Title Branched-chain ester biosynthesis in ripening apple fruit

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Abstract

In apple fruit, aroma is an essential element of organoleptic quality and it can suffer in response to a number of pre- and post-harvest cultural treatments. Of the several classes of odor-active compounds, esters are the most important, but little is known regarding pathways of biosynthesis. This research presents evidence for a 'new' pathway for ester biosynthesis in apple that uses the starting products pyruvate and acetyl-CoA for the synthesis of precursors to branched-chain (BC) and certain short, straight-chain (SC) esters. The initial step in the pathway involves the formation of citramalic acid from pyruvate and acetyl-CoA by citramalate synthase (CIM). Citramalic acid then provides for the formation of α-keto-β-methylvalerate and its tramsaminated product isoleucine via α-ketobutyrate, and also for the BC ester precursors 2-methylbutanol or 2-methylbutanoate. The hypothesized pathway also provides for the formation of 3-, 4-, and 5-carbon fatty acids via the process of single-carbon elongation of α-keto acids, which are metabolized to short-chain fatty acids. These short-chain fatty acids are proposed to contribute to SC ester formation. Analysis of ripening fruit revealed that citramalic acid increased about 120-fold as ester production increased during ripening. At the same time, the content of isoleucine increased more than 20-fold, while other amino acids remained steady or declined. Apple disc feeding studies documented the incorporation of ^{13}C-labeled acetate into citramalic acid and isoleucine and into esters derived from 2-methylbutanoate, 2-methylbutanol, propanoate, and butanoate, supporting the hypothesized pathway. Furthermore, two novel genes were identified from apple, the sequence of which suggests that they are members of the 2-isopropylmalate synthase (*IPMS*) gene family. Purified His-tag protein from these genes was found to form citramalate and 2-ethylmalate from the α-keto acids pyruvate and α-ketobutyrate, respectively, when acetyl-CoA was added. Substrate specificity for α-keto acids in decreasing order was α-ketobutyrate, pyruvate, and α-ketovalerate and is characteristic of CIM. Therefore, the two genes (*MdCIM1* and *MdCIM2*) are apparently alleles coding for CIM, which initiates carbon flux into esters via the 'citramalate pathway'. The sequence of MdCIM1 differed from that of MdCIM2 by two amino acids, yet MdCIM2 was essentially non-functional, possessing only a fraction of

the activity of MdCIM1. The hypothesized pathway constitutes a conceptual shift in the regulation of ester biosynthesis in that it implies synthetic, rather than catabolic pathways are responsible for ester precursor supply.