Abstract

Overexpression studies on MYB transcription factors have helped to unravel the regulation of the anthocyanin and flavonoid biosynthesis pathway. The R2R3-MYB transcription factor ODORANT1 (ODO1) has been shown to activate expression of $EPSP$ synthase, that encodes an important enzyme in the floral shikimate pathway leading towards volatile benzenoids in $Petunia$ hybrida cv Mitchell flowers. Cyclic expression of $ODO1$ in petals limbs coincides with the rhythmic emission of benzenoids. We investigated whether flower specific overexpression of $ODO1$, using the $FLORAL$ BINDING $PROTEIN1$ promotor, resulted in higher volatile emission. We observed that the overexpression was not very efficient, leading to an at most three fold increase in expression. The volatile profile of these flowers was altered in a way that could reflect the flux through the pathway, but was not markedly enhanced. Constitutive expression of $ODO1$ in Arabidopsis did not result in enhanced expression of the endogenous $EPSP$ Synthase.
Benzenoids and phenylpropanoids are dominantly present in the scent of flowers (Knudsen et al., 1993). These molecules are all derived from the general phenylpropanoid pathway, which is also responsible for the biosynthesis of a wide range of other important secondary metabolites such as anthocyanins, flavonoids, lignins, and coumarins. Many genes for enzymes in the phenylpropanoid pathway and its subsequent branches are regulated by MYB transcription factors.

MYB transcription factors contain a DNA-binding domain similar to that of the proto-oncogene \(c-myb\), and have been identified in all eukaryotes. The structural characteristic of MYB proteins is the DNA-binding domain, the signature of all transcription factor families. Additionally, MYBs can contain regions with the features of activator domains. MYBs from animals generally contain three repeats (R1, R2, and R3) in their DNA-binding domain, but that of plants usually consists of two imperfect repeats of approximately 50 residues (R2, R3) (Martin et al., 1991; Stracke et al., 2001).

In vertebrates, three different \(MYB\) genes are present in the genome, while invertebrates such as Drosophila contain a single member of the MYB family (reviewed in Davidson et al., 2005). In chicken, mice and humans, all three vertebrate \(MYB\) genes have been implicated in oncogenesis. Drosophila \(MYB\) is implicated in cell cycle progression (reviewed in Davidson et al., 2005). In plants, MYB proteins can be classified into three subfamilies depending on the number of repeats in the MYB domain: one, two or three. The family with two repeats, the R2R3-MYB proteins is by far the largest, for \(Oryza sativa\) (Jia et al., 2004), and maize (\(Zea mays\); (Rabinowicz et al., 1999), a minimum of 80 R2R3-MYB genes has been reported. In Arabidopsis, 126 R2R3-MYB transcription factors have been described (Kranz et al., 2000; Stracke et al., 2001). The first identified plant MYB gene was \(C1\) from \(Zea mays\) that is required for the synthesis of anthocyanins in the aleurone (Paz-Ares et al., 1987). The C1 family (\(C1, Pf\)) of MYB transcription factors interact with basic helix-loop-helix (bHLH) type of transcription factors of the R family (\(R, B, Lc, Sn\)). Together they control the expression of the structural genes of the anthocyanin pathway in maize (reviewed in Koes et al., 1994). Both transcription factors need to be present in order to activate the anthocyanin genes (Roth et al., 1991). In petunia, a similar interaction has been found between the MYB transcription factor AN2 and the basic helix-loop-helix AN1; together with a WD40 protein they control the transcription of a subset of structural genes from the anthocyanin pathway in petals, while in the anthers another MYB, AN4 has the same role (Quattrocchio et al., 1993; Quattrocchio, 1994; Quattrocchio et al.,
1999; Spelt et al., 2000). Up until now, MYB genes have been shown to regulate different branches of the phenylpropanoid metabolism. To sum up some examples: in Arabidopsis, upregulation of the MYB transcription factor *PAP1* activated phenylpropanoid biosynthetic genes (Borevitz et al., 2000), while in snapdragon flavonoid biosynthesis genes are activated by AmMYB305 (Sablowski et al., 1994). However, regulation by repression has also been reported; *AtMYB4* represses the synthesis of sinapate esters, UV protection pigments (Jin et al., 2000). Lignification in pine is regulated by MYB transcription factors; *PtMYB1* activates the *PAL2* promotor in differentiating xylem (Patzlaff et al., 2003a), and overexpression of *PtMYB4* increased transcript levels of lignin biosynthetic genes (Patzlaff et al., 2003b). In strawberry, the *FaMYB1* transcription factor plays a key role in regulating the transcription of late flavonoid biosynthesis genes (Aharoni et al., 2001). Recently the MYB transcription factor *ODORANT1* has been shown to regulate gene expression in the floral shikimate pathway; its down regulation resulted in a dramatic decrease of emitted volatile benzenoids (Verdonk et al., 2005). Next to their role in the phenylpropanoid pathway, MYB transcription factors have also been implicated in several other processes such as trichome development (Wang et al., 2004), lateral meristem formation (Schmitz et al., 2002), cold acclimation (Zhu et al., 2005) the formation of conical epidermal cells in petals (Noda et al., 1994; Mur, 1995), and the maintenance of the circadian rhythm, which is regulated by at least three MYB proteins (Hazen et al., 2005).

Several approaches to manipulate the flavonoid pathway and other branches of phenylpropanoid pathway using MYB transcription factors have been described. The enhanced expression of the endogenous Arabidopsis MYB-like transcription factor *PAP1* resulted in the massive accumulation of lignin, hydroxycinnamic acid esters and flavonoids (Borevitz et al., 2000). By studying the transcriptome and the metabolome of these *PAP1* overexpressing plants, several genes with unidentified or putative functions, as well as eight new anthocyanin molecules were identified (Tohge et al., 2005). Another approach is the introduction of known transcriptional activators into other species. In tobacco, and Arabidopsis, the introduction of the maize *C1* and *R* genes resulted in induced anthocyanin amounts (Lloyd et al., 1992). By engineering the expression of *C1* and *R* in cultured maize cell lines, that do normally not express them, two anthocyanins that are predominantly present in differentiated plant tissues accumulated (Grotewold et al., 1998). The introduction of the maize *Lc* and *C1* genes in the fruit of tomato upregulated the flavonoid pathway in tomato flesh, a tissue that normally does not produce any flavonoids (Bovy et al., 2002).
In this study, we present the attempts to overexpress the R2R3-MYB transcription factor *ODORANT1* in petunia Mitchell, to manipulate the floral benzenoid pathway, and eventually alter the scent quantity and quality of flowers. We chose for a flower specific overexpression in petunia using the promotor of the *FLORAL BINDING PROTEIN 1* (*FBP1*) to drive the expression of *ODO1*. Furthermore, to investigate whether the shikimate pathway could also be upregulated in other plant species, a CaMV 35S promotor driven *ODO1* was introduced into Arabidopsis plants.

**Results**

*Flower specific overexpression of ODORANT1*

*ODORANT1* expression in petunia Mitchell cycles, with maximum levels when the floral volatile benzenoids are produced; i.e. during the evening and night (Verdonk et al., 2003; Chapter 2, Fig. 3 and 4). It is first expressed in the late stages of development when the flower is fully opened (Verdonk et al., 2005, Chapter 4, Fig. 1). *FLORAL BINDING PROTEIN1* (*FBP1*) is constitutively and highly expressed throughout flower development, and also when the flower is fully opened (Angenent et al., 1993). By using the *FBP1* promotor to drive *ODO1* expression, we aimed to change the rhythmic scent emission to become continuous. The choice was made to transform petunia Mitchell and not the low-fragrant petunia W138 cultivar, because W138 has up until now never been successfully transformed (R. Koes, pers. comm.).

The 1.1 kb *FBP1* promotor fragment was isolated by (Angenent et al., 1993), who also used it to drive the expression of the Arabidopsis *SUPERMAN* gene in transgenic petunia and tobacco (Kater et al., 2000). For our construct, the *SUPERMAN* open reading frame (ORF) was replaced with that of *ODO1* so that it would be expressed from the *FBP1* promotor (Fig. 1A). Petunia Mitchell leaf discs were treated with *Agrobacterium tumefaciens* containing the *FBP1-ODO1-nos* construct. Twenty independent lines were generated, and the presence of the construct was confirmed by PCR (data not shown).

During the open stages of the flower, the expression of *ODO1* is very high in the evening and night, but it is also still expressed early in the light period (Chapter 3, Verdonk et al., 2005; Chapter 4). *FBP1* is also expressed in the tip of early stage flower buds (Angenent et al., 1993). Therefore, we analyzed the expression of *ODO1* in young flower buds (maximal 2-3 cm long). In those tissues, the endogenous expression of *ODO1* is at low level, and therefore an increase in expression from the transgene should be detectable.
The tips of young flower buds (2-3 cm) of 18 independent transgenic lines and of the wild type Mitchell were harvested at 13:00 h, RNA was extracted and an RNA gel blot analysis was performed. We chose this timepoint because ODO1 expression would be at its lowest point in its diurnal cycle (Verdonk et al., 2005; Chapter 4, Fig. 1B). In the young bud tissue, quantification of ODO1 expression relative to FBP1 revealed that in 10 of the 18 transgenic lines, the expression was more than 1.5 fold elevated over the expression levels in Mitchell (Fig 2). In five lines the expression was lower than in Mitchell; relative ODO1 expression varied between 0.5 to 3.1 fold over all 18 transgenic lines. Because we performed this blot only once, statistical significance of these results could no be determined. Headspace analysis was performed with fully opened flowers of six transgenic lines that had a significant increase of ODO1 expression (Fig. 2 and 3), and the volatile profile was compared with that of Mitchell. We performed the measurements on plants that had been grown in the greenhouse, but the measurements themselves were performed in a growth chamber. Volatiles of three cut flowers placed in a vial were trapped from 11:00 h until 15:30, the time that Mitchell normally produces very low levels of volatiles (Verdonk et al., 2003; Chapter 2; Verdonk et al., 2005; Chapter 4). The quantitative emission of five volatiles was determined: benzyl alcohol (BOH), phenylethylalcohol (PE), benzyl benzoate (BB), methyl benzoate (MeBA), and isoeugenol (IE).
The differences in emission between Mitchell and the transgenic lines were not significant for all of the volatiles, for all six transgenic lines (Fig 3). There was no correlation between the lines with increased ODO1 expression and the lines that emitted more volatile benzenoids. Lines 2-1, 2-10 and 2-18 had a clear increase of ODO1 expression (1.9 to 2.3 fold), but no increased volatile emission (Fig. 3). Then, two lines (2-5 and 2-14) with increased ODO1 expression that had higher emission for PE, IE, and MeBA. Finally, line 2-9 had the highest ODO1 expression compared with Mitchell, but only PE emission was elevated.
Overexpression of ODORANT1 in Arabidopsis

Arabidopsis thaliana is a model plant for different fields of study. Its genome has been fully sequenced, and the availability of numerous T-DNA insertion lines (www.arabidopsis.org) caused an enormous leap forward in the understanding of plant physiological processes. In the field of volatile production the model plant has hardly been used to study floral scent, mainly because Arabidopsis has small flowers that produces only low amounts of volatiles (Vainstein et al., 2001). Furthermore, the floral scent of Arabidopsis consists of sesquiterpenes and monoterpenes, with a complete absence of benzenoids (Chen et al., 2003). To investigate whether it is possible to scale up the flux through the shikimate pathway and perhaps subsequent benzenoid biosynthesis, transgenic Arabidopsis lines were generated with ODO1 driven by the constitutive CaMV 35S promotor (Fig 1B). To achieve this, the construct that previously had been used in bombardment experiments (Verdonk et al., 2005, Chapter 4), was used to transform Arabidopsis thaliana ecotype Colombia plants. Twenty-two independent transformants were obtained, and the presence of the transgene was confirmed by a PCR on genomic DNA (data not shown). Using RNA gel blots, the expression of the transgene in the leaves of the transgenic plants was analyzed. In two independent lines (2.1 and 27.3), ODO1 was indeed expressed (Fig 4).

Figure 3: Quantified emission of volatile benzenoids and the expression of ODO1 by Mitchell (M) and six FBP1-ODO1 overexpression lines (2-1, 2-5, 2-9, 2-10, 2-14 and 2-18). The ratio of ODO1 expression of FBP1 expression as depicted in figure 2 are placed in relation to the volatile measurements. All values were calculated as a ratio of the value measured for Mitchell. For benzyl alcohol, 1.0 corresponds with 1.12 µg / g flower / h; for phenylethylalcohol: 0.7 µg / g flower / h; for benzyl benzoate: 0.011 µg / g flower / h; for isoeugenol: 2.11 µg / g flower / h; and for methyl benzoate: 2.61 µg / g flower / h. Bars annotated with different letters indicate significant differences among lines (analysis of variance, $P < 0.1$ according to least significant differences post-hoc analysis; M: n=6, 2-1: n=2, 2-5: n=3, 2-9: n=2, 2-10: n=2, 2-14: n=4, 2-18: n=2). All experiments were performed with greenhouse-grown plants.
Because ODO1 has been shown to activate transcription of EPSPS (Verdonk et al., 2005, Chapter 4), we investigated whether the transgenic lines that expressed the ODO1 gene, had increased EPSPS transcript levels. The levels of EPSPS transcripts in Arabidopsis leaves were very low, and for the ODO1 expressing lines no increase was detected (Fig. 4). To test whether there would be an effect on the EPSPS activity, we sprayed the plants with a glyphosate solution since overexpression of petunia EPSPS in soybean and cotton resulted in glyphosate resistant plants (reviewed in Herrmann and Weaver, 1999). However, all Arabidopsis plants died.

Discussion

Flower specific ODO1 overexpression

The overexpression of ODO1 in the FBP1-ODO1 transgenic lines is not really convincing based on the one experiment performed. The ratio of ODO1 expression over FBP1 in the tips of 2-3 cm long buds was determined, and a maximal increase of 3.1 fold was observed (Fig. 2). To be conclusive about whether there really is an increased expression of ODO1, the RNA analysis of the overexpression lines will have to be repeated. The choice to drive the ODO1 expression by the FBP1 promotor could also be a problem. Although the expression of FBP1 is high, it could be that higher expression levels are needed. The microarray experiment described in Chapter 3 can perhaps provide candidates with a constitutive high expression in petals. The acyl-coenzyme-A synthetase for example, has high expression levels throughout the day without an apparent cycle (Chapter 3, table 1).

Still, an increase in expression of ODO1 was observed, but it is hard to explain the differences in emitted benzenoids by the overexpression lines, because they do not correlate with the overexpression. The ODO1 expression quantification however, was only based on one experiment. Therefore, the emission levels could perhaps better be observed alone. Nevertheless, the flux through the pathway is not reflected. IE and MeBA are products that are not synthesized further, and their emission was found to be significantly higher in two lines. While BOH, PE, and BB are emitted volatiles, they are also precursors for other
benzenoids, BOH and BB emissions by the transgenic lines were never significantly different of Mitchell, but for PE, in three different lines, the emission was significantly elevated. When other structural enzymes of the benzenoid pathway will be identified, their activities during the diurnal cycle could perhaps explain this. For now, we can only conclude that there seems to be a slight effect on some of the emitted volatiles. It is clear that these experiments will have to be repeated, and perhaps another overexpression approach will have a better chance to succeed. Effects on another branch of the pathway, anthocyanin biosynthesis, are not expected in the petals, but the tubes and the anthers could have a higher supply of precursors for anthocyanin biosynthesis. We did not test this hypothesis however.

**Constitutive overexpression in Arabidopsis**

Introduced expression of ODO1 in Arabidopsis did not result in higher transcript of EPSPS. When ODO1 was over expressed in petunia leaves, an increased EPSPS activity was observed (Verdonk et al., 2005, Chapter 4, Figure 6C). It is possible that the EPSPS promotor is activated by a complex of transcription factors, as has been observed for the DFR promotor in petunia petals, which is activated by a complex of a MYB, a bHLH and a WD40 transcription factor (Koes et al., 2005). In petunia leaves, this other transcriptional activators could have been present, and form a complex with ODO1 that activated the EPSPS promotor. In Arabidopsis, a similar complex might not have been formed, and therefore, no increase of the endogenous EPSPS transcript was observed. Overexpression of ODO1 in closer related species like tobacco or tomato might have more chance to succeed, but on the other hand, the introduction of the flavonoid pathway regulators C1 (a MYB) and Lc (a bHLH) from maize, a monocot, into tomato, a dicot, resulted in high accumulation of flavonols (Bovy et al., 2002). The introduction of C1, alone in tobacco however did not result in a higher flavonoid content, but when R (an bHLH) was also introduced, the anthocyanins accumulated massively (Lloyd et al., 1992). Similar mechanisms may play a role here as well. Future studies to find interactors of ODORANT1 could elucidate the transcriptional activation mechanism that is required to activate the shikimate pathway. If ODO1 acts alone, it would be interesting to see if the two closest related Arabidopsis MYBs, AtMYB42 and AtMYB85 can activate the shikimate pathway genes.

Constitutive overexpression of ODORANT1 was also attempted in petunia Mitchell plants, but from two different attempts, no plants with increased expression of ODO1 were obtained (data not shown). Furthermore, compared with other transformations performed at the same time, the yield of transgenic plants was surprisingly low (first attempt 2 lines,
second attempt 5 lines). Perhaps the ODO1 expression at early stages of development has a negative effect on the plants. The experiments with the FBPI-ODO1 overexpressing lines will have to be repeated to be conclusive, but there seems to be an effect of ODO1 on the emission of volatile benzenoids. The transformants that were yielded seemed to have a tendency for low ODO1 expression; therefore, an inducible promotor system might also be a tool to consider for the future.

Materials and methods

Construction of overexpression constructs of ODO1 cDNA

The cDNA of ODO1 was excised from the phage library according to the manufacturers protocol (Stratagene, La Jolla, CA, USA). Then, the cDNA was cut out of the pBluescript SK(-) vector with SacI and EcoRV, and cloned between the SacI and SmaI site of the pSp72 vector generating the pSp72-ODO1 construct. The SUPERMAN-insert was cut out of the FBPI-SUPERMAN-nos construct, using BsmHI, then it was replaced with the BamHI-BamHI insert containing the ODO1 ORF fragment from the pSp72-ODO1 vector, generating the FBPI-ODO1-nos construct so that ODO1 was driven by the FBPI promoter and terminated by the nopaline synthase terminator (Fig 1A). The EcoRi-BamHI fragment containing the ODO1 ORF from the pSp72 vector was also cloned behind the 35S promotor in pGreen 1K (www.pgreen.ac.uk), which was modified to contain the 35S cassette from pMON999, as described by (Brandwagt et al., 2002) so that ODO1 was driven by the 35S promotor and terminated by the nos terminator (Fig 1B).

DNA isolation and PCR analysis of transgenic plants

Arabidopsis and petunia plants were grown on 0.5 x MS (Duchefa, Haarlem, The Netherlands) plates with a pH of 5.8; supplemented with 50 µg/ml kanamycin. Seedlings (0.5 g) were frozen and homogenized using a pestle in an eppendorf after which 400 µl of extraction buffer (7 M Urea; 0.3 M NaCl; 50 mM Tris-Cl pH 8; 20 mM EDTA; 1% laurosylsarcosine) was added. Two hundred µl buffer saturated Phenol (pH 7.5) and 200 µl CHCl3 were added, the mixture was shaken vigorously and incubated for 15 min at room temperature. The mixture was centrifuged for 5 min, and 300 µl of the water phase was precipitated with 10% 4.4 M NH4Ac, pH 5.2 and 90% isopropanol for 5 min. After centrifuging for 5 min, the pellet was taken up in 100 µl Milli-Q (Millipore, Billerica, MA, USA) and precipitated in 10% 3M Kac, pH 5.5 and 1 volume of isopropanol. The pellet was washed with 70 % Ethanol, and taken up in 20 µl 0.1 µg/µl RNAse A solution. Two µl of this solution was used for PCR. The presence of the 35S-ODO1 construct was confirmed using a forward primer in the ODO1 ORF (5’-CAAAATGGGGATTGATCC-3’), and reverse primer in the nopaline synthase terminator (5’-GACTCTAATCATAAAAACCCA-3’). The presence of the FBPI-ODO1 construct was confirmed by using a forward primer in the FBPI promoter (5’-GAGGGGCTTTAATGCAGATGG-3’), and a reverse primer in the ODO1 ORF (5’-GCATCACTAAGAAGGCCCC-3’).

RNA isolation and RNA gel-blot analysis of transgenic Arabidopsis lines overexpressing ODORANT1

Plants were grown in a growth chamber at 13 h light. After 4 weeks, leaves were harvested for RNA extraction. Leaf tissue (0.5 g) was frozen and homogenized using a pestle in a micro centrifuge tube. Then 1 ml of extraction buffer was added (38% phenol; 0.8 M Guanide Thiocyanate; 0.4 M Ammonium Thiocyanate; 0.1 M NaAc; 5% glycerol; pH 5). After 5 min of extraction 250 µl of CHCl3 was added and the mixture was shaken vigorously for 15 s. The mixture was centrifuged for 5 min, and the water phase was precipitated with one volume iso-propanol, and incubated for 20 min at room temperature. Then, the mixture was centrifuged for 10 min, and the pellet was washed with 70% Ethanol, after which it was dissolved into RNase free Milli-Q. The concentration was determined spectrophotometrically (Nanodrop Technologies, Rockland, DE, USA). For the isolation of RNA of the flower buds of the overexpression lines, one or two young buds of 2-3 cm were harvested and ground in liquid nitrogen in a 12 ml Polypyrrole tube (Cat No.163270, Greiner Bio One, Kremsmuenster, Austria) using a glass rod, using five times the volumes described above. Ten µg of RNA was taken up in 5 volumes reaction mixture (60% DMSO; 20% 6 M glyoxal (deionized); 12% 10 x BTPE; 4.8% Glycerol and 2% Ethidium Bromide (10 mg/ml)), and incubated at 55 °C for 1 h. The RNA was separated on a BTPE gel (for 1 liter 10 x BTPE: 60 g/l Bis-Tris; 30 g PIPES; 20 ml 0.5 M EDTA, pH 8). After that, the gel was blotted onto Hybond XL (Amersham,
Uppsala, Sweden) in 10 mM NaOH. The hybridization and preparation of radioactive probes and the quantification were performed as described previously (Laxalt et al., 2001).

**Volatile measurements**

Volatiles were measured and quantified as described previously by Verdonk et al., (2005; Chapter

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**References**


Overexpression of ODORANT1 in petunia and Arabidopsis
