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

Itay Gonda1,2, Einat Bar1, Vitaly Portnoy1, Shery Lev1, Joseph Burger1, Arthur A. Schaffer1, Ya’akov Tadmor1, Shimon Gepstein2, James J. Giovannoni3, Nurit Katzir1 and Efraim Lewinsohn1,*

1 Institute of Plant Sciences, Newe Ya’ar Research Center, Agricultural Research Organization, PO Box 1021, Ramat Yishay 30095, Israel
2 Faculty of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel
3 United States Department of Agriculture and Boyce Thompson Institute for Plant Research, Cornell University, Tower Road, Ithaca, NY 14853, USA

* To whom correspondence should be addressed. E-mail: twefraim@agri.gov.il

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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-[13C6]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).
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; Manriquez 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 than 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 keto acid to the α-keto acid, and the keto group is converted into an amino group (Percudani and Paracchi, 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).
dwellling 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; Vuralghan 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 photosynthesis, 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, Malatras 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 (Tiemann 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-15]C3 Phenylalanine was from Cambridge Isotope Laboratories, Inc. (http://www.isotope.com/cil/products/searchproducts.cfm).

Plant material
Melons (C. melo L. var. ‘Dolce’, ‘Védrantais’, ‘Noy Yizre’el’, ‘Tam Dew’, ‘Archet’, 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 ABsolutive™ 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’-AAATGGGACACGCTCTCAACTACTC-3′ reverse primer 5’-AACAAAGAATACAGAAGGCTC-3′; (ii) CmBCAT1: forward primer 5’-TGTAGGAAGACTGTGATTTCGAC-3′ reverse primer 5’-TCCCTACAGCGATTTGG-3′; (iii) cyclophilin (accession no. D7632830) forward primer 5’-GATGGAGCTCTAACGCRTGAC-3′, reverse primer 5’-CCTCCCTTGGCACATGAAATATTAG-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 acid 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 polydimethylsiloxiane/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 cm³) 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 of seasand (−50+70 mesh) and 0.5 g of polyvinylpolypyrrolidone (PVPP) 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, 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).

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 EBLASTM2 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 PyML method using ClustalW (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/mitopred.html), TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP), and iPSORT (http://ipsort.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 were injected into a GC-MS 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.
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-13C6]phenylalanine. The 13C6 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.
Exogenous L-isoleucine increased the levels of 2-methylbutyl 3-methylbutyl acetate (Fig. 2C, Supplementary Table S1).

In order to account biochemically for the results of the amino acid transaminase activities accepting various amino acids as substrates performed. The formation of the corresponding \( \alpha \)-keto acid was assessed using a derivatization method (see Materials and methods), because the \( \alpha \)-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 \( \alpha \)-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, or no addition of \( \alpha \)-keto glutarate, 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

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 \( \alpha \)-keto acid feeding experiments, melon crude cell-free extracts were tested for transaminase enzymatic activities. The keto acid, \( \alpha \)-keto glutarate, was used as the amine acceptor in the reactions. First, small-scale transaminase assays with \( ^{1} \)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-[\( ^{1} \)H]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-[\( ^{1} \)H]isoleucine was used as a substrate. Much lower but still significant transaminase enzymatic activities were observed when L-[\( ^{1} \)H]methionine (37.7 ± 4.8 pkat mg protein\(^{-1}\)) and L-[\( ^{1} \)H]phenylalanine (5.7 ± 1.2 pkat mg protein\(^{-1}\)) were utilized as substrates. No activity was detected in control reactions devoid of \( \alpha \)-keto glutarate, 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 carried out. 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). 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 sapiens 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 Expsys). *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: EEE52220.1) 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. sapiens* 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 *vivro*. 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.

**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 \textit{CmArAT1} and \textit{CmBCAT1} (Fig. 4A). The expression patterns of both \textit{CmArAT1} and \textit{CmBCAT1} indicate up-regulation during fruit ripening, in both flesh and rind tissues (Fig. 4A, B). The expression level of \textit{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 \textit{CmBCAT1} dramatically increased (90-fold in flesh and 108-fold in rind) as compared with the levels at 25 DAA.

The expression of \textit{CmArAT1} and \textit{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 \textit{CmBCAT1} and \textit{CmArAT1} was lower in the non-climacteric cultivars ‘Tam Dew’ and ‘Piel De Sapo’. The non-climateric ‘Rochet’ cultivar displayed substantial expression of \textit{CmBCAT1} and \textit{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 \textit{et al.}, 1990; Wyllie and Leach, 1992; Wyllie \textit{et al.}, 1995, Beaulieu and Grimm, 2001; Jordán \textit{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.

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**Fig. 4.** Expression patterns of \textit{CmBCAT1} (left panels) and \textit{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.
Transamination of amino acids into melon volatiles

(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 Druwart, 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 benzoate. 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-[1-14C]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; Ibda et al., 2006; Manriquez 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-Ulloa 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-14C]-leucine and [U-14C]-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-13C6]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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