More than meets the nose

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CHAPTER 6

GENERAL DISCUSSION

Towards understanding regulation of floral scent production: from the *Petunia* genome to the transcriptome and beyond

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To attract different pollinators, wild *Petunias* display a variety in floral traits, such as color, morphology, nectar and floral volatile benzenoid and phenylpropanoid (FVBP) emission. FVBPs biosynthesis and emission by wild *Petunia* species differ in complexity, quantity and timing. Timely emission of a complex blend of FVBPs requires strict regulation. The network of transcriptional regulators known to date only partly explains the regulation of this pathway. Starting from the established role of the R2R3-MYB transcription factor ODORANT1 (ODO1), we have not only added transcription factors to the regulatory network, but also biosynthetic genes and transporters to the FVBP pathway. While trying to identify new relevant genes we noted that their expression patterns are not sufficient to explain the tight regulation of FVBP biosynthesis. Rhythmic changes in protein levels, probably regulated by posttranslational modifications, add another dimension to the regulation of FVBPs biosynthesis in *Petunia*.

Regulation of FVBPs biosynthesis in *PinfS6* and *PaxiN*

Genomic and transcriptional differences have been studied extensively to explain the diversity of floral traits in wild *Petunia*. For example, a transcriptional network of activators and repressors regulates biosynthesis of flower color in *Petunia* [1]. Independent mutations of one of these regulators, *ANTHOCYANIN2* (*an2*), led to loss of color in *P. axillaris* [2]. Loss of FVBP biosynthesis in *P. exserta* was attributed to loss of *ODO1* and *CINNAMATE-CoA LIGASE* (*CNL*) [3,4]. While *P. exserta* presumably is derived from *P. axillaris* and hence lost its ability to produce FVBPs, *P. axillaris* is derived from *P. integrifolia* and gained a more abundant and complex FVBP blend [3,5]. Gaining floral scent is not a straightforward mutation of a single gene or few genes (CHAPTER 2). The recently sequenced genomes of *P. integrifolia inflata S6* (*PinfS6*) and *P. axillaris axillaris N* (*PaxiN*) made an excellent tool to start unraveling FVBP biosynthesis in this thesis (CHAPTER 1).

P. integrifolia is pollinated by bees and *P. axillaris* is pollinated by hawk moths. Emission of FVBPs coincides with pollinator activity. *PaxiN* emits FVBPs at the onset of dark period when hawk moths are active, whereas *PinfS6* mainly emits FVBPs during daytime when bees are active (CHAPTER 2) [6]. In addition, the composition of FVBPs matches with pollinator preferences. The hawk moth *Manduca sexta* has stronger antennal responses to *P. axillaris* floral scent than to *P. integrifolia* [6]. *PinfS6* mainly emits large amounts of benzaldehyde during daytime, and only small amounts of isoeugenol and eugenol (CHAPTER 2). *PaxiN* gained the ability to emit methylbenzoate (MeBA), benzylbenzoate, phenylacetaldehyde and phenylethylbenzoate. Gain of active *S-ADENOSYL-L-METHIONINE : BENZOIC ACID / SALICYLIC ACID CARBOXYL METHYL-TRANSFERASE* (*BSMT*) and *BENZOYL-COA:BENZYLALCOHOL / 2-PHENYLETHANOL BENZOYLTRANSFERASE* (*BPBT*) promoters in *PaxiN* explains the gain of MeBA,

benzylbenzoate and phenylethylbenzoate biosynthesis [4]. However, it does not explain the increase in isoeugenol and eugenol biosynthesis and emission, nor the gain of phenylacetaldehyde biosynthesis by *PaxiN* compared to *PinfS6* [4]. Interestingly, while mutating *ODO1* is an important mechanism for loss of FVBP biosynthesis, *ODO1* is expressed in both *PaxiN* and *PinfS6*. Apparently, multiple genes underlie the difference in FVBP biosynthesis and emission between these two species. Increased expression of most shikimate, arogenate and C_6-C_3 biosynthetic genes seems to lead to increased FVBP biosynthesis by *PaxiN* compared to *PinfS6*. And increased FVBP emission in *PaxiN* compared to *PinfS6* could be caused by increased expression of the transcription factor *PH4* (CHAPTER 2).

Finally, not only the composition and amount of FVBPs is different, but *PinfS6* and *PaxiN* also have different rhythms. Emission of benzaldehyde by *PinfS6* starts earlier, during daytime, than by *PaxiN (*CHAPTER 2). In the hybrid *Petunia* collection another example of differences in the timing and amount of benzaldehyde emission was studied. *P. hybrida* cv. V26 has purple flowers that emit more benzaldehyde, at an earlier time point, than the white flowers of *P. hybrida* cv. W115 (Nur Fariza binti M.Shaipulah, PhD thesis, UvA, unpublished). Studying the promoters showed that the *ODO1* promoter of V26 has only one evening element (EE) compared to two evening elements in the W115 *ODO1* promoter. Expressing *ODO1* under the control of *ODO1* promoters with only one functional EE led to earlier expression of *ODO1* transcript levels in W115. This earlier expression of *ODO1* led to earlier and increased emission of benzaldehyde (Nur Fariza binti M.Shaipulah, PhD thesis, UvA, unpublished). Surprisingly, in *PaxiN* and *PinfS6* the rhythm of *ODO1* expression and transcript levels are similar: expression increases at 08:00 h and decreases at 20:00 h (CHAPTER 2). *PaxiN* and *PinfS6*, like *P. hybrida* cv. V26, both contain one intact and one mutated EE in the promoter of *ODO1*. Instead, differences in daytime expression of the R2R3 MYB EMISSION OF BENZENOIDS II (*EOBII*) seem to correlate with the timing of benzaldehyde emission by *PaxiN* and *PinfS6*. In both *PinfS6* and *PaxiN* expression of *EOBII* increases during the dark period. However, during daytime transcript levels of *EOBII* decrease drastically in *PaxiN*, while in *PinfS6 EOBII* transcript levels remain high. Transcript levels of *EOBII* in *PinfS6* are never lower than at least half of peak *EOBII* transcript levels. We therefore argued in CHAPTER 2 that high expression of both *EOBII* and *ODO1* are required for benzaldehyde biosynthesis. Silencing of *EOBII* indeed leads to reduced benzaldehyde emission, while the effect of silencing *ODO1* on benzaldehyde emission is unknown [7,8]. In both *P. hybrida* cv. V26 and W115, *EOBII* is expressed high during the day and lower at the onset of the dark period. Still benzaldehyde is emitted during this period of lower *EOBII* transcript levels in W115 (Nur Fariza binti M.Shaipulah, PhD thesis, UvA, unpublished). From our data it is not clear whether the hypothesis that EOBII has a role in the timing of benzaldehyde biosynthesis should be accepted or rejected. Therefore it remains to be investigated if

there is a minimal *EOBII* transcript level required at the time of benzaldehyde biosynthesis, and what this minimal transcript level is, or if high *EOBII* transcript levels at a different time points are sufficient for benzaldehyde biosynthesis. Increasing *EOBII* transcript levels in *PaxiN* during daytime (11:00 h – 17:00 h) could answer the question if low *EOBII* transcript levels in *PaxiN* delays benzaldehyde biosynthesis.

PaxiN starts emitting FVBPs at the onset of the dark period, although *PaxiN ODO1* transcript levels are characteristic for daytime FVBP biosynthesis as in *PinfS6* (CHAPTER 2). This shows that not only ODO1 is responsible for the timing of FVBP biosynthesis in *Petunia*, but that apparently something else is causing a delay in FVBP biosynthesis in *PaxiN*. The circadian clock gene LHY directly regulates the circadian rhythm of FVBP biosynthesis in *Petunia* by, among other things, repressing the *ODO1* promoter. LHY also binds to EEs in the promoters of *EPSP1* and *IGS1* [9]. In theory the timing of the precursor supply to the FVBP pathway could be altered by mutations in the EE of the *EPSPS1* promoter. However, the promoters of *EPSPS1* and *IGS1* have the same number of EEs in *PaxiN* and *PinfS6*. Besides changes in *cis* elements such as the EE, the presence or lack of a (unknown) repressor or activator of FVBP biosynthesis in *PaxiN* or *PinfS6* during daytime could cause the difference in timing of FVBP biosynthesis. As discussed in the previous paragraph, the role of EOBII in delaying benzaldehyde biosynthesis in *PaxiN*, and maybe FVBPs biosynthesis in general, should be investigated first. The RNA-seq experiment on *PaxiN* and *PinfS6* was performed at the onset of the dark period, which made the data very suitable to study what causes the differences in amount and composition of FVBP biosynthesis and emission by these species (CHAPTER 2) [4]. However, at this time point *PaxiN* and *PinfS6* are both emitting FVBPs, which makes this dataset not suitable to study what is causing the difference in rhythm. RNA-seq should be performed at a time point when FVBP biosynthesis is low in *PaxiN*, but *ODO1* is expressed and *PinfS6* is emitting benzaldehyde (for example at 11:00 h). To find the factor that is delaying FVBP biosynthesis in *PaxiN*, this data should be compared to *PinfS6* at the same time point and to *PaxiN* a later time point, when *PaxiN* is making FVBPs (for example 20:00 h). If such a factor is identified it would be interesting to see what role it plays in timing of FVBP biosynthesis in other *Petunia* species and hybrid cultivars. It could help to explain why the number of EE in the promoter of *ODO1* is not always corresponding to the rhythm of FVBP biosynthesis in *Petunia* (Nur Fariza binti M.Shaipulah, PhD thesis, UvA, unpublished).

Transcriptional regulation of the different branches in the FVBP pathway

To summarize, many factors underlie the differences in FVBP biosynthesis between *PaxiN* and *PinfS6* (CHAPTER 2). An unknown factor in *PaxiN* delays FVBP biosynthesis, despite daytime *ODO1* expression (CHAPTER 2). The increase in FVBP emission by *PaxiN* compared to *PinfS6* can be explained by increased expression of *PH4* (CHAPTER 2). MeBA, benzylbenzoate and phenylbenzoate biosynthesis by *PaxiN* is explained by the gain of active *BSMT* and *BPBT* promoters [4]. Finally, the general increase of FVBP biosynthesis by *PaxiN* is caused by increased expression of shikimate, arogenate and C_6-C_3 pathway biosynthetic genes (CHAPTER 2). It cannot be exclude that the slight increase in expression of *ODO1* in *PaxiN* is the reason for this general increase in expression of FVBP genes [4]. Interestingly, when *ODO1* is silenced in the fragrant *P. hybrida* cv. W115, expression of shikimate, arogenate and C_6-C_3 pathway genes is reduced (CHAPTER 3). Also the other transcription factors in the ODO1-regulatory network, EOBI, EOBII and LHY (Figure 1), regulate these specific parts of the FVBP pathway. For example, silencing of *EOBII* leads to reduced expression of *CS*, *CM*, *PAL*, *CFAT* and *IGS* [8]. Moreover, EOBI and EOBII both activate the promoters of *IGS1* and *PAL1* [8,10]. And LHY binds to EE in the promoters of *EPSPS1* and *IGS1* [9]. We found that ODO1 directly regulates *DAHPS1*, *EPSPS1*, *PAL1*, *CCoAOMT1*, *CCoAOMT2* and *IGS1* (CHAPTER 3). Especially *EPSPS1*, *PAL1* and *IGS1* thus seem to be under strong regulation of this transcriptional network. By regulating *EPSPS1* and *PAL1*, the transcriptional network directly regulates important biosynthetic steps in the precursor supply for FVBP biosynthesis. In addition we have identified *CINNAMYL ALCOHOL DEHYDROGENASE 1* and *2* (*CAD1* and *CAD2*), two enzymes in the C_6-C_3 branch upstream of isoeugenol and eugenol biosynthesis, to be regulated by ODO1 (CHAPTER 3). Emission of vanillin, eugenol and isoeugenol was reduced upon silencing of *CAD1*. The reduction in emission of vanillin could either indicate that vanillin is synthesized from a later intermediate in the C_6-C_3 branch than previously assumed, or that more vanillin is incorporated in lignin upon reduced precursor supply [11] (CHAPTER 3).

The role of the ODO1, EOBI, EOBII and LHY transcriptional network in regulating the C_6-C_2 and C_6-C_1 branches of the FVBP pathway seems less prominent. ODO1 negatively regulates the expression of *BSMT1*, *BSMT2*, *PAAS2* and *PAAS3*, while EOBII positively regulates *BPBT* expression (CHAPTER 3 and [8]). Changes in the promoters of *BSMT* and *BPBT* are responsible for the gain of MeBA, benzylbenzoate and phenylbenzoate biosynthesis by *PaxiN* compared to *PinfS6*. Still, the gain of these active promoters in *PaxiN* compared to *PinfS6* is not sufficient to explain the high amounts of these volatiles in *PaxiN* [4]. Comparing the expression levels of *ODO1*, *LHY*, *EOBI* and *EOBII* in *PaxiN* to *PinfS6* (CHAPTER 2) indicates that these transcription factors are not responsible for the high amounts of MeBA, benzylbenzoate and

phenylbenzoate biosynthesis in *PaxiN*. Besides MeBA, benzylbenzoate and phenylbenzoate, *PaxiN* also gained the ability to make phenylacetaldehyde. Although *PinfS6* does not make phenylacetaldehyde it does express *PAAS*, but to a lower extent than *PaxiN* (CHAPTER 2). Similar to *BSMT*, *PAAS* is negatively regulated by ODO1 (CHAPTER 3). Finally, *CNL1* is not regulated by ODO1 but is higher expressed in *PaxiN* than in *PinfS6* (CHAPTER 2&3). Despite this, ChIP-seq experiments showed that ODO1 binds to *CNL1* (CHAPTER 3). CNL is an interesting gene, it has be pointed to as a hotspot for evolution of FVBP biosynthesis [12]. In *P. exserta* multiple defects in *CNL1* has led to inactivation [4]. Still, in *PinfS6* benzaldehyde is made in large amounts, showing that CNL1 is functional (CHAPTER 2).

To recapitulate, it seems that the C_6-C_2 and C_6-C_1 branches of the FVBP pathway are not under strong regulation of the ODO1, LHY, EOBI and EOBII transcriptional network. This suggests that other regulators are in charge. These unknown regulators could be identified by yeast-1-hybrids (Y1H) with the promoters of the most interesting C_6-C_2 and C_6-C_1 biosynthetic genes, namely *CNL1*, *BSMT1*, *BPBT1* and *PAAS1*. In addition, the promoters of these genes could be used to identify common binding sites. Using the putative promoters (2000 bp) of all *CNL*, *BSMT*, *BPBT* and *PAAS* copies of *PaxiN* did not lead to motif discovery by MEME (http://meme-suite.org/tools/meme, motif width 6-10). TCCCTCCACC was discovered as a consensus motif in the *PinfS6* promoters. This motif has similarities to MYB and WRKY binding sites in *Arabidopsis*, although these similarities were not significant (http://meme-suite.org/tools/tomtom, Arabidopsis DAP motifs database). Interestingly, the *PaxiN CNL1* and *BSMT5* promoters do contain an EE, similar to the EEs found in the *IGS* and *EPSPS* promoter (AAGATATTT). This EE is located 1995 bp upstream of the start codon in the *CNL1* promoter. This EE sequence is mutated in the *PinfS6 CNL1* promoter (tAGtTATT). Perhaps this EE present in the *PaxiN*, but not in the *PinfS6* promoter of *CNL1*, is contributing to the delay of benzaldehyde biosynthesis in *PaxiN* compared to *PinfS6*. Including promoters of more C_6-C_2 and C_6-C_1 biosynthetic genes in motif analysis could increase the chance of discovering common motifs. Still, a Y1H experiment to discover transcription factors binding to the *CNL1*, *BSMT1*, *BPBT1* and *PAAS1* promoters is more powerful than motif analysis. It is unclear if the genes in the C_6-C_2 and C_6-C_1 branch are regulated similarly and have the same promoter motifs. Moreover, motifs can usually be bound by a class of transcription factors and do not point to a specific transcription factor.

Discovering atypical transcriptional regulators of FVPB biosynthesis

So far all transcription factors with a characterized role in FVBP regulation in *Petunia* are MYB transcription factors. MYBs regulate besides secondary metabolism, also specialized cell and tissue development in plants [13]. The biological role of MYB subgroups is usually well conserved within angiosperms [14]. Gene expression patterns and evolution of target promoter sequences determine the function of specific MYB transcription factors within and between plant species [14]. Interaction with different basic-helix-loop-helix (bHLH) or WD40 transcription factors can specify the function of MYB transcription factors [14-17]. In these MYB-bHLH-WD40 (MBW) protein-complexes bHLH proteins are often redundant, and conserved WD40 proteins are shared between complexes [13]. A good example of the regulation of phenylpropanoid biosynthesis by MBW protein-complexes in *Petunia* is the biosynthesis of color [1,18]. We have used ODO1 to identify transcription factors regulating FVBP biosynthesis. With RNA-seq we have found WRKYs and heat shock transcription factors (HSTF) / heat shock factors (HSF) that are regulated by ODO1 (CHAPTER 3), and with yeast-2-hybrid (Y2H) we have found two proteins of unknown function to interact with ODO1 (CHAPTER 5). We did not identify bHLH or WD40 transcription factors, the usual interacting partners of MYBs regulating phenylpropanoid biosynthesis. However, we did identify another MYB transcription factor of unknown function with the RNA-seq experiment described in CHAPTER 3. It could be that ODO1 does not require interactions with bHLH and WD40 proteins to function, like some other MYB transcription factors [13]. The Y2H results are not sufficient to exclude interaction of ODO1 with bHLH or WD40 protein. However, it does show that interaction of ODO1 with such proteins is not so common or strong that it is easily detected by Y2H.

WRKYs are often associated with stress responses of plants. Nevertheless, WRKY transcription factors are also key regulators of secondary metabolism [19]. Although MYB, bHLH and WD40 proteins are generally considered the key regulators of phenylpropanoid biosynthesis, there are numerous examples of WRKYs regulating phenylpropanoid biosynthesis [19]. Even more, recently WRKYs have been shown to interact with MBW protein complexes in regulating pigmentation [20]. In *Arabidopsis* a MBW complex regulates expression of *WRKY44*, a regulator of anthocyanin biosynthesis [21]. Still, it cannot be excluded that the WRKYs that we have identified, WRKY3 and WRK4, are regulated by both ODO1 and biotic stress. WRKY3 and WRKY4 show high similarity to two WRKYs in *N. tabacum* that are induced by pathogen infection and salicylic acid (SA) [22]. Biotic stress is known to induce phenylpropanoid biosynthesis [23,24]. SA can activate *PAL* and tobacco mosaic virus can induce CAFFEIC ACID/5-HYDROXYFERULIC ACID 3/5-O-METHYLTRANSFERASE (*COMT*) and *CCoAOMT* [25-27]. A volatile from the COMT and CCoAOMT pathway, eugenol, has been shown to have antiviral properties against tomato yellow leaf curl virus [28]. In addition, when

infiltrating *Petunia* petals with *Agrobacterium tumefaciens* for transient transformation purposes we noticed a decrease in FVBP emission and an increase in MeSA emission (data not shown). MeSA is one of the few FVBPs that is not regulated by ODO1 [7]. The precursor of MeSA, salicylic acid, is induced upon pathogen infection and can activate PAL [26,27]. Generally biotic stress induces phenylpropanoid biosynthesis. However, we noticed a decrease in the emission of most FVBPs upon *A. tumefaciens* infection (data not shown). We did not measure internal FVBPs pools. It could be that upon *A. tumefaciens* infection FVBP biosynthesis remains the same or even increases and that only FVBP emission is reduced. In this case antiviral compounds such as eugenol could remain at the site of infection. Reduced emission of FVBPs and increased internal pools of FVBPs are seen with increased temperatures, a form of abiotic stress [29,30]. It could also be that phenylpropanoid intermediates are redirected from FVBP biosynthesis to other pathways with roles in plant defense. Either way, additional research is required to start understanding if and how biotic stress effects FVBP biosynthesis and emission.

Global climate change has initiated research on the effect of temperature on FVBP biosynthesis and potentially pollination. FVBP biosynthesis and emission by *Petunia* is not only sensitive to the circadian clock and light, but also to temperature [29]. In *P. axillaris* FVBP emission increases while the internal FVBP pool decreases upon increasing temperatures [31]. Whereas in *P. hybrida* cv. P720, Blue Spark and W115 FVBP emission decreased while the internal FVBP pool increased upon elevated temperatures [29,30]. In addition, the expression of the MYB transcription factor *EOBV*, but not *ODO1*, *EOBI* and *EOBII*, increased upon elevated temperatures in *P. hybrida* cv. P720 and Blue Spark [30]. Interestingly, it has been shown that heat stress can have different effects depending on whether it is day or night, for instance in grapevine, on phenylpropanoid biosynthesis [32]. HSFs and heat shock treatment can suppress the induction of *PAL* by wounding [33,34]. Heat shock proteins are not only considered an interaction point between different stresses but also in cell differentiation, development and life span [29,35,36]. Careful analysis is required to understand the relationship between heat stress and the FVBP pathway in *Petunia*. The dark and light period should be taken into consideration, and could be responsible for the inconsistency in the effect of temperature on FVBP biosynthesis in previous studies. The HSTF and HSF regulated by ODO1 could play an important role in integrating temperature stress into FVBP regulation. ODO1 could in this case regulate the time-dependent effect of heat stress on FVBP biosynthesis and emission.

In our search for regulators of FVBP biosynthesis we found some surprising candidates. We expected to find MYB, WD40 and bHLH transcription factors when looking for regulators of phenylpropanoid biosynthesis. WRKY and HSTF /HSF were not the first type of transcription factors we expected to find. Research of WRKYs has mostly focuses on stress response, but WRKYs are also known to have a role of in

phenylpropanoid biosynthesis [19,20]. Temperature is known to affect FVBP biosynthesis and emission in *Petunia*. As heat shock proteins are known to be masters in integrating pathways, the roles of HSTF and HSF in this matter are worthwhile studying. Heat shock treatment and heat shock proteins have been implicated to modulate the effect of biotic stress on phenylpropanoid biosynthesis [37-39]. Besides these WRKYs and heat stress transcription factors, we also have discovered a MYB regulated by ODO1 and two proteins of unknown function to interact with ODO1. Unfortunately, we were unable to obtain lines in which these genes were silenced by RNAi and have thus been unable to elucidate the role of these genes in FVBP biosynthesis.

Transforming *Petunia* transformations

RNAi has been used many times to successfully silence genes in *Petunia*. For example, *ODO1* was strongly silenced in *P. hybrida* cv. W115 [7]. This silenced line was used in CHAPTER 3 to comprehend the role of ODO1 in regulating FVBP biosynthesis. However, this same technique failed in silencing most genes in this thesis. RNAi only reduced gene expression in CAD1-ir lines and some CAD2-ir lines (CHAPTER 3). The silencing of two transporters, five transcription factors and two genes of unknown function was not successful (CHAPTER 3 and 5). Once *Petunia* was at the start of discovering RNAi, but nowadays the world is rapidly been taken over by CRISPR/Cas. CRISPR/Cas enables precise, heritable insertions and deletions in eukaryote genomes. The potential of CRISPR/Cas for mutating genes in *Petunia* is promising, based on successes in other plant species such as *Arabidopsis*, *N. benthamiana*, tomato and rice [40-42]. In the Netherlands a consortium of breeding companies and universities has combined efforts to develop an efficient CRISPR/Cas system for *Petunia* (http://www.sia-projecten.nl/projectenbank/project/petunia-and-crisprcas-technology). Recently a study on CRISPR/Cas in *Petunia* has been published [40]. CRISPR/Cas9 mutation of a gene essential for chlorophyll biosynthesis was studied, because of easy phenotypic detection. Efficiencies of 55,6-87,5% were reported for CRISPR/Cas in *Petunia* [40]. In our laboratory the first experiments to mutate biosynthetic and regulatory genes resulted in less than 5% success rate (data not shown). Although CRISPR/Cas will be the standard for mutating genes in *Petunia* in the near future, additional research will be required to reach that point. When developing CRISPR/Cas for *Petunia*, tissue specificity of the technique should be taken into account. In our laboratory, and the study published on CRISPR/Cas in *Petunia*, the 35S promoter is used. However, the activity of the 35S promoter in embryogenic cells is minimal, leading to the preferences for housekeeping genes such as ubiquitin to obtain homozygous lines [41-43]. Even more, the success of specific promoters for CRISPR/Cas differs between plant species [42].

For all RNAi silencing experiments performed in this thesis we have used the CaMV 35S promoter. The 35S promoter was proven successful in silencing for example the transcription factor *ODO1* and biosynthetic gene *KAT1* [7,44]. However, for one of the unknown genes from CHAPTER 5 no transgenic plants were obtained. The disadvantage of the 35S promoter is, that when a gene is essential, no transgenic plants might be obtained or silencing might be repressed. The shikimate pathway does not provide only precursors for the phenylpropanoid pathway, but is essential for phenylalanine, tyrosine and tryptophan biosynthesis in plants. Moreover, phenylpropanoids are the precursor for lignin. If a gene is essential for development or growth, using a flower or petal specific promoter for silencing can increase the changes of creating a viable plant with silenced flowers. Recently, *ADT1* and *PhABCG1* were successfully silenced in *Petunia* petals by RNAi using a petal-specific linalool synthase promoter from *Clarkia breweri* [45,46]. Using this promoter in future studies could increase the success rate of RNAi in petal tissue. Compared to silencing genes by RNAi, constitutive mutation of genes by CRISPR/Cas could give even more problems when genes are essential for growth and development.

Previously silencing of EOBII by RNAi in *Petunia* led to failure of flower opening [47]. Therefore to study the effect of EOBII on FVBP biosynthesis virus induced gene silencing (VIGS) was used [8]. The technique depends on simultaneously silencing of chalcone synthase to visualize successful silencing. This requires VIGS to be carried out in *Petunia* species with colored flowers [48]. The disadvantage is that *Petunia* species with white flowers, such as *P. axillaris* and *P. hybrida* cv. W115, produce a more complex blend of FVBPs and are usually used in studies on FVBP biosynthesis. For example, *P. hybrida* cv. P720 used in the EOBII study does not produce methyl benzoate, the most abundant volatiles in *P. axillaris* and *P. hybrida* cv. W115 [8,48]. We have syringe-infiltrated *P. hybrida* W115 petals with *Agrobacterium tumefaciens* harboring RNAi constructs to study petal specific gene silencing. We co-infiltrated petals with 35S:AN2, and used color as a visual cue for successful infiltration and gene expression. As mentioned above we observed that *A. tumefaciens* infiltration affects FVBP emission (data not shown). Therefore we did not continue with this technique. The role of PPY-AT in FVBP biosynthesis in *Petunia* has been studied by vacuum infiltration of *Petunia* petals with *A. tumefaciens* harboring a RNAi construct [49]. In this study FVBP emission was only compared between petals infiltrated with the RNAi construct or empty construct. In the VIGS experiment FVBP emission of *EOBII* silenced petals was only directly compared to mock treated petals. However, when looking at emission of FVBPs by untreated petals in the same article, VIGS also seems to reduce FVBP emission [48]. In summary, although VIGS and *A. tumefaciens* transient silencing allows rapid analysis of gene silencing in a petal specific manner, there are common disadvantages. In both techniques FVBP emission is altered by the experimental procedure, which in the best case only reduces the strength of the methods. In the

worst case this could lead to wrong interpretation on the role of certain genes in FVBP biosynthesis. Besides this, the transient nature of these techniques does not allow analysis in homozygous generations. Therefore CRSPR/Cas mutation is the preferred method for studying the role of genes in FVBP biosynthesis. For genes with also a potential role in plant development and growth petal-specific silencing of genes by RNAi is the most reliable option since petal specific mutation of genes by CRISPR/Cas is not available.

Regulating the transcriptional network

Volatiles can attract pollinators, but are also costly to produce and can attract herbivores and florivores [50,51]. Therefore FVBP emission by *Petunia* coincides with pollinator activity and reproductive state of the flower. Rhythmic, petal and developmental stage specific expression of *ODO1* regulates this precise biosynthesis of FVBPs in *Petunia* [7,52]. Pollinators and florivores can evolutionary shape the regulation of FVBP biosynthesis. Light, temperature and biotic stress can temporarily change FVBP biosynthesis. There is a lot of literature on the timing and evolution of transcriptional regulation of FVBP biosynthesis. And recently the interest on the effect of temperature on FVBP biosynthesis and emission is growing due to global warming [29-31]. The discovery of two WRKYs regulated by ODO1 and with a potential role in defense (CHAPTER 3) shows that research on the effect of biotic stress on FVBP biosynthesis and emission is needed. Little is known about defense against biotic stress in flowers, but a recent study showed that jasmonate signaling regulating floral defense is flower specific in wild tobacco [53].

We propose that the transcriptional network around ODO1 allows integration of these different environmental, developmental and circadian signals into precise regulation of FVBP biosynthesis and emission (Figure 1). The selective pressure of pollinators and florivores evolutionary shapes the transcriptional network. For example, in *P. exserta* the mutation of *ODO1* resulted in loss of FVBP biosynthesis [3]. Increased expression of *PH4* in *PaxiN* probably underlies increased emission of FVBPs (CHAPTER 2). Light and circadian clock dependent FVBP emission is controlled by activation or inhibition of the *ODO1* promoter by EOBI&EOBII and LHY, respectively. This regulates the daily timing of FVBP biosynthesis and emission. Interaction of ETHYLENE RESPONSE FACTOR 6 (ERF6) with EOBI inhibits activation of the *ODO1* promoter [54]. In *Petunia* ethylene signaling leads to senescence of flowers [55]. It was suggested that FVBP biosynthesis is also regulated by ethylene [56], limiting FVBP biosynthesis and pollinator attraction to the developmental stage when flowers need to be pollinated. Also gibberellic acid (GA) was shown to be involved in the developmental timing of FVBP biosynthesis. *ODO1*, *EOBI* and *EOBII* transcript levels were reduced when a GA biosynthetic gene was overexpressed [57]. Suppression of

DELLA1, a negative regulator of GA biosynthesis, only down regulated expression of *EOBI* and *EOBII* [57]. The circadian clock, light, GA and ethylene signaling thus regulate the timing of FVBP biosynthesis. The LHY, EOBI, EOBII and ODO1 transcriptional network, which integrate these factors in timely FVBP biosynthesis, has been extensively studied. Still, many questions remain. For example, what is responsible for the delay in FVBP biosynthesis in *PaxiN* compared to *PinfS6*, despite similar *ODO1* expression rhythms? How does light regulate EOBI and EOBII? And what does the transcriptional regulation of FVBP biosynthesis by GA look like?

As discussed above temperature is known to effect emission of FVBPs in *Petunia*. However, literature on this matter is contradicting and the role of EOBV in this has not been characterized [29-31]. Also the two heat shock transcription factors regulated by ODO1 discovered in CHAPTER 3 need further characterization. The effect of biotic stress on FVBP biosynthesis and emission is even less studied. The WRKYs regulated by ODO1 (CHAPTER 3), and two putative interactors of EOBII with high similarity to proteins with a function in virus response (CHAPTER 5), require additional research. The reduction in FVBP emission upon VIGS and *A. tumefacians* transient silencing experiments suggest that biotic stress could affect FVBP biosynthesis and emission. Interestingly, a review studying BSMT-type enzymes in plants suggest that floral benzenoid biosynthesis might be derived from plant defense related benzenoid biosynthesis [58]. Emission of one of the products of this enzyme, MeSA, was increased upon *A. tumefaciens* infiltration of *Petunia* petals (data not shown). Are these biotic defense components in the FVBP pathway of *Petunia* rudimentary, do they still have a function in defense or have they evolved to roles in pollinator attraction? Do temperature and biotic stress interfere with the core transcriptional network of FVBP biosynthesis around ODO1 in *Petunia*? Or as suggested by the model in its current form, are signals from ODO1, temperature and biotic stress combined in transcriptional regulation of FVBP biosynthesis downstream of this core transcriptional network (Figure 1)? It is clear that when we zoom out from the network of transcription factors regulating FVBP biosynthesis, that there is still a lot to discover on how different signals are combined into accurate responses to environmental signals and precise timing of FVBP biosynthesis and emission in *Petunia*.

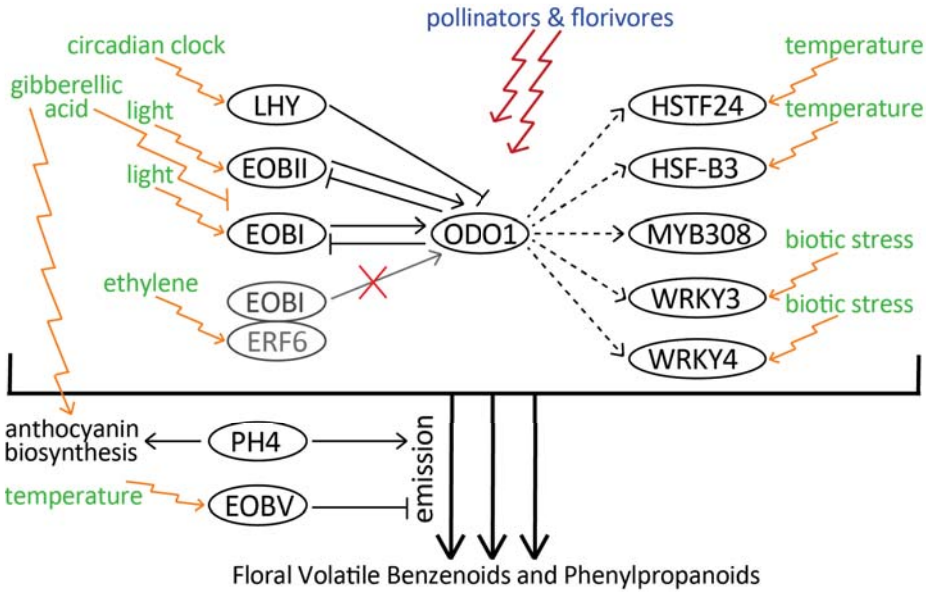

Figure 1. Model of environmental, developmental and circadian signals integrated in the transcriptional network regulation floral volatile benzenoid and phenylpropanoid (FVBP) biosynthesis in *Petunia*. Transcription factors are circled. Arrows or blunt arrows depict activation or repression of transcription factors, respectively. In green are environmental, developmental or circadian signals that regulate expression of the transcription factors. Pollinators and florivores drive the evolution of transcription factors regulating FVBPs. Abbreviations: ODO1: ODORANT1; EOB: EMISSION OF BENZENOIDS; LHY: LATE ELONGATED HYPOCOTYL; ERF6: ETHYLENE RESPONSIVE FACTOR6; HSTF24; HEAT SHOCK TRANSCRIPTION FACTOR24; HSF-B3: HEAT SHOCK FACTOR-B3.

Regulating the regulator

So far we have mainly spoken about transcriptional regulation of FVBP biosynthesis, and only touched on the field of posttranslational regulation when looking for interacting partners of ODO1 and EOBII. With the pODO1:GFP-ODO1 expressing *Petunia* lines generated for the ChIP-seq experiment we have made a start with studying often overlooked events: posttranslational regulation of transcription factors.

While many genes in the FVBP pathway are expressed rhythmically this does not necessarily mean that the protein levels change rhythmically too. In *Arabidopsis*, a large-scale study showed that of the many genes with diurnal expression rhythm only a few actually have diurnal cycling protein levels [59]. In *Petunia* the activity of BSMT did not have such a strong diurnal pattern as the transcript levels of *BSMT* [60]. We observed with confocal microscopy and anti-GFP western blotting that GFP-ODO1 protein levels

are low in the morning and high at the onset of the dark period, similar to transcript levels of *ODO1* (CHAPTER 4). In addition, Fariza Shaipulah showed by confocal microscopy that the GFP signal of GFP-ODO1 is weak in the morning and strong at the onset of the dark period. In addition she showed that shifting *ODO1* expression forward, shifts the onset of GFP fluorescence from GFP-ODO1 forward too (Nur Fariza binti M.Shaipulah, PhD thesis, UvA, unpublished). Cycling of ODO1 protein levels suggests active degradation by for example ubiquitination. Proteins that do follow the rhythmic cycling of their transcript levels are believed to be key regulators, determining the timing of a pathway [61]. Rhythmic protein levels of ODO1 regulating the timing of a few biosynthetic genes in the FVBP pathway could be sufficient for correct timing of FVBP biosynthesis. PAL, the first committed enzyme to C_6-C_1 and C_6-C_3 biosynthesis, has rhythmic activity levels [60]. *PAL* is one of the genes that is directly regulated by ODO1 and by EOBI and EOBII (CHAPTER 3) [8,10]. This rhythmic activity of PAL can thus regulate rhythmic precursor supply and hence rhythmic FVBP emission. It would be interesting to see if protein levels of the other genes directly regulated by ODO1 and regulated by EOBI, EOBII and/or LHY are also rhythmic. This would be especially interesting for *EPSPS1*, *PAL1* and *IGS1* as noted before. Biosynthetic genes in the FVBP pathway that do not have such a key role in the timing of FVBP biosynthesis do not need to have rhythmic protein levels.

The cycling of ODO1 at the protein level is thus important for regulating the timing of FVBP biosynthesis. However, we have very little insight on how ODO1 abundance or activity is regulated at the protein level. Repression of the *ODO1* promoter by LHY, and activation by EOBI and EOBII, regulates timing of *ODO1* expression (Figure 2). An important assumption for this is that EOBI and EOBII protein levels closely follow their transcript levels, as is expected if EOBI and EOBII are key regulators of timely FVBP emission. Protein levels of LHY are rhythmic in *Arabidopsis* [62]. In addition to LHY, the direct binding of ODO1 proteins to the *ODO1* DNA sequence could also reduce transcription of *ODO1* (CHAPTER 3) (Figure 2). What composes the transcriptional input of rhythmic ODO1 proteins levels is thus fairly well understood. However, what regulates fast degradation of ODO1 proteins to ensure rhythmic levels is unknown. Ubiquitination and phosphorylation are known to posttranslationally regulate MYB and bHLH transcription factors, and are good candidates to start studying post-translational regulation of ODO1 [63]. In addition we have found two uncharacterized interacting partners of ODO1 (IPO1 and IPO2) (CHAPTER 5). Interesting, IPO2 has the same molecular size as the posttranslation modification of GFP-ODO1 as seen on anti-GFP western blots (CHAPTER 4). IPO1 and IPO2 could change the activity of ODO1 by changing the binding of ODO1 to DNA or by changing the localization of ODO1 (CHAPTER 5).

To better understand how FVBP biosynthesis is regulated in *Petunia*, more focus on proteins involved is needed in the future. Transcript levels do not translate

one to one in protein levels and activity. There are many post-translational modifications that can regulate the level and activity of a protein and hence regulate FVBP biosynthesis. The answers to what is required for strong activation of promoters by ODO1 and EOBII [52,64], what is delaying FVBP biosynthesis in *PaxiN* (CHAPTER 2) and how different environmental signals are integrated in a FVBP biosynthesis output might be all waiting the proteome.

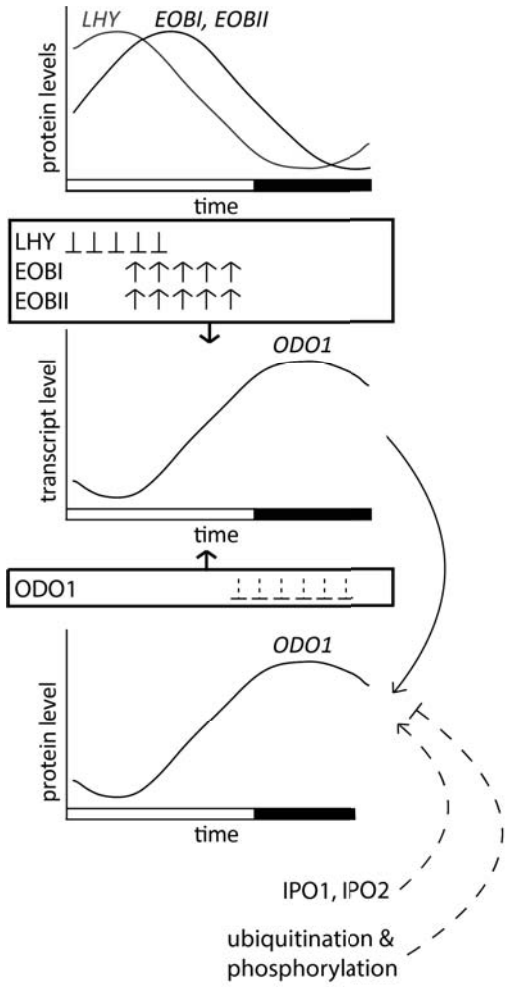

Figure 2. A model for transcriptional and posttranslational regulation of ODO1. *ODO1* transcript levels are positively regulated by EOBI and EOBII and negatively regulated by LHY and ODO1. ODO1 transcript levels in turn regulate ODO1 protein levels. ODO1 protein levels and activity are also potentially regulated by IPO1, IPO2 ubiquitination and/or phosphorylation. Arrows represent activation, and blunt arrows represent inactivation. Steps indicated by dashed lines are uncharacterized. Abbreviations: ODO1: ODORANT1; EOB: EMISSION OF BENZENOIDS; LHY: LATE ELONGATED HYPOCOTYL; IPO: INTERACTING PARTNER OF ODO1.

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