Floral scent production by Petunia hybrida

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CHAPTER 6.

General discussion

Floral scent mostly consists of three different chemical classes of molecules: terpenoids, benzenoids and fatty acid derivatives. In addition, nitrogen and sulfur containing molecules can be part of the bouquet. Floral scent plays an important role in the interaction with pollinators. Night-pollinated species, such as tobacco and petunia rely for the most part on scent to attract pollinators.

Petunia is an excellent model system to study the production of floral volatile benzenoid molecules. The wild species *Petunia axillaris* is pollinated by hawk moths, which are attracted by a complex mixture of volatiles, mainly benzenoids (Hoballah et al., 2005). To study benzenoid biosynthesis, we used the *Petunia hybrida* cv Mitchell, a doubled haploid originating from a complex hybrid between *P. axillaris* and the petunia cultivar 'Rose of Heaven'. It emits a blend of benzenoids similar to that of *P. axillaris*, but it has several advantages over the wild species: it exhibits superior fertility, growth, tissue culture and transformation abilities. Therefore, it has been a model plant for many laboratories, and has been well characterized at the genetic and physiological level (reviewed in Gerats and Vandenbussche, 2005).

Benzenoids are derived from the shikimate pathway, the chain of reactions that connects primary metabolism with secondary metabolism. The shikimate pathway not only provides precursors for the biosynthesis of benzenoids, but also for all other phenylpropanoids, for example flavonoids and lignins (reviewed in Herrmann and Weaver, 1999). To study the characteristics of benzenoid biosynthesis in petunia Mitchell, we used a targeted metabolomics approach. By measuring the headspace of whole flowers and separate tissues, we determined that a mixture of at least twelve different benzenoids is produced, mostly in the petals. The emission of this mixture has a circadian rhythm with maximum emission at night, during the 5 to 8 days when the flower is open (Verdonk et al., 2003, Chapter 2). These findings were used to generate a dedicated microarray for the identification of genes involved in the production and regulation of volatile benzenoids. A collection of approximately 800 random clones from a cDNA library of petal tissue, harvested approximately 2 h before scent production was spotted onto microarray slides. The transcriptome of petunia Mitchell petals harvested in the afternoon was compared with that

of petals harvested in the morning, and with that of petals from the low-scent producing petunia W138 cultivar. From these experiments, we learned that the regulation of benzenoid biosynthesis is at the level of transcription. The data presented in Chapter 3 illustrate that a targeted approach can yield interesting information. We identified several genes that are (putatively) involved in volatile biosynthesis and several other genes for which the function still needs to be established.

Underwood (2003) used a microarray approach to analyze changes in gene expression during ethylene-induced senescence of petunia Mitchell flowers. For this, more than 3000 unique ESTs from three different cDNA libraries (different developmental stages, ethylene-treated, and pollinated flowers) were sequenced and amplified for the construction of microarrays. Then the transcriptome of whole flowers with and without ethylene treatment was compared. Several genes (e.g. *PAL, BSMT, SAMS*) that we now know are related to benzenoid production were found to be down regulated by ethylene. For our experiments we amplified only 800 random cDNAs from the tissue responsible for the production of scent, at the time when volatile biosynthesis had commenced and we identified more benzenoid synthesis related genes than Underwood (2003). This illustrates the advantage of a targeted transcriptomics approach, made feasible by our knowledge of the volatile emission pattern. Since our main interest was the production of volatile benzenoids by the petal limbs, we only analyzed the transcriptional differences in petal limbs just before benzenoids are produced. More extensive comparison of the transcriptome of early and late developmental stages of the petal, as well as the comparison of the low-scent producing petunia W138 cultivar, may still identify genes that are not differentially expressed during benzenoid formation, but are important for this process.

One of the differentially expressed genes provided the opportunity to investigate the transcriptional regulation of the biosynthesis of benzenoids, because it encoded an R2R3-MYB transcription factor. Analysis of its expression pattern revealed that it perfectly correlated with the developmental, circadian and tissue-specific benzenoid biosynthesis. Through silencing its expression, we learned that it was a regulator of the floral shikimate pathway that provides phenylpropanoid precursors for the biosynthesis of floral benzenoids (Verdonk et al., 2005; Chapter 4). Phylogenetic analysis of ODO1 in comparison with other MYBs involved in phenylpropanoid biosynthesis place ODO1 in a new subgroup of MYB transcription factors, together with two MYBs from Arabidopsis and one of *Pimpinella brachycarpa* (Verdonk et al., 2005, Chapter 4, Figs 2 and 3), all with unknown functions. MYB transcription factors are highly conserved in their DNA binding domain with a few variable residues (Stracke et al., 2001). In this new subgroup of MYBs however, there is a

high conservation in these residues. It is likely that they are functionally related. The functional relation of MYBs of different species has been shown for MYBs that act in the anthocyanin pathway. The anthocyanin pathway is regulated by a complex of a MYB transcription factor and a basic Helix-loop-helix transcription factor. These two transcription factors are highly conserved in their DNA binding domains, and their introduction in other species has been applied successfully (reviewed in Chapter 5). It remains to be determined whether *ODO1* homologs could have a function in the regulation of the floral shikimate pathway.

Similarities between the regulation of different branches of the floral phenylpropanoid pathways

During different floral developmental stages, the shikimate pathway provides precursors for benzenoid, anthocyanin and flavonoid biosynthesis. In the early stages of flower development, the precursors are used for the biosynthesis of anthocyanins and flavonoids, which are stored in the vacuole (reviewed in Grotewold, 2004). Petunia Mitchell produces no anthocyanins in the petals because of a mutation in the regulatory *AN2* gene. It does produce anthocyanins in the tubes, which are visible as characteristic purple stripes (Quattrocchio et al., 1999). In the *ODO1*-silenced lines, the content of flavonoids in the petals and anthocyanins in the tubes was identical to that of the wild type. Therefore, the regulation of the shikimate pathway by *ODO1* seems to be specific for provision of precursors for the biosynthesis of floral volatile benzenoid molecules. This is corroborated by results from a cross of the violet-colored V26 line with *ODO1* silenced Mitchell lines. V26 has dark colored petals, and produces a scent dominated by benzenoids (Chapter 4, figure 9, 10). The hybrids with reduced *ODO1* expression had a strong reduction in benzenoid emission (Chapter 4, Figure 11). However, there was no difference in the color of the flower (Chapter 4, figure 10) and the coloration was independent of *ODO1* expression levels.

In other plant species the flux through the different branches of the phenylpropanoid pathway may be regulated differently. In Carnation (*Dianthus caryophyllus*), the silencing of a gene in the anthocyanin pathway, *flavone-3-hydroxylase* (*F3H*), not only dramatically changed the color of the flowers, but the flowers also emitted more methyl benzoate than the control wild type plants (Zuker et al., 2002). It would be interesting to measure the volatile profiles of petunia mutants in the anthocyanin pathway. One could argue that the mutation *AN2* might be the cause for the presence of volatile benzenoids in petunia Mitchell. The absence of anthocyanins synthesis might cause the precursors to be redirected to benzenoid production. This does not hold however, because the petunia V26 cultivar is

An2^{+} (Quattrocchio et al., 1999), and produces floral benzenoids in similar amounts as petunia Mitchell.

The developmental separation of color (flavonoids, anthocyanins) and scent (benzenoids) production is not fully understood. It can be assumed that the precursors are diverted towards color during the early stages of development, and towards benzenoids once the flower is open. *Chalcone synthase* (*CHS*), a key enzyme of flavonoid biosynthesis (Koes et al., 1989), is expressed only in the early stages of floral development (Chapter 4, fig 8C). When the flower opens and starts to produce benzenoids, *CHS* expression diminishes, and the precursors can then be used to produce volatile benzenoids. ODO1 could be a key enzyme that regulates the flux towards the phenylpropanoid pathway. Enhanced expression of *ODO1* coincides with the decrease of *CHS* expression (Chapter 4, Fig 8C). *CHS* expression is reduced by high *trans*-cinnamic acid (*t*-CA) levels (Loake et al., 1991) and these high *t*-CA levels could indirectly be caused by ODO1 activity. However, the *t*-CA levels in petals are around 10^{-6} M (Underwood et al., 2005) and this is below the levels needed for repression (> 10^{-4} M). There must be at least one other regulator of the floral shikimate pathway, because *ODO1* silenced W115 x V26 hybrids did not have a more intense color. It is possible that ODO1 competes with another MYB in a complex that activates CHS transcription.

There is a growing number of examples that the same WD40 and bHLH regulators in petunia control distinct processes by interacting with specific MYB proteins (Koes et al., 2005). Together with different MYB proteins, they can activate anthocyanin synthesis, but also control acidification of the vacuole, and the color and the morphogenesis of the seed coat (Spelt et al., 2002). It is likely that *ODO1* can also bind to the same bHLH and WD40 regulators to activate the shikimate pathway genes when benzenoids are produced. It would be interesting to see whether ODO1 indeed interacts with bHLH and WD40 proteins. At the same time, transcriptional regulators of ODO1 itself will have to be identified to study how *ODO1* expression is regulated. It remains to be seen how the shikimate pathway is regulated during the first stages of floral development, because two genes that have an important role in the production of phenylpropanoid precursors, (*PAL*) *Phenylalanine Ammonia Lyase* and *EPSP* (*5-enolpyruvylshikimate-3-phosphate*) *synthase*, are already expressed during those stages, while *ODO1* expression was below our detection levels (Chapter 4, Fig 12). Another question that comes to mind is what regulates the expression of the structural benzenoid biosynthesis genes. Since *benzoic acid/salicylic acid methyltransferase* (*PhBSMT*) and *benzyl alcohol/phenylethanol benzoyl transferase* (*PhBPBT*) appear to be regulated at the substrate level (Chapter 3), it would be interesting

to know if that also holds for other, still hypothetical, benzenoid biosynthesis genes like the *3-ketoacylthiolase* and the *acyl coenzyme A synthetase*. Another interesting observation was the reversed circadian rhythm in expression, compared to Arabidopsis, of a group of genes (*PAL, SHM, PP2C*), while other known circadian regulated genes (*DEAD/DEAH box helicase, Aquaporin*) were not. The cause for this reversed circadian expression could be elucidated using *ODO1*. Analysis of the *ODO1* promotor could shed some light on that question. In Arabidopsis, so-called evening and morning elements that have been identified in the promotors of circadian genes, only differ one nucleotide of each other (Michael and McClung, 2002). Interactors of similar elements in the *ODO1* promotor can be identified and used to study the circadian rhythm.

The use of genetics in pathway elucidation

The increased expression of *BSMT* in the *ODO1* silenced lines indicates that this is caused by low levels of its substrate, benzoic acid (BA). Moreover, W138 flowers that emit only traces of methyl benzoate have a high *BSMT* expression (J.C. Verdonk, unpublished data). This observation raises the question whether the restoration of high levels of BA in the petals of W138 will give rise to the production of methyl benzoate. The group of Cris Kuhlemeier at the University of Bern, Switzerland, has successfully generated a population of RILs of *Petunia inflata inflata* and W138, as well of *P. axillaris parodii* and W138. Two different scent loci designated *SCE1* and *SCE2* were identified, both on chromosome VII (Stuurman et al., 2004). Interestingly, introduction of the *P. i. inflata* and *P. a. parodii SCE1* QTL alleles in petunia W138 resulted in a *P. axillaris*-like fragrance production. Thus, it seems that the structural genes responsible for the production of volatile benzenoids are present in petunia W138, although precursor biosynthesis is lacking. The identity of the genes responsible for the *SCE1* and *SCE2* QTLs is not known yet, but we predict that one of them is *ODO1* and that the introduction of *ODO1* in low-fragrant cultivars like W138 will have the same effect as the introduction of the *P. i. inflata* or *P. a. parodii SCE1* QTL alleles.

Manipulation of floral scent

Although silencing of *ODO1* proved its involvement in scent production, the identification of *ODO1* as a shikimate pathway regulator gave us the opportunity to test the possibility to upregulate this pathway towards phenylpropanoid. Successful approaches to manipulate the phenylpropanoid content of several plant species by overexpression of involved genes have been reported for, amongst others, anthocyanins, flavonoids and lignins (Reviewed in Chapter 5). We attempted flower specific overexpression of *ODO1* using a *FLORAL BINDING PROTEIN1* (*FBP1*) promoter in petunia, and constitutive overexpression of

ODO1 using the Cauliflower Mosaic Virus 35S promotor in Arabidopsis (Chapter 5). The levels of *ODO1* expression were not highly increased, however. In petunia Mitchell flowers, a small increase in some of the volatiles was observed, but further studies will have to be done to be conclusive about this. The *FBP1* promotor that was used to drive the expression of *ODO1* might not be strong enough. In transgenic Arabidopsis, although the *ODO1* transcript was detected, an effect on the expression level of the shikimate pathway gene *EPSPS* that is transcriptionally activated by *ODO1* in petunia (Verdonk et al., 2005; Chapter 4), was not observed. Furthermore, Arabidopsis flowers of *ODO1* expressing plants with expression of *ODO1* did not emit any benzenoids (data not shown). It is possible that *ODO1* does not act alone to activate the shikimate pathway, and that co-factors are missing in Arabidopsis.

Manipulation of the biosynthesis of floral volatile phenylpropanoids and benzenoids has until now only been achieved by silencing of involved genes (Underwood et al., 2005; Verdonk et al., 2005). The engineering of floral scent has great potential; it could for example be used to improve crop yields. Fruit orchards in the United States, for example, are critically dependent on bee pollination, and a reduction in the number of visits by bees has caused a corresponding decrease in fruit yield (Vainstein et al., 2001). Enhancing volatile emission may attract more bees and thus prevent such losses. The manipulation of floral scent of ornamentals is also interesting because of their large economic importance; over $30-40 billion dollar worldwide (Zuker et al., 1998), and because many modern varieties lack a distinct scent. Finally, the metabolic engineering of fragrance could increase protection against pathogens and pests (Dudareva and Pichersky, 2000). However, there are several pitfalls that have to be overcome in successfully engineering the scent of flowers.

Genetic manipulation of ornamentals is not as well developed as for most food crops, and when the technique is available, this may not apply for the elite varieties. In petunia there are large differences between the ability of a cultivar to be genetically transformed, for example, it seems impossible to transform petunia W138 Furthermore, each crop represents a small segment of a market that consists of hundreds of varieties representing many different species. Finally, regulatory costs are still too high to allow profitable introduction of genetically modified ornamentals.

The first report of the manipulation of floral scent was the introduction of the *Clarkia breweri* gene encoding *S-Linalool Synthase* (*LIS*) in *Petunia hybrida* cv Mitchell (Lucker et al., 2001). The transcript of *LIS* was detected in all tissues of the plant, but almost all

linalool, a monoterpene, was converted into a glycosylated non-volatile form. With this study, a major problem in the manipulation of secondary metabolites was demonstrated. Similar results were obtained in kiwi fruits that were expressing a stilbene synthase; in these transgenic lines only the glycosylated form of the end product was detected (Kobayashi et al., 2000). It is possible that the introduced molecule is phytotoxic to the cells and therefore is converted into a glycosylated (non toxic) form of molecule. Therefore, developmental, or tissue specific overexpression approaches will have to be considered, when attempting to enhance the production of potentially toxic volatiles in plant cells.

Another problem with the manipulation of floral volatiles is that scent is not stored like color, but emitted throughout the lifespan of the flower. In contrast with color manipulation, a small increase in scent will be difficult to notice. In order to have a lasting effect, there needs to be a continuous production of scent molecules. Petunia Mitchell plants, for example, have been reported to produce 20-140 µg of methyl benzoate and between 30 and 60 µg isoeugenol at the peak of emission (Reviewed in Schuurink et al., 2005). To be able to emit such high quantities of volatile benzenoids, a massive supply of precursors is needed, and the flux through the pathway has to be substantially elevated.

The group of David Clark at the University of Florida generated knock down lines for *PhBSMT*. In these plants, the level of methyl benzoate was decreased markedly (Negre et al., 2003; Underwood et al., 2005). The levels of the other volatiles, however, did not change. Therefore, it seems that benzoic acid (BA) is not freely available for conversion into other volatiles. The BA levels of these transgenic lines were not determined, but we predict that it accumulated, since the levels of other volatiles did not change. The overexpression of *PhBSMT* in petunia Mitchell did not lead to more methyl benzoate production (D. Clark pers. comm.). It has already been shown that BA is the limiting factor for the production of methyl benzoate (Kolosova et al., 2001). Therefore, to achieve increased MeBA production next to overexpression *PhBSMT* it will also be necessary to increase the amount of BA.

BPBT has a more central position in the benzenoid pathway than BSMT. Benzyl benzoate, the most abundant product formed by BPBT, is a key intermediate, together with benzaldehyde, between phenylalanine and BA (Boatright et al., 2004). Transgenic RNAi lines with decreased *PhBPBT* expression had a strikingly altered volatile profile (R. Dexter and D. Clark, pers. comm., see chapter 3) that supports this role. It will be interesting to see what the effect of overexpression of *PhBPBT* is. In the future, the silencing of other structural genes will help us to improve our knowledge of the benzenoid pathway. The

introduction of modifications in the pathway could alter the flux of the available substrate through the pathway. An illustrative example could be the introduction of modifying enzymes such as isoeugenol methyl transferase (IEMT) from *Clarkia breweri* (Wang and Pichersky, 1998). This might result in the conversion of the substantial pool of isoeugenol into isomethyleugenol, and the traces of eugenol into methyleugenol, generating flowers with spectacular different fragrance. Isoeugenol is a very abundant volatile in the headspace of petunia (Underwood et al., 2005; Verdonk et al., 2005), Chapter 4) and is well perceived by humans compared with for example methyl benzoate.

In conclusion, further characterization of ODO1 will help us to understand the regulation of floral benzenoid biosynthesis, but also the regulation of the shikimate pathway. The importance of all shikimate pathway derived secondary metabolites (e.g. flavonoids, benzenoids, lignins, coumarins, stilbenes) is high, manipulation of these metabolites could lead to interesting applications.

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