Fruit Flavor Formation in Wild and Cultivated Strawberry

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Abstract

In recent years we have used various genomics tools to investigate ripening in strawberry, in particular the process of fruit flavor biogenesis. The combination of biochemical analysis, generation of a strawberry Expressed Sequence Tags (EST) collection and gene expression analysis using cDNA microarrays resulted in the identification of several genes playing a key role in the formation of volatile flavors during strawberry fruit ripening. Genes associated with the biosynthesis of lipid-derived flavors, such as esters, and those involved in the formation of mono- and sesquiterpenes have been isolated. In this report we summarize the main results obtained by functional characterization of the genes identified. Moreover, differences in volatile profiles between the wild, diploid strawberry and the cultivated, octaploid strawberry allowed us to obtain insight into the molecular processes which resulted in the formation of a certain flavor component and the loss of another.

INTRODUCTION

The flavor and aroma of fruit is determined by a complex mixture of hundreds of compounds. For example, more than 300 compounds contribute in different levels to the characteristic flavor associated with ripe strawberry fruit. They include metabolites derived from several chemical classes, including acids, aldehydes, ketones, alcohols, esters, sulfur compounds, furans, phenols, terpenes, epoxides and lactones. Their concentration will often vary among the different fruit tissues and will represent 10 to 100 ppm of the total fruit material. They are generally formed when metabolism of the fruit changes to catabolism in which high molecular weight structures such as proteins, polysaccharides and lipids are converted to ripening metabolites. Early research on fruit flavors focused on identifying components present in the different fruit species. A second objective was to characterise which volatiles were key elements conveying the characteristic odor unique to a particular fruit. Later, researchers began investigating their biogenesis and the effect that processing and storage imposed on them. In recent years, additional information on fruit flavors and their biosynthesis was obtained by using molecular approaches. A few genes which directly influence fruit flavor formation have been described. They include the isolation and characterisation of the alcohol acyltransferases from ripe strawberry and melon fruit (*SAAT*, Aharoni et al., 2000; *CMAAT*, Yahyaoui et al., 2002), an *O*-methyltransferase from strawberry (*FaOMT*, Wein et al., 2002) and the functional characterisation of four terpene synthases from lemon peel (Lucker et al., 2002).

In this study we further characterised genes involved in ester-formation in various fruit species including *VAAT*, the putative orthologue of *SAAT* from the wild strawberry. Interestingly, despite their extensive similarity in sequence, the recombinant SAAT and VAAT enzymes show a marked difference in terms of substrate preference. Genes encoding enzymes which catalyse mono- and sesquiterpene biosynthesis have also been cloned from both the wild and cultivated strawberry. Comparative studies between the wild and the cultivated strawberry volatile profiles, genes and recombinant enzymes provided the first details on how these components are formed and the reasons behind their differential production in the two strawberry genotypes.

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RESULTS AND DISCUSSION

Characterisation of Genes Associated with Biosynthesis of Lipid Derived Flavors

Lipid and amino acid derived flavor compounds are of great importance to the flavor of fruit. A major class of lipid derived compounds in strawberry as well as in other fruit are esters. Esters are generated by alcohol acyltransferases, using alcohol and acyl CoA substrates. We have previously identified and characterised the *SAAT* gene which encodes the enzyme catalysing the esterification reaction in the cultivated strawberry (*Fragaria* x *ananassa*; Aharoni et al., 2000).

To extend this analysis, we have searched for genes encoding ester-forming enzymes in a variety of other fruit species (Beekwilder et al., 2004). Most soft fruit species produce volatile esters during ripening and in some cases specific esters will have major influence on the typical flavor associated with a certain fruit (e.g. in banana, apple and melon). Some fruit species have been reported to generate more than 100 ester types during ripening, as for example strawberry. However, each of these fruits has a markedly different spectrum of esters and this prompt us to examine what factors determine the difference in ester profiles between fruit. We therefore cloned and characterised putative orthologous of the *SAAT* gene from several other fruit species, including the wild strawberry (*Fragaria vesca*), banana and lemon and compared their activity.

The activity of the recombinantly produced ester-forming enzymes on a large range of substrates was analysed, and aligned to the ester-profile in the headspace of the fruits. It was observed that there are clear differences among the enzymes from different source, but there is no clear relation between enzyme preference and the ester profile of the corresponding fruit. Interestingly, the ester-forming enzyme from wild strawberry (VAAT) showed an extremely high homology to SAAT in terms of sequence, but had a markedly different preference for alcohol and CoA substrates.

We further analysed the role of enzyme-availability vs. substrate availability in the formation of esters in vivo, by using transgenic petunia plants. Petunia plants constitutively expressing the *SAAT* gene under the control of the 35S CaMV promoter were generated. Upon analysis of transgenic petunia leaves and flowers, no difference in emitted volatiles could be observed. However, when alcohols were supplied as an external substrate, *SAAT*-expressing petunia leaves could produce several esters, while non-transformed control plants could not. These findings highlight the importance of basic catabolic processes, which influence the availability of flavor precursors during ripening. Our data suggest that substrate availability is a crucial factor determining fruit flavor composition and more specifically the ester profile of a given fruit species.

Characterisation of Genes Associated with Biosynthesis of Terpene Flavors

A remarkable difference exists between the fruit of the diploid wild species and the modern, cultivated species not only in terms of fruit size and yield but also in their flavor and aroma profile. We noticed a remarkable difference in terpene composition between the wild strawberry species and the cultivated ones (Fig. 1a). While the terpenoid profile of cultivated strawberry varieties is dominated by the monoterpene linalool and the sesquiterpene nerolidol, fruit of wild strawberry species emit mainly olefinic monoterpenes, myrtenyl acetate and myrtenol which are not detected in the cultivated species. The mechanism by which fruit flavors are gained and lost during evolution and domestication are largely unknown. Through cDNA microarray analysis we identified the *FaNES1* gene in the cultivated strawberry and showed that the recombinant FaNES1 enzyme produced in *Escherichia coli* cells is capable of generating both linalool and nerolidol when supplied with geranyl diphosphate (GDP) or farnesyl diphosphate (FDP), respectively (Fig. 1b). The same terpene synthase enzyme (Tps) was also capable of generating linalool and nerolidol when overexpressed in transgenic Arabidopsis and potato plants (Aharoni et al., 2003).

Characterization of additional genes, that are very similar to *FaNES1* from both the wild and cultivated strawberry species (*FaNES2* and *FvNES1*), showed that *FaNES1*

is exclusively present and highly expressed in the cultivated (octaploid) varieties and encodes a protein truncated at its N-terminus. Extensive green fluorescent protein localization experiments suggested that through a change in subcellular localization the FaNES1 enzyme encountered both GDP and FDP, allowing it to produce linalool and nerolidol. The change in localization of the protein was coupled to changes in steady state mRNA levels of the corresponding transcript. Conversely, an insertion mutation affected the expression of a different terpene synthase from the cultivated species (termed *FaPINS*) which encodes an enzyme capable of catalyzing the biosynthesis of the typical wild species monoterpenes and caused the loss of these compounds in the cultivated strawberries. The findings uncover molecular evolutionary mechanisms used by plants to generate diversity in flavors, which may be specifically selected for in domesticated species.

Flavor compounds in similarity to most other secondary metabolites produced by plants are further modified by for example hydroxylation, glycosylation and methylation. The loss of one flavor component influences the fruit flavor profile by the fact that it is no longer available as substrate for such modifications. This phenomenon was demonstrated by the cloning and characterization of a cytochrome P450 gene (*PINH)*, encoding the enzyme catalyzing the C10 hydroxylation of the monoterpene α-pinene to myrtenol, a typical wild strawberry flavor compound. In the wild strawberry, only low levels of myrtenol could be detected most probably due to conversion by a putative alcohol acyltransferase (such as SAAT and VAAT) to its corresponding acetate ester. High levels of myrtenyl acetate could indeed be detected in the profile of wild strawberry (Fig. 1a).

In conclusion, the cloning and characterisation of a series of genes from different pathways associated with fruit flavor in strawberry provided us with the basic information needed for understanding the complex process of flavor formation in ripening fruit.

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Figures

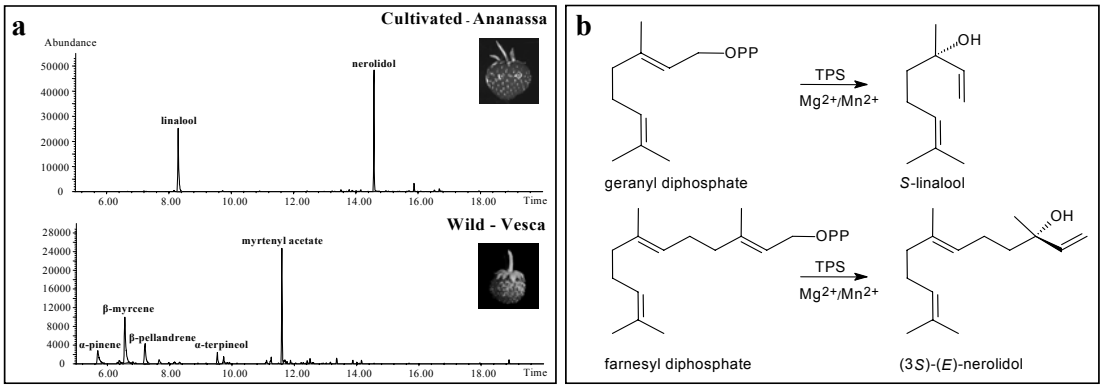

Fig. 1. Terpenoid production in cultivated and wild strawberry during ripening.
a. Terpenoids detected by headspace analysis of ripe fruits. Low levels of the monoterpene alcohol myrtenol (not depicted here) could be detected in the wild species; b. Reactions catalysed by terpene synthases (TPS) for the formation of the monoterpene alcohol linalool and the sesquiterpene alcohol nerolidol.