Amino-Acid Transaminases – Key Enzymes in the Formation of Amino-Acid Derived Aroma Compounds in Melons

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Research Thesis

In Partial Fulfillment of The
Requirements for the Degree of
Master of Science in Biology

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Submitted to the Senate of
the Technion - Israel Institute of Technology

Tamuz, 5769, Haifa
June , 2009

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This research thesis was done under the supervision of Dr. Efraim Lewinsohn and Dr. Nurit Katzir in the Newe Ya'ar Research Center, Agricultural Research Organization and of Prof. Shimon Gepstein, the Faculty of Biology, Technion.

The generous financial help of: grant No. IS-3877-06 from BARD, the United States-Israel Binational Agricultural Research and Development Fund, the EU within the plant metabolomics project META-PHOR (FOOD-CT-2006-036220) and the Agricultural Research Organization Director's fund is gratefully acknowledged.

FIGURES AND TABLES

Table of Contents

Table of Contents - continued

ABSTRACT

The melon (*Cucumis melo* L., Cucurbitaceae) is a highly polymorphic species and many diverse cultivars are grown throughout the world. In 2006 the total fruit crop of melons in Israel was 47.9 thousand tons and more than a million tons in the USA (cantaloupes and honeydews). The flavor is one of the most important characteristics of the melon fruit quality, and melon aroma plays a crucial role in determining the unique flavor of each cultivar. The aroma of melons is composed of many volatile compounds biosynthetically derived from amino-acids, fatty-acids, carotenoids and terpenes. Amino-acids are known precursors of aroma compounds in melons as well as in other organisms. In cheese-dwelling micro-organisms and in yeasts the biosynthetic pathway of amino acid derived aroma compounds is well understood, and in the majority of cases the initial step involves transamination of the amino acid to form an α-keto acid intermediate. However, in plants, the initial steps in the biosynthetic pathway starting with the catabolism of the amino acids into aldehydes have received little attention. One report in tomato fruits showed that L-phenylalanine is first decarboxylated *en route* to the formation of aroma compounds. Another study in petunia and rose petals demonstrated that L-phenylalanine is converted to phenylacetaldehyde in one enzymatic step. It was previously unknown which is the degradation pathway of L-phenylalanine to phenylacetaldehyde in melon fruits. The main goal of my research was to assess the mechanism by which amino acids are incorporated into volatile aroma compounds in melon fruit.

The level of free amino acids in the fruit was measured during the development of four melon cultivars. It was found that in climacteric cultivars the free amino acids content increases in the ripe fruit. Incubation of melon fruit cubes with amino acids enhanced the formation of aroma compounds similar to the side chain of the exogenous amino acid supplied, as detected by GC-MS. When L-[$^{13}C_6$]phenylalanine was supplied to melon fruit cubes it also incorporated into volatile aroma compounds, as evident by GC-MS. Additional incubation experiments of melon fruit cubes with α-keto acids also led to enhanced formation of aroma compounds similar to the side chain of the exogenous keto acid supplied. Amino acid transaminase activities were extracted from the flesh of mature melon fruits (cv. 'Dulce'). These activities converted L-isoleucine, L-leucine, L-valine, L-methionine or L-phenylalanine into

their respective α-keto acids, utilizing α-ketoglutarate as the amine acceptor. In data-mining of the Melon EST Database two novel genes were isolated and characterized (*CmArAT1*and *CmBCAT1*) encoding 45.6 and 42.7 KDa proteins respectively. When expressed in *E. coli*, CmArAT1 and CmBCAT1 had aromatic amino acid transaminase activity and branched-chain amino acid transaminase activity, respectively. The expression patterns of *CmBCAT1* and *CmArAT1* were examined using qRT-PCR revealing increased expression during fruit ripening in flesh and rind tissues. My results indicate that in melon tissues, the catabolism of amino-acids into aroma volatiles occurs through a transamination mechanism first (a pathway known in microorganisms), rather than decarboxylation or direct aldehyde synthesis, as has been demonstrated in other plants.

SYMBOLS AND ABBREVIATIONS

AA	– Amino acids
AAT	– alcohol acetyltransferase
ArAA	– Aromatic amino acid
ArAT	– Aromatic amino acid transaminase
Arg	– Arginine
Asn	– Aspargine
Asp	– Aspartate
BCAA	– Branched chain amino acid
BCAT	– Branched chain amino acid transaminase
bis-Tris propane	– 1,3-bis(tris(hydroxymethyl)methylamino)propane
BSTFA	– *N,O*-bis(Trimethylsilyl)trifluoroacetamide
cDNA	– Complementary deoxyribonucleic acid
DAA	– Days after anthesis
DNA	– Deoxyribonucleic acid
DTT	– dithiothretiol
EBI	– European bioinformatics institute
E. coli	– *Escherichia coli*
EDTA	– Ethylenediaminetetraacetic acid
EST	– Expressed sequence tag
Expasy	– Expert protein analysis system
F.W.	– Fresh weight
GC-MS	– Gas chromatography mass spectrometry
Glu	– Glutamate
HMM	– Hidden Markov model
4-HPPA	– 4-Hydroxy phenylpyruvte
IPTG	– isopropylthio-β-galactoside
KI	– Kovats index
KIC	– Ketoisocaproate
KIV	– Ketoisovalerate
KMV	– Ketomethylvalerate
LB	– Luria Bertani
Lys	– Lysine

MTBE – Methyl *tert*-butyl ether

PCR – Polymerase chain reaction

PhyML – Phylogenetic maximum likelihood

PLP – pyridoxal 5'-phosphate

PPA – Phenylpyruvate

PVP – Polyvinylpyrrolidone

PVPP – Polyvinylpolypyrrolidone

qRT-PCR – quantitive real time polymerase chain reaction

SE – Standard error

SPME – Solid phase micro extraction

TAT – Tyrosine aminotransferase

Tris-HCl – 2-Amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride

Tyr – Tyrosine

U-14C – Unilabeled ^{14}C

Units

°C – degree Celsius

m/z – mass / charge

m – meter

µg – microgram

µl – microliter

mCi – milliCurie

mg – milligram

ml – milliliter

mM – millimolar

mmole – millimole

ng – nanogram

min – minute

pkat – picokatal (=picomole/second)

v/v – volume/volume

w/v – weight/volume

LITERATURE SURVEY

The melon

The melon (*Cucumis melo* L.) is a member of the Cucurbitaceae family which includes many species and genera such as cucumber (*Cucumis sativus* L.), watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), pumpkin and zucchini (*Cucurbita pepo* L., *Cucurbita maxima* Dush., *Cucurbita moschata* Dush.) (Whitaker and Davis, 1962). The melons are an old crop thought to have originated in the tropical Africa / Middle-East region, and spread to the Mediterranean, the Indian sub-continent and throughout Asia (Seymour and McGlasson, 1993). Today, melon is one of the most common agricultural crops in Israel, USA and worldwide. In 2006 the total fruit crop of melons in Israel was 47.9 thousand tons (CBS, 2008). Melon is a highly polymorphic species that comprises a broad array of wild and cultivated genotypes that can be subgrouped according to different traits (Mallick and Masui, 1968). Smith and Welch (1964) divided the various cultivars by the fruit rind characters: *reticulates* – cultivars with netted rind, and *inodorus* – cultivars with smooth rind. Other common division is based on the ripening physiology of the fruit: climacteric and non-climacteric cultivars. The ripening of climacteric fruits is characterized by a burst of respiration, usually associated with increase in ethylene production followed by upregulation of specific ripening associated genes and down regulation of other genes. As a result, changes in color, firmness, texture, taste and aroma of the fruits take place to complete ripening (Dangl *et al.*, 2000). The climacteric cultivars are usually more aromatic than the non-climacteric, though the latter have longer shelf-life. Melons are also classified based on other fruit traits such as, sugar and acid content, rind color, flesh color, shape and size, seeds characters and the level of total aroma (Seymour and McGlasson, 1993; Pitrat *et al.*, 2000; Burger *et al.*, 2006, 2009).

Secondary metabolites

Plants produce a vast and diverse assortment of organic compounds that do not seem to directly participate in plant development. These compounds, traditionally referred to as secondary metabolites, are distributed among the plant kingdom. Their functions, although still unknown for their majority, are being elucidated with increasing frequency. In contrast, primary metabolites are found in all plants and

perform metabolic roles that are essential and usually evident. However, no clear border separates between primary and secondary metabolites, and they can not readily be distinguished on the basis of chemical structure or biosynthetic origin (Croteau *et al.*, 2000). More than 100,000 secondary metabolites have been identified to date, and it is postulated that this is only 10% of the total secondary metabolites produced by plants. Secondary metabolites can be divided into groups based on their chemical structure: terpenoids (mono-, sesqui-, di-, tri- tetra and polyterpenes), phenolic compounds and alkaloids and other nitrogen containing compounds (Wink, 1988).

Although their name "secondary" suggest that they are less essential for plant biology, secondary metabolites often have key roles in ecological interactions that can be crucial for the plant survival (Wink, 1988). For example, sparteine (an alkaloid) is an anti-viral compound. Citronellal, cineol and many other essential oil terpenes have antibacterial activity. Chlorogenic acid, geraniol, saponins and a large number of secondary metabolites antifungal. Monoterpenes, sesquiterpene lactones and non-protein amino acids play important roles in allelopathy. Many alkaloids and terpenes are toxic to insects and some isoflavones and glucosinolates are toxic to vertebrates. Many color, taste, aroma and active compounds play an important role in attraction of pollinators and seed dispersers (Wink, 1988). Still, the functions of the majority of secondary metabolites are unknown (Lewinsohn and Gijzen, 2009).

Flavor and aroma

Flavor is defined as the combination of taste and odor. However, flavor is also influenced by other sensations such as pain, heat, cold and tactile sensations, often referred to as the ‘texture’ of foods (Thomson, 1987). Humans can detect five tastes: sweet, salty, sour, bitter and umami (Temussi, 2006). In contrast, humans can distinguish between thousands of odor molecules due to a multigene family encoding 500–1,000 olfactory receptors (Buck and Axel, 1991; Rouquier et al., 2000). These receptors are located in the olfactory epithelium in the nasal cavity and detect odorants reaching this area through either the nasal or retronasal routes (Figure 1) (Thomson, 1987). In the nasal route, the air that contains the volatile molecules is directly taken in through the nose to reach the olfactory receptors, while in the retronasal route the volatile molecules are taken in through the oral cavity and reach

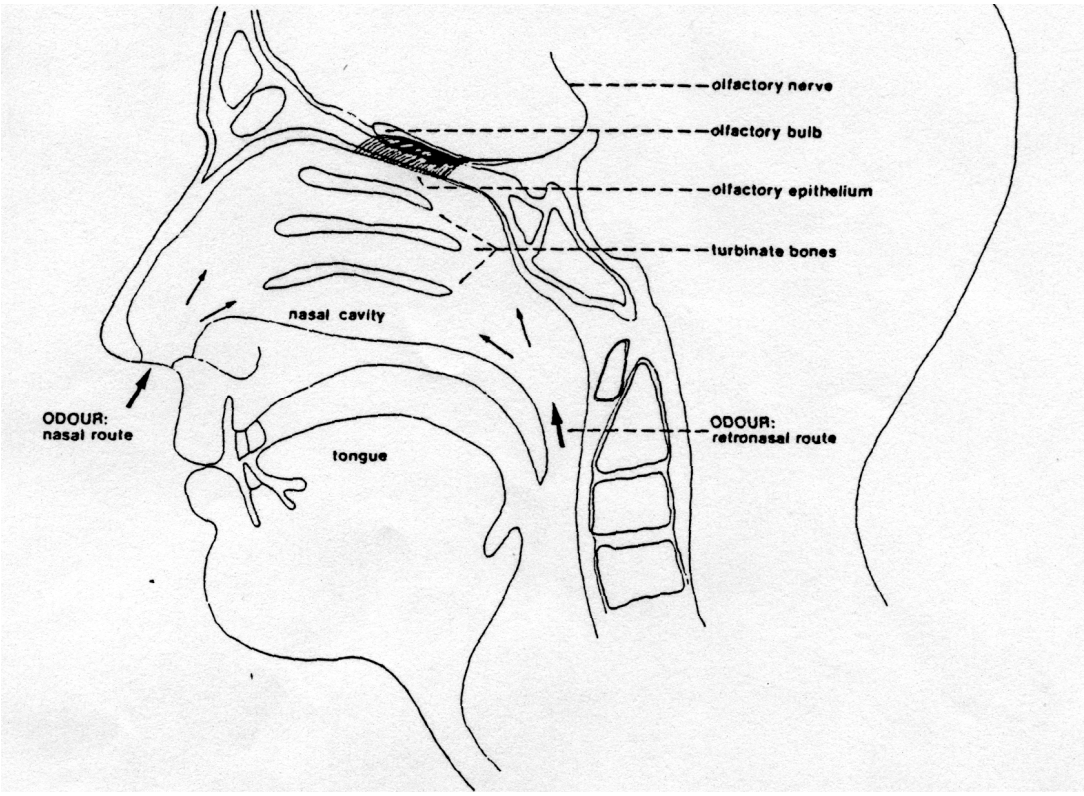


Figure 1. Taste and smell sensors in humans.

Taste and olfactory sensory stimulation are integrated with a variety of sensory inputs including visual, tactile, and nutrient-sensing from the gastrointestinal tract to generate the overall flavor perception of specific foods (from Thomson, 1987).

the olfactory receptors *via* cavities in the throat (Thomson, 1987). The odorant must possess certain molecular properties in order to produce an olfactory impression: a certain degree of lipophilicity, sufficiently high vapor pressure so it can be transported to the olfactory system, some water solubility to permeate the thin layer of mucus. Furthermore, it must be present at a sufficiently high concentration to be able to interact with one or more of the olfactory receptors (Schwab *et al.*, 2008).

The aromas of fruits and vegetables are determined by unique complex combinations of volatile compounds (Thomson, 1987; Kuentzel and Bahri, 1990; Schwab *et al.*, 2008). Although different fruits often share many aroma characteristics, each fruit has distinctive aromas that are determined by the proportions of key volatiles and the presence or absence of unique components (Kuentzel and Bahri, 1990; Tucker, 1993). The most important plant aroma compounds include, among others, amino acid-derived compounds, lipid-derived compounds, phenolic derivatives, mono- and sesquiterpenes (Croteau and Karp, 1991; Schwab *et al.*, 2008).

The aroma of melons

Acceptable aroma is one of the important characteristics of melon quality (Seymour and McGlasson, 1993). Unlike sugar production, which stops as the fruit is harvested, formation of aroma molecules actively continues as long as the melons are harvested when ripen (Wyllie *et al.*, 1995; Beaulieu and Grimm, 2001). In aromatic melon varieties, volatile esters are prominent together with sulfur containing aroma compounds, sesquiterpenes, norisoprenes, short-chain alcohols and aldehydes (Wyllie and Leach**,** 1992; Seymour and McGlasson, 1993; Shalit *et al.*, 2001; Jordán *et al.*, 2001; Beaulieu and Grimm, 2001; Shalit *et al.*, 2001; Aubert and Bourger, 2004; Portnoy *et al.*, 2008). Non-aromatic varieties often have much lower levels of total volatiles, and lack volatile esters (Shalit *et al.*, 2001; Aubert and Bourger, 2004; Burger *et al.*, 2006). Young melon fruits display low levels of aroma volatiles and only when the fruit ripens gains its final and unique aroma profile (Horvat and Senter, 1987; Beaulieu and Grimm, 2001; Shalit *et al.*, 2001).

Specific characteristics of the melon aroma

Schieberle *et al.* (1990) described 12 odorants that significantly contribute to the melon aroma including ethyl-2-methylpropanoate, methyl-2-methylbutanoate and ethyl butanoate which account for fruity and sweet aroma notes. Wyllie and Leach (1992) measured the amounts of 5 different thioesters in 26 different melon cultivars. They found that in almost all cases at least one of the thioesters is significantly abundant at a level regarding its odor threshold to make a marked contribution to the fruit flavor. More recently, Jordán *et al.* (2001) performed a gas-chromatography olfactometry experiment and revealed 25 odorants with significant contribution to melon aroma. They included ethyl isobutyrate, isobutyl acetate, ethyl-2-methylbutyrate, benzyl alcohol, ethyl-3-(methylthio)propinioate and benzyl butyrate that confer sweet, fruity and cantaloupe-like aroma notes. Thus, each cultivar has a unique aroma profile that is composed by the absolute quantities of the volatiles and the relative proportions between them. Differences in these profiles are keys in forming the unique flavor and aroma of each cultivar.

Melon aroma – biosynthesis and genetics aspects

In the past few years, research dealing with the biosynthesis of the aroma compounds of melons has been published (Shalit *et al.*, 2001; El-Yahyaoui *et al.*, 2002; El-Sharkawy *et al.*, 2005; Manríquez *et al.*, 2006; Ibdah *et al.*, 2006; Portnoy *et al.*,

2008). Manríquez *et al.* (2006) identified and characterized two distinctive alcohol dehydrogenase genes (ADH) (*Cm-ADH1* and *Cm-ADH2*) accounting for the formation of various alcohols from aldehyde substrates in the melon fruit. Shalit *et al.* (2001) found an alcohol acetyltransferase (AAT) activity in melon cell free extracts involved in acetate esters formation. El-Yahyaoui *et al.* (2002) characterized two genes encoding alcohol acetyltransferase (*Cm-AAT1* and *Cm-AAT2*) responsible for the biosynthesis of different volatile esters, and El-Sharkawy *et al.* (2005) characterized two additional melon AAT's (*Cm-AAT3 and Cm-AAT4*). The expression of all of these genes correlates with the ethylene production of the fruit and is inhibited in transgenic melons unable to produce ethylene (El-Yahyaoui *et al.*, 2002; El-Sharkawy *et al.*, 2005; Manríquez *et al.*, 2006). Ibdah *et al.* (2006) identified a carotenoid cleavage dioxygenase gene (*CmCCD1*) that generates geranylacetone, pseudoionone, β-ionone, α-ionone and pseudoionone from various carotenoids substrates. Portnoy *et al.* (2008) discovered two sesquiterpene synthases genes (*CmTpsNY* and *CmTpsDul*) from two different cultivars ('Noy Yizre'el' and 'Dulce'), each of them utilized the same substrate (farnesyl diphosphate) to generate different products. CmTpsNY, isolated from 'Noy Yizre'el' cultivar, generates δ-cadinene, γ-cadinene and α-copaene *in vitro* which are the major sesquiterpenes accumulated in 'Noy Yizre'el' rind fruit tissue. CmTpsDul, isolated from 'Dulce' cultivar, generates only α-farnesene *in vitro*, the sole sesquiterpene accumulated in the rind tissue of 'Dulce' fruits. All of these 9 genes were found to be upregulated in the last stages of fruit development (El-Yahyaoui *et al.*, 2002; El-Sharkawy *et al.*, 2005; Manríquez *et al.*, 2006; Ibdah *et al.*, 2006; Portnoy *et al.*, 2008), in parallel manner to the accumulation of the melon volatiles (Seymour and McGlasson, 1993; Beaulieu and Grimm, 2001; Shalit *et al.*, 2001).

Amino acids derived aroma compounds – general aspects

Free amino acid content increases during melon ripening (Wyllie *et al.*, 1995) and aroma volatiles (esters in particular) derived from amino acids are major contributors to the aroma of melons (Schieberle *et al.*, 1990; Wyllie *et al.*, 1995; Jordán *et al.*, 2001; Beaulieu and Grimm, 2001; Aubert and Bourger, 2004) and other fruits (Buttery *et al.*, 1987; Wyllie and Fellman, 2000; Baldwin *et al.*, 2000; Matich and Rowan, 2007), as well of cheese and milk products (Yvon and Rijnen, 2001; Liu *et al.*, 2008) and yeast-fermented products like beer and bread (Dickinson *et al.*, 2003;

Perpète *et al.*, 2006). The amino acids that are further catabolized to aroma volatiles are usually the aromatic amino acids (L-phenylalanine, L-tyrosine and L-tryptophan), the branched chain amino acids (L-leucine, L-isoleucine and L-valine) and sulfur containing amino acids (L-methionine and L-cysteine) (Yvon and Rijnen, 2001; Liu *et al.*, 2008).

Reports regarding the formation of amino acid-derived volatiles in cheese-dwelling micro-organisms are available. The first step of the pathway is relatively well understood, and in the majority of the cases it initially involves transamination (Figure 2) followed by decarboxylation (Yvon *et al.*, 2000; Yvon and Rijnen, 2001). Further observations have indicated that this initial step leads to the formation of aroma compounds (Yvon and Rijnen, 2001; Liu *et al.*, 2008) (Figure 3. green route). It had also been found that yeasts utilize the same pathway (Dickinson *et al.*, 2003; Perpète *et al.*, 2006) as was suggested in the beginning of the preceding century by Ehrlich (Ehrlich. 1907) (Figure 2).

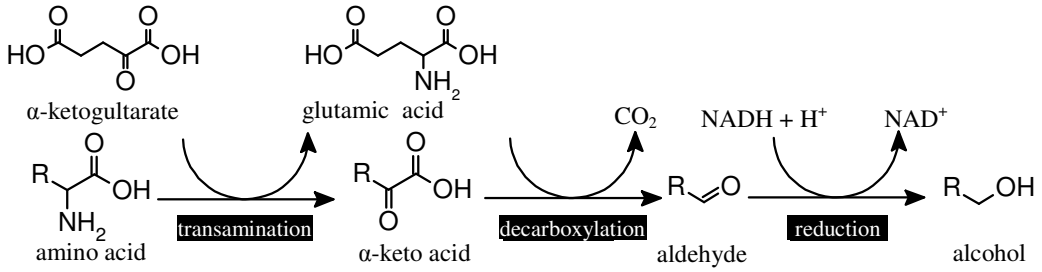


Figure 2. Ehrlich pathway for volatiles formation in yeasts. The biosynthetic pathway of amino acids derived aroma compound in yeasts (Ehrlich 1907; Dickinson *et al.*, 2003).

The biosynthesis of amino acids derived aroma compounds in plants

The last two steps in the formation of volatile esters (reduction to alcohol by ADH, and ester formation by AAT) in plants have been extensively investigated, both in melons (Shalit *et al.*, 2001, El-Yahyaoui *et al.*, 2002; El-Sharkawy *et al.*, 2005, Manríquez *et al.*, 2006) and in other plants (Pérez *et al.*, 1993; 1996; Beekwilder *et al.*, 2004; Boatright *et al.*, 2004; Larkov *et al.*, 2008; Zaks *et al.*, 2008). However, the knowledge on the initial step of the pathway that involves the catabolism of amino acids for the formation of aldehydes is limited.

Studies preformed in tomato (*Solanum lycopersicum* L.) fruit indicated that the catabolism of L-phenylalanine into aroma volatiles is initially mediated by aromatic

amino acid decarboxylases (*LeAADC1A*, *LeAADC1B* and *LeAADC2*), releasing an amine intermediate (Tieman *et al.*, 2006) (Figure 3, red route, solid arrows). Amino

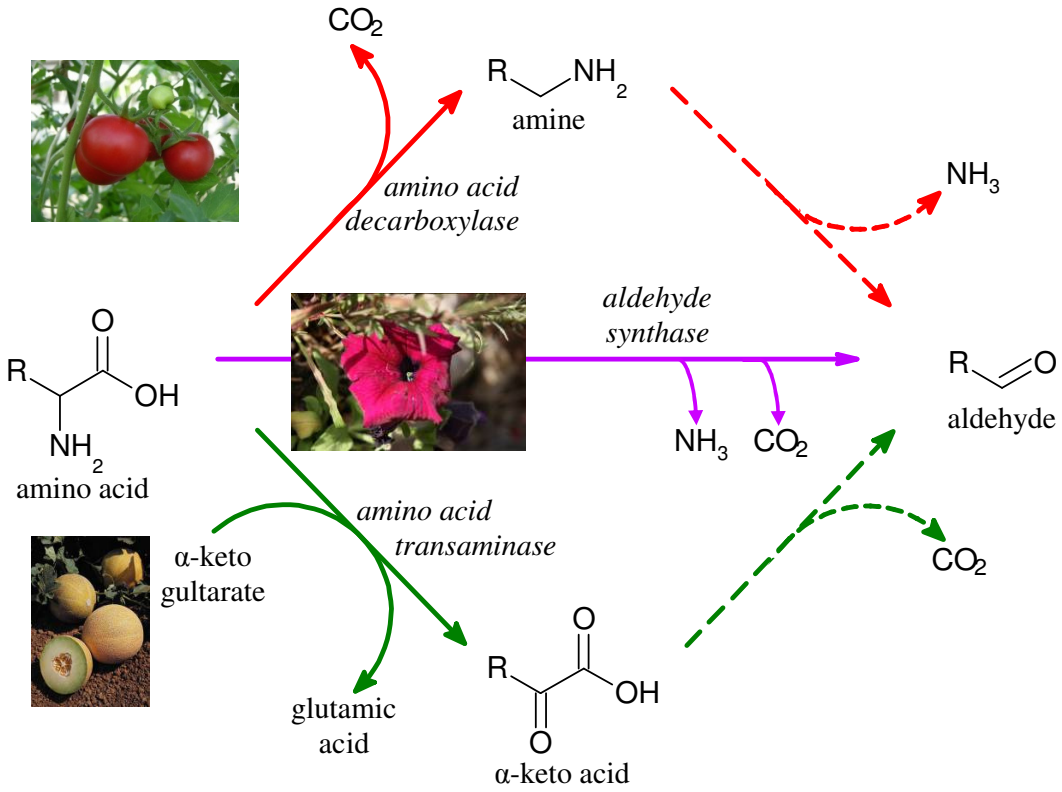


Figure 3. Biosynthetic routes for amino acids degradation to volatile aldehydes in plants. Red route: decarboxylation first followed by deamination with an amine intermediate, catalyzed by decarboxylases and a yet unidentified deaminases, as demonstrated in tomato (Tieman *et al.*, 2006). Purple route: aldehyde synthesis in a single enzymatic step with no free intermediates as was demonstrated in petunia and rose petals (Kaminaga *et al.*, 2006). Green route: transamination followed by decarboxylation with an α-keto acid intermediate, catalyzed by two enzymes as was demonstrated in this work, and in cheese dwelling micro-organisms (Rijnen *et al.*, 1999). Solid arrows indicate that the enzyme activity and gene have been identified. Dashed arrows indicate that the proposed plant enzymes and genes are yet to be identified (Schwab *et al.*, 2008), although the α-keto acid decarboxylases occur in microorganisms (Yvon and Rijnen 2001).

acid decarboxylases are pyridoxal-5'-phosphate (PLP)-dependant enzymes that release CO_2 and the corresponding amine derivative. It has been proposed that deamination of the product, phenethylamine, releases phenylacetaldehyde, but this step has not been confirmed (Figure 3, red route, dashed arrows). Transgenic tomatoes overexpressing *LeAADC1A* or *LeAADC2* emitted higher levels of phenylalanine derived volatiles

(Tieman *et al.*, 2006). In contrast, it seems that a different biosynthetic route takes place in petals of petunia (*Petunia hybrida*) and rose (*Rosa hybrida*), in which one enzyme (either *PhPAAS* or *RhPAAS*, respectively) is able both to decarboxylate and deaminate L-phenylalanine to release phenylacetaldehyde (Kaminaga *et al.*, 2006) (Figure 3, purple route). Flowers of transgenic petunia plants with suppressed phenylacetaldehyde synthase gene expression did not emit phenylacetaldehyde and 2-phenylethanol as compared with non-silenced controls (Kaminaga *et al.*, 2006). Preliminary experiments using melon fruit cell-free extracts failed to detect L-phenylalanine decarboxylases or phenylacetaldehyde synthase activities in radioactive *in vitro* assays (see below).

Amino acid transaminases

Amino acid transaminases are PLP-dependant enzymes which are present both in prokaryotic and in eukaryotic organisms (Percudani and Peracchi, 2003). Amino acid transaminases have been deeply investigated throughout the last 70 years, when the first amino acid transaminase was identified in 1937 from muscle tissue (cited in Cooper *et al.*, 1985). Amino acid transaminases transfer the amino group of the amino acid to the α-keto acid, and the keto group of the α-keto acid to the amino acid (Figure 2). The resulting products are usually L-glutamate (when the α-keto acid substrate is α-ketoglutarate, the most common amine acceptor), and the α-keto acid derived from the respective amino acid substrate. Bacteria utilize different amino acid transaminases for the last step in the biosynthesis of both aromatic amino acids (ArAA's) and branched-chain amino acids (BCAA's) (Figure 4A). In plants, however, while BCAA's are synthesized in similar fashion to that of bacteria (Figure 4A), ArAA's synthesis involve amino acid transamination not for the formation of the amino acid itself but one step prior to it, producing arogenate from prephenate (Siehl, 1999; Singh, 1999) (Figure 4B). In addition to their involvement in amino acids formation, amino acid transaminases have important roles in photorespiration, C4 photosynthesis, nitrogen assimilation and transport, tocopherol biosynthesis as well as in secondary metabolism (Mizukami and Ellis, 1991; Hagelstein *et al.*, 1997; Lea and Ireland, 1999; Malkin and Niyogi, 2000; Siedow and Day, 2000, Lopukhina *et al.*, 2001, Malatrasi *et al.*, 2006; Schuster *et al.*, 2006; Knill *et al.*, 2008). However, their role in biosynthesis of plant volatiles has not been fully documented so far.

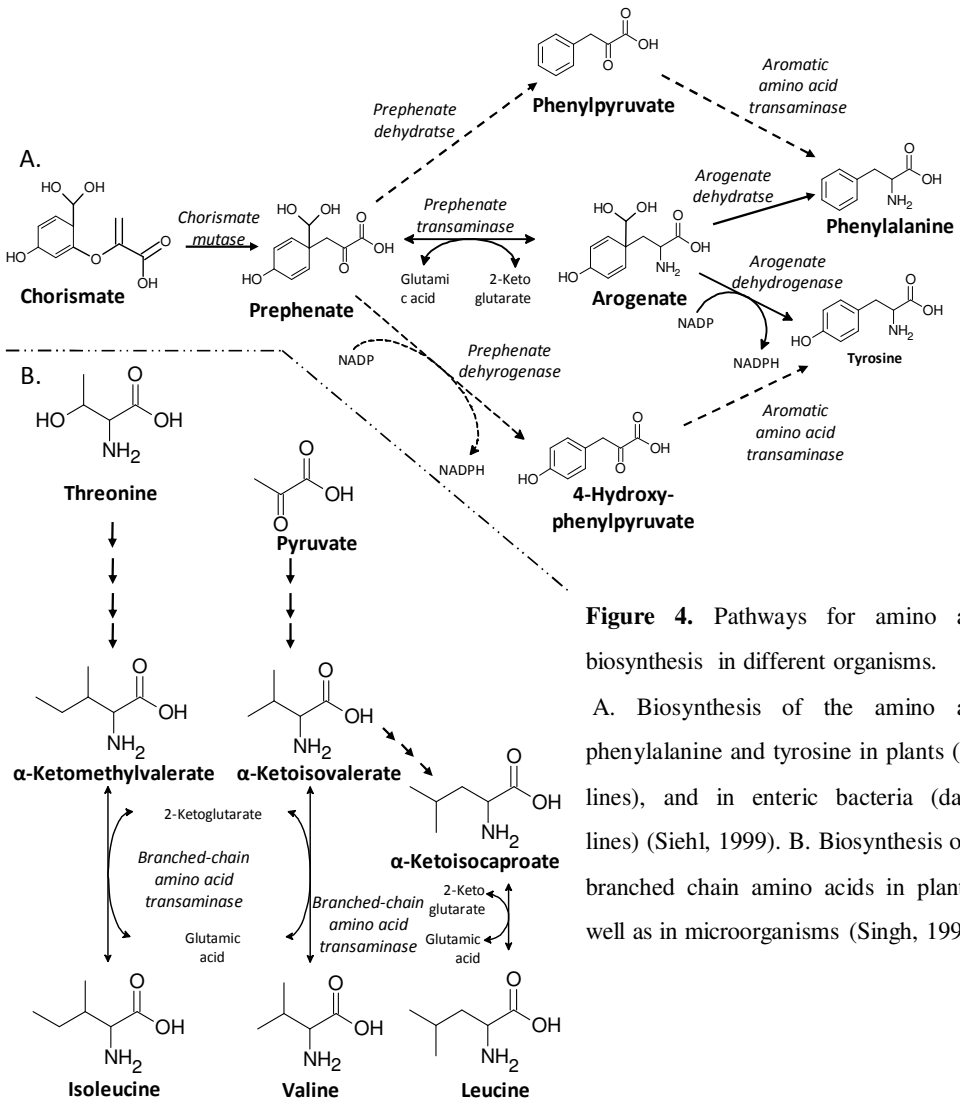


Figure 4. Pathways for amino acids biosynthesis in different organisms.

A. Biosynthesis of the amino acids phenylalanine and tyrosine in plants (solid lines), and in enteric bacteria (dashed lines) (Siehl, 1999). B. Biosynthesis of the branched chain amino acids in plants as well as in microorganisms (Singh, 1999).

Amino acid transaminases gene family

Transaminases are the largest gene family of the PLP-dependent enzymes (Percudani and Peracchi, 2003). Amino acid transaminases can be classified into four subgroups: subgroup I is the largest and most diverse one that includes aspartate, alanine, tyrosine, histidinol-phosphate and phenylalanine transaminases. Subgroup II includes acetylornithine, ornithine, ω-amino acid, 4-aminobutyrate and diaminopelargonate transaminases. Subgroup III includes D-alanine and the branched-chain amino acid transaminases, and subgroup IV that includes serine and phosphoserine transaminases (Mehta *et al.*, 1993; Jensen and Gu, 1996). This grouping is based on aa sequence similarities of the transaminase gene products. More recent studies based on spatial

structures (in addition to sequence similarities), include in the same family but a broader range of PLP-dependant enzymes, besides PLP-dependent transaminases. In this broader approach classification some transaminase subgroups are more closely related to other PLP-dependant enzymes such as decarboxylases, rather than to other transaminases (Christen and Mehta, 2001). In computerized databases (e.g. Pfam, Panther) the family division is based largely on conserved domains spatial structures, and it is slightly different in its division and nomenclature. According to both the Pfam (http://pfam.sanger.ac.uk/) and the Panther classification system (http://www.pantherdb.org/), the aromatic amino acid transaminases belong to the aminotransferase related class I (Panther) or I and II (Pfam), as was classified earlier (Mehta *et al.*, 1993; Jensen and Gu, 1996, Christen and Mehta, 2001). However, the branched-chain amino acid transaminases are members of the so called aminotransferases class IV, in contrast to subgroup III as assigned by Mehta *et al.* (1993) and Jensen and Gu (1996). In this research I used the Panther classification system in order to determine subfamily division of the enzymes discussed.

While many databases contain increasing numbers of annotated BCAT's and ArAT's from plants, the number of those genes that have been functionally characterized is limited. Only one plant ArAT (annotated as TAT – tyrosine aminotransferase) from arabidopsis was functionally expressed and characterized (Lopukhina *et al.*, 2001). Six BCAT's transcripts were isolated from arabidopsis (*AtBCAT1-6*) and 5 (AtBCAT1,2,3,5,6) of them rescue BCAT yeast mutants in complementation assays (Diebold *et al.*, 2002). AtBCAT3 has broad substrates specify acceptance, and it cam catalyzed the transamination of both the branched-chain amino acids and methionine (Knill *et al.*, 2008). AtBCAT4 was shown to possess methionine transaminase activity *in vitro* participating in glucosinolates biosynthesis (Schuster *et al.*, 2006). ArAT and BCAT genes from a wide range of organisms, such as mammals, bacteria, yeasts and metazoans were identified and characterized (Dickinson *et al.*, 2003; Grange *et al.*, 1985; Dietrich *et al.*, 1991; Seralini *et al.*, 1995; Davoodi *et al.*, 1998; Blankenfeldt *et al.*, 1999; Rijnen *et al.*, 1999; Yvon *et al.*, 2000; Yennawar *et al.*, 2001), including a BCAT gene from barley that was heterologously expressed in yeast BCAT mutants, resulted in complementation and phenotype rescue (Malatrasi *et al.*, 2006). In my work I checked the levels of free amino acids in the melon fruit during development. I ascertained the chemical origin of amino acids derived aroma compounds. I found amino acids transaminase activities in cell-free extracts of ripe melon flesh, and show

that the enzymatic products of these reactions (α-keto acids) are intermediates in aroma compounds formation in melon fruit. I characterized two novel amino acid transaminase genes from melon, and assessed their role in biosynthesis of amino-acid derived volatiles in the fruit.

WORKING HYPOTHESIS

Based on preliminary experiments the following major hypothesis guided me in this research:

- Melon fruits utilize a different pathway for amino acid degradation *en route* to volatile aroma compounds as compared to other plants.

RESEARCH GOALS

The main goal of my research was to determine by which route melons degraded amino acids *en route* to volatile aroma compound. In detail:

- Validate the chemical origin of some important constituents of the melon aroma, thought to be derived from amino acids, by feeding of melon cubes with labeled and unlabeled putative precursors.
- Identify the first catabolic step to take place in melon fruit toward the formation of aroma volatiles using cell-free extracts prepared from melon cubes.
- Find genes encoding those enzymes in the Melon EST Database based on sequence similarities to other plant amino acids degrading enzymes.
- Characterize those genes both for their functionality and expression pattern.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise indicated. The L-[ring-$^{13}C_6$]phenylalanine was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

Plant material

Melons (*Cucumis melo* L. var. Dulce) were sown in April 2008 in the open field at the Newe-Ya'ar Research Center in northern Israel and grown under commercial conditions in the summer of 2008 with drip irrigation and fertilization, or sown in August 2008, in the greenhouse at the Newe-Ya'ar Research Center in the autumn of 2008 with drip irrigation and fertilization. Fruit samples were collected 12 and 25 days after anthesis (DAA), at harvest (ripe stage - after change of color and development of abscission zone) and seven days post harvest.

Free amino acids levels measurements

Melon cubes were put in vials containing 10 ml ethanol, and the liquid level was marked. The vials were sealed and incubated in 100 °C for 1 hour. Then the ethanol evaporated had been compensated. From each vial 100 µl were transferred into a new vial. To each sample 400 µl of 20 mM ninhydrin (in ethanol) were added, and then incubated at 100 °C for 20 minutes. After chilling, 5 ml of a "diluter" solution consisting of 100mM K_3PO_4/H_2O/acetone (1/1/2, v/v/v) was added, and the absorbance was measured at 570 nm. The levels of free amino acids were determined according to L-leucine standard.

Incubation experiments and volatile analyses

Melon cubes (about 4 g each) cut from the flesh of mature fruit were put in sterile Petri dish plates. On each cube, 500 µl of a solution containing 30 mM amino or α-keto acids were applied on the top of the cube. The plate was covered and incubated overnight at room temperature. Then, each cube was frozen in liquid nitrogen and ground into a uniform powder using a chilled mortar and pestle. One g of the powder was placed in a 10 ml SPME vial containing 0.7 g solid NaCl. To each vial 2 ml of a 20 % (w/v) NaCl solution and 0.2 µg of 2-heptanone (that was used as internal

standard) were added. The vial was then sealed and stored at 4°C, for no longer than 1 week until analysed. Headspace sampling was conducted according to Davidovich-Rikanati *et al.*, (2008) with slight modifications. A 65 µm fused silica fiber coated with polydimethylsiloxane/divinylbenzene (PDMS/DVB) (Supelco Inc., Bellefonte, PA, USA) was used. The sample was preheated to 30°C, agitated for 5 min at 500 RPM and then the fiber was inserted into the vial and exposed to the sample headspace. After 25 min the SPME syringe was introduced into the injector port of the GC-MS apparatus for further analysis (see below).

GC-MS analyses

Volatile compounds were analyzed on a GC-MS apparatus (Agilent Technologies CA, USA) equipped with an Rtx- 5 SIL MS (30 m * 0.25 mm * 0.25 µm) fused-silica capillary Column (Restek Co.). Helium (0.8 ml / min) was used as a carrier gas. The injector temperature was 250°C, set for splitless injection. The oven was set to 50°C for 1 min, and then the temperature was increased to 180°C at a rate of 5°C / min, then to 260°C at 20°C / min. Thermal desorption was allowed for 10 min. The detector temperature was 280°C. The mass range was recorded from 41 to 250 m/z, with an electron energy of 70 eV. A mixture of straight-chain alkanes (C7–C23) was injected into the column under the aforementioned conditions for determination of retention times. The identification and quantification of the volatiles was done according to Davidovich-Rikanati *et al.* (2008) with the exception that for quantification the area used in the calculation was of a major peak rather than the TIC area multiplied by a correction factor ratio.

Preparation of cell-free extracts from melon flesh tissues

Fruit flesh was cut into small pieces (about 2 cm^3) and frozen at -20°C for no longer than 6 months until use. The frozen pieces were placed in a chilled mortar and ground with a pestle in the presence of sea-sand (-50 +70 mesh) and 0.5 g polyvinylpolypyrrolidone (PVPP) until a uniform powder was obtained. Ice-cold extraction buffer [50 mM bis-tris propane pH 8.5, 10% (w/v) D-sorbitol, 10 mM dithiothreitol, 5 mM $Na_2S_2O_5$, 25 µM pyridoxal 5′-phosphate (PLP), and 0.1% (w/v) polyvinylpyrrolidone (PVP-40)] was added (4:1 v/w) and the suspension was further extracted for an additional 30 s. The slurry was centrifuged at 26,000 *g* for 20 min at

4°C. The supernatant (crude extract) was put inside a dialysis bag, and placed in solid ground sucrose at 4°C overnight. The concentrated slurry (about 5 ml) was desalted on a 15 mm x 85 mm P-6 column (BioRad Labs. Inc., Hercules, CA, USA), and the protein containing fractions were merged and stored at -20°C until enzymatic activity determinations. Protein was determined using the Bradford reagent (Bradford, 1976).

Amino-acid transaminase assays

Small-scale radioactive assay

Enzymatic assays were performed by mixing 20 µl of desalted concentrated crude extract (about 25 µg protein) with 50 mM bis-tris propane pH 8.5 buffer containing 10 mM α-keto glutaric acid, 5 mM L-amino-acid {L-[3,4-^{3}H] isoleucine; L-[4,5-^{3}H]leucine; L-[4,5-$^{3}H_2$]phenylalanine; L-[3,5-^{3}H]tyrosine (GE Healthcare); L-[methyl-$^{3}H_3$] methionine, specific activities: 0.01mCi/mmol}, 1 mM dithiothreitol, 225 µM PLP, 10% (w/v) D-sorbitol, in a total volume of 100 µl. The reactions were incubated for 2 hours (unless otherwise indicated) at 30°C. After incubation, each sample was acidified with 20 µl 1 N HCl, and incubated for 30 min at 30°C. The samples were extracted with 750 µl ethyl acetate, vigorously shaken and centrifuged at 20,000 *g* to allow for phases separation. The organic phase (550 µl) was transferred to 5 ml scintillation vials containing 3 ml of scintillation liquid (Ultima Gold™, Perkin Elmer, Shelton, CT, USA). The radioactivity was quantified using a liquid scintillation analyzer (Tri-Carb 2800TR, Perkin Elmer, Shelton, CT, USA). Product amounts were calculated on the basis of the specific activity of the substrate and the counting efficiency of the machine.

Large scale GC-MS assay

Assays were performed by mixing 1 ml of the concentrated and desalted melon crude extract (200 µl for bacterial lysate extracts), with 50 mM bis-tris propane pH 8.5 buffer containing 5 mM α-keto glutaric acid, 5 mM L-amino-acid. 1 mM dithiothreitol, 225 µM PLP, 10% (w/v) D-sorbitol, to a total volume of 2 ml (400 µl for bacterial lysate extracts). The reactions were incubated overnight at 30°C. After incubation each sample was acidified with 40 µl 10 N HCl (8 µl for bacterial lysate extracts), and incubated for 30 min at 30°C. The samples were extracted with methyl *tert*-butyl ether (MTBE) and the MTBE was evaporated to dryness. For tri-methyl

silyl derivatization, the samples were dissolved in 100 µl pyridine and incubated for 90 min at 37°C. Then, 100 µl *N,O*-bis(Trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco Inc., Bellefonte, PA, USA) were added and the samples were incubated for another 90 min at 37°C. The samples were transferred into glass test tubes and stored at 4°C until analysis in GC-MS as follows. A 1 µl aliquot of the concentrated derivatized extract was injected into a GC-MSD system (Agilent, http://www.home.agilent.com) equipped with an Rtx–5 SIL MS column (30 m * 0.25 mm * 0.25 µm) fused-silica capillary Column (Restek Co.). Helium (0.8 ml / min) was used as a carrier gas with splitless injection. The injector temperature was 250°C, and the detector temperature was 280°C. The following conditions were used: initial temperature, 50°C for 1 min, followed by a ramp from 50 to 200°C at a rate of 5°C / min, then to 300°C at 20°C / min. A quadrupole mass detector with electron ionization at 70 eV was used to acquire the MS data in the range of 41 to 250 m/z. The reaction products as their silyl derivatives were identified by comparison of spectral data with α-keto acids authentic standards treated in an identical way as the reaction products. Each of the biosynthetic and standards derivatized products displayed more than one peak in the GC-MS chromatogram, since the BSTFA can generate more than one tri-methyl silyl derivative from a single α-keto acid due to partial derivatization. The phenomenon of partial derivatization was previously reported using BSTFA (Díaz-Cruz *et al.*, 2003).

Heterologous expression of *CmArAT1* and *CmBCAT1* in Escherichia coli and preparation of bacterial lysates

The plasmids used for expression were pBK-CMV carrying the inserts under the control of *Lac* promoter (Figure 5). The clones from the melon EST Database harboring the *CmArAT1*and *CmBCAT1* genes were extracted, sequenced and transformed into chemically competent JM 109 *E. coli* bacteria (Promega). Then, the cultures were incubated overnight in 3 ml LB medium containing 5 mM kanamycin at 37°C with shaking at 225 RPM, and then transferred to 50 ml LB medium and incubated for another 6 hours at 37°C with shaking at 225 RPM. At this stage, isopropylthio-β-galactoside (IPTG) was added to a final concentration of 1 mM, and bacteria were incubated for another night at 30°C with shaking. The bacteria were then centrifuged and the pellet was suspended in 1 ml of lysis buffer containing 50

mM bis-tris propane pH 8.5, 10% (w/v) D-sorbitol, 1 mM dithiothretiol, 25 µM PLP and 100 µg/ml lysozyme (Sigma grade VI from chicken egg, 60,000 units / mg protein). The samples were then vigorously mixed and incubated on ice for 20 min. Then, the cells were frozen with liquid nitrogen and thawed twice. After the cells lysed, the suspensions were centrifuged (26,000 *g* for 20 min at 4°C). The supernatants were desalted on a P-6 column (BioRad Labs. Inc., Hercules, CA, USA), the protein containing fractions were collected and stored at -20°C until used.

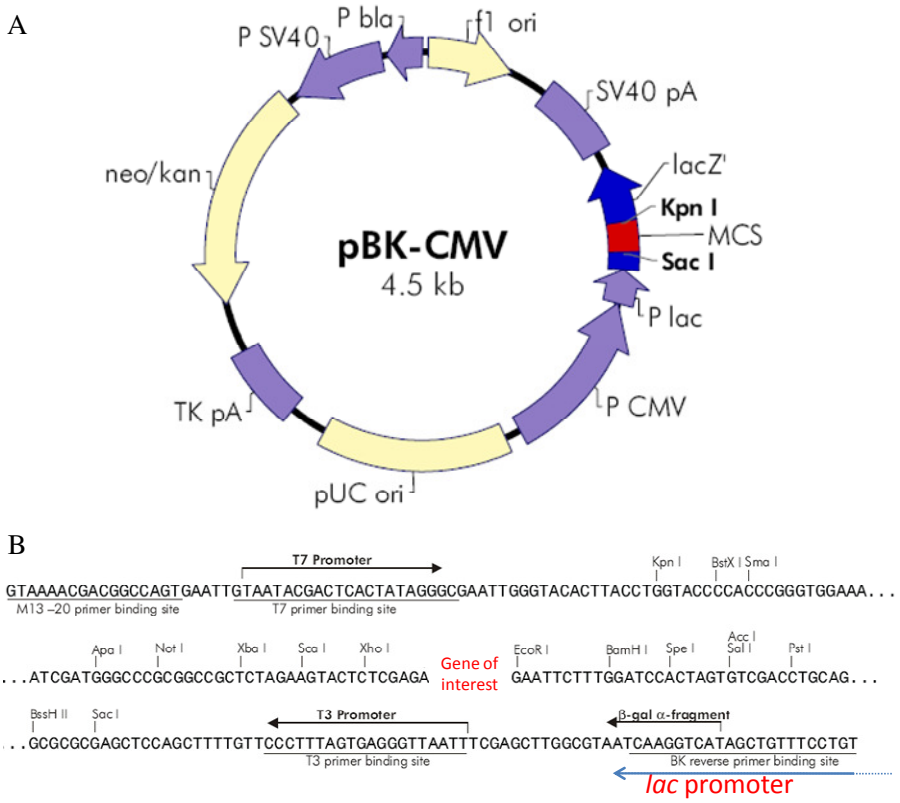


Figure 5. Map of the plasmid used for the heterologous expression.
A. general map of pBK-CMV plasmid. B. Zoom into the multi cloning site, show the exact location of the inserts, cloned in the making of the EST library.

cDNA synthesis for qRT-PCR

Total RNA was isolated as previously described (Portnoy *et al.*, 2008). RNA samples (20 µg) were treated with RNAse-free DNAse I (20 units) (EPI-CENTER®, USA) for 15 min at 37°C. First strand cDNA was synthesized from 1 µg of total RNA by the use of Verso™ cDNA kit (ABgene®'s Inc., Epsom, Surrey, UK), using a blend of

random hexamers and anchored oligo-dT primers (3:1). The cDNA was then diluted into a total volume of 100 µl of 3 mM Tris-HCl, pH 7.2, 0.2 mM EDTA solution.

Quantitative real-time PCR analysis

qRT- PCR was performed on an ABI PRISM® 7000 Sequence Detection System using SYBR® Green PCR Master Mix (Applied Biosystems, USA). Amplifications were conducted using the ABsolute™ QPCR SYBR® Green Mixes (ABgene®'s Inc., Epsom, UK). A 2-µl aliquot of cDNA was used for each qRT-PCR reaction. Thermo-cycling was initiated by 15-min incubation at 95° C, followed by 40 cycles (90°C, 15 s; 60°C, 1 min). A melting-curve analysis was performed for each reaction to confirm the specificity of amplification. The relative quantification of gene expression was performed using the housekeeping gene *cyclophilin* from melon as a reference. qRT-PCR was performed in duplicate for each primer combination. Three biological samples of each developmental stage were tested. Ct values were determined by the ABI Prism 7000 SDS software and exported into MS Excel workbook (Microsoft Inc., Redmond, WA) for statistical analysis. Real-time efficiencies (E) were calculated from the slopes of standard curves for each gene ($E = 10^{[-1/slope]}$) (Ramakers *et al.*, 2003). The relative expression ratio (R) was calculated according to Pfaffl (2001) while the control was the sample of the respective young fruit (12 DAA).

The following primers (0.2 µm final concentration) were used:
(1) *CmArAT1*: forward primer 5'- AAATGGACACAGCTTCAACTATC -3'
and reverse primer 5'- AACAAGAATAAGCAGAAGGGTC -3';
(2) *CmBCAT1*: forward primer 5'- ATGATGAGAGCTGTGATTTTGAC -3'
and reverse primer 5'- TCCCATAACGGCTCATTTG -3';
(3) *cyclophilin* (housekeeping gene, accessions no. DV632830) forward primer:
5'- GATGGAGCTCTACGCCGATGTC -3'
and reverse primer 5'- CCTCCCTGGCACATGAAATTAG-3'.

Bioiformatic analyses

Sequence homologies were based on the EBI align tool (http://www.ebi.ac.uk/Tools/emboss/align/), as preformed by the Needle program in

EBLOSUM62 matrix. Multiple sequence alignment was done with EBI MUSCLE tool (http://www.ebi.ac.uk/Tools/muscle/), and shaded with Boxshade version 3.21 (http://www.ch.embnet.org/software/BOX_form.html). The phylogenetic tree was constructed using the PyhML method using ClustalW alignment (without alignment curation), and visualized utilizing the TreeDyn "A la carte" mode (http://www.phylogeny.fr/). Family and subfamily division noted in the text and in Figure 13, were determined by the "HMM sequence scoring" tool of the Panther classification system (http://www.pantherdb.org/)

N-terminus targeting predictions were done using the following tools: Predotar (http://urgi.versailles.inra.fr/predotar/predotar.html), MITOPRED (http://bioapps.rit.albany.edu/MITOPRED/), Mitoprot (http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html), Target 1.1 (http://www.cbs.dtu.dk/services/TargetP/), iPSORT (http://psort.ims.u-tokyo.ac.jp/), LOCTree (http://cubic.bioc.columbia.edu/services/loctree/).

RESULTS

The free amino acids levels in melon fruits increase during fruit development

To estimate the availability of free amino acids to volatiles production, a preliminary experiment was carried out in which the level of free amino acids of 4 melon cultivars (2 climacteric and 2 non-climacteric) was measured using ninhydrin reagent. The climacteric cultivars ('Dulce' and 'Noy Yizre'el) show significant increases of total free amino acids levels in the late stages of 30 and 35 DAA compared to the earlier developmental stages (12 and 25 DAA), and especially the cultivar 'Dulce' (Figure 5). The free amino acids level in the non-climacteric cultivars ('PI' and 'Rochet') increased more moderately as compared to the climacteric cultivars (Figure 5).

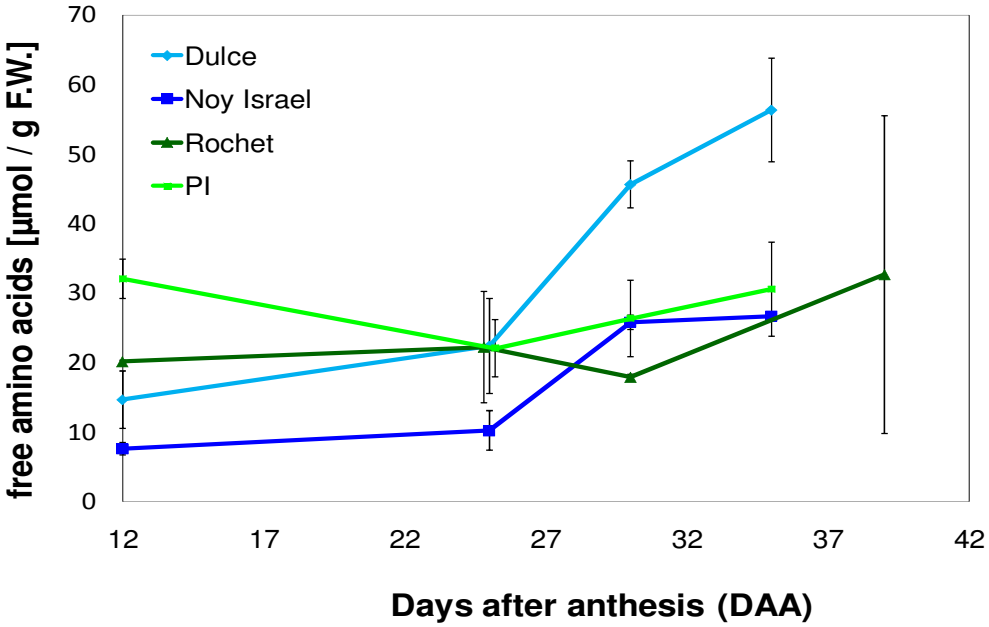
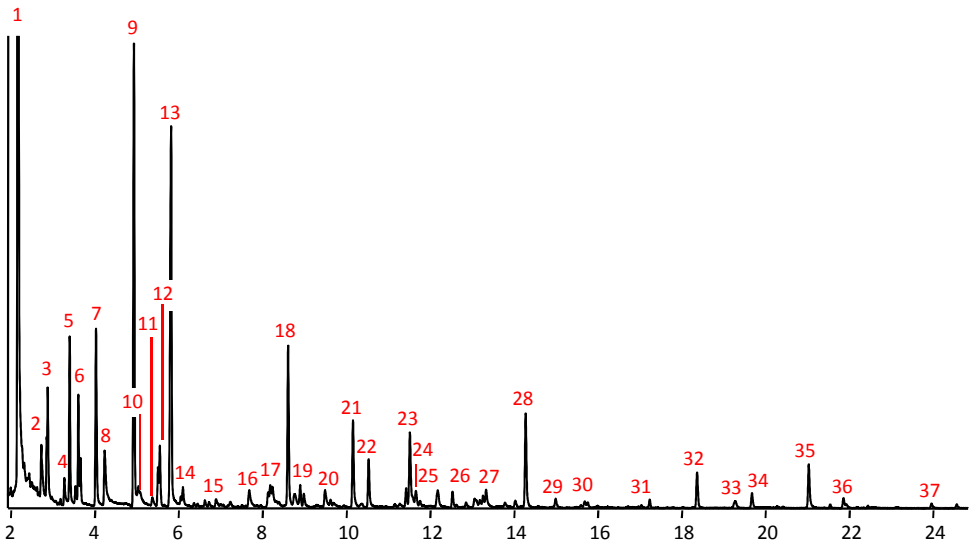


Figure 6. Free amino acids levels in melon fruits during development**.** The levels of total free amino acids in the fruits during development of 4 melon cultivars. The results are mean of 1-3 biological repeats ± SD.

Exogenous amino and α-keto acids enhance the formation of aroma compounds in melon cubes

Melons contain a vast number of aroma compound (see Figure 7). To assess the biosynthetic origin of aroma compounds in melon, cubes of ripe flesh fruits were incubated with exogenous amino acids, and the volatiles in the cubes were monitored. Incubation with a specific amino acid resulted in enhanced levels of



1-ethyl acetate
2-ethyl propanoate
3-propyl acetate
4-2-methylbutanol
5-ethyl isobutanoate
6-isobutyl acetate
7-ethyl butanoate
8-unknown
9-ethyl-2-methylbutanoate
10-2-hexenal (E)
11-hexanol
12-2-methylbutyl acetate / 3-methylbutyl acetate
13-2-heptanone – I.S.
14-ethyl valerate / heptanal
15-S-methyl-2-methylbutanthioate
16-benzaldehyde
17-ethyl-2(methylthio) acetate
18-ethyl hexanoate
19-hexyl acetate
20-1,8-cineole
21-2,3-butanedioldiacetate
22-unknown
23-ethylheptanoate / ethyl 3-(methylthio)propanoate
24-nonanal
25-methyl octanoate
26-unknown
27-benzyl acetate
28-ethyl octanoate
29-1-cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl
30-ethylphenyl acetate
31-ethyl nonanoate
32-6-methoxy-2-ethoxy-7-methyl-7H-purine
33-unknown
34-ethyl decanoate
35-neryl acetone
36-beta ionone
37-ethyl dodecanoate

Figure 7. Gas-chromatogram of melon aroma volatiles of the 'Dulce' cultivar.
Only major peaks are numbered.

volatiles with a similar structure to the side chain of the amino acid supplied (Figures 8, 9 and Table 1). Exogenous L-isoleucine increased the levels of 2-methyl butanal, 2-methyl butanol, 2-methyl 2-butenal, methyl 2-methyl butanoate, ethyl 2-methylbutanoate, 2-methylbutyl acetate, ethyl tiglate, propyl 2-methylbutanoate, 2-methylbutyl propionate and *S*-methyl-2-methylbutaethioate by 3.4 to 81.5 fold. The compounds 2-methyl butanoic acid and 2-methylbutyl 2-methylbutanoate, were

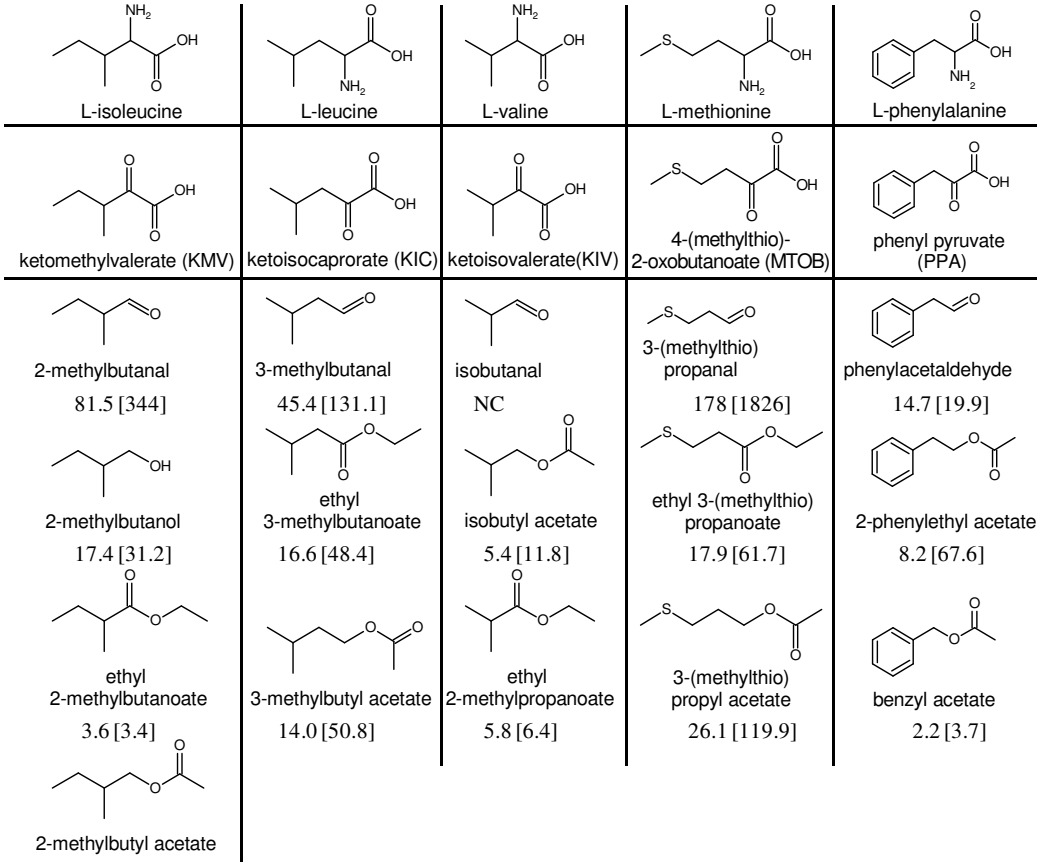
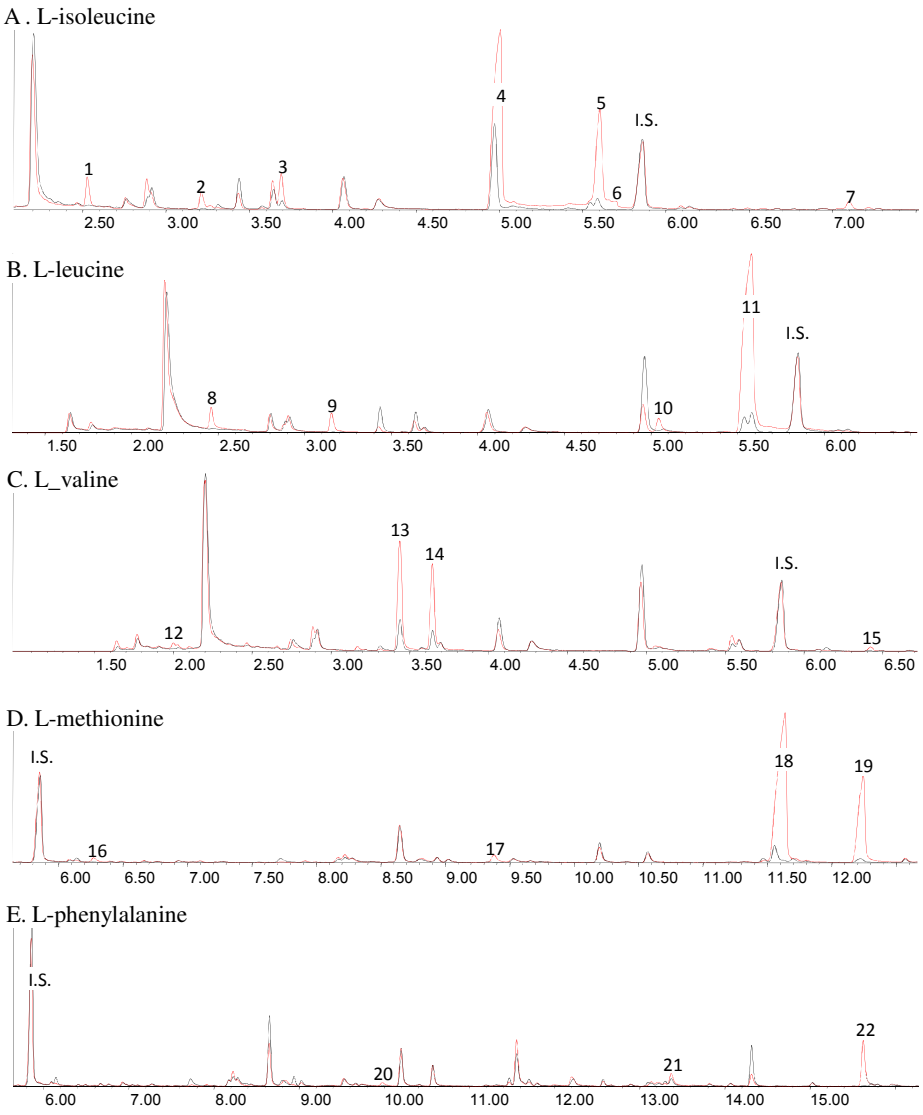


Figure 8. Melon volatiles derived from amino and keto acid feeding experiments. Melon cubes were incubated overnight with 30 mM aqueous solutions of the respective amino or α-keto acid, and compared to control incubations (water). The acids supplied are shown at the top two rows. Numbers represent the fold increase in the corresponding compound when the respective amino acid was supplied. Numbers in brackets depict the fold increase in the levels of the compound when the respective α-keto acid was supplied. The volatiles were identified and quantified using SPME coupled to GC-MS. NC indicates a novel compound that was absent in the controls.

detected in melons cubes incubated with L-isoleucine but absent in control (DDW) incubations. All of the above volatiles display the typical L-isoleucine side chain. Exogenous L-leucine enhanced the levels of: 3-methylbutanal, 3-methylbutanol, ethyl isovalerate and 3-methylbutyl acetate (14.0 to 100.7 fold). These volatiles display the typical L-leucine side chain. Exogenous L-valine enhanced the levels of ethyl isobutanoate, isobutyl acetate and isobutyl isobutanoate (5.3 to 51 fold). Moreover, isobutanal and methyl isobutanoate were present in L-valine fed cubes but undetected in the controls.



1- 2-methylbutanal
2- 2-methylbutanol
3- methyl-2-methylbutanoate
4- ethyl-2-methylbutanoate
5- 2-methylbutyl acetate
6- 2-methylbutanoic acid
7- S-methyl-2-methylbuthanthioate
8- 3-methylbutanal
9- 3-methylbutanol
10- methyl-3-methylbutanoate
11- 3-methylbutyl acetate
12- isobutanal
13- ethyl isobutanoate
14- isobutyl acetate
15- isobutyl isobutanoate
16- 3-(methylthio)propanal
17- methyl 3-methylthiopropinioate
18- ethyl 3-methylthiopropinioate
19- 3-(methylthio)propyl acetate
20- phenylacetaldehyde
21- benzyl acetate
22- ethyl phenylacetate

Figure 9. Gas-chromatograms of the aroma volatiles of melon cubes incubated with amino acid solutions.

Traces indicated incubation with 30 mM amino acid (red) or water (black). All chromatograms are representative for 4 to 10 biological repeats. Samples were analyzed with SPME method. 2-Heptanone was used as internal standard (0.1ppm). I.S. – internal standard.

Table 1. Amino acid-derived aroma volatiles in incubation experiments of melon cubes with different L-amino acids

Compound name	Identification with	Solution RT	H_2O	L-leucine	L-isoleucine	L-valine	L-methionine	L-phenylalanine	Q-ion
			Volatile quantity [ng / g fw]						
Valine derived aroma volatiles									
isobutanal	MS	2.00	n.d.	n.d.	0.9±0.1	11.0±2.9	n.d.	0.2±0.2	72
methyl isobutanoate	MS,KI	2.66	n.d.	n.d.	n.d.	10.6±4.0	n.d.	n.d.	43
ethyl isobutanoate	MS,KI	3.44	15.3±1.9	4.3±0.0	7.0±1.1	82.3±9.8	7.8±0.0	19.4±0.0	71
isobutyl acetate	MS,KI	3.65	35.7±3.1	16.9±0.1	71.0±22.5	207.9±29.2	26.3±1.1	51.9±6.1	43
isobutyl isobutanoate	MS	6.42	0.2±0.2	n.d.	0.2±0.2	10.2±1.9	n.d.	n.d.	71
Leucine derived aroma volatiles									
3-methylbutanal	MS,KI	2.47	3.1±0.6	145.3±0.1	5.2±2.3	10.1±3.2	12.9±7.1	3.5±0.6	71
3-methylbutanol	MS,KI	3.17	0.8±0.3	80.6±0.9	0.9±0.4	5.3±2.1	1.3±0.2	2.8±0.7	55
ethyl isovalerate	MS,KI	5.06	1.4±0.2	23.1±0.0	1.0±0.1	5.7±1.3	0.9±0.3	1.7±0.1	88
3-methylbutyl acetate	MS,KI	5.54	20.8±5.1	290.4±4.9	3.5±2.0	30.9±0.1	14.6±3.3	22.5±3.4	43
Isoleucine derived aroma volatiles									
2-methylbutanal	MS,KI	2.53	0.5±0.3	1.6±0.0	32.5±14.4	1.9±0.1	0.5±0.1	0.8±0.2	57
2-methylbutanol	MS,KI	3.22	2.0±0.9	9.0±0.5	34.9±5.4	3.3±0.5	2.1±0.4	3.0±0.5	70
2-methyl 2-butenal	MS	3.27	0.5±0.1	n.d.	21.6±12.6	0.7±0.1	0.2±0.2	0.4±0.0	84
methyl 2-methylbutanoate	MS,KI	3.69	10.2±4.1	3.9±0.0	71.8±10.1	9.7±2.6	8.6±0.1	10.4±0.9	88
ethyl 2-methylbutanoate	MS,KI	4.99	157.4±24.1	56.8±0.0	563.5±35.3	163.1±32.9	103.9±0.7	120.8±4.8	102
2-methylbutyl acetate	MS,KI	5.61	29.5±6.4	11.8±2.3	394.1±178.6	28.1±3.7	19.9±2.5	28.1±5.6	70
2-methyl butanoic acid	MS,KI	5.71	n.d.	n.d.	34.1±1.3	1.0±1.0	n.d.	1.0±1.0	74
ethyl tiglate	MS	7.04	1.6±0.1	0.4±0.1	5.4±2.1	1.4±0.3	0.8±0.1	1.3±0.1	83
S-methyl-2-methylbutaethioate	MS***	7.10	0.9±0.1	n.d.	48.9±29.8	1.0±0.7	3.5±0.2	0.8±0.2	85
propyl 2-methylbutanoate	MS,KI	7.22	1.0±0.1	0.1±0.1	8.9±3.4	1.0±0.3	0.7±0.0	0.5±0.0	103
2-methylbutyl propionate	MS,KI	7.91	0.7±0.7	n.d.	3.2±2.7	n.d.	n.d.	n.d.	70
2-methylbutyl 2-methylbutanoate	MS,KI	11.64	n.d.	n.d.	4.8±1.8	n.d.	n.d.	n.d.	85
Methionine derived aroma volatiles									
dimethyl disulfide	MS,KI	3.31	0.4±0.4	1.1±0.1	2.4±0.6	3.4±0.9	5.6±1.1	3.2±0.8	94
3-(methylthio) propanal	MS,KI	6.30	0.1±0.0	0.1±0.0	0.1±0.0	n.d.	17.7±8.4	0.1±0.0	104
3-(methylthio) propanol	MS,KI	8.16	n.d.	n.d.	n.d.	n.d.	8.2±1.6	n.d.	106
ethyl (methylthio) acetate	MS,KI	8.24	8.2±3.7	3.4±0.3	3.1±0.0	7.5±1.1	13.8±3.7	20.5±2.0	134
methyl 3-methylthiopropinioate	MS,KI	9.39	0.6±0.2	0.1±0.1	0.3±0.1	0.3±0.1	17.2±2.3	1.1±0.4	134
ethyl 3-methylthiopropinioate	MS,KI	11.55	27.3±10.5	10.3±0.3	5.0±1.4	13.3±2.0	487.4±75.1	53.0±11.6	74
3-(methylthio) propylacetate	MS,KI	12.22	8.2±1.3	2.5±0.1	3.9±1.3	4.4±0.6	214.0±6.3	19.2±1.5	43
Phenylalanine derived aroma volatiles									
benzaldehyde	MS,KI	7.72	1.0±1.0	4.8±0.1	6.2±2.4	3.9±1.0	3.0±1.1	3.0±0.9	106
benzyl alcohol	MS,KI	9.73	2.5±0.9	3.9±0.0	2.5±0.2	2.8±0.0	2.6±0.3	6.1±0.9	79
phenylacetaldehyde	MS,KI	9.98	0.6±0.6	0.6±0.0	0.2±0.1	0.2±0.0	0.3±0.1	8.8±1.9	91
phenethyl alcohol	MS,KI	11.97	1.1±1.1	0.6±0.2	n.d.	n.d.	n.d.	4.4±0.6	91

Table 1 - continued

Compound name	Identification with	Solution RT	H_2O	L-leucine	L-isoleucine	L-valine	L-methionine	L-phenylalanine	Q-ion
			Volatile quantity [ng / g fw]						
benzyl alcohol	MS,KI	13.38	22.2±11.5	6.2±0.0	14.7±8.8	11.7±5.7	15.3±4.0	48.9±28.1	108
ethyl benzoate	MS,KI	13.62	0.8±0.2	1.5±0.4	0.1±0.1	0.9±0.2	0.9±0.1	3.2±1.0	105
methyl phenylacetate	MS,KI	13.74	n.d.	n.d.	n.d.	n.d.	n.d.	0.4±0.1	91
ethyl phenylacetate	MS,KI	15.62	1.5±0.5	1.1±0.0	0.4±0.0	1.1±0.3	0.7±0.2	68.3±4.3	91
phenethyl acetate	MS,KI	15.96	1.2±0.5	0.2±0.0	0.3±0.2	0.1±0.1	0.4±0.2	9.7±4.2	104

Values are in ng g^{-1} fw. The concentration of all amino-acids solutions was 30 mM, similar results were obtained when using 5 mM (not shown). pH of all solutions was 7.0. Data shown are average of two biological replications ± SE. RT – retention time, MS – mass spectrum, KI – Kovats index, n.d. – not detectable. Q-ion – ion used for quantification. ***-identification according to Kourkoutas et. al., (2006).

All these compounds display the typical L-valine side chain. Exogenous L-methionine increased the levels of almost all of the melon volatiles that contain sulfur atoms such as dimethyl disulfide, 3-(methylthio)-propanal, *S*-methyl-3-methylbutaethioate, ethyl (methylthio) acetate, methyl 3-methylthiopropionate, ethyl 3-methylthiopropionate and 3-(methylthio) propylacetate by 1.7 to 177 fold. The compound 3-(methylthio) propanol was only present after incubation with L-methionine and absent in control incubations. Exogenous L-phenylalanine increased the levels of benzaldehyde, benzyl alcohol, phenylacetaldehyde, phenethyl alcohol, benzyl acetate, ethyl benzoate, ethyl phenylacetate, phenethyl acetate by 2.4 to 42.8 fold. Methyl phenylacetate was present in L-phenylalanine fed cubes but undetected in the controls. All of the above compounds contain an aromatic ring reminiscent of L-phenylalanine.

The biochemical origin of the aromatic volatiles was further confirmed by incubation of melon cubes with 5 mM L-[ring-$^{13}C_6$]phenylalanine. The $^{13}C_6$ label incorporated (up to 50%, Figure 10) into the same volatiles whose levels increased when non-labeled L-phenylalanine was previously administered (Figures 7 and 8).

To assess whether these conversions take place through transamination *via* α-keto acid intermediates (Figure 3, green route), cubes of ripe melon fruits were incubated with the corresponding α-keto acids. All α-keto acids were effective substrates for the formation of the respective aroma compounds. In most of the cases, the α-keto acid had a more pronounced effect than the amino acid itself, on the formation of the aforementioned aroma compounds (Table 2). The L-isoleucine derived aroma compounds levels were enhanced up to 344 fold when incubated with ketomethylvalerate (KMV, the α-keto acid derived from L-isoleucine). The levels of L-leucine derived aroma compounds increased up to 160 fold when incubated with ketoisocaproate (KIC, the α-keto acid derived from L-leucine). The levels of L-valine derived aroma compounds levels increased up to 100 fold when incubated with ketoisovalerate (KIV, the keto acid derived from L-valine). The levels of L-methionine derived aroma compounds were enhanced up to 1820 fold when incubated with 4-methylthio-2-oxobutyrate (MTOB, the α-keto acid derived from L-methionine). Finally, the levels of L-phenylalanine derived aroma compounds increased up to 293 fold when incubated with phenylpyruvic acid (PPA, the α-keto acid derived from L-phenylalanine). The values presented in Figure 8 and in Tables 1 and 2 are the means of two biological repeats; similar results were obtained in

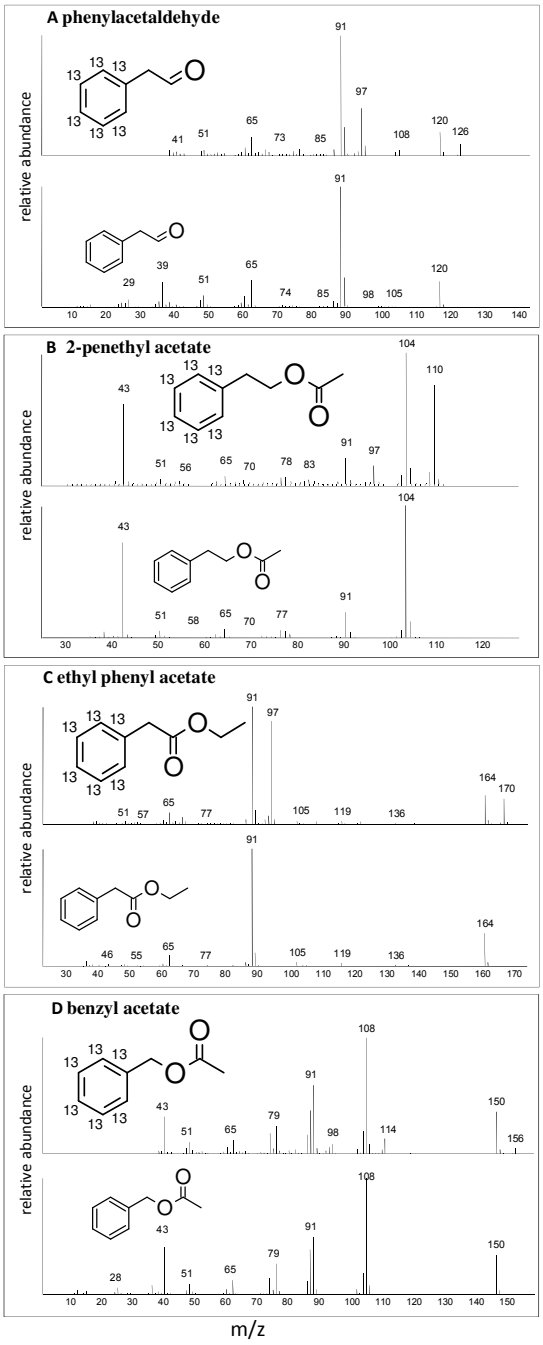


Figure 10. Incorporation of L-[ring-$^{13}C_6$]phenylalanine into melon volatiles.

Mass spectra of four volatiles (out of 8) containing aromatic rings that were detected in the ^{13}C-phe fed melon cubes. A. phenylacetaldehyde, B. 2-phenethyl acetate, C. ethyl phenyl acetate, D. benzyl acetate. The upper spectra in each panel represent the masses of the biosynthetic volatile, and the lower spectra in each panel are from the computerized Wiley 7 N library.

additional experiments in which slightly different incubation conditions were used. The compositions of the volatiles in the solutions (devoid of melon cubes) of amino and keto acids are shown in table 3. Exept for the benzaldehyde in the L-phenylalanine and PPA solutions, phenylacetaldehyde in the L-phenylalanine solution, and dimethyl disulfide in the L-methionine and MTOB solutions, no other volatiles were detected above the levels detected in the melon cubes.

Table 2. Amino acid-derived aroma volatiles in incubation experiments of melon cubes with different α-keto-acids

Compound name	Identification with	Solution RT	H_2O	KIC	KMV	KIV	MTOB	PPA
			Volatile quantity [ng / g fw]					
Valine derived aroma volatiles								
Isobutanal	MS	2.00	n.c.	1.7±0.0	2.4±0.4	48.6±3.4	0.9±0.1	1.2±0.4
methyl isobutanoate	MS,KI	2.66	n.c.	n.c.	n.c.	6.4±1.0	n.c.	n.c.
ethyl isobutanoate	MS,KI	3.44	15.3±1.9	3.2±1.0	7.6±4.2	90.2±10.0	5.3±0.0	8.0±0.9
isobutyl acetate	MS,KI	3.65	35.7±3.1	18.5±2.3	54.2±18.0	422.6±20.6	18.5±1.7	25.8±1.3
isobutyl isobutanoate	MS	6.42	0.2±0.2	n.c.	n.c.	20.1±0.1	n.c.	n.c.
Leucine derived aroma volatiles								
3-methylbutanal	MS,KI	2.47	3.1±0.6	419.6±34.4	19.5±6.5	36.1±3.1	9.4±0.2	13.1±1.7
3-methylbutanol	MS,KI	3.17	0.8±0.3	128.2±9.7	3.6±0.2	12.4±2.1	4.5±0.8	2.3±0.6
ethyl isovalerate	MS,KI	5.06	1.4±0.2	67.8±4.0	1.0±0.2	2.8±0.2	1.0±0.0	0.5±0.0
3-methylbutyl acetate	MS,KI	5.54	20.8±5.1	1056.6±52.7	5.6±3.1	126.2±32.3	16.2±3.4	7.8±0.4
Isoleucine derived aroma volatiles								
2-methylbutanal	MS,KI	2.53	0.5±0.3	2.2±0.5	137.7±3.4	5.7±0.6	1.4±0.0	1.9±0.2
2-methylbutanol	MS,KI	3.22	2.0±0.9	3.2±0.1	62.3±8.6	4.3±0.7	2.3±0.0	3.4±0.2
2-methyl 2-butenal	MS	3.27	0.5±0.1	n.c.	7.7±0.3	0.4±0.1	0.2±0.0	0.5±0.2
methyl 2-methylbutanoate	MS,KI	3.69	10.2±4.1	2.6±1.0	51.9±14.6	7.3±0.7	7.1±0.8	6.9±0.4
ethyl 2-methylbutanoate	MS,KI	4.99	157.4±24.1	37.5±14.1	537.7±73.0	102.1±6.3	97.3±5.0	69.7±5.1
2-methylbutyl acetate	MS,KI	5.61	29.5±6.4	8.6±4.7	377.9±17.6	20.7±6.3	16.7±3.0	18.6±3.0
2-methyl butanoic acid	MS,KI	5.71	n.c.	n.c.	57.6±20.9	n.c.	0.6±0.6	n.c.
ethyl tiglate	MS	7.04	1.6±0.1	0.3±0.1	4.0±1.3	0.6±0.0	0.6±0.1	0.5±0.1
S-methyl-2-methylbutaethioate	MS***	7.10	0.9±0.1	n.c.	13.2±6.4	0.3±0.1	3.3±0.5	0.2±0.1
propyl 2-methylbutanoate	MS,KI	7.22	1.0±0.1	0.1±0.1	5.1±1.5	0.6±0.0	0.6±0.0	0.6±0.1
2-methylbutyl propionate	MS,KI	7.91	0.7±0.7	3.8±0.3	2.5±0.7	n.c.	n.c.	n.c.
2-methylbutyl 2-methylbutanoate	MS,KI	11.64	n.c.	0.5±0.5	8.1±0.6	n.c.	n.c.	n.c.
Methionine derived aroma volatiles								
dimethyl disulfide	MS,KI	3.31	0.4±0.4	0.7±0.1	1.6±0.7	0.8±0.0	4.8±1.0	2.4±0.5
3-(methylthio) propanal	MS,KI	6.30	0.1±0.0	0.3±0.0	0.5±0.4	0.3±0.2	182.6±5.5	0.3±0.1
3-(methylthio) propanol	MS,KI	8.16	n.c.	2.0±2.0	n.c.	n.c.	168.2±18.1	n.c.
ethyl (methylthio) acetate	MS,KI	8.24	8.2±3.7	7.8±5.2	3.1±0.7	8.8±1.0	17.5±2.0	9.8±2.3
methyl 3-methylthiopropinioate	MS,KI	9.39	0.6±0.2	0.5±0.3	0.3±0.0	0.6±0.0	81.3±0.8	0.5±0.1
ethyl 3-methylthiopropinioate	MS,KI	11.55	27.3±10.5	21.3±13.2	12.5±2.3	29.2±8.2	1679.2±11.4	27.4±9.4
3-(methylthio) propylacetate	MS,KI	12.22	8.2±1.3	5.1±1.9	5.5±0.7	7.0±1.5	983.3±202.3	4.3±0.9
Phenylalanine derived aroma volatiles								
benzaldehyde	MS,KI	7.72	1.0±1.0	4.3±1.5	2.9±1.4	4.8±1.1	7.3±0.5	228.1±69.4
benzyl alcohol	MS,KI	9.73	2.5±0.9	2.5±0.1	0.8±0.8	2.2±0.3	3.0±0.3	51.7±4.1
phenylacetaldehyde	MS,KI	9.98	0.6±0.6	0.6±0.0	0.3±0.0	0.7±0.1	0.5±0.1	175.8±15.3
phenethyl alcohol	MS,KI	11.97	1.1±1.1	n.c.	n.c.	0.3±0.3	n.c.	144.5±3.7
benzyl alcohol	MS,KI	13.38	22.2±11.5	8.3±1.1	4.7±1.5	7.0±3.2	5.0±2.5	82.5±1.6
ethyl benzoate	MS,KI	13.62	0.8±0.2	0.8±0.0	0.2±0.0	0.6±0.1	1.2±0.0	1.9±0.0

Table 2 - continued

Compound name	Identification with	Solution RT	H_2O	KIC	KMV	KIV	MTOB	PPA
			Volatile quantity [ng / g fw]					
methyl phenylacetate	MS,KI	13.74	n.c.	n.c.	n.c.	n.c.	n.c.	8.4±0.2
ethyl phenylacetate	MS,KI	15.62	1.5±0.5	0.5±0.2	0.4±0.2	1.3±0.2	0.6±0.3	325.0±22.7
phenethyl acetate	MS,KI	15.96	1.2±0.5	0.1±0.1	0.1±0.0	0.1±0.1	0.1±0.0	81.3±5.4

Values are in ng g^{-1} fw. The oncentration of all α-keto-acids solutions was 30mM. pH of all solutions was 7.0. All data is average of two biologic repeats ± SE. KMV - Ketomethylvalerate (keto-isoleucine). KIC – Ketoisocaproate, (keto-leucine), KIV - Ketoisovalerate (keto valine), MTOB - 4-methylthio-2-oxobutanoate (keto-methionine), PPA – phenylpyruvate (keto phenylalanine), RT – retention time, MS – mass spectrum, KI – Kovats index, n.d. – not detectable. ***-identification according to MS detected by Kourkoutas et. al., 2006.

Table 3. Levels of volatiles found in the control solutions incubated overnight without melon cubes

Solution (30 mM)	Volatiles detected [ng ml^{-1}]	
L-val	none	
KIV	none	
L-leu	none	
KIC	3-methylbutanal	8.96
L-ile	none	
KMV	2-methylbutanal	0.8
	2-methylbutanol	0.26
L-met	dimethyl disulfide	33.4
MTOB	dimethyl disulfide	289.2
	3-(methylthio) propanal	2.72
L-phe	benzaldehyde	7.92
	phenylacetaldehyde	10.48
PPA	benzaldehyde	10853
	phenylacetaldehyde	8.82
	benzyl alcohol	12.96

Soluble protein extracts from mature melon fruits display amino acid transaminase activities accepting various amino acids as substrates

In order to account for the results of the amino and α-keto acid feeding experiments, I searched for transaminase enzyme activities in melon crude cell-free extracts. Soluble protein was extracted from the flesh of ripe melon fruits, and the extracts were assayed for amino acid transaminase activity with various amino acids as substrates. The keto acid, α-keto glutarate, the most ubiquitous amine acceptor of aminotransferases, was used as the amine acceptor in the reactions. First, small-scale assays with ^{3}H-labeled amino acids were preformed. Amino acid transaminase activity was readily measured in the cell free extracts. The highest activity (1430.2 ± 26.6 pkat mg protein $^{-1}$) was observed when L-[$^{3}H_2$]leucine was used as a substrate, while a substantial but lower level of 843.7±1.9 pkat mg protein $^{-1}$ was observed when L-[$^{3}H_2$]isoleucine was used as a substrate (Figure 11). Much lower but still significant transaminase enzymatic activities were observed when L-[$^{3}H_3$]methionine (37.7±4.8 pkat mg protein $^{-1}$) and L-[$^{3}H_2$]phenylalanine (5.7±1.2 pkat mg protein $^{-1}$) were utilized as substrates (Figure 11). No activity was detected in control reactions devoid of α-keto glutarate, reactions without protein added or reactions when heat-inactivated enzyme was used. Attempts to identify L-phenylalanine decarboxylase activity were unsuccessful.

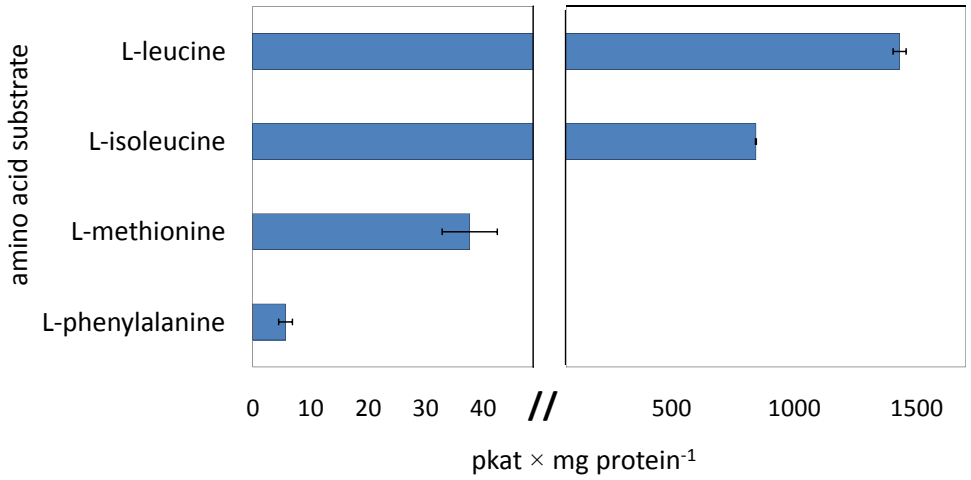


Figure 11. Amino acid transaminase activities in soluble protein melon crude extracts (radioactive assays). The activities were measured with scintillation counter (see materials and methods). Values are means of two replications ± SE.

To identify the products generated in the transaminase reactions, GC-MS analyses of larger scale assays were preformed. The production of the corresponding α-keto acid was assessed using a derivatization method (see materials and methods), because the α-keto acids formed cannot be detected directly using GC-MS. All the different amino acids substrates supported the transamination reactions (Figure 11) forming the corresponding α-keto acids. The products generated were KMV for L-isoleucine, KIC for L-leucine, KIV for L-valine, MTOB for L-methionine and PPA for L-phenylalanine. No activity was detected in any of the control assays that included the replacement of active enzyme with heat-inactivated protein, omission of protein, no addition of α-keto glutarate, or when no L-amino acid substrate was added. Interestingly, when the co-factor PLP was omitted from both the extraction and reaction buffers, the activity remained unaffected.

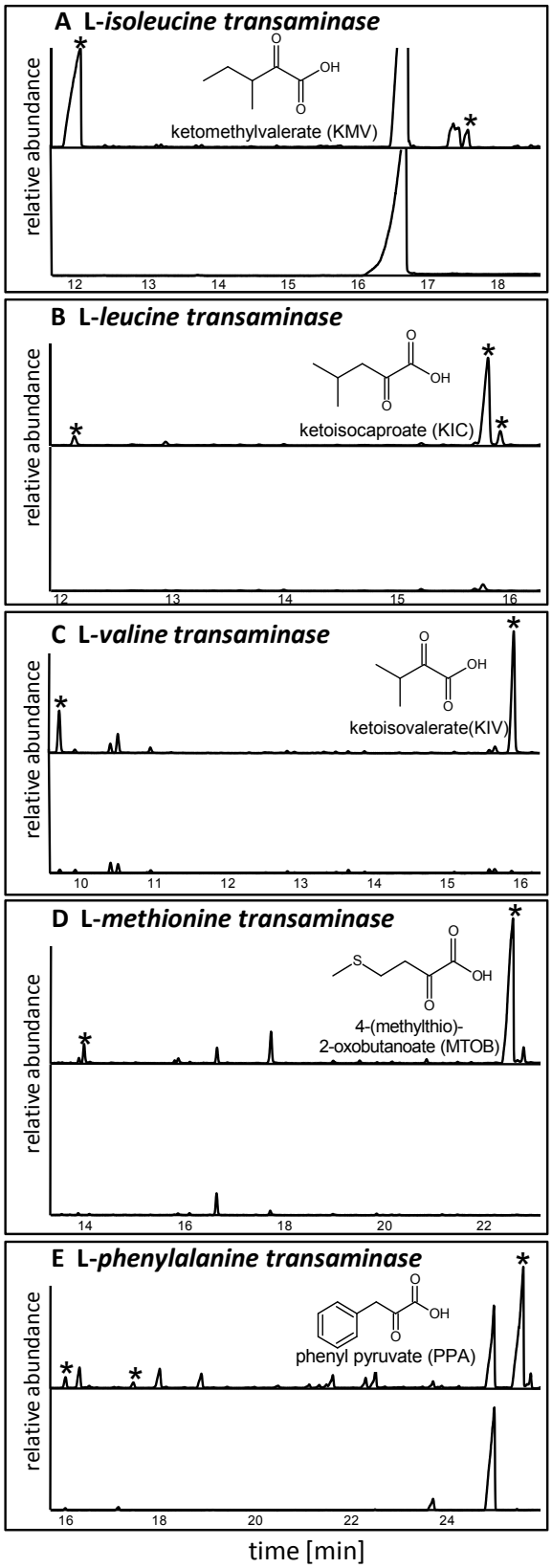


Figure 12. Amino acid transaminase activities in soluble protein melon crude extracts. GC-MS chromatograms of the enzymatic products after derivatization with BSTFA. The substrates of the reactions were: A. L-isoleucine, B. L-leucine, C. L-valine, D. L-methionine, E. L-phenylalanine. The upper chromatograms in each panel depict the full reactions and the lower chromatograms are the control reaction assays lacking α-keto glutarate. Similar chromatograms were obtained in control assays that lacked protein or amino acid substrates, or when heat-inactivated enzyme was used (not shown). Asterisks represent peaks that are typical for the tri-methyl silyl derivates of the α-keto acids generated. All chromatograms are typical for two replications.

The genes *CmArAT1* and *CmBCAT1* encode aromatic amino acid transaminase and branched-chain amino acid transaminase enzymes, respectively

The melon EST Database (http://www.icugi.org/) contain comprehensive annotations of clones from a collection of Expressed Sequence Tag (EST) libraries, most of them from the fruit. This database has proven to be a good source for identifying genes affecting quality characteristics of melon, including aroma traits (Ibdah *et al.*, 2006; Portnoy *et al.*, 2008). Data mining of this database revealed one unigene (comprising 3 clones originating from ripe fruit), with strong similarity to tyrosine transaminases. The 3 clones were overexpressed in JM109 competent cells and one of the clones (DV634148 later termed *CmArAT1*), encoded a protein possessing L-phenylalanine and L-tyrosine transaminase activities (see below). *CmArAT1* is a full-length clone coding for a 412 aa protein with an estimated molecular weight of 45.6 KDa, as predicted by Expasy. CmArAT1 belongs to the aminotransferase related subgroup I gene family, and to the tyrosine aminotransferase subfamily according to the Panther classification system (see Figure 13) (http://www.pantherdb.org/). As judged by the EBI align tool, CmArAT1 is moderately similar to the *Arabidopsis thaliana* tyrosine aminotransferase 1 (AtTAT1) (44% identity), the only other plant tyrosine transaminase gene that has been previously functionally identified (Lopukhina *et al.*, 2001). Interestingly CmArAT1 is 33 % identical to the *Rattus norvegicus* tyrosine aminotransferase (RnTAT), 32% identical to the *Homo sapien*s tyrosine aminotransferase (HsTAT), and 30% identical to the *Trypanosoma cruzi* tyrosine aminotransferase (TcTAT), that also have been functionally identified (Grange *et al.*, 1985; Dietrich *et al.*, 1991; Montemartini *et al.*, 1993; Seralini *et al.*, 1995). The Lys 245 residue inferred in PLP binding is conserved in all functionally expressed aromatic amino acid aminotransferases, including CmArAT1 (Figure 14A). Bioinformatic analysis of the N-terminus of CmArAT1 using several tools reveals no clear identifiable sequence for sub-cellular targeting of the gene product.

Further exploration of the database seeking for branched chain amino acid transaminases yielded a unigene comprising 14 clones derived from ripe fruits of different cultivars and chosen for further analyses. The unigene had significant similarity to other members of the BCAT gene family. Three of the clones that had substantial 5' or 3' sequence information were transformed to *E. coli* cells and grown

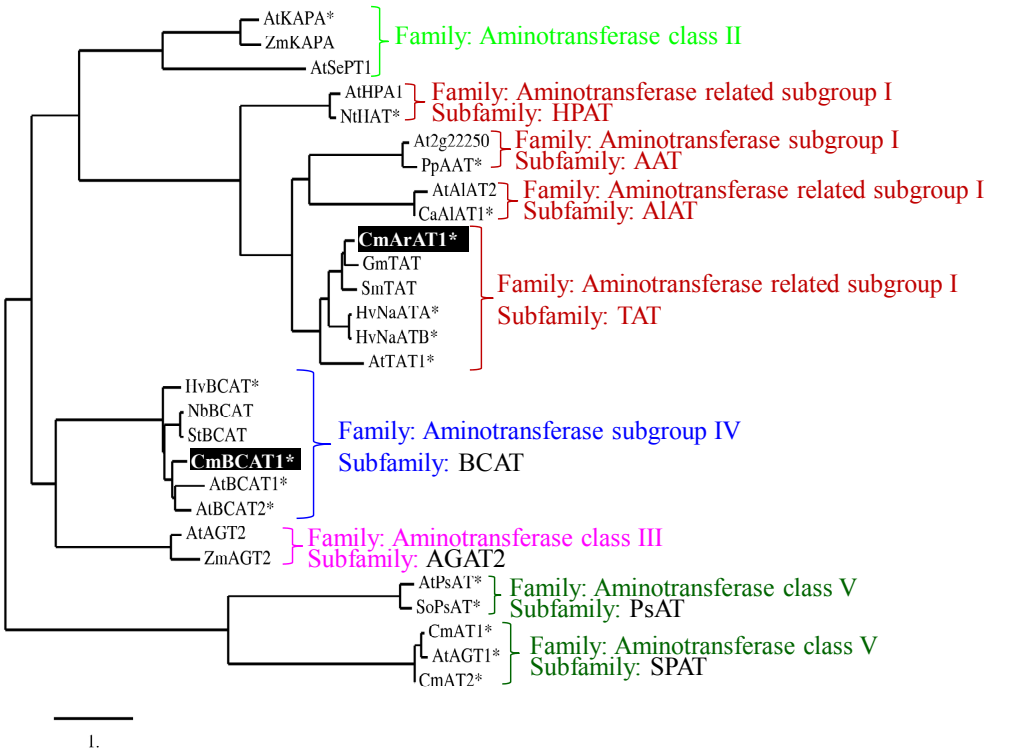


Figure 13. Phylogenetic tree of plant amino acid transaminases. Amino acid sequences of representative members of the "amino acid transaminase related" gene superfamily were compared utilizing the "A la carte" mode of Phylogeny.fr program (http://www.phylogeny.fr/). Family and subfamily divisions were determined by the "HMM sequence scoring" tool of the Panther classification system (http://www.pantherdb.org/). The novel melon CmArAT1 and CmBCAT1 sequences are in black shaded characters. AT-aminotransferase, AAT- aspartate aminotransferase, HPAT-histidinol-phosphate aminotransferase, TAT-tyrosine aminotransferase, AlAT – alanine aminotransferase, BCAT-branched-chain aminoacids transaminase, AGAT2-alanine-glyoxylate aminotransferase 2, PsAT- phosphoserine aminotransferase, SPAT- serine-pyruvate aminotransferase. Genes accession numbers and organism names are given in table 4. Asterisks represent genes that have been functionally identified by heterologous expression.

Table 4. Protein accession numbers for genes in Figure 13			
Name in the text	**UniprotKB accession no.**	**Genebank accession no.**	**organism**
SmTAT	A9X448	ABC60050	*Salvia miltiorrhiza*
GmTAT	Q4ZJF7	AAY21813	*Glycine max* (L.) Merr.
HvNaATA	Q9SMG7	BAA87052	*Hordeum vulgare* L.
HvNaATB	Q9ST03	BAA87054	*Hordeum vulgare* L.
PpAAT	Q5F4K8	CAF31327	*Pinus pinaster* Ait.
SoPsAT	P52877	BAA12206	*Spinacia oleracea* L.
CmAT2	Q6V1W4	AAQ56195	*Cucumis melo* L.
CmAT1	Q6V1W7	AAQ56192	*Cucumis melo* L.
AtTAT1	Q9SUR6	AAK82963	*Arabidopsis thaliana* (L.) Heynh.
AtBCAT1	Q93Y32	CAB93130	*Arabidopsis thaliana* (L.) Heynh.
AtBCAT2	Q9M439	CAB93128	*Arabidopsis thaliana* (L.) Heynh.
AtSePT1	Q9LSZ9	AAK92764	*Arabidopsis thaliana* (L.) Heynh.
AtPSAT	Q96255	BAA13640	*Arabidopsis thaliana* (L.) Heynh.
AtAGT2	Q940M2	AAD48837	*Arabidopsis thaliana* (L.) Heynh.
AtKAPA	Q2QKD2	AAY82238	*Arabidopsis thaliana* (L.) Heynh.
AtHPA1	Q949X3	AAK92767	*Arabidopsis thaliana* (L.) Heynh.
NtHPA	O82030	CAA70403	*Nicotiana tabacum* L.
AtAlAT2	Q9LDV4	AAF82781	*Arabidopsis thaliana* (L.) Heynh.
AtAGT1	Q56YA5	AAC26854	*Arabidopsis thaliana* (L.) Heynh.
At2g22250	Q3E6N9	Q3E6N9	*Arabidopsis thaliana* (L.) Heynh.
CaAlAT1	Q6VEJ5	AAR05449	*Capsicum annuum* L.
ZmKAPA	B6TFA9	ACG35792	*Zea mays* L.
ZmAGT2	B6U6Q5	ACG45038	*Zea mays* L.
NbBCAT	B6RFK8	ABW76425	*Nicotiana benthamiana* Domin.
StBCAT	Q9SNY8	AAF07192	*Solanum tuberosum* L.
HvBCAT	Q70KX9	CAE00460	*Hordeum vulgare* L.

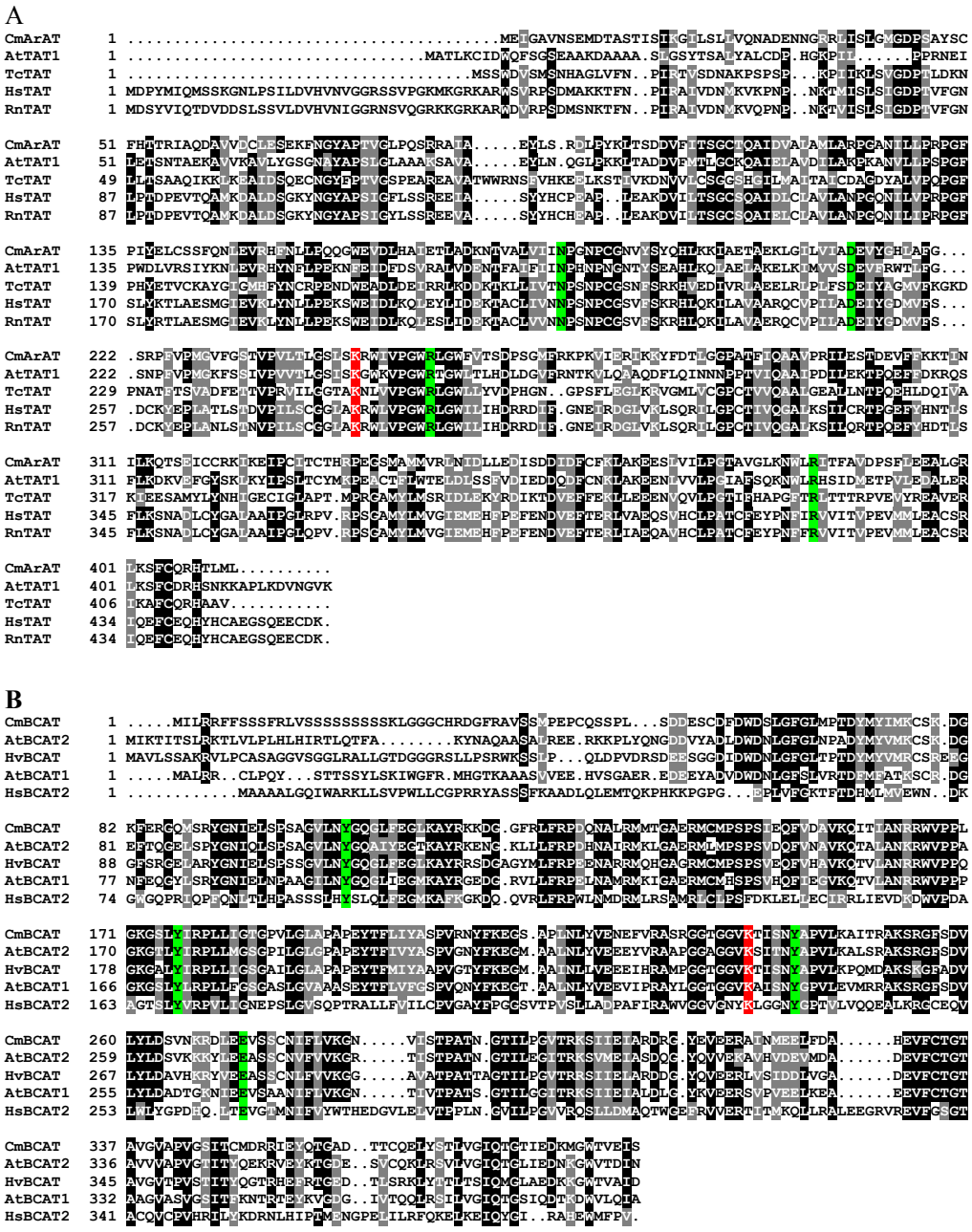


Figure 14. Amino acid sequence multiple alignments of CmArAT1 (A) and CmBCAT1 (B). AA conserved in four genes or more are shaded in black. AA similarities are shaded in gray. Red background indicates the conserved Lys residues that covalently bind the PLP cofactor and green background indicates conserved amino acids proposed to possess crucial roles in catalysis, as determined by the X-ray structures of TcTAT and HsBCAT2. Alignments were performed using the EBI 'MUSCLE' tool and shaded using BoxShade version 3.21. CmArAT1: *Cucumis melo* aromatic amino acids transaminase (GeneBank accession no. 1206059); AtTAT1: *Arabidopsis thaliana* tyrosine aminotransferase 1 (AAK82963); TcTAT: *Trypanosoma cruzi* tyrosine aminotransferase (AAA02975); HsTAT: *Homo sapiens* tyrosine

aminotransferase (AAI30535); RnTAT: *Rattus norvegicus* tyrosine aminotransferase (CAA26519); CmBCAT1: *Cucumis melo* branched-chain amino acid transaminase 1 (1206082); AtBCAT1/2; *Arabidopsis thaliana* branched-chain amino acids transaminases 1 and 2 (CAB93130, CAB93128); HvBCAT: *Hordeum vulgare* branched-chain amino acid aminotransferase (CAE00460); HsBCAT2: *Homo sapiens* branched-chain amino acid aminotransferase (AAH01900).

in LB medium (that contains amino acids) and transferred to a SPME vial containing 1 mM IPTG for induction of protein expression. After overnight incubation the vials were analyzed by GC-MS that revealed the presence of 3-methylbutanal and 2-methylbutanal in the headspace of bacteria harboring the DV63340 insert (later termed *CmBCAT1*) (Figure 15). These latter aldehydes are derived from L-leucine and L-isoleucine, respectively.

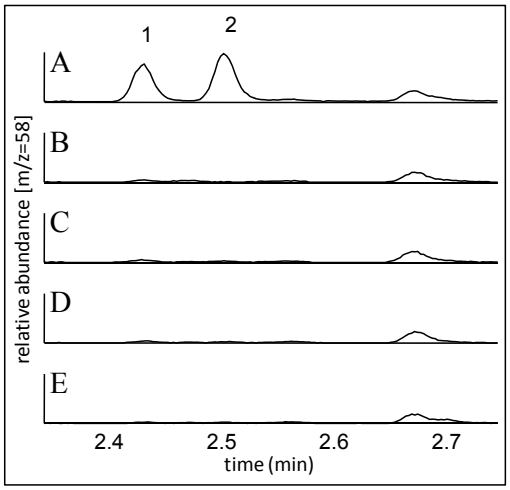


Figure 15. Functional screening for a gene coding for branched chain amino acid transaminase. Single ion (m/z=58) chromatograms of volatiles emitted by *E. coli* overexpressing clones putatively coding for *CmBCAT1*: A. Genebank accession no. DV633470, later termed *CmBCAT1*, B. *E. coli* harboring no plasmids, C. DV63385, D. DV634650, E. DV634486 (annotated as a hypothetical protein, used as control). The putative gene products generate α-keto acids from potential substrates present in the medium. α-Keto acids are not detectable under these GC-MS conditions. Peaks 1 and 2 depict 3-methylbutanal and 2-methylbutanal, putatively generated by endogenous bacterial enzymes, that decarboxylate the α-keto acid derivatives of L-leucine and L-isoleucine respectively.

CmBCAT1 codes a 389 aa protein with an estimated molecular weight of 42.7 KDa (predicted by Expasy). CmBCAT1 belongs to the aminotransferase related subgroup IV gene family, and to the branched-chain aminotransferases subfamily as predicted by the Panther classification system (see Figure 13). CmBCAT1 was 62 % identical to the *Hordeum vulgare* branched chain amino acid aminotransferase 1 (HvBCAT1),

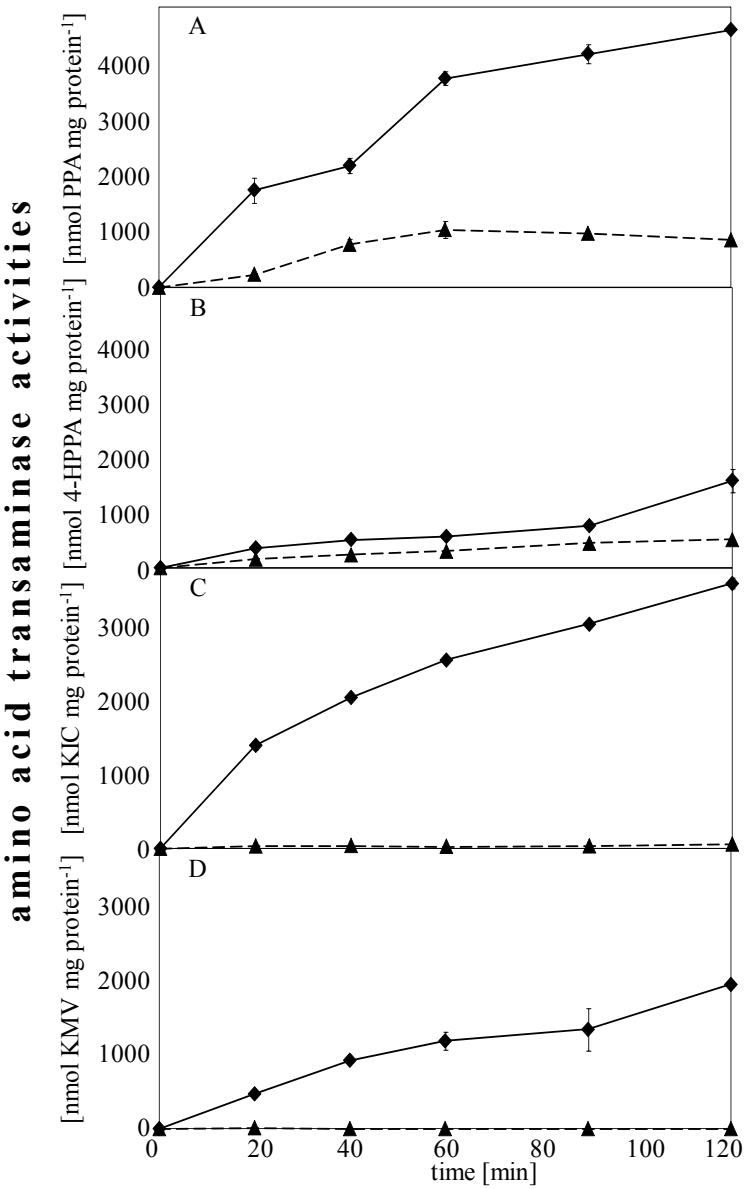


Figure 16. Functional expression of *CmArAT1* and CmBCAT1 using *in-vitro* radioactive assays. Accumulation of PPA, due to L-phenylalanine transaminase activity (A) and 4-HPPA due to L-tyrosine transaminase activity (B) of desalted lysates from *E. coli* overexpressing *CmArAT1* (solid line, diamonds), or a control insert DV633368 (dashed line, triangles). Accumulation of KIC, due to L-leucine transaminase activity (C) and KMV due to L-isoleucine transaminase activity (D) of desalted lysates from *E. coli* overexpressing *CmBCAT1* (solid line, diamonds), or a control insert DV633368 (dashed line, triangles). Values are means of two replications ± SE. PPA: phenylpyruvate, 4-HPPA: 4-hydroxy phenyl pyruvate, KIC: ketoisocaproate, KMV: ketomethylvalerate.

61% identical to *Arabidopsis thaliana* branched chain amino acid transaminase 2 (AtBCAT2), and 59% identical to the *Arabidopsis thaliana* branched chain amino acid transaminase 1 (AtBCAT1), that have been functionally identified (Diebold *et al.*, 2002; Schuster *et al.*, 2006; Malatrasi *et al.*, 2006). CmBCAT1 was also 30% identical to *Homo sapien*s branched chain amino acid aminotransferase 2 (HsBCAT2) that has also been functionally identified (Davoodi *et al.*, 1998). The Lys 236 residue inferred in PLP binding is conserved in all functionally expressed branched-chain amino acid aminotransferases including CmBCAT1 (Figure 14B). Bioinformatic analysis of the N-terminus of CmBCAT1 using several different tools (Predotar, MitoprotII, pSORT and TargetP) indicated targeting of the *CmBCAT1* gene product to the mitochondria while LocTree indicates chloroplast targeting.

Functional expression of *CmArAT1* and *CmBCAT1* in Escherichia coli using bacterial lysates

In order to functionally determine the biochemical roles of *CmArAT1* and *CmBCAT1*, the recombinant genes products were assayed for amino acids transaminase activity *in vitro*. Bacterial lysates (desalted) derived from the recombinant *E. coli* expressing *CmArAT1* catalyzed the transamination of the aromatic amino acids L-phenylalanine and L-tyrosine (Figure 16AB). The control lysates (desalted) from *E. coli* expressing inserts of clone DV633368 (control) catalyzed those reactions but at a much lower level (Figure 16AB), probably due to bacterial endogenous protein. Bacterial lysates (desalted) derived from the recombinant *E. coli* expressing *CmBCAT1* catalyzed the transamination of the branched-chain amino acids L-isoleucine and L-leucine (Figure 16CD), and L-valine (only in GC-MS assay, data not shown), and at much lower rates L-methionine and L-phenylalanine (data not shown). Lysates (desalted) from *E. coli* expressing inserts of clone DV633368 (control) catalyzed those reactions at a much lower level (Figure 16C), probably due to bacterial endogenous protein. The identification of all products was confirmed by larger scale assays analyzed by GC-MS (not shown).

Expression of *CmArAT1* and *CmBCAT1* during fruit development

To better understand the regulation of *CmArAT1* and *CmBCAT1* during fruit physiologic maturation and their possible roles in melon quality determination, their expression during fruit development was monitored. The expression patterns of both

CmArAT1 and *CmBCAT1* indicate up-regulation during fruit ripening, both in flesh and rind tissues as determined by qRT-PCR (Figure 17). Young flesh tissues displayed very low expression levels of both *CmArAT1* and *CmBCAT1*. The expression level of *CmArAT1* at the ripe stage raised 26 fold as compared to the levels detected 25 days after anthesis (Figure 17). In melon rind tissues, a similar pattern of expression was observed, but the increase in the level of expression was 46 fold. In a similar fashion, the expression levels of *CmBCAT1* in the flesh tissues increased 90 fold as compared to the levels exhibited 25 days post anthesis, and in the peel it increased by 108 fold.

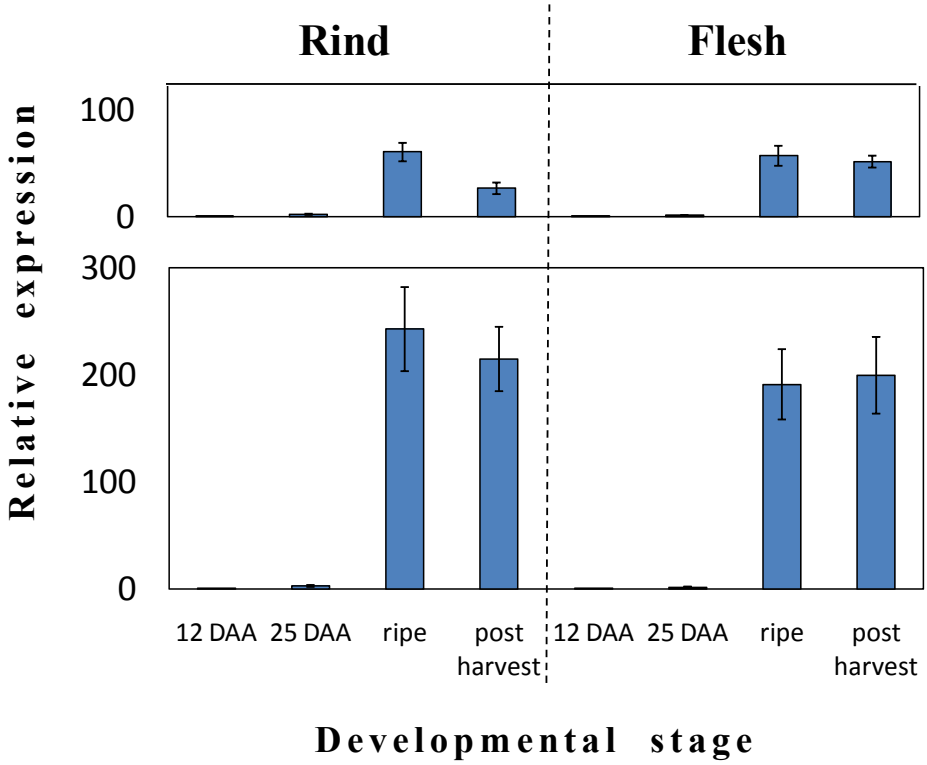


Figure 17. Expression patterns of amino acid transaminase genes in melon tissues during fruit development. Real-time PCR analysis of *CmArAT1* (upper panel) and *CmBCAT1* (lower panel). RNA was extracted separately from the rind and flesh at various stages of fruit development as indicated. Expression levels were normalized with the internal control *cyclophilin* and are plotted relatively to the expression of the 12 DAA samples. Values represent the mean of three biological repeats ± SE derived from three replicates. DAA – days after anthesis.

DISCUSSION

Many of the important volatile constituents of the aroma of melon are likely derived from amino acids, as suggested by their chemical structures (Yabumoyo and Jennings, 1977; Schieberle *et al.*, 1990; Wyllie and Leach, 1992; Wyllie *et al.*, 1995, Beaulieu and Grimm, 2001; Jordán *et al.*, 2001). Wyllie *et al.* (1995) found that the level of certain free amino acids (alanine, methionine, valine, leucine and isoleucine) in the fruit increased during the fruit development. My findings on the level of total free amino acids during fruit development (Figure 6) are in accordance with these data, especially concerning the climacteric cultivars ('Dulce' and 'Noy Israel').

I found that a large number of melon volatiles originate from amino-acids upon incubation of melon fruit cubes with exogenous amino acid solutions (Figures 6, 7, Table 1). These include many of the volatiles that strongly contribute to the full aroma of melons (Schieberle *et al.*, 1990; Wyllie and Leach, 1992; Wyllie *et al.*, 1995; Beaulieu and Grimm, 2001; Jordán *et al.*, 2001; Aubert and Bourger, 2004), such as ethyl isobutanoate, isobutyl acetate, methyl 2-methylbutanoate, ethyl 2-methylbutanoate, 2-methylbutyl acetate, ethyl 3-methylthiopropionate, 3-(methylthio) propyl acetate, benzyl alcohol and benzyl acetate (Figures 6, 7, Table 1). Some of the compounds mentioned above are also important contributors to the aroma of many other fruits, such as tomato, banana and apple (Buttery *et al.*, 1987; Wyllie and Fellman, 2000; Baldwin *et al.*, 2000; Matich and Rowan, 2007). Radiolabeled aromatic and branched-chain amino acids generated radioactive volatiles in banana fruit disks (Tressl and Drawert, 1973). Deuterated L-isoleucine was converted into the corresponding 2-methylbutyl volatile derivatives in apples (Rowan *et al.*, 1996; Matich and Rowan, 2007). Feeding of exogenous L-isoleucine into strawberry peduncles resulted in enhanced levels of the structurally corresponding 2-methylbutyl volatile derivatives in fruits (Pérez *et al.*, 2002). My experiments confirm that amino acids are important precursors for aroma compounds in melon similarly to what is known in other fruits. However, the exact biochemical route of these conversions was unknown, although it is clear that both decarboxylation and deamination steps are required (Figures 2, 3, Schwab *et al.*, 2008).

α-Keto acids are key intermediates to volatiles in cheese-dwelling microorganisms (Yvon and Rijnen 2001). In order to ascertain if α-keto acids are efficient substrates for the formation of melon volatiles**,** feeding experiments using melon fruit cubes

were performed. The results indicate that α-keto acids are efficient precursors of melon aroma compounds (Figure 8 and Table 2). Indeed, the same aroma compounds whose levels were enhanced by the incubation with the amino acids, were also enhanced by incubation with the respective α-keto acids. These experiments strongly suggest that α-keto acids are key intermediates in the formation of melon volatiles in a similar pathway to that described in microorganisms (Figure 3, green route). The greater efficiency of α-keto acids as compared to amino acids in enhancing volatile levels might be due to more efficient uptake or metabolism of the α-keto acids as compared to the amino acids. Nevertheless, it could also be that the rate of amino acids deamination into α-keto acids might be rate-limiting for the production of the aforementioned volatiles.

Although my results indicate that α-keto acids are precursors of melon volatiles, I noticed that substantial levels of benzaldehyde were present in the PPA solutions used for the incubation experiments (Table 3). Therefore, it is possible that benzaldehyde, in addition to PPA, is a precursor of some of the aromatic volatiles formed such as benzyl alcohol, benzyl acetate and ethyl benzoate. Nevertheless, it is unlikely that the volatiles containing an aromatic ring with two carbons in their side chain, such as phenylacetaldehyde and phenethyl acetate are derived from benzaldehyde. It was demonstrated in petunia petals that benzaldehyde is not a precursor for phenylacetaldehyde (Boatright *et al.*, 2004). Similarly, the increases in dimethyl disulfide observed, could be due to the presence of this compound both in the L-methionine and the MTOB solutions (Table 3), and not due to enzymatic conversions. Still, the presence of this contaminant cannot solely explain the enhanced levels of other sulfur-containing aroma compounds.

Although microbial transaminases have clear roles in the formation of volatiles (Figure 3, green route), the role of amino acid transaminases in the formation of plant volatiles had not been evaluated. Melon cell-free extracts exhibited significant levels of amino acid transaminase activities (Figures 8 and 9). These results strengthen the hypothesis that the conversion of amino acids to aroma compounds in melons is initiated by transamination. I cannot exclude the possibility that other amino acid catabolic pathways such as those described in tomato fruit (Tieman *et al.*, 2006) and petunia flowers (Kaminaga *et al.*, 2006) (Figure 3, red and purple routes) are also operational in melons. Still, experiments utilizing crude melon fruit extracts failed to

reveal substantial amino acid decarboxylase or aldehyde synthase activities when supplied with L-[$^{3}H_2$]phenylalanine as a substrate (not shown).

In this research two novel melon genes were identified, namely *CmArAT1* and *CmBCAT1*, that encode enzymes possessing aromatic amino acid transaminase and branched-chain amino acid transaminase activities, respectively (Figure 16). Some of the amino acids considered crucial for activity in the *Trypanosoma cruzi* tyrosine aminotransferase (TcTAT) based on its X-ray crystal structure (Blankenfeldt *et al.*, 1999), are also conserved in CmArAT1 (Figure 14A). They include the Lys245 that covalently anchors the co-factor PLP, as well as Asn185, Asp213 and Arg253 residues which help stabilize the bound PLP, and Arg284, one of the amino acids involved in the binding of the incoming amino acid (Figure 14A). Moreover, the key amino acids in the human branched-chain aminotransferase 2 (HsBCAT2) (Yennawar *et al.*, 2001), are also conserved in CmBCAT1 (Figure 14B). They include the Lys236 that covalently anchors PLP, Tyr105 and Arg178 that are important in catalysis and in stabilizing the dimeric structure, and Tyr241 and Glu272 that anchor the PLP ring. Despite the high sequence similarity of CmBCAT1 to PLP dependent enzymes, and although I am not aware of any amino acid transaminase enzyme activity that does not utilize PLP, I did not succeed in showing exogenous PLP dependence of CmBCAT1 (data not shown).

Many of the enzymes involved in the degradation of branched-chain amino acids for the production of acetyl-CoA are mitochondrial (Anderson *et al.*, 1998; Singh, 1999; Diebold *et al.*, 2002). The N-terminus bioinformatics analyses of CmBCAT1 predicted mitochondrial localization, further supporting a catabolic role for CmBCAT1 *en route* to aroma compounds. The bioinformatics analysis of CmArAT1 was not informative enough to predict organellar targeting. More experimental work is needed to unequivocally determine the sub-cellular localization of CmBCAT1 and CmArAT1.

The expression of both *CmArAT1* and *CmBCAT1* were monitored during fruit development. The levels of expression of both genes were low at early stages of fruit development, and sharply increased upon maturation, both in fruit rind and flesh tissues (Figure 17). These patterns of expression are in accordance with the pattern of accumulation of amino acid derived aroma compounds in melon fruit (Beaulieu and Grimm, 2001; Shalit *et al.*, 2001). Interestingly, other genes involved in volatiles formation in melons such as alcohol-acetyl transferases, alcohol dehydrogenases,

carotenoid cleavage dioxygenases and sesquiterpene synthases are also upregulated upon fruit maturation (El-Yahyaoui *et al.*, 2002; El-Sharkawy *et al.*, 2005; Manríquez *et al.*, 2006; Ibdah *et al.*, 2006; Portnoy *et al.*, 2008).

Although it is well established that amino acids serve as precursors of plant volatiles, only two reports dealing with the initial biosynthetic step of this route are available, and indicate that different plant tissues utilize different enzymatic paths to synthesize similar compounds. In tomato fruit, L-phenylalanine is first decarboxylated to form an amine intermediate *en route* to phenylacetaldehyde (Figure 3, red route) (Tieman *et al.*, 2006), while in petunia flowers one bifunctional enzyme decarboxylates and deaminates L-phenylalanine directly releasing phenylacetaldehyde (Figure 3, purple route) (Kaminaga *et al.*, 2006). The feeding experiments I show here (Figures 6, 7 and Tables 1 and 2), together with the measurements of L-phenylalanine transaminase activity in cell-free extracts (Figure 12E), strengthen the hypothesis that L-phenylalanine first undergoes transamination, releasing PPA, *en route* to phenylacetaldehyde and other L-phenylalanine-derived aroma compounds in melon fruit (Figure 3, green route).

No prior enzymatic evidence for the involvement of BCAT activity in the formation of plant volatiles is available, although exogenous [U-^{14}C]-L-leucine and [U-^{14}C]-L-valine were incorporated into KIC and KIV in banana slices (Tressl and Drawert, 1973). The feeding experiments (Figure 8, Tables 1 and 2), and the presence of BCAT activity in cell-free extracts (Figure 12A,B,C), indicate that transamination is likely the initial step in the metabolism of branched-chain amino acids into aroma compounds in melon fruit (Figure 3, green route).

CmArAT1 and CmBCAT1 are upregulated during fruit development (Figure 17) and their functional ArAT and BCAT roles (Figure 16), lead us to propose that CmArAT1 and CmBCAT1 are key enzymes in the formation of aroma compounds in melon fruit, and have important roles in regulating this biosynthetic process. This route is apparently novel in plants but similar to the route that reportedly takes place in cheese-dwelling microorganisms (Yvon and Rijnen, 2001). α-Keto acid decarboxylases subsequently release volatile aldehydes (Figure 3, green route) or carboxylic acids through the α-keto acid dehydrogenase complex in cheese-dwelling microorganisms (Yvon and Rijnen, 2001; Liu *et al.*, 2008). Candidate unigenes that might code for the aforementioned enzymes are apparent in the Melon EST Database.

Still, more work is needed to ascertain if these genes have any involvement in the biosynthesis of melon fruit volatiles.

CONCLUSIONS

- A large number of important aroma compounds in melon fruit originate from degradation of amino acids.
- Melon fruits utilize amino acid transaminases as the first enzymatic step in the degradation of amino acids for the formation of volatile aroma compounds.
- CmArAT1 and CmBCAT1 are key enzymes in the formation of aroma compounds in melon fruit.
- *CmArAT1* and *CmBCAT1* might have important roles in regulating these aroma formation processes in melon fruits.

LITERATURE CITED

Aubert C, Bourger N (2004) Investigation of volatiles in Charentais cantaloupe melons (*Cucumis melo* Var. cantalupensis) characterization of aroma constituents in some cultivars. J Agric Food Chem **52:**4522-4528

Baldwin E A, Scott J W, Shewmaker C K, Schuch W (2000) Flavor trivia and tomato aroma: biochemistry and possible mechanisms for control of important aroma components. HortSci **35:**1013-1022

Beaulieu J C, Grimm C C (2001) Identification of volatile compounds in cantaloupe at various developmental stages using solid phase microextraction. J Agric Food Chem **49:**1345-1352

Beekwilder J, Alvarez-Huerta1 M, Neef E, Verstappen F W A, Bouwmeester J H, Aharoni A (2004) Functional characterization of enzymes forming volatile esters from strawberry and banana. Plant Physiol **135:**1865-1878

Blankenfeldt W, Montemartini-Kalisz M, Kalisz H M, Hecht H J, Nowicki C (1999) Crystal structure of *Trypanosoma cruzi* tyrosine aminotransferase: substrate specificity is influenced by cofactor binding mode. Protein Sci **8:**2406-2417

Boatright J, Negre F, Chen X, Kish C M, Wood B, Peel G, Orlova I, Gang D, Rhodes D, Dudareva N (2004) Understanding *in vivo* benzenoid metabolism in petunia petal tissue. Plant Physiol **135:**1993-2011

Bradford M M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem **72**: 248-254

Buck L, Axel R (1991) A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell **65:**175-187

Burger Y, Sa'ar U, Paris H S, Lewinsohn E, Katzir N, Tadmor Y, Schaffer A A (2006) Genetic variability for valuable fruit quality traits in *Cucumis melo*. Isr J Plant Sci **54:**233-242

Burger Y, Paris H S, Cohen R, Katzir N, Tadmor Y, Lewinsohn E, Schaffer A A, (2009) Genetic diversity of *Cucumis melo*. Hort Rev (*In Press*).

Buttery R G, Teranishi R, Ling L C (1987) Fresh tomato aroma volatiles: a quantitative study. J Agric Food Chem **35:**540-544

Central Bureau of Statistics, (2008) Statistical abstract of Israel, pp. 741

Cooper A J L (1985) L-Glutamate-L-amino acid transaminases. *In* Meister A, ed, Methods in Enzymology. Glutamate, Glutamine, Glutathione, and Related Compounds. vol. 113, pp. 63-65

Christen P, Mehta P K (2001) From cofactor to enzymes. The molecular evolution of pyridoxal-5'-phosphate-dependent enzymes. Chem Rec **1:**436–447

Croteau R, Karp F (1991) Origin of natural odorants. *In* Muller P M, Lamparsky D, Eds, Perfumes. Art, Science and Technology. Elsevier Applied Science: London, U.K., pp 101-126

Croteau R, Kutchan T M, Lewis N G (2000) Natural products (secondary metabolites). *In* Buchanan B B, Gruissem W, Jones L R, Eds, Biochemistry & Molecular Biology of Plants. American Society of Plant Physiologists: Rockville, Maryland, pp 767-729.

Dangl J L, Dietrich R A, Thomas H (2000) Senescence and programmed cell death. *In* Buchanan B B, Gruissem W, Jones L R, Eds, Biochemistry & Molecular Biology of Plants. American Society of Plant Physiologists: Rockville, Maryland, pp 1044-1100.

Davidovich-Rikanati R, Lewinsohn E, Bar E, Iijima Y, Pichersky E, Sitrit Y (2008) Overexpression of the lemon basil α-zingiberene synthase gene increases both mono- and sesquiterpene contents in tomato fruit. Plant J **56:**228-238

Davoodi J, Drown P M, Bledsoe R K, Wallin R, Reinharti G D, Hutson S M (1998) Overexpression and characterization of the human mitochondrial and cytosolic branched-chain aminotransferases. J Biol Chem **273:**4982-4989

Díaz-Cruz M S, López de Alda M J, López R, Barcelo D (2003) Determination of estrogens and progestogens by mass spectrometric techniques (GC/MS, LC/MS and LC/MS/MS). J Mass Spectrom **38:**917-923

Dickinson J R, Salgado L E J, Hewlins M J E (2003) The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*. J Biol Chem **278:** 8028-8034

Diebold R, Schuster J, Däschner K, Binder S (2002) The branched-chain amino acid transaminase gene family in arabidopsis encodes plastid and mitochondrial proteins. Plant Physiol **129:**540-550

Dietrich J B, Lorber B, Kern D (1991) Expression of mammalian tyrosine aminotransferase in *Saccharomyces cerevisiae* and *Escherichia coli*: Purification to homogeneity and characterization of the enzyme overproduced in the bacteria.

Eur J Biochem **201:**399-407

El-Sharkawy I, Manríquez D, Flores F B, Regad F, Bouzayen M, Latché A, Pech J C (2005) Functional characterization of a melon alcohol acyl-transferase gene family involved in the biosynthesis of ester volatiles. Identification of the crucial role of a threonine residue for enzyme activity. Plant Mol Biol **59:**345-362

El-Yahyaoui F, Wongs-Aree C, Latché A, Hackett R, Grierson D, Pech J C (2002) Molecular and biochemical characteristics of a gene encoding an alcohol acyl-transferase involved in the generation of aroma volatile esters during melon ripening. Eur J Biochem **269:**2359-2366

Ehrlich F (1907) Über die bedingungen der fuselölbildung und über ihren zusammenhang mit dem eiweissaufbau der hefe. Ber Dtsch Chem Ges **40:**1027-1047

Grange T, Guénet C, Dietrich J B, Chasserot S, Fromont M, Befort N, Jami J, Beck G, Pictet R (1985) Complete complementary DNA of Rat tyrosine aminotransferase messenger RNA: deduction of the primary structure of the enzyme. J Mol Biol **184:**347-350

Hagelstein P, Sieve B, Klein M, Jans H, Schultz G (1997) Leucine synthesis in chloroplasts: Leucine/isoleucine aminotransferase and valine aminotransferase are different enzymes in spinach chloroplasts. J Plant Physiol **150:**23-30

Horvat R J, Senter S D (1987) Identification of additional volatile compounds from cantaloupe. J food sci **52:**1097-1098

Ibdah M, Azulay Y, Portnoy V, Wasserman B, Bar E, Meir A, Burger Y, Hirschberg J, Schaffer AA, Katzir N, Tadmor Y, Lewinsohn E (2006) Functional characterization of *CmCCD1*, a carotenoid cleavage dioxygenase from melon. Phytochem **67:**1579-1589

Jensen R A, Gu W (1996) Evolutionary recruitment of biochemically specialized subdivisions of family I within the protein superfamily of aminotransferases. J Bacteriol **178:**2161–2171

Jordán M J, Shaw P E, Goodner K L (2001) Volatile components in aqueous essence and fresh fruit of *Cucumis melo* cv. athena (muskmelon) by GC-MS and GC-O. J Agric Food Chem **49:**5929-5933

Kaminaga Y, Schnepp J, Peel G, Kish C M, Ben-Nissan G, Weiss D, Orlova I, Lavie O, Rhodes D, Wood K, Porterfield D M, Cooper A J L, Schloss J V, Pichersky E, Vainstein A, Dudareva N (2006) Plant phenylacetaldehyde synthase Is

a bifunctional homotetrameric enzyme that catalyzes phenylalanine decarboxylation and oxidation. J Biol Chem **281:**23357- 23366

Knill T, Schuster J, Reichelt M, Gershenzon J, Binder S (2008) Arabidopsis branched-chain aminotransferase 3 functions in both amino acid and glucosinolate biosynthesis. Plant Physiol **146:**1028-1039

Kuentzel H, Bahri D (1990). Synthetic ingredients of food flavourings. *In* Ashurts P R, Ed, Food Flavorings; Blackie Academic & Professional, Glasgow and London, pp. 115-157

Kourkoutas D, Elmore J S, Mottram D S (2006) Comparison of the volatile compositions and flavour properties of cantaloupe, Galia and Honeydew muskmelons. Food Chem **97:**95-102

Larkov O, Zaks A, Bar E, Lewinsohn E, Dudai N, Mayer A M, Ravid U (2008) Enantioselective monoterpene alcohol acetylation in *Origanum*, *Mentha* and *Salvia* species. Phytochem **69:**2565-2571

Lea P J, Ireland R J (1999) Nitrogen metabolism in higher plants. *In* Singh B K, ed, Plant Amino Acids. Biochemistry and Biotechnology. American Cyanamid company: Princeton, New Jersey, pp 1-49

Lewinsohn E, Gijzen M (2009) Phytochemical diversity: The sounds of silent metabolism. Plant Sci **176:**161–169

Liu M, Nauta A, Francke C, Siezen R J (2008) A comparative genomics of enzymes in flavor-forming pathways from amino acids in lactic acid bacteria. Appl Environ Microbiol **74:**4590–4600

Lopukhina A, Dettenberg M, Weiler E W, Holländer-Czytko H (2001) Cloning and characterization of a coronatine-regulated tyrosine aminotransferase from arabidopsis. Plant Physiol **126:**1678-1687

Malatrasi M, Corradi M, Svensson J T, Close T J, Gulli M, Marmiroli N (2006) A branched-chain amino acid aminotransferase gene isolated from *Hordeum vulgare* is differentially regulated by drought stress. Theor Appl Genet **113:**965-976

Mallick M F R, Masui M (1968) Origin, distribution and taxonomy of melons. Scientia Hortic **28:**251-261

Malkin R, Niyogi K (2000) Photosynthesis. *In* Buchanan B B, Gruissem W, Jones L R, Eds, Biochemistry & Molecular Biology of Plants. American Society of Plant Physiologists: Rockvill, Maryland, pp 568-630

Manríquez D, El-Sharkawy I, Flores F B, El-Yahyaoui F, Regad F, Bouzayen M, Latché A, Pech J C (2006) Two highly divergent alcohol dehydrogenases of melon exhibit fruit ripening-specific expression and distinct biochemical characteristics. Plant Mol Biol **61:**675-685

Matich A, Rowan D (2007) Pathway analysis of branched-chain ester biosynthesis in apple using deuterium labeling and enantioselective gas chromatography-mass spectrometry. J Agric Food Chem **55:**2727-2735

Mehta P K, Hale T I, Christen P (1993) Aminotransferases: demonstration of homology and division into evolutionary subgroups. Eur J Biochem **214:**549-561

Mizukami H, Ellis B E (1991) Rosmarinic acid formation and differential expression of tyrosine aminotransferase isoforms in *Anchusa officinalis* cell suspension cultures. Plant Cell Reports **10:**321-324

Montemartini M, Santome J A, Cazzulo J J, Nowicki C (1993) Purification and partial structural and kinetic characterization of tyrosine aminotransferase from epimastigotes of *Trypanosoma cruzi*. J Biochem **292:**901-906

Percudani R, Peracchi A (2003) A genomic overview of pyridoxal-phosphate dependent enzymes. EMBO reports **4:**850-854

Pérez A G, Sanz C, Olias J M (1993) Partial purification and some properties of alcohol acetyltransferase from strawberry fruits. J Agric Food Chem **41:**1462-1466

Pérez A G, Sanz C, Olias R, Rios J J, Olias J M (1996) Evolution of strawberry alcohol acetyltransferase activity during fruit development and storage. J Agric Food Chem **44:**3286-3290

Pérez A G, Olías R, Luaces P, Sanz C (2002) Biosynthesis of Strawberry Aroma Compounds through Amino Acid Metabolism. J Agric Food Chem **50:** 4037-4042

Perpète P, Duthoit O, De-Maeyer S, Imray L, A Lawton I, Stavropoulos K E, Gitonga V W, Hewlins M J E, Dickinson J R (2006) Methionine catabolism in *Saccharomyces cerevisiae*. FEMS Yeast Res **6:**48–56

Pitrat M, Hanelt P, Hammer K (2000) Some comments on interspecific classification of cultivars of melon. *In* Proceeding of cucurbitaceae 2000, 7th Eucarpia Meeting on Cucurbit Genetics and Breeding. Katzir N, Paris H S eds., Acta Horticulturae **510:**29-36

Portnoy V, Benyamini Y, Bar E, Harel-Beja R, Gepstein S, Giovannoni J J, Schaffer A A, Burger J, Tadmor Y, Lewinsohn E, Katzir N (2008) The molecular

and biochemical basis for varietal variation in sesquiterpene content in melon (*Cucumis melo* L.) rinds. Plant Mol Biol **66:**647-661

Pfaffl M W (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res **29:**2002-2007

Ramakers C, Ruijter J M, Deprez R H, Moorman A F (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett **339:**62-66

Rijnen L, Bonneau S, Yvon M (1999) Genetic characterization of the major lactococcal aromatic aminotransferase and its involvement in conversion of amino acids to aroma compounds. Appl and Environ Microbiol **65:**4873-4880

Rouquier S, Blancher A, Giorgi D (2000) The olfactory receptor gene repertoire in primates and mouse: evidence for reduction of the functional fraction in primates. Proc Natl Acad Sci **97:**2870–2874

Rowan D D, Lane H P, Allen J M, Fielder S, Hunt M B (1996) Biosynthesis of 2-methylbutyl, 2-methyl-2-butenyl, and 2-methylbutanoate esters in Red Delicious and Granny Smith apples using deuterium-labeled substrates. J Agric Food Chem **44:**3276-3285

Schieberle P, Ofner S, Grosch W (1990) Evaluation of potent odorants in cucumbers (*Cucumis sativus*) and muskmelons (*Cucumis melo*) by aroma extract dilution analysis. J Food Sci **55:**193-195

Schuster J, Binder S (2005) The mitochondrial branched-chain aminotransferase (AtBCAT-1) is capable to initiate degradation of leucine, isoleucine and valine in almost all tissues in *Arabidopsis thaliana*. Plant Mol Biol **57:**241-254

Schuster J, Knill T, Reichelt M, Gershenzon J, Binder S (2006) Branched-chain aminotransferase4 is part of the chain elongation pathway in the biosynthesis of methioninehionine-derived glucosinolates in *Arabidopsis*. Plant Cell **18:**2664-2679

Schwab W, Davidovich-Rikanati R, Lewinsohn E (2008) Biosynthesis of plant-derived flavor compounds. Plant J **54:**712-732

Seralini G E, Luu-The V, Labrie F (1995) Cloning and expression of human tyrosine aminotransferase cDNA. Biochim Biophys Acta **1260:**97-101

Seymour G B, McGlasson W B (1993). Melons. *In* G B Seymour, J E Taylor, G A Tucker eds., Biochemistry of Fruit Ripening. Chapman & Hall, London pp. 273-290

Shalit M, Katzir N, Tadmor Y, Larkov O, Burger Y, Shalechet F, Lastochkin E, Ravid U, Amar O, Edelstein M, Karchi Z, Lewinsohn E, (2001) Acetyl CoA:

alcohol acetyl transferase activity and aroma formation in ripening melon fruits. J Agric Food Chem **49:**794-799

Siedow J N, Day D A (2000) Respiration and photorespiration. *In* Buchanan B B, Gruissem W, Jones L R, Eds, Biochemistry & Molecular Biology of Plants. American Society of Plant Physiologists: Rockville, Maryland, pp 767-729.

Siehl D L (1999) The biosynthesis of tryptophan, tyrosine, and phenylalanine from chorismate. *In* Singh B K, ed, Plant Amino Acids. Biochemistry and Biotechnology. American Cyanamid company: Princeton, New Jersey, pp 171-204

Singh B K, Shaner D L (1995) Plant physiologists biosynthesis of branched chain amino acids: from test tube to field. Plant Cell **7:**935-944

Singh B K (1999) Biosynthesis of valine, leucine and isoleucine. *In* Singh B K, ed, Plant Amino Acids. Biochemistry and Biotechnology. American Cyanamid company: Princeton, New Jersey, pp 171-204

Smith P G, Welch J E (1964) Nomenclature of vegetables and condiment herbs grown in United States. Proceeding of the American society for Horticultural Science, **84:**534-548

Temussi P (2006). The history of sweet taste: not exactly a piece of cake. J Mol Recognit **19**:188–99

Thomson D M H (1987). The meaning of flavor. *In* G G Birch, M G Lindley, eds., Developments in Food Flavors. Elsevier, London, pp 1-21

Tieman D, Taylor M, Schauer N, Fernie A R, Hanson A D, Klee H J (2006) Tomato aromatic amino acid decarboxylases participate in synthesis of the flavor volatiles 2-phenylethanol and 2-phenylacetaldehyde. Proc Natl Acad Sci **103:**8287-8292

Tressl R, Drawert F (1973) Biogenesis of banana volatiles. J Agric Food Chem **21:**560-565

Tucker G A (1993). Introduction. *In* G B Seymour, J E Taylor, G A Tucker eds., Biochemistry of Fruit Ripening. Chapman & Hall, London pp. 1-51

Whitaker T W, Davis G N, (1962) Cucurbits: botany, cultivation and utilization. Interscience publishers Inc. New York.

Wink M (1988) Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivores. Theor Appl Genet **75:**1432-2242

Wyllie S G, Leach D N (1992) Sulfur-containing compounds in the aroma volatiles of melons (*Cucumis melo*). J Agric Food Chem **40:**253-256

Wyllie S G, Leach D N, Wang Y, Shewfelt R L (1995) Key aroma compounds in melons. Their development and cultivar dependence. *In* Roussef R L, Leahy M M, Eds, Fruit Flavors; ACS Symposium Series 596, American Chemical Society: Washington, DC, pp 248-257

Wyllie S G, Fellman J K (2000) Formation of volatile branched chain esters in bananas (*Musa sapientum* L.). J Agric Food Chem **48:**3493-3496

Yennawar N, Dunbar J, Conway M, Hutsonb S, Farber G (2001) The structure of human mitochondrial branched-chain aminotransferase. Acta Cryst **D57:**506-515.

Yabumoyo K, Jennings W G (1977) Volatile constituents of cantaloupe, *Cucumis melo*, and their biogenesis. J Food Sci **42:**32-37

Yvon M, Chambellon E, Bolotin A, Roudot-Algaron F (2000) Characterization and role of the branched-chain aminotransferase (BcaT) isolated from *Lactococcus lactis* subsp. Cremoris NCDO 763. Appl and Environ Microbiol **66:**571-577

Yvon M, Rijnen L (2001) Cheese flavour formation by amino acid catabolism. Inter Dairy J **11:**185-201

Zaks A, Davidovich-Rikanati R, Bar E, Inbar M, Lewinsohn E (2008) Biosynthesis of linalyl acetate and other terpenes in *Mentha aquatica* var. *citrata*. Israel J Plant Sci **56:**233-244

תקציר

המלון (*Cucumis melo* L.) הינו מין השייך למשפחת הדלועיים הכוללת בין היתר את המלפפון, האבטיח, הקישוא והדלעת. המלון הוא מין הכולל מגוון רחב של זני בר ותרבות, ומשמש כגידול חקלאי ברחבי העולם. בשנת 2006 היבול הכולל של מלונים בישראל עמד על 49.7 אלף טון, ועל יותר ממיליון טון בארה"ב. טעמו המלא של הפרי (flavour) המושפע מחוש הטעם וחוש הריח שלנו, הוא אחד מהמאפיינים החשובים הקובעים את איכות המלון. חוש הטעם של האדם מסוגל להבחין אך ורק בחמישה טעמים שונים (מתוק, מלוח, מר, חמוץ ואוממי), המושפעים מנוכחות סוכרים וממתיקים, חומצות שונות, תרכובות מרירות ומינרלים שונים. לעומת זאת, חוש הריח יכול להבחין בין אלפי סוגי ריח שונים בעזרת קולטנים אולפקטוריים הממוקמים בשכבת האפיתל האולפקטורי באף. לכל חומר סף הרחה (odor treshhold) שונה הקובע את הכמות המינימלית הדרושה ממנו באוויר על-מנת שנוכל להריחו. ישנם חומרים נדיפים אשר דרושה מהם כמות מועטה בכדי שנבחין בנוכחותם, ואילו חומרים אחרים לא יורגשו כלל גם בכמות גבוהה מאוד. כמו פירות וירקות רבים אחרים, גם הארומה האופיינית של המלון וזניו השונים מורכבת מתערובת ייחודית של חומרים נדיפים רבים בכמויות שונות ויחסים מסוימים, ורק הרכב זה ייתן את הארומה והטעם האופייניים לכל פרי וזן.

חומרי ארומה במלון כוללים בין היתר מונו- וססקוויטרפנים, נגזרות פנוליות, נגזרות של חומצות שומן ונגזרות של חומצות אמיניות. חלק מחומרי הארומה החשובים ביותר במלון הינם אסטרים אשר מקורם בחומצות אמיניות. בחיידקי גבינות ובשמרי מזון המסלול הביוסינטטי של יצירת חומרים אלו ידוע ומובן כמעט בשלמותו הן ביוכימית והן מולקולארית. בצמחים, שני השלבים האחרונים במסלול הביוסינטטי המוביל ליצירת אסטרים אלו נחקר באינטנסיביות גם במלון וגם בצמחים נוספים במחקר ביוכימי ומולקולרי. לעומתם, השלב הראשון המתמקד בתהליך הפירוק של חומצות אמיניות בדרך ליצירת חומרים נדיפים קיבל תשומת לב מועטה בלבד. בחיידקי גבינות ובשמרי מזון שלב אנזימטי זה לרוב הינו טרנסאמינציה של החומצה אמינית לקבלת תוצר ביניים של חומצה קטונית. בריאקציה זו האנזים פועל על שני סובסטרטים (חומצה אמינית וחומצה קטונית, לרוב אלפא-קטו-גלוטאראט) ומחליף בין השייר האמיני של החומצה האמינית והשייר הקטוני של החומצה הקטונית. מנגד, בשני מחקרים בודדים שבוצעו בצמחים, נמצא כי מסלול אחר פועל ליצירת החומרים הנדיפים. בפירות של עגבנייה נמצא כי החומצה האמינית פנילאלאנין עוברת קודם דה-קרבוקסילציה, ומשחררת תוצר ביניים אמיני, שככל הנראה עובר לאחר מכן דה-אמינציה ליצור של חומרים נדיפים נוספים. במחקר נוסף נמצא כי בעלי כותרת של פרחי פטוניה ו-וורד אנזים אחד מבצע בשלב אנזימטי אחד גם דה-קרבוקסילציה וגם דה-אמינציה של פנילאלאנין, ומשחרר פנילאצטאלדהיד. עד היום לא היה ידוע כיצד מפורקת החומצה האמינית פנילאלאנין

ומיוצר פנילאצטאלדהיד במלון. המטרה העיקרית של מחקר זה הייתה לגלות מהו המסלול הביוכימי בו משתמש פרי המלון בכדי לפרק חמצות אמיניות ולייצר חומרים נדיפים.

כניסוי ראשוני נבדקה רמת החומצות האמיניות החופשיות בפירות של זני מלון שונים לאורך תהליך ההבשלה. נמצאו הבדלים ניכרים בין הזנים השונים, כאשר הזנים הקלימקטריים מראים עליה בתכולת החומצות האמיניות החופשיות, בעוד שבזנים הלא-קלימקטריים התמונה פחות ברורה. במחקר זה הודגרו קוביות של ציפת מלון בתמיסות של חומצות האמיניות לאוצין, איזולאוצין, ואלין, פנילאלאנין ומתיונין, ונבדקו הרכב וכמות החמרים הנדיפים בעזרת מכשיר GC-MS. נמצא כי רמתם של חומרי ארומה הדומים במבנם לזה של החומצה האמינית ששימשה בניסוי עלתה מאוד ביחס לקוביות בתמיסת הביקורת (מים). קוביות מלון שהודגרו בתמיסות של חומצות קטוניות הנגזרות מהחומצות האמיניות הנ"ל, ייצרו חומרי ארומה הדומים במבנם לזה של החומצה הקטונית ברמה גבוהה אף יותר מזו שייצרו הקוביות שהודגרו בתמיסות החומצות האמיניות. קוביות מלון שהודגרו עם החומצה האמינית פנילאלאנין המסומנת עם שישה אטומי פחמן כבדים (^{13}C) בטבעת הארומטית ייצרו חומרי ארומה בעלי טבעת ארומטית מסומנים, אשר ניתן לזהותם בעזרת ספקטרום המסות שלהם.

מיצויים אל-תאיים של חלבון מסיס שהופקו מציפת פרי בשל הראו פעילות טרנסאמינציה עם החומצות האמיניות המוזכרות לעיל (פרט לואלין) בניסויים רדיואקטיביים *in-vitro*. מכיוון שלא ניתן להבחין בחומצות קטוניות במכשיר ה- GC-MS באופן ישיר, מומשה שיטת דריבטיזציה באמצעות ריאגנט BSTFA בכדי לזהות את תוצר הפעילות האנזימטית. ניסויים אנזימטיים שבוצעו בנפח גדול יותר ונבדקו ב- GC-MS בשיטה זו, זיהו את תוצרי הפעילות האנזימטית כחומצות הקטוניות הנגזרות מהחומצות האמיניות ששימשו כסובסטרטים (כולל ואלין). הסובסטרט הקטוני בכלל הריאקציות (הן בניסויים הרדיואקטיביים והן בניסויים שנבדקו ב- GC-MS) היה אלפא-קטו-גלוטאראט.

חיפוש במאגר המידע הביואינפורמטי של המלון (Melon EST Database) העלה שני גנים מועמדים לקודד לשני אנזימי טרנסאמינאזות. האחד פועל על החומצות האמיניות הארומטיות (פנילאלאנין, טירוזין וטריפטופן) (*CmArAT1*) , והשני פועל על החומצות האמיניות מסועפות השרשרת (לאוצין, איזולאוצין וואלין) (*CmBCAT1*). גנים אלו מקודדים לחלבונים בעלי משקל מולקולרי של 45.6 KDa ושל 42.7 Kda בהתאמה. גנים אלו בוטאו בחיידקי *E. coli* והתוצר החלבוני הופק לצורך בדיקת פעילות של אמינוטרנספרזות *in-vitro* הן בניסוי עם סובסטרט רדיואקטיבי, והן בניסוי עם סובסטרט "קר" שנבדק ב- GC-MS. תוצאות הניסויים הוכיחו כי *CmArAT1* מקודד לאנזים בעל פעילות טרנסאמינאציה של חומצות אמיניות ארומטיות (פנילאלאנין וטירוזין), ו- *CmBCAT1* מקודד לאנזים בעל פעילות טרנסאמינאציה של חומצות אמיניות מסועפות שרשרת. כדי להבין את תפקידם הביולוגי של שני גנים אלו נבדקה תבנית הביטוי שלהם במהלך התפתחות הפרי. נמצא כי הביטוי של *CmArAT1* בציפת הפרי

הבשל עולה פי 26 ביחס לביטויו בפרי לאחר 25 ימים ממועד ההפריה, ופי 46 בקליפת הפרי. ביטויו של *CmBCAT1* בפרי הבשל עולה פי 90 לעומת רמת הביטוי שלו לאחר 25 ימים ממועד ההפריה, ואילו בציפת הפרי רמת הביטוי שלו עולה פי 108.

תוצאות המחקר מצביעות על כך כי פירוק חומצות אמניות בדרך ליצירת חומרי ארומה בפרי המלון נעשה בצורה דומה לזו המוכרת מחיידקי גבינות ושמרי מזון. החומצה האמינית קודם עוברת תהליך של טרנסאמינציה להפיכתה לחומצה קטונית, ורק אח"כ מוסרת הקבוצה הקרבוקסילית לקבלת האלדהיד הנדיף. מקרה זה שונה מהמקרים המוכרים עד היום בצמחים (פרי העגבנייה ועלי כותרת של פרחי פטוניה ו-וורד). עוד עולה כי אנזימי הטרנסאמינאזות שבודדו (CmArAT1 ו- CmBCAT1) הינם אנזימי מפתח בתהליך יצירת החומרים הנדיפים העולים בצורה חדה עם הבשלת הפרי.

Amino-Acid Transaminases - אנזים מפתח ביצירת חומרי ארומה במלון שמקורם בחומצות אמיניות

חיבור על מחקר

לשם מילוי חלקי של הדרישות לקבלת התואר
מגיסטר למדעים בביולוגיה

איתי גונדה

הוגש לסנט הטכניון - מכון טכנולוגי לישראל

תמוז התשס"ט חיפה יוני 2009

Amino-Acid Transaminases - אנזים מפתח ביצירת חומרי ארומה במלון שמקורם בחומצות אמיניות

המחקר נעשה בהנחיית דר' אפרים לוינסון ודר' נורית קציר ממרכז מחקר נווה יער, מנהל המחקר החקלאי, ופרופ"ח שמעון גפשטיין מהפקולטה לביולוגיה בטכניון.

אני מודה לקרנות הבאות: קרן בארד דו-לאומית ישראל-ארה"ב למחקר ופיתוח חקלאי (גראנט מס' IS-3877-06), האיחוד האירופי בתוך פרוייקט מטה-פור למטבולומיקה של צמחים (FOOD-CT-2006-036220), ולקרן מנהל המחקר החקלאי, על התמיכה הנדיבה בהשתלמותי.

Amino-Acid Transaminases - אנזימי מפתח
ביצירת חומרי ארומה במלון
שמקורם בחומצות אמיניות

איתי גונדה