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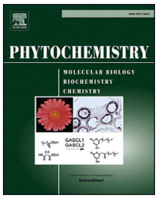

Differential metabolism of L–phenylalanine in the formation of aromatic volatiles in melon (*Cucumis melo* L.) fruit

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ABSTRACT

Studies on the active pathways and the genes involved in the biosynthesis of L-phenylalanine-derived volatiles in fleshy fruits are sparse. Melon fruit rinds converted stable-isotope labeled L-phe into more than 20 volatiles. Phenylpropanes, phenylpropenes and benzenoids are apparently produced via the well-known phenylpropanoid pathway involving phenylalanine ammonia lyase (PAL) and being (*E*)-cinnamic acid a key intermediate. Phenethyl derivatives seemed to be derived from L-phe via a separate biosynthetic route not involving (*E*)-cinnamic acid and PAL. To explore for a biosynthetic route to (*E*)-cinnamaldehyde in melon rinds, soluble protein cell-free extracts were assayed with (*E*)-cinnamic acid, CoA, ATP, NADPH and $MgSO_4$, producing (*E*)-cinnamaldehyde *in vitro*. In this context, we characterized *CmCNL*, a gene encoding for (*E*)-cinnamic acid:coenzyme A ligase, inferred to be involved in the biosynthesis of (*E*)-cinnamaldehyde. Additionally we describe *CmBAMT*, a SABATH gene family member encoding a benzoic acid:*S*-adenosyl-L-methionine carboxyl methyltransferase having a role in the accumulation of methyl benzoate. Our approach leads to a more comprehensive understanding of L-phe metabolism into aromatic volatiles in melon fruit.

1. Introduction

The aroma of melon (*Cucumis melo* L.) fruit is determined by the combination of hundreds of volatiles imparting both agreeable and disagreeable odors and constituting the full bouquet of flavors often associated with melon consumption (Beaulieu and Grimm, 2001; Gonda et al., 2016). Melon is a very polymorphic cultivated species and this polymorphism is also reflected in the different aromas displayed by the many varieties and accessions known (Aubert and Bourger, 2004; Burger et al., 2006; Kourkoutas et al., 2006; Shalit et al., 2001; Tang et al., 2015). As is the case with many other fruits, melon volatiles are often derived from nutritionally important metabolites such as essential amino and fatty acids and carotenoids (Goff and Klee, 2006; Gonda et al., 2010, 2013; Ibdah et al., 2006). In this context, aroma volatiles derived from amino acids are major contributors to the unique aroma of melons (Buttery et al., 1982; Jordán et al., 2001; Pang et al., 2012; Wyllie et al., 1994). We have previously showed that the essential amino

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Abbreviations: AAT, alcohol acyl-transferase; BAMT, *S*-adenosyl-L-methionine: benzoic acid carboxyl methyltransferase; CCR, cinnamoyl-CoA reductase; CNL, (*E*)-cinnamic acid-CoA ligase; CoA, coenzyme A; PAL, L-phenylalanine ammonia-lyase; PPP, phenylpropanoid pathway; RIL, recombinant inbred line; SAM, *S*-adenosyl-L-methionine.

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acids L-phe, L-methionine, L-leucine, L-isoleucine and L-valine, are metabolized into aroma compounds in melon fruit (Gonda et al., 2010, 2013), and some of these conversions are apparently initiated by the action of aminotransferases generating the respective α-keto-acids. Volatiles displaying aromatic rings are mostly derived from L-phe and include benzenoids (C_6—C_1), phenethyl compounds (C_6—C_2), phenylpropanes (C_6—C_3), phenylpropenes (C_6=C_3) as well as hydroxylated and methoxylated phenylpropenes (Fig. 1; Freilich et al., 2015; Gonda et al., 2016). Among the most important L-phe-derived aroma compounds are (*E*)-cinnamaldehyde and (*E*)-cinnamyl acetate that impart key cinnamon notes as well as phenethylderivatives and methylbenzoate that impart floral notes (Schwab et al., 2008). L-Phe derived volatiles such as benzyl acetate and 2-phenethyl acetate, are key contributors to the peculiar melon aroma as determined in organoleptic studies (Buttery et al., 1982; Hayata et al., 2003; Jordán et al., 2001; Pang et al., 2012; Perry et al., 2009; Wyllie et al., 1994).

It is clear that more than one biosynthetic route is involved in the conversion of L-Phe into plant volatiles. It is well known that plants generally utilized the well-defined “phenylpropanoid pathway” (PPP) to generate both volatiles and non-volatile derivatives such as lignins, stilbenes and flavonoids (Vogt, 2010). The initial step of the PPP involves the action of L-phe ammonia-lyase (PAL), generating (*E*)-cinnamic acid, followed by the action of (*E*)-cinnamic acid 4-hydroxylase (C4H) and *p*-coumaric acid coenzyme A (CoA) ligase (4CL) to generate the key intermediates *p*-coumaric acid and *p*-coumaryl-CoA (Fig. 1). CoA ligation of (*E*)-cinnamic acid, rather than *p*-coumaric acid, has been demonstrated in several plant tissues catalyzed by specific (*E*)-cinnamic acid CoA-ligase (CNL) enzymes (Gaid et al., 2012; Klempien et al., 2012; Lee et al., 2012). Yet, the role of the PPP in the generation of melon fruit volatiles has not been examined in detail. Genes and enzymes responsible for the last steps of the biosynthesis of the phenylpropenes eugenol (and its derivatives) and estragole, in strawberry and apple, have been characterized recently (Atkinson, 2016). Still, the initial biosynthetic steps of the PPP volatiles in fleshy fruits have received little attention.

Previous studies have indicated that L-phe can be converted into phenylpyruvate by an aromatic amino acid aminotransferase (*CmArAT1*) that is expressed and active in melon fruit tissues (Gonda et al., 2010). CmArAT1 preferentially utilizes *p*-hydroxyphenylpyruvate as an amine acceptor generating L-tyrosine, similarly to the Arabidopsis orthologue (Yoo et al., 2013). The phenylpyruvate generated probably undergoes decarboxylation to phenylacetaldehyde by a yet unidentified decarboxylase and is then converted to phenethyl alcohol and its esters. Conversely, in tomato fruit L-phe follows a decarboxylation-first pathway, generating 2-phenethylamine followed by deamination to generate phenylacetaldehyde (Tieman et al., 2006) in a distinct route as compared to melons. Interestingly, in other plant tissues such as petunia and rose petals, a phenylacetaldehyde synthase enzymes convert L-phe into phenylacetaldehyde in one catalytic step (Farhi et al., 2010; Kaminaga et al., 2006). It is presently unknown if melon fruits solely utilize the deamination-first pathway or may utilize other L-phe conversion pathways for the biosynthesis of aromatic volatiles.

We show here the differential incorporation of isotopically labeled L-phe and (*E*)-cinnamic acid into different volatile compounds demonstrating that parallel metabolic roadways are active in melon for the formation of fruit volatiles. We additionally document the ability of melon fruit tissues to support the enzymatic production of (*E*)-cinnamaldehyde from (*E*)-cinnamic acid in cell-free extracts. An existing transcriptomic and metabolic data infrastructure based on a recombinant inbred line (RIL) melon population was used for gene mining by correlation-based-analyses to facilitate the discovery and characterization of two melon genes encoding enzymes involved in the biosynthesis of L-phe-derived aroma volatiles. They include *CmCNL* which encodes an enzyme that ligates (*E*)-cinnamic acid and CoA to form cinnamoyl-CoA *en route* to cinnamyl acetate, and *CmBAMT* which encodes a protein that generates methyl benzoate from benzoic acid using SAM as a methyl donor.

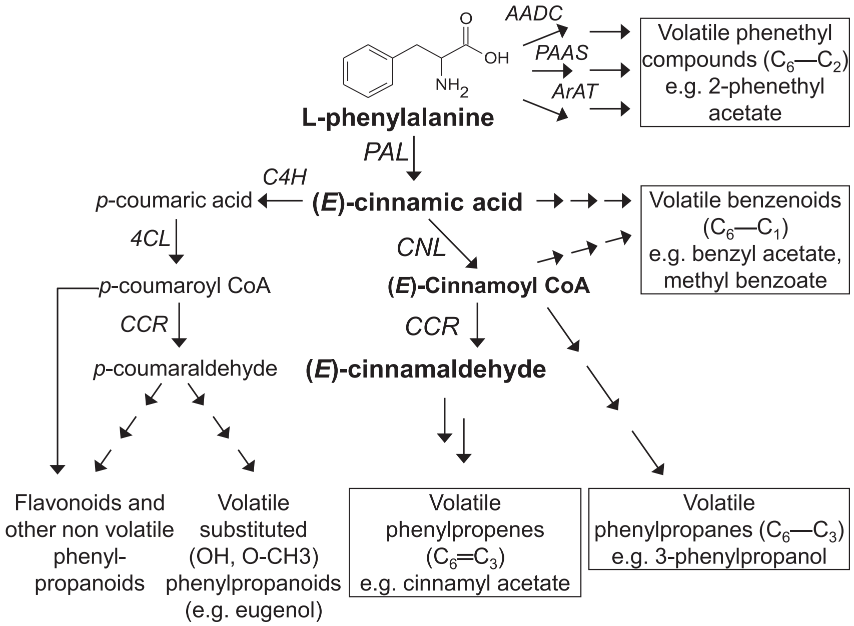

Fig. 1. Conversion of L-phenylalanine to various phenolic volatiles and other phenylpropanoids in fruits. Enzyme names are shown in italics. Boxes represent volatile groups found in melon fruit. PAL: phenylalanine ammonia lyase; CNL: (*E*)-cinnamic acid CoA ligase; CCR: cinnamoyl CoA reductase; C4H - (*E*)-cinnamic acid 4-hydroxylase; 4CL - *p*-coumaric acid CoA ligase; CoA – coenzyme A; ArAT – aromatic amino acid aminotransferase; AADC - aromatic amino acid decarboxylase; PAAS – phenylacetaldehyde synthase.