Volatile esters are flavor components of the majority of fruits. The last step in their biosynthesis is catalyzed by alcohol acyltransferases (AATs), which link alcohols to acyl moieties. Full-length cDNAs putatively encoding AATs were isolated from fruit of wild strawberry (Fragaria vesca) and banana (Musa sapientum) and compared to the previously isolated SAAT gene from the cultivated strawberry (Fragaria × ananassa). The potential role of these enzymes in fruit flavor formation was assessed. To this end, recombinant enzymes were produced in Escherichia coli, and their activities were analyzed for a variety of alcohol and acyl-CoA substrates. When the results of these activity assays were compared to a phylogenetic analysis of the various members of the acyltransferase family, it was clear that substrate preference could not be predicted on the basis of sequence similarity. In addition, the substrate preference of recombinant enzymes was not necessarily reflected in the representation of esters in the corresponding fruit volatile profiles. This suggests that the specific profile of a given fruit species is to a significant extent determined by the supply of precursors. To study the in planta activity of an alcohol acyltransferase and to assess the potential for metabolic engineering of ester production, we generated transgenic petunia (Petunia hybrida) plants overexpressing the SAAT gene. While the expression of SAAT and the activity of the corresponding enzyme were readily detected in transgenic plants, the volatile profile was found to be unaltered. Feeding of isoamyl alcohol to explants of transgenic lines resulted in the emission of the corresponding acetyl ester. This confirmed that the availability of alcohol substrates is an important parameter to consider when engineering volatile ester formation in plants.

Volatile esters are produced by virtually all soft fruit species during ripening. They play a dual role in the ripe fruit, serving both as "biological bribes" for the attraction of animals and as protectants against pathogens. In some fruits, like apple (Malus domestica), pear (Pyrus communis), and banana (Musa sapientum), esters are the major components in their characteristic aroma. In other fruits, like strawberry (Fragaria × ananassa), they contribute as notes to the blend of volatiles that constitute the aroma. Often, a single fruit emits a large spectrum of esters; for instance, more than 100 different esters have been detected in ripe strawberry fruit (Zabetakis and Holden, 1997). Most volatile esters have flavor characteristics described as fruity (Burdock, 2002). However, they are not only produced and emitted by fruit but also are part of floral scents (Dudareva et al., 1998b). They are very often emitted by vegetative parts of plants, either constitutively or in response to stress or insect infestation (Mattiacci et al., 2001). Esters therefore play a major role in the interaction between plants and their environment. Formation of volatile esters is not restricted to the plant kingdom and is also common in microorganisms such as yeast and fungi. For example, isoamyl acetate and ethyl acetate are produced during beer fermentation and are known to affect flavor quality (Verstrepen et al., 2003).

Alcohol acyltransferase (AAT) enzymes catalyze the last step in ester formation by transacylation from an acyl-CoA to an alcohol. Combinations between different alcohols and acyl-CoAs will result in the formation of a range of esters in different fruit species. The most likely precursors for the esters are lipids and amino acids. Their metabolism during ripening will therefore play an important role in determining both the levels and type of esters formed. For example, in strawberry, the amino acid Ala has been implicated in the formation of ethyl esters during ripening (Perez et al., 1992). However, levels of amino acid precursors could not explain in all cases the formation of the corresponding ester, and it has therefore been suggested that selectivity of enzymes preceding AATs in the biosynthetic pathway also plays a role in determining ester composition (Wyllie and Fellman, 2000). In addition, fatty acids, generated by the oxidative degradation of linoleic and linolenic acids, contribute to ester biosynthesis in fruit. Degradation of fatty acids results in the production of volatile aldehydes, which in turn are utilized by alcohol dehydrogenases to form alcohols. The latter are subsequently converted to hexyl and hexenyl esters (Perez et al., 1996; Shalit et al., 2001). Such a pathway has been demonstrated by Wang et al. (2001), who altered the levels of fatty acids in transgenic tomato (Lycopersicon esculentum) fruit, leading to dramatic changes in the aroma profile. Ripening related processes, such as changes in cell wall composition, might also contribute intermediates to ester biogenesis. In tomato, evidence was provided that
activity of pectin methyl-esterase (which catalyzes demethylation of pectins) regulates levels of methanol in the fruit (Frenkel et al., 1998). The accumulating methanol may then be utilized to generate methyl esters in ripe fruit.

Due to their key role in ester biosynthesis, the activity of AAT enzymes was the subject of several early investigations on extracts of various fruit species, including banana, strawberry, and melon (Cucumis melo; Wylie and Fellman, 2000; Shalit et al., 2001; Olias et al., 2002). In each of these studies, the AAT activity was shown to be ripening induced, and the substrate specificity of the AATs toward tested substrates (both to alcohols and acyl-CoAs) appeared to be broad. In recent years, fruit-expressed genes encoding enzymes with AAT activity have been isolated and characterized from strawberry and melon. Aharoni et al. (2000) utilized cDNA microarrays for gene expression profiling during strawberry fruit development and identified the \textit{SAAT} gene, which showed a strong induction upon ripening. Moreover, the recombinant SAAT enzyme could catalyze the formation of esters typically found in strawberry, using aliphatic, medium-chain alcohols (e.g. octanol) in combination with various chain length (up to C\textsubscript{10} tested) acyl-CoAs.

In this study, we characterize the substrate preference of fruit-expressed members of the BAHD family. We cloned full-length cDNAs for enzymes from wild strawberry (\textit{Fragaria vesca}) and banana and compared them to SAAT. Recombinant expression in \textit{Escherichia coli} and assays on their ability to use different alcohol substrates provided evidence for a role of these enzymes in the biosynthesis of esters that contribute to fruit flavor. Finally, we report the first attempt to use the \textit{SAAT} gene for metabolic engineering of plants, with the aim to alter the profile of emitted volatiles.

**RESULTS**

Further Characterization of the Cultivated Strawberry SAAT Enzyme

The activity of recombinant SAAT enzyme was tested using a range of additional substrates that had not been tested by Aharoni et al. (2000). Substrate preference of the purified recombinant enzyme was tested in assays using \textsuperscript{14}C-labeled acyl-CoAs as cosubstrates for a range of alcohol substrates. Substrate preference is used here as a measure to compare the activity of an enzyme for different substrates at a fixed substrate concentration. In these tests, geraniol proved to be an excellent substrate. The activity with geraniol exceeded that with octanol (77\% of activity with geraniol), the best substrate in previous tests (Table I). Other alcohols including terpene alcohols (nerol, perilla alcohol, carveol, and farnesol) and aromatic alcohols (cinnamyl alcohol and eugenol) were also tested. The activity of SAAT with nerol (54\%) and perilla alcohol (45\%) is significant, while the other substrates were only poorly used (Table I). The activity of SAAT with butyryl- and hexanoyl-CoA in combination with a subset of alcohols was also studied (Table II). With the larger acyl-CoA cosubstrates, SAAT again preferred geraniol and C\textsubscript{6} to C\textsubscript{9} aliphatic alcohols as substrates. However, relatively higher activity was observed with butanol and hexanol in combination with butyryl- and hexanoyl-CoA (81\% and 76\%, respectively), compared to acetyl-CoA (11\%), while decanol is less preferred in combination with the higher CoAs. This shows that SAAT can use a surprising diversity of acyl-CoAs and alcohols, including terpene alcohols, in addition to the aliphatic alcohols that are usually available in fruit.
Table 1. Substrate specificity of the SAAT, VAAT, and BanAAT recombinant enzymes toward different types of alcohols
Comparison of esterification activity of each of the enzymes with different alcohols (20 mM) and 14C acetyl-CoA (0.1 mM) as acyl donor.

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>SAAT</th>
<th>VAAT</th>
<th>BanAAT</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>5 ± 2e</td>
<td>11 ± 0.6</td>
<td>0.3 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>3 ± 2e</td>
<td>11 ± 0.9</td>
<td>0.1 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>1-Propanol</td>
<td>12 ± 2c</td>
<td>18 ± 0.1</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>2-Propanol</td>
<td>6 ± 1e</td>
<td>1 ± 0.1</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>1-Butanol</td>
<td>11 ± 1e</td>
<td>38 ± 0.1</td>
<td>1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>2-Butanol</td>
<td>15 ± 0.1</td>
<td>1 ± 0.1</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>Isoamylalcohol</td>
<td>17 ± 1d</td>
<td>21 ± 0.1</td>
<td>1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>1-Hexanol</td>
<td>40 ± 2c</td>
<td>39 ± 0.1</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>cis-2-hexen-1-ol</td>
<td>28 ± 3d</td>
<td>39 ± 0.0</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>cis-3-hexen-1-ol</td>
<td>19 ± 1d</td>
<td>45 ± 0.0</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>trans-2-hexen-1-ol</td>
<td>43 ± 4d</td>
<td>100 ± 0.7</td>
<td>31 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>trans-3-hexen-1-ol</td>
<td>6 ± 0.4d</td>
<td>23 ± 0.0</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>1-Heptanol</td>
<td>70 ± 25d</td>
<td>14 ± 0.6</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>1-Octanol</td>
<td>77 ± 16e</td>
<td>6 ± 0.0</td>
<td>44 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>1-Nonanol</td>
<td>66 ± 1d</td>
<td>3 ± 0.1</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>1-Decanol</td>
<td>37 ± 1d</td>
<td>1 ± 0.1</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>Furfuryl alcohol</td>
<td>3 ± 0.4d</td>
<td>9 ± 0.0</td>
<td>0.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Benzyllalcohol</td>
<td>3 ± 0.2d</td>
<td>11 ± 0.1</td>
<td>2 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>2-Phenyl-ethylalcohol</td>
<td>7 ± 1d</td>
<td>12 ± 0.4</td>
<td>1 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>Linalool</td>
<td>1 ± 0.0e</td>
<td>1 ± 0.9</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>Geraniol</td>
<td>100 ± 6</td>
<td>13 ± 0.0</td>
<td>88 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Nerol</td>
<td>54 ± 2</td>
<td>3 ± 0.1</td>
<td>46 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Carveol</td>
<td>2 ± 0.1</td>
<td>2 ± 0.0</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>Eugenol</td>
<td>7 ± 0.0</td>
<td>7 ± 0.7</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>Farnesol</td>
<td>11 ± 1.0</td>
<td>2 ± 0.4</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>Perilla alcohol</td>
<td>45 ± 2</td>
<td>8 ± 0.1</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>2-Ethoxyethanol</td>
<td>2 ± 0.3</td>
<td>4 ± 0.1</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>Cinnamyl alcohol</td>
<td>10 ± 2</td>
<td>9 ± 0.1</td>
<td>100 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

*aThe added alcohol. bMolecular structure of the alcohol added. cActivity calculated as nanomoles product formed per hour per microgram protein. The highest activity for each enzyme was set at 100%, and the numbers indicated signify the percentage of activity relative to the highest activity. For SAAT, 100% was 3.0 nmol substrate h⁻¹ µg protein⁻¹; for VAAT 100% activity was 1.3 nmol substrate h⁻¹ µg protein⁻¹; for BanAAT 100% activity was 3.4 nmol substrate h⁻¹ µg protein⁻¹. dValues have been calculated from data published previously (Aharoni et al., 2000). eValues have been calculated from previously published data and have been confirmed independently in the same experiment from which the novel data have originated. fNot tested.
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Table II. Substrate specificity of the SAAT recombinant enzyme for different types of alcohols in combination with acetyl-CoA, butyryl-CoA, and hexanoyl-CoA

Comparison of esterification activity of each of the enzymes with different alcohols (20 mM) and 14C acetyl-CoA, butyryl-CoA, or hexanoyl-CoA (0.1 mM) as acyl donor.

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Acetyl-CoA</th>
<th>Butyryl-CoA</th>
<th>Hexanoyl-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Butanol</td>
<td>11 ± 2*</td>
<td>81 ± 11</td>
<td>67 ± 12</td>
</tr>
<tr>
<td>2-Butanol</td>
<td>15 ± 1</td>
<td>75 ± 1</td>
<td>50 ± 5</td>
</tr>
<tr>
<td>1-Hexanol</td>
<td>40 ± 2</td>
<td>73 ± 23</td>
<td>65 ± 4</td>
</tr>
<tr>
<td>cis-2-hexenol</td>
<td>28 ± 2</td>
<td>66 ± 13</td>
<td>62 ± 2</td>
</tr>
<tr>
<td>cis-3-hexenol</td>
<td>19 ± 5</td>
<td>43 ± 5</td>
<td>n.t.(^b)</td>
</tr>
<tr>
<td>trans-2-hexenol</td>
<td>43 ± 4</td>
<td>81 ± 15</td>
<td>100 ± 12</td>
</tr>
<tr>
<td>1-Octanol</td>
<td>77 ± 16</td>
<td>100 ± 3</td>
<td>69 ± 3</td>
</tr>
<tr>
<td>1-Nonanol</td>
<td>66 ± 1</td>
<td>58 ± 3</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>1-Decanol</td>
<td>37 ± 1</td>
<td>6 ± 2</td>
<td>15 ± 6</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>3 ± 1</td>
<td>7 ± 8</td>
<td>14 ± 0.2</td>
</tr>
<tr>
<td>2-Phenylethyl alcohol</td>
<td>7 ± 1</td>
<td>6 ± 4</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>Geraniol</td>
<td>100 ± 6</td>
<td>89 ± 5</td>
<td>75 ± 3</td>
</tr>
</tbody>
</table>

*Activity calculated as nanomoles product formed per hour per microgram protein. The highest activity for each enzyme was set at 100%, and the numbers indicated signify the percentage of activity relative to the highest activity. \(^b\) n.t., Not tested.

Characterization of the Wild Strawberry (VAAT) and Banana (BanAAT) Alcohol Acyl Transferases

Acyl transferase genes related to the SAAT gene from strawberry were identified in a number of fruit species, as described in the supplemental data (available at www.plantphysiol.org). From this collection, a gene from wild strawberry (termed VAAT) and from banana (termed BanAAT) were selected for further analysis. An alignment of the amino acid sequences encoded by the cDNAs of SAAT, VAAT, and BanAAT is shown in Figure 1A.

The esters produced in wild and cultivated strawberries are comparable (Pyysalo et al., 1979). Therefore, it was of interest to compare the SAAT enzyme to its closest ortholog, VAAT, in more detail. First, sequence comparison (Fig. 1A) reveals that the sequences of both enzymes are closely related (86% identical). Diversity is concentrated in two regions in particular, around positions 60 and 430.

Also the substrate preference of the recombinant VAAT and SAAT enzymes were compared (Table I). As reported before (Aharoni et al., 2000), the SAAT enzyme has a preference for C6 to C10 aliphatic alcohols. For VAAT, the optimal size of aliphatic alcohols was clearly smaller than for SAAT; only C6 alcohols (23%–100%) and n-butyl alcohol (38%) were good substrates, and methyl and ethyl alcohols (11%), geraniol (13%), phenylenethyl alcohol (12%), and benzyl alcohol (11%) were used to limited extent. Longer aliphatic alcohols like octanol, which are favored by SAAT (77%), were hardly used at all by VAAT (6%). So, although there is an overlap between both enzymes for C6 alcohols, SAAT and VAAT have shifted preferences.

The \( K_m \) and \( V_{max} \) values for octanol and acetyl-CoA were determined for VAAT to compare these to the published values of SAAT (Aharoni et al., 2000). The \( K_m \) value of VAAT for octanol, using 10 mM acetyl-CoA as a cosubstrate, is 0.6 mm (\( V_{max} = 89 \text{ nmol h}^{-1} \text{ µg} \text{ protein}^{-1} \)), which is about 10-fold lower than that of SAAT (\( K_m = 5.6 \text{ mM} \); \( V_{max} = 26 \text{ nmol h}^{-1} \text{ µg}^{-1} \)). The catalytic efficiency of VAAT with octanol (expressed as \( k_{cat}/K_m \)) is \( 1.0 \times 10^3 \text{ M}^{-1} \text{ s}^{-1} \), which is considerably higher than that of SAAT (32 M\(^{-1}\) s\(^{-1}\)). The \( K_m \) value for acetyl-CoA (using 20 mM octanol as cosubstrate) is dramatically (200-fold) higher for VAAT (\( K_m = 2 \text{ mM} \); \( V_{max} = 350 \text{ nmol h}^{-1} \text{ µg}^{-1} \)) than for SAAT (\( K_m = 0.01 \text{ mM} \); \( V_{max} = 45 \text{ nmol h}^{-1} \text{ µg}^{-1} \)). The catalytic efficiency of SAAT with acetyl-CoA (3.1 \( \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \)) is about 20-fold higher than VAAT (1.2 \( \times 10^3 \text{ M}^{-1} \text{ s}^{-1} \)).

Recombinant expression in \( E. \) coli was also achieved for BanAAT, and activity of the produced enzyme could be tested. In Table I, results with a limited set of alcohol substrates in combination with acetyl-CoA are compared to those obtained with SAAT and VAAT. The BanAAT enzyme showed an activity profile similar to SAAT, as it used geraniol, nerol, and the C6 and C8 alcohols quite efficiently. Cinnamyl alcohol was the best substrate for BanAAT, while the SAAT and VAAT enzymes were only very modestly active with this substrate.

Expression of SAAT in Transgenic Petunia

The activity of SAAT was also studied in planta using petunia plants. Leaves of petunia do not emit any esters, while flowers emit exclusively esters originating from benzyl-alcohol and methanol, in addition to nonester aromatic compounds (Verdonk et al., 2003). Plants were transformed with a cDNA encoding the strawberry SAAT gene under control of the double 35S-cauliflower mosaic virus promoter. All regenerated plants were screened for the presence of the SAAT gene by PCR. PCR-positive plants were transferred to greenhouse conditions for further examination. Transgenic plants exhibited normal development, compared to nontransformed and empty-vector control plants, which went through the same regeneration process.

Total RNA was extracted from young leaves of two nontransformed plants, three plants transformed with an empty binary vector, and six plants transformed with SAAT. Expression of the SAAT transcript was analyzed by northern-blot analysis (Fig. 2). All analyzed SAAT-transformed plants expressed the gene, but clear differences in expression level were observed. Plants S216A and S218A (low expressers) and S152A and S157A (high expressers) were selected for further analysis. T1-generation transgenic plants were self-fertilized, and the T2 progeny was again analyzed by northern-blot analysis (Fig. 2). As expected, clear differences in expression level were observed. From each line, three PCR-positive plants were selected and compared to three nontransformed plants and three plants transformed with vector pBinPLUS without an insert.
Figure 1. (Legend appears on following page.)
In Vitro Enzyme Activity of SAAT Produced by Petunia

Alcohol acyltransferase activity in leaves of the plants was analyzed in vitro, using acetyl-CoA and geraniol as substrates. On three different days, extracts from leaf material were assayed by adding geraniol and acetyl-CoA. Formation of geranyl acetate was analyzed using gas chromatography-mass spectrometry (GC-MS; Fig. 3). When the SAAT gene was not present (Control and pBinPLUS lines), hardly any geranyl acetate could be detected (Fig. 3A), and plants with low SAAT-RNA levels (S216A and S218A) produced no significant increase in the amount of geranyl acetate. High-expressing plants (S152A and S157A) produced 10 to 40 times more geranyl acetate than low-expressers and control plants (Fig. 3B, peak 3; Fig. 4A). It was concluded that SAAT enzyme was expressed in active form in these plants.

The Volatile Profile of Petunia Plants Producing SAAT

As a next step, the in vivo effect of the presence of the SAAT gene on volatile release of petunia was tested. The headspace volatiles were measured in flowers of all six lines (S157A, S152A, S218, S216, pBinPLUS, and Control) for 2 d. No changes in the emission from flowers of endogenous petunia esters (benzyl benzoate and methyl benzoate; Verdonk et al., 2003) were observed, nor were any esters or other volatile compounds that were novel to petunia detected (data not shown). Also in green parts, no changes in the emitted volatiles could be observed (Fig. 5, A and B).

Activity of Petunia Expressed SAAT with Externally Supplied Alcohols

To test if external substrates could be used by intact plant material, experiments were set up feeding
alcohol as a substrate. Initially, geraniol was used, but quantitative comparison of these experiments was hampered by experimental problems. These problems are likely related to the poor solubility of geraniol, which at 6 mM could only be temporarily suspended in water. Plant parts that were in contact with the surface of the geraniol suspension showed severe necrosis, thus strongly affecting supply to the upper plant parts. When petunia leaves were fed with a solution of isoamyl alcohol (which is soluble in water at 6 mM), no necrosis was observed and much more reproducible results were obtained.

Explants of petunia plants expressing the SAAT enzyme produced abundant amounts of isoamyl acetate when fed with an isoamyl alcohol solution. This production depended on the presence of expressed SAAT enzyme (Peak 1, Fig. 5, C and D). In Figure 4B, results on isoamyl acetate production of all six lines recorded at three different days are presented. High expressers (S157A and S152A) reproducibly emitted about 10-fold more isoamyl acetate than control leaves or low-expressor leaves. The SAAT enzyme apparently finds no suitable alcohol substrate in petunia unless this is fed to the plant.

When feeding alcohols to the petunia flowers, no changes in the emission of endogenous petunia esters (benzyl benzoate and methyl benzoate; Verdonk et al., 2003) were observed (results not shown). Remarkably, in the leaf headspace, a few novel esters were detected when isoamyl alcohol was fed independently from the presence of the SAAT gene. The major esters (peaks 2 and 3, Fig. 5C) were identified as 3-methyl butanoyl 2-methyl butanoate and 3-methyl butanoyl 3-methyl butanoate (collectively referred to as isoamyl methylbutyrate), while a minor peak (peak 4, Fig. 5C) was identified as isoamyl benzoate.

**DISCUSSION**

The Role of BAHD Family Members in Fruit Flavor Formation

Esters are important for the flavor of a number of fruits (Morton and MacLeod, 1990). Wild and cultivated strawberries produce linear esters like ethyl hexanoate, octyl acetate, and hexyl butyrate, while banana flavor is dominated by isoamyl acetate and butyl acetate. Biosynthesis of esters in fruit is mediated by enzymes from the BAHD family. Two fruit-expressed genes (SAAT from strawberry and CM-AAT1 from melon) from this family have been earlier implicated in the biosynthesis of volatile esters (Aharoni et al., 2000; Yahyaoui et al., 2002). In this paper, two other genes that are related to the SAAT gene have been isolated from wild strawberry and banana. Several lines of evidence suggest involvement of these genes in the formation of fruit flavor.

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**Figure 2.** Northern-blot analysis of RNA samples derived from petunia plants. RNA was extracted from wild-type (Control 2 and Control 5) plants, plants transformed with empty vector pBinPLUS (pBIN24A, pBIN69A, and pBIN27A), and plants transformed with the pBIN-SAAT construct (S152A, S216A, S249A, S157A, S136A, and S218A) and analyzed for expression of the SAAT gene using amplified SAAT cDNA as a probe. In the last lane, an equal amount of strawberry RNA was loaded. The bottom section represents a control hybridization with a petunia ubiquitin probe.

**Figure 3.** In vitro formation of geranyl acetate by leaf extracts of wild-type petunia (A) and the SAAT expressing line S157A (B), when supplied with geraniol and acetyl-CoA. Depicted are GC-MS chromatograms of m/z 69. The 100% detector response corresponds to $2 \times 10^6$ ion counts. Major volatiles detected are marked with numbers: Peak 1 is geraniol; peak 2 is nerol, a contaminant of the geraniol sample; peak 3 is geranyl acetate.
As a first criterion, the expression pattern of the VAAT and BanAAT genes was considered, based on literature data. The VAAT gene (represented by clone 5.1.R2) was shown by RNA-gel blots of wild-strawberry fruit RNAs to be strongly induced in the transitions between green and breaker fruit and between breaker and red fruit (Nam et al., 1999). In a similar way, the BanAAT gene expression (represented by cDNA clone UU130) was reported to be upregulated in banana pulp after ripening was initiated with ethylene (Medina-Suarez et al., 1997).

As a second criterion, the sequence homology to known acyltransferases was considered. As is clear from the presence of conserved sequences in the genes, they are part of the BAHD family (St-Pierre and De Luca, 2000). Four groups within this family have been defined by Hoffmann et al. (2003), based on sequence homology. One group (A; Fig. 1B) comprises the Taxus acyltransferases involved in taxol biosynthesis, a second group (B) consists of the anthocyanin acyltransferases, a third group (C) of genes encodes enzymes with unrelated substrates, including SAAT, DAT, and BEAT, and a fourth group (D) comprises the hydroxycinnamoyltransferases. An additional fifth group, group E containing agamatine coumaroyltransferases, has been identified by Burhenne et al. (2003). Lastly, a group comprising the melon CM-AAT1 and wound-induced enzymes from C. brewerri (BEBT) and Arabidopsis (CHAT) can be identified as group F. In Figure 1B, the BanAAT and VAAT enzymes have been added.
Figure 5. Total volatile emission from petunia leaves. GC-MS chromatograms (detector response; 100% of $1.5 \times 10^6$ total ion counts) of head space volatiles released in vivo by petunia leaves over a period of 24 h. A, Leaves of wild-type petunia leaves feeding on water. B, Leaves of line S157A on water. C, Leaves of wild-type petunia on an aqueous 6.5 mM isoamyl alcohol solution. D, Leaves of line S157A on an aqueous 6.5 mM isoamyl alcohol solution. The major volatile compounds detected are marked with numbers: peak 1 is isoamyl acetate; peak 2 is isoamyl 2-methyl butyrate; peak 3 is isoamyl 3-methylbutyrate; and peak 4 is isoamyl benzoate.
to a phylogenetic tree including enzymes of the BAHD family with known activity and putative enzymes expressed in fruit (see supplemental data). The VAAT enzyme is very closely related to the SAAT enzyme and falls into group C. The BanAAT enzyme is exceptional and does not match any of the sequence groups.

As a third criterion, the ability of fruit-expressed acyltransferases to produce the esters found in the fruits they were obtained from was tested. This was done by expressing the isolated cDNAs in E. coli, and testing the ability of the recombinant and partially purified enzymes to form esters from a broad range of alcohols in combination with acetyl-CoA. The SAAT and VAAT enzymes are clearly able to produce a number of the esters found in both wild and cultivated strawberries, such as hexyl acetate and octyl acetate. This further supports a role for the SAAT and VAAT enzymes in fruit flavor formation.

Also for the BanAAT enzyme, our data provide evidence for a role in flavor formation in ripening banana fruit, as recombinant BanAAT produced the characteristic banana volatiles such as isoamyl acetate and butyl acetate. However, these esters were made with considerably lower efficiency than octyl acetate, cinnamoyl acetate, and geranyl acetate (Table I). An in situ analysis of AAT activity in banana fruit also showed a preference for C6 alcohols over C3 and C4 alcohols, as is the case for recombinant BanAAT, while longer alcohols like octanol were not tested (Wyllie and Fellman, 2000). This suggests that BanAAT corresponds to the major enzyme responsible for ester formation in banana fruit. Its low efficiency for synthesizing isoamyl acetate could explain the presence of significant amounts of isoamyl alcohol (in addition to isoamyl acetate) in ripe banana volatiles (Shiota, 1993), which could be important for its volatile blend. The expression profile and substrate usage of the banana and wild strawberry enzymes point to a role for these enzymes in fruit flavor formation. However, definite proof for this role has to come from reverse genetics strategies, which should show that fruits carrying knockout mutations or antisense-expressing constructs of BAHD genes have clearly reduced emission of esters.

A Comparison of Substrate Preferences of Related and Unrelated Enzymes

A comparison of substrate preference can be made between the SAAT, VAAT, and BanAAT enzymes and related enzymes known from the literature. The closest relative to SAAT for which activity measurements have been published is the rose alcohol acyl transferase (RhAAT; Shalit et al., 2003). The substrate preference of RhAAT is quite similar to that of SAAT. Both RhAAT and SAAT prefer geraniol in combination with acetyl-CoA (Table II). For RhAAT, this preference very likely relates to the presence of geranyl acetate in rose flower scent. For SAAT, there is no obvious relevance for this preference to the formation of strawberry fruit flavor, since geranyl acetate has not been reported to occur in ripe strawberries. VAAT is even closer related to SAAT than RhAAT, but its activity is quite distinct from both enzymes. The VAAT enzyme hardly accepts octanol, nonanol, or decanol as substrates in combination with acetyl-CoA, although geraniol is accepted. VAAT is much more active on short alcohol substrates (C4 to C6, but also ethanol and methanol), which are not preferred by SAAT, at least in combination with acetyl-CoA (Table I). These observations are in agreement with the substrate preferences reported for enzyme extracts of wild and cultivated strawberry fruits (Olias et al., 2002). Remarkably, the requirements of SAAT with respect to alcohols combined with butyryl- and hexanoyl-CoA are less stringent (Table II). Butanol, for instance, is hardly used with acetyl-CoA, while it is readily accepted with butyryl-CoA and hexanoyl-CoA. This is particularly relevant since butyrylbutyrate and butylhexanoate have been identified in strawberries (Zabetakis and Holden, 1997).

It is difficult to compare absolute kinetic parameters for these enzymes in a way relevant for the in vivo situation. The catalytic efficiency of SAAT for octanol is much (30 times) lower than that of VAAT. On the other hand, the substrate preference data (Table I) indicate that SAAT turns over 2 nmol octanol per hour per μg protein, while VAAT turns over only 0.078 nmol octanol per hour per μg protein, suggesting that SAAT is 25-fold more efficient for this substrate. These seemingly contradictory observations can be explained by the difference in affinity for the second substrate, acetyl-CoA, of both enzymes. Substrate preference measurements were carried out at 0.1 mM acetyl-CoA, which ensures saturated substrate binding by SAAT (10 times higher than K_m, which is 0.01 mM), but not by VAAT (20 times lower than K_m, which is 2 mM). Obviously, this has strong impact on the observed substrate preferences. The in vivo concentration of acetyl-CoA in strawberry fruit is not known, but for green leaves it has been reported to range from 0.05 to 1.4 mM, depending on the number of chloroplasts present (Bao et al., 2000). In view of the low amount of plastids in strawberry fruit, the acetyl-CoA concentration in this fruit is probably at the lower end of this range, which is why it was estimated that a concentration of 0.1 mM acetyl-CoA in the substrate preference assay was appropriate.

The banana enzyme BanAAT is only distantly related to the SAAT and VAAT; members of group C of the BAHD family (Fig. 1, A and B). However, BanAAT shares most enzymatic properties with SAAT and RhAAT (Shalit et al., 2003) and prefers octanol and geraniol. The VAAT preferences are more related to those of CM-AAT1 (Yahyaoui et al., 2002), which does not accept nonanol, and BEAT and BEBT (Dudareva et al., 1998a; D’Auria et al., 2002), which produce benzyl acetate. However, these enzymes belong to group F, while VAAT belongs to group C. These cases...
illustrate that predicting substrate usage based on sequence grouping remains difficult. A similar lack of relationship between primary structure and enzymatic properties has recently been reported for the hydroxycinnamoyltransferases (Burhenne et al., 2003).

Pichersky and Gang (2000) have suggested a special form of convergent evolution in which new enzymes with the same function evolve independently in separate plant lineages from a shared pool of related enzymes with similar but not identical functions. Such a situation seems to be the case with the fruit AATs that are involved in the formation of volatiles. There is a strong overlap in substrate specificity of unrelated members of the BAHD gene family (for instance SAAT and BanAAT) and differences in specificity between closely related members such as SAAT and VAAT.

**Engineering of Volatile Esters in Plants**

The engineering of volatile emission from plants has been described mainly for terpenoids. Lewinsonh et al. (2001) transformed a tomato variety, with a low endogenous level of linalool in the fruit, with C. brevleri linalool synthase under control of the fruit-specific E8 promoter. This resulted in fruit with strongly elevated levels of S-linalool. The same gene was used to introduce the emission of linalool from carnation flowers (Lavy et al., 2002). Interestingly, when Lucker et al. (2001) attempted to realize linalool emission from petunia, a plant that does not normally emit significant amounts of terpenes, hardly any change in volatile emission was observed. Overexpressing the C. brevleri linalool synthase resulted instead in accumulation of linalool to significant amounts in the form of a nonvolatile glucopyranoside conjugate. Thus, endogenous enzymes in petunia seem to sequester the volatile linalool as a nonvolatile conjugate. Leaves of Arabidopsis plants constitutively expressing a linalool synthase from strawberry did produce linalool but also its glycosylated and hydroxylated derivatives (Aharoni et al., 2003).

The pathway eventually leading to ester formation has been engineered by Speirs et al. (1998). By overexpressing alcohol dehydrogenase in tomato fruit, levels of hexanol and cis-3-hexenol were increased, at the expense of aldehydes. These fruits were recognized by trained taste panels as having a more intense ripe fruit flavor. By expressing SAAT in petunia, we aimed to engineer the emission of esters, which could convey a fruity aroma to the plant. However, as in the case of engineering linalool production in petunia, engineering emission of volatile esters in plants appears not to be trivial. Both expression of SAAT and the activity of its corresponding enzyme could be detected in the transgenic plants generated, and activity was found to be inherited in the T2 generation. However, no new esters could be detected in the headspace of transgenic lines, and no increase in the ester benzylibenzoate, which is normally emitted by wild-type petunia flowers, was observed. Feeding alcohols to petunia explants demonstrated that the bottle-neck for ester production was the availability of alcohols. Clearly, the success of engineering of ester formation will also depend heavily on substrate availability.

Our results indicate the presence of alcohol acyltransferase activity also in nontransgenic petunia plants. When fed with isoamyl alcohol, the wild-type plant converted part of the alcohol into isoamyl methylbutyrate and isoamyl benzoate (Fig. 4C). This strongly suggests that there is an alcohol acyltransferase active in the petunia background. The fact that benzyl benzoate has been found in considerable amounts in wild-type petunia flowers (Verdonk et al., 2003) also suggests the presence of such an endogenous enzyme. Very low levels of isoamyl acetate were found in wild-type plants when fed with isoamyl alcohol (Fig. 4C), indicating that the putative endogenous acyltransferase hardly uses acetyl-CoA, while it can use methylbutyryl-CoA and benzoyl-CoA. Probably both acetyl-CoA and benzoyl-CoA are available in petunia, and while the endogenous enzyme uses only benzoyl-CoA, the ectopic SAAT enzyme uses acetyl-CoA.

To engineer ester production into plants, it should be considered that a supply of alcohol substrate is required, either from an endogenous source or by introducing additional genes. The SAAT enzyme can take many different alcohol substrates, which are derived from several pathways. Linear alcohols like octanol could be provided by stimulating fatty acid metabolism, for instance by expressing lipoxygenase genes, or by amino acid catabolism. Terpene alcohols like geraniol could be provided by introducing a gerniol synthase (Iijima et al., 2004). However, by engineering the alcohol supply, other side-products may be induced. This is illustrated by our finding of previously undetected isoamyl methylbutyrate and isoamyl benzoate in the wild-type petunia headspace when feeding isoamyl alcohol (Fig. 5C). Engineering of ester formation in a background that already contains alcohols may avoid these problems. In fruits of tomato and strawberry, for instance, alcohols are quite abundantly available (Zabetakis and Holden, 1997; Speirs et al., 1998). Another possibility is to vary culturing conditions, for example by introducing stress, which will affect the concentration of alcohols (Mattiacci et al., 2001). Our findings parallel those described by Schwab (2003), who reviewed several reports on biochemical analysis of heterologously expressed enzymes where poor specificity was observed for enzymes involved in the production of secondary metabolites during fruit ripening, (e.g. in flavor and color formation). It was concluded that metabolome diversity is also caused by low enzyme specificity and directly related to availability of suitable substrates. Future work will therefore have to take into account the entire metabolic pathway, rather than a single enzyme, to engineer emission of novel volatiles in plants.
MATERIALS AND METHODS

Plant Material and Petunia Transformation

For cloning the full-length cDNAs, we used ripe fruit of wild strawberry (Fragaria vesca L., PR line 92189) and pulp of ripe banana (Musa sapientum). The petunia (Petunia hybrid (Vilm.) variety W115 was used for generating transgenic plants, which were grown at 18°C with 18/6 h light/dark conditions in the greenhouse.

RNA Isolation and RNA-Gel Blots

RNA isolation from the various fruit tissues was performed according to Aspil et al. (2000) and from leaves of petunia using the method of Vervoord et al. (1989). RNA-gel blots were generated (10 µg of either petunia leaf or ripe strawberry fruit total RNA) and hybridized as described previously (Aharoni et al., 2000). The SAAT gene probe (780-bp fragment) was generated by PCR using the oligonucleotides AAP157 5′-TCACATCAGTATGCTCC-3′ and AAP158 5′-GCTGGAGGTCAAGTACG-3′. The blot was rehybridized with a petunia cDNA probe showing homology to a gene encoding a ubiquitin protein (identified in a petunia expressed sequence tag library described in Aharoni et al., 2000) that was amplified by PCR from a pBlueScript vector (Stratagene, La Jolla, CA) using the T7 and T3 oligonucleotides.

SAAT Overexpression Construct and Plant Transformation

The entire SAAT coding region (1,393 bp) was amplified from pSETB (Aharoni et al., 2000) using oligonucleotides AAP165 (5′-CGATTCGCCGAGAAATATGACG-3′) and AAP166 (5′-CTGCAGACCTTGACGCCGACTAAATG-3′). The amplified fragment was cleaved with BglII and Sall restriction enzymes and inserted in a sense orientation into BamHI and Sall restriction sites, located between a double 35S-cauliflower mosaic virus promoter and a nopaline synthesize terminator, in the pFLAP10 subcloning vector (provided by A. Boyy, Plant Research International, Wageningen, The Netherlands). From the pFLAP10 vector, the fragment was excised with PacI and Ascl restriction digestive and introduced to the pBluesIPJ binary vector (Van Engelen et al., 1995) containing the kanamyic resistance selection marker (ntplII). Petunia was transformed with the LBA4404 Agrobacterium tumefaciens strain as described by Luckner et al. (2001). Fifteen lines rooting vigorously on media containing kanamycin (50 µg/mL) were transferred to the greenhouse. Plants transformed with the empty pBluesIPJ binary vector and nontransformed wild-type ones were also regenerated in a similar way in vitro on nonselective medium and utilized for the experiments.

Cloning Full-Length Genes, Sequencing, and Phylogenetic Analysis

To obtain full-length cDNAs we used the SMART RACE cDNA amplification kit (CLONTECH, Palo Alto, CA) according to the manufacturer instructions with slight modifications to the annealing temperatures (normally 5°C–10°C lower than recommended) or number of cycles (up to 35 cycles). PCR, restriction digests, plasmid DNA isolation, and gel electrophoresis were performed using standard protocols. All fragments were purified from the gel using the GFX purification kit (Amersham, Little Chalfont, UK). Cloning of PCR fragments was either performed using the PCR SCRIPT (Stratagene, La Jolla, CA) or pCR 4Blunt-TOPO (Invitrogen, Chalfont, UK). Cloning of PCR fragments was either performed using the 35 cycles). PCR, restriction digests, plasmid DNA isolation, and gel electrophoresis were performed using standard protocols. All fragments were purified from the gel using the GFX purification kit (Amersham, Little Chalfont, UK). Cloning of PCR fragments was either performed using the PCR SCRIPT (Stratagene, La Jolla, CA) or pCR 4Blunt-TOPO (Invitrogen, Chalfont, UK). Cloning of PCR fragments was either performed using the 35 cycles). PCR, restriction digests, plasmid DNA isolation, and gel electrophoresis were performed using standard protocols. All fragments were purified from the gel using the GFX purification kit (Amersham, Little Chalfont, UK). Cloning of PCR fragments was either performed using the PCR SCRIPT (Stratagene, La Jolla, CA) or pCR 4Blunt-TOPO (Invitrogen, Chalfont, UK). Cloning of PCR fragments was either performed using the PCR SCRIPT (Stratagene, La Jolla, CA) or pCR 4Blunt-TOPO (Invitrogen, Chalfont, UK). Cloning of PCR fragments was either performed using the PCR SCRIPT (Stratagene, La Jolla, CA) or pCR 4Blunt-TOPO (Invitrogen, Chalfont, UK). Cloning of PCR fragments was either performed using the PCR SCRIPT (Stratagene, La Jolla, CA) or pCR 4Blunt-TOPO (Invitrogen, Chalfont, UK).

Expression and Partial Purification of the Recombinant Proteins

A modified expression vector (pRSET B; Invitrogen) described earlier (Aharoni et al., 2000) was used for the expression of VAAT and SAAT in Escherichia coli cells (BL21 Gold DE3 strain; Stratagene). The oligonucleotides used for cloning to pRSET B introduced restriction sites at both ends of the fragments, which were compatible to the BamHI and SalI restriction sites in the vector. The oligonucleotides AAP165 and AAP167 (several above) introduced a BamHI and a SalI, respectively, to pIAAT; AAP232 (5′-CGATTCGCCGAGGTCAAGTACG-3′) and AAP227 (5′-ACTCTACCTCTCCCAAGTCT-3′) were introduced a BamHI and a XhoI, respectively, to pIAAT. To obtain an in-frame N-terminus fusion of the recombinant proteins to the His-tag, the A and T nucleotides of the ATG codon were removed, resulting in the elimination of the starting Met and insertion of a Pro in the protein sequences. Vectors pRSET-SAAT, pRSET-VAAT, and pRSET-BanAAAT were transformed in E. coli BL21 CodonPlus-RIL. Fresh overnight cultures were diluted 100-fold in 50 mL Luria-Bertani medium supplied with ampicillin (50 µg/mL) and Ck (1%) and grown until A600 was 0.6. Cells were recovered by centrifugation and resuspended in Luria-Bertani medium supplied with ampicillin and isopropylthio-β-galactoside (1 mM). After overnight growth at 16°C, cells were harvested by centrifugation and resuspended in 1 ml ice-cold buffer B (50 mM Tris-HCl, pH 8.0, and 10 mM 2-mercaptoethanol). Ten micromolar lysosome was added, and cells were sonicated on ice for 5 times 10 s, with 5 s breaks and an amplitude of 14 using an MSE Soniprep 150 sonicator (MSE Scientific Instruments, Crawley, UK). Sonicated cells were centrifuged at 4°C and 14,000g for 5 min. The supernatant was used for purification of His-tagged proteins on a Ni-NTA spin column (Qiagen, Valencia, CA) as prescribed by the manufacturer. Resulting eluates were adjusted to contain 2 mM diithiothreitol, and were immediately used for activity assays.

Substrate Preference Assays

Activity of recombinant proteins with different alcohol substrates was assayed using 14C radiolabeled acetyl-CoA (Amersham) or 14C-butyryl-CoA or 14C-hexanoyl-CoA (Campro, Veenendaal, The Netherlands). The reactions were made in duplicate. In a total reaction volume of 100 µL, 2 µL acetyl-CoA (5 mm), 0.2 µL 14C-labeled acetyl-CoA, 85.8 µL 50 µM Tris-HCl buffer, pH 8.3, and 10 µL purified enzyme were mixed slowly in a cold Eppendorf (Westbury, NY) tube. The reactions were started by adding 2 µL alcohol, solved at 1 mM in hexane, and incubated at 30°C. After 20 min incubation at 30°C, the reactions were stopped by cooling on ice for 15 min. The esters formed were extracted with 700 µL hexane. The hexane phase was mixed with 4.5 ml scintillation cocktail (Ultima Gold, Packard Bioscience, Groningen, The Netherlands) and analyzed by scintillation counting. The enzymatic activity was determined as nmoles substrate turned over per hour per microgram protein. Km and Vmax determinations for recombinant VAAT protein were performed exactly as described in Aharoni et al. (2000) for SAAT. Km values were determined by dividing Vmax values by the protein concentration of the purified enzyme in the reaction mixture, taking into account the calculated molecular mass of about 50 kD.

Enzyme Activity Assays with Plant Material

One gram of leaf material was ground in a pestle and mortar and immediately mixed into 1 mL buffer A containing 50 mM Tris-HCl, pH 8.0.
and 1 mM dithiothreitol. The material was centrifuged for 5 min at 13,000 g, and the supernatant was used for assays. The assay was performed in a capped vial at 30°C and contained 50 μL of supernatant, 300 μL buffer A, 25 μL 8 mM acetyl-CoA solution, and 25 μL 1.6 mM geraniol suspension. After 30 min, 400 μL saturated CaCl2 solution was added by injection through the septum. Products were analyzed using solid-phase microextraction. Volatiles released into the vial headspace (at 35°C with stirring) were subsequently trapped for 10 min by exposing a 100-μm polydimethylsiloxane fiber (Supelco, Bellefonte, PA) to the headspace. Desorption was performed for 1 min, and products were analyzed by GC-MS, as described by Verhoeven et al. (1997).

Headspace Analysis
To analyze the headspace of plant parts, three flowers or three stem explants with a single node and two to three young leaves were cut from a single plant and weighed and cut ends were placed together in a covered 10-mL beaker covered with aluminum foil and containing 2 mL of water. For headspace analysis (performed basically as described by Bouwmeester et al., 1999), beakers with the detached plant parts were placed in closed glass cuvettes (500 mL) for 24 h at 21°C in a light regime of 9 h light, 8 h darkness, and 7 h light. A vacuum pump was used to draw air through the glass cuvet at approximately 100 mL/min, with the incoming air being purified through a glass cartridge (140 × 4 mm) containing 150 mg of Tenax TA (20/35 mesh, Alltech, Deerfield, IL). At the outlet, the volatiles emitted by the detached leaves were trapped on a similar cartridge. Volatiles were collected during 24 h. Cartridges were eluted using 3 × 1 mL of redistilled pentane:diethyl ether (4:1). Of the (nonconcentrated) samples, 2 μL was analyzed by GC-MS using a gas chromatograph (5890 series II, Hewlett-Packard) equipped with a 30-m × 0.25-mm i.d., 0.25-μm film thickness column (5MS, Hewlett-Packard, Palo Alto, CA) and a mass-selective detector (model 5972A, Hewlett-Packard). The GC was programmed at an initial temperature of 45°C for 1 min, with a ramp of 10°C/min up to 220°C and final time of 5 min. The injection port (splitless mode), interface, and MS source temperatures were 250°C, 350°C, and 270°C, respectively, and the He inlet pressure was controlled with an electronic pressure control unit to achieve a constant column flow of 1.0 mL/min. The ionization potential was set at 70 eV, and scanning was performed from 30 to 250 atomic mass units. Data were quantified and compared to injected standards.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AJ001450.1, Z93116, AX025506, AX025504, AX025475, AX025508, AX025510, AY534530, AX025477, AX025516, AX025512, AY345331, AX025518, QP2WRR, BAA94345, AAC93311, QM6E2, QPFWPS, QM6E0, QLL769, AAFO787, AAON9979, AA273661, CAA94432, AAN09796, CA1B1466, AC114474, AAOF3701, BQ06456, pBIN24A, pBIN69A, pBIN27A, S152A, S216A, S249A, S157A, S136A, and S182A.

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LITERATURE CITED

Characterization of Enzymes Forming Fruit Esters


