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RESEARCH PAPER

Branched-chain and aromatic amino acid catabolism into aroma volatiles in *Cucumis melo* L. fruit

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Abstract

The unique aroma of melons (*Cucumis melo* L., Cucurbitaceae) is composed of many volatile compounds biosynthetically derived from fatty acids, carotenoids, amino acids, and terpenes. Although amino acids are known precursors of aroma compounds in the plant kingdom, the initial steps in the catabolism of amino acids into aroma volatiles have received little attention. Incubation of melon fruit cubes with amino acids and α-keto acids led to the enhanced formation of aroma compounds bearing the side chain of the exogenous amino or keto acid supplied. Moreover, L-[$^{13}C_6$]phenylalanine was also incorporated into aromatic volatile compounds. Amino acid transaminase activities extracted from the flesh of mature melon fruits 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. Two novel genes were isolated and characterized (*CmArAT1* and *CmBCAT1*) encoding 45.6 kDa and 42.7 kDa proteins, respectively, that displayed aromatic and branched-chain amino acid transaminase activities, respectively, when expressed in *Escherichia coli*. The expression of *CmBCAT1* and *CmArAT1* was low in vegetative tissues, but increased in flesh and rind tissues during fruit ripening. In addition, ripe fruits of climacteric aromatic cultivars generally showed high expression of *CmBCAT1* and *CmArAT1* in contrast to non-climacteric non-aromatic fruits. The results presented here indicate that in melon fruit tissues, the catabolism of amino acids into aroma volatiles can initiate through a transamination mechanism, rather than decarboxylation or direct aldehyde synthesis, as has been demonstrated in other plants.

Key words: Amino acid metabolism, amino acid transaminase, aromatic amino acids, branched-chain amino acids, *Cucumis melo*, melon aroma, volatile aroma compounds.

Introduction

The aromas of fruits and vegetables are determined by unique 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 a distinctive aroma that is determined by the proportions of key volatiles as well as the presence or absence of unique components (Kuentzel and Bahri, 1990; Tucker, 1993). The most important aroma compounds include, among others, amino acid-derived compounds, lipid-derived compounds, phenolic derivatives, and mono- and sesquiterpenes (Croteau and Karp, 1991; Schwab *et al.*, 2008).

Melon (*Cucumis melo* L., Cucurbitaceae) is a highly polymorphic species that comprises a broad array of wild and cultivated genotypes differing in fruit traits such as climactericity, sugar and acid content, and secondary metabolites associated with colour, taste, and aroma (Seymour and McGlasson, 1993; Burger *et al.*, 2006, 2009).

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In climacteric aromatic melon varieties, volatile esters are prominent, together with sulphur-containing aroma compounds, sesquiterpenes, norisoprenes, short-chain alcohols, and aldehydes (Wyllie and Leach, 1992; Shalit *et al.*, 2001; Portnoy *et al.*, 2008). Non-aromatic varieties often have much lower levels of total volatiles, and lack the volatile esters (Shalit *et al.*, 2001; Burger *et al.*, 2006). Volatiles derived from amino acids are major contributors to melon aroma (Schieberle *et al.*, 1990; Wyllie *et al.*, 1995; Beaulieu and Grimm, 2001; Jordán *et al.*, 2001). Both aromatic and non-aromatic varieties possess amino acid-derived volatiles. In the aromatic varieties these volatiles are mostly esterified and their levels are usually higher than in the non-aromatic varieties. In the non-aromatic varieties they occur as aldehydes and alcohols.

The last two steps in the formation of volatile esters 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; Aharoni *et al.*, 2000; Beekwilder *et al.*, 2004; Boatright *et al.*, 2004; Larkov *et al.*, 2008; Zaks *et al.*, 2008). These steps involve alcohol dehydrogenase and alcohol acetyltransferase activities that convert volatile aldehydes to their respective alcohols and esters, and these activities are related to climactericity.

However, our knowledge of the initial steps of the catabolic pathways that generate aldehydes from amino acids is currently being accumulated (Fig. 1). Studies performed in tomato (*Solanum lycopersicum* L., Solanaceae) fruit indicated that catabolism of L-phenylalanine into aroma volatiles is initially mediated by decarboxylation, followed by deamination (Tieman *et al.*, 2006) (Fig. 1, red route). In contrast, it seems that a different biosynthetic route takes place in petunia (*Petunia hybrida*, Solanaceae) and rose (*Rosa hybrida*, Rosaceae) petals, in which one enzyme is able both to decarboxylate and to deaminate L-phenylalanine to release phenylacetaldehyde (Kaminaga *et al.*, 2006) (Fig. 1, purple route).

Reports regarding the formation of amino acid-derived volatiles in cheese-dwelling microorganisms and several strains of *Saccharomyces cerevisiae* are available. The first step of the pathway is relatively well understood, and in the majority of the cases it was found initially to involve transamination (Fig. 1, green route) followed by decarboxylation (Yvon *et al.*, 2000; Yvon and Rijnen, 2001; Dickinson *et al.*, 2003; Vuralhan *et al.*, 2003) as was hypothesized by Ehrlich more then a century ago (cited in Dickinson *et al.*, 2003). In both bacteria and yeasts the decarboxylation can occur either by keto acid decarboxylases to release aldehydes, or by the α-keto acid dehydrogenase complex to release a carboxylic acid (Dickinson *et al.*, 2000, 2003; Liu *et al.*, 2008). The substrates for the transamination reactions are specific amino acids and α-keto acids (usually α-ketoglutarate) (Yvon and Rijnen, 2001; Schuster *et al.*, 2006). Transaminases are pyridoxal-5′-phosphate (PLP)-dependent enzymes that reversibly transfer the amino group of the amino acid to the α-keto acid, and the keto group is converted into an amino group (Percudani and Peracchi, 2003) (Fig. 1, green route). The resulting products are usually L-glutamate and the α-keto acid derived from the respective amino acid substrate. The direction of the reaction *in vivo* is usually determined by substrate availability (Mathews *et al.*, 2000). Further observations in cheese-

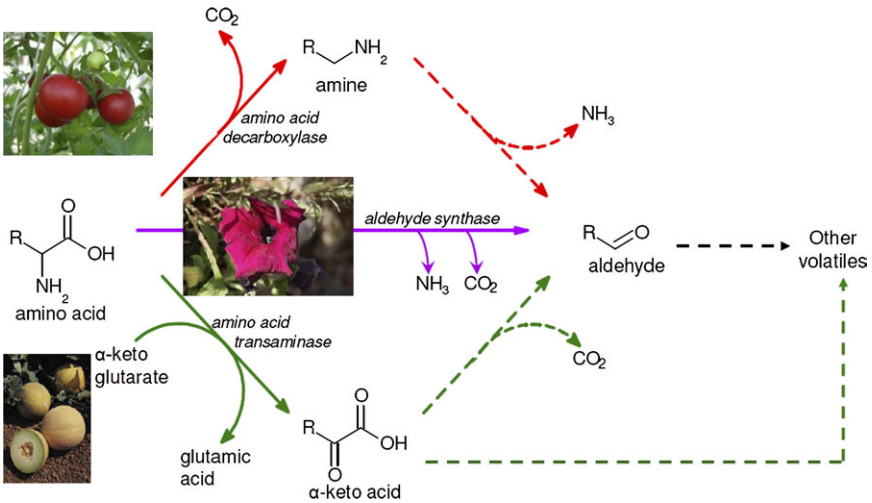

Fig. 1. Biosynthetic routes for amino acid degradation to volatiles in plants and microorganisms. Red route: decarboxylation first followed by deamination with an amine intermediate, catalysed by amino acid decarboxylases and deaminases, as partially demonstrated in tomato fruit (Tieman *et al.*, 2006). 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, catalysed by two separate enzymes as partially demonstrated in this work, in most cheese-dwelling microorganisms (Rijnen *et al.*, 1999), as well as in several strains of *Saccharomyces cerevisiae* (Dickinson *et al.*, 2003; Vuralhan *et al.*, 2003). Solid arrows indicate that the enzyme activity and genes 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 have been identified in microorganisms (Yvon and Rijnen 2001; Vuralhan *et al.*, 2003).

dwelling microorganisms and yeasts have indicated that transamination is the initial step that leads to the formation of aroma compounds (Dickinson *et al.*, 2000; Yvon and Rijnen, 2001; Vuralhan *et al.*, 2003; Liu *et al.*, 2008). In plants, the involvement of transaminases in volatile biosynthesis has not been documented. Plant transaminases are important enzymes in photorespiration, C4 photosynthesis, nitrogen assimilation, and transport, amino acid and 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).

It is shown here that the initial step in the formation of amino acid-derived aroma compounds in ripe melon fruit can follow the pathway found in cheese-dwelling microorganisms and yeasts (Rijnen *et al.*, 1999; Yvon *et al.*, 2000; Yvon and Rijnen, 2001; Dickinson *et al.*, 2003; Liu *et al.*, 2008), rather than those documented in other plants (Tieman *et al.*, 2006; Kaminaga *et al.*, 2006). For that, the levels of aroma compounds derived from exogenous L-phenylalanine, L-methionine, L-isoleucine, L-leucine, and L-valine in melon fruit cubes were monitored. Evidence indicating that the α-keto acids are intermediates in these conversions is provided here. Additionally, amino acid transaminase activities were found in cell-free extracts derived from ripe melon fruit. Two new melon genes, *CmArAT1* and *CmBCAT1*, were identified and characterized following bacterial expression, and shown to encode enzymes possessing aromatic amino acid transaminase (ArAT) and branched-chain amino acid transaminase (BCAT) activities, respectively. Both genes are expressed during late stages of ripening in aromatic fruits, while ripe fruits of non-aromatic cultivars display lower expression levels. Observations that help in rationalizing the formation of melon aroma volatiles from amino acids via transamination are summarized.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich Co. (http://www.sigmaaldrich.com/israel.html) unless otherwise indicated. L-[ring-$^{13}C_6$]Phenylalanine was from Cambridge Isotope Laboratories, Inc. (http://www.isotope.com/cil/products/searchproducts.cfm).

Plant material

Melons (*C. melo* L. vars 'Dulce', 'Védrantais', 'Noy Yizre'el', 'Tam Dew', 'Rochet', and 'Piel De Sapo') were grown in the open field with drip irrigation and fertilization under commercial conditions in the summer of 2008 at the Newe-Ya'ar Research Center in northern Israel and/or in the in the autumn of 2008 in greenhouses. Fruit samples were collected 12 d and 25 d after anthesis (DAA) and at harvest (ripe stage—after change of colour and development of the abscission zone) as indicated.

cDNA synthesis for quantitative real-time PCR (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 U) (EPI-CENTER®, USA) for 15 min at 37 °C. First-strand cDNA was synthesized from 1 μg of total RNA by the use of a Verso™ cDNA kit (ABgene®'s Inc., http://www.abgene.com/), 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.

qRT-PCR analysis

qRT- PCR was performed on an ABI PRISM® 7000 Sequence Detection System using SYBR® Green PCR Master Mix (Applied Biosystems, http://www3.appliedbiosystems.com/AB_Home/index.htm). Amplifications were conducted using the ABsolute™ QPCR SYBR® Green Mixes (ABgene®'s Inc.). A 2 μl aliquot of cDNA was used for each qRT-PCR. Thermocycling 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., http://www.microsoft.com/en/us/default.aspx) 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: (i) *CmArAT1*: forward primer 5′-AAATGGACACAGCTTCAACTATC-3′, reverse primer 5′-AACAAGAATAAGCAGAAGGGTC-3′; (ii) *CmBCAT1*: forward primer 5′-ATGATGAGAGCTGTGATTTTGAC-3′, reverse primer 5′-TCCCATAACGGCTCATTTG-3′; (iii) *cyclophilin* (accession no. DV632830): forward primer 5′-GATGGAGCTCTACGCCGATGTC-3′, reverse primer 5′-CCTCCCTGGCACATGAAATTAG-3′.

Incubation experiments and volatile analyses

Melon cubes (~4 g each) cut from the flesh of mature fruit were put in sterile Petri dish plates and 500 μl of a solution of 5 mM or 30 mM amino or α-keto acids as indicated were applied on top of each 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. A 1 g aliquot of the powder was placed in a 10 ml glass vial containing 0.7 g of 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. Solid-phase microextraction (SPME) sampling was conducted according to Davidovich-Rikanati *et al.* (2008) with slight modifications. A 65 μm fused silica fibre coated with polydimethylsiloxane/divinylbenzene (PDMS/DVB) (Supelco Inc., http://www.sigmaaldrich.com/analytical-chromatography.html) was used. The sample was pre-heated to 30 °C, agitated for 5 min at 500 rpm, and then the fibre 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 gas chromatography–mass spectrometry (GC-MS) apparatus for further analysis (see below).

GC-MS analyses

Volatile compounds were analysed on a GC-MS apparatus (Agilent Technologies, http://www.home.agilent.com/agilent/home.jspx?cc=US&lc=eng) equipped with an Rtx-5 SIL MS (30 m×0.25 mm×0.25 μm) fused-silica capillary column (Restek Co., http://www.restek.com/). Helium (0.8 ml min^{-1}) 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^{-1}, then to 260 °C at 20 °C min^{-1}. Thermal desorption was allowed for 10 min. The detector temperature was 280 °C. The mass range was recorded from 41 *m/z* to 250 *m/z*, with 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 the major peak area multiplied by a correction factor ratio.

Preparation of cell-free extracts from melon flesh tissues

Fruit flesh was cut into small pieces (~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 seasand (–50+70 mesh) and 0.5 g of 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 (DTT), 5 mM $Na_2S_2O_5$, 25 μM 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 (~5 ml) was desalted on a 15 mm×85 mm P-6 column (BioRad Labs Inc., http://www.bio-rad.com/), and the protein-containing fractions were merged and stored at –20 °C until enzymatic activity determinations. Protein was determined using the Bradford reagent and bovine serum albumin (BSA) as a standard (Bradford, 1976).

Amino acid transaminase assays

Small-scale radioactive assay: Enzymatic assays were performed by mixing 20 μl of desalted concentrated crude extract (~25 μg of protein) with 50 mM BIS-TRIS propane pH 8.5 buffer containing 10 mM α-ketoglutarate, 5 mM L-amino acid {L-[3,4-^{3}H] isoleucine; L-[4,5-^{3}H]leucine; L-[4,5-$^3H_2$]phenylalanine; L-[3,5-^{3}H]tyrosine (GE Healthcare, http://www.gehealthcare.com/ilen/); L-[methyl-$^3H_3$] methionine (Perkin Elmer, http://www.perkinelmer.com/), specific activities: 0.01 mCi/mmol}, 1 mM DTT, 225 μM PLP, 10% (w/v) D-sorbitol, in a total volume of 100 μl. The reactions were incubated for 2 h (unless otherwise indicated) at 30 °C. After incubation, each sample was acidified with 20 μl of 1 N HCl, and incubated for 30 min at 30 °C. The samples were extracted with 750 μl of ethyl acetate, vigorously shaken, and centrifuged at 20 000 *g* to allow separation of the phases. The organic phase (550 μl) was transferred into 5 ml scintillation vials containing 3 ml of Ultima Gold™ scintillation liquid (Perkin Elmer, http://www.perkinelmer.com/). Radioactivity was quantified using a liquid scintillation analyser (Tri-Carb 2800TR, Perkin Elmer, http://www.perkinelmer.com/). 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 incubated as described for the small-scale assays except that the radiolabelled substrate was omitted and the volume was increased to 2 ml for fruit-derived cell-free extracts and 0.2 ml for the bacterial lysates. Samples were then acidified with 40 μl of 10 N HCl (8 μl for bacterial lysates), and incubated for 30 min at 30 °C. The samples were extracted with methyl *tert*-butyl ether (MTBE), the MTBE evaporated to dryness, and the residue dissolved in 100 μl of pyridine and incubated for 90 min at 37 °C. For tri-methyl silyl derivatization, 100 μl of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco Inc., http://www.sigmaaldrich.com/analytical-chromatography.html) were added. The samples were incubated for another 90 min at 37 °C, transferred into glass test tubes, and stored at 4 °C until GC-MS analysis. 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 (30 m×0.25 mm×0.25 μm) fused-silica capillary column (Restek Co., http://www.restek.com/). Helium (0.8 ml min^{-1}) 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 °C to 200 °C at a rate of 5 °C min^{-1}, then to 300 °C at 20 °C min^{-1}. A quadrupole mass detector with electron ionization at 70 eV was used to acquire the MS data in the range of 41 *m/z* to 250 *m/z*. The reaction products as their silyl derivatives were identified by comparison of spectral data with authentic α-keto acid standards treated in an identical way to the reaction products.

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

The pBK-CMV expression plasmids carrying the inserts under the control of the *Lac* promoter were used. The 'Dulce' melon *CmArAT1* and *CmBCAT1* were sequenced and transformed into chemically competent JM 109 *E. coli* (Promega, http://www.promega.com/). Then, the cultures were incubated overnight in 3 ml of Luria–Bertani (LB) medium containing 5 mM kanamycin at 37 °C with shaking, and then transferred to 50 ml of LB medium and incubated for another 6 h at 37 °C with shaking. Then, isopropyl-β-D-thiogalactopyranoside (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 suspended in 1 ml of lysis buffer containing 50 mM BIS-TRIS propane pH 8.5, 10% (w/v) D-sorbitol, 1 mM DTT, 25 μM PLP, and 100 μg ml^{-1} lysozyme (Sigma grade VI from chicken egg, 60 000 U mg^{-1} 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) and the supernatants desalted on a P-6 column (BioRad Labs. Inc., http://www.bio-rad.com/). The protein-containing fractions were pooled and stored at –20 °C until used.

Bioinformatic analysis

Sequence homologies were based on the EBI align tool (http://www.ebi.ac.uk/Tools/emboss/align/), as performed by the Needle program in EBLOSUM62 matrix. Multiple sequence alignment was done with the 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 divisions were determined by the 'HMM sequence scoring' tool of the Panther classification system (http://www.pantherdb.org/).

N-terminus targeting predictions were done using 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), TergetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/), and iPSORT (http://psort.ims.u-tokyo.ac.jp/).

Results

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

To obtain a broad picture of the biosynthetic origin of aroma compounds in melon, ripe melon flesh cubes were incubated with exogenous amino acids, and the volatiles

accumulated were monitored. Incubation with a specific amino acid resulted in enhanced levels of volatiles with a similar structure to the side chain of the amino acid supplied (Fig. 2, Supplementary Table S1 available at *JXB* online). Exogenous L-phenylalanine (5 mM) increased the levels of aromatic volatiles such as benzaldehyde, benzyl alcohol, phenylacetaldehyde, phenethyl alcohol, and ethyl phenylacetate acetate (Fig. 2A). Higher (30 mM) levels of L-phenylalanine brought about much higher levels of the latter compounds and an increase of additional aromatic compounds (Fig. 2C, Supplementary Table S1). The biochemical origin of the aromatic volatiles was further ascertained by incubation of melon cubes with 5 mM L-[ring-$^{13}C_6$]phenylalanine. The $^{13}C_6$ label was incorporated

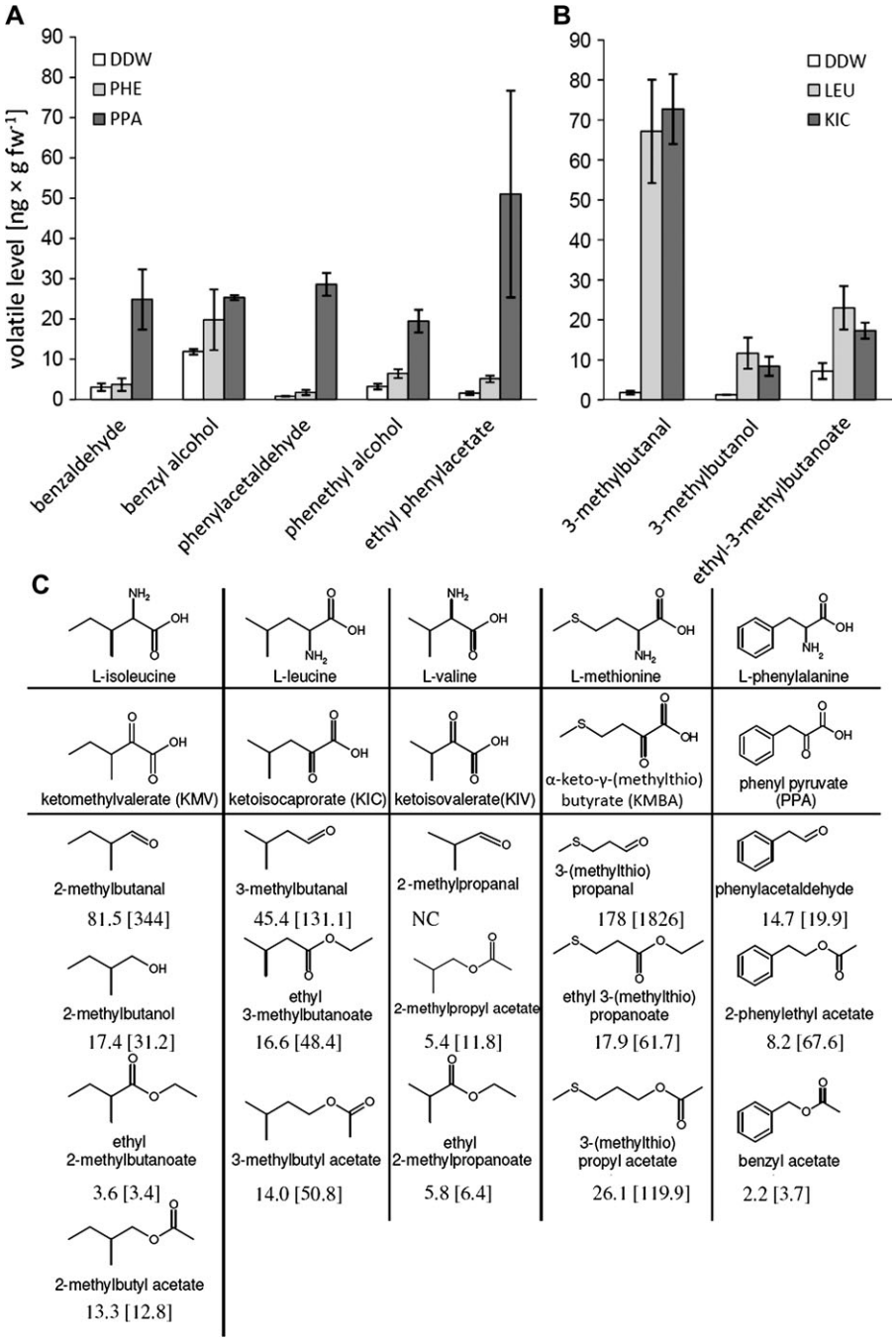

Fig. 2. Melon volatiles derived from amino and α-keto acids. (A) Volatile levels in melon cubes incubated overnight with either 5 mM L-phenylalanine (PHE), phenylpyruvate (PPA), or control (DDW). (B) Volatiles enhanced in melon cubes incubated overnight with either 5 mM L-leucine (LEU), ketoisocaproate (KIC), or control (DDW). (C) Volatiles enhanced after overnight incubation of melon cubes with 30 mM aqueous solutions of the respective amino or α-keto acid, and compared with control incubations (water). The acids supplied are shown in the top two rows. Numbers represent the fold increase in the corresponding compound when the respective amino acid was supplied. Numbers in parentheses depict the fold increase in the levels of the compound when the respective α-keto acid was supplied. NC indicates a novel compound that was absent in the controls. The experiments were replicated 5–10 times, with comparable results.

(up to 50%, Supplementary Fig. S1 at *JXB* online) into the same aromatic volatiles whose levels increased when non-labelled L-phenylalanine was administered (Fig. 2A, C, Supplementary Table S1).

Exogenous 5 mM L-leucine enhanced the levels of: 3-methylbutyl alcohol and its derivatives such as 3-methylbutanal and ethyl 3-methylbutanoate (Fig. 2B). Addition of 30 mM L-leucine caused the additional accumulation of 3-methylbutyl acetate (Fig. 2C, Supplementary Table S1). Exogenous L-isoleucine increased the levels of 2-methylbutyl alcohol derivatives, including an acid, aldehydes, esters (such as ethyl 2-methylbutanoate and 2-methylbutyl acetate), and a thioester. Exogenous L-valine enhanced the levels of 2-methylpropyl alcohol derivatives, such as 2-methylpropyl acetate. Exogenous L-methionine increased the levels of almost all of the melon volatiles that contain sulphur, including ethyl 3-methylthiopropionate and 3-(methylthio)propylacetate.

Subsequently, melon fruit cubes were incubated with the respective α-keto acids, that were in most cases more efficient substrates for the formation of the related aroma compounds than the corresponding amino acids (Fig. 2, Supplementary Table S2 at *JXB* online). Thus, the levels of L-phenylalanine-, L-leucine-, L-isoleucine-, and L-valine-derived aroma compounds increased up to 293-, 160-, 344-, and 100-fold when incubated with phenylpyruvate (PPA), ketoisocaproate (KIC), ketomethylvalerate (KMV), and ketoisovalerate (KIV), respectively (Fig. 2, Supplementary Table S2). Finally, the levels of L-methionine-derived aroma compounds were enhanced up to 1820-fold when incubated with α-keto-γ-(methylthio)butyrate (KMBA) (Fig. 2, Supplementary Table S2).

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

In order to account biochemically for the results of the amino and α-keto acid feeding experiments, melon crude cell-free extracts were tested for transaminase enzymatic activities. The keto acid, α-ketoglutarate, was used as the amine acceptor in the reactions. First, small-scale transaminase assays with ^{3}H-labelled amino acids were performed. Amino acid transaminase activity was readily measured in fruit flesh 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. 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. No activity was detected in control reactions devoid of α-ketoglutarate, reactions without protein added, or reactions when heat-inactivated enzyme was used.

To identify the products generated in the transaminase reactions, GC-MS analyses of larger scale assays were performed. The formation 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 acid substrates supported transamination reactions (Supplementary Fig. S2 at *JXB* online) forming the corresponding α-keto acids. The products generated were KMV for L-isoleucine, KIC for L-leucine, KIV for L-valine, KMBA 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-ketoglutarate, or when no L-amino acid substrate was added.

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

The Melon EST Database (http://www.icugi.org/) contains 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 three clones originating from ripe fruit), with strong similarity to tyrosine transaminases. The clones were overexpressed in JM109 competent *E. coli* 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 amino acid protein with an estimated mol. wt of 45.6 kDa, as predicted by Expasy. CmArAT1 belongs to the aminotransferase gene family-related subgroup I, and to the tyrosine aminotransferase (TAT) subfamily according to the Panther classification system (see Supplementary Fig. S3 at *JXB* online) (http://www.pantherdb.org/). As judged by the EBI align tool, CmArAT1 is similar (89% identity) to the to *Ricinus communis* putative TAT (GenBank: EEF39128.1); 85% identical to the *Arabidopsis thaliana* putative TAT (GenBank: AAN71911.1), and 86% identical to the *Glycine max* putative TAT (GenBank: AAY21813.1). CmArAT1 is moderately similar (44% identity) to the *A. thaliana* TAT 1 (AtTAT1) (Supplementary Fig. S4A at *JXB* online), the only plant aromatic amino acid transaminase gene that has been functionally identified and found to act on L-tyrosine (Lopukhina *et al.*, 2001). Interestingly CmArAT1 is 33% identical to the *Rattus norvegicus* TAT (RnTAT), 32% identical to the *Homo sapien*s TAT (HsTAT), and 30% identical to the *Trypanosoma cruzi* TAT (TcTAT), that have also been functionally identified for TAT activity (Grange *et al.*, 1985; Dietrich *et al.*, 1991; Montemartini *et al.*, 1993; Seralini *et al.*, 1995). *CmArAT1* is similar to many sequences present in ESTs derived from ripening fruit such as apple, kiwifruit, and orange, but their functionality has not been determined. Bioinformatic analysis of the N-terminus of CmArAT1 reveals no clear identifiable sequence for subcellular targeting.

Further exploration of the database searching for BCATs yielded a unigene comprising 14 clones derived from ripe fruits of different cultivars that was similar to other members of the BCAT gene family. Three of the clones that had substantial 5′ or 3′ sequence information were transformed into *E. coli* cells and grown in LB medium (that contains amino acids). The cultures were transferred to a glass vial containing 1 mM IPTG for induction of protein expression. After an overnight incubation the vials were analysed by GC-MS using SPME, which revealed the presence of 3-methylbutanal and 2-methylbutanal in the headspace of bacteria harbouring the DV633470 insert (later termed *CmBCAT1*) (Supplementary Fig. S5 at *JXB* online). These latter aldehydes are derived from L-leucine and L-isoleucine, respectively.

CmBCAT1 codes for a 389 amino acid protein with an estimated mol. wt of 42.7 kDa (predicted by Expasy). CmBCAT1 belongs to the aminotransferase subgroup IV gene family, and to the BCAT subfamily as predicted by the Panther classification system (see Supplementary Fig. S3 at *JXB* online). CmBCAT1 is 79% identical to the *R. communis* putative BCAT (GenBank: EEF52220.1) and 79% identical to the *Vitis vinifera* hypothetical protein (XM_002285475), but these two latter genes have not been functionally characterized. CmBCAT1 is also 62% identical to the *Hordeum vulgare* BCAT 1 (HvBCAT1), 61% identical to *A. thaliana* BCAT 2 (AtBCAT2), and 59% identical to the *A. thaliana* BCAT 1 (AtBCAT1), that have been functionally identified and shown to encode proteins possessing BCAT activities (Supplementary Fig. S4B at *JXB* online) (Diebold *et al.*, 2002; Malatrasi *et al.*, 2006; Schuster *et al.*, 2006). CmBCAT1 was also 30% identical to *H. sapien*s BCAT 2 (HsBCAT2) that has also been functionally identified (Davoodi *et al.*, 1998). *CmBCAT1* was similar to fruit ESTs from apple, tomato, and grape, but the annotations and functionality of these accessions are unknown. Bioinformatic analysis of the N-terminus of CmBCAT1 using Predotar, MitoprotII, pSORT, and TargetP tools indicated targeting of CmBCAT1 to the mitochondria.

Functional expression of CmArAT1 *and* CmBCAT1 *in* E. coli

In order to determine functionally the biochemical roles of *CmArAT1* and *CmBCAT1*, the recombinant proteins were assayed for amino acid transaminase activities *in vitro*. Lysates derived from bacteria overexpressing *CmArAT1* catalysed the transamination of the aromatic amino acids L-phenylalanine and L-tyrosine (Fig. 3A, B). Lysates from bacteria expressing inserts of clone DV633368 (control) catalysed those reactions but at much lower levels (Fig. 3A, B), probably due to bacterial endogenous protein. Lysates derived from *E. coli* overexpressing *CmBCAT1* catalysed the transamination of the branched-chain amino acids L-isoleucine, L-leucine (Fig. 3C, D), and L-valine (tested only in GC-MS assays; data not shown). Lysates from bacteria overexpressing inserts of clone DV633368 (control) catalysed those reactions at a much lower level, probably due to bacterial endogenous activity (noticeable in longer incubations; not shown). The identification of all products was ascertained by larger scale GC-MS assays (not shown). Interestingly, when omitting the cofactor PLP from both the extraction and reaction buffers, the activity of *CmBCAT1* remained unaffected.

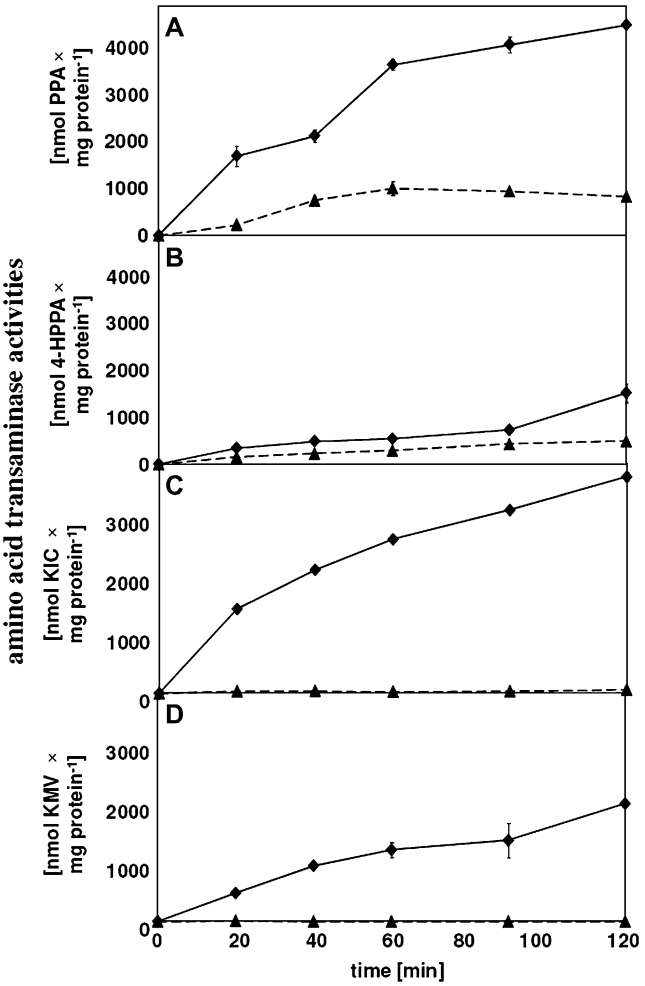

Fig. 3. Functional expression of *CmArAT1* and *CmBCAT1*. Accumulation of PPA, due to L-phenylalanine transaminase activity (A) and 4-HPPA due to L-tyrosine transaminase activity (B) of lysates from *E. coli* overexpressing *CmArAT1* (solid line, diamonds) or a control insert DV633368 (dashed line, triangles). Accumulation of KIC, due to L-leucine transaminase activity (C) and KMV due to L-isoleucine transaminase activity (D) of 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.

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

To understand better the regulation of *CmArAT1* and *CmBCAT1* during fruit physiological maturation, their expression was monitored during plant and fruit development. The climacteric cultivar 'Dulce' was used in these

experiments. Vegetative tissues such as roots, and young and old leaves display low expression levels of both *CmArAT1* and *CmBCAT1* (Fig. 4A). The expression patterns of both *CmArAT1* and *CmBCAT1* indicate up-regulation during fruit ripening, in both flesh and rind tissues (Fig. 4A, B). The expression level of *CmArAT1* in the ripe flesh increased 26-fold as compared with the levels detected 25 d after anthesis (Fig. 4B). In melon rind tissues, a similar pattern of expression was observed, but the increase was 46-fold. Similarly, the expression levels of *CmBCAT1* dramatically increased (90-fold in flesh and 108-fold in rind) as compared with the levels at 25 DAA.

The expression of *CmArAT1* and *CmBCAT1* in ripe fruits of different melon cultivars was also tested. The highest levels of expression were observed in the aromatic climacteric cultivars 'Dulce', 'Védrantais', and 'Noy-Yizre'el'. In contrast, expression of *CmBCAT1* and *CmArAT1* was lower in the non-climacteric cultivars 'Tam Dew' and 'Piel De Sapo'. The non-climateric 'Rochet' cultivar displayed substantial expression of *CmBCAT1* and *CmArAT1* (Fig. 4C).

Discussion

Many of the important volatile constituents of the aroma of melons are 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). It was found in this work that a large number of melon volatiles originate from amino acids upon incubation of melon fruit cubes with exogenous amino acid solutions

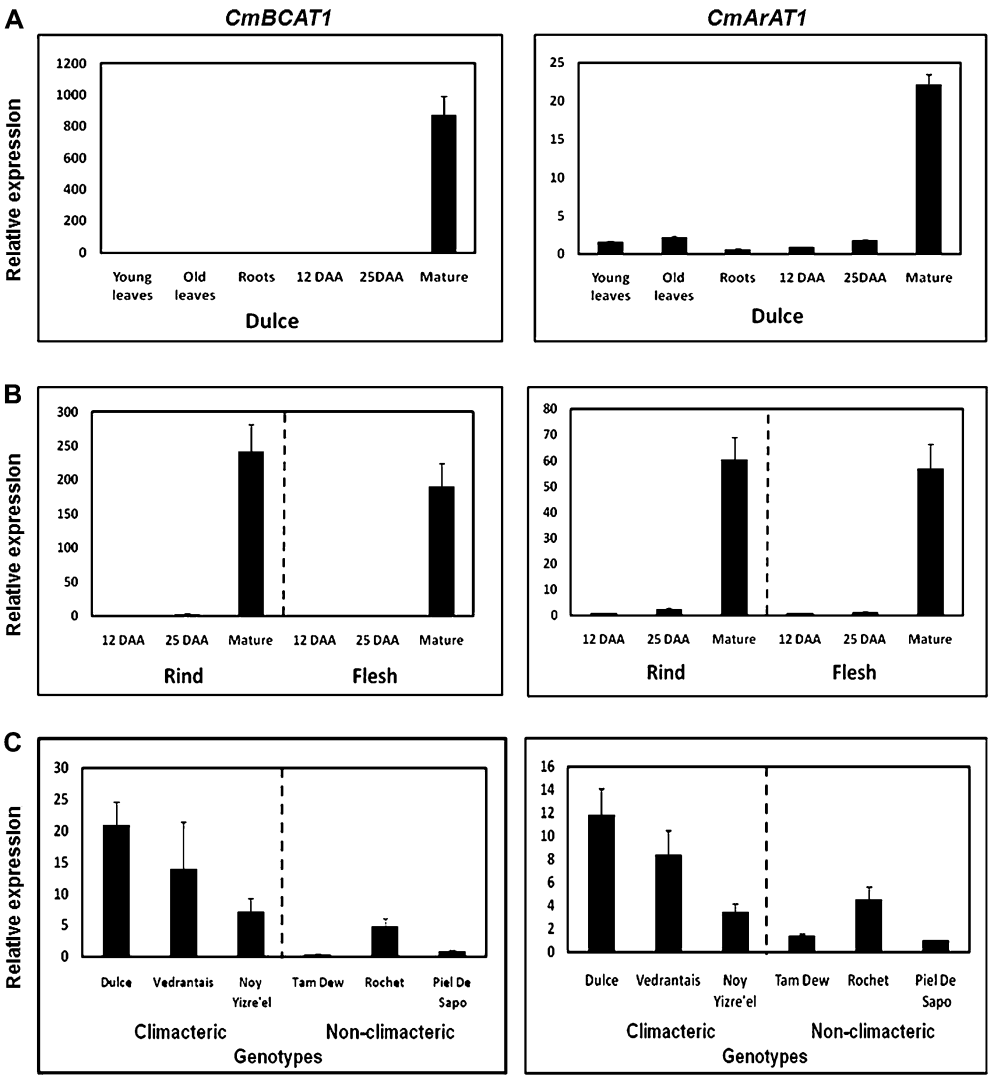

Fig. 4. Expression patterns of *CmBCAT1* (left panels) and *CmArAT1* (right panels) in melon tissues as determined by real-time PCR analysis. (A) Vegetative and fruit tissues of 'Dulce' melons. (B) Rind and flesh tissues of 'Dulce' fruit at different stages of development. (C) Ripe fruits of three climacteric cultivars ('Dulce', 'Védrantais', and 'Noy Yizre'el') and three non-climacteric cultivars ('Tam Dew', 'Rochet', and 'Piel De Sapo'). Expression levels were normalized with the internal control *cyclophilin* and plotted relative to the expression of the 12 DAA 'Dulce' (A and B) or to mature fruit of 'Piel De Sapo' samples (C). Values represent the mean of three biological samples ±SE derived each from three technical replications.

(Fig. 2, Supplementary Fig. S1, Supplementary Table S1 at *JXB* online). 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), such as ethyl 2-methylpropanoate, 2-methylpropylacetate, methyl 2-methylbutanoate, ethyl 2-methylbutanoate, 2-methylbutyl acetate, ethyl 3-methylthiopropionate, 3-(methylthio)propylacetate, and benzyl alcohol (Fig. 2, Supplementary Fig. S1, Supplementary Table S1 at *JXB* online). 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; Baldwin *et al.*, 2000; Wyllie and Fellman, 2000; Matich and Rowan, 2007). Radiolabelled 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). The incubation 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 (Fig. 1, Schwab *et al.*, 2008).

α-Keto acids are key intermediates to volatiles in cheese-dwelling microorganisms and yeasts (Yvon and Rijnen 2001; Dickinson *et al.*, 2003). In order to ascertain if α-keto acids are efficient substrates for the formation of melon volatiles, feeding experiments were performed. The results indicate that α-keto acids are more efficient precursors of melon aroma compounds (Fig. 2, Supplementary Table S2 at *JXB* online). 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 following a similar pathway to that described in microorganisms (Fig. 1, green route). The greater efficiency of α-keto acids as compared with amino acids in enhancing volatile levels might be due to more efficient uptake or metabolism of the α-keto acids as compared with the amino acids. Nevertheless, it could also be that the rate of amino acid deamination into α-keto acids might be rate-limiting for the production of the aforementioned volatiles.

Although the aforementioned results indicate that α-keto acids are precursors of melon volatiles, substantial levels of benzaldehyde were present in the PPA solutions used for the incubation experiments (Supplementary Table S3 at *JXB* online). 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 benzonate. Nevertheless, it is unlikely that the volatiles containing an aromatic ring with two carbons in their side chain, such as phenylacetaldehyde and 2-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 disulphide observed could be due to the presence of this compound in both the L-methionine and the KMBA solutions (Supplementary Table S3), and not due to enzymatic conversions. Still, the presence of this contaminant cannot solely explain the enhanced levels of other sulphur-containing aroma compounds in L-methinine-fed melon cubes.

Although microbial transaminases have clear roles in the formation of volatiles (Fig. 1, 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 (Supplementary Fig. S2 at *JXB* online). These results strengthen the hypothesis that the conversion of amino acids into aroma compounds in melons is initiated by transamination. It cannot be excluded that other amino acid catabolic pathways such as those described in tomato fruit (Tieman *et al.*, 2006), and in rose and petunia flowers (Kaminaga *et al.*, 2006) (Fig. 1, 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).

Two novel melon genes were identified, namely *CmArAT1* and *CmBCAT1*, that encode enzymes possessing ArAT and BCAT activities, respectively (Fig. 3). Some of the amino acids considered crucial for activity in the *Trypanosoma cruzi* TAT (TcTAT) based on its X-ray crystal structure (Blankenfeldt *et al.*, 1999) are also conserved in CmArAT1 (Supplementary Fig. S4A at *JXB* online). They include the Lys245 that covalently anchors the cofactor 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 (Supplementary Fig. S4A). Moreover, the key amino acids in HsBCAT2 (Yennawar *et al.*, 2001) are also conserved in CmBCAT1 (Supplementary Fig. S4B). 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 fact that CmBCAT1 has a high sequence similarity to PLP-dependent enzymes, and although the authors are not aware of any amino acid transaminase enzyme activity that does not utilize PLP, attempts to show the dependence of CmBCAT1 on exogenous PLP were unsuccessful (data not shown). The current hypothesis is that the unaltered activity may be due to PLP that was tightly bound to the enzyme and was not released during the extraction process.

It is difficult to determine the actual role of a specific amino acid transaminase *in vivo* based on estimations of its kinetic properties *in vitro*. As the reaction is fully reversible, the actual direction of the reaction *in vivo* is primarily directed by substrate and product intracellular concentrations (Mathews *et al.*, 2000). BCATs have been

shown to be able to catalyse the last step in the biosynthesis of branched-chain amino acids (Singh, 1999), but it seems that ArATs are not involved in aromatic amino acid biosynthesis in plants, a pathway that is mediated by arogenate, independently to ArAT activity (Siehl, 1999). Still, the biosynthesis of both branched and aromatic amino acids in plants seemingly takes place in the plastids, as judged by vast amounts of biochemical and molecular evidence (Siehl, 1999). In contrast, 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; Schuster and Binder, 2005). 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 determine unequivocally the subcellular localization of CmBCAT1 and CmArAT1.

The expression of both *CmArAT1* and *CmBCAT1* was monitored during plant development. The levels of expression of both genes were low in vegetative tissues and at early stages of fruit development, and sharply increased upon maturation, both in fruit rind and in flesh tissues (Fig. 4A, B). 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). This is in accordance to the expression patterns of other genes involved in volatile formation in melons such as alcohol-acetyl transferases, alcohol dehydrogenases, carotenoid cleavage dioxygenases, and sesquiterpene synthases which are also up-regulated upon fruit maturation (El-Yahyaoui *et al.*, 2002; El-Sharkawy *et al.*, 2005; Ibdah *et al.*, 2006; Manríquez *et al.*, 2006; Portnoy *et al.*, 2008). In contrast, branched-chain amino acids are primarily biosynthesized in vegetative, especially young tissues (Singh, 1999). The low levels of expression of both of these genes in vegetative tissues and in immature fruit and their dramatic up-regulation upon fruit maturation further supports important roles in amino acid catabolism.

The observation that climacteric cultivars generally display higher *CmArAT1* and *CmBCAT1* expression levels, as compared with non-climacteric cultivars (Fig. 4C), strengthens the hypothesis that these genes play crucial roles in the biosynthesis of melon aroma volatiles. Although non-climacteric melon fruits often accumulate amino acid-derived volatiles, they are generally present at lower levels, as compared with climacteric melon varieties (Shalit *et al.*, 2001; Obando-Ulloaa *et al.*, 2008, 2009). The varietal differences in the expression patterns of *CmArAT1* and *CmBCAT1* (Fig. 4C) further support their involvement in volatile biosynthesis in melon fruit.

Conclusion

Although it is well established that amino acids serve as precursors of plant floral and fruit volatiles, only two studies dealing with the initial 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 (Fig. 1, red route) (Tieman *et al.*, 2006), while in petunia and rose petals one bifunctional enzyme decarboxylates and deaminates L-phenylalanine directly releasing phenylacetaldehyde (Fig. 1, purple route) (Kaminaga *et al.*, 2006). The feeding experiments (Fig. 2, Supplementary Fig. S1 and Supplementary Tables S1, S2 at *JXB* online) together with the measurements of L-phenylalanine transaminase activity in cell-free extracts (Supplementary Fig. S2A) 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 (Fig. 1, 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 and branched-chain aroma volatiles in banana slices (Tressl and Drawert, 1973). The feeding experiments (Fig. 2, Supplementary Tables S1, S2 at *JXB* online) and the presence of BCAT activity in cell-free extracts (Supplementary Fig. S2B–D) indicate that transamination is probably the initial step in the metabolism of branched-chain amino acids into aroma compounds in melon fruit (Fig. 1, green route).

The expression of *CmArAT1* and *CmBCAT1* is much higher in ripe fruits of aromatic melon cultivars as compared with vegetative tissues and non-ripe fruits as well as compared with ripe fruits of non-climacteric varieties (Fig. 4A–C). Considering their functional ArAT and BCAT roles (Fig. 3), it seems 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 during fruit maturation. This route is apparently novel in plants but similar to the route that reportedly takes place in cheese-dwelling microorganisms and yeasts (Yvon and Rijnen, 2001; Dickinson *et al.*, 2003) and predicted by Ehrlich already in 1907 (cited in Dickinson *et al.*, 2003). α-Keto acid decarboxylases might release volatile aldehydes (Fig. 1, green route) or carboxylic acids through the α-keto acid dehydrogenase complex in cheese-dwelling microorganisms and yeasts (Dickinson *et al.*, 2000, 2003; Yvon and Rijnen, 2001; Vuralhan *et al.*, 2003; Liu *et al.*, 2008). It might be possible that some of the esters evolved after incubation of melon disks with α-keto acids could be generated from the corresponding acyl-CoA moieties, generated by the action of the branched-chain α-keto acid dehydrogenase complex (Li *et al.*, 2003). Additional candidate unigenes that might code for some of the aforementioned enzymes are apparent in the Melon EST Database, but their functionality and their actual involvement in the biosynthesis of aroma compounds in melon are still unknown.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Incorporation of L-[ring-$^{13}C_6$]phenylalanine into melon volatiles.

Supplementary Fig. S2. Amino acid transaminase activities in melon soluble protein extracts.

Supplementary Fig. S3. Phylogenetic tree of plant amino acid transaminases.

Supplementary Fig. S4. Amino acid sequence multiple alignments of CmArAT1 (A) and CmBCAT1 (B).

Supplementary Fig. S5. Functional screening of genes coding for branched-chain amino acid transaminases.

Supplementary Table S1. Amino acid-derived VOCs in incubation experiments of melon cubes with different L-amino acids.

Supplementary Table S2. Amino acid-derived VOCs in incubation experiments of melon cubes with different α-keto-acids.

Supplementary Table S3. Levels of volatiles found in the control solutions incubated overnight without melon cubes.

Supplementary Table S4. Accession numbers of genes appear in Supplementary Figure S1.

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References

Anderson MD, Che P, Song J, Nikolau BJ, Wurtele ES. 1998. 3-Methylcrotonyl-coenzyme A carboxylase is a component of the mitochondrial leucine catabolic pathway in plants. *Plant Physiology* **118,** 1127–1138.

Aharoni A, Keizer LC, Bouwmeester HJ, *et al*. 2000. Identification of the SAAT gene involved in strawberry flavor biogenesis by use of DNA microarrays. *The Plant Cell* **12,** 647–662.

Baldwin EA, Scott JW, Shewmaker CK, Schuch W. 2000. Flavor trivia and tomato aroma: biochemistry and possible mechanisms for control of important aroma components. *HortScience* **35,** 1013–1022.

Beaulieu JC, Grimm CC. 2001. Identification of volatile compounds in cantaloupe at various developmental stages using solid phase microextraction. *Journal of Agricultural and Food Chemistry* **49,** 1345–1352.

Beekwilder J, Alvarez-Huerta M, Neef E, Verstappen FWA, Bouwmeester JH, Aharoni A. 2004. Functional characterization of enzymes forming volatile esters from strawberry and banana. *Plant Physiology* **135,** 1865–1878.

Blankenfeldt W, Montemartini-Kalisz M, Kalisz HM, Hecht HJ, Nowicki C. 1999. Crystal structure of *Trypanosoma cruzi* tyrosine aminotransferase: substrate specificity is influenced by cofactor binding mode. *Protein Science* **8,** 2406–2417.

Boatright J, Negre F, Chen X, Kish CM, Wood B, Peel G, Orlova I, Gang D, Rhodes D, Dudareva N. 2004. Understanding *in vivo* benzenoid metabolism in petunia petal tissue. *Plant Physiology* **135,** 1993–2011.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* **72,** 248–254.

Burger Y, Paris HS, Cohen R, Katzir N, Tadmor Y, Lewinsohn E, Schaffer AA. 2009. Genetic diversity of *Cucumis melo*. *Horticultural Reviews* **35,** 165–198.

Burger Y, Sa'ar U, Paris HS, Lewinsohn E, Katzir N, Tadmor Y, Schaffer AA. 2006. Genetic variability for valuable fruit quality traits in *Cucumis melo*. *Israel Journal of Plant Science* **54,** 233–242.

Buttery RG, Teranishi R, Ling LC. 1987. Fresh tomato aroma volatiles: a quantitative study. *Journal of Agricultural and Food Chemistry* **35,** 540–544.

Croteau R, Karp F. 1991. Origin of natural odorants. In: Muller PM, Lamparsky D, eds. *Perfumes. Art, science and technology*. London: Elsevier Applied Science, 101–126.

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. *The Plant Journal* **56,** 228–238.

Davoodi J, Drown PM, Bledsoe RK, Wallin R, Reinharti GD, Hutson SM. 1998. Overexpression and characterization of the human mitochondrial and cytosolic branched-chain aminotransferases. *Journal of Biological Chemistry* **273,** 4982–4989.

Dickinson JR, Harrison SJ, Dickinson JA, Hewlinsi MJE. 2000. An investigation of the metabolism of isoleucine to active amyl alcohol in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **275,** 10937–10942.

Dickinson JR, Salgado LEJ, Hewlins MJE. 2003. The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **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 Physiology* **129,** 540–550.

Dietrich JB, 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. *European Journal of Biochemistry* **201,** 399–407.

El-Sharkawy I, Manríquez D, Flores FB, Regad F, Bouzayen M, Latché A, Pech JC. 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 Molecular Biology* **59,** 345–362.

El-Yahyaoui F, Wongs-Aree C, Latché A, Hackett R, Grierson D, Pech JC. 2002. Molecular and biochemical characteristics of a gene encoding an alcohol acyl-transferase involved in the generation of aroma volatile esters during melon ripening. *European Journal of Biochemistry* **269,** 2359–2366.

Grange T, Guénet C, Dietrich JB, 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. *Journal of Molecular Biology* **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. *Journal of Plant Physiology* **150,** 23–30.

Ibdah M, Azulay Y, Portnoy V, *et al*. 2006. Functional characterization of *CmCCD1*, a carotenoid cleavage dioxygenase from melon. *Phytochemistry* **67,** 1579–1589.

Jordán MJ, Shaw PE, Goodner KL. 2001. Volatile components in aqueous essence and fresh fruit of *Cucumis melo* cv. athena (muskmelon) by GC-MS and GC-O. *Journal of Agricultural and Food Chemistry* **49,** 5929–5933.

Kaminaga Y, Schnepp J, Peel G, *et al*. 2006. Plant phenylacetaldehyde synthase is a bifunctional homotetrameric enzyme that catalyzes phenylalanine decarboxylation and oxidation. *Journal of Biological Chemistry* **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 Physiology* **146,** 1028–1039.

Kuentzel H, Bahri D. 1990. Synthetic ingredients of food flavourings. In: Ashurts PR, ed. *Food flavorings*. Glasgow: Blackie Academic & Professional, 115–157.

Larkov O, Zaks A, Bar E, Lewinsohn E, Dudai N, Mayer AM, Ravid U. 2008. Enantioselective monoterpene alcohol acetylation in *Origanum, Mentha* and *Salvia* species. *Phytochemistry* **69,** 2565–2571.

Lea PJ, Ireland RJ. 1999. Nitrogen metabolism in higher plants. In: Singh BK, ed. *Plant amino acids. Biochemistry and biotechnology*. Princeton, NJ: American Cyanamid Company, 1–49.

Li L, Thipyapong P, Breeden DC, Steffens JC. 2003. Overexpression of a bacterial branched-chain α-keto acid dehydrogenase complex in *Arabidopsis* results in accumulation of branched-chain acyl-CoAs and alteration of free amino acid composition in seeds. *Plant Science* **165,** 1213–1219.

Liu M, Nauta A, Francke C, Siezen RJ. 2008. A comparative genomics of enzymes in flavor-forming pathways from amino acids in lactic acid bacteria. *Applied and Environmental Microbiology* **74,** 4590–4600.

Lopukhina A, Dettenberg M, Weiler EW, Holländer-Czytko H. 2001. Cloning and characterization of a coronatine-regulated tyrosine aminotransferase from arabidopsis. *Plant Physiology* **126,** 1678–1687.

Malatrasi M, Corradi M, Svensson JT, Close TJ, Gulli M, Marmiroli N. 2006. A branched-chain amino acid aminotransferase gene isolated from *Hordeum vulgare* is differentially regulated by drought stress. *Theoretical and Applied Genetics* **113,** 965–976.

Malkin R, Niyogi K. 2000. Photosynthesis. In: Buchanan BB, Gruissem W, Jones LR, eds. *Biochemistry and molecular biology of plants*. Rockville, MD: American Society of Plant Physiologists, 568–630.

Manríquez D, El-Sharkawy I, Flores FB, El-Yahyaoui F, Regad F, Bouzayen M, Latché A, Pech JC. 2006. Two highly divergent alcohol dehydrogenases of melon exhibit fruit ripening-specific expression and distinct biochemical characteristics. *Plant Molecular Biology* **61,** 675–685.

Mathews CK, van Holde K, Ahern KG. 2000. *Biochemistry*, 3rd edn. San Francisco: Benjamin Cummings.

Matich A, Rowan D. 2007. Pathway analysis of branched-chain ester biosynthesis in apple using deuterium labeling and enantioselective gas chromatography–1mass spectrometry. *Journal of Agricultural and Food Chemistry* **55,** 2727–2735.

Mizukami H, Ellis BE. 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 JA, Cazzulo JJ, Nowicki C. 1993. Purification and partial structural and kinetic characterization of tyrosine aminotransferase from epimastigotes of *Trypanosoma cruzi*. *Journal of Biochemistry* **292,** 901–906.

Obando-Ulloa JM, Moreno E, García-Mas J, Nicolai B, Lammertyn J, Monforte AJ, Fernández-Trujillo JP. 2008. Climacteric or non-climacteric behavior in melon fruit aroma volatiles. *Postharvest Biology and Technology* **49,** 27–37.

Obando-Ulloaa JM, Nicolai B, Lammertyn J, Bueso MC, Monforte AJ, Fernández-Trujillo JP. 2009. Aroma volatiles associated with the senescence of climacteric or non-climacteric melon fruit. *Postharvest Biology and Technology* **52,** 146–155.

Percudani R, Peracchi A. 2003. A genomic overview of pyridoxal-phosphate dependent enzymes. *EMBO Reports* **4,** 850–854.

Pérez AG, Olías R, Luaces P, Sanz C. 2002. Biosynthesis of strawberry aroma compounds through amino acid metabolism. *Journal of Agricultural and Food Chemistry* **50,** 4037–4042.

Pérez AG, Sanz C, Olias JM. 1993. Partial purification and some properties of alcohol acetyltransferase from strawberry fruits. *Journal of Agricultural and Food Chemistry* **41,** 1462–1466.

Pérez AG, Sanz C, Olias R, Rios JJ, Olias JM. 1996. Evolution of strawberry alcohol acetyltransferase activity during fruit development and storage. *Journal of Agricultural and Food Chemistry* **44,** 3286–3290.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29,** 2002–2007.

Portnoy V, Benyamini Y, Bar E, *et al*. 2008. The molecular and biochemical basis for varietal variation in sesquiterpene content in melon (*Cucumis melo* L.) rinds. *Plant Molecular Biology* **66,** 647–661.

Ramakers C, Ruijter JM, Deprez RH, Moorman AF. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience Letters* **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. *Applied and Environmental Microbiology* **65,** 4873–4880.

Rowan DD, Lane HP, Allen JM, Fielder S, Hunt MB. 1996. Biosynthesis of 2-methylbutyl, 2-methyl-2-butenyl, and 2-methylbutanoate esters in Red Delicious and Granny Smith apples using deuterium-labeled substrates. *Journal of Agricultural and Food Chemistry* **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. *Journal of Food Science* **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 Molecular Biology* **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 methionine-derived glucosinolates in *Arabidopsis*. *The Plant Cell* **18,** 2664–2679.

Schwab W, Davidovich-Rikanati R, Lewinsohn E. 2008. Biosynthesis of plant-derived flavor compounds. *The Plant Journal* **54,** 712–732.

Seralini GE, Luu-The V, Labrie F. 1995. Cloning and expression of human tyrosine aminotransferase cDNA. *Biochimica et Biophysica Acta* **1260,** 97–101.

Seymour GB, McGlasson WB. 1993. Melons. In: Seymour GB, Taylor JE, Tucker GA, eds. *Biochemistry of fruit ripening*. London: Chapman & Hall, 273–290.

Shalit M, Katzir N, Tadmor Y, *et al*. 2001. Acetyl CoA: alcohol acetyl transferase activity and aroma formation in ripening melon fruits. *Journal of Agricultural and Food Chemistry* **49,** 794–799.

Siedow JN, Day DA. 2000. Respiration and photorespiration. In: Buchanan BB, Gruissem W, Jones LR, eds. *Biochemistry and molecular biology of plants*. Rockville, MD: American Society of Plant Physiologists, 767–729.

Siehl DL. 1999. The biosynthesis of tryptophan, tyrosine, and phenylalanine from chorismate. In: Singh BK, ed. *Plant amino acids. Biochemistry and biotechnology*. Princeton, NJ: American Cyanamid Company, 171–204.

Singh BK. 1999. Biosynthesis of valine, leucine and isoleucine. In: Singh BK, ed. *Plant amino acids. Biochemistry and biotechnology*. Princeton, NJ: American Cyanamid Company, 227–249.

Thomson DMH. 1987. The meaning of flavor. In: Birch GG, Lindley MG, eds. *Developments in food flavors*. London: Elsevier, 1–21.

Tieman D, Taylor M, Schauer N, Fernie AR, Hanson AD, Klee HJ. 2006. Tomato aromatic amino acid decarboxylases participate in synthesis of the flavor volatiles 2-phenylethanol and 2-phenylacetaldehyde. *Proceedings of the National Academy of Sciences, USA* **103,** 8287–8292.

Tressl R, Drawert F. 1973. Biogenesis of banana volatiles. *Journal of Agricultural and Food Chemistry* **21,** 560–565.

Tucker GA. 1993. Introduction. In: Seymour GB, Taylor JE, Tucker GA, eds. *Biochemistry of fruit ripening*. London: Chapman & Hall, 1–51.

Vuralhan Z, Morais MA, Tai S, Piper MDW, Pronk JT. 2003. Identification and characterization of phenylpyruvate decarboxylase genes in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* **69,** 4534–4541.

Wyllie SG, Fellman JK. 2000. Formation of volatile branched chain esters in bananas (*Musa sapientum* L.). *Journal of Agricultural and Food Chemistry* **48,** 3493–3496.

Wyllie SG, Leach DN. 1992. Sulfur-containing compounds in the aroma volatiles of melons (*Cucumis melo*). *Journal of Agricultural and Food Chemistry* **40,** 253–256.

Wyllie SG, Leach DN, Wang Y, Shewfelt RL. 1995. Key aroma compounds in melons. Their development and cultivar dependence. In: Roussef RL, Leahy MM, eds. *Fruit flavors; ACS Symposium Series 596*. Washington, DC: American Chemical Society, 248–257.

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

Yabumoyo K, Jennings WG. 1977. Volatile constituents of cantaloupe, *Cucumis melo*, and their biogenesis. *Journal of Food Science* **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. *Applied and Environmental Microbiology* **66,** 571–577.

Yvon M, Rijnen L. 2001. Cheese flavour formation by amino acid catabolism. *International Dairy Journal* **11,** 185–201.

Zaks A, Davidovich-Rikanati R, Bar E, Inbar M, Lewinsohn E. 2008. Biosynthesis of linalyl acetate and other terpenes in lemon mint (*Mentha aquatica* var. *citrata*, Lamiaceae) glandular trichomes. *Israel Journal of Plant Sciences* **56,** 233–244.