ODORANT1 Regulates Fragrance Biosynthesis in Petunia Flowers

Julian C. Verdonk, Michel A. Haring, Arjen J. van Tunen, and Robert C. Schuurink

Department of Plant Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, 1098 SM Amsterdam, The Netherlands

Floral scent is important to plant reproduction because it attracts pollinators to the sexual organs. Therefore, volatile emission is usually tuned to the foraging activity of the pollinators. In Petunia hybrida, volatile benzenoids determine the floral aroma. Although the pathways for benzenoid biosynthesis have been characterized, the enzymes involved are less well understood. How production and emission are regulated is unknown. By targeted transcriptome analyses, we identified ODORANT1 (ODO1), a member of the R2R3-type MYB family, as a candidate for the regulation of volatile benzenoids in Petunia hybrida cv W115 (Mitchell) flowers. These flowers are only fragrant in the evening and at night. Transcript levels of ODO1 increased before the onset of volatile emission and decreased when volatile emission declined. Downregulation of ODO1 in transgenic P. hybrida Mitchell plants strongly reduced volatile benzenoid levels through decreased synthesis of precursors from the shikimate pathway. The transcript levels of several genes in this pathway were reduced by suppression of ODO1 expression. Moreover, ODO1 could activate the promoter of the 5-enol-pyruvylshikimate-3-phosphate synthase gene. Flower pigmentation, which is furnished from the same shikimate precursors, was not influenced because color and scent biosynthesis occur at different developmental stages. Our studies identify ODO1 as a key regulator of floral scent biosynthesis.

INTRODUCTION

Plants use combinations of floral form, color, and scent to attract pollinators that enable or facilitate reproduction. These pollination syndromes have been extensively studied from the ecological level to individual scent molecules and from an evolutionary perspective. Studies on adaptive evolution with near-isogenic lines of monkeyflowers (Mimulus lewisi and M. cardinalis) showed that pollinator preference could shift with the change in color of the flowers (Bradshaw and Schemske, 2003). Co-evolution can occur between floral scent emission and flower architecture to accommodate different insects for pollination. For instance, related species with phenotypically dissimilar flowers, Clarkia breweri and C. concinna, produce different scents because they depend on different insects for pollination (Raguso and Pichersky, 1995). Also, Petunia integrifolia, a violet-flowering species with small, broad flowers lacking strong scent, is visited by bees during the day, whereas P. axillaris, a white-flowering species with slender flowers, which is visited by hawkmoths (Manduca contracta and M. diffusa subsp petuniae) at night, starts producing a strong scent at dusk (Ando et al., 2001).

Hawkmoth-pollinated flowers are white with usually long (>3 cm) narrow tubes that provide rewarding nectar. These flowers produce scent at night, and this scent is dominated by nitrogenous compounds, terpenoids, and benzenoids (Knudsen and Tollsten, 1993; Raguso et al., 2003). The P. axillaris-derived, white-flowering P. hybrida (Mitchell) predominantly emits volatile benzenoids in the evening and night (Kolosova et al., 2001; Verdonk et al., 2003). These volatile benzenoids are mostly produced by the petals (Verdonk et al., 2003), and emission decreases upon pollination (i.e., when the pollen tubes reach the base of the style). This communication between the various flower organs is mediated by ethylene (Negre et al., 2003).

Benzenoid metabolism in petunia petal tissue has recently been modeled with the aid of isotope labeling and metabolic flux analyses (Boatright et al., 2004). This model indicates that all volatile benzenoids in petunia are derived from Phe. Phenylacetaldehyde and phenylethylalcohol are synthesized from Phe, whereas the other volatile benzenoids originate from trans-cinnamic acid, which is made from Phe by the activity of Phe ammonia lyase (PAL). Shortening of the side chain of trans-cinnamic acid occurs through the β-oxidative and non-β-oxidative pathways, with benzaldehyde and benzylbenzoate as key intermediates between Phe and benzoic acid. Benzoic acid, the precursor of volatile methylbenzoate, is synthesized in the evening and night (Kolosova et al., 2001), in tune with methylbenzoate emission. Benzylbenzoate also accumulates rhythmically, with a maximum at night (Boatright et al., 2004).

Several genes encoding enzymes involved in benzenoid biosynthesis have been identified from C. breweri (Dudareva et al., 1998; D’Auria et al., 2002), snapdragon (Antirrhinum majus).
(Murfitt et al., 2000), and rose (Rosa hybrida) (Lavid et al., 2002). Only two corresponding cDNAs have been identified from petunia petals, S-adenosyl-L-Met:benzoic acid carboxyl methyltransferase (BSMT) and benzoyl-CoA:benzyl alcohol/phenylethanol benzoyltransferase (BPBT) (Negre et al., 2003; Boatright et al., 2004). The steady state transcript levels of petunia BPBT change rhythmically and peak in the afternoon, preceding the peak levels of its product benzylbenzoate (Boatright et al., 2004). BSMT activity in petunia shows rhythmic fluctuations with its product, methylbenzoate, but the fine tuning of methylbenzoate emission seems to be at the level of its precursor, benzoic acid, the levels of which increase almost fivefold during the night when methylbenzoate emission is highest (Kolosova et al., 2001). How benzoic acid synthesis is regulated is unknown, although some control might be at the level of PAL activity, which also fluctuates rhythmically in petunia (Kolosova et al., 2001). PAL is also regulated at the transcript level in petunia petals (Verdonk et al., 2003).

In this study, we describe the identification of an R2R3 MYB-type transcription factor, ODORANT1 (ODO1), from the scent producing P. hybrida cv Mitchell (W115), which controls the synthesis of volatile benzenoids. Suppression of ODO1 expression by RNA interference (RNAi) revealed that it specifically regulates the shikimate pathway in petals toward benzenoid production. This results in a strongly reduced emission of volatile benzenoids, the main compounds of the floral scent. Because flower pigmentation and volatile production occur at different developmental stages, downregulation of ODO1 does not influence flower color.

RESULTS

Identification and Expression of ODO1

Because our previous study showed that several genes of the shikimate pathway, which provides precursors for benzenoid biosynthesis, were regulated at the transcript level in P. hybrida Mitchell petals (Verdonk et al., 2003), we took a transcriptomics approach to identify genes involved in the regulation of volatile benzenoid synthesis. Using a dedicated, petal-specific cDNA microarray (Verdonk et al., 2003), we compared the transcriptome of P. hybrida Mitchell flowers that were producing scent with that of flowers just about to produce scent and with that of flowers of P. hybrida cultivar W138, which emits very low levels of volatile benzenoids (Stuurman et al., 2004). cDNAs of genes with increased transcript levels just before the production of scent and those of genes with very low transcript levels in P. hybrida W138 were sequenced. Among these were several genes from the shikimate pathway, including 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (DAHPS), 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS), chorismate mutase (CM), and two PAL genes (PAL1 and PAL2). In addition, we identified a cDNA encoding a BPBT, which is involved in benzylbenzoate production, one of the volatile benzenoids emitted by Mitchell. This gene has previously been shown to be transcriptionally regulated in petunia flowers (Boatright et al., 2004).

Among the cDNAs of the differentially regulated genes, we identified one cDNA encoding a transcription factor that was upregulated in the fragrant Mitchell flowers but not in flowers of the nonfragrant W138. We tentatively named this gene ODORANT1 (ODO1). RNA gel blot analysis of petals from plants grown in growth chambers showed a temporal expression pattern of ODO1 consistent with a role in regulating floral scent. We detected two transcripts (Figure 1A; 1.4 and 1.7 kb), which corresponded to two cDNAs with different 3’ untranslated region lengths (see Supplemental Table 1 online). ODO1 transcript levels started to increase between 1300 and 1600 h preceding the increase in volatile benzenoid emission, as shown for methylbenzoate, isoeugenol, and benzylbenzoate (Figures 1A and 1B). Transcript levels of ODO1 increased steadily, peaked at 2200 h, and were back at their lowest level early the next morning.

![Figure 1. Petal-Specific Expression of ODO1 Correlates with Scent Emission.](image-url)

Light (L) and dark (D) periods are indicated at the top of the figure. Hybridization with 18S rRNA is shown to illustrate the loading of the gels. These experiments (A) to (D) were performed with plants from growth chambers.

(A) RNA gel blot analyses of ODO1 in Mitchell petals harvested at 3-h intervals. The asterisks indicate the two transcripts that were detected.

(B) Bar graph depicting the emission of three selected volatile benzenoids measured for 1 h around the same time points (mean and SE, n = 3).

(C) Organ- and tissue-specific expression. R, roots; S, stems; L, leaves; Sp, sepals; PT, petal tube; PL, petal limb; A, anthers; St, stigma.

(D) RNA gel blot analysis of ODO1 in Mitchell (M) and W138 at 1800 h.
(1000 h) when volatile emission was also at its lowest. Expression of \textit{ODO1} was restricted to the petals, with the highest expression in the limb (Figure 1C), which is the major site of scent emission (Verdonk et al., 2003). RNA gel blot analysis confirmed that transcript levels of \textit{ODO1} in the nonfragrant W138 were only 10% of those in Mitchell (Figure 1D).

\textbf{ODO1 Is a Member of the R2R3-Type MYB Family of Proteins}

\textit{ODO1} encodes a putative protein of 294 amino acids (see Supplemental Table 1 online) with high similarity to proteins of the R2R3-type MYB family (Stracke et al., 2001). Although the N-terminal R2R3 domain contains the conserved motifs and amino acids involved in binding certain variable core motifs in DNA (Romero et al., 1998), the C terminus is not similar to any sequences in the National Center for Biotechnology Information (NCBI) or The Arabidopsis Information Resource (TAIR) databases. Phylogenetic analyses (Figure 2; see Supplemental Figure 1 online) place \textit{ODO1} apart from MYBs involved in regulation of anthocyanin and phenylpropanoid biosynthesis. Its closest relatives are a MYB from \textit{Pimpinella brachicarpa} and two MYBs from \textit{Arabidopsis thaliana} (\textit{AtMYB42} and \textit{AtMYB85}), whose functions are unknown. The two \textit{Arabidopsis} MYBs form a subgroup separate from the other 126 R2R3 MYB genes in \textit{Arabidopsis} (Stracke et al., 2001; Petroni et al., 2002). Sixteen of the more variable amino acids in the R2R3 domain (Stracke et al., 2001) are highly conserved in these four proteins (Figure 3), which puts \textit{ODO1} apart from other known petunia MYBs and defines a new subgroup in this family of transcription factors. The domain for interactions with R-like basic-helix-loop-helix proteins (Zimmermann et al., 2004) is absent in the R2R3 domain of \textit{ODO1}.

\textbf{Silencing of ODO1 Leads to Diminished Volatile Benzenoid Production}

To investigate the role of \textit{ODO1} in regulating floral scent, we suppressed its expression in Mitchell through RNAi. We used the constitutive 35S promoter of \textit{Cauliflower mosaic virus} to drive an inverted repeat of the unique C terminus of \textit{ODO1}, which would only suppress accumulation of \textit{ODO1} transcripts. Genomic DNA gel blots with the same region of the C terminus of \textit{ODO1} as a probe indicated that, under stringent conditions, no other DNA sequences similar to \textit{ODO1} could be detected in the petunia genome (see Supplemental Figure 2 online). All independent transformants were morphologically normal and were assayed for volatile emissions and for \textit{ODO1} transcript levels in their petals. From these analyses, it was clear that downregulating \textit{ODO1} reduced emission of volatile benzenoids, as illustrated by the gas chromatography time-of-flight mass spectrometry chromatograms for RNAi line 3 and Mitchell (Figure 4A). The RNAi line 3 clearly emits much lower levels of benzenoids than the parental Mitchell. Subsequent quantitative analyses of the volatiles emitted from four independent transgenic lines, from Mitchell, and a transgenic, nonsilenced, control line (line 40) showed that emission of all benzenoids (of which the majority is presented in Figure 4B) was reduced, with the exception of methyl salicylate and benzyl alcohol, which was only significantly reduced in RNAi line 12. The inhibitory effect of \textit{ODO1} suppression on volatile emission was more pronounced for compounds that are downstream in the biosynthetic pathway (see Figure 5B) (e.g., isoeugenol and vanillin). To eliminate the possibility that emission but not production of the volatile benzenoids was affected by \textit{ODO1} suppression, we performed solid phase micro-extraction analysis of petal extracts and analyzed the products trapped on the solid phase micro-extraction fiber with gas chromatography–mass spectrometry (Verdonk et al., 2003). These analyses showed that the four RNAi lines had reduced stores of all volatiles that were emitted (see Supplemental Figure 3 online), indicating that \textit{ODO1} regulates biosynthesis rather than emission. In all the lines where volatile emission was reduced, the expression of \textit{ODO1} was also reduced (Figure 5A), whereas in line 40, where \textit{ODO1} was not suppressed, volatile emission was normal (Figures 4B and 5A).

\textbf{ODO1 Controls the Shikimate Pathway and Benzoic Acid Synthesis}

Which steps in the biosynthesis of volatile benzenoids are controlled by \textit{ODO1}? Synthesis of benzenoids starts with the
shikimate pathway that produces L-Phe, which is then converted to the first benzenoid precursor, trans-cinnamic acid, by PAL (Figure 5B). RNA gel blot analyses showed that transcript levels of enzymes in the shikimate pathway (e.g., DAHPS, EPSPS, and CM) as well as PAL1 and 2 were much lower in the RNAi flowers than in Mitchell or the control line 40 (Figure 5A). Because benzoic acid is probably formed from trans-cinnamic acid (Ribnicky et al., 1998; Jarvis et al., 2000; Boatright et al., 2004) and shown to be accumulating with a rhythm that correlates with methylbenzoate emission (Kolosova et al., 2001), we investigated whether benzoic acid levels were affected by KD oxidase. Indeed, benzoic acid levels were 12 times lower in RNAi line 3 than in Mitchell (11.9 ± 5.9 g/g fresh weight; n = 3, P < 0.01). We then addressed the question whether ODO1 would be able to activate transcription of shikimate pathway genes. To test this, we cloned the Mitchell EPSPS promoter (Benfey et al., 1990) to drive the Escherichia coli uidA (β-glucuronidase [GUS]) gene and introduced this construct into Mitchell leaf cells by particle bombardment in the presence and absence of a 35S promoter-ODO1 construct. Figure 5C shows that ODO1 is able to activate the EPSPS promoter (n = 10, P = 0.017) in petunia leaf cells. The specificity of ODO1 for the EPSPS promoter was demonstrated by the absence of significant activation of the dihydroflavonol 4-reductase (DFR) promoter–GUS reporter (P = 0.65). This DFR-GUS construct could be activated by another petunia R2R3-type MYB, anthocyanin promoter AN1 to activate DFR (Spelt et al., 2000). The EPSPS promoter was not significantly activated by the combination of AN1 and AN2 (P = 0.39), indicating some level of specificity of floral R2R3-type MYB proteins (Figure 5C).

**Microarray Analyses**

To determine whether genes, other than those from the shikimate pathway, were modulated in the ODO1-suppressed plants, we performed experiments with dedicated petunia microarrays. These experiments with petal limbs from wild-type Mitchell and RNAi line 3 confirmed that genes from the shikimate pathway (DAHPS and EPSPS) were downregulated (Table 1). PAL was also downregulated but only 1.4-fold (P < 0.01; see Supplemental Table 3 online). Additionally, we discovered that the following genes from the S-adenosyl Met (SAM) cycle were also downregulated: SAM-synthase, Met synthase, hydroxymethyltransferase, and adenosylhomocysteinase. We confirmed the suppression of SAM-synthase by RNA gel blot analysis for several independent RNAi lines (Figure 5A). This result suggested that the SAM: S-adenosyl homocysteine (SAH) ratio in petal limbs, which is a measurement of the methylation status, was altered. However, the SAM:SAH ratios were 22.1 (± 1.1) in Mitchell and 22.4 (± 6.9) in RNAi line 3, respectively, revealing no significant difference (P = 0.72, n = 3), and the same was true for the SAM levels (P = 0.43). Transcript levels of sucrose synthase were also significantly lower in the RNAi plants, indicating that there is less demand for sucrose, probably because of reduced activity of the shikimate pathway. An aquaporin-like protein, a DEAD/DEAH box helicase, and a mitochondrial 26S rRNA were also downregulated.

Among the upregulated genes were two enzymes catalyzing the biosynthesis of volatile benzenoids, BBPBT and BSMT (Table 1). For BSMT, we were able to confirm this upregulation in the RNAi lines by RNA gel blot analysis, but not for BBPBT, probably because of differences in specificity between the microarray and RNA gel blot analysis (Figure 5A). Four other genes putatively involved in the biosynthesis of secondary metabolites, isoflavone reductase-like, NAD-dependent epimerase/dehydratase, peroxidase, and an alcohol dehydrogenase, were also upregulated in RNAi line 3. One transcription factor, a C3HC4-type zinc finger protein, of which the function is unknown, was upregulated in the RNAi line, as were protein phosphatase 2C and an absic acid stress-related protein. Two different proteases were upregulated in the RNAi lines, suggesting a higher turnover of proteins when ODO1 expression is suppressed. Finally, two genes with
high similarity to Arabidopsis proteins with unknown function were also upregulated in the RNAi line.

**ODO1 Suppression Does Not Affect Color**

Because *ODO1* suppression downregulates genes in the shikimate pathway, one could envision that *ODO1* suppression would prevent the accumulation of flavonols and colored compounds like anthocyanins in flowers (Figure 5B). *Trans-cinnamic* acid is also a precursor of flavonols (Koes et al., 1994) that accumulate in the white Mitchell petals (Van der Meer et al., 1992), where flavonol-synthesizing enzymes are expressed. Because of a mutation in the regulatory *AN2* gene in Mitchell (Quattrocchio et al., 1999), anthocyanin production is reduced in petal limbs but not in the tubes. Nonetheless, *ODO1* RNAi lines still exhibited the characteristic purple anthocyanin stripes in the tubes (Figure 6A), and thin layer chromatography (TLC) analysis revealed that flavonols accumulated in the limbs to the same extent as in Mitchell (Figure 6B). An explanation for this phenomenon could be that flavonols and anthocyanins are produced early in flower...
Figure 5. Modulation of ODO1 Expression Alters Expression of Volatile Benzenoid Genes.

(A) RNA gel blot analysis of Mitchell (M) and RNAi lines 1, 3, 12, 35, and 40 (control) at 1700 h for ODO1, DAHPS, EPSP synthase (EPSPS), CM, two PAL genes (PAL1 and 2), BSMT, BPBT, and SAM-synthase (SAMS). Transcript levels of FLORAL BINDING PROTEIN 1 (FBP1), a MADS box protein involved in specification of floral organ identity (Angenent et al., 1992), are shown to indicate the loading of the gels.

(B) Schematic representation of the shikimate pathway that leads to the biosynthesis of Phe and its derivatives trans-cinnamic acid, coumaric acid, caffeic acid, and ferrulic acid. Solid arrows indicate well-described enzymatic reactions, whereas broken arrows indicate routes that are still hypothetical. E-4P, erythrose 4-phosphate; PEP, phosphoenolpyruvate. Numbers indicate the following enzymes: 1, DAHP synthase; 2, EPSP synthase; 3, CM; 4, PAL; 5, chalcone synthase.

(C) Activation assays. The columns and error bars denote the mean and standard error of the activity of the EPSPS-GUS and DFR-GUS reporter constructs after bombardment with 35S-ODO1 or a mix of 35S-AN1 and 35S-AN2. Reporter gene activity, measured as glucuronidase enzyme activity, is expressed in relative arbitrary units and was normalized to luciferase enzyme activity (LUC) expressed from a cobombarded reference construct, which contained the luciferase gene driven by the 35S promoter.
development. Indeed, expression of chalcone synthase (CHS), a key enzyme in colored compound production, is highest in developing flower buds, whereas ODO1 is first expressed in mature flowers (Figure 6C). Flavonols and anthocyanins are sequestered in the vacuoles and are stable during the lifespan of the flowers (Jonsson et al., 1984).

ODO1 Suppression Affects Scent Production in P. hybrida cv V26

To investigate whether ODO1 can regulate scent production in another petunia cultivar, we made crosses between the violet P. hybrida cultivar V26, which emits a different blend of volatile benzenoids than Mitchell (see Supplemental Figure 4 online), and the white RNAi lines 3 and 35 and between V26 and Mitchell. The pink flowers from the F1 plants of crosses between Mitchell and V26 produced a volatile profile that was intermediate in quantity in comparison with Mitchell (see Supplemental Figure 4 online). The F1 progeny of crosses between the RNAi line 35 and V26 maintained their pink flowers (see Supplemental Figure 5 online), whereas emission of all benzenoids was significantly reduced compared with the cross between Mitchell and V26. This is illustrated for phenylacetaldehyde, which is emitted in higher amounts by V26 than Mitchell, and isoeugenol, which is emitted in higher amounts by Mitchell than V26 (Figure 7A; see Supplemental Figure 4 online). The reduced emission of volatile benzenoids in the RNAi line 35 V26 progeny correlated with a reduction in ODO1 expression (Figure 7B) compared with the progeny of Mitchell × V26. Thus, ODO1 expression is required for scent production in at least one other colored petunia cultivar.

DISCUSSION

ODO1 Expression Precedes Volatile Benzenoid Emission

In this study, the power of a targeted transcriptomics approach to unravel the regulation of a specific biosynthetic process in plants is illustrated by the discovery of the MYB transcription factor ODO1. Through differential hybridization of a highly specific cDNA-microarray of petunia petal tissue that had just started to emit volatile benzenoids (Verdonk et al., 2003), we were not only able to identify ODO1, but also a set of cDNAs encoding biosynthetic enzymes such as BPBT, EPSPS, DAHPS, CM, and PAL. This confirmed our previous result that several genes in the shikimate pathway are transcriptionally regulated (Verdonk et al., 2003) and corroborated the finding that the biosynthetic gene BPBT is regulated at the transcript level (Boatright et al., 2004).

Table 1. Microarray Analysis of Genes Modulated upon ODO1 Suppression in RNAi Line 3a

<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Annotation</th>
<th>BLAST E-Value</th>
<th>Ratio</th>
<th>P-Value</th>
<th>c</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF082893</td>
<td>Met synthase</td>
<td>1E-128</td>
<td>-2.5</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>CAB78435</td>
<td>Hydroxymethyltransferase</td>
<td>1E-134</td>
<td>-2.0</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>M18745</td>
<td>Sucrose synthase</td>
<td>1E-132</td>
<td>-1.8</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>BAA03709</td>
<td>S-adenosyl-L-homocysteine hydrolase</td>
<td>1E-141</td>
<td>-1.6</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>AF452015</td>
<td>Aquaporin-like protein TIP1;1</td>
<td>0</td>
<td>-1.6</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Z11899</td>
<td>Mitochondrion mmp26 gene for rRNA large subunit (26S)</td>
<td>1E-102</td>
<td>-1.6</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>M21084</td>
<td>EPSP synthase</td>
<td>0</td>
<td>-1.6</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>X82214</td>
<td>S-adenosylmethionine-synthetase</td>
<td>1E-156</td>
<td>-1.6</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>AY705977</td>
<td>ODO1</td>
<td>0</td>
<td>-1.6</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>NM_130144</td>
<td>DEAD/DEAH box helicase</td>
<td>1E-54</td>
<td>-1.5</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>CO805162</td>
<td>DAHP synthase</td>
<td>0</td>
<td>-1.5</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>U31094</td>
<td>P21 Thiol protease homolog</td>
<td>1E-138</td>
<td>1.5</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>NM_127325</td>
<td>Arabidopsis expressed protein</td>
<td>1E-18</td>
<td>1.6</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>U12439</td>
<td>Abscisic stress ripening-related protein</td>
<td>4E-46</td>
<td>1.6</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>AF349916</td>
<td>Short chain-type alcohol dehydrogenase</td>
<td>3E-99</td>
<td>1.6</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>AY171099</td>
<td>Cys protease</td>
<td>5E-97</td>
<td>1.8</td>
<td>0.018</td>
<td></td>
</tr>
<tr>
<td>Y12689</td>
<td>Isoflavone reductase-like</td>
<td>5E-75</td>
<td>1.8</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>NM_120336</td>
<td>Arabidopsis expressed protein</td>
<td>1E-18</td>
<td>1.8</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>NM_123585</td>
<td>CSHC4-type zinc finger protein</td>
<td>9E-25</td>
<td>1.9</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>AY611496</td>
<td>BPBT</td>
<td>0</td>
<td>1.9</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>NM_113287</td>
<td>NAD-dependent epimerase-dehydratase protein</td>
<td>2E-56</td>
<td>2.1</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>NM_122675</td>
<td>Protein Phosphatase 2C</td>
<td>5E-95</td>
<td>2.2</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>AY233465</td>
<td>BSMT1</td>
<td>0</td>
<td>2.5</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>AK10885</td>
<td>Peroxidase</td>
<td>2E-29</td>
<td>4.4</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

a Sequenced cDNAs from the dedicated microarray that were upregulated or downregulated by 1.5-fold are shown.
b Corresponding GenBank accession numbers with the highest similarity are shown.
c P-values (adjusted for multiple testing) denote the significant difference in the average ratios (Ratio) of ODO1-suppressed plants over wild-type Mitchell plants in three independent experiments.
The expression pattern of ODO1, as determined by RNA gel blot analyses, displays all characteristics predicted from a scent regulator. First, it is only expressed in the tube and limb of the petals (Figure 1C), the two tissues responsible for scent production (Verdonk et al., 2003), which specifically express BSMT and BPBT (Negre et al., 2003; Boatright et al., 2004). Second, its increase in transcript levels precedes the emission of volatile benzenoids (Figures 1A and 1B). Third, its transcript levels decrease when scent emission decreases (Figures 1A and 1B), thus showing a rhythmic expression pattern that correlates with scent production. Fourth, its rhythmic expression correlates with the rhythmic expression of EPSPS, DAHPS, CM, and PAL (Verdonk et al., 2003), and fifth, its transcript levels in the nonfragrant W138 are only 10% of those in Mitchell (Figure 1D).

**ODO1 Is Distinct from Other Floral MYB Proteins**

ODO1 belongs to the large family of R2R3-type proteins (Figure 2), but its C terminus seems to be unique because it shows no similarity to available sequences in the NCBI databases. Two MYBs from Arabidopsis (AtMYB42 and AtMYB85) and one from *P. brachicarpa* show the highest overall percentage similarity with ODO1. Remarkably, 16 of the highly variable amino acids in the R2R3 domain (Stracke et al., 2001) are conserved in these four proteins. This puts these four MYBs apart from those regulating anthocyanin and phenylpropanoid production.

Since the discovery of the first R2R3-type MYB gene in plants, the anthocyanin regulatory gene C1 in maize (Paz-Ares et al., 1987), other R2R3-type MYB genes in plants have also been shown to regulate anthocyanin synthesis (Sainz et al., 1997; Borevitz et al., 2000; Kobayashi et al., 2002) and phenylpropanoid metabolism (Hemrn et al., 2001; Stracke et al., 2001); to...
activate PAL promoters (Sablowski et al., 1994; Yang et al., 2001); and to determine cell fate (Lee and Schiefelbein, 2001; Wang et al., 2004). However, we have shown that ODO1 is a MYB that exerts influence over multiple genes involved in generating precursors for volatile benzenoid biosynthesis (Figure 5A). Two MYBs have been identified in fragrant red roses (Guterman et al., 2002), which also produce volatile benzenoids. However, these MYBs have the highest similarity with GhMYB9 and PsMYB26, which are phylogenetically unrelated to ODO1 and which are likely to regulate phenylpropanoid genes involved in flavonoid synthesis (Figure 2).

**ODO1 Regulates the Floral Shikimate Pathway toward Benzenoids**

Suppression of ODO1 expression in Mitchell had only one principal biochemical consequence (i.e., reduced emission—through lower production—of volatile benzenoids) (Figures 4A and 4B). All measurable emitted volatile benzenoids, with the exception of methyl salicylate were severely reduced in the RNAi lines that showed silencing of ODO1. This suggests that ODO1 either regulates many biosynthetic genes coordinately or that it regulates the flow through the biosynthetic pathway by precursor regulation. The observation that benzenoids that are produced further downstream in the pathway (Figure 5B) are more strongly reduced by ODO1 suppression suggested the latter. RNA gel blot and microarray analyses confirmed that suppression of ODO1 expression resulted in downregulation of several genes from the shikimate pathway (Figure 5A, Table 1) and two newly identified members of the PAL family (PAL1 and PAL2) in petals. The expression of two genes directly involved in volatile production, BPBT and BSMT, was not reduced by ODO1 suppression (Figure 5A, Table 1).

Evidence that ODO1 transcriptionally regulates the shikimate pathway was provided by the capacity of ODO1 to activate the EPSPS promoter in Mitchell leaves (Figure 5C). Because the shikimate pathway is essential for plant life, this pathway will be regulated in tissues other than petals by transcription factors other than ODO1. This is likely to be so for the EPSPS promoter, which has been shown to contain multiple transcription initiation sites in leaves, but predominantly one site in petals (Gasser et al., 1988). At least one other transcription factor, the zinc finger protein EPF1, binds to the EPSPS promoter (Takatsuji et al., 1991).

**ODO1 Suppression Reduces Benzoic Acid Levels**

The transcriptional downregulation of the shikimate pathway resulted in reduced levels of benzoic acid, which were 12 times lower in RNAi line 3 than in Mitchell. This indicates that the transcriptional downregulation of the shikimate pathway has a direct effect on the levels of metabolites formed through this pathway and, therefore, on the levels of volatile benzenoids (Figure 4B), which are synthetized from these metabolites (Figure 5B). Thus, the rhythmic regulation of the shikimate pathway by ODO1 is likely to result in the rhythmic accumulation of benzoic acid levels, regardless of the pathway through which it is formed, either non-β-oxidative or β-oxidative (Boatright et al., 2004). Benzoic acid accumulation is tightly and rhythmically controlled in snapdragon (A. majus) and Nicotiana suaveolens, where it precedes the rhythmic volatile emission (Kolosova et al., 2001). This suggests a similar regulation of benzoic acid levels in these two species. It has been reported that the large amounts of benzylibenzoate that accumulate in Mitchell petals can be an intermediate between Phe and benzoic acid (Boatright et al., 2004). We have not investigated benzylbenzoate levels in petals, but the emission of benzylbenzoate from our Mitchell is substantial (5 μg/t/g flower). Benzylbenzoate emission is severely reduced by ODO1 suppression (Figure 4B), whereas BPBT is not affected (Figure 5A) or even slightly upregulated according to the microarray analysis (Table 1). The BPBT transcript levels correlate closely to benzylbenzoate accumulation that occurs rhythmically, similar to benzoic acid (Kolosova et al., 2001; Boatright et al., 2004), suggesting that the substrates for BPBT are also reduced in the RNAi lines.

Surprisingly, ODO1 suppression resulted in increased BSMT transcript levels (Figure 5A, Table 1). Because it has been shown that feeding Phe to petals reduced BSMT transcript levels (Boatright et al., 2004), it might very well be that Phe levels were lower in the RNAi lines leading to higher BSMT transcript levels. Apparently, BSMT activity in the RNAi lines was not reduced because methyl salicylate emission was not significantly altered by ODO1 suppression (Figure 4B). Because salicylic acid can be formed not just from benzoic acid, but also from chorismate (Wildermuth et al., 2002) (see Figure 5B), an early intermediate in Phe biosynthesis, this intermediate might be less affected by ODO1 suppression. Moreover, salicylic acid is the preferred substrate of BSMT (Negre et al., 2003). In general, volatile emission of compounds further downstream in the biosynthetic pathway (Figure 5B) was more affected by ODO1 suppression.

The microarray analyses also revealed that transcripts of genes from the SAM cycle were less abundant in the ODO1-suppressed lines (Table 1, Figure 5A). However, SAM and SAH levels were not affected, showing that the overall methylation status of the petals had not changed. The expression of SAM-synthase is upregulated in Mitchell at the onset of scent production (Verdonk et al., 2003). Because fewer benzenoid precursors are present as substrate for methylation when ODO1 expression is suppressed, it is likely that additional SAM production is not required and that, therefore, SAM-synthase is not upregulated in the RNAi line, as is the case in the Mitchell wild type.

The benzoic acid levels that we measured were twice as high as reported previously (Kolosova et al., 2001) probably because of different culture and measurement methods. Our petunia plants were grown under a 16-h light regime instead of a 12-h light regime and emitted twice the amount of methylbenzoate than those reported previously (Kolosova et al., 2001; Negre et al., 2003), reflecting the higher amounts of benzoic acid. In addition, differences in methylbenzoate emission as a result of measurement methods cannot be excluded because we measured excised flowers instead of flowers on living plants. Moreover, we noticed that shorter measurement periods (1 h) resulted in the detection of approximately fourfold higher amounts of methylbenzoate than with longer measuring periods of 20 h (Figure 1B versus 4B), whereas the measured amount of
isoeugenol was the same for both measurement periods. Negre et al. (2003) measured methylbenzoate over a period of 12 h, which might have resulted in an underestimation of the emitted amounts. Our Mitchell plants also emitted isoeugenol, which was only detected inside petunia petals by Boatright et al. (2004), suggesting that the higher levels of precursors in our plants led to higher production of isoeugenol and, therefore, emission. We are convinced that isoeugenol is emitted by Mitchell because we measured isoeugenol emission from flowers on living plants (Verdonk et al., 2003) and from cut flowers (Figures 1B and 4). However, we cannot exclude the possibility that the cultivar Mitchell (W115) that we used is different from the cultivar Mitchell used by Negre et al. (2003) and Boatright et al. (2004).

Color and Scent Production Occur at Different Developmental Stages

Because flavonols and anthocyanins are synthesized from coumaric acid (Figure 5B), one of the precursors upstream of the precursors for vanillin and isoeugenol, we envisioned that color could be affected by ODO1 suppression as well. However, several lines of evidence show that this is not the case. First, the RNAi lines still had the characteristic purple stripes in their tubes (Figure 6A). Second, the RNAi lines produced similar amounts of flavonols as Mitchell (Figure 6B). Third, suppression of ODO1 expression in a cross between RNAi line 3 and the violet V26 did not result in loss of color in the limbs, in spite of a significant reduction in volatile benzenoid emission (Figure 7; see Supplemental Figure 5 online). Fourth, expression of the anthocyanin biosynthesis gene CHS occurs early during flower development before scent emission starts (Figure 6C). Expression of genes involved in anthocyanin biosynthesis in petunia petals have been shown to peak early during flower development just before opening of the flower bud, when enzyme activities peak, and to diminish at later stages (Gerats et al., 1983; Koes et al., 1989). Moreover, PAL1 and EPSPS are also expressed early during flower development, but expression increases when ODO1 is expressed (see Supplemental Figure 6 online). This indicates that color and scent are produced at different stages of development and are thus likely to be controlled differently. An interaction between color and scent production seems to exist in carnation (Dianthus caryophyllus) because modification of flower color (anthocyanins) by suppression of an enzyme in flavonoid biosynthesis increased the emission of methylbenzoate (Zuker et al., 2002).

We conclude that in P. hybrida Mitchell, ODO1 is required for the regulation of the floral shikimate pathway that provides precursors for volatile benzenoids. Although we have shown that ODO1 is capable of activating the EPSPS promoter directly (Figure 5C), it remains to be investigated whether ODO1 interacts with specific sequences in promoters either alone or in concert with other regulatory proteins or whether it influences the activity of other transcription factors. Perhaps it would be useful to focus on anthocyanin biosynthesis in petunia petals, which is regulated by the R2R3-type MYB AN2, the basic-helix-loop-helix-protein AN1, and the WD40 protein AN11 (De Vetten et al., 1997; Spelt et al., 2000, 2002). The transcriptional network controlling scent production and emission can now be investigated using ODO1 as a starting point.

METHODS

Plant Material and Transformation

Petunia hybrida cv Mitchell (also referred to as cultivar W115; P. axillaris × [P. axillaris × P. hybrida cv Rose of Heaven], P. hybrida cv W138, and P. hybrida cv V26) plants were grown as previously described (Verdonk et al., 2003). Plants bearing at least three mature flowers were used in all experiments. Transgenic petunias were obtained via Agrobacterium tumefaciens (strain GV3101 carrying plasmid pMP90)—mediated transformation, by dipping leaf cuttings in bacterial cultures (overnight at 28°C, 10−1 dilution). Transgenic calli were selected on MS medium containing 150 mg/mL kanamycin, from which plants were subsequently regenerated (Lucker et al., 2001). Rooting plants were tested for the presence of the neomycin phosphotransferase II gene and of the RNAi construct using PCR. PCR-positive plants were transferred to the greenhouse. Plants were self-pollinated, and the progeny showed segregation for kanamycin resistance and volatile benzenoid production.

Selection and Identification of ODO1

The construction, labeling, and analysis of the petal-specific DNA microarrays (with 834 cDNAs) have been described previously (Verdonk et al., 2003; Kant et al., 2004). These microarrays also contained 176 petunia DNA fragments and 427 tomato (Lycopersicon esculentum) ESTs for unrelated experiments and were not included in the analyses. Three experiments compared Mitchell petal limbs from 900 h with those from 1500 h, petal limbs from 1200 h with those from 1500 h, and Mitchell petal limbs from 1500 h with W138 (a nonfragrant cultivar) petal limbs from 1500 h. cDNAs that were coordinately upregulated (K-means clustering, Spotfire software; Spotfire, Sommerville, MA) with scent emission and that were not upregulated in W138 were sequenced. We found 153 differentials between 900 h and 1200 h, 193 differentials between 1200 h and 1500 h, and 253 differentials between Mitchell and W138. We sequenced 192 cDNAs because the clustering patterns indicated that several cDNAs were represented more than once, which was indeed the case even in our subselection (see Supplemental Tables 2 and 3 online). The sequences were compared with sequences in the NCBI and TAIR databases using the TBLASTX algorithm. One of them was identified as a MYB-homolog, ODO1, the subject of the studies presented here. In addition, DAHP5, EPSPS, CM, PAL1 and 2, and BPBT were also selected from these microarray experiments. For determining tissue-specific ODO1 expression, a mature Mitchell plant from the growth chamber was dissected at 1500 h and directly frozen in liquid nitrogen. For the developmental analysis, buds were tagged when they were 0.5 cm long and flowers were tagged just before opening. These buds and flowers were harvested at 1500 h when they reached the right stage and frozen in liquid nitrogen. RNA gel blot analysis was performed as described previously (Verdonk et al., 2003). Specific 3′ untranslated region probes were used for PAL1 and 2.

Phylogenetic Analysis

Trees were generated from a ClustalW 1.8 alignment (see Supplemental Figure 1 online) using PAUP version 3.1.1 (D.L. Swofford, PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1.1; computer program distributed by the Illinois Natural History Survey, Champaign, IL) with bootstrap analysis (n = 100) using the heuristic type of search and retaining trees with a frequency of 50% (Jeanmougin et al., 1998). Trees were made monophyletic using human c-Myb as an outgroup and visualized as cladograms using Treeview software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).
Microarray Experiments with RNAi Plants

Petal limbs of Mitchell and ODO1 RNA line 3 were harvested at 1500 h from plants grown in the growth chamber. For each experiment, four to five petal limbs were pooled for RNA extraction. The RNA was additionally purified with the RNeasy kit (Qiagen, Valencia, CA). Twenty-five micrograms of total RNA was labeled with Cy3 and Cy5 as previously described (Verdonk et al., 2003). Incorporation of the Cy3 and Cy5 dyes was determined with a spectrophotometer (Nanodrop Technologies, Wilmington, DE). The data in this article represent three hybridizations of three independent replicates, including dye swaps. Normalization, calculation of the average ratios and standard error, calculation of significance, and control of the false discovery rate were all done as previously described by Kant et al. (2004) (see Supplemental Tables 2 and 3 online).

After the analysis, clones were selected on the basis of three criteria: the significance of the adjusted P-values (α < 0.05), the average signal-to-noise ratio of the spots had to be greater than five, and the minimal treatment to control ratio was set to >1.5 or <1.5 on the basis of our RNA gel blot control experiments, which were performed with the same RNA standard and as previously described (Kant et al., 2004). Each plant line of synthetic external standards of known concentration and the internal control, kanamycin resistance to plant cells; VIB, Gent, Belgium, who are kindly acknowledged in this study.

For cotton GhMYB9, AAQ62541. For human HsC-MYB, M15024. For tomato LeTHM16, X99210. For rice OsMYB51, AJ311051. For Arabidopsis AtMYB101 (At2g32460), X90379; AtMYB108 (At3g06490), AF262733; AtMYB15 (At3g23250), X90384; AtMYB20 (At1g66230), AF062869; AtMYB42 (At1g12350), AF175999; AtMYB85 (At4g22680), AF175993; AtMYB101 (At3g24840), X90379; AtMYB108 (At3g06480), AF262733; AtAP1 (At1g66650), AF325123 and AtAP2 (AT1G66390), AF325124. For cotton GhMYB9, AAQ62541. For human HeC-MYB, M15024. For tomato LeTHM16, X90210. For rice OsMYB51, AJ311051. For

Benzoic Acid, Flavonol, and SAM/SAH Analyses

Benzoic acid levels were determined essentially as described by Zhang and Zuo (2004) with the following modifications. Flowers from 1800 h were boiled in 2 N HCl for 10 min after addition of 1% C-benzoic acid as the internal standard, followed by extraction with ethyl acetate. Derivatized samples were analyzed and quantified by gas chromatography–mass spectrometry as described for the emitted volatiles. Flavonols in the ethyl acetate extract were separated on silica-TLC plates containing F254 as described by Koos et al. (1995) and visualized under UV light (254 nm). Three independent experiments were performed. SAM and SAH levels were determined in extracts of petal limbs essentially as described by Struyts et al. (2000). One petal limb was ground in liquid nitrogen before addition of 1.5 ml of ice-cold mill-Q water (Millipore, Billerica, MA). This extract was subsequently centrifuged at 13,000 g at 4°C and the supernatant used for the analyses.

Transient Expression Assays by Particle Bombardment

The EPSPS promoter was PCR amplified from genomic Mitchell DNA with the following forward and reverse primers: 5′-GGTACAAGATTTGAT-TAGTCGGG-3′ and 5′-GATATCCCTTGAGCATGTTTG-3′, followed by PCR reamplification with two nested primers: 5′-ggatctTAAATGGATG-3′ and 5′-catgcaattGTTGAAAGATGTTA-GTC-3′ (the lowercase letters represent the adapters). The amplified fragment was used to replace the actin promoter XbaI-NcoI in pDMC207 (McElroy et al., 1995) so that the EPSPS promoter would drive GUS. The open reading frame of ODO1 was cloned in pGreen, which was modified to contain the 35S cassette from pMON999, so that ODO1 was driven by the 35S promoter and terminated by the nopaline synthase terminator. The transient expression assays by particle bombardment of Mitchell leaves were done as previously described (Quattrocchio et al., 1993; de Vetten et al., 1997) with the modification that 1-μm gold particles were used in stead of tungsten. Gold particles for five bombardments were coated with 4 μg of reporter plasmid plus 1 μg effector plasmid or 1 μg pBluescript KS+ plasmid (Stratagene, La Jolla, CA). Reporter gene activity, measured as glucuronidase enzyme activity, was normalized to luciferase enzyme activity (LUC) expressed from a cobombarded reference construct, which contained the luciferase gene driven by the 35S promoter. Relative activities were calculated by setting the ratio of EPSPS-GUS/LUC to 1 and subsequently expressing the other ratios relative to this. This was done separately for each experiment. Before statistical analyses, the relative data were arcsin-square root transformed to achieve normality. Data were evaluated using analysis of variance followed by the least significant difference post-hoc analysis.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under the following accession numbers: AY705977 for ODO1 and AY705976 for PAL1. Partial sequences of PAL2, CM, and DAHPS were submitted to the GenBank dbEST database under accession numbers CO805160, CO805161, and CO805162, respectively. The accession numbers for other sequences in this manuscript are as follows. For snapdragon AmMIXTA, X79108, and AmMYB305, JQ09958. For Arabidopsis AtMYB5 (At3g13540), U26935; AtMYB15 (At3g23250), X90384; AtMYB20 (At1g66230), AF062869; AtMYB42 (At1g12350), AF175999; AtMYB85 (At4g22680), AF175993; AtMYB101 (At3g24840), X90379; AtMYB108 (At3g06480), AF262733; AtAP1 (At1g66650), AF325123 and AtAP2 (AT1G66390), AF325124. For cotton GhMYB9, AAQ62541. For human HeC-MYB, M15024. For tomato LeTHM16, X90210. For rice OsMYB51, AJ311051.
Acknowledgments

The authors wish to thank Alan Musgrave (University of Amsterdam, The Netherlands) for critically reading the manuscript and Francesca Quattrocchio (Free University, Amsterdam, The Netherlands) for assistance with the particle bombardment experiments. Merijn Kant (University of Amsterdam) is kindly acknowledged for analyzing the microarray data. We thank Eduard Stuys (Free University, Academic Hospital, Amsterdam, The Netherlands) for measuring SAM and SAH. Ludek Tivkosky and Harold Lemereis are acknowledged for their excellent care of the petunia plants.

Received October 26, 2004; accepted February 22, 2005.

References


Jarvis, A.P., Schaaf, O., and Oldham, N.J. (2000). 3-Hydroxy-3-phenylpropanoic acid is an intermediate in the biosynthesis of benzoic acid and salicylic acid but benzoaldehyde is not. Planta 212, 119–126.


Negre, F., Kish, C.M., Boatright, J., Underwood, B., Shibuya, K., Wagner, C., Clark, D.G., and Dudareva, N. (2003). Regulation of...


