334	56435496	gatccctcgatgtctgcaag	gcccacccacagtgaaagta	336	27, 29	59
PDC 3	CO754399	50889650	tggaccaccaaggttagcat	cattccagcagctctttgct	215	33	57
PDC 4	CN945515	48418328	gcggtgagattgtggagtct	accctcagaggctcatcctt	293	26, 27	57
PDC 5	CV567449	54464858	tctgttccaccacatccaga	gtgtacccgccgttgtttat	307	28	57
PDC 6	CN491928	46609400	ggatcgattggttggtctgt	tcttctcctccgttgctgtt	340	Failed	57
PDC 6	CN491928	46609400	gacggcccttacaatgtgat	gggcggttattagcagttga	245	Failed	57
BCAT 1	DT003328	71825936	caccgaagttggttgagcag	gagaacacgccttccttctg	290	35	57
BCAT 2	CV082955	51561944	gagtacgatcgtgcctctcg	cacaggagcaacaccaacag	363	30, 32	57

Table 2 (cont'd).

Target	Accession no.	GenBank gi no.	Forward primer sequence	Reverse primer sequence	Size (bp)	Cycles	Temp. (°C)
BCAT 3	CO541320*	50353369	gggagaactcagtccggtta	gggatttgcccatctgtacc	300	31	57
BCAT 4	CX024412	56433559	gtttgggagggtcttcgagt	attacgacgggtggtagctg	333	31	57
BCAT 5	CN581127	46992677	tgcaactcctggtggtactg	ccttcactacgagcaacgtc	258	32	57
BCAT 6	CO867683	51097833	cctgctccggagtacacatt	ctggaatccttcgctacgag	369	29	57
BCAT 7	CN912350	48384850	cgatcacatggttcatcgag	actgcatagaaagcgggtgt	279	33	57
BCAT 8	CV882612	55857820	agagggagggttctggtgtt	gaggttgtcccattcgatgt	293	32	59
BCAT 9	CO903152	51293455	cctgacacaacatgggacac	gttggtgcaggcatacacag	289	34	59
BCAT 10	JK045129	336041423	cccgagtccaaatcattcat	tcgaagggtaccaggttgag	352	28, 31	59
BCAT 11	DT003206	71825814	ggtctcaaggcaatcaggac	gctgcatccagaaagaggac	326	Failed	57
BCAT 11	DT003206	71825814	cttgcagccatgacttctga	tcctgattgccttgagacct	325	Failed	57

Table 2 (cont'd).

Target	Accession no.	GenBank gi no.	Forward primer sequence	Reverse primer sequence	Size (bp)	Cycles	Temp. (°C)
TD1	CN878598	48264838	ggcaggtgctctttctcttg	ggctgtatgcacaccaacac	304	29	59
TD2	CN945640	48418453	gcctccattctcatcacctc	gatggccacgtcatacacct	323	31	59
18s rRNA	DQ341382	85717895	gagaaacggctaccacatcc	gagcgtaggcttgctttgag	390	18	59

* Forward primer spans two ESTs, CO541320 and CO899974

Table 3. The liquid chromatography coupled with tandem mass spectrometry (MS/MS) conditions for 21 amino acids including deuterated methionine (Methionine-d_3) were optimized for *m/z*, cone voltage, collision voltage, and retention time. The parent mass corresponds to amino acid molecular mass plus hydrogen ion and the daughter ion is used for MS/MS, specific *m/z* for selection of the amino acid. The data acquisition was split into two functions, function no. 1 (0 to 1.8 min) and function no. 2 (1.8 to 6.0 min).

Compound	Mass of parent > daughter ions (*m/z*)	Cone voltage (V)	Collision voltage (V)	Retention time (min)	Function no.
Alanine	89.9 > 44.0	18	15	1.53	1
Arginine	175.0 > 69.9	26	20	3.91	2
Asparagine	132.9 > 86.9	26	20	1.13	1
Aspartate	133.9 > 73.8	18	15	1.09	1
Cysteine	122.0 > 75.9	20	15	1.28	1
Glutamine	146.9 > 130.0	18	15	1.22	1
Glutamate	147.9 > 83.9	18	15	1.18	1
Glycine	75.9 > 30.2	18	40	1.3	1
Histidine	156.0 > 110.0	18	15	3.51	2
Isoleucine	132.0 > 69.0	18	15	3.06	2
Leucine	132.0 > 30.0	18	15	2.88	2
Lysine	146.9 > 84.0	18	15	3.73	2
Methionine	149.9 > 104.0	18	15	2.21	2
Phenylalanine	166.0 > 120.0	18	15	3.2	2

Table 3 (cont'd).

Compound	Mass of parent > daughter ions (*m/z*)	Cone voltage (V)	Collision voltage (V)	Retention time (min)	Function no.
Proline	116.0 > 69.9	26	15	1.51	1
Serine	105.9 > 59.8	18	15	1.18	1
Threonine	120.0 > 56.9	26	25	1.35	1
Tryptophan	205.0 > 188.0	18	15	3.57	2
Tyrosine	182.0 > 136.1	18	15	2.19	2
Valine	118.0 > 71.9	18	15	2.14	2
Methionine-d_3	153.0 > 107.0	18	15	2.21	2

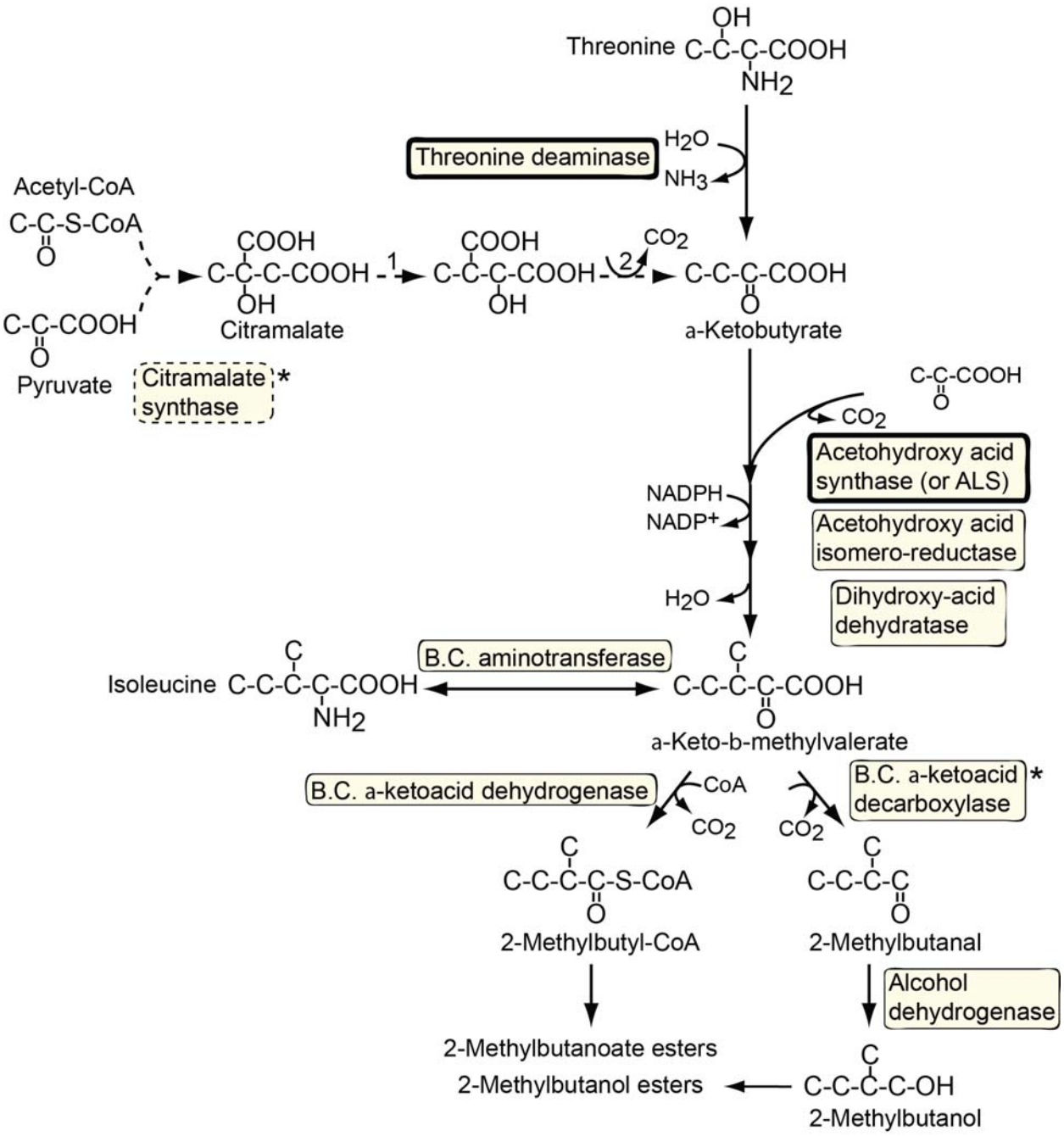
Threonine
Threonine deaminase
H_2O
NH_3
Acetyl-CoA
Citramalate
Pyruvate
Citramalate synthase*
a-Ketobutyrate
CO_2
Acetohydroxy acid synthase (or ALS)
NADPH
$NADP^+$
Acetohydroxy acid isomero-reductase
H_2O
Dihydroxy-acid dehydratase
Isoleucine
B.C. aminotransferase
a-Keto-b-methylvalerate
B.C. a-ketoacid dehydrogenase
CoA
CO_2
B.C. a-ketoacid decarboxylase*
2-Methylbutyl-CoA
2-Methylbutanal
Alcohol dehydrogenase
2-Methylbutanoate esters
2-Methylbutanol esters
2-Methylbutanol

Figure 2.

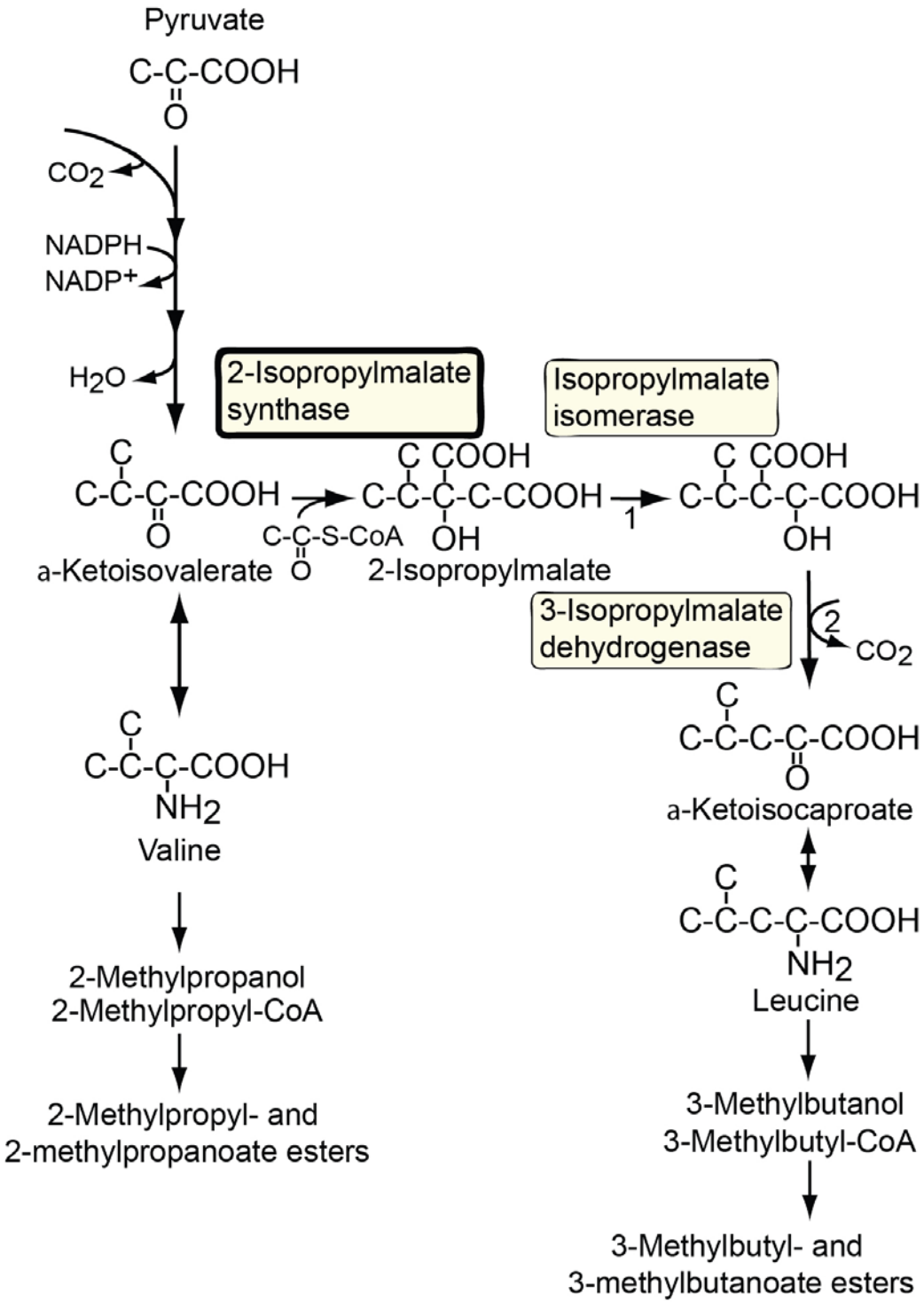

Figure 2 (cont'd).

Figure 2. Putative (dashed lines) and demonstrated (solid lines) pathways involved in branched-chain ester biosynthesis. Genes in bold indicates that they are regulated by feedback inhibition, threonine deaminase is inhibited by isoleucine, but stimulated by valine, acetohydroxy acid synthase [a.k.a. acetolactate synthase, (ALS)] is inhibited by valine and leucine, and 2-isopropylmalate synthase is inhibited by leucine. * Indicates that gene is found in bacteria, but not in plants. Hydrogens in carbon-hydrogen bonds are not shown. Numbers 1 and 2 in the putative pathway are considered to share the same enzyme in the leucine biosynthesis isopropylmalate isomerase and 3-isopropylmalate dehydrogenase, respectively. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

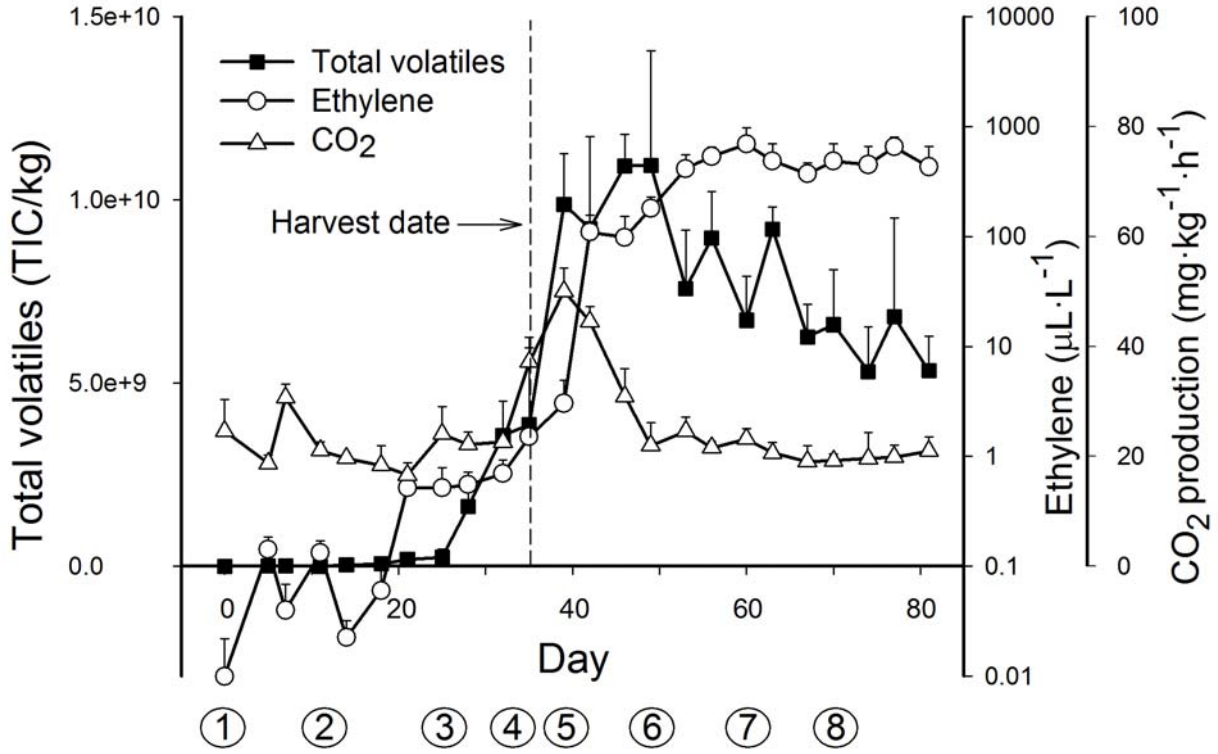

Figure 3. Internal ethylene, total volatiles [in total ion count (TIC)], and CO_2 production in pre-climacteric through post-climacteric 'Jonagold' apples. Fruit were examined from 2 Sept. 2004 (day 0) to 23 Nov. 2004 (day 81). Fruit were collected from the field until 7 Oct. 2004 (day 35), and thereafter maintained at room temperature (21±1 ºC). Each symbol represents the average of four replications. Vertical bars represent mean +SD. Eight time points (day 0, 11, 25, 32, 39, 49, 60, 70) were selected for amino acid and gene expression analyses based on distinct physiological stages.

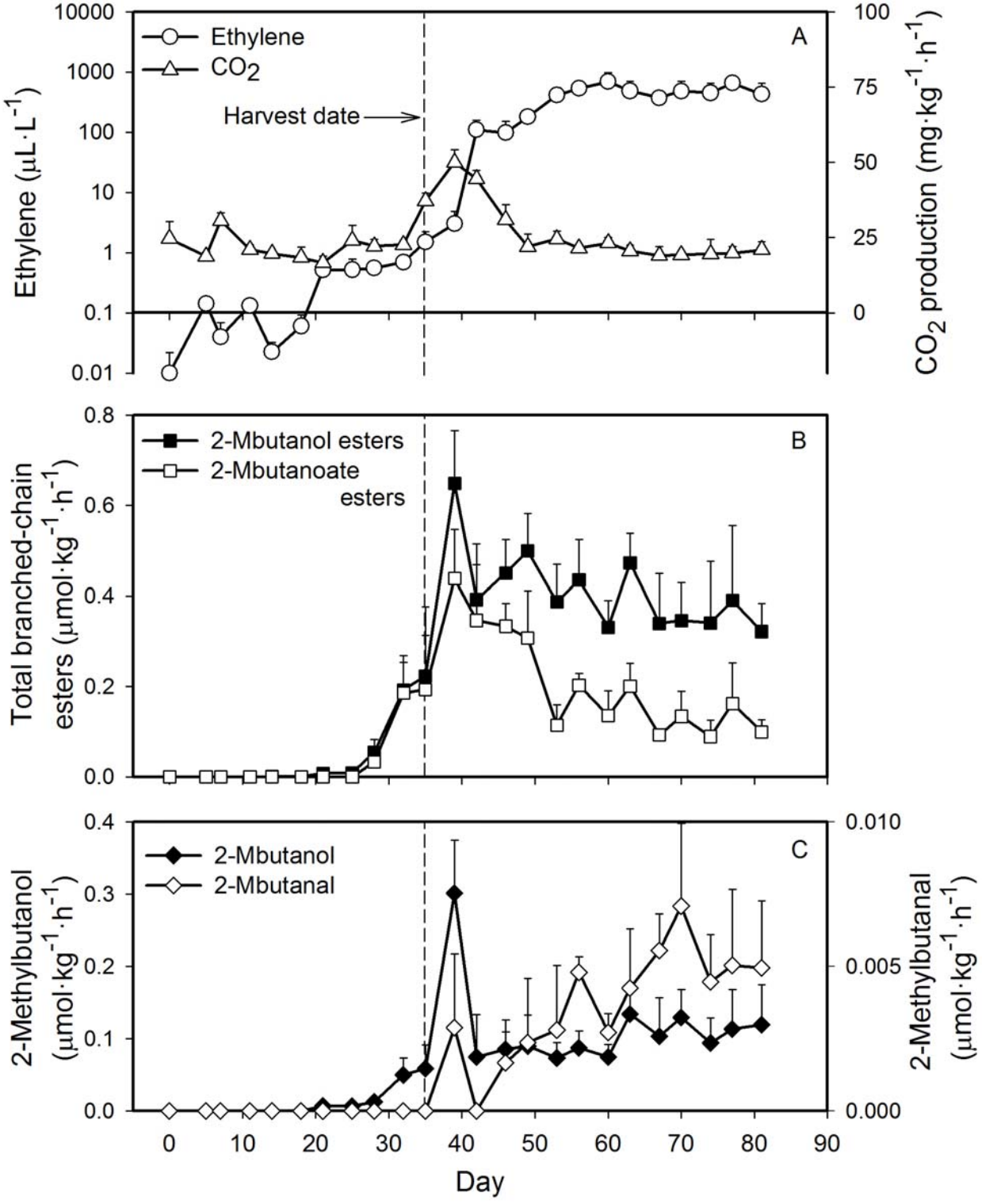
Ethylene
CO2
Harvest date
A
Ethylene (µL·L-1)
CO2 production (mg·kg-1·h-1)
10000
1000
100
10
1
0.1
0.01
100
75
50
25
0
2-Mbutanol esters
2-Mbutanoate esters
B
Total branched-chain esters (µmol·kg-1·h-1)
0.8
0.6
0.4
0.2
0.0
2-Mbutanol
2-Mbutanal
C
2-Methylbutanol (µmol·kg-1·h-1)
2-Methylbutanal (µmol·kg-1·h-1)
0.4
0.3
0.2
0.1
0.0
0.010
0.005
0.000
0
10
20
30
40
50
60
70
80
90
Day

Figure 4.

Figure 4. Patterns of 2-methylbutanol and 2-methylbutanoate esters, 2-methylbutanol, and 2-methylbutanal production during ripening and senescence of 'Jonagold' apple. The volatile profile was tracked from early September (day 0) until late November (day 81). The fruit were collected from the field until 7 Oct. 2004 (day 35) and thereafter maintained at room temperature (indicated by dashed vertical line). A. Internal ethylene and CO_2 production. B. Total 2-methylbutanol and 2-methylbutanoate esters production. C. 2-Methylbutanol and 2-methylbutanal production. Each symbol represents the average of four replications. Vertical bars represent mean +SD.

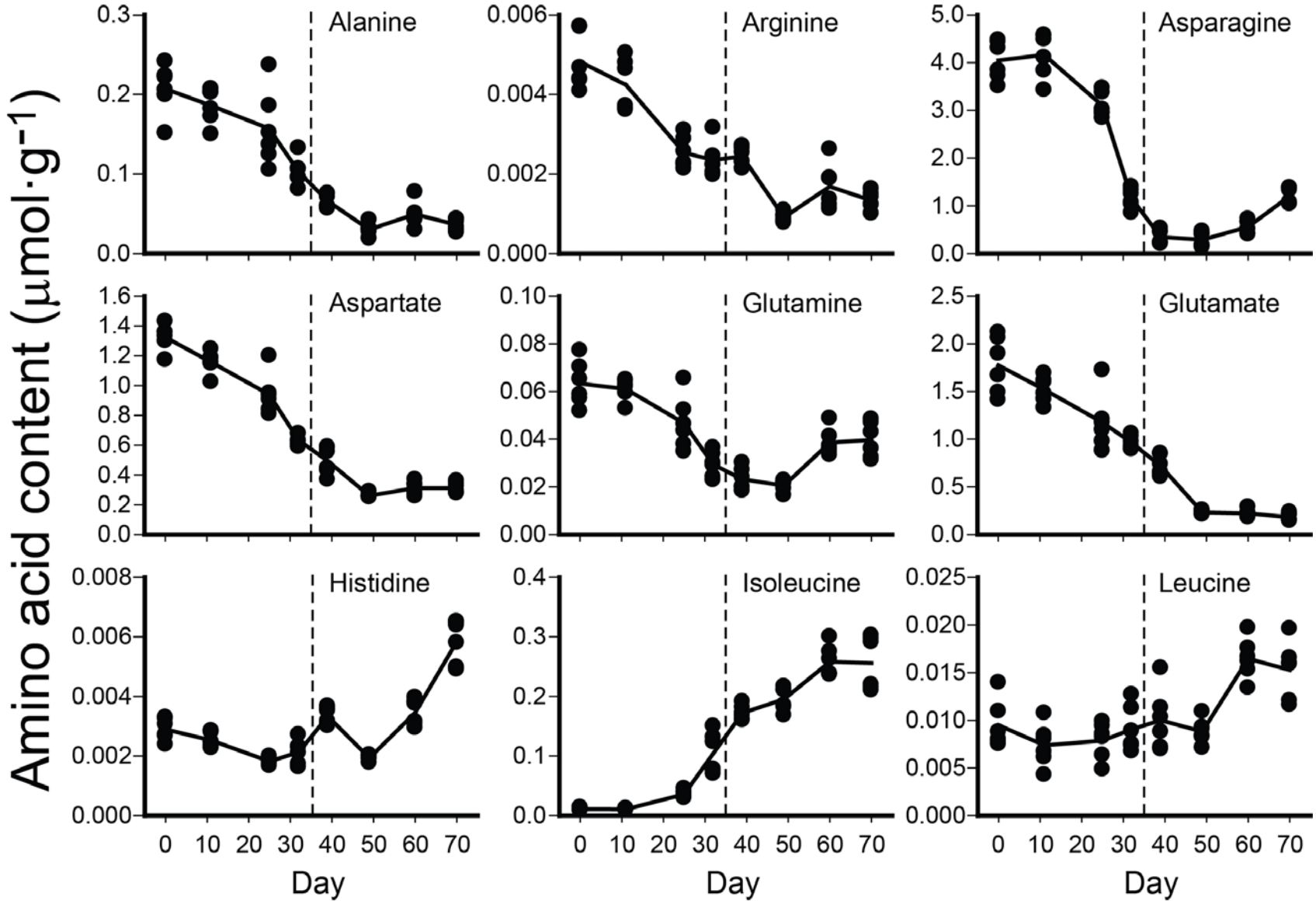
Amino acid content (μmol·g⁻¹)
Alanine
Arginine
Asparagine
Aspartate
Glutamine
Glutamate
Histidine
Isoleucine
Leucine
Day
Day
Day

Figure 5.

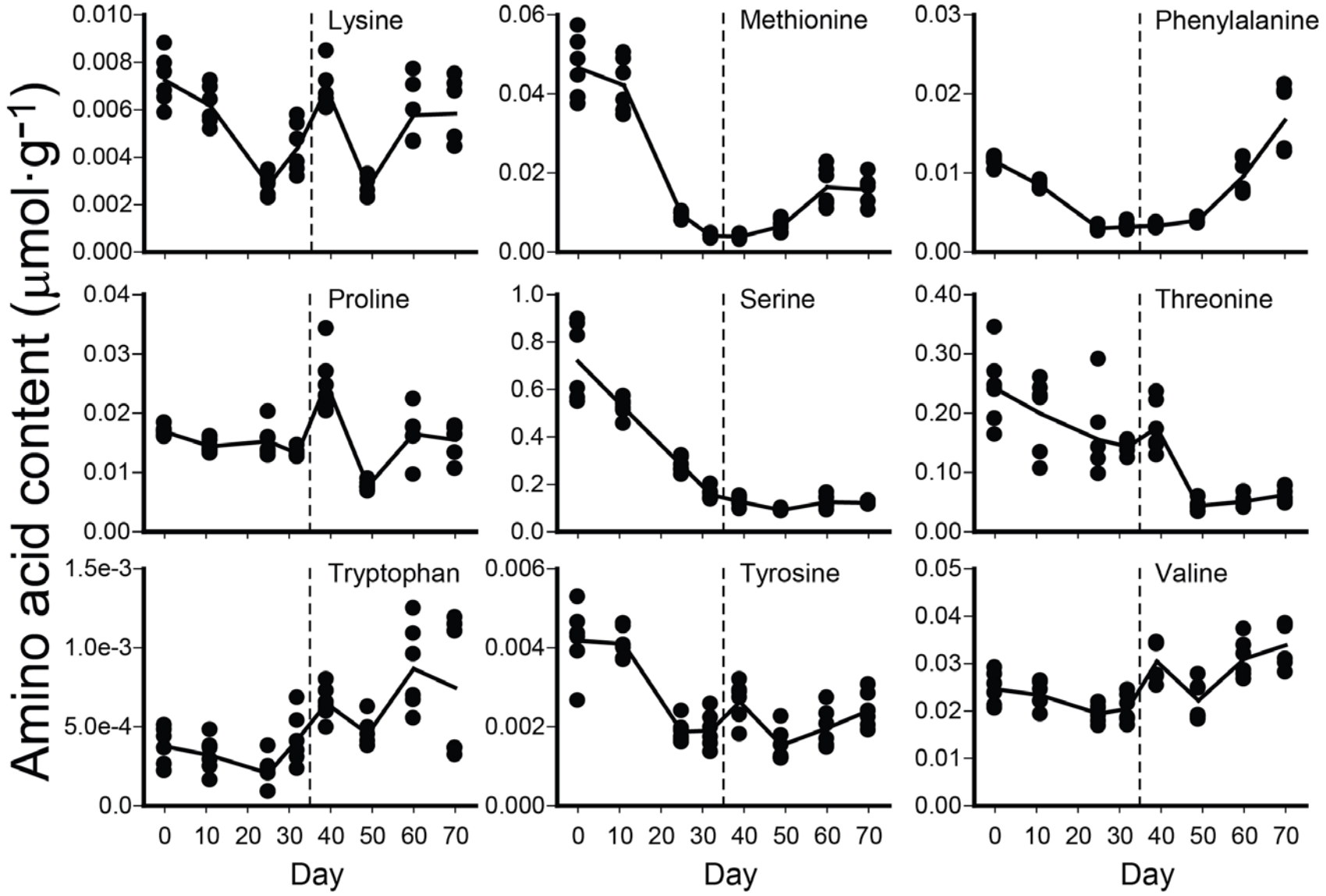

Figure 5 (cont'd).

Figure 5. Ontogeny of amino acid content in pre-climacteric through post-climacteric ‘Jonagold’ apples. Fruit were examined from 2 Sept. 2004 (day 0) to 23 Nov. 2004 (day 81). Fruit were collected from the field until 7 Oct. 2004 (dashed-line, day 35), and thereafter maintained at room temperature (21±1 ºC). Eight time points (day 0, 11, 25, 32, 39, 49, 60, 70) were selected for amino acid analysis based on distinct physiological stages. There were total of six replications, two biological and three technical replications.

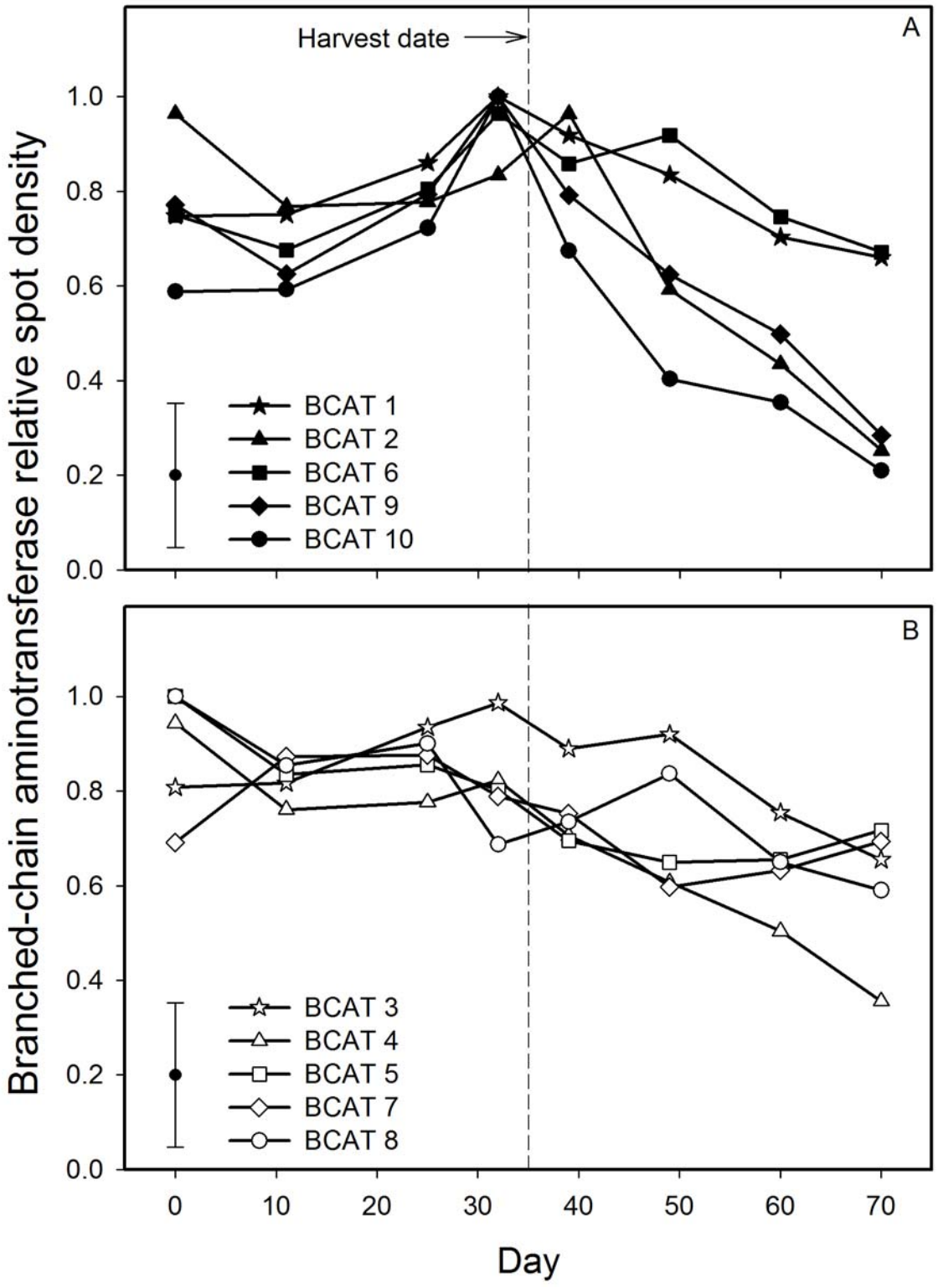
Harvest date
A
B
Branched-chain aminotransferase relative spot density
1.0
0.8
0.6
0.4
0.2
0.0
BCAT 1
BCAT 2
BCAT 6
BCAT 9
BCAT 10
BCAT 3
BCAT 4
BCAT 5
BCAT 7
BCAT 8
0
10
20
30
40
50
60
70
Day

Figure 6.

Figure 6. Expression of putative branched-chain aminotransferase (*BCAT*) genes for 'Jonagold' apple fruit ripened at room temperature performed by semi-quantitative RT-PCR. The graph's panels A and B were separated by expression pattern purpose only. The values are based on spot density relative to maximum value. 18S ribosomal RNA was used as a control. All data are normalized relative to control gene spot density. The control gene spot density ranged between 0.78–0.98. Each symbol represents the average of two replicate measurements. The average pooled standard deviation is 0.15.

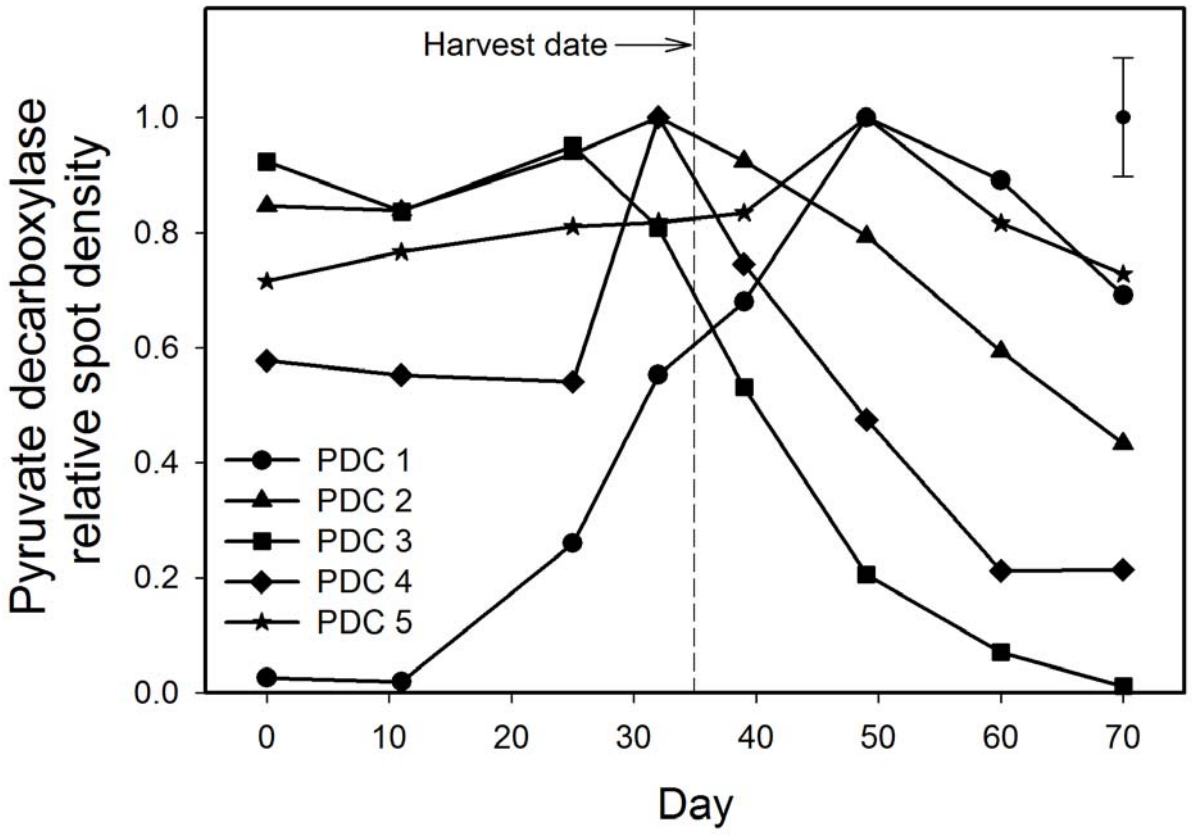

Figure 7. Expression of putative pyruvate decarboxylase (*PDC*) genes for 'Jonagold' apple fruit ripened at room temperature performed by semi-quantitative RT-PCR. The values are based on spot density relative to maximum value. 18S ribosomal RNA was used as a control. All data are normalized relative to control gene spot density. The control gene spot density ranged between 0.78–0.98. Each symbol represents the average of two replications. The average pooled standard deviation is 0.10.

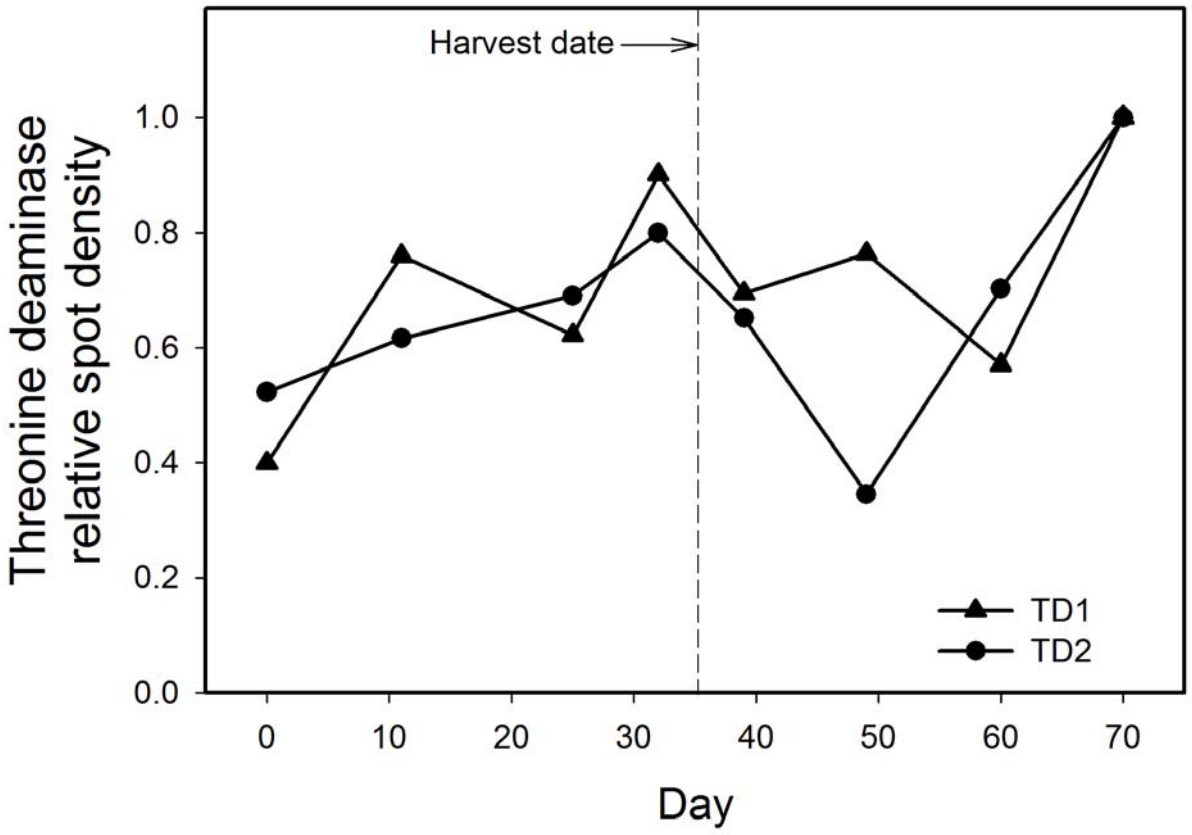

Figure 8. Expression of putative threonine deaminase (*TD*) genes for 'Jonagold' apple fruit ripened at room temperature performed by semi-quantitative RT-PCR. The value is based on spot density relative to maximum value. 18S ribosomal RNA was used as a control. All data are normalized relative to control gene spot density. The control gene spot density ranged between 0.78–0.98.

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CHAPTER IV

BIOSYNTHESIS OF ISOLEUCINE VIA CITRAMALATE PATHWAY: CHARACTERIZING CITRAMALATE SYNTHASE AS THE FIRST COMMITTED STEP IN A NEW BIOSYNTHETIC PATHWAY FOR ESTER SYNTHESIS IN RIPENING APPLE FRUIT

Introduction

Esters are aroma impact compounds produced by many horticultural fruits and contribute importantly to the sensory quality of apple (*Malus ×domestica*) fruit. Esters normally account for 80% to 95% of the total volatiles emitted by apple (Paillard, 1990). The esters hexyl acetate, butyl acetate, and 2-methylbutyl acetate are abundantly produced and considered to confer typical apple aroma characteristics (Dimick and Hoskin, 1983; Fellman et al., 2000); however, the volatile profile can contain dozens of esters. Surprisingly, despite the importance of aroma in the quality of fruits, the biochemistry of ester formation is poorly understood.

The esters are largely composed of either straight-chain (SC) or branched-chain (BC) alkyl (alcohol-derived) and alkanoate (acid-derived) groups. Alkyl group precursors normally range from 1 to 6 carbons in length and the alkanoate group precursors range from 2 to 8 carbons (Paillard, 1990). The final step of ester formation is from the condensation of an alcohol and a Co-A derivative of a carboxylic acid by alcohol acyltransferase (AAT) (Ueda and Ogata, 1977). The AAT gene has been extensively studied for its importance in ester biosynthesis in several horticultural crops including apple (Aharoni et al., 2000; Balbontín et al., 2010; Günther et al., 2011; Harada et al., 1985; Jayanty et al., 2002; Souleyre et al., 2005; Yahyaoui et al., 2002). It is generally accepted that AAT will influence the ester profile by its substrate specificity and availability, but control of ester synthesis probably lies at the level of ester precursor formation (Ferenczi et al., 2006; Wyllie and Fellman, 2000).

Ester precursors are suggested to be produced primarily by degradative processes. BC ester precursors have been proposed to be derived from branched-chain amino acid (BCAA) degradation (Gonda et al., 2010; Rowan et al., 1996; Tressl and Drawert, 1973; Wyllie and Fellman, 2000). It is known that the BCAAs, isoleucine, valine, and leucine can be

transaminated to produce their respective α-keto acids, α-keto-β-methylvalerate, α-keto-isovalerate, and α-ketoisocaproate, respectively, by branched-chain aminotransferase (BCAT) (Figure 9). The α-keto acids, in turn, are metabolized into the alcohol and acid ester precursors. In apples, esters related to isoleucine metabolism predominate, while those from valine can be detected only occasionally and usually at low levels, and little-to-no esters are produced from leucine (Ferenczi, 2003; Mattheis et al., 1998; Ortiz et al., 2010; Plotto et al., 2000; Sugimoto, 2007). During apple fruit ripening, isoleucine has been reported to accumulate, but the other BCAAs have not (Defilippi et al., 2005; Nie et al., 2005; Sugimoto et al., 2011). Feeding studies in apple fruit have demonstrated that exogenously fed isoleucine is metabolized to 2-methylbutanol and 2-methylbutanoate ester (Rowan et al., 1996; 1998). These data were taken to indicate that isoleucine is degraded into α-keto-β-methylvalerate via BCAT to form ester precursors. BCAT expression increases during ripening leading to the formation of BC esters (Gonda et al., 2010; Pérez et al., 2002). However, BC esters can also be produced directly from exogenously supplied BC α-keto acids (Gonda et al., 2010). Since the BC α-keto acids are in approximate equilibrium with their respective BCAAs (Tewari et al., 2000), it may be reasonable to expect that, for apple, the pool of isoleucine roughly mirrors the pool of its respective BC α-keto acid. Therefore, the accumulation of isoleucine in apples during ripening may well be a by-product of the accumulation of α-keto-β-methylvalerate. To our knowledge, no data are currently available on α-keto-β-methylvalerate levels in apple.

In plants, isoleucine is synthesized from threonine via aspartate, which originates from the tricarboxylic acid (TCA) cycle (Azevedo et al., 1997). In this pathway, threonine is deaminated to α-ketobutyrate by threonine deaminase (TD) (Binder, 2010) (Figure 9). Threonine degradation to α-ketobutyrate is reported to be the only pathway for isoleucine formation in

plants based on evidence for autotrophy in *Nicotiana plumbaginifolia* (Colau et al., 1987; Sidorov et al., 1981). α-Ketobutyrate is metabolized to α-keto-β-methylvalerate, the isoleucine precursor, by three enzymes acting in succession: acetohydroxy acid synthase [AHAS, a.k.a. acetolactate synthase, (ALS)], acetohydroxy acid isomero-reductase, and dihydroxy-acid dehydratase. These enzymes also catalyze the formation of α-ketoisovalerate from pyruvate to synthesize valine. Leucine synthesis, however, begins with the valine precursor, α-ketoisovalerate, which is acted upon by a succession of three enzymes [2-isopropylmalate synthase (IPMS), 2-isopropylmalate isomerase (IPMI), and 3-isopropylmalate dehydrogenase (IPMDH)] to form the leucine precursor α-ketoisocaproate.

Biosynthesis of all three BCAAs is responsive to feedback regulation. TD is inhibited by isoleucine, but stimulated by valine; ALS is inhibited by valine and leucine, and IPMS is inhibited by leucine (Eisenstein, 1991; Singh and Shaner, 1995; Wessel et al., 2000). Given that isoleucine is under feedback regulation, the explanation for the accumulation of isoleucine in ripening apple fruit is not obvious. Sugimoto et al. (2011) found that isoleucine was the only amino acid to increase extensively in apple fruit and they proposed the existence of an alternative pathway for α-ketobutyrate formation in ripening apple fruit, whose first step involves the formation of citramalic acid.

An alternative pathway for isoleucine biosynthesis, a ‘citramalate pathway’, has been described in several strains of bacteria (Gray and Kornberg, 1960; Hochuli et al., 1999; Risso et al., 2008; Westfall et al., 1983). In this pathway, acetyl-CoA and pyruvate are substrates for the formation of citramalic acid by citramalate synthase (CIM), which is a member of the 2-isopropylmalate (IPMS) family (Figure 9). Citramalate, in turn, is then converted to α-ketobutyrate by IPMI and IPMDH, which are the same enzymes that follow IPMS in the

pathway for leucine biosynthesis (Xu et al., 2004). In *Methanococcus jannaschii* and *Leptospira interrogans*, the CIM protein, CimA (gi: 15669582) and LiCMS (gi: 24215050), respectively, have been characterized for their activity and specificity (Howell et al., 1999; Xu et al., 2004). In *Saccharomyces cerevisiae*, the citramalate pathway has been suggested to be present (Vollbrecht, 1974) and CIM activity is evident in baker's yeast (Losada et al., 1964) and in *Saccharomyces carlsbergensis* (Sai and Uemura, 1969), but no nucleotide or amino acid sequence for CIM has been identified as yet in the yeast genome database. In plants, Kroumova and Wagner (2003) reported the involvement of one-carbon fatty acid biosynthesis (1-C FAB) in the formation of sugar esters in some, but not all, members of the Solanaceae [e.g., tobacco (*Nicotiana tabacum*) and petunia (*Petunia ×hybrida*)] and they suggested that the citramalate pathway enables 1-C FAB. However, there has been no molecular characterization of CIM in any plant species, nor, in fact, in any eukaryote, to our knowledge.

The reactions of the citramalate pathway can synthesize various chain length fatty acids, from 3 to 12 carbons in length and they can be straight or branched (Kandra et al., 1990; Kroumova et al., 1994; Kroumova and Wagner, 2003; Oku and Kaneda, 1988). In yeast, 1-C FAB of α-ketobutyrate has been demonstrated in the synthesis of 2-ethylmalate and 2-propylmalate (Strassman and Ceci, 1967) and, respectively from these compounds, the formation of butanol and pentanol (Ingraham et al., 1961). The citramalate pathway has been proposed to be involved in the formation of esters possessing 2-methylbutyl and 2-methylbutanoate groups and esters with SC groups 3 to 5 carbons in length in apple (Sugimoto et al., 2011). It was suggested as providing an explanation for the observed accumulation of isoleucine during apple ripening. Intriguingly, Hulme (1954) isolated citramalate from the peel of the mature apple fruit and demonstrated that its concentration increased in ripening fruit (Hulme and Wooltorton,

1958). The presence of citramalic acid suggests that the citramalate route of ester biosynthesis is at least feasible. Hulme (1954) proposed citramalic acid participated in the mitochondrial reactions of the TCA cycle, but no evidence has been forthcoming to support this hypothesis.

CIM belongs to an acyltransferase family (EC 2.3.3), members of which share a common reaction between α-keto acids and acetyl-CoA. Other enzymes that belong to the acyltransferase family include citrate synthase, homocitrate synthase, malate synthase, IPMS, and methylthioalkylmalate synthase (MAM). Each differs in substrate specificity, preferring oxaloacetate, α-ketoglutarate, glyoxylate, α-ketoisovalerate, and various ω-methylthio-α-ketoalkanoates, respectively (Textor et al., 2004), and contribute to primary and secondary metabolic pathways.

In *Leptospira interrogans*, a spirochaete that causes Weil's disease, LiCMS protein has a sequence similar to a characterized MtIPMS (*Mycobacterium tuberculosis*), but unlike IPMS, its activity is specific to pyruvate as an α-keto acid substrate (Ma et al., 2008; Xu et al., 2004). In Arabidopsis, four genes, *IPMS*1 (At1g18500), *IPMS*2 (At1g74040), *MAM*1 (At5g23010), and *MAM*3 (At5g23020) have been characterized (de Kraker et al., 2007; Textor et al., 2004; 2007). AtIPMS and AtMAM catalyze the first enzymatic steps in leucine biosynthesis and in glucosinolate formation, respectively. The AtIPMS enzymes and AtMAM enzymes carry out similar reactions, but differ in substrate specificity. The two AtIPMS enzymes have broad substrate specificity, but prefer α-ketoisovalerate as a substrate. AtMAM enzymes, on the other hand, prefer ω-methylthio-α-ketoalkanoic acid. In addition, Textor et al. (2007) reported that AtMAM3 has a low level of citramalate synthase activity. It has been proposed that the *AtMAM* genes evolved from *AtIPMS* (de Kraker and Gershenzon, 2011).

The amino acid sequence identity is approximately 60% between AtIPMS and AtMAM proteins (de Kraker et al., 2007). AtIPMS and AtMAM proteins are considered to be localized in the chloroplast, although this has only been demonstrated for AtMAM3 (Textor et al., 2007). The yeast IPMS proteins Leu4 and Leu9 are in the mitochondria and the cytosol, respectively (Beltzer et al., 1988; Casalone et al., 2000). AtIPMS and AtMAM proteins share conserved motifs of GxGERxG for possible acetyl-CoA binding (de Kraker et al., 2007) and a HxH[DN]D motif, which is involved in binding of the divalent metal cofactor (de Kraker and Gershenzon, 2011; Koon et al., 2004). Perhaps the most significant difference between AtIPMS and AtMAM is the presence of an additional 130 amino acid sequence in the region of the C-terminal region in AtIPMS. In yeast, this C-terminal region is involved in leucine feedback inhibition and is called the R-region (Cavalieri et al., 1999). AtIPMS enzymes are inhibited by leucine (de Kraker et al., 2007) and LiCMS is inhibited by isoleucine (Zhang et al.., 2009). The truncated C-terminal region in AtMAMs eliminates feedback inhibition by leucine (de Kraker and Gershenzon, 2011).

Extensive previous work has lead to the conclusion that catabolic pathways are primarily responsible for ester biosynthesis (Gonda et al., 2010; Rowan et al., 1996; Tressl and Drawert, 1973; Wyllie and Fellman, 2000). The lack of supportive molecular and biochemical data suggests that a reassessment of this conceptual model is appropriate. The existence of new data implicating CIM during ripening raises the possibility that one or more uncharacterized synthetic pathways may exist (Sugimoto et al., 2011). The objective of this work was to explore the potential for a bacterial-like citramalate pathway to contribute to isoleucine biosynthesis and the synthesis of BC and SC esters. In this work, we describe the identification of MdCIM1 and MdCIM2, novel members of the IPMS gene family, demonstrate the function of the coded

protein as being typical of a citramalate synthase, demonstrate gene expression patterns consistent with the patterns of isoleucine and citramalic acid, and determine isotopic labeling patterns in BC and SC esters, citramalate, citraconate, 2-ethylmalate, and isoleucine, consistent with a functioning citramalate pathway. Our work demonstrates the existence of a new, primarily synthetic rather than catabolic, pathway for the synthesis of important esters in apple.

Materials and Methods

Isotope feeding study with ^{13}C acetic acid.

We studied the incorporation of ^{13}C-labeled acetate (1-^{13}C or 2-^{13}C or 1, 2-^{13}C) into methyl esters and other metabolite synthesized by peel discs of 'Jonagold' apple fruit. Methanol was added to the incubation solution to enhance the synthesis of methyl esters, which are normally present at extremely low levels in 'Jonagold' apples. Thus, methanol esters detected could be considered as being synthesized de-novo. Further, the methyl group cannot be simply metabolized from acetate and was, therefore, not expected to interfere with the interpretation of labeled carbon incorporation from ^{13}C-acetate.

Application of stable acetic acid isotopes. Filter paper discs (11.5 mm dia.) were cut and placed on a glass side and wetted with approximately 20 μL of a solution (adjusted to pH 7 with 1 M KOH with or without 0.1M MES) containing unlabeled acetic acid (20 mM) or 1-^{13}C or 2-^{13}C or 1,2-^{13}C acetic acid (20 mM) (99 atom % ^{13}C enrichment, Sigma-Aldrich) with methanol (20 mM). Peel discs 11.5 mm diameter were removed randomly from the surface of fruit using a stainless-steel cork borer and trimmed to 1- to 2-mm thickness using a stainless-steel scalpel. The apple discs were placed skin-side-up onto the wetted filter paper, and approximately 200 μL

additional incubation solution was added to the apple discs/filter paper unit. Five discs were placed on each glass and the slide was placed into a 30-mL glass vial sealed with a cap housing a valved septum (Mininert valve 20/400mm; Sigma-Aldrich). The samples were incubated for 24 to 26 hours at 22 °C. The incorporation of ^{13}C into headspace volatiles was analyzed by gas chromatography (GC) coupled with time-of flight mass spectrometry (TOFMS) as described below. There were four biological replicates and each replicate was derived from a single fruit. In the first two biological replicates, discs were incubated in solutions containing MES buffer and in the other two biological replicates, discs were incubated in a non-MES solution. The presence of buffer did not affect the volatile profile or incorporation of label. Following analysis of volatiles, apple discs were freeze dried and derivatized acids were quantified using GC/MS as described below. The data for headspace volatiles were from apple discs incubated without MES buffer and the data for soluble metabolites (derivatized acids) were from apple discs incubated with MES.

Headspace volatile analysis. Headspace ester, aldehyde, and alcohol content was measured by GC/TOFMS. The GC (HP-6890, Hewlett Packard Co., Wilmington, DE) was equipped with a mass selective detector (Pegasus II, LECO Corp., St. Joseph, MI). Headspace volatiles were sampled using a 1-cm long, solid-phase micro extraction (SPME) fiber (65 μm PDMS-DVB, Supelco Co., Bellefonte, PA). Following a 3 min sorption time, the SPME fiber was immediately transferred to a GC splitless injection port (230 °C) and desorbed for 2 min. Conditions of GC separation and TOFMS analysis were as previously described (Song et al., 1997). In brief, desorbed volatiles were trapped on-column using a liquid nitrogen cryofocussing trap. Separation of volatiles was by capillary column (HP-5MS, 29 m x 0.25 mm i.d., 0.25 μm film thickness,

Agilent Technologies, Santa Clara, CA,). The temperature of the GC was ramped from 40 to 240 °C at a rate of 40 °C·min^{-1}, the flow rate of the helium carrier gas was 1 mL·min^{-1}, and the GC was operated in splitless mode. Identification of compounds was by comparison of the mass spectrum with authenticated reference standards and/or with spectra in the National Institute for Standard and Technology Version 05 (NIST 05) mass spectrum library. The quantification was by calibrating with a known amount of an authenticated, high purity standard mixture of 72 volatilized alcohols, aldehydes, and esters as previously described (Song et al., 1997).

Soluble metabolite analysis. Apple discs were freeze dried and placed into 15-mL polypropylene tubes containing three 4-mm stainless steel balls and ground using a vibrating grinder (GenoGrinder 2000, SPEX CertiPrep, Inc., Metuchen, NJ) operated at 700 strokes/min. Approximately 0.2 to 0.25 mL of solution containing acetonitrile, 2-propanol, and water in a ratio of 3:3:2 (v/v/v) was added to 0.020 to 0.025 g of ground tissue and the tissue was extracted for 5 min at 22 °C. The extracts were centrifuged at 14,000 x g for 5 min and 100 µL of the supernatant was transferred to a clean 1.5-mL microfuge tube. The transferred supernatant was completely vacuum dried, 50 µL of methoxyamine hydrochloride in pyridine (10 mg·mL^{-1}) was added, and the solution was incubated at 50 °C overnight. The incubated solution was derivatized by adding 100 µL of N-methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) and incubated overnight at 22 °C. Metabolites were quantified by comparison of instrument response to that of an authenticated, high purity derivatized standard mixture of 10 acids containing 50 µM each of valine, threonine, isoleucine, leucine, aspartic acid, citramalic acid, α-ketobutyric acid, α-keto-β-methylvaleric acid, 2-isopropylmalic acid (Sigma-Aldrich, St. Louis, MO), and citraconic acid (Fisher Scientific USA, Pittsburgh, PA).

Derivatized acid analysis was performed using a mass spectrometer (Agilent 5973, Agilent Technologies, Santa Clara, CA) coupled to a GC (Agilent 6890). The injection port temperature was 280 °C. Separation of volatiles was by capillary column (DB-5MS, 30 m x 0.25 mm i.d., 0.25 μm film thickness, Agilent, Santa Clara, CA). The GC was programmed as follows: 80 °C for 2 min, ramped 30 °C·min^{-1} to 130 °C, then 15 °C·min^{-1} to 300 °C, and held at 300 °C for 3 min. The flow rate of the helium carrier gas was 1.2 mL·min^{-1}. The GC was operated in either split (1:20 ratio) or splitless mode and selective ion monitoring (SIM) was used depending on the need for sensitivity and/or specificity. Injection volumes were 0.2 μL for split mode and either 0.2 μL or 1 μL for splitless modes for scanning and SIM mass spectral analysis, respectively. Identification of compounds was by comparison of the mass spectrum and GC retention time with those of derivatized standards and comparison to the NIST 05 mass spectrum library using commercial software Agilent ChemStation software. The quantification was by comparison with a known amount of derivatized standards as described previously. Where no standard was available, 2-ethylmalic acid was quantified by estimation of the instrument response factor based on the retention time of adjacent peaks for which standards were available.

Isotopic analysis. Isotopomers of headspace volatiles and soluble acids were quantified as previously described. Propanol, propanal, methyl propanoate, and butanal were quantified by integrating extracted ion chromatogram peaks for the molecular masses *m/z* 60, 58, 88, and 72, respectively. 2-Methylbutanol and methyl 2-methylbutanoate were quantified by integrating extracted ion chromatogram peaks for *m/z* 70 and 88, respectively. Methyl butanoate was quantified by integrating the chromatographic peak for *m/z* 74, a fragment corresponding to

•$CH_2C(=OH+)OCH_3$ generated by the McLafferty rearrangement. This mass, and its heavier isotopologs, were used to distinguish whether ^{13}C was incorporated into C-1 and/or C-2 of methyl butanoate. This was done to determine whether the butanoate portion of the methyl butanoate is synthesized via one- or two-carbon FAB. The acids isoleucine, citramalic acid, citraconic acid, 2-ethylmalate, and the mixture of 2-isopropylmalate and 2-propylmalate were quantified by integrating peaks of *m/z* 302, 433, 301, 447, and 461, respectively. These masses correspond to the molecular masses of the *tert*-butyldimethylsilyl derivatives minus 57 Da, corresponding to loss of the *tert*-butyl group from the derivative. The isotope enrichment was assessed by integrating peaks corresponding to the heavier isotopologs, and mole% enrichments were calculated by correcting for natural isotope content by unlabeled acetate control on the mass distribution as described by Biemann (1962). The mass isotopolog distribution is shown as unlabeled mass fraction (M), one ^{13}C-labeled mass fraction (M+1), two ^{13}C-labeled mass fraction (M+2), so on, and up to five ^{13}C-labeled mass fraction (M+5) are reported. The expected position of the isotopic carbon from labeled acetate in the various compounds of interest is described in the proposed pathway (Figure 9). The amounts of threonine, α-ketobutyrate, and α-keto-β-methylvalerate were in low abundance and could not be quantified reliably in this study.

Developmental changes in CIM and IPMS gene expression and citramalic acid content.

To determine the developmental pattern of gene expression and citramalic acid content, eight developmental stages were selected based on physiological changes during ripening. These stages are: stage 1 (day 0), early preclimacteric; stage 2 (day 11), late preclimacteric and onset of trace ester biosynthesis; stage 3 (day 25), onset of autocatalytic ethylene and rapid increase of ester biosynthesis; stage 4 (day 32), half-maximal ester biosynthesis and engagement of the

respiratory climacteric; stage 5 (day 39), near maximal ester biosynthesis, peak in respiratory activity, and onset of rapid tissue softening; stage 6 (day 49), end of maximal ester biosynthesis, conclusion of the respiratory climacteric, and completion of tissue softening; stage 7 (day 60), midpoint in the decline in ester biosynthesis, maximal ethylene production, and onset of senescence; and stage 8 (day 70), postclimacteric minimum in ester production and extensive fruit senescence.

Plant material. 'Jonagold' apples were harvested for examination every three to four days from research plots at the Michigan State University Clarksville Horticultural Experiment Station, Clarksville, Mich., from 2 Sept. 2004 (day 0) until ripening was fully engaged on 7 Oct. 2004 (day 35). On each occasion, fruit were held overnight in the laboratory to equilibrate to laboratory temperature (21±1 °C). During the holding period, fruit were covered with ventilated, black, 0.1-mm thick plastic bags to avoid desiccation and responses to intermittent laboratory light before analysis. All fruit (approximately 200) remaining on the trees were then harvested and transported to the laboratory on 7 Oct. 2004 (day 35) after it was apparent that ripening was underway. This was done to avoid damage in the field due to freezing and fruit drop. Thereafter, these fruit were maintained at room temperature, 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 23 Nov. 2004 (day 81).

On each evaluation date, 20 apples were randomly chosen and the internal ethylene content of each was measured. Of these, the fourteen fruit having an internal ethylene content nearest the median were selected for further analysis. The four fruit having ethylene levels closest to the median were chosen for analysis of CO_2 production and ester emission as described

by Sugimoto et al. (2008). Of the remaining 10 fruit, maturity analysis (percent red coloration, background color, °Brix, and starch index) was performed on each date as described by Mir et al. (2001). From these fruit, the skin and 2 to 3 mm of underlying cortex tissue were removed and immediately frozen in liquid nitrogen and stored at –80 °C. Two replicates were created, each consisting of pooled tissue samples from five fruit. The pooled tissue samples were used for soluble metabolite analyses and gene expression studies.

Isolation of RNA. Approximately 5 g of frozen 'Jonagold' apple tissue was ground using liquid nitrogen chilled mortar and pestle. Ground tissue was extracted using hot borate/phenol followed by LiCl precipitation (López-Gómez and Gómez-Lim, 1992). To compare gene expression in different tissues, leaf, root, and stem tissues from 'Jonagold' apple trees grown in a commercial orchard were collected. Root tissue was collected from a tree with scion rooting. Tissue samples (3 g) were subsequently ground and extracted as described for fruit tissue.

Microarray printing, design, labeling, and statistical analysis. Custom cDNA microarray slides were created using a robotic printing device in the Genomics Technology Support Facility (GTSF) of the Genomics Core in Michigan State University, East Lansing, MI. Approximately 10,000 unsequenced cDNA gene fragments were generated from the lambda phage cDNA library from 'Mutsu' apple fruit described by Gao et al. (2005) using a mass excision kit and protocols as described by the manufacturer (ZAP-cDNA synthesis kit, Stratagene, LaJolla, CA). In addition to the unsequenced cDNA fragments, 116 apple ESTs that were available in the GenBank (kindly donated by Dr. Schuyler Korban, University of Illinois, Urabana-Champaign) were also included on the array. The microarray printing procedure, experimental design, the

microarray protocol used for labeling, hybridization and washing, image scanning, and statistical analyses have been described previously by Sugimoto et al. (2008). Gene fragments undergoing greater than a four-fold change in expression and/or identified as undergoing significant changes in expression ($P<0.00025$) relative to day 0 were sequenced. Assigned identity for *MdCIM* was initially *IPMS* based on BLAST analysis of the predicted amino acid sequence against the NCBI non-redundant protein and Arabidopsis protein databases. Following protein characterization, the designation *MdCIM* was adopted and is used uniformly hereafter.

Determination of mRNA transcript levels by reverse transcription polymerase chain reaction (RT-PCR). The expression of *MdCIM*, *MdIPMS*, and the 18S ribosomal RNA (18s rRNA) gene was measured using RT-PCR analysis. For each analysis, there were two biological and two technical replications. cDNA synthesis and PCR reactions were performed using commercially available kits according to manufacturer (Invitrogen, Carlsbad, CA) directions. Before creating cDNA, total RNA was treated with DNase using an RNase-free DNase kit according to the manufacturer (Qiagen Inc, Valencia, CA). DNase-treated total RNA (1.0 µg) was reverse transcribed using oligo(dT)12-18 primer or random hexamer and SuperScript II as described by the manufacturer (Invitrogen). cDNA created with oligo (dT)12-18 primer was used for 18s rRNA and *MdCIM* expression and cDNA created with random hexamer was used for 18s rRNA and *MdIPMS* expression analysis. The cDNA (1.0 µL) was used as a template in a 50-µL PCR reaction containing 10 µM of the forward and reverse gene-specific primers. Primer sequences, the expected size of the PCR product, optimum cycle number, and optimum temperature for primer binding are listed in Table 4. The PCR reaction was performed as follows: 1) 5 min at 95 °C, 2) 30 s at 95 °C, 3) 30 s at 57–59 °C, 4) 30 s at 72 °C, repeating 18–33 cycles from steps

2–4, and final elongation 5 min at 72 °C. The amplified PCR products were separated by electrophoresis on a 1.5% (w/v) agarose gel, visualized with 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 number, 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. For each PCR run, the target cycle number was bracketed by 2 to 3 cycles to ensure that transcript synthesis had not plateaued. PCR products were cleaned using a PCR purification kit (QIAquick, Qiagen) and sequenced at the GTSF to verify identity. All the PCR generated sequences were 98% to 100% identical to original sequence. A partial sequence of the 18s rRNA gene (gi: 85717895) was used as an internal control for PCR analyses. The spot density for the 18s rRNA varied approximately ±10% across the eight developmental stages.

Citramalic acid analysis. The same eight developmental stages as described previously were used for citramalic acid analysis. Approximately 5 g of frozen apple tissue from each of the two replicates were ground into fine powder using a liquid nitrogen cooled mortal and pestle. The ground tissue was further divided into three technical replicates, each containing 0.5 g of tissue. The powdered tissue was transferred into 1 mL of preheated water, mixed vigorously, and incubated at 90 to 95 °C for 10 min. After centrifugation at 5000 x g for 5 min, the supernatant was filtered through a 0.22 μm filter (Millex GS, Millipore, Billerica, MA) and the cleared filtrate diluted into one fifth of its original concentration with water and was used for analysis.

Citramalic acid and 2-isopropylmalic acid (Sigma-Aldrich Corporation) were dissolved in water to make individual stock solutions of 1 mM. A master mixture was created with both

acids at a final concentration of 50 µM. A series of five working standards ranging from 1 to 40 µM was prepared by serial dilutions from the 50 µM master mixture and used to create calibration curves.

Citramalic acid was analyzed using a mass spectrometer (Quattro Premier XE, Waters Corp., Milford, MA) coupled to an ultra performance liquid chromatograph (UPLC) (Acquity, Waters Corp., Milford, MD). The column (Thermo Betasil C18, 2.1 x 150 mm, 5-µm particles, Thermo Fisher Scientific Inc., Waltham, MA) was held isothermally at 50 °C. The injection volume was 5 µL and solvents were 1 mM N, N-dimethylhexylammonium acetate (pH 7.7) in water (mobile phase A) and methanol (mobile phase B) and the flow rate was 0.4 $mL \cdot min^{-1}$. The gradient program was as follows: 80% mobile phase A and 20% mobile phase B at start, increasing to 75% mobile phase B after 3 min, decreasing to 20% mobile phase B at 4 min for re-equilibration of the column. The total run time was 5 min. Mass spectra were acquired using electrospray ionization in negative ion mode. The capillary voltage was set at 3.25 kV, the cone gas flow rate was 40 $L \cdot h^{-1}$, the desolvation gas flow rate was 600 $L \cdot h^{-1}$, the source temperature was 120 °C, and the desolvation temperature was 350 °C. Data were collected and quantified with proprietary software (MassLynx 4.0 and QuanLynx; Waters).

MdCIM and *MdIPMS* cloning, identification, and sequencing.

Total RNA extracted from ripe 'Jonagold' fruit skin as described previously was used to identify nucleotide sequences. All the primers used for identifying genomic and coding sequences are listed in Table 5. First-strand cDNA was synthesized using 4 µg of total RNA, oligo(dT)12-18 primer, and SuperScript II (Invitrogen) according to manufacturer instructions. Gene fragments were amplified by PCR containing cDNA, 10 µM of the forward and reverse

gene-specific primers (GSP), and Phusion high-fidelity DNA polymerase (New England Biolabs Inc., Ipswich, MA) in a 50-µL PCR reaction buffer as described by the manufacturer. The PCR products were gel purified by QIAquick gel extraction kit (Qiagen). The gel purified PCR products was used to add dATP in the 50-µL reaction buffer containing 0.2 mM dATP and PCR reaction solutions as provided by the manufacturer ($MgCl_2$, PCR 10x buffer, and Taq DNA polymerase) (Invitrogen) and incubated for 15 min at 72 °C. The mixture was ligated into a modified pCR 2.1-TOPO vector (Invitrogen), and transformed to TOP10 *E. coli* cells (Invitrogen). The transformed colonies were screened by restriction analysis and sequenced at the GTSF.

MdCIM mRNA sequence. An *MdCIM* clone was obtained from the 'Mutsu' cDNA library created as described previously (Sugimoto et al., 2008). The clone was sequenced at GTSF using several internal primers to obtain a full length sequence of the mRNA, which was about 2000 nucleotides long. The mRNA likely contained the open reading frame (ORF) based on analysis by the NCBI ORF finder program and translated protein size was 473 amino acids. However, this was much shorter than two characterized Arabidopsis IPMS proteins (de Kraker et al., 2007), AtIPMS1 (631 a.a.) and AtIPMS2 (631 a.a.) and two MAM proteins (Textor et al., 2004; 2007) AtMAM1 (506 a.a.) and AtMAM3 (503 a.a.). The alignment of nucleotide sequence of *MdCIM* with *AtIPMS1* and *2* and *AtMAM1* and *2* revealed that the 5' end of the sequence likely contained the start codon and that the sequence was shorter than *AtIPMS* and *AtMAM*. To verify the length of nucleotide sequence, 3' RACE was performed.

3' RACE of MdCIM. 3' RACE was performed using commercially available kit (3 'RACE system for rapid amplification of cDNA ends, Invitrogen) according to manufacturer. In brief, first-strand cDNA was synthesized using 4 µg of total RNA, SuperScript II, and an adapted primer. cDNA (2.0 µL) was used as templates in a 50-µL PCR reaction containing primer CIM GSP (no. 3, Table 5) and an abridged universal amplification primer provided by manufacturer, in addition to the Taq DNA polymerase. PCR products were gel purified, cloned into a modified pCR 2.1-TOPO vector, which was used to transform DH5α *E. coli* cells and the insert was sequenced by the GTSF.

MdCIM gDNA sequence. To determine the genomic sequence of *MdCIM*, approximately 100 mg of DNA was isolated from young buds of 'Jonagold' and 'Mutsu' using a kit (DNeasy plant minikit, Qiagen) as described by manufacturer. gDNA was used as a template in a 50-µL PCR reaction containing 10 µM of the ORF forward and reverse primer nos. 1 and 2. PCR products were gel purified and cloned directly to cloning vector (Zero Blunt TOPO PCR cloning kit, Invitrogen). Inserts in the transformed colonies were sequenced at the GTSF.

MdIPMS mRNA sequences. There were five apple ESTs (GenBank nos. CN488984, DT000542, DT001339, DT002187, DT001294) annotated as *IPMS* in the GenBank database. The respective clones were kindly donated by Dr. Schuyler S. Korban. Of these five clones, only DT001294 and DT001339 were of sufficient length to be used for further analysis. However, the 5' end of the sequence was missing in both clones. Therefore, we performed 5' RACE as described below.

5' RACE of MdIPMS. 5' RACE was performed as described by Scotto-Lavino et al. (2006) with modification. In brief, first-strand cDNA was synthesized using 5 µg of total RNA, SuperScript II, and primer IPMS GSP no. 19 (Table 5). A 5' end tailed cDNA was created from the cDNA by incubating with dATP and terminal deoxynucleotidyl transferase (Invitrogen). Two PCR reactions were performed using Phusion high-fidelity DNA polymerase (New England Biolabs): the first PCR reaction contained primers IPMS GSP1 (no. 20), Qt (no. 16), and Qo (no. 17), and the second nested PCR contained the first PCR reaction as a template and primers IPMS GSP2 (no. 21) and Qi (no. 18). The nested PCR products were gel purified, cloned into a modified pCR 2.1-TOPO vector, transformed to DH5α *E. coli* cells, and the insert was sequenced at the GSTF.

Once the tentative cDNA sequence was identified from 5' RACE, the ORF was obtained by PCR reaction containing forward and reverse primers nos. 12 to 15. The gel purified PCR products were cloned into a modified pCR 2.1-TOPO vector generating two *MdIPMS*-pCR 2.1, transformed to DH5α *E. coli* cells, and sequenced. We isolated two IPMS sequences, named *MdIPMS*1 and *MdIPMS*2, and the original sequences correspond to the clones DT001339 and DT001294, respectively.

MdCIM and MdIPMS protein characterization.

All the primers used for expressing proteins are listed in Table 5. The cloning procedures were described previously in identifying sequences of *MdCIM* and *MdIPMS.*

Isolation of RNA and cDNA cloning in expression vector. Total RNA from ripe 'Jonagold' apple skin was used to create cDNA. PCR was performed using forward and reverse primer nos. 4 to 7. To generate full length ORF and truncated ORF lacking a putative chloroplast transit peptide

(ChloroP; Emanuelsson et al., 1999), forward primers nos. 4 and 5 were used with the combinations of ORF reverse primers nos. 6 and 7 either with or without containing stop codon. dATP was added to the gel purified PCR product, cloned into an expression vector (pBAD-TOPO, Invitrogen) to create *MdCIM1*- and *MdCIM2*-pBAD-TOPO, which were used to transform TOP10 *E. coli* cells. The insert was sequenced at the GTSF.

The protein expression of the pBAD-TOPO construct was very poor so the PET 101/D-TOPO (Invitrogen) expression vector containing C-terminal 6xHis tag was used. The *MdCIM* ORF was generated using *MdCIM*-pBAD-TOPO as a template in a PCR reaction using forward and reverse primer nos. 8 to 11. The ORF for the *MdIPMS* genes was generated using *MdIPMS*-pCR 2.1 as a template in a PCR reaction using primer nos. 22 to 27. The forward primer nos. 8 to 10, 22, 24, 25, and 27 were used to generate full length ORF and truncated ORFs (lacking a putative chloroplast transit peptide) as previously described. For CIM, only the full length protein was expressed and the truncated protein failed to express despite using two different forward primers nos. 9 and 10. For IPMS, both full length and truncated protein were expressed. The gel purified PCR products were cloned directly into the PET 101/D-TOPO vector, generating full length *MdCIM*-PET 101/D and full and truncated length *MdIPMS*-PET 101/D. Vectors were used to transform TOP10 *E. coli* cells, and the insert sequenced by the GTSF. The screened constructs were used to transform to BL21(DE3) *E. coli* cells (Invitrogen) for protein expression analysis.

Protein expression in E. coli. MdCIM- or *MdIPMS*-PET 101/D-TOPO in BL21(DE3) cells were grown in 250 mL Luria-Bertani medium containing antibiotic carbenicillin (50 $\mu g \cdot mL^{-1}$) at 37 °C for 1-2 h until the OD_{600} reached 0.6. The expression of the gene was induced by adding

isopropyl β-D-1-thiogalactopyranoside (IPTG) optimized to final concentrations of 0.25 mM and 1 mM for CIM and IPMS, respectively, and the incubation was continued for 6 to 7 hours at 22 °C. After incubation, the cells were harvested by centrifugation at 5000 x g for 10 min in 4 °C and the bacterial pellets were stored at –80 °C.

Expressed His-tag protein purification. Bacterial pellets which had been thawed on ice were homogenized with 10 mL lysis buffer (50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 0.1% TritonX-100, 20 mM imidazole), 200 µL lysozyme (50 $mg \cdot mL^{-1}$, Hoffmann-La Roche Ltd., Basel, Switzerland), 5 µL Benzonase (Sigma-Aldrich), and 1 tablet of EDTA-free protease inhibitor (Hoffmann-La Roche Ltd.) for 20 min at 4 °C. The cells were disrupted using a sonicator (Misonix Sonicator S-4000 Ultrasonic Processor, Qsonica, LLC., Newtown, CT) fitted with a Microtip probe (Qsonica, LLC.). The cells were lysed using using pulsed sonication (10 s) followed by 10 s with no sonication and repeated for a total process time of 1.5 min. The sonicator power setting was 4 to 5 W. The disrupted cells were precipitated by centrifugation at 12,000 x g for 15 min at 4 °C. The bacterial lysate was added to a chromatography column (Econo-Pac, Bio-Rad Laboratories, Inc., Hercules, CA) packed with 1.5 mL Ni-NTA agarose (Qiagen) previously rinsed with lysis buffer. Binding of the His-tag protein to the nickel resin was facilitated by mixing for 1 to 2 min. The column was washed with washing buffer of the same composition as the lysis buffer except that it contained 35 mM imidazole. The His-tag protein was eluted with 3 mL elution buffer of the same composition as the lysis buffer except that it contained 300 mM imidazole. The eluent was transferred to a desalting column (Econo-Pac 10 DG column, Bio-Rad Laboratories) and 4 mL of desalting

buffer (50 mM Tris buffer pH 8.0, 1 mM $MgCl_2$, and 10% glycerol) was applied as described by de Kraker et al. (2007). The desalted eluent was used for enzyme assays.

The protein concentration was determined by Bradford assay 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-tag protein was separated by electrophoresis on a 10% SDS-PAGE gel, stained using Coomassie Brilliant Blue R-250, photographed, and sequenced at the Research Technology Support Facility (RTSF) of the Proteomics Core at Michigan State University using liquid chromatography tandem MS.

End-point enzyme assay (DTNB) for CIM and IPMS. The enzyme assays for CIM and IPMS were performed as described by de Kraker et al. (2007) with modifications. An aliquot of 5 to 20 µL of the protein preparation was added to an enzyme assay mixture (100 mM Tris buffer pH 8.0, 4 mM $MgCl_2$, 0.25 mM acetyl-CoA, 10 mM α-keto acids) for a final volume of 150 µL and incubated for 20 min at 22 °C. The reaction was stopped by the addition of 200 µL ethanol and color developed by adding 200 µL of a fresh 1 mM solution of Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB] (Sigma-Aldrich) in 100 mM Tris buffer pH 8.0. The mixture was left for 2 to 3 min for DTNB to react with free thiol groups released from acetyl-CoA and form a yellow color. After full color development, the absorbance of the mixture was measured against water at 412 nm. The absorbance was adjusted by subtracting the background of the identical enzyme assay mixture without α-keto acids. For α-keto-γ-(methylthio)butyric acid, the results were further corrected for the slight reactivity between its thiol group and DTNB using reactions containing the α-keto acid, but not acetyl-CoA . The molar extinction coefficient (ε_{412}=14140 $M^{-1} \cdot cm^{-1}$) was used to calculate enzyme activity (Kohlhaw, 1988). The enzyme

product was derivatized, analyzed with GC/MS, and the identity confirmed with an authenticated standard as described previously.

Enzyme kinetics. Enzyme assays for kinetic analyses were DTNB end-point assays run at 22 °C using 5 to 15 µL aliquots of protein eluent. Absorbance of the reaction increased linearly for at least 30 min. For individual assays, absorbance was recorded every 5 min for 20 min. Correction for background absorbance was as previously described. K_m and V_{max} were determined by regression analysis of the Lineweaver-Burke plot. Each assay was repeated 2 to 3 times. The concentration of acetyl-CoA was fixed at 250 µM while the concentration of α-keto acids (pyruvate, α -ketobutyrate, α -ketoisovalerate) ranged from 0.33 to 16 mM. For MdCIM1 and 2 and MdIPMS1, the concentration of the α-keto acids was fixed at 10 mM while the acetyl-CoA concentrations ranged from 16.6 to 200 µM. For MdIPMS2, the concentration of α-ketobutyrate was fixed at 10 mM and α-ketoisovalerate at 5 mM while the acetyl-CoA concentration ranged from 16.6 to 200 µM. The concentrations of the substrates were selected so that the reciprocals of the substrate concentrations were evenly distributed on the Lineweaver-Burke plot to prevent uneven weighting during curve-fitting.

Determination of pH and amino acid feedback regulation. The optimum pH range for MdCIM1 and MdIPMS1 and MdIPMS2 was determined using several buffers spanning pH 5.5 to 10.5. Buffers included potassium or sodium phosphate with citrate (pH 5.5 to 7.0), potassium or sodium phosphate (pH 6.0 to 8.0), Tris (pH 7.0 to 9.0), 2-amino-2-methyl-propane (pH 9.0 to 10.5), replacing Tris pH 8.0. The rate of reaction was determined by end-point DTNB assay as previously described.

To determine if the enzyme is inhibited by BCAAs, the activity was tested by addition of valine, leucine, isoleucine, and threonine at concentrations of 0, 0.05, 0.1, 0.15, 0.3, 0.5, 1, 2.5, and 10 mM in the previously described enzyme assay mixture. Pyruvate was used as the substrate for MdCIM and α-ketoisovalerate was used for MdIPMS1 and 2. The rate of reaction was determined by end-point enzyme assay as previously described. Data are expressed as a percentage of the activity of control reaction containing no inhibitor. Each reaction was repeated twice.

Subcellular localization of MdCIM and MdIPMS. The ORFs of *MdCIM1*, *MdCIM2*, *MdIPMS1* and *MdIPM2* were generated from the *MdCIM*-pBAD-TOPO and *MdIPMS*-PCR 2.1 constructs using forward and reverse primer nos. 28 to 36. The gel purified PCR products were cloned directly into pDONR207 by BP clonase recombination reactions as described by the manufacturer (Invitrogen). The product of the BP recombination reactions was used to transform DH5α *E. coli* cells and the insert was sequenced at the GTSF. *MdCIM*1 and 2 and *MdIPMS*1 and 2 in the pDONR207 construct were transferred to the destination vector pEarleyGate 101 (35S-Gateway-YFP-HA tag-OCS 3', ABRC stock no. CD3-683) (Earley et al., 2006) using LR clonase recombination reactions as described by the manufacturer (Invitrogen). The product of LR recombination reactions were used to transform DH5α *E. coli* cells, generating *MdCIM*- or *MdIPMS*-pEarleyGate 101, and the insert was sequenced at the GTSF. *MdCIM*- or *MdIPMS*-pEarleyGate 101 constructs were used to transform *Agrobacterium tumefaciens* strain EHA105.

Approximately 4- to 5-week-old tobacco (*Nicotiana tabacum* cv. Samson) plants grown at 22 °C in a growth chamber were used for transient expression assays. *MdCIM*- or *MdIPMS*-

pEarleyGate 101 in Agrobacterium was infiltrated into leaves and the infiltrated areas were analyzed after three days by confocal microscopy as described by Reumann et al. (2009).

Protein sequence alignment and phylogenetic tree. Predicted protein sequences were aligned using a MultAlin program (Corpet, 1988) and the formatting was with ESPript (Gouet et al., 1999). The phylogenetic tree for *IPMS* and *CIM* genes was created using the Neighbor-Joining method (Saitou and Nei, 1987). All IPMS and CIM sequences were obtained from NCBI database. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 32 amino acid sequences from yeast, bacteria, and plants. Signal sequences, regulatory C-terminal region, alignment gaps, and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

Results

^{13}C-acetate feeding study. We fed stable isotope acetate (1-^{13}C, 2-^{13}C, and 1, 2- ^{13}C acetate) to ripening apple fruit discs to determine whether label could be incorporated into citramalate and its potential metabolites. The predicted carbon labeling position for metabolites in the citramalate and related metabolic pathways is shown in Figure 9. Carbons derived from C-1 and C-2 position of acetate are indicated as open and solid symbols adjacent to the carbon atoms, respectively.

The labeled carbon from ^{13}C-labeled acetate was incorporated into citramalate, citraconate, and isoleucine, increasing the molecular mass by one when fed with 1-^{13}C or 2-^{13}C acetate and increasing mass by two when fed with 1, 2-^{13}C acetate (Figure 10). ^{13}C-label was also incorporated into propanal, propanol, and methyl propanoate only when 2-^{13}C acetate was

provided. Similarly, the 2-methylbutanoate portion of methyl 2-methylbutanoate and 2-methylbutanol were only labeled with 2-^{13}C acetate. Interestingly, 1, 2-^{13}C acetate incorporation into 2-ethylmalate brought about an increase up to four mass units, which was interpreted as being indicative of multiple cycle of acetyl-CoA addition and subsequent one-carbon chain elongation. Label was also incorporated into 2-propylmalate and 2-isopropylmalate (Figure 10). These compounds share the same molecular mass and were not separated clearly by GC/MS, however, enrichment of the M+3, M+4, and M+5 isotopomers is not predicted to be labeled in 2-isopropylmalate and likely primarily 2-propylmalate. Label (M+2 and M+3) from 2-^{13}C acetate and label (M+3, M+4, and M+5) from 1, 2-^{13}C acetate provides further evidence that chain elongation is occurring. The incorporation of label acetate into methyl butanoate was consistent with its synthesis from 2-ethylmalate (Figure 11). To determine if citramalate pathway contributes to the butanoate portion of methyl butanoate, we evaluated label incorporation into *m/z* 74, generated by the McLafferty rearrangement. If the butanoate portion of methyl butanoate is synthesized via 1-C FAB pathway, labeling will be incorporated only from the C-2 position of acetic acid, whereas via 2-C FAB, mixed enrichment of C-1 and C-2 position labeling pattern would be expected. The mole fraction of M+2 methyl butanoate was enriched by 2- and 1, 2-^{13}C acetate, but the mole fraction of M+1 was enriched by all three isotopes of acetate. The mass increase in methyl butanoate by two mass units when fed with both 2- and 1, 2-^{13}C acetate may indicate that butanoate is synthesized by both pathways. Similarly, M+1 and M+2 butanal was enriched by 1- and 1, 2-^{13}C acetate and by all three ^{13}C acetate (Figure 11). There was also evidence of label in the M+4 isotopomer of butanal. The small fraction of labeling in the M+4 isotopomer of butanal from 1, 2-^{13}C acetate may indicate this ester precursor is synthesized in part by a route other than 1-C FAB.

Citramalic acid analysis and citramalate synthase gene expression. Citramalic acid content increased during ripening in concert with increased BC esters, propyl and propanoate esters, and isoleucine (Figure 12). Citramalic acid content was very low in unripe fruit and began to increase on day 25 (0.004 $mg \cdot g^{-1}$) and increased further as ripening progressed. Citramalic acid levels remained high even during senescence (0.12 $mg \cdot g^{-1}$ on day 60). The citramalic acid concentration increased about 120-fold during ripening compared to the unripe stage.

Based on microarray data, the expression of *MdCIM* began to increase on day 11, increasing to 32-fold higher than day 0 by day 39 and remaining high during senescence (Figure 12). The expression pattern was similar to the patterns of isoleucine and citramalic acid accumulation. Semi-quantitative PCR analysis yielded similar results to the microarray data for *MdCIM* (Figure 13). The expression pattern for *MdIPMS*1 and *MdIPMS*2 differed from *MdCIM*. *MdIPMS*1 expression rose somewhat during the early stages of ripening, but overall, was relatively constant from day 0 to 70. The expression of *MdIPMS*2 did not change during ripening. To determine whether expression of the *MdCIM* gene is fruit and/or ripening specific, semi-quantitative PCR analysis was performed using tissues from different organs and unripe (day 0) and ripe (day 49) fruit. *MdCIM* was fruit specific and only expressed during the ripening stage (Figure 14).

MdCIM and MdIPMS sequence analysis. Genomic sequence of *MdCIM* revealed a total of eight introns within the ORF (data not shown). Genomic sequence analysis of *MdCIM* indicated that there were, in fact, two distinct *MdCIM* genes which differed in the length of the 4th intron. The longer 4th intron sequence was 224 bp and the shorter sequence was 101 bp. The gene which had

a longer intron sequence was named *MdCIM*1 and that with a shorter intron sequence was named *MdCIM*2. The *MdCIM*1 and *MdCIM*2 ORF nucleotide sequences differed only in two locations. The first nucleotide was located in the N-terminal region position and the second one in the C-terminal region. The nucleotide difference in these two regions yielded differences in the predicted amino acid sequences in both locations such that lysine^{36} and gluamine^{387} of MdCIM1 corresponded to proline^{36} and glutamate^{387}, respectively, in MdCIM2 (Figure 15). The inferred coding sequences of *MdCIM*1 and 2 were 1422 bp, much shorter than previously characterized Arabidopsis *MAM* genes (about 1700 bp).

The two *IPMS* genes, *MdIPMS*1 and *MdIPMS*2, were also cloned and had coding sequences of 1905 bp and 1890 bp, respectively (data not shown). The length of these coding sequences were similar to those of Arabidopsis *IPMS* genes (about 2000 bp). The two *MdIPMS* genes had 93% similarity to one another at the nucleotide level. There was essentially no similarity (0%) between the nucleotide sequences of *MdIPMS*1 or 2 and the two *MdCIM* genes, but there was some similarity (approximately 65%) at the amino acid level. When tblastx was performed, the MdCIM sequences had its highest similarity with *Solanum pennellii* LpIPMSa (AF004165), scoring E-value of $2 \cdot 10^{-175}$. Overall, MdCIM and MdIPMS both had a higher similarity with *Brassica insularis* IPMS1 (DQ143886) and Arabidopsis IPMS1 and IPMS2, with similarity scores ranging from 0.0 to $7 \cdot 10^{-171}$. When comparing MdCIM against Arabidopsis MAMs, the score was $3 \cdot 10^{-162}$ with MAM1 and $2 \cdot 10^{-158}$ with MAM3.

Alignment of MdCIM and MdIPMS proteins with known plant IPMS and MAM and *Leptospira* CIM proteins revealed two shared domains, containing motifs of GxGERxG and HxH[DN]D (Figure 15). Based on ChloroP analysis (Emanuelsson et al., 1999), MdCIM1 and 2 were predicted to possess a 56 amino acid chloroplast targeting region and MdIPMS1 and 2 were

predicted to contain a 66 amino acid chloroplast targeting region. Unlike MdIPMS1 and 2 and AtIPMSs, MdCIM did not have an R-region in the C-terminus region, a region that is reported to confer leucine feedback inhibition (Cavalieri et al., 1999). The phylogenic tree analysis based on CIM and IPMS protein sequences from bacteria, yeast, and plants clusters MdCIM with the MAM subgroup (Figure 16). The two MdIPMS proteins cluster within the Rosaceae family and belong to IPMS group.

MdCIM and MdIPMS protein characterization. As noted, MdCIM1 and 2 proteins were not produced in the bacterial expression system unless the putative chloroplast targeting regions were left intact. The MdIPMS proteins were expressed with or without the chloroplast targeting regions, but had greater activity with the chloroplast target sequences removed. Therefore, the full length MdCIM1 and 2 proteins and MdIPMS1 and 2 proteins without a signal sequence were used for SDS-PAGE gel and enzyme analysis. The predicted protein size excluding 6x His-tag (0.8 kD) was around 52 kD for full length MdCIM1 and 62 kD for truncated MdIPMSs, which was confirmed with SDS-PAGE gel analysis (Figure 17). The lower bands on the gel were the expected protein size. The upper bands on the gel were non-target proteins.

MdCIM2 had very low overall activity (approximately eight times lower activity than that of MdCIM1) and was highly specific for pyruvate, having essentially no activity with other α-keto acids evaluated. It was therefore excluded from comparative analysis. MdCIM1, MdIPMS1 and MdIPMS2 had a relatively high level of activity with α-ketobutyrate, the activity of which was used as a reference for comparing substrate preferences (Table 6). The substrate preference of MdCIM1 differed from MdIPMS1 and 2. The two MdIPMS proteins were most active with α-ketoisovalerate. On the other hand, MdCIM1 did not have much activity with α-

ketoisovalerate, but had a much greater preference for pyruvate than the two MdIPMS proteins. Interestingly, MdCIM1 had a small activity with oxaloacetate. The products of the enzyme assays for MdCIM1 with pyruvate and MdIPMS1 with α-ketoisovalerate were verified to be citramalic acid and 2-isopropylmalic acid, respectively, by GC/MS analysis and comparison with authenticated standards (Figure 18).

The K_m values of MdCIM and the two MdIPMS proteins for acetyl-CoA were relatively similar, ranging from 6.6 to 13.5 μM (Table 7). However, MdCIM1 had a somewhat lower K_m for acetyl-CoA when pyruvate, rather than α-ketobutyrate, was used as a co-substrate. MdCIM1 had a lower K_m for α-ketobutyrate than either MdIPMS1 or 2. The two MdIPMS proteins had low K_m values for their namesake reaction using α-ketoisovalerate compared to that of MdCIM1 for pyruvate. The V_{max} of the reactions using α-ketobutyrate was similar for all three proteins, but those of the namesake reactions differed as much as four-fold, with the V_{max} of MdCIM1 with pyruvate being lowest. The enzymatic efficiency of the three proteins was similar for α-ketobutyrate, however, MdCIM had a relatively low enzyme efficiency with pyruvate compared to the efficiencies of the reaction of MdIPMS 1 and 2 with α-ketoisovalerate.

The optimum pH range for MdCIM1 was around 9.0 to 9.5 and for the two MdIPMS proteins, it was between 8.0 and 9.0 (Figure. 19). Both MdCIM and MdIPMS enzymes had very low activity at pH 6.0, but activity gradually increased until pH 8.0 to 9.0, then decreased sharply after pH 10.0.

Transient expression of MdCIM1, MdIPMS1 and 2 proteins fused with YFP in tobacco indicated that these proteins are targeted to chloroplast (Figure 20). MdIPMS proteins without predicted chloroplast target region had no fluorescence in the chloroplast (data not shown).

MdCIM was not subject to inhibition by any of the BCAA, but was stimulated slightly (120%) at low concentrations of threonine and inhibited (70%) at higher concentrations of threonine (Figure 21). MdIPMS1 and MdIPMS2 activity was reduced by 70% and 40%, respectively, by the presence of as little as 0.05 mM leucine and further decreased with increasing leucine concentration. MdIPMS2 was somewhat more strongly inhibited by leucine than MdIPMS1. The activity of the two MdIPMS proteins was inhibited by high concentrations of isoleucine and valine, decreasing 60% to 75%, whereas MdCIM1 activity was not influenced by these two amino acids. Interestingly, both MdIPMS proteins were negatively impacted by threonine, in that activity decreased by as the threonine concentration increased. The maximal level of inhibition (approximately 80%) found at 10 mM threonine was similar the maximal level of inhibition by leucine. Unlike MdCIM1, the activity of MdIPMS1 and 2 were not stimulated by low threonine concentrations.

Discussion

^{13}C-acetate feeding study and citramalic acid. A 'citramalate pathway' has been demonstrated by isotopic feeding studies in several strains of bacteria by determination of the labeling position during metabolic flux analysis (Ekiel et a., 1984; Feng et al., 2009; Gray and Kornberg, 1960; Hochuli et a., 1999; Nesbakken et al., 1988; Risso et al., 2008). The patterns for label incorporation observed in the current study for citramalic acid, citraconic acid, isoleucine, 2-methylbutanol, and 2-methylbutanoate from ^{13}C-acetic acid feeding are similar to those from bacterial studies characterizing citramalate synthase and its related metabolic pathway. Labeling patterns were uniformly consistent with those predicted for exogenously supplied acetate for the pathway depicted in Figure 9. The data suggest that some portion of BC ester biosynthesis in

apple is via a citramalate pathway that functions in addition to the established threonine-dependent pathway of isoleucine synthesis. The data do not argue for incorporation of label from ^{13}C-acetic acid via threonine, which would yield a gain in the mass of isoleucine more than two mass units when fed ^{13}C-acetic acid if the acid was extensively incorporated into threonine via the TCA cycle (Jolchine, 1962). Moreover, the isotope enrichment pattern showed no evidence of dilution for citramalic acid, citraconic acid, isoleucine, and methyl 2-methylbutanoate, suggesting that this pathway may be the primary route of synthesis for 2-methylbutanol and 2-methylbutanoate esters. The existence of a citramalate pathway in ripening apple would explain why threonine content decreases even as the content of citramalate (Hulme and Wooltorton, 1958), and isoleucine (Defilippi et al., 2005; Nie et al., 2005; Sugimoto et al., 2011) increases markedly. Further support for a citramalate-dependent pathway for isoleucine synthesis can be argued from the fact that isoleucine inhibits TD (Eisenstein, 1991; Wessel et al., 2000), and thereby acts as a means of negative feedback regulation of its own synthesis. For yeast, suppressing TD has the effect of causing an accumulation of threonine in yeast mutants (Martinez-Force and Benitez, 1994), however, as noted, threonine did not accumulate in ripening apple fruit. The labeling data taken in conjunction with these findings support the suggestion that synthesis of isoleucine and its derivative esters may be accomplished at least in part via the citramalate pathway that by-passes threonine in the synthesis of α-ketobutyrate. Importantly, the relative contributions of the two routes of α-ketobutyrate synthesis are as yet unknown.

Pattern of labeled acetate incorporation detected in propanal, propanol, and methyl propanoate was consistent with the notion that propanol is produced from α-ketobutyrate (Guymon et al., 1961; Vollbrecht, 1974). Atsumi and Liao (2008) enhanced propanol and butanol production in *E. coli* by transformation with *CIM* from *Methanococcus jannaschii*. The

introduction of the citramalate pathway was shown to create a route to synthesize α-ketobutyrate utilizing the products of glycolysis rather than those from the TCA cycle, which would flow through threonine. The engineered citramalate pathway in *E. coli* resulted in an increase in propanol and butanol production by 9- and 22-fold, respectively. An active citramalate pathway in apple should therefore have a similar impact on its volatile profile. Citralamate-derived propanol and butanol would contribute to the synthesis of propanol and butanol esters, respectively, which are commonly and abundantly produced during apple ripening (Ferenczi, 2003; Sugimoto, 2007).

The formation of 2-ethylmalate has not been previously demonstrated in apple. However, in yeast, 2-ethylmalate synthase activity has been described (Strassman and Ceci, 1967). Ingraham et al. (1961) reported that butanol and pentanol are formed from α-ketovalerate and α-ketocaproate, respectively, from α-ketobutyrate by a pathway analogous to the citramalate pathway. The pattern of 1,2-^{13}C acetate incorporation into 2-ethylmalate (M+4) and 2-propylmalate (M+3, M+4, and M+5) in the current study is consistent with an active chain-elongation process such as that described in these previous reports for yeast.

Currently, both BC and SC ester biosynthesis in fruit is uniformly represented as being via degradative pathways (Bartley et al., 1985; Gonda et al., 2010; Pérez et al., 2002; Rowan et al., 1996; 1998; 1999; Tressl and Drawert, 1973; Wyllie and Fellman, 2000). This conclusion stems from an extensive history of feeding studies. However, labeling studies presented here have demonstrated that both BC and SC esters are synthesized from acetate and are consistent with carbon flux through citramalate and chain elongation of α-keto acids catalysed by CIM and IPMS. The isotope incorporation in C3 and C4 esters as well as BC esters suggests that the proposed citramalate pathway acts as a synthetic route for ester formation in ripening apple fruit.

Citramalic acid has been detected in apple, pear, banana, citrus, and tomato (Degu et al., 2011; Fraser et al., 2007; Rudell et al., 2008; Ulrich, 1970). Hulme (1954) isolated citramalic acid from the peel of the mature 'Edward VII' apple fruit and observed its absence in pulp tissue and postulated that citramalic acid participated in the TCA cycle, although no data have supported this suggestion apart from the current demonstrated activity with oxaloacetate. Hulme and Wooltorton (1958) studied the changes of citramalic acid content in 'Bramley's Seedling' apple during 100 days of storage at 15 °C and reported that there was no citramalic acid detected at the time of harvest, but it increased to 0.1 $mg \cdot g^{-1}$ after 25 days, and maximized at 0.25 $mg \cdot g^{-1}$ after 100 days. The pattern of citramalic acid accumulation with ripening in 'Jonagold' was similar to that reported by Hulme and Wooltorton (1958), but their maximum content in 'Bramley's Seedling' at day 100 was about twice the content in 'Jonagold'. The difference may be due to differences in cultivar, storage temperature, or in extraction method. The 'Jonagold' skin samples in the current study contained a small portion of pulp, which may have decreased the total amount of the citramalic acid.

In the present study, the highly correlated increases in *MdCIM* expression, citramalic acid production, isoleucine and BC ester production argue for ripening-specific activation of the citramalate pathway in apple. The fruit-specific expression of *MdCIM* gene further supports the role of MdCIM in ester biosynthesis.

MdCIM and MdIPMS characterization. Apple genome consists of 17 chromosomes and are considered to be evolved from nine ancestral chromosomes due to genome wide duplication (Velasco et al., 2010). It is possible that one of *MdIPMS* and *MdCIM* is evolved from *MdIPMS* during gene duplication, the former retained its original function and the latter changed its

function as suggested by Ohno (1970). This is similar to in *AtMAM*s which is suggested to be evolved from *AtIPMS* based on phylogenetic analyses and enzyme activities (Benderoth et al., 2009; de Kraker et al., 2007; de Kraker and Gershenzon, 2011). While *MdCIM* is located on chromosome 6, MdIPMS1 and 2 are located on chromosomes 2 or 17. Chromosomes 2, 6, and 17 originate independently from the nine ancestral chromosomes 7 and 9, 6, and 3, respectively (Velasco et al., 2010), suggesting that *MdCIM* evolution occurred similarly during the genome wide duplication.

The K_m of MdCIM1 for pyruvate was much higher than for previously reported bacterial CIM enzymes, but that for acetyl-CoA was markedly lower. In *Methanococcus jannaschii* the K_m was 850 μM and 140 μM for pyruvate and acetyl-CoA, respectively (Howell et al., 1999), and in *Leptospira interrogans* the K_m was 60 μM and 1118 μM for pyruvate and acetyl-CoA, respectively (Ma et al., 2008). The enzyme or catalytic efficiency of MdCIM1 was slightly higher for α-ketobutyrate than pyruvate. 'Cox Orange Pippin' contains about 1.40 $\mu g \cdot g^{-1}$ pyruvate (Hulme et al., 1964) and 'Jonathan' contains about 0.26 $\mu g \cdot g^{-1}$ (Willis and McGlasson, 1968). LaRossa et al. (1987) determined that α-ketobutyrate is toxic to *Salmonella typhimurium* and Höfgen et al. (1995) detected high levels of pyruvate but trace amounts of α-ketobutyrate in potato leaf. In apple fruit, it is possible that pyruvate is likely more abundant than α-ketobutyrate. Unfortunately, there is no report for α-ketobutyrate concentration in fruit, but one might suggest that MdCIM1 has a significant activity with pyruvate based on the information of the abundance of pyruvate in apple tissue. We were unable to detect α-ketobutyrate in apple tissue extracts (see below).

MdCIM1 had an activity with several substrates, including α-ketovalerate. This result is different from the bacterial CIMs, which were essentially pyruvate-specific and did not have

much activity with other α-keto acids (α-ketoisovalerate, α-ketobutyrate, glyoxylate) tested (Howell et al., 1999; Ma et al., 2008; Xu et al., 2004). The relatively high specificity for pyruvate in *Leptospira interrogans* may stem from the fact that isoleucine is synthesized exclusively via the citramalate pathway (Zou et al., 2007). The two pathways to synthesize isoleucine via α-ketobutyrate in apple may serve differing functions. While the threonine pathway may serve to synthesize normal metabolic needs, MdCIM may have evolved to perform a specialized metabolic function in fruit. In some ways, this specialization is similar to that for MAM which, is involved the relatively unique pathways of glucosinolate biosynthesis acting as anti-herbivore defenses (Textor et al., 2004; 2007).

The two MdIPMS enzymes were considered to be highly specific for α-ketoisovalerate based on their catalytic efficiency for this substrate. High specificity for α-ketoisovalerate is consistent with yeast (Kohlhaw, 1988; Ulm et al., 1972), bacteria *Mycobacterium tuberculosis* (de Carvalho and Blanchard, 2006), *Neurospora* (Webster and Gross, 1965), *Salmonella typhimurium* (Kohlhaw et al., 1969), and Arabidopsis (de Kraker et al., 2007). The two MdIPMS enzymes also had substantial activity with other substrates such as α-ketobutyrate and α-ketovalerate, similar to IPMS from the other species noted. In apple, the lack of MdIPMS1 and 2 activity with pyruvate differed from that in yeast, bacteria, and Arabidopsis where IPMS had low level of activity (de kraker et al., 2007; Kohlhaw, 1988; Kohlhaw et al., 1969; Webster and Gross, 1965).

Yeast produce propanol, butanol, and amyl alcohols and the citramalate pathway has been proposed to contribute to their production (Guymon et al., 1961; Ingraham et al., 1961; Vollbrecht, 1974). The fact that MdCIM1 and MdIPMS1 and 2 have a similar, high level of activity with α-ketobutyrate and α-ketovalerate suggests they support metabolism similar to that

in yeast (Strassman and Ceci, 1967). The production of 2-ethylmalate and 2-propylmalate by MdCIM1 and the two MdIPMS proteins suggests that both enzymes may contribute to chain-elongation. It may be that MdCIM provides the entry point for carbon into this cyclical pathway and that both MdCIM and MdIPMS carry out the additional chain-elongation steps in conjunction with IPMI and IPMDH.

In general, BCAA metabolism is tightly regulated by feedback inhibition by the three BCAAs (Eisenstein, 1991; Singh and Shaner, 1995; Wessel et al., 2000). The fact that the two MdIPMS enzymes were both strongly inhibited by low levels of leucine supports their role in the formation of this BCAA. The concnetration of leucine needed to achieve maximal inhibition of MdIPMS (0.10 to 0.3 mM) was similar to that for *Neurospora* (Webster and Gross, 1965) and yeast (Ulm et al., 1972), but lower than that for Arabidopsis, which was maximally inhibited at 1 mM leucine (de Kraker et al., 2007). The lack of an effect of valine and isoleucine on MdIPMS activity until the concentration reached 1.0 mM was also consistent with IPMS enzymes from *Neurospora* and yeast (Ulm et al., 1972; Webster and Gross, 1965). The inhibition of MdIPMS1 and 2 by threonine has not been previously reported to our knowledge in plants or microorganisms (Wiegel and Schlegel, 1977). The slight stimulation of MdCIM1 activity by low levels of threonine and inhibition at higher concentrations has also not been reported for CIM. Threonine is present at low levels in apple fruit and does not change dramatically during ripening (Sugimoto et al., 2011), so a rationale an effect of threonine on the synthetic pathways of all three BCAAs is not obvious. The possible utility to the fruit for threonine suppression of MdCIM activity is to favor either citramalate or threonine pathway in a mechanism similar to the feedback regulation of TD by isoleucine.

The difference in amino acid sequence of the two MdCIM proteins and two MdIPMS proteins at the C-terminal end indicated approximately 150 amino acids were missing from MdCIMs. The C-terminal portion of the protein has been designated the R-region (Cavalieri et al., 1999) because this region is essential for feedback regulation of LiCMS by isoleucine (Zhang et al., 2009) and AtIPMS by leucine (de Kraker et al., 2007). The removal of the R-region from AtIPMS1 and 2 eliminates feedback inhibition by leucine (de Kraker and Gershenzon, 2011). The absence of the R-region in MdCIM is similar to AtMAM proteins, which lack feedback regulation (de Kraker and Gershenzon, 2011). The lack of an R-region in MdCIM1 explains the lack of inhibition by BCAAs.

The substantially lower activity of MdCIM2 and its high specificity for pyruvate compared to MdCIM1 is striking in that the only amino acid sequence difference was the change in the C-terminal glutamine^{387} to glutamate^{387} in MdCIM2. This amino acid has not been demonstrated to be important in activity although this residue is conserved across Arabidopsis and tomato. However, histidine^{386}, the amino acid next to glutamine^{387}, is important in binding of acetyl-CoA and this maybe related to the reduction of activity in MdCIM2 (Ma et al., 2008). The targeting of MdCIM and MdIPMS proteins to the chloroplast was consistent with the previously reported IPMS in spinach (*Spinacia oleracea*) (Hagelstein and Schultz, 1993) and AtMAM3 (Textor et al., 2007). Plastid undergoes conversion to chromoplast during fruit ripening (Bathgate et al., 1985; Thomson, 1966), so MdCIM may be targeted to chromoplast in some apple cultivars where chlorophyll is lost during ripening.

Conclusion

^{13}C-acetate feeding of ripening apple discs has demonstrated that several metabolites directly or indirectly related to ester synthesis could originate from citramalate synthesis. In addition, fruit and ripening-specific expression of a gene that, based on substrate specificity of the expressed protein, codes for MdCIM1 has been demonstrated. Further, the capacity of MdCIM1 and MdIPMS1 and 2 to contribute to one-carbon elongation of α-keto acids provides for a novel means of fatty acid synthesis in plants. *MdCIM1* expression increases synchronously with the production of esters and, more to the point, with increases in the concentration of citramalic acid and isoleucine. These data strongly implicate the existence of a citramalate pathway in the skin of ripening apple fruit as proposed by Sugimoto et al. (2011). The establishment of an active citramalate pathway together with the presence of an additional one-carbon chain elongation step demonstrated a novel route for BC and SC ester formation in ripening fruit that is synthetic, rather than catabolic in nature. This finding represents a conceptual departure from previous conclusions regarding ester biosynthesis, which have almost exclusively considered ester biosynthesis, and in particular, BC ester biosynthesis to be a degradative process.

Table 4. GenBank gi number, forward and reverse primer sequence (5'→3'), expected PCR gene fragment size (bp), optimum cycle number, and optimum annealing temperature 'Temp' for isopropylmalate synthase (*MdIPMS*1 and 2), citramalate synthase (*MdCIM*1) genes, and the 18S ribosomal RNA (18s rRNA) gene from apple for semi-quantitative RT-PCR.

Target	GenBank gi no.	Forward primer sequence	Reverse primer sequence	Size (bp)	Cycle	Temp (° C)
IPMS1	48408575	agggtttaggaggcgactgt	cggagagtggtgtcgaagat	322	29	59
IPMS2	71823902	cgcatctccgaccctaacta	acctgctccttgctcttcct	386	33	59
CIM1	n.a.	caccgtgaaggcactatgaa	ggctcggagacaatccttct	456	27	57
18s rRNA	85717895	gagaaacggctaccacatcc	gagcgtaggcttgctttgag	390	18	59

Table 5. Primer list used to clone genomic and coding sequences and to express proteins of isopropylmalate synthase (*MdIPMS*1 and 2) and citramalate synthase (*MdCIM*1 and 2) genes from 'Jonagold' apple.

Primer no.	Experiment	Primer name	Primer sequence (5'->3')
1	Genomic	CIM forward	caccatggccttctcagcagaaaat
2	DNA	CIM reverse	caccgtgaaggcactatgaa
3	3 'RACE	CIM GSP	agctgaacaagactgccgatca
4	*MdCIM*-	CIM full forward	atggccttctcagcagaaaatc
5	pBAD-TOPO	CIM short forward	tgctccgctctctcccaatc
6		CIM full reverse (-stop)	aatggccacctgataaaccaatg
7		CIM full reverse (+stop)	ttaaatggccacctgataaacca
8	*MdCIM*-	CIM full forward	caccatggccttctcagcagaaaat
9	PET101/D-	CIM short forward	caccatgtgctccgctctct
10	TOPO	CIM short forward2	caccatggcctgctccgctctctcccaat
11		CIM full reverse	aatggccacctgataaaccaatg
12	ORF/mRNA	IPMS1 forward	aaccatggcggctgtctgcacaa
13		IPMS1 reverse	tcatgcagacaccggcgttagtt
14		IPMS2 forward	cttctgctcaaaccaggaacca
15		IPMS2 reverse	tcatgcagacactttgtttcgttca

Table 5 (cont'd).

Primer no.	Experiment	Primer name	Primer sequence (5'->3')
16	5' RACE	Qt	ccagtgagcagagtgacgaggactcgagctcaagcttttttttttttttttt
17		Qo	ccagtgagcagagtgacg
18		Qi	gaggactcgagctcaagc
19		IPMS GSP	ctgatggataccactttcatgtgc
20		IPMS GSP1	ccaacaatagccttgtgtggct
21		IPMS GSP2	tgcaacccagtgtactcttcca
22	*MdIPMS*-	IPMS1 full forward	caccatggcggctgtctgcacaaa
23	PET 101/D-	IPMS1 full reverse	tgcagacaccggcgttagttcc
24	TOPO	IPMS1 short forward	caccatggcgtgctctcaaactgacaaccccaaa
25		IPMS2 full forward	caccatggcaactgtctgcacgca
26		IPMS2 full reverse	tgcagacactttgtttcgttca
27		IPMS2 short forward	caccatggcgtgctctcaaactgacaaccccaaa

Table 5 (cont'd).

Primer no.	Experiment	Primer name	Primer sequence (5'->3')
28	*MdCIM*-, *MdIPMS*-pDONR207/YFP	CIM full forward	ggggacaagtttgtacaaaaaagcaggcttcaccatggccttctcagcagaaaatc
29		CIM short forward	ggggacaagtttgtacaaaaaagcaggcttcaccatggcctgctccgctctctcccaatc
30		CIM reverse	ggggaccactttgtacaagaaagctgggtcaatggccacctgataaaccaat
31		IPMS1 full forward	ggggacaagtttgtacaaaaaagcaggcttcaccatggcggctgtctgcacaaacc
32		IPMS1 short forward	ggggacaagtttgtacaaaaaagcaggcttcaccatggcgtgctctcaaactgacaaccc
33		IPMS1 reverse	ggggaccactttgtacaagaaagctgggtctgcagacaccggcgttagttcc
34		IPMS2 full forward	ggggacaagtttgtacaaaaaagcaggcttcaccatggcaactgtctgcacgcacc
35		IPMS2 short forward	ggggacaagtttgtacaaaaaagcaggcttcaccatggcgtgctctcaaactgacaaccc
36		IPMS2 reverse	ggggaccactttgtacaagaaagctgggtctgcagacactttgtttcgttca

Table 6. Substrate preference for citramalate synthase (MdCIM1) and isopropylmalate synthase (MdIPMS1 and 2) proteins under saturating substrate conditions. The enzyme assay was performed by Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB] endpoint assay using Tris buffer pH 8.0 containing 4 mM $MgCl_2$, 0.25 mM acetyl-CoA, and 10 mM substrates and incubating at 22 °C for 20 min. Then the enzyme mixture was measured for the absorbance against water at 412 nm. The absorbance was corrected by subtracting the background of the identical enzyme assay mixture without α-keto acids. For α-keto-γ-(methylthio)butyric acid, the results were further corrected for the slight reactivity between its thiol group and DTNB using reactions containing the α-keto acid, but not acetyl-CoA. Experiments were repeated three times for each substrate/enzyme combination.

	Relative Conversion (%) ± SD		
Substrate	MdCIM1	MdIPMS1	MdIPMS2
α-Ketobutyrate	100 ± 2.8	100 ± 2.5	100 ± 1.4
α-Ketoisovalerate (IPM)	6.3 ± 1.0	309.8 ± 3.0	327.1 ± 2.1
α-Keto-β-methylvalerate	0	4.9 ± 0.1	4.2 ± 0.5
α-Ketoisocaproate	0	0.3 ± 0.1	1.2 ± 0.1
α-Ketohexanoic acid	0	2.4 ± 0.2	1.2 ± 0.1
α-Ketooctanoic acid	1.2 ± 0.5	3.1 ± 0.6	1.2 ± 0.1
Oxaloacetate	18.0 ± 1.6	3.5 ± 0.1	8.4 ± 0.0
Glyoxylate	0.3 ± 0.1	2.8 ± 0.1	0
α-Ketoglutarate	0	0.3 ± 0.1	0.6 ± 0.1
α-Keto-γ-(methylthio)butyric acid	0	3.8 ± 0.1	0
Pyruvate (CIM)	50.4 ± 2.3	3.8 ± 0.2	3.0 ± 0.4
α-Ketovaleric acid	21.4 ± 1.0	149.0 ± 2.8	146.4 ± 2.3

Table 7. Kinetic parameters for citramalate synthase (MdCIM) and 2-isopropylmalate synthase (MdIPMS). The enzyme assay was performed by Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB] endpoint assay using Tris buffer pH 8.0 containing 4 mM $MgCl_2$, 0.25 mM acetyl-CoA, and 10 mM substrates at 22 °C. The absorbance was recorded from every 5 min until 20 min had elapsed. The enzyme mixture was measured for the absorbance against water at 412 nm. The absorbance was corrected by subtracting the background of the identical enzyme assay mixture without α-keto acids. K_m and V_{max} were determined by regression analysis of the Lineweaver-Burke plots for each substrate. There were two or three replications performed for each regression analysis.

Enzyme	Substrate	K_m ± SE (μM)	V_{max} ± SE ($\mu mol \cdot min^{-1} \cdot g^{-1}$)	K_{cat} ± SE (s^{-1})	K_{cat}/K_m ±SE ($M^{-1} \cdot s^{-1}$)
MdCIM1	Pyruvate	2,446 ± 187	259 ± 27	0.22 ± 0.02	92 ± 3
	Acetyl-CoA	6.6 ± 0.9	383 ± 36	0.33 ± 0.03	53,169 ± 10,928
	α-Ketobutyrate	3,559 ± 509	447 ± 214	0.39 ± 0.18	104 ± 37
	Acetyl-CoA	11.3 ± 0.2	576 ± 75	0.50 ± 0.06	43,917 ± 4,796
MdIPMS1	α-Ketoisovalerate	1,004 ± 44	1,059 ± 313	1.09 ± 0.32	1,106 ± 371
	Acetyl-CoA	9.0 ± 1.9	900 ± 214	0.93 ± 0.22	102,341 ± 7,298
	α-Ketobutyrate	5,734 ± 796	478 ± 140	0.49 ± 0.14	82 ± 12
	Acetyl-CoA	13.5 ± 1.6	288 ± 37	0.30 ± 0.03	22,083 ± 243
MdIPMS2	α-Ketoisovalerate	706 ± 39	748 ± 89	0.77 ± 0.09	1,093 ± 91
	Acetyl-CoA	8.6 ± 1.0	742 ± 90	0.77 ± 0.09	90,742 ± 9,753
	α-Ketobutyrate	6,287 ± 562	544 ± 19	0.56 ± 0.02	91 ± 11
	Acetyl-CoA	10.4 ± 1.7	362 ± 61	0.37 ± 0.06	37,143 ± 6,932

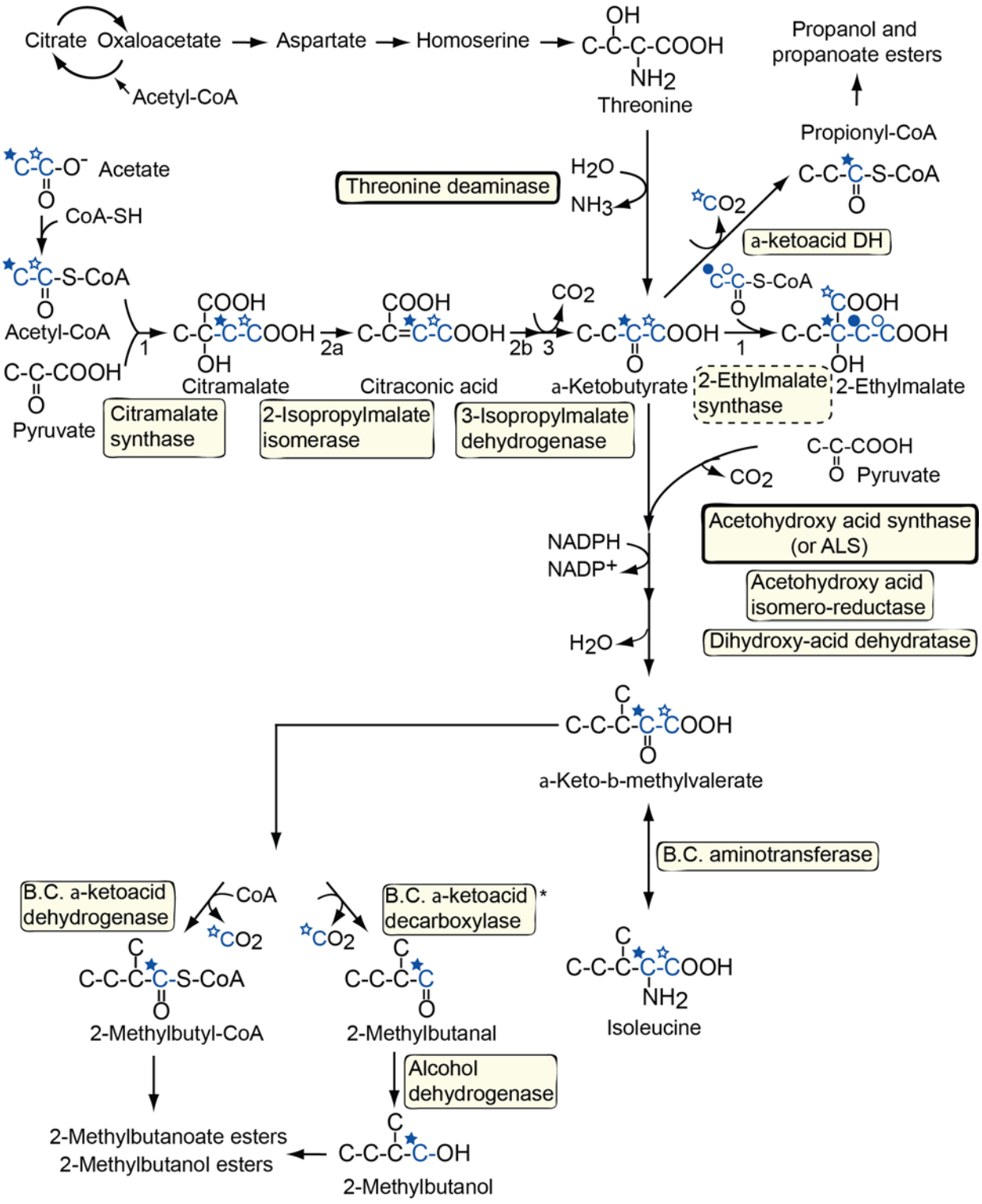
Citrate
Oxaloacetate
Aspartate
Homoserine
Threonine
Acetyl-CoA
Propanol and propanoate esters
Propionyl-CoA
Acetate
CoA-SH
Threonine deaminase
a-ketoacid DH
Acetyl-CoA
Pyruvate
Citramalate
Citraconic acid
a-Ketobutyrate
2-Ethylmalate
Citramalate synthase
2-Isopropylmalate isomerase
3-Isopropylmalate dehydrogenase
2-Ethylmalate synthase
Acetohydroxy acid synthase (or ALS)
Acetohydroxy acid isomero-reductase
Dihydroxy-acid dehydratase
a-Keto-b-methylvalerate
B.C. aminotransferase
B.C. a-ketoacid dehydrogenase
B.C. a-ketoacid decarboxylase
2-Methylbutyl-CoA
2-Methylbutanal
Isoleucine
Alcohol dehydrogenase
2-Methylbutanoate esters
2-Methylbutanol esters
2-Methylbutanol

Figure 9.

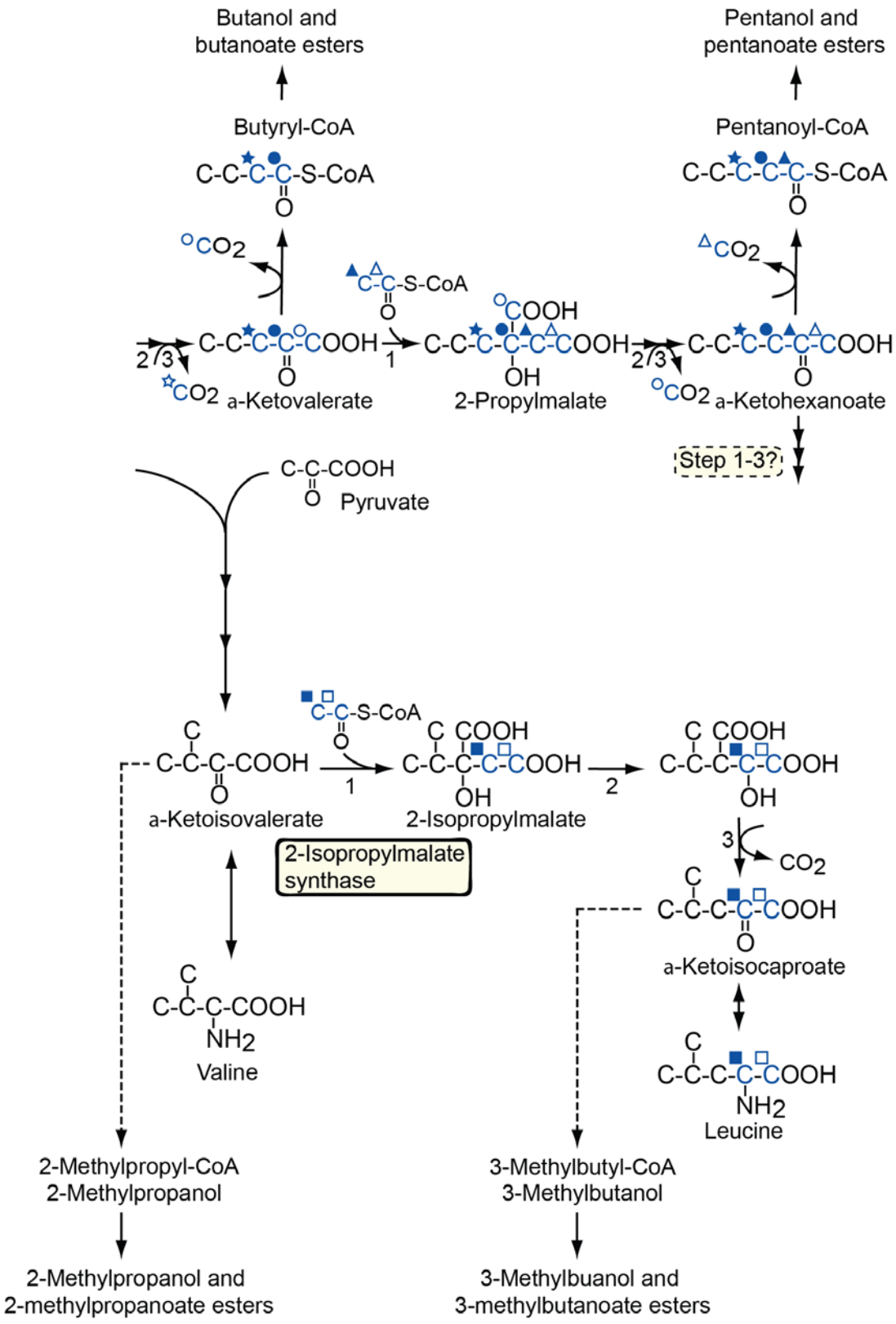

Figure 9 (cont'd).

Figure 9. Pathways involved in branched-chain ester biosynthesis in apple. Genes in bolded boxes are regulated by feedback inhibition. Of these, threonine deaminase is inhibited by isoleucine, but stimulated by valine, acetohydroxy acid synthase [a.k.a. acetolactate synthase, (ALS)] is inhibited by valine and leucine, and 2-isopropylmalate synthase is inhibited by leucine. The trivial name of 2-ethylmalate synthase, in dashed box, is tentatively given in yeast (Canovas et al., 1965). An asterisk indicates the gene is found in bacteria, but not in plants. Hydrogens in carbon-hydrogen bonds are not shown. Enzymatic steps numbered 2 and 3 in the citramalate pathway are considered to be the same enzymes used in leucine biosynthesis, namely 2-isopropylmalate isomerase and 3-isopropylmalate dehydrogenase, respectively. Carbons derived from the C-1 and C-2 positions of acetyl-CoA are indicated with open and solid symbols adjacent to the carbon atoms, respectively. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

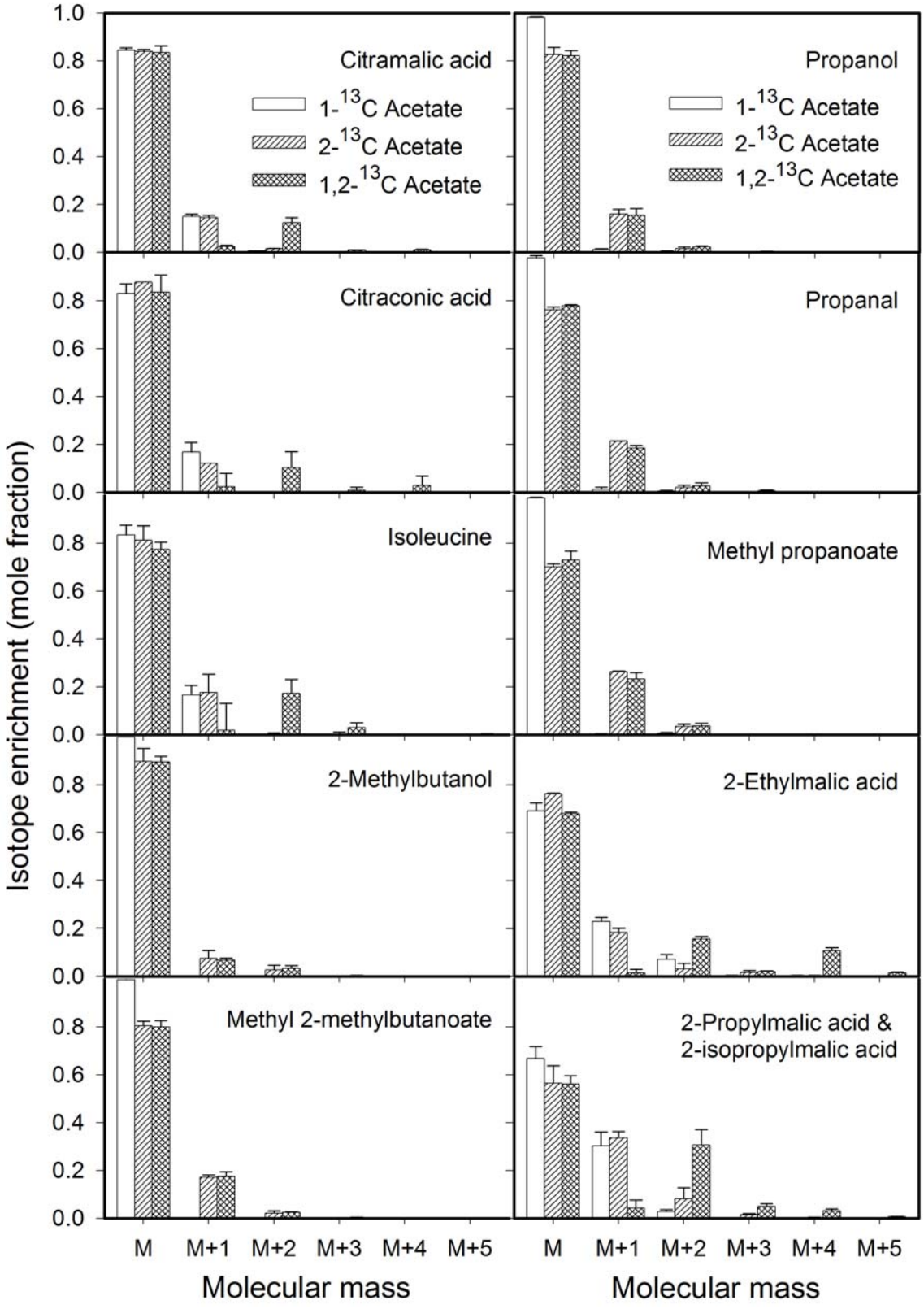
Isotope enrichment (mole fraction)
1.0
0.8
0.6
0.4
0.2
0.0
Citramalic acid
1-13C Acetate
2-13C Acetate
1,2-13C Acetate
Propanol
Citraconic acid
Propanal
Isoleucine
Methyl propanoate
2-Methylbutanol
2-Ethylmalic acid
Methyl 2-methylbutanoate
2-Propylmalic acid &
2-isopropylmalic acid
M
M+1
M+2
M+3
M+4
M+5
Molecular mass

Figure 10.

Figure 10. Mass isotopolog distribution of acids, alcohols, aldehydes, and esters from apple discs fed with 1-^{13}C acetate, 2-^{13}C acetate, and 1, 2-^{13}C acetate. The isotope distribution (in mole fraction) is expressed as unlabeled mass (M) and one mass unit heavier than the unlabeled mass (M+1) up to 5 mass units heavier (M+5) than the unlabeled standard. Except for citraconic acid analysis where 2-^{13}C acetate incorporation yielded only one sample that was quantifiable for the acid, there were two biological replicates for each data point. Vertical bars represent the standard deviation of the mean.

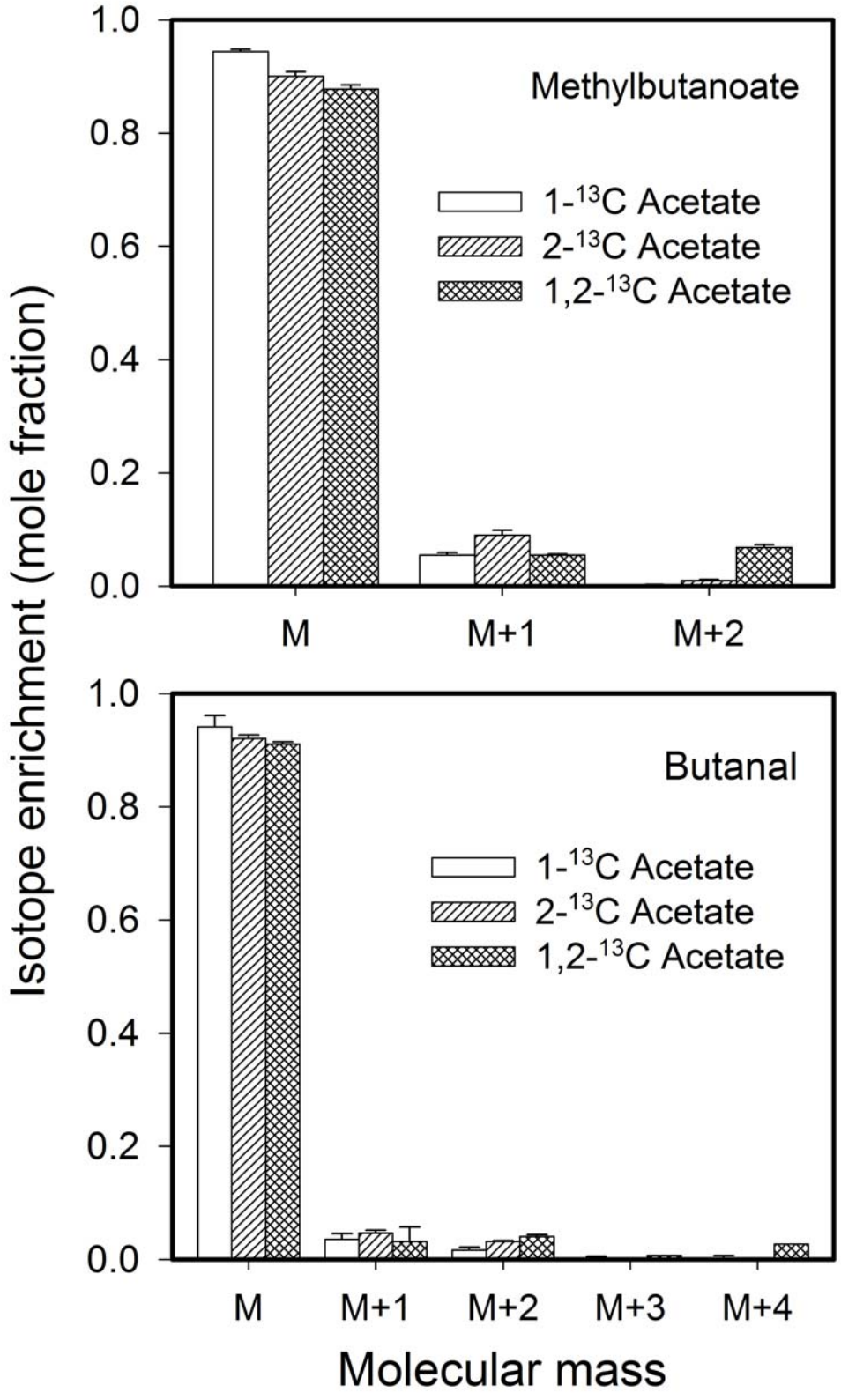
Methylbutanoate
1-13C Acetate
2-13C Acetate
1,2-13C Acetate
Butanal
1-13C Acetate
2-13C Acetate
1,2-13C Acetate
Isotope enrichment (mole fraction)
1.0
0.8
0.6
0.4
0.2
0.0
M
M+1
M+2
M+3
M+4
Molecular mass

Figure 11.

Figure 11. Mass isotopolog distribution of butanal and methylbutanoate from apple discs fed with 1-^{13}C acetate, 2-^{13}C acetate, and 1, 2-^{13}C acetate. The isotope distribution (in mole fraction) is expressed as unlabeled mass (M) and one mass unit heavier than the unlabeled mass (M+1) up to 4 mass units heavier (M+4) than the unlabeled standard. Vertical bars represent standard deviation of the mean of two biological replications.

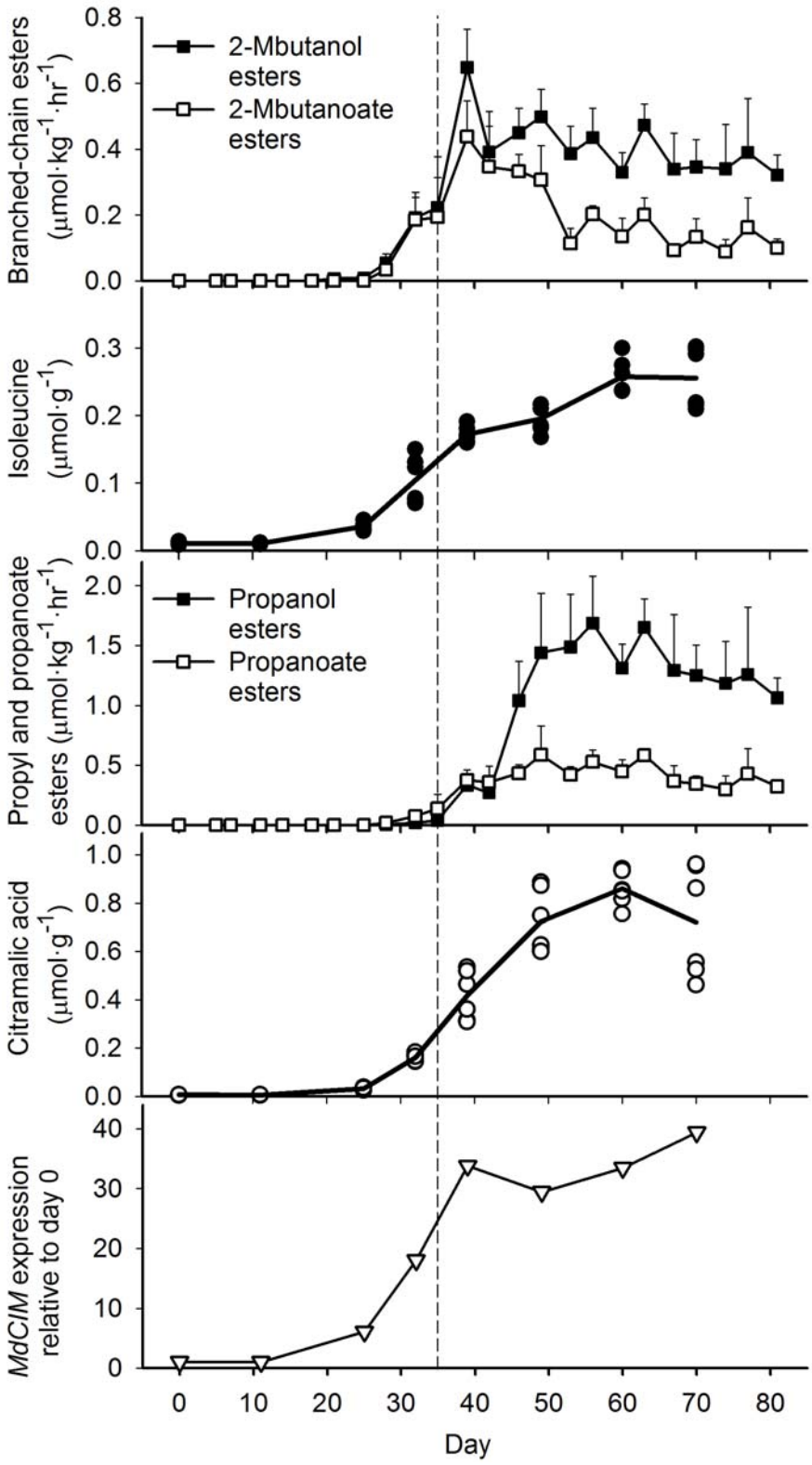
Branched-chain esters (µmol·kg-1·hr-1)
2-Mbutanol esters
2-Mbutanoate esters
Isoleucine (µmol·g-1)
Propyl and propanoate esters (µmol·kg-1·hr-1)
Propanol esters
Propanoate esters
Citramalic acid (µmol·g-1)
MdCIM expression relative to day 0
Day

Figure 12.

Figure 12. Developmentally-dependent changes in the production of branched-chain esters, propyl and propanoate esters, citramalic acid, and isoleucine and in the gene expression of citramalate synthase (*MdCIM*) based on microarray analysis for 'Jonagold' apple fruit during ripening and senescence. The fruit were examined from 2 Sept. 2004 (day 0) to 23 Nov. 2004 (day 81). Fruit were collected from the field until 7 Oct. 2004 (dashed-line, day 35), and thereafter maintained at room temperature (21±1 °C). Eight time points (day 0, 11, 25, 32, 39, 49, 60, 70) were selected for acids and gene expression analysis based on distinct physiological stages. Each symbol represents the average of four biological replications for ester analysis, six (two biological and three technical) replications for acid analysis, and two biological replications for gene expression. Vertical bars represent the standard deviation of the mean. The gene expression for citramalate synthase is based on microarray data and is depicted relative to day 0.

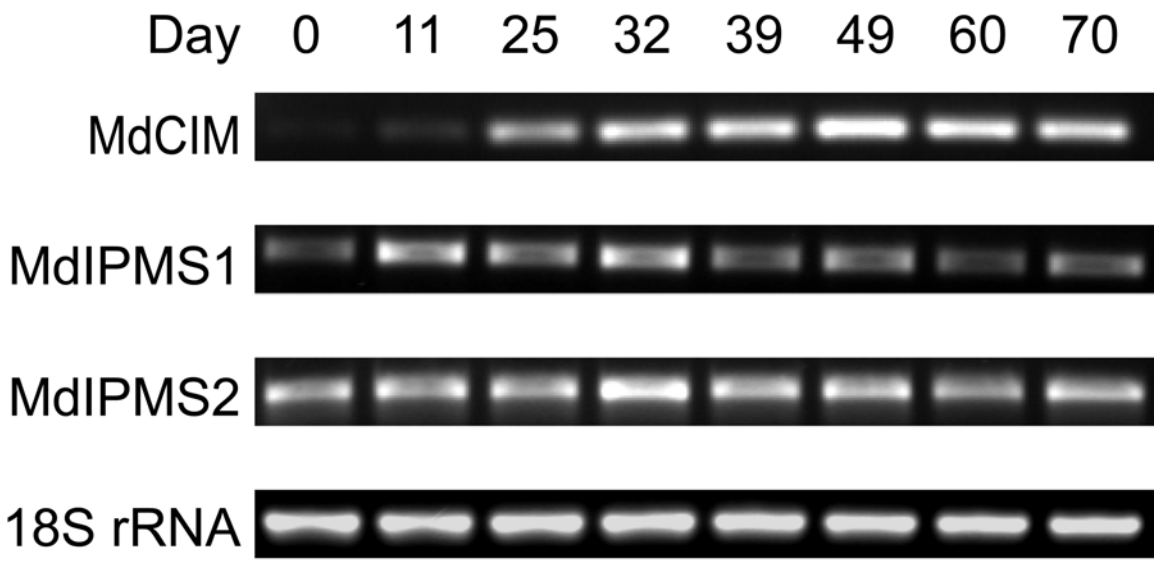

Figure 13. RT-PCR analysis of citramalate synthase (*MdCIM*) and 2-isopropylmalate synthase (*MdIPMS*) expression for 'Jonagold' apple fruit during ripening. Eight time points (day 0, 11, 25, 32, 39, 49, 60, 70) were selected based on distinct physiological stages during which fruit were collected from 2 Sept. 2004 (day 0) to 12 Nov. 2004 (day 70). Total RNA was isolated from fruit at each time point and 18s rRNA was used as a control. The optimum PCR cycles were 27, 29, and 33 for *MdCIM* (likely a combination of *MdCIM*1 and *MdCIM*2), *MdIPMS*1, and *MdIPMS*2 respectively. Experiments were repeated twice and essentially identical results were obtained.

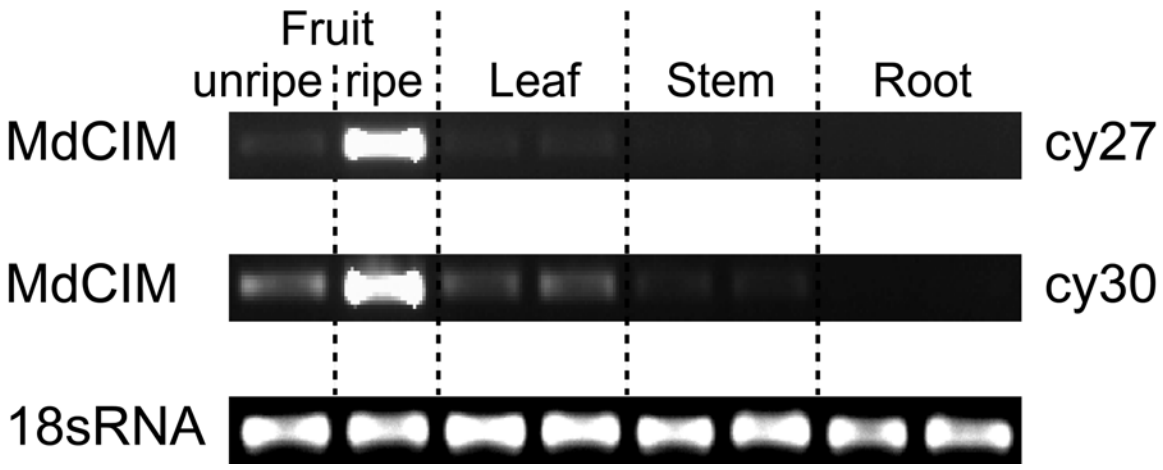

Figure 14. RT-PCR analysis of citramalate synthase (designated *MdCIM*, but likely a combination of *MdCIM*1 and *MdCIM*2) gene expression in different organs of 'Jonagold' apple. Tissues were unripe (day 0) and ripe (day 49) fruit, root, stem, and leaf. 18s rRNA was used as a control. Two biological replicates were performed for leaf stem and root tissue, shown as two lanes. Data are presented for 27 and 30 PCR cycles (cy27 and cy30, respectively) to better reveal low levels of expression for unripe fruit and leaf tissue.

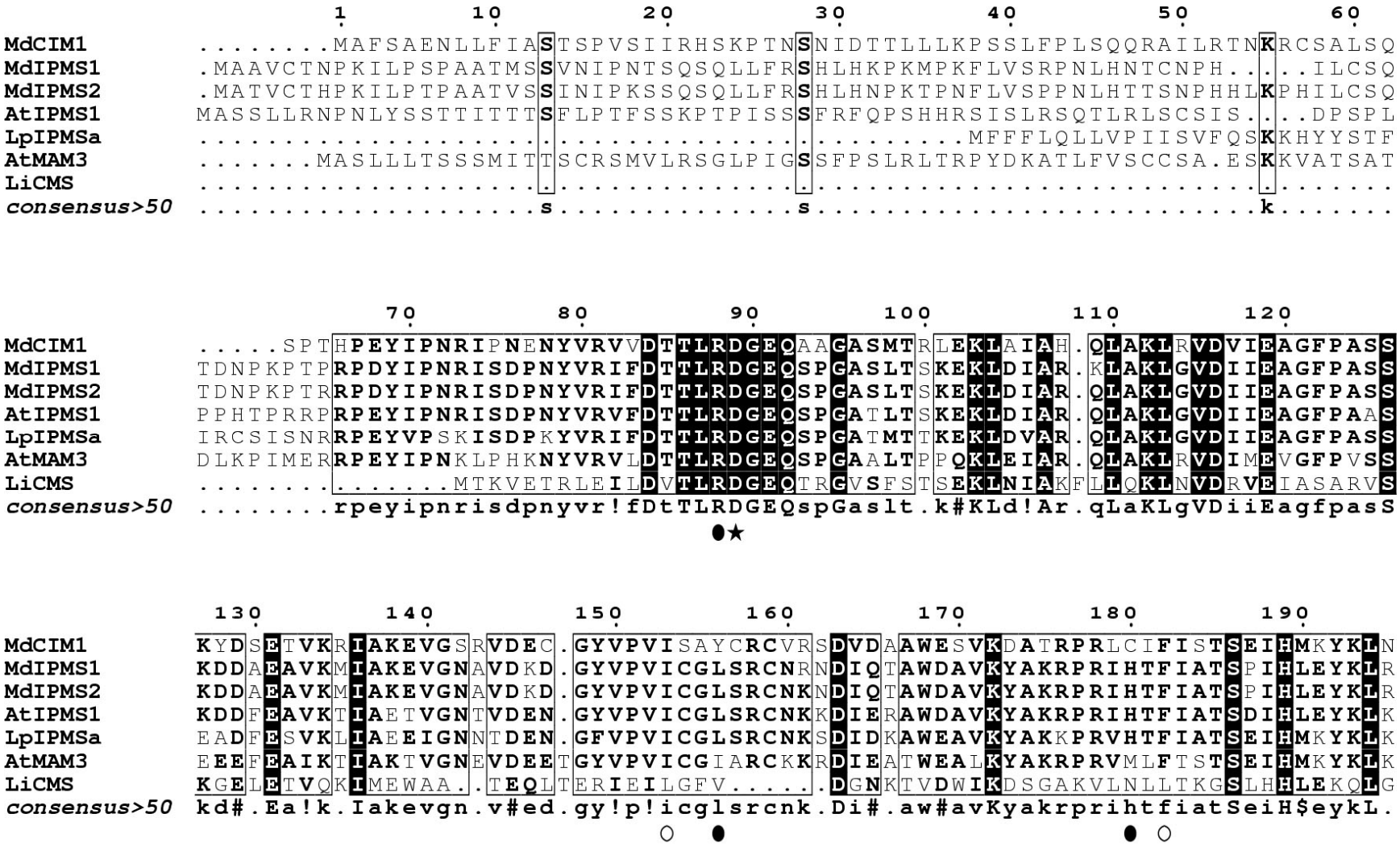
MdCIM1
MdIPMS1
MdIPMS2
AtIPMS1
LpIPMSa
AtMAM3
LiCMS
consensus>50

Figure 15.

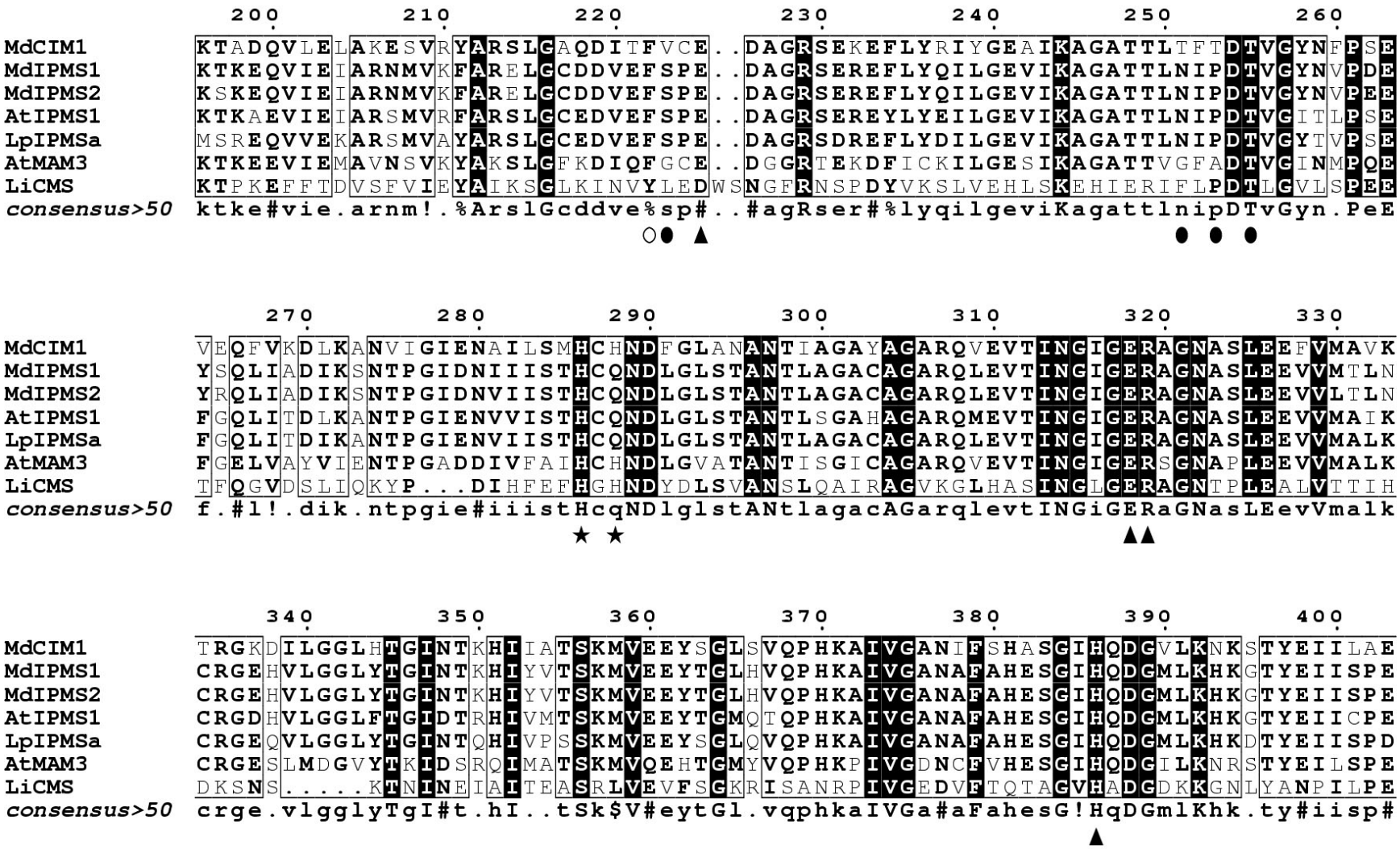

Figure 15 (cont'd).

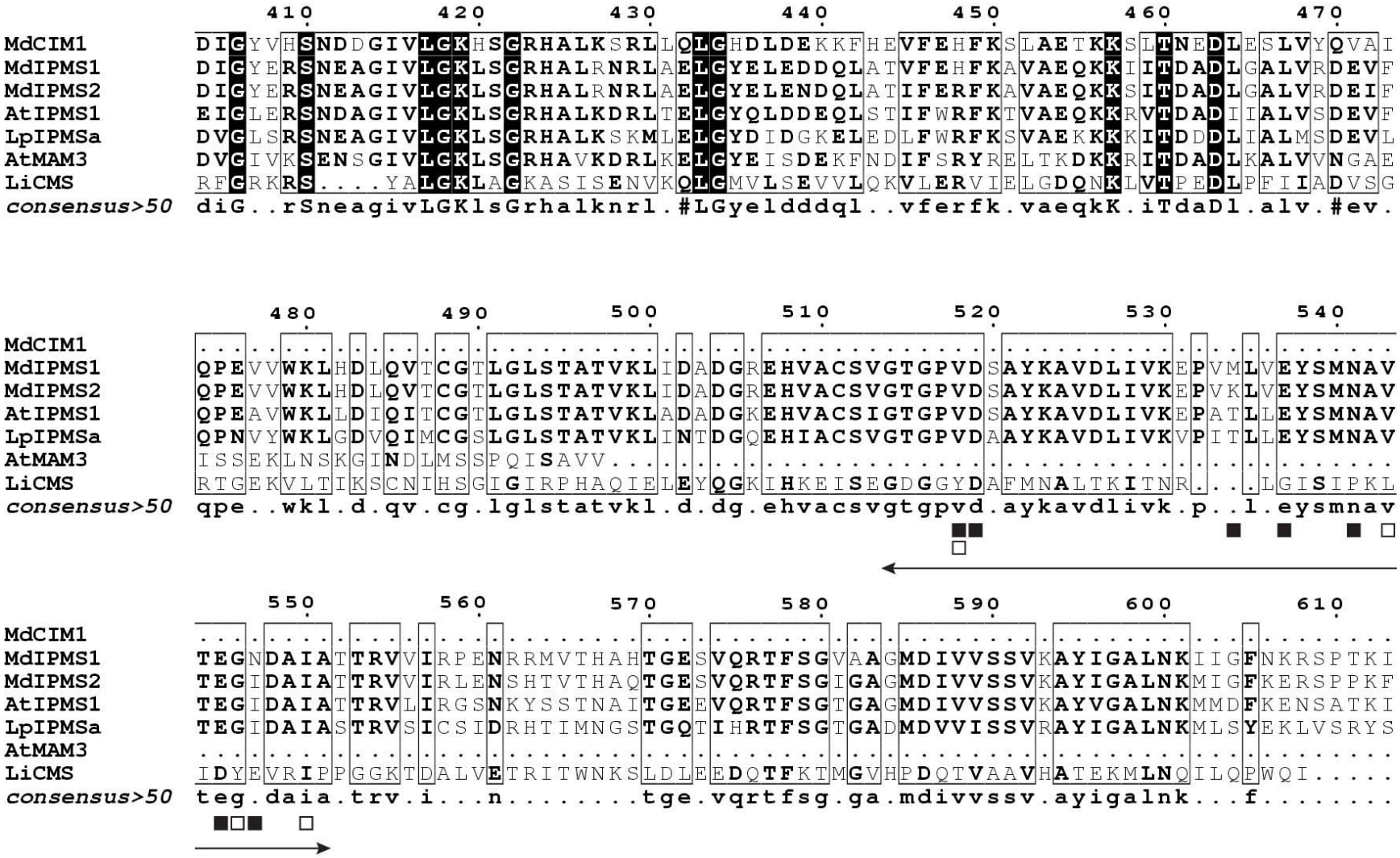

Figure 15 (cont'd).

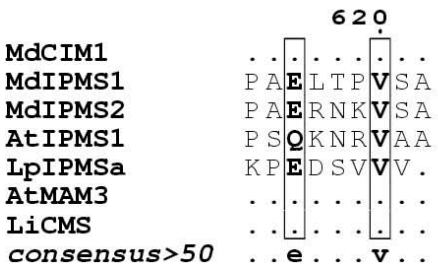

Figure 15. Amino acid sequence alignment of citramalate synthase (CIM or CMS), 2-isopropylmalate synthase (IPMS), and methylthioalkylmalate synthase (MAM) genes from apple (*Malus ×domestica*), *Arabidopsis thaliana* IPMS1 (At1g18500) and MAM3 (At5g23020), *Lycopersicon pennellii* (AF004165), and *Leptospira interrogans* (gi: 24215050). The highlighted dark background shows the conserved sequences across the species. The amino acids that are considered to interact with a metal cofactor are marked with stars, the residues that interact with acetyl-CoA with closed triangles, the residues that interact with α-ketoisovalerate with closed circles, and the residues that interact with pyruate with open circles. The predicted regulatory region is underlined with a double-headed arrow between the sequences 514-551. The amino acids thought to interact with isoleucine are marked with open squares and the residues that interact with leucine are marked with closed squares for feedback inhibition (de Kraker and Gershenzon, 2011; Ma et al., 2008; Zhang et al., 2009). The predicted cleavage sites for chloroplast target region are indicated by arrows; the site MdCIM1 was predicted to be between amino acids 56 and 57, and for MdIPMS1 and 2 was between amino acids 59 and 60 based on ChloroP analysis (Emanuelsson et al., 1999).

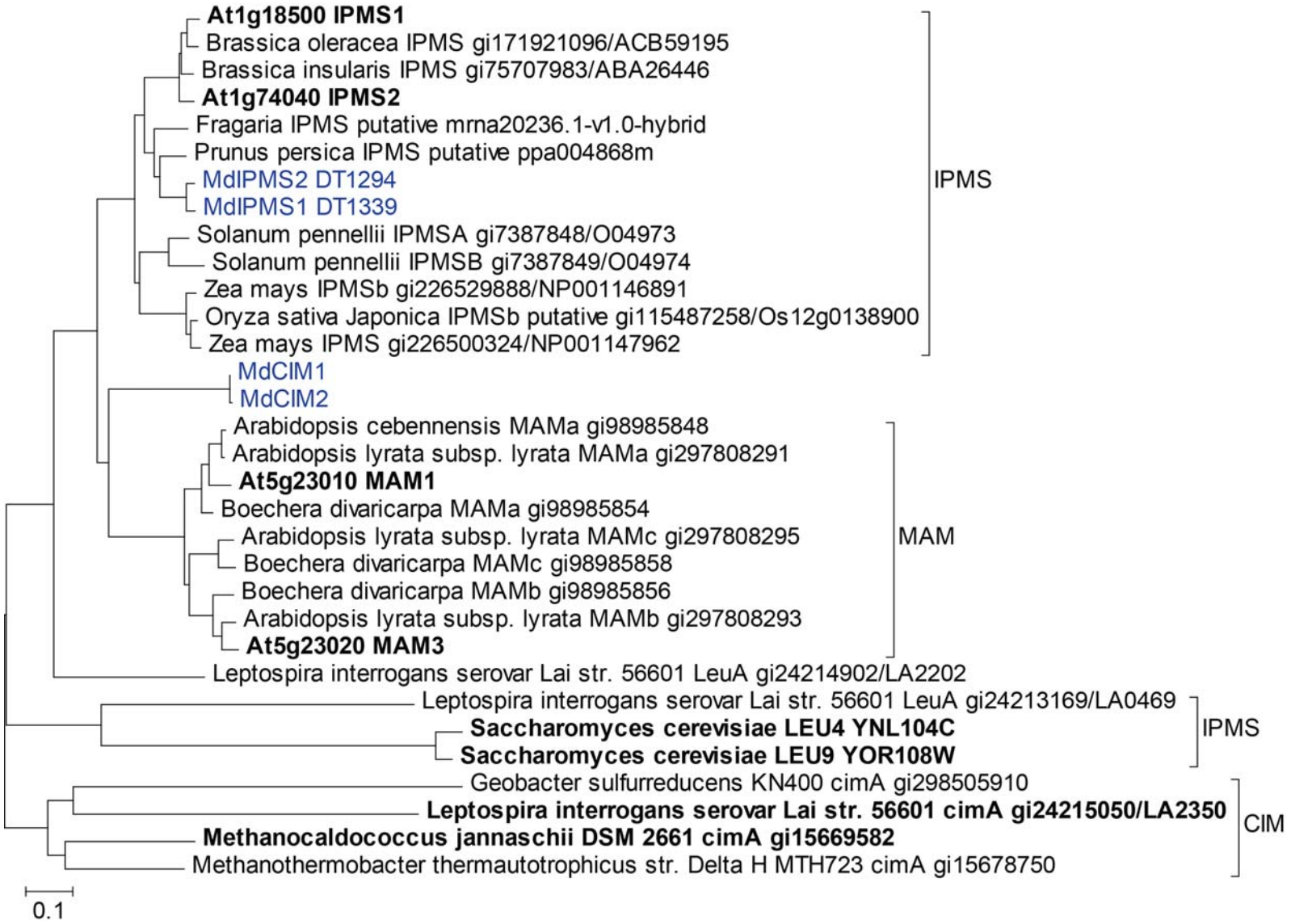
At1g18500 IPMS1
Brassica oleracea IPMS gi171921096/ACB59195
Brassica insularis IPMS gi75707983/ABA26446
At1g74040 IPMS2
Fragaria IPMS putative mrna20236.1-v1.0-hybrid
Prunus persica IPMS putative ppa004868m
MdIPMS2 DT1294
MdIPMS1 DT1339
Solanum pennellii IPMSA gi7387848/O04973
Solanum pennellii IPMSB gi7387849/O04974
Zea mays IPMSb gi226529888/NP001146891
Oryza sativa Japonica IPMSb putative gi115487258/Os12g0138900
Zea mays IPMS gi226500324/NP001147962
IPMS
MdCIM1
MdCIM2
Arabidopsis cebennensis MAMa gi98985848
Arabidopsis lyrata subsp. lyrata MAMa gi297808291
At5g23010 MAM1
Boechera divaricarpa MAMa gi98985854
Arabidopsis lyrata subsp. lyrata MAMc gi297808295
Boechera divaricarpa MAMc gi98985858
Boechera divaricarpa MAMb gi98985856
Arabidopsis lyrata subsp. lyrata MAMb gi297808293
At5g23020 MAM3
MAM
Leptospira interrogans serovar Lai str. 56601 LeuA gi24214902/LA2202
Leptospira interrogans serovar Lai str. 56601 LeuA gi24213169/LA0469
Saccharomyces cerevisiae LEU4 YNL104C
Saccharomyces cerevisiae LEU9 YOR108W
IPMS
Geobacter sulfurreducens KN400 cimA gi298505910
Leptospira interrogans serovar Lai str. 56601 cimA gi24215050/LA2350
Methanocaldococcus jannaschii DSM 2661 cimA gi15669582
Methanothermobacter thermautotrophicus str. Delta H MTH723 cimA gi15678750
CIM
0.1

Figure 16.

Figure 16. Phylogenetic tree for 32 2-isopropylmalate synthase (IPMS) and citramalate synthase (CIM) sequences from selected bacteria, yeast, and plants. Bold print indicates characterized proteins. The accession number and the annotation are indicated along with species. The alighment was performed using the Neighbor-Joining method (Saitou and Nei, 1987). All IPMS and CIM sequences were obtained from the NCBI database. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and indicate the number of amino acid substitutions per site. Signal sequences, the R-region, alignment gaps, and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

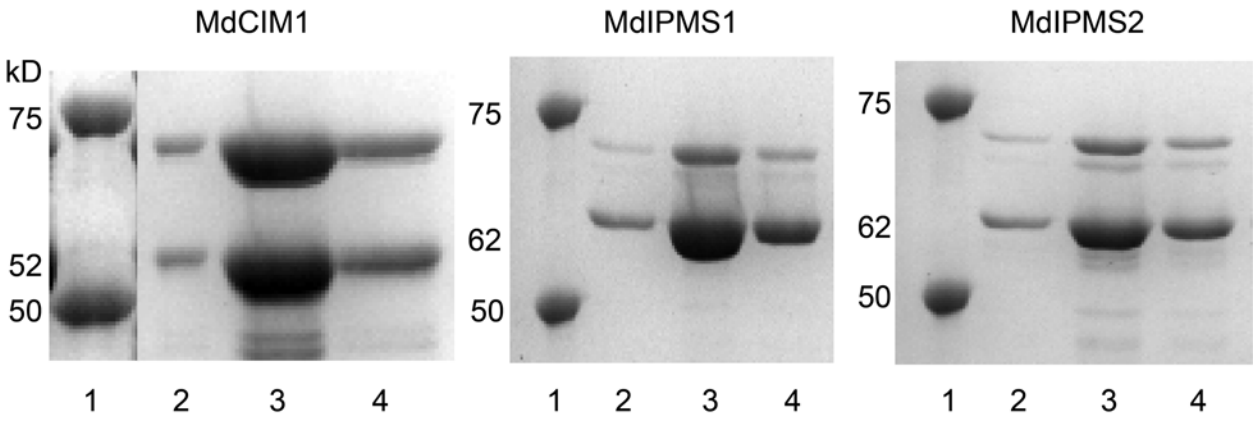

Figure 17. Protein size of 2-isopropylmalate synthase (MdIPMS) and citramalate synthase (MdCIM) as determined by electrophoresis on a 10% SDS-PAGE gel and stained using Coomassie Brilliant Blue R-250. MdCIM1 was expressed as full length ORF and MdIPMS1 and 2 were expressed without the chloroplast targeting sequence. The predicted protein size excluding 6x His-tag (0.8 kD) was around 52 kD for full length MdCIM1 and 62 kD for truncated MdIPMS1 and 2. The lane 1 on each gel contains 50 and 75 kD protein standards for size estimation. Lanes 2 to 4 are replications of purified proteins differing in concentration during protein purification process. The upper bands on the gel are the non-specific proteins.

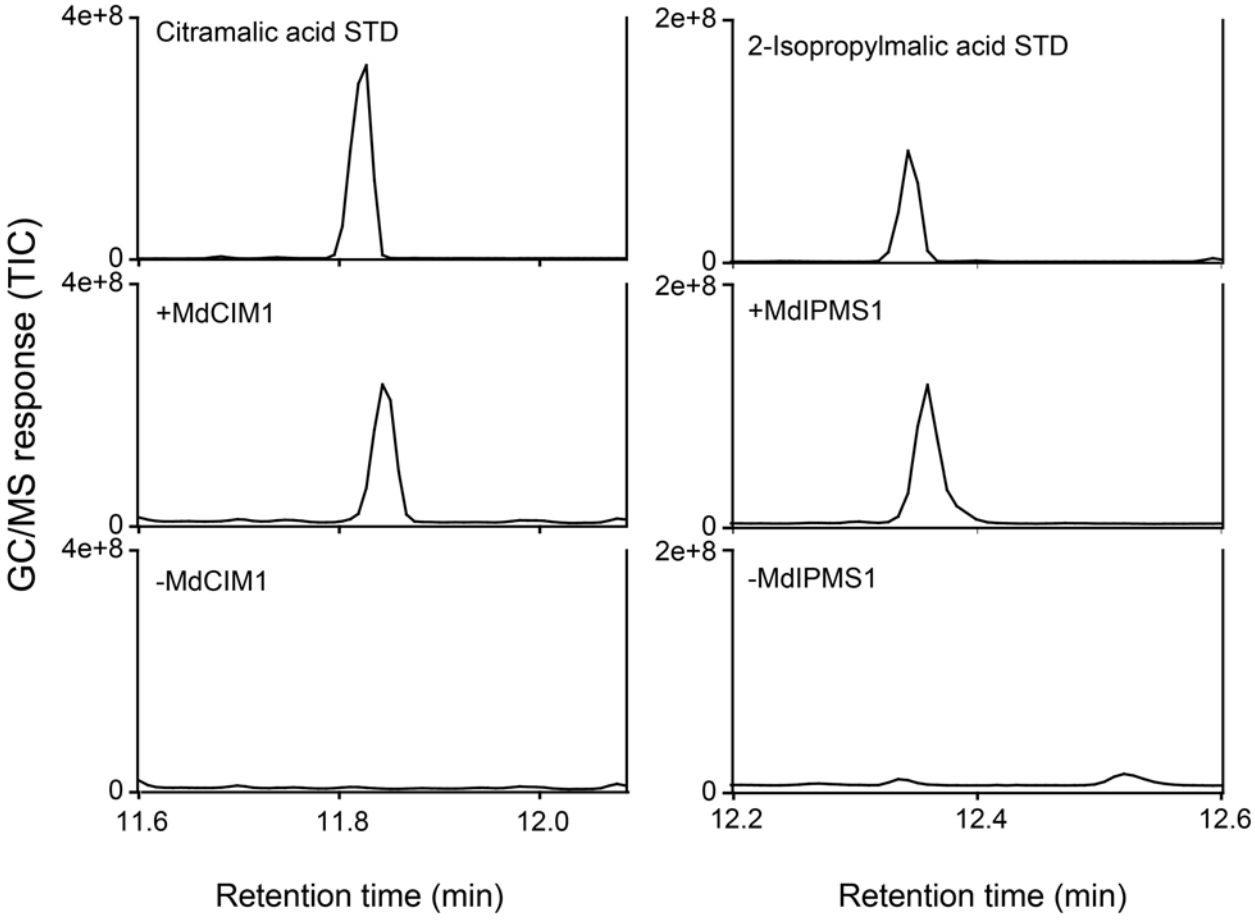

Figure 18. Gas chromatography coupled with mass spectrometry (GC/MS) analysis of the reaction products of citramalate synthase (MdCIM1, left) using pyruvate as a substrate and 2-isopropylmalate synthase (MdIPMS1, right) using α-ketoisovalerate as a substrate following incubation at 22 °C for 120 min. Data are expressed as total ion count (TIC). Assay mixtures and authenticated standards were derivatized and analyzed by GC/MS. The top graphs are the chromatograms of a standard for the expected assay product, the middle graphs are the chromatograms of the actual enzyme assay product, and the bottom graphs are chromatograms of the assay mixture without added enzymes.

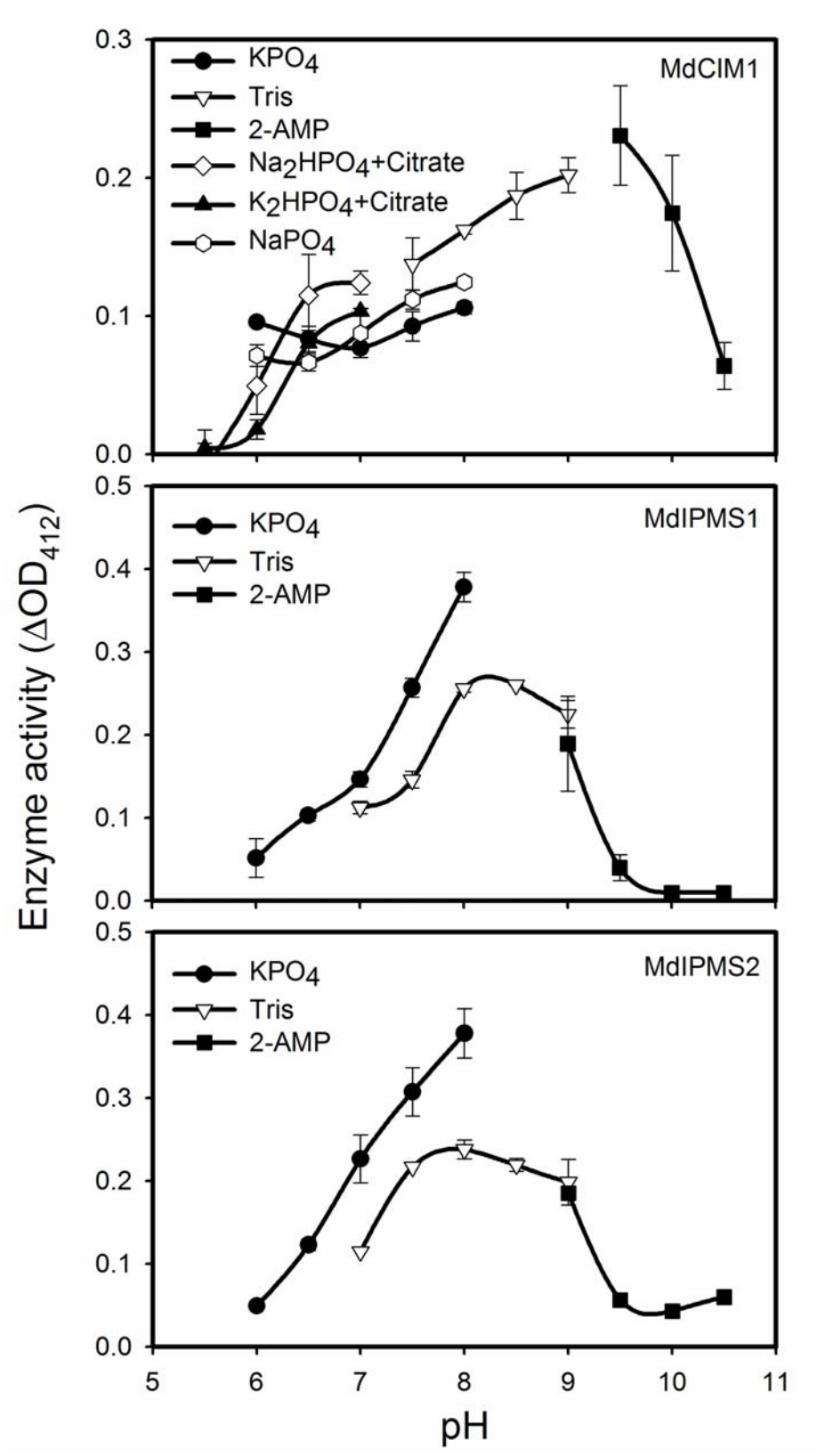
KPO4
Tris
2-AMP
Na2HPO4+Citrate
K2HPO4+Citrate
NaPO4
MdCIM1
MdIPMS1
MdIPMS2
Enzyme activity (ΔOD412)
pH
0.0
0.1
0.2
0.3
0.4
0.5
5
6
7
8
9
10
11

Figure 19.

Figure 19. Activity of citramalate synthase (MdCIM1) and 2-isopropylmalate synthase (MdIPMS1 and 2) as affected by pH. pH was adjusted using potassium or sodium phosphate with citrate (pH 5.5 to 7.0), potassium or sodium phosphate (pH 6.0 to 8.0), Tris (pH 7.0 to 9.0), and 2-amino-2-methyl-propane (2-AMP, pH 9.0 to 10.5). The enzyme assay was performed by Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB] assay and the absorbance was corrected by subtracting the background of the identical enzyme assay mixture without α-keto acids. Pyruvate and α-ketoisovalerate was used as substrates for MdCIM1 and the two MdIPMS proteins, respectively. Experiments were repeated twice for each data points. Vertical bar represents ± SD of the mean.

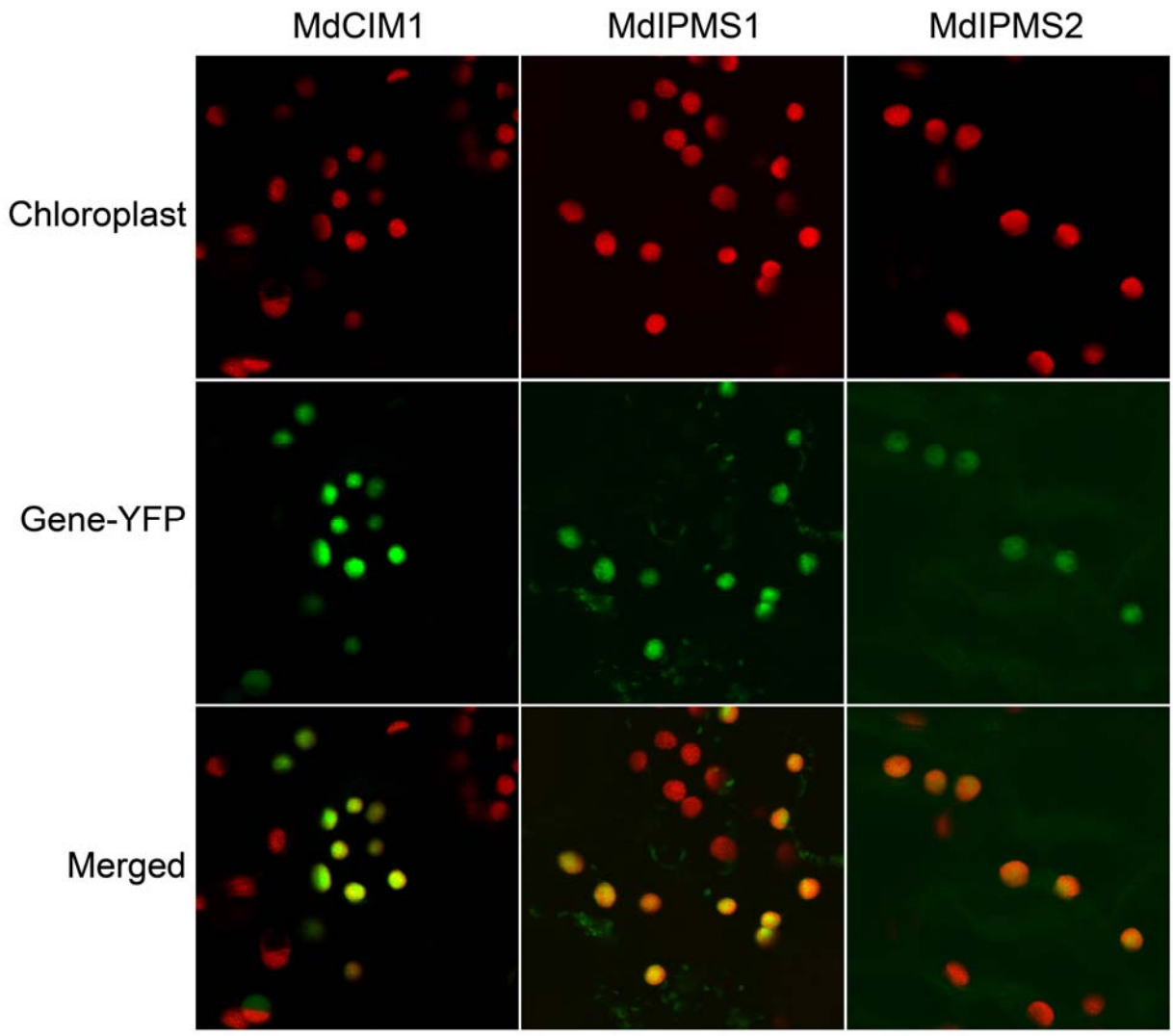

Figure 20. Transient expression of citramalate synthase (MdCIM1) and 2-isopropylmalate synthase (MdIPMS1 and 2) in tobacco (*Nicotiana tabacum* cv. Samson). MdCIM1 and MdIPMS1 and 2 sequences fused with YFP were infiltrated into tobacco leaves and the infiltrated areas were analyzed after three days by confocal microscopy as described by Reumann et al. (2009). Top row shows the chloroplast, middle row shows the protein fused with YFP, and the bottom row shows the overlay image.

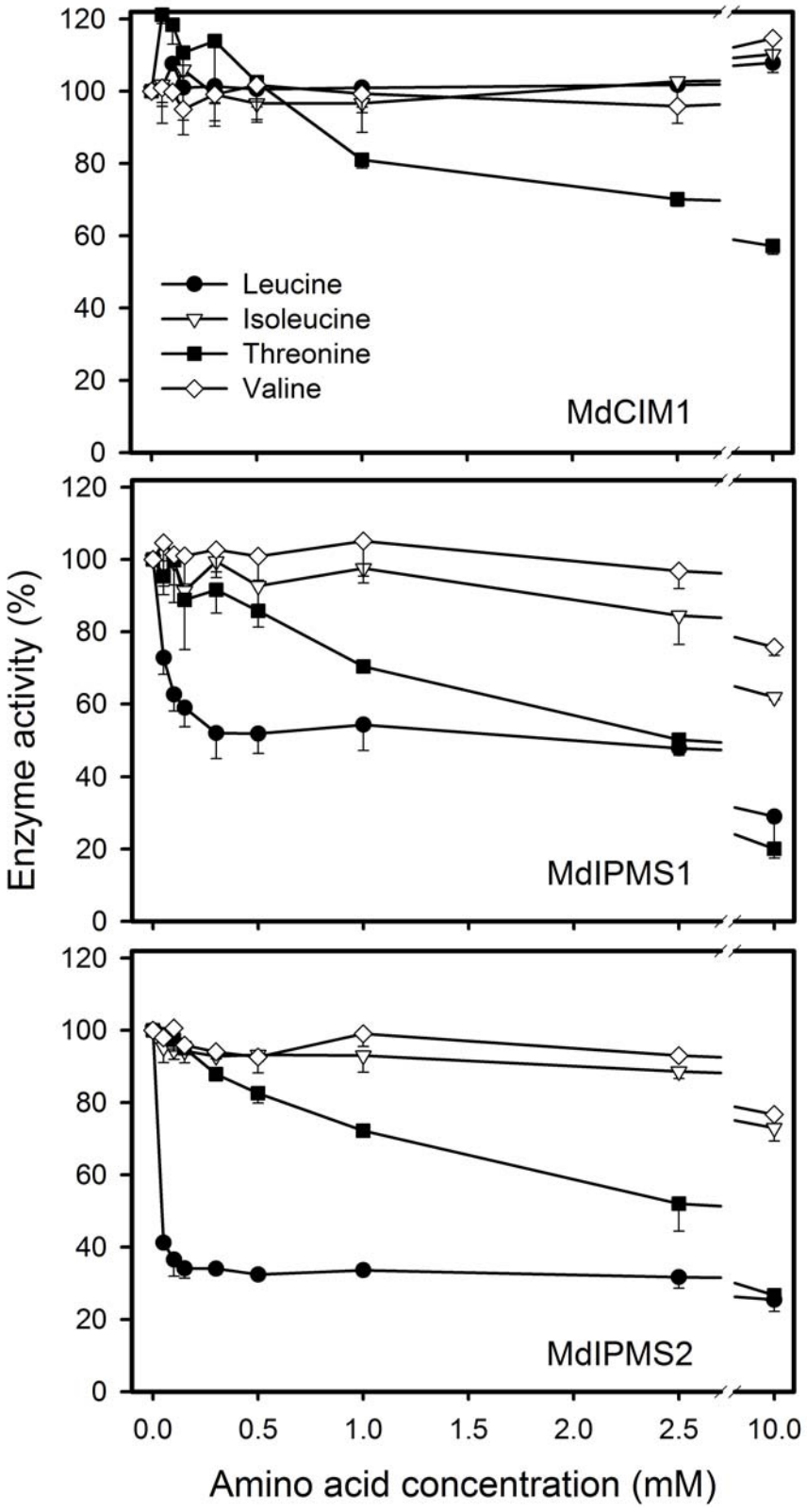
Leucine
Isoleucine
Threonine
Valine
MdCIM1
MdIPMS1
MdIPMS2
Enzyme activity (%)
Amino acid concentration (mM)
0
20
40
60
80
100
120
0.0
0.5
1.0
1.5
2.0
2.5
10.0

Figure 21.

Figure 21. Activity of citramalate synthase (MdCIM1) and 2-isopropylmalate synthase (MdIPMS1 and 2) proteins as affected by branched-chain amino acids and threonine. Amino acids were added in an enzyme assay mixture at different concentrations (0, 0.05, 0.1, 0.15, 0.3, 0.5, 1, 2.5, 10 mM). The substrates pyruvate and α-ketoisovalerate were used for MdCIM and MdIPMSs, respectively, and incubated at 22 °C for 20 min. The enzyme assay was performed by Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB] end-point assay and the absorbance was corrected by subtracting the background of the identical enzyme assay mixture without α-keto acids. The activity of the assay mixture lacking added amino acids was taken as 100%. Vertical bar represent the standard deviation from the mean for two replicate assays.

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APPENDIX

CHARACTERIZING THE VOLATILE PROFILE AND STORABILITY OF APPLE FRUIT FROM THE GENEVA *MALUS* CORE COLLECTION

Introduction

The genus *Malus* is genetically diverse, but cultivated apples have a very narrow genetic base (Way et al., 1991). A narrow genetic base has the potential to make commercial apple production vulnerable to potentially catastrophic losses and limits the potential diversity of traits that might be manipulated through selection. For this reason, the USDA has made increasing the genetic diversity of apple a programmatic goal and in 1987, developed the wild apple germplasm collection at the New York Agricultural Experiment Station at Geneva (Forsline et al., 2010). The genetic diversity of the collection was intended to be broad enough to act as a source for a number of desirable horticultural traits including resistance to biotic and abiotic stresses, fruit quality, and other attributes useful for the development of new apple cultivars (Forsline et al., 2010). Phenotypic data for the collections can be accessed on the Germplasm Resources Information Network (GRIN) http://www.ars.usda.gov/Aboutus/docs.htm?docid=6169. The trees and fruit from the trees are described with 25 priority descriptors such as categorized as morphology (e.g. shape, size, color), phenology, production, and growth (e.g., tree vigor and habit) as listed in Forsline et al. (2010). These categories provide useful information for breeding to reduce production costs, to increase marketability of the fruit and improve other traits, most usually focusing on disease resistance during production (Janick et al., 1996). Unfortunately, the database is lacking in postharvest traits and information on disorders associated with storage even though apple cultivars are known differ substantially in their postharvest quality traits and susceptibility to postharvest disorders.

Of the fruit traits important to consumers, little attention has previously been given to aroma and, in fact, only very limited information for this trait exists in the GRIN database. In part, this reflects the emphasis of breeding program on screening apple accessions for disease or

pest resistance (Crosby et al., 1992; Janick, 2006; Janisiewicz et al., 2008; Myers et al., 2007) while neglecting organoleptic quality despite the consumer's demand for better flavor (Baldwin, 2002). In the GRIN system, the descriptors of fruit flesh flavor are categorized under morphology and fruit are classified as "aromatic", "sweet", "subacid", "acid", and "astringent". There is a need to expand the available information of aromatic component since aroma plays a significant role in determining the overall apple flavor. Inclusion of phenotypic data describing the production of aroma active molecules would improve the GRIN database in this regard. The needed aroma information should optimally include both qualitative and quantitative analyses of the odor-active volatiles, since these differ dramatically in type and amount even between cultivars within a single *Malus* species (Kakiuchi et al., 1986).

In ripening apple fruit, esters are the primary aroma impact compounds that influence aroma and normally account for 80% to 95% of the total volatile emission (Paillard, 1990). Typical esters include hexyl acetate, butyl acetate, and 2-methylbutyl acetate and are autonomously produced in abundance during ripening (Paillard, 1990). In general, they are perceived as fruity and floral (Plotto et al., 2000). Despite the typical esters, some cultivars predominate in specific esters. For example, acetate esters predominate in 'Calville blanc' and 'Golden Delicous'; butanoate esters are found in high amounts in 'Bell de Boskoop' and ' Canada Blanc'; propanoate esters are abundant in 'Richard' and 'Reinette du Mans' (Paillard, 1990); ethyl esters are plentiful in 'Starking', 'Delicious', and 'Bisbee Delicious' (Guadagni et al., 1971; Mattheis et al., 1991a, 1991b; Paillard, 1990), and pentyl esters in 'Redchief Delicious', while low in amount, are higher than is typically found in most apple cultivars (Rudell et al., 2002). Also, as ripening progresses from preclimacteric to postclimacteric, the amounts of

individual compounds also change over time, altering the ester composition (Ferenczi, 2003; Mattheis et al., 1991a).

Each ester has its own odor note and threshold. The unique ester composition in the fruit serves as an aroma fingerprint that is specific to each cultivar. Some aroma compounds for instance, enhance the intensity of "sweet flavor" and can affect the perception of sweetness in fruits (Young et al., 1996) while others add additional flavor notes (Williams et al., 1977). Unsaturated aldehydes and their esters, which are normally in very low abundance in intact fruit, may also contribute as a 'green' odor (Olias et al., 1993; Salas et al., 2006).

The esters of intact apple fruit are composed of alkyl (alcohol-derived) and alkanoate (acid-derived) groups, typically 1- to 8-carbons in length. The alkanoate groups enter into the ester forming reaction as CoA thioesters in a reaction catalyzed by alcohol acyl-CoA transferase (AAT, EC2.3.1.84) (Ueda and Ogata, 1977). These alkyl and alkanoate groups can be straight or branched. Several hypotheses have been suggested to propose pathways by which ester precursors are formed but little is known about their biosynthetic pathways. Precursors of straight-chain (SC) esters have been suggested to be formed from fatty acids by a combination of β-oxidation and lipoxygenase activities (Drawert, 1975; Sanz et al., 1997; Yahia, 1994). Precursors of branched-chain (BC) esters have been suggested to be formed from the metabolism of branched-chain amino acids (BCAA) (Gonda et al., 2010; Pérez et al., 2002; Rowan et al., 1996, 1998; Tressl and Drawert, 1973). Aroma characterization will take an advantage of these biochemically and metabolically variable resources for physiological, biochemical and molecular investigations.

The objective of this research was to investigate the diversity of volatile esters produced by each apple germplasm lines during ripening and to provide a cursory evaluation of sensory

attributes and storability in refrigerated air. The information will serve to expand the GRIN system database and to assist in the development of new breeding lines. The characterization of flavor and esters and storage disorder during cold storage should help guide selections of breeding material for new varieties from this collection. Characterization of the volatile profiles of important members of the Geneva *Malus* collection has the potential to provide foundational information to support further physiological, biochemical, and molecular investigations.

Materials and Methods

Plant Material. 184 lines from the 4-copy Geneva *Malus* Core Collection and an additional 12 Kazakhstan apple lines (all *M. sieversii*) were harvested on nine dates between 24 Aug. 2005 and 24 Oct. 2005 from the USDA Plant Genetic Resources Unit's *Malus* Germplasm Repository at Cornell University, Geneva, NY. These 184 lines consisted of 39 taxa: 20 taxa were primary species (48 accessions), eight were cultivated species (88 accessions), nine were secondary species (12 accessions), one consisted of unidentified species (2 accessions), and one consisted of uncharacterized hybrid species (34 accessions). Apples were collected at a mature stage, initially estimated based on the GRIN harvest code, which classified lines relative to the timing for 'Delicious' maturation as: 1) extreme early, >60 days before 'Delicious'; 2) very early, 50 to 60 days before 'Delicious'; 3) early, 30 to 50 days before 'Delicious'; 4) mid-early, 20 to 30 days before 'Delicious'; 5) mid,10 days before 'Delicious'; 6) mid/late, same time as 'Delicious'; 7) late, 10 days later than 'Delicious'; 8) very late, 20 to 30 days later than 'Delicious'; 9) extremely late, >30 days later than 'Delicious' and by starch index (see below) at the time of harvest. The harvested apples were packed and secured in a specialized corrugated cardboard box with polyurethane padding with holes for individual apples to avoid bruising

during shipping. The packed apples were shipped overnight to Michigan State University for evaluation. Samples for each of the 184 lines consisted of 3 to 50 fruit depending on fruit size and availability on the tree. On each occasion, fruit were held overnight in the laboratory to equilibrate to laboratory temperature (21±1 °C) and covered with ventilated, black, 4-mil thick plastic bags to avoid desiccation and responses to intermittent laboratory light before analysis. Objective measurements included fresh weight (mass), internal ethylene concentration (IEC), starch index, firmness, °Brix, and volatile profile. Fruit from each line were also informally given subjective flavor evaluations by the investigators. The perceptions of the flavor included as tart, astringent, sweet, nutty, floral, fruity, bland, acid, lemony, alcoholic, spicy, anise-like, and other unusual characters. Volatile compounds were analyzed (see below) when fruits were assessed to be fully ripe based on IEC, starch index, background color and/or volatile production.

For storage analysis, fruit with a mass greater than 30 g (60 lines) were stored in air at 0 °C and a relative humidity greater than 95%. After 3 and 6 months storage, fruit were transferred from cold storage to room temperature (21±1 °C) and assayed for IEC, firmness retention and physiological disorders one day after removal from cold storage. Samples sizes varied from 10 to 15 fruit, depending on availability.

Volatile analysis. For volatile measurement, a gas chromatograph (GC) (HP-6890, Hewlett Packard Co., Wilmington, DE) coupled with time-of-flight mass spectrometer (TOFMS) (Pegasus III, LECO Corp., St. Joseph, MI) was used. Fruit were held at 21 °C in a 1-L Teflon container (Savillex Corporation, Minnetonka, MN) fitted with a gas-sampling port. The number of apples held in the container varied from 1 to 20 fruit depending on the fruit size and the analysis was performed on a minimum of 10 g total fruit mass. Sampling ports were sealed with

a Teflon-lined half-hole septum (Supelco Co., Bellefonte, PA) and apples were incubated at 22 °C for 20 min. Headspace volatiles were sampled using a 1-cm long, solid-phase micro extraction (SPME) fiber (65 μm PDMS-DVB, Supelco Co., Bellefonte, PA). Following a 3 min sorption time, the SPME fiber was immediately transferred to the GC for desorption and separation of volatiles. The injection port was operated in splitless mode and held isothermally at 230 °C; the desorption time was 2 min. Desorbed volatiles were trapped on-column using a liquid nitrogen cryofocussing trap. The GC was equipped with a TOFMS for detection, identification and quantification of volatiles. Conditions of GC separation and TOFMS analysis were as previously described (Song et al., 1997). Identification of compounds was by comparison of the mass spectrum with authenticated reference standards and/or with spectra in the National Institute for Standard and Technology (NIST) mass spectrum library (Version 05) when no standard was available. Volatile compounds were quantified by calibrating with a known amount of an authenticated, high purity standard mixture of 28 volatilized alcohols, aldehydes, and esters as previously described (Song et al., 1997). Where no standard was available, quantization was by estimation of the instrument response factor based on the Kovats index for the compound of interest (Bartelt, 1997). Detected volatiles emitted during 20 min incubation in the Teflon container was used for data analysis.

Measurement of IEC. The IEC was determined by withdrawing a 1-mL gas sample from the interior of the apples and subjecting the gas sample to GC (Carle Series 400 AGC; Hach Company, Loveland, CO) analysis as previously described (Mir et al., 2001). Due to limitations on the interior gas space, the IEC could only be determined on fruit with a mass greater than 5 g. The GC, fitted with a 6-m long, 2-mm i.d. stainless steel column packed with activated alumina,

was equipped with a flame ionization detector. The ethylene detection limit was approximately 0.005 $\mu L \cdot L^{-1}$. The ethylene concentration was calculated relative to a certified standard (Matheson Gas Products Inc., Montgomeryville, PA) containing 0.979 $\mu L \cdot L^{-1}$ ethylene, 4.85% CO_2 and 1.95% O_2, balanced with N_2.

Measurement of starch. The starch index at harvest was determined by cutting fruit in half through the seed cavity along the plane perpendicular to the longitudinal axis as described by Mir et al. (2001). The cut flesh was dipped into an iodine solution containing 10 g KI and 40 g I_2 per 4 L of water. Color development was allowed to proceed for at least 1 min. Starch index (1 – 8) was determined by comparison to the Cornell Starch Chart (Blanpied and Silsby, 1992) where 1 = 100% core and flesh stain (unripe), 3 = 0% core stain and 100% flesh stain, 6 = 0% core and 40% flesh stain, and 8 = 0% core or flesh stain (ripe).

Measurement of firmness. Firmness was determined only on those lines with a fruit mass greater than 20 g. Firmness was determined using a drill-stand-mounted Effegi penetrometer (FT-327; McCormick Fruit Tree Inc., Yakima, WA) fitted with an 11-mm diameter probe as described by Mir et al. (2001). The penetrometer was calibrated at 53.4 N (12 lb) using a top-loading balance. Two skin discs (2 to 3 cm in diameter) and 3 to 5 mm of underlying cortex tissue were removed from opposite sides of each fruit. The penetrometer probe was pressed into the tissue of the cut surface to a depth of 8 to 9 mm in a single smooth motion requiring approximately 1 s. Data were recorded as pounds and converted to Newtons by multiplying by 4.45 N/lb.

Cluster analysis. Hierarchical cluster analysis was performed on all the esters detected in this study using Cluster 3.0 (de Hoon et al., 2004), which was developed from the original program by Eisen et al. (1998) and TreeView software (Eisen et al., 1998). The uncentered correlation and centroid linkage method was used.

Results and discussion

Ripening profiles. The number of different esters detected (ester count) was used as a measure of ester diversity. Ester diversity tended to increase with increasing fruit weight (Figure 22). The ester count was linearly correlated with the cubed root of fruit weight (R^2=0.64). The relationship between fruit size and ester diversity supported classification of the lines into five fruit size categories (small, small-medium, medium, medium-large, and large) for comparison. Fruit mass ranged from 0.3 g to 342.3 g (Table 1). Lines of *M.* ×*domestica* used for commercial fruit production were all in the large fruit category.

The cubed root of fruit weight was also correlated with °Brix, internal ethylene, and firmness. On average, smaller fruit tended to have a higher °Brix (R^2=0.76), lower ethylene (R^2=0.86) and higher firmness (R^2=0.98) than larger fruit (Table 8). The lower IEC in smaller fruit may, in part, be related to enhanced diffusion characteristics associated with smaller fruit size. A number of the early-ripening varieties had relatively high IEC compared to late ones. IEC for very early to mid-early ripening varieties (harvest code 2 to 4) were 128 $uL \cdot L^{-1}$ on average, whereas mid to very late ripening varieties (harvest code 5 to 8) were 80 $uL \cdot L^{-1}$. This finding was similar to that from previous studies (Hansen, 1945, Oraguzie et al., 2007). The source of diversity in apple taxa is not known. Harada et al. (2000) suggested that the rates of ethylene production in apple are influenced by genotype of 1-aminocyclopropane-1-carboxylate synthase.

However, this relationship did not hold up in subsequent studies (Oraguzie et al., 2007; Sun et al., 2009).

There was no correlation between the higher ethylene producing lines and low firmness retention (R^2=0.020) nor on the lateness of varieties (based on harvest code) and high firmness retention (R^2=0.005). In our study, medium-sized fruit had a higher firmness than larger fruit, averaging 98 N compared to 70 N for large fruit. De Salvador et al. (2006) studied the relationship between fruit size and firmness and reported that smaller fruit had a higher firmness than larger fruit in 'Golden Delicious' and 'Red Chief' apples. Vincent (1989) suggested that firmness is correlated with fruit density and is partially related to the amount of air space in the flesh. Goffinet et al. (1995) reported that fruit size is positively correlated with cortex cell number. The cell density of members of the Geneva *Malus* collection is not known.

The starch index of most of the apple lines declined over time (data not shown), although starch degradation remained incomplete for several lines. About 5% of the lines evaluated emitted high volatiles and copious amounts of ethylene even though when the starch index was below 5 (data not shown). These lines were members of *M. ×domestica*, *M. ×hybrid*, and *M. sieversii* taxa. In some cases, fruit with a starch index of five and below became mealy, greasy, and obviously overripe while still retaining significant levels of starch. This finding is not commonly observed in commercial varieties until fruit reach a starch index in the range of 7 to 8 (Beaudry et al., 1993; Brookfield et al., 1997; de Castro et al., 2007).

Esters. A total of 67 different esters including three with unsaturated alkyl groups were detected in the 184 lines of apple fruit (Table 9). Three unsaturated esters, 2-, 3-, 5-hexenyl acetate were detected, but are not listed in Table 9. Free acids and formate esters were occasionally detected

but not quantified. Ester subgroups tended to be unevenly distributed within the matrix. For instance, pentyl alcohol-derived esters were abundant only in combination with acetate, but not with longer-chain acid moieties. Ester abundance tended to be greatest for esters containing alkyl and alkanoate moieties composed of 2, 4, and 6 carbons or branched moieties derived from 2-methylbutanol or 2-methylbutanoate. Esters derived from 2-methylpropanol, 2-methylpropanoate, methanol, heptanol, heptanoate, octanol, and octanoate were generally in low abundance and less frequently detected. Esters containing unsaturated subgroups were relatively rare and of these, only acetate esters were detected (data not shown).

The three esters most frequently detected were butyl acetate (179 lines), hexyl acetate (168 lines), and hexyl 2-methylbutanoate (164 lines). For ester classes segregated by the alkanoate group, acetate esters were most often found in greatest abundance. The three most abundant acetates, in decreasing order were ethyl acetate, butyl acetate, and 2-methylbutyl acetate. Two of these esters, butyl acetate and 2-methylbutyl acetate, together with hexyl acetate (the fourth most abundant acetate ester in this study) are considered to be primary contributors to apple aroma (Fellman et al., 2000). For esters classed by the alkyl moiety, ethyl esters tended to be the most abundant, although butyl and hexyl esters, which were slightly less abundant, were found more frequently.

We ranked the top 15 lines that produced predominantly either alkyl and alkanoate ester groups (Table 10). Interestingly, ‘Cox’s Orange Pippin’, ‘Empire’ and ‘Delicious’ produced higher levels of unsaturated esters. Overall, within commercial lines, ‘Delicious’ was one of the top ester producing lines, ranking no. 4, in terms of ester abundance, and had a high ester diversity, ranking no. 2 (Table 11). The data also indicated that not all high ester producing lines

had high ester counts. For example, 'Ingol', which ranked no. 2 in ester abundance, predominantly produced acetate, ethyl-, and propyl esters, but ranked no. 88 in ester diversity.

The esters detected were consistent with the type and numbers of esters previously characterized (Dimick and Hoskin, 1983; Lo Scalzo et al., 2001; Paillard, 1990). In addition, we found five 'new' esters not previously reported: propyl 2-methylpropanote, 2-methylbutyl 2-methylpropanoate, heptyl butanoate, octyl 2-methylbutanoate, and methyl octanoate. Six esters (octyl butanoate, propyl pentanoate, pentyl pentanoate, propyl heptanoate, 2-methylpropyl octanoate, and pentyl octanoate) identified previously (Dimick and Hoskin, 1983; Lo Scalzo et al., 2001; Paillard, 1990) were not detected in this study. The five 'new' esters were not likely taxa-specific, as they were found in several *M.* ×*domestica* lines. While there were some exceptions, larger fruit tended to have a greater diversity of esters (Figure 22). Large fruit averaged 46 different esters per variety compared to small fruit, which averaged 10 ester types per variety.

Although ester formation seems to be fundamentally a function of precursor availability, diversity in the ester profile may be partially explained by the activity of AAT, which combines an alcohol with an acid-derived CoA to form the esters. The *AAT* gene has been identified in several horticultural crops such as apple, strawberry, melon, banana, and mountain papaya (Aharoni et al., 2000; Balbontín et al., 2010; Harada et al., 1985; Jayanty et al., 2002; Souleyre et al., 2005; Yahyaoui et al., 2002). AAT has features that have the potential to influence the ester profile: while some isozymes can utilize a broad range of alcohol and acyl-CoA precursors they can also exhibit a marked substrate preference (El-Sharkawy et al., 2005; Yahyaoui et al., 2002). The reaction velocity is affected by carbon chain length, and architecture (e.g. straight- or branched-chain) of acyl-CoAs, or alcohol substrates (Aharoni et al., 2000; Olias et al., 2002;

Ueda et al., 1992; Yahyaoui et al., 2002). Also, AAT substrate preference differs by fruit species and, within a species, between cultivars (Holland et al. 2005).

There were several apple lines that produced predominantly SC and a small amount of BC esters, while others produced more nearly equal amounts of SC and BC esters (Table 12). The high SC or BC ester producing lines did not associate with particular species or country of origin. This may relate to the complexity of the *Malus* ancestry where several 'species' are likely hybrids. Of the lines grown for commercial fruit consumption, 'Cox's Orange Pippin' could be classified as a high SC line and 'Jonathan' as a BC line. The extreme range in the ratios of SC and BC esters suggests that these two ester classes are synthesized via differing pathways or are differentially regulated. The utility of this sort of information lies in its potential to be used for further metabolic study. Since ester formation is primarily a function of precursor availability rather than AAT activity (Echeverría et al., 2004; Wyllie and Fellman, 2000), genetic and biochemical evaluation of apple lines with distinct ester profiles should be informative. For instance, an Italian cultivar 'Annurca' produces primarily SC ester (96.8%) and little BC esters (Lo Scalzo et al., 2001). Most of the BC esters produced by apple are derived from 2-methylbutanol or 2-methylbutanoate. Although these compounds are known to be product of isoleucine metabolism (Rowan et al., 1996; Sugimoto et al., 2011), the relationship between BC esters and isoleucine accumulation has not been investigated. The diversity of BC and SC ester production detected in this study suggests the regulation of ester precursor supply may be explored using this source. The involvement of β-oxidation, the lipoxygenase pathway, and BCAA metabolism for ester formation has been suggested based on the results of various substrate feeding studies, as yet the mechanism of the regulation of ester biosynthesis is not fully understood and further molecular and biochemical characterization is warranted.

When the esters were classified according to their alkyl and alkanoate groups, cluster analysis for the esters across the 184 lines suggested that several classes of esters were more closely associated than others, implying regulatory linkages (Figure 23). Unsaturated esters do not cluster with any of the other ester types, which is suggestive of independent regulation. This is supported by what is known about the pathway of formation of the unsaturated C-6 aldehyde precursors (Feussner and Wasternack, 2002), which, as far as is known, would be exclusively via the lipoxygenase pathway. Additionally, a seven-member alkanoate cluster (propanoate, 2-methylbutanoate, butanoate, 2-methylpropanoate, pentanoate, heptanoate and hexanoate esters), clustered separately from a six-member alkyl ester cluster (propyl, 2-methylbutyl, butyl, 2-methylpropyl, pentyl, and hexyl esters). With the exception of heptyl esters, the members of these two clusters are acid and alcohol equivalents of the same carbon backbone. However, the separate clustering according to acid and alcohol precursors suggests differing metabolic regulation of corresponding acid and alcohol precursors for these ester classes.

Within the alkanoate cluster, propanoate, 2-methylbutanoate, and butanoate esters clustered together. The co-regulation of propanoate, 2-methylbutanoate, and butanoate esters is consistent with the biology of the proposed 'citramalate pathway' for ester synthesis in apple (Sugimoto et al., 2011). In the proposed pathway, citramalate formation leads to the production of precursors for the formation of esters composed of 2-methylbutanol and 2-methylbutanoate subgroups, and 3- to 5- and longer-carbon SC subgroups.

Alcohols. Ester diversity is dependent on the diversity of the precursors apples synthesize. The alcohol precursors were not uniformly found in fruit of different size categories (Figure 24). Small fruit tended to produce methanol and unsaturated alcohols, whereas larger fruit tended to

form BC alcohols and 3- to 6-carbon SC alcohols. All the alcohols that we detected were similar to those reported previously (Dimick and Hoskin, 1983; Mattheis et al., 1991a; Paillard, 1990). In addition, the alcohols 2-butanol, 3-pentanol, 1-penten-3-ol, 2-methyl-2-propanol, and 2-penten-ol were also tentatively identified, but not quantified. The alcohols detected include some that have not been reported in apple fruit, but have been detected in other fruits, namely 1-penten-3-ol and 2-methyl 2-propanol found in tangerine (Miyazaki et al., 2011) and 2-penten-ol found in yellow passion fruit (Werknoff et al., 1998) and kiwi (Takeoka et al., 1986). On the other hand, 3-penten-2-ol, which has an apple-like aroma and found in 'Starkspur Golden' fruit (Vanoli et al., 1995) was not detected.

Large fruit had more diversity and a higher production of esters and alcohols, but did not tend to produce the more peculiar aromas. The taste of commercial apples was perceived as generally balanced with sweetness, acidity, and florals, and fruity notes. Cultivars perceived as only sweetness tended to have a bland taste. Small fruit were more astringent than large fruit. Unusual taste/aromas included broccoli-like, tomato-like, and butter (crayon)-like (Table 13). Dimethyl sulfide was detected in Vilmorin (accession no. 271831) and likely contributes to a broccoli-like taste, since this compound is an aroma impact compound in the *Brassica* family (Buttery et al., 1976). Some of the breeding lines developed for scab resistance (Crosby et al., 1992), which included several 'PRI' lines, 'Co-op 15', 'Redfree', 'Prima', 'Jonafree', and 'Dayton', and had odd flavors or scents, or perceived an imbalance of sweetness and acidity. Several varieties had unusual aromas and associated peculiarities in the aroma profile. Two varieties, Kerr (accession no. 588866) and PRI 2050-2 (accession no. 589819) produced lilac alcohol. This compound is a derivative of a terpenoid metabolism and is found in lilac (*Syringa vulgaris*) flowers and kiwifruit (*Actinidia arguta*) flowers (Matich et al., 2003). Kerr fruit was

the only fruit to possess a noticeable lychee-like aroma. Kerr produced rose oxide, which is found in rose (Flament et al., 1993; Shalit et al., 2003) and geranium leaf (Wüst et al., 1998). Rose oxide contributes to rose scent, has a low odor threshold (Wüst and Mosandl, 1999), and is considered to be an aroma impact compound in lychee (*Litchi chinesi*) fruit (Ong and Acree, 1998), but is also found in grape (*Vitis vinifera*) (Luan et al., 2005).

Storage. The 60 germplasm lines in the Geneva Core collection that had fruit mass greater than 30 g included *M. asiatica* (1 line), *M. ×domestica* (37 lines), *M. ×hybrid* (20 lines), *M. prunifolia* (1 line), and *M. sieversii* (1 line). The IEC for these fruit ranged from 1.7 to 925.3 $\mu L \cdot L^{-1}$ after 3 months storage and 2.7 to 868.3 $\mu L \cdot L^{-1}$ after 6 months storage (Table 14). Approximately 63% of the apples stored for 3 months and 47% of those lines stored for 6 months had an IEC greater than 100 $\mu L \cdot L^{-1}$. Several apple lines had significantly lower ethylene levels than most commercial varieties. Low ethylene production has been associated with good keeping quality in terms of retention of firm flesh texture (DeEll et al., 2001; Johnson and Colgan, 2003; Watkins, 2001), however, in our study, the correlation between firmness and IEC after 3- and 6-month storage was poor (3-month storage: R^2=0.0075, 6-months storage: R^2=0.0043). Soluble solids content ranged from 11.8% to 19.3% for fruit held for 3 months and 11% to 18% for fruit held for 6 months in refrigerated storage. Some fruit retained a slight amount of starch; approximately 23% (14 lines) of the germplasm lines had a starch index below 8 after 3-month storage and by 6 months only 2 lines had a starch index below 8. Firmness ranged from 28.0 to 97.9 N for fruit stored 3 months and 37.8 to 79.2 N for fruit held 6 months in refrigerated storage. About 30% of the 3-month fruit had a firmness level of 62.3 N or greater; this percentage decreased to approximately 21% after 6 months.

Approximately 60% (36 lines) of the 3-month stored fruit were free from storage disorders; however, after 6 months, only 22% (13 lines) had no disorders. Common disorders included soft scald, internal browning, bitter pit, sun scald, senescent breakdown, and superficial scald. Out of six taxa evaluated, detailed storage disorder data are only shown for *M. ×domestica* and *M. ×hybrid* lines (Figure 25). Over all, *M. ×domestica* lines had less chilling injury and superficial scald occurrence than *M. ×hybrid* lines after 3- and 6-months storage. *M. asiatica* had no disorders whereas *M. prunifolia* and *M. sieversii* all had superficial scald after 3- and 6-months storage.

Bitter pit was only found in *M. ×domestica* lines and no bitter pit was detected in *M. ×hybrid* lines even after 6 months. This observation was consistent with previous findings that cultivar has a significant effect on bitter pit incidence (Volz et al., 2006). There were five commercial varieties among the 13 lines with no disorders: 'Marshall McIntosh', 'Empire', 'Gala', 'Fuji Red Sport Type2', and 'Golden Delicious', despite the report of susceptibility to bitter pit in 'Golden Delicious' (Scott et al., 1985), and to soft scald in 'Fuji', 'McIntosh', and 'Golden Delicious' (Watkins et al., 2004). After 6 months, over 70% of the stored fruit lines were lost due to senescent breakdown and decay.

Conclusion

Our data expands on the data available in the GRIN system. It is hoped the information of ester character provided here will lead to the development of new breeding lines with better flavor quality and that the data on storage disorders will lead to the identification of useful germplasm for the development of disorder-free cultivars. This evaluation of the patterns of esters appears to support recent insights into the metabolic pathways of precursor biosynthesis

during ripening, and more utility in physiological, biochemical and molecular studies is certainly possible. In that some of the varieties contained peculiar aroma notes, it is conceivable that the development of new, interesting flavor lines having commercial value is possible. We are optimistic that the use of the collected data will aid in the development of fruit with good flavor and will benefit both growers and consumers in an increasingly competitive world market for apple.

Table 8. Range of values for four maturity indices for 184 lines of the Geneva *Malus* Core Collection and Kazakhstan apple lines (all *M. sieversii*) used to determine the optimum ripening stage for volatiles analysis. Fruit were harvested on nine dates between 24 Aug., 2005 and 24 Oct., 2005 from the USDA Plant Genetic Resources Unit's *Malus* Germplasm Repository at Cornell University, Geneva, NY. Apples were collected at a mature stage. Measurements were taken one day after one day equilibration to room temperature and/or waited until when fruits were assessed to be at an optimum ripe stage for volatile analysis. Fruit were classified into five categories based on the mass and the number of accessions that belongs to the category is shown. Maturity indices include internal ethylene content, °Brix, starch index (1 – 8) based on the Cornell starch chart, and firmness. n.d. indicates not determined.

			Maturity index			
Fruit classification	Mass (g)	No. of accession	Ethylene ($\mu L \cdot L^{-1}$)	°Brix	Starch index	Firmness (N)
small	<2	35		14-32	5–8	n.d.
small–medium	2–10	20	0.02–176.6	13–32	1–8	n.d.
medium	10–40	27	0.05–607.1	11–20.5	3–8	47.6–126.8
medium–large	40–100	25	0.19–761.1	11.5–19	2–8	49.4–138.0
large	>100	77	1.09–782.2	11–17.5	3–8	24.0–109.0

Table 9. Matrix of esters detected organized by acid and alcohol moieties for 184 lines of the Geneva *Malus* Core Collection and Kazakhstan apple lines (all *M. sieversii)*. Each box indicates the ester derived from corresponding alcohol (left column) and acid (top row), e.g. the alcohol ethanol and acid acetic acid indicates the ester ethyl acetate. Numbers in the upper portion of each box indicate the sum of all ester concentrations in the sampling chambers ($nmol \cdot L^{-1}$) and that in the lower portion of each box indicate the percentage of the 184 lines producing that ester in detectable quantities. Prop, 2-Mprop, But, 2-Mbut, Pent, Hex, Hep, Oct, 2-Mpropanol, and 2-Mbutanol indicate propanoic, 2-methylpropanoic, butanoic, 2-methylbutanoic, pentanoic, hexanoic, heptanoic, octanoic, 2-methylpropanol, and 2-methylbutanol, respectively. n.d. indicates not detected.

Alcohol	Acid moiety								
moiety	Acetic	Prop	2-Mprop	But	2-Mbut	Pent	Hex	Hep	Oct
Methanol	187	21	1	214	132	1	19	n.d.	0
	16%	23%	2%	51%	64%	14%	44%	n.d.	12%
Ethanol	6,096	1,570	70	2,093	1,227	35	377	4	50
	74%	62%	39%	79%	75%	38%	72%	20%	35%
Propanol	1,538	354	11	451	303	n.d.	58	n.d.	2
	67%	66%	49%	77%	74%	n.d.	70%	n.d.	26%
2-Mpropanol	259	25	1	35	14	n.d.	4	n.d.	n.d.
	60%	26%	4%	52%	55%	n.d.	30%	n.d.	n.d.
Butanol	3,602	673	32	933	409	12	375	3	32
	97%	77%	65%	83%	82%	62%	80%	34%	53%
2-Mbutanol	2,384	102	2	47	52	n.d.	41	n.d.	n.d.
	84%	61%	21%	73%	64%	n.d.	65%	n.d.	n.d.

Table 9 (cont'd).

Alcohol	Acid moiety								
moiety	Acetic	Prop	2-Mprop	But	2-Mbut	Pent	Hex	Hep	Oct
Pentanol	504	110	2	32	27	n.d.	12	n.d.	n.d.
	74%	50%	24%	68%	65%	n.d.	59%	n.d.	n.d.
Hexanol	1,809	264	18	277	1,053	8	301	n.d.	18
	91%	76%	71%	79%	89%	53%	77%	n.d.	14%
Heptanol	10	n.d.	n.d.	1	2	n.d.	n.d.	n.d.	n.d.
	27%	n.d.	n.d.	11%	21%	n.d.	n.d.	n.d.	n.d.
Octanol	3	n.d.	n.d.	n.d.	1	n.d.	n.d.	n.d.	n.d.
	9%	n.d.	n.d.	n.d.	4%	n.d.	n.d.	n.d.	n.d.

Table 10. The top 15 highest ester producing lines categorized by the alkanoate (acid) and alkyl (alcohol) subgroups from 184 lines of the Geneva *Malus* Core Collection and Kazakhstan apple lines (all *M. sieversii*). Each row shows the plant common name, taxon, and the accession no. There are some species that do not have a common name and are indicated "unnamed". Acet, Prop, But, Pent, Hex, Hep, Oct, 2-Mprop, 2-Mbut, 2-Mpropyl, and 2-Mbutyl indicate acetates, propanoates, butanoates, pentanoates, hexanoates, heptanoates, octanoates, 2-methylpropanoates, 2-methylbutanoates, 2-methylpropyl, and 2-methylbutyl, respectively.

Alkanoate esters									
Rank	Acet	Prop	But	Pent	Hex	Hep	Oct	2Mprop	2Mbut
1	E29-56	Delicious	Keepsake	Viking	Viking	Viking	Viking	Viking	Delicious
	hybrid	domestica	domestica	domestica	domestica	domestica	domestica	domestica	domestica
	590071	589841	589894	589434	589434	589434	589434	589434	589841
2	Ingol	Keepsake	Murray	Zelenova Sotchu	Delicious	Delicious	KAZ95 18-10	Zelenova Sotchu	PRI 1279-9
	domestica	domestica	domestica	domestica	domestica	domestica	sieversii	domestica	hybrid
	589441	589894	589486	589546	589841	589841	633800	589546	589791
3	Liberty	PRI 1279-9		Delicious	Zelenova Sotchu	Halberstadter Ju	E7-47	KAZ96 08-16	Cortland
	domestica	hybrid	domestica	domestica	domestica	domestica	hybrid	sieversii	domestica
	588943	589791	594106	589841	589546	132759	590069	613998	588848

Table 10 (cont'd).

Alkanoate esters									
Rank	Acet	Prop	But	Pent	Hex	Hep	Oct	2Mprop	2Mbut
4	PRI 1850-4	Marshall	PRI 1279-9	Halberst-	Dorsett	KAZ96		Halberstadter	Halberstadter
		McIntosh		adter Ju	Golden	08-16		Ju	Ju
	hybrid	domestica	hybrid	domestica	domestica	sieversii	kirghisorum	domestica	domestica
	589792	588998	589791	132759	589913	613998	589380	132759	132759
5	E7-47	Cortland	Marshall	KAZ95	PRI 1279-9	Zelenova	KAZ96	KAZ95	Liberty
			McIntosh	18-02P-33		Sotchu	02-01P-11	05-01P-22	
	hybrid	domestica	domestica	sieversii	hybrid	domestica	sieversii	sieversii	domestica
	590069	588848	588998	633801	589791	589546	633803	633918	588943
6	KAZ96	Wijcik	Wijcik	KAZ96	KAZ96	KAZ95	KAZ95		Wijcik
	09-02	McIntosh	McIntosh	08-16	09-05	18-10	18-02P-33		McIntosh
	sieversii	domestica	domestica	sieversii	sieversii	sieversii	sieversii	ioensis	domestica
	614000	590186	590186	613998	633921	633800	633801	590008	590186

Table 10 (cont'd).

Alkanoate esters									
Rank	Acet	Prop	But	Pent	Hex	Hep	Oct	2Mprop	2Mbut
7	PRI 1312-6	Murray	Zelenova Sotchu	Wijcik McIntosh		Murray	Zelenova Sotchu	KAZ95 18-10	PRI 1312-6
	hybrid	domestica	domestica	domestica	domestica	domestica	domestica	sieversii	hybrid
	590079	589486	589546	590186	594106	589486	589546	633800	590079
8	Dolgo	Halberst-adter Ju	Delicious	KAZ95 18-10	KAZ96 02-01P-11	E29-56	Bushy Grove	Murray	Tolman Sweet
	hybrid	domestica	domestica	sieversii	sieversii	hybrid	domestica	domestica	domestica
	588870	132759	589841	633800	633803	590071	135999	589486	588805
9	KAZ96 08-16	Empire	Tolman Sweet	Cortland	KAZ96 08-16	PRI 1279-9	KAZ96 08-16	Marshall McIntosh	Kimball McIntosh 2-4-4-4
	sieversii	domestica	domestica	domestica	sieversii	hybrid	sieversii	domestica	domestica
	613998	588842	588805	588848	613998	589791	613998	588998	589122

Table 10 (cont'd).

Alkanoate esters									
Rank	Acet	Prop	But	Pent	Hex	Hep	Oct	2Mprop	2Mbut
10	Delicious	Tolman	Empire		E31-10	E7-47	Halberst-	Bushy	Keepsake
	Sweet						adter Ju	Grove	
	domestica	domestica	domestica	domestica	hybrid	hybrid	domestica	domestica	domestica
	589841	588805	588842	594106	590072	590069	132759	135999	589894
11	Keepsake	PRI 1176-1	KAZ95	Marshall	E7-47	KAZ96	PRI 1279-9	Tolman	
			18-02P-33	McIntosh		02-01P-11		Sweet	
	domestica	hybrid	sieversii	domestica	hybrid	sieversii	hybrid	domestica	domestica
	589894	590085	633801	588998	590069	633803	589791	588805	594106
12	PRI 1279-9	E29-56	Viking	PRI	E29-56			PRI 1279-9	Murray
				1279-9					
	hybrid	hybrid	domestica	hybrid	hybrid	kirghisorum	kirghisorum	hybrid	domestica
	589791	590071	589434	589791	590071	589380	590043	589791	589486

Table 10 (cont'd).

Alkanoate esters									
Rank	Acet	Prop	But	Pent	Hex	Hep	Oct	2Mprop	2Mbut
13	Ein Shemer		E31-10	Murray	Laxton's Fortune	Tolman Sweet	Northern Spy		Fuji Red Sport Type2
	domestica 280401	domestica 594106	hybrid 590072	domestica 589486	domestica 589646	domestica 588805	domestica 588872	domestica 594106	domestica 588844
14	Northern Spy	Zelenova Sotchu	Rambo-Red Summer	PRI 1850-4	KAZ95 18-10	KAZ95 18-02P-33		Redfree	E29-56
	domestica 588872	domestica 589546	domestica 588798	hybrid 589792	sieversii 633800	sieversii 633801	domestica 594106	domestica 594111	hybrid 590071
15	orientalis 594095	KAZ96 08-16 sieversii 613998	Halberst-adter Ju domestica 132759	KAZ95 05-01P-22 sieversii 633918	x soulardii 589391	Ein Shemer domestica 280401	Dorsett Golden domestica 589913	Liberty domestica 588943	KAZ96 08-16 sieversii 613998

Table 10 (cont'd).

Alkyl esters						
Rank	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
1	E29-56 hybrid 590071	E29-56 hybrid 590071	Delicious domestica 589841	Ingol domestica 589441	Delicious domestica 589841	PRI 1279-9 hybrid 589791
2	Murray domestica 589486	Liberty domestica 588943	Sweet Delicious domestica 588955	Golden Delicious domestica 590184	PRI 1754-2 hybrid 589794	Laxton's Fortune domestica 589646
3	domestica 594106	Dolgo hybrid 588870	PRI 1176-1 hybrid 590085	Laxton's Fortune domestica 589646	Laxton's Fortune domestica 589646	Jonafree domestica 589962
4	Spokane Beauty domestica 589006	PRI 1850-4 hybrid 589792	Redspur Delicious domestica 589255	PRI 1346-2 hybrid 589785	Ein Shemer domestica 280401	Northern Spy domestica 588872
5	PRI 2482-100 hybrid 589795	Ingol domestica 589441	PRI 1346-2 hybrid 589785	Northern Spy domestica 588872	PRI 1346-2 hybrid 589785	domestica 594106

Table 10 (cont'd).

Alkyl esters						
Rank	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
6	PRI 1850-4	KAZ96 08-16	E29-56	Calville Blanc	Northern Spy	Golden Delicious
	hybrid 589792	sieversii 613998	hybrid 590071	domestica 589596	domestica 588872	domestica 590184
7	PRI 1279-9	KAZ96 09-02	Ein Shemer	Delicious	PRI 1176-1	Co-op 15
	hybrid 589791	sieversii 614000	domestica 280401	domestica 589841	hybrid 590085	hybrid 589805
8		Keepsake	PRI 1754-2	Mollie's Delicious	Cox's Orange Pippin	Jonsib Crab
	orientalis 594095	domestica 589894	hybrid 589794	domestica 588981	domestica 588853	hybrid 589824
9	Trebu Seklandzis	E7-47	PRI 1279-9	Burgundy	E29-56	PRI 1346-2
	domestica 262966	hybrid 590069	hybrid 589791	domestica 588835	hybrid 590071	hybrid 589785
10		PRI 1312-6	Ingol	Jonafree	Jonafree	Delicious
	halliana 594112	hybrid 590079	domestica 589441	domestica 589962	domestica 589962	domestica 589841

Table 10 (cont'd).

Alkyl esters						
Rank	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
11		PRI 1279-9	Fuji Red Sport Type2	Ein Shemer	Redspur Delicious	Calville Blanc
	asiatica	hybrid	domestica	domestica	domestica	domestica
	594099	589791	588844	280401	589255	589596
12	E31-10	Viking	Winter Majetin	E29-56	PRI 1279-9	Smith Jonathan
	hybrid	domestica	domestica	hybrid	hybrid	domestica
	590072	589434	589645	590071	589791	589845
13	KAZ96 05-05	Empire	Northern Spy	E.8	Smith Jonathan	Anna
	sieversii	domestica	domestica	domestica	domestica	domestica
	633919	588842	588872	590179	589845	280400
14	Ein Shemer	Florina	Smith Jonathan	Prima	Prima	KAZ96 08-16
	domestica	orientalis	domestica	domestica	hybrid	hybrid
	280401	594095	588747	589845	589181	589181

Table 10 (cont'd).

Alkyl esters						
Rank	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl
15		Halberstadter Ju	Co-op 15	PRI 1279-9	Golden Delicious	E7-47
	domestica	domestica	hybrid	hybrid	domestica	hybrid
	323617	132759	589805	589791	590184	590069

Alkyl esters					
Rank	Heptyl	Octyl	2-Mpropyl	2-Mbutyl	Unsaturated
1	Ein Shemer		PRI 1176-1	Delicious	Laxton's Fortune
	domestica	domestica	hybrid	domestica	domestica
	280401	594106	590085	589841	589646
2	Ottawa5	Burgundy	Delicious	PRI 1176-1	Cox's Orange Pippin
	hybrid	domestica	domestica	hybrid	domestica
	589349	588835	589841	590085	588853

Table 10 (cont'd).

Alkyl esters					
Rank	Heptyl	Octyl	2-Mpropyl	2-Mbutyl	Unsaturated
3		Dorsett Golden	Sweet Delicious	Fuji Red Sport Type2	PRI 1279-9
	domestica 594106	domestica 589913	domestica 588955	domestica 588844	hybrid 589791
4	Dorsett Golden	KAZ96 05-05	Calville Blanc	Idared	E7-47
	domestica 589913	sieversii 633919	domestica 589596	domestica 588841	hybrid 590069
5	Delicious	E14-32	Ein Shemer	Northern Spy	Delicious
	domestica 589841	hybrid 589572	domestica 280401	domestica 588872	domestica 589841
6	Jonafree	Ein Shemer	Smith Jonathan	Jonsib Crab	
	domestica 589962	domestica 280401	domestica 589845	hybrid 589824	domestica 619168

Table 10 (cont'd).

Alkyl esters					
Rank	Heptyl	Octyl	2-Mpropyl	2-Mbutyl	Unsaturated
7	PRI 1279-9	Ottawa5	E29-56	Sweet Delicious	Robusta 5
	hybrid 589791	hybrid 589349	hybrid 590071	domestica 588955	x robusta 588825
8	Co-op 15	PRI 1732-2	Redspur Delicious	Smith Jonathan	Empire
	hybrid 589805	hybrid 589946	domestica 589255	domestica 589845	domestica 588842
9	Burgundy	PRI 1316-1	Northern Spy	PRI 1312-6	Roberts Crab
	domestica 588835	hybrid 589776	domestica 588872	hybrid 590079	hybrid 437057
10	PRI 1346-2	KAZ96 08-16		Esopus Spitzenburg	Landsberger Reinette
	hybrid 589785	sieversii 613998	domestica 619168	domestica 588785	domestica 589565
11	PRI 1316-1	Jonafree		Redspur Delicious	Sweet Delicious
	hybrid 589776	domestica 589962	ioensis 590008	domestica 589255	domestica 588955

Table 10 (cont'd).

Alkyl esters					
Rank	Heptyl	Octyl	2-Mpropyl	2-Mbutyl	Unsaturated
12	E29-56	Cox's Orange Pippin	E7-54 Blanc	Calville Russet	Rosemary
	hybrid	domestica	hybrid	domestica	domestica
	590071	588853	590070	589596	589648
13	Prima	Co-op 15	Jonsib Crab	Golden Delicious	Winter Majetin
	hybrid	hybrid	hybrid	domestica	domestica
	589181	589805	589824	590184	589645
14		PRI 1279-9	Florina	Kansas K14	
	sieversii	asiatica	hybrid	domestica	hybrid
	613998	594107	589791	588747	588804
15	PRI 1754-2	Irish Peach	Burgundy	Liberty	Virginiagold
	hybrid	domestica	domestica	domestica	domestica
	589794	104727	588835	588943	588778

Table 11. Lines from the Geneva *Malus* Core Collection and Kazakhstan apple (all *M. sieversii*) having highest (top 20) ester abundance (top table) and ester diversity (bottom table). Concentration is abbreviated as 'conc.'. 'Conc. rank' is based on the ranking from high (1) to low (184) amount of ester produced. 'Count rank' is similarly ranked based on the number of esters detected. The maximum ester count is 67 including 3 unsaturated esters.

Conc. rank	Accession no.	Common name	Taxon	Conc. $nmol \cdot L^{-1}$	Count rank
1	590071	E29-56	hybrid	950	4
2	589441	Ingol	domestica	793	88
3	588943	Liberty	domestica	757	47
4	589841	Delicious	domestica	715	2
5	589792	PRI 1850-4	hybrid	652	56
6	590069	E7-47	hybrid	638	13
7	590079	PRI 1312-6	hybrid	622	27
8	589894	Keepsake	domestica	617	73
9	589791	PRI 1279-9	hybrid	614	3
10	614000	KAZ96 09-02	sieversii	610	35
11	613998	KAZ96 08-16	sieversii	584	13
12	588870	Dolgo	hybrid	566	27
13	280401	Ein Shemer	domestica	447	4
14	594106		domestica	430	1
15	588842	Empire	domestica	419	47
16	589255	Redspur Delicious	domestica	389	16

Table 11 (cont'd).

Conc. rank	Accession no.	Common name	Taxon	Conc. nmol·L^{-1}	Count rank
17	588844	Fuji Red Sport Type 2	domestica	385	35
18	588872	Northern Spy	domestica	384	67
19	588955	Sweet Delicious	domestica	371	16
20	589646	Laxton's Fortune	domestica	350	73

Count. rank	Accession no.	Common name	Taxon	Ester count	Conc. rank
1	594106		domestica	61	14
2	589841	Delicious	domestica	59	4
3	589791	PRI 1279-9	hybrid	58	9
4	590071	E29-56	hybrid	56	1
4	589795	PRI 2482-100	hybrid	56	42
4	280401	Ein Shemer	domestica	56	13
5	589805	Co-op 15	hybrid	55	37
5	588848	Cortland	domestica	55	29
5	437042	P.13	domestica	55	56

Table 11 (cont'd).

Count. rank	Accession no.	Common name	Taxon	Ester count	Conc. rank
6	590072	E31-10	hybrid	54	65
6	589913	Dorsett Golden	domestica	54	68
6	262966	Trebu Seklandzis	domestica	54	53
7	613998	KAZ96 08-16	sieversii	53	11
7	590069	E7-47	hybrid	53	6
7	588798	Rambo-Red Summer	domestica	53	67
8	589122	Kimball McIntosh 2-4-4-4	domestica	52	62
8	588955	Sweet Delicious	domestica	52	19
8	589255	Redspur Delicious	domestica	52	16
8	590070	E7-54	hybrid	52	63
8	589807	PRI 1773-6	hybrid	52	74

Table 12. Twenty lines from the 184 lines of the Geneva *Malus* Core Collection and Kazakhstan apple lines (all *M. sieversii*) having either in straight-chain (SC) esters or branched-chain (BC) esters based on the ratio of SC/BC ester production. SC esters are the sum of all SC alcohol and acid derived esters and BC ester are the sum of either BC alcohol or acid derived esters.

Straight-chain ester producing lines					
Accession no.	Common name	Taxon	SC ester $nmol \cdot L^{-1}$	BC ester $nmol \cdot L^{-1}$	Ratio SC/BC
594095		orientalis	284.64	1.18	241.52
594099		asiatica	216.20	1.00	215.15
589792	PRI 1850-4	hybrid	630.26	21.67	29.09
633919	KAZ96 05-05	sieversii	103.16	6.38	16.18
588778	Virginiagold	domestica	104.09	7.48	13.91
589441	Ingol	domestica	739.41	53.97	13.70
589380		kirghisorum	105.59	8.11	13.02
588870	Dolgo	hybrid	525.34	40.83	12.87
588835	Burgundy	domestica	234.56	19.85	11.82
262966	Trebu Seklandzis	domestica	188.57	19.90	9.48
104727	Irish Peach	domestica	306.82	34.26	8.95
590069	E7-47	hybrid	572.99	64.26	8.92
589962	Jonafree	domestica	205.34	23.87	8.60
590071	E29-56	hybrid	851.00	98.99	8.60
588853	Cox's Orange Pippin	domestica	154.43	21.90	7.05

Table 12 (cont'd).

Straight-chain ester producing lines					
Accession no.	Common name	Taxon	SC ester $nmol \cdot L^{-1}$	BC ester $nmol \cdot L^{-1}$	Ratio SC/BC
613998	KAZ96 08-16	sieversii	507.58	76.52	6.63
614000	KAZ96 09-02	sieversii	527.35	82.22	6.41
589894	Keepsake	domestica	525.34	91.45	5.74
633918	KAZ95 05-01P-22	sieversii	145.35	28.64	5.07
588943	Liberty	domestica	631.72	124.68	5.07

Branched-chain ester producing lines					
Accession no.	Common name	Taxon	SC ester $nmol \cdot L^{-1}$	BC ester $nmol \cdot L^{-1}$	Ratio SC/BC
594111	Redfree	domestica	60.43	32.37	1.87
589122	Kimball McIntosh 2-4-4-4	domestica	111.80	60.41	1.85
589780	PRI 384-1	hybrid	59.17	33.05	1.79
588841	Idared	domestica	163.71	91.54	1.79
588772	Monroe	domestica	104.05	62.82	1.66

Table 12 (cont'd).

Branched-chain ester producing lines					
Accession no.	Common name	Taxon	SC ester $nmol \cdot L^{-1}$	BC ester $nmol \cdot L^{-1}$	Ratio SC/BC
588866	Kerr	hybrid	37.94	23.48	1.62
590070	E7-54	hybrid	105.53	66.23	1.59
588837	Gravenstein Washington Red	domestica	70.36	44.45	1.58
135999	Bushy Grove	domestica	89.30	57.96	1.54
589807	PRI 1773-6	hybrid	89.79	58.94	1.52
589824	Jonsib Crab	hybrid	171.84	114.21	1.50
589382		domestica	60.12	41.71	1.44
483257	Reinette Simirenko	domestica	60.00	43.10	1.39
590085	PRI 1176-1	hybrid	200.63	145.14	1.38
589775	PRI 2382-1	hybrid	27.21	20.59	1.32
589415	Hoopesii	x platycarpa	21.78	17.87	1.22
589786	PRI 77-1	hybrid	13.80	14.16	0.97
590185	Jonathan	domestica	20.65	21.53	0.96
589490	Trent	domestica	35.53	37.83	0.94
588838	Nova Easygro	domestica	44.22	59.30	0.75

Table 13. Twenty lines from the 184 lines of the Geneva *Malus* Core Collection and an additional 12 Kazakhstan apple lines (all *M. sieversii*) having unusual tastes, scents, or fruit flesh color.

Accession no.	Common name	Taxon	Notes
271831	Vilmorin	yunnanensis	broccoli, cucumber, vegetative taste, dimethyl sulfide
588772	Monroe	domestica	tomato scent, green
588778	Virginiagold	domestica	mildly tart, simple aroma profile Primarily hexyl & butyl acetate
588835	Burgundy	domestica	perfume
588866	Kerr	hybrid	litchee taste and scent
588922	Yellow Autumn Crab	x sublobata	quince scent, mildly astringent, orange-yellow flesh, grassy
589253	Carmine Crab	x atrosanguinea	spicy hot
589421	M. rockii	sp.	very astringent
589646	Laxton's Fortune	domestica	caraway seed, unusual scent, plastic,
589726	Britegold	domestica	papaya, banana
589776	PRI 1316-1	hybrid	off-flavor, tropical fruit
589780	PRI 384-1	hybrid	feces scent
589819	PRI 2050-2	hybrid	fruity, mild buttery scent, has lilac alcohol

Table 13 (cont'd).

Accession no.	Common name	Taxon	Notes
589933		fusca	tart like rhubarb
589975		fusca	green flesh
589996		coronaria	buttery skin scent
590079	PRI 1312-6	hybrid	melon
633801	KAZ95 18-02P-33	sieversii	has long-chain alcohols, floral
633802	KAZ96 08-17	sieversii	grapy, berry
633918	KAZ95 05-01P-22	sieversii	juicy, fruity, pink flesh

Table 14. Storage quality analysis (internal ethylene content, °Brix, starch index (1-8), firmness, and number of fruit loss due to decay after storage) for 60 lines of the Geneva *Malus* Core Collection sorted by fruit size and to test for storability. The measurements were taken after 3 month (3M) and 6 month (6M) in air at 0 °C. Fruit were classified into five categories based on the mass and the number of accessions that belongs to the category is shown.

3M Storage quality index						
Fruit mass (g)	No. of accession	Ethylene ($\mu L \cdot L^{-1}$)	°Brix	Starch index	Firmness (N)	Fruit loss (%)
30–60	6	1.7–307.6	12.7–19.3	6.9–8	61.0–97.9	0
60–100	10	50.0–536.9	13.3–15.6	7–8	30.7–86.3	0
100–150	23	7.4–925.3	11.8–16.3	6.2–8	35.2–89.0	0
150–200	14	44.6–325.9	13–15.8	7.8–8	35.2–81.0	4
>200	7	14.9–681.3	14–16.8	7.2–8	28.0–62.7	11

6M Storage quality index						
Fruit mass (g)	No. of accession	Ethylene ($\mu L \cdot L^{-1}$)	°Brix	Starch index	Firmness (N)	Fruit loss (%)
30–60	6	7.4–326.8	16.5–18	7.7–8	61.9–79.2	45
60–100	10	41.7–555.2	13.3–15	8	42.3–68.5	7
100–150	23	2.7–868.3	11–17.7	7.5–8	38.7–73.9	16
150–200	14	2.9–251.6	11.8–16.2	8	40.1–69.9	20
>200	7	90.6–538.8	12.3–16	8	37.8–50.3	39

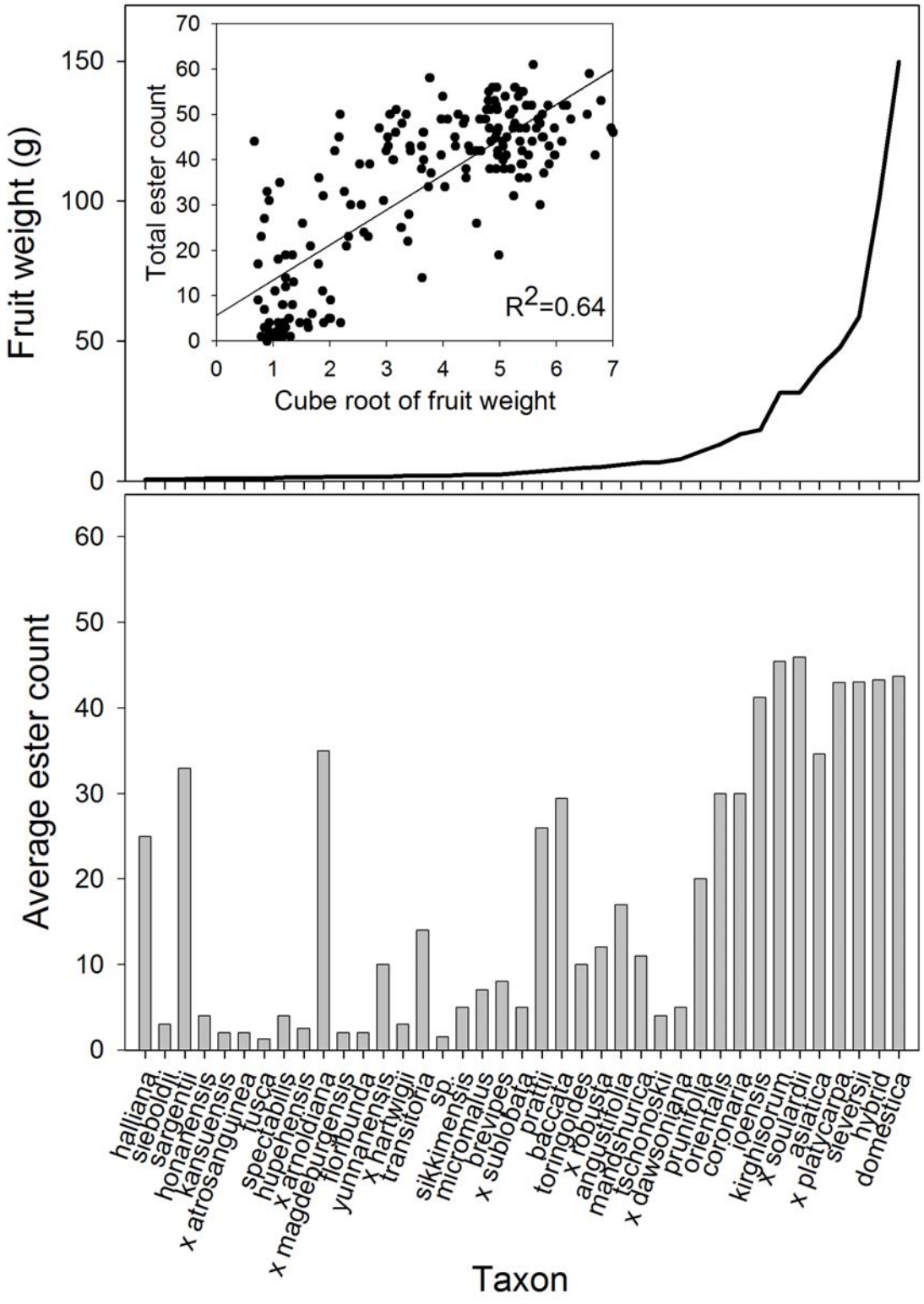
Fruit weight (g)
150
100
50
0
Total ester count
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7
Cube root of fruit weight
R2=0.64
Average ester count
60
50
40
30
20
10
0
halliana
sieboldii
sargentii
honanensis
kansuensis
x atrosanguinea
fusca
spectabilis
hupehensis
x arnoldiana
x magdeburgensis
floribunda
yunnanensis
x hartwigii
transitoria
sp.
sikkimensis
micromalus
brevipes
x sublobata
pratii
baccata
toringoides
x robusta
angustifolia
mandshurica
tschonoskii
x dawsoniana
prunifolia
orientalis
coronaria
ioensis
kirghisorum
x soulardii
asiatica
x platycarpa
sieversii
hybrid
domestica
Taxon

Figure 22.

Figure 22. Ester count by taxon based on presence or absence of esters during ripening of the Geneva *Malus* Core Collection and Kazakhstan apple (all *M. sieversii*) and the regression between ester count and the cubed root of fruit weight. Total of 39 taxa including primary, cultivated, secondary, an unidentified, and uncharacterized hybrid species (Forsline et al., 2010) are listed in order by fruit weight ranging from small to large fruit. The maximum number of ester count is 67 including 3 unsaturated esters.

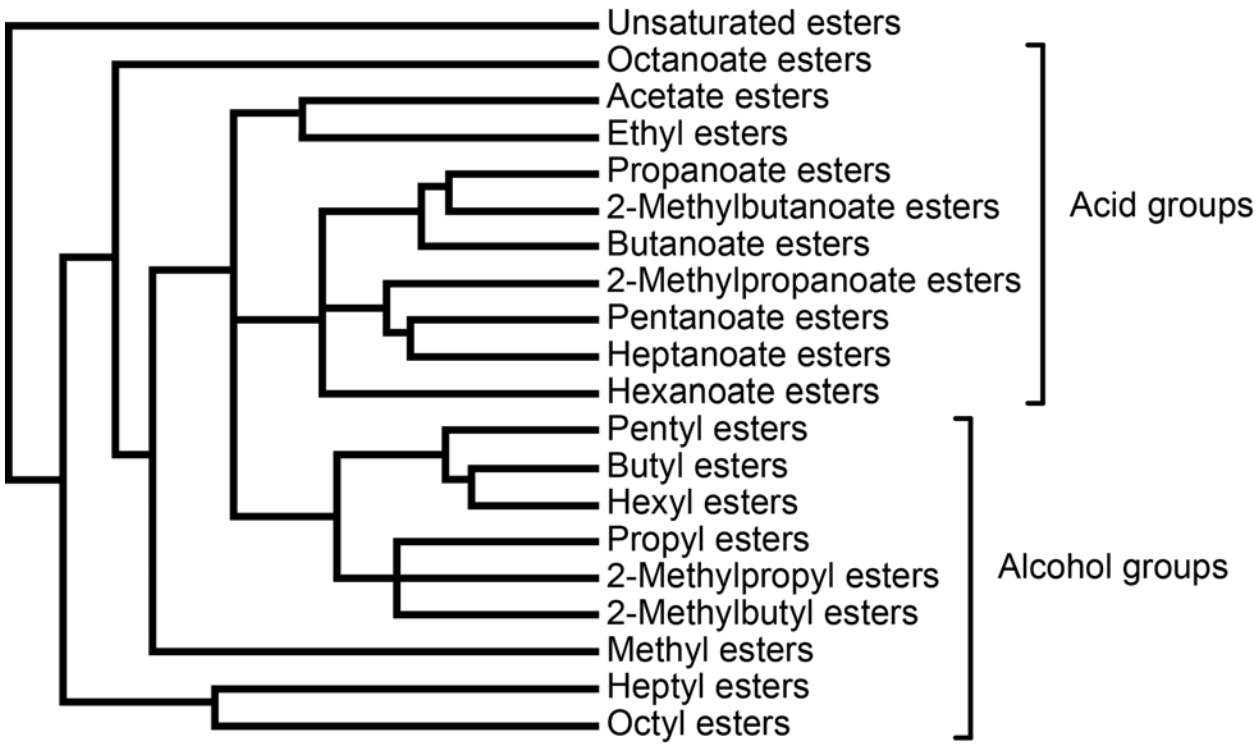

Figure 23. Cluster analysis of the esters classified according to alkyl (alcohol) and alkanoate (acid) groups across the Geneva *Malus* Core Collection and Kazakhstan apple (all *M. sieversii*). Esters that comprise the alkyl or alkanoate classifications are found in Table 2 with the exception for the unsaturated esters, which include 2-, 3-, and 5-hexenyl acetate.

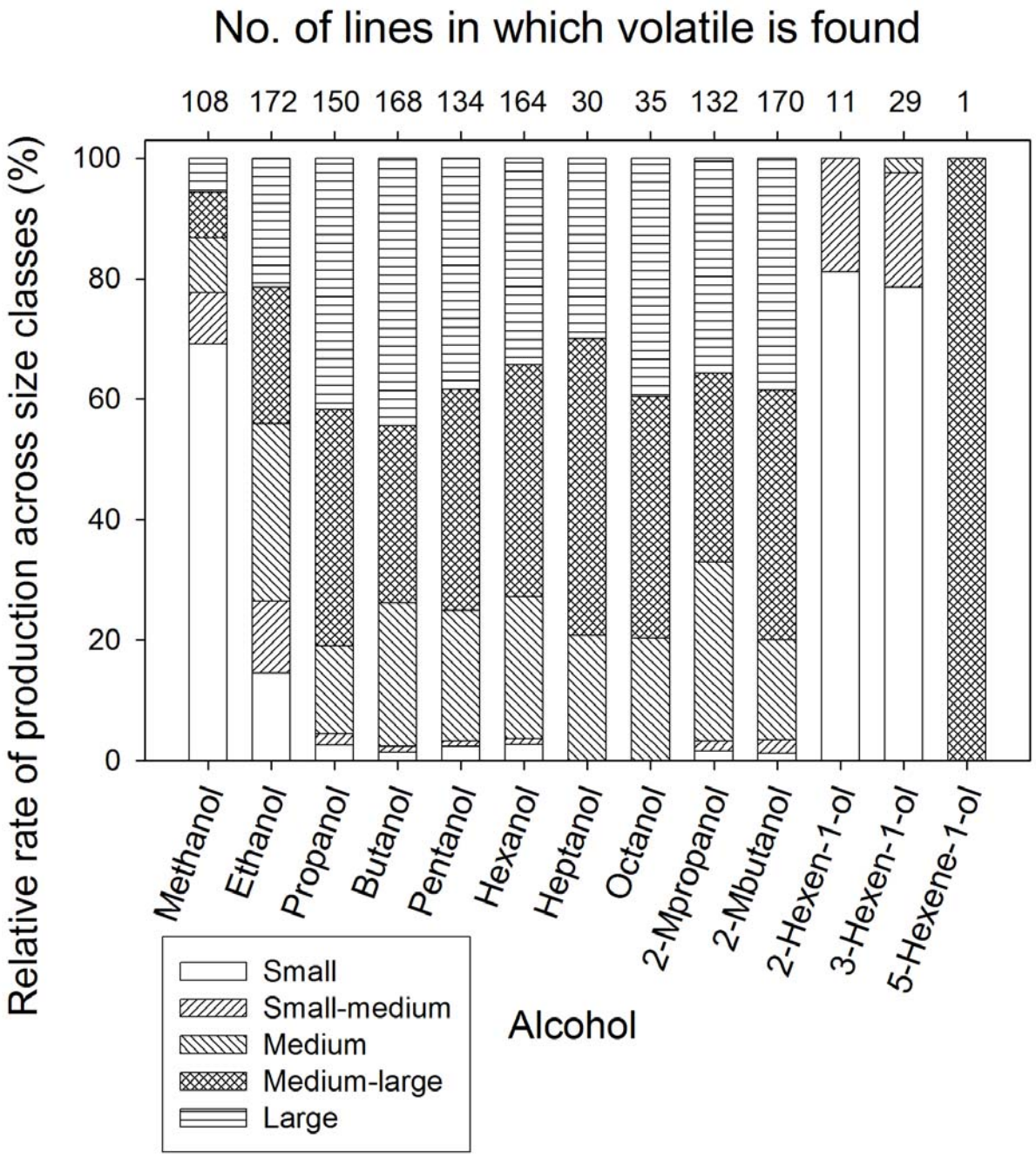

Figure 24. Relative rate of alcohol production across the Geneva *Malus* Core Collection and Kazakhstan apple (all *M. sieversii*) categorized by fruit weight ranging from small to large fruit. The numbers on the top indicates the number of lines in which volatile is found.

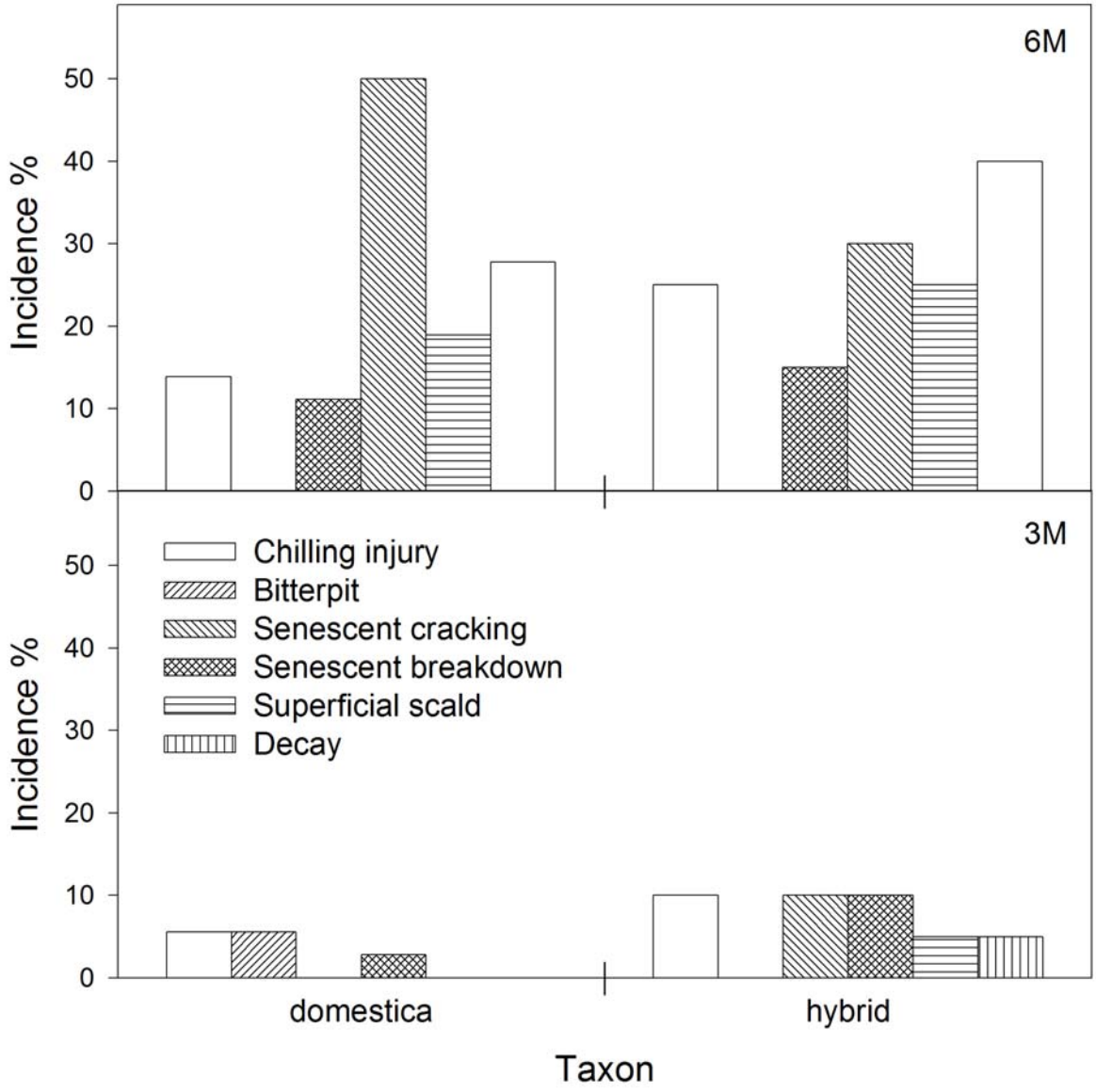

Figure 25. Storage disorders of *M.* ×*domestica* (37 lines) and *M.* ×*hybrid* (20 lines) of the Geneva *Malus* Core Collection after 3 months (3M) and 6 months (6M) in air at 0 °C.

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