The biochemistry and genetics of floral scent production as part of the petunia pollination syndrome

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CHAPTER 6

General discussion

1. Circadian rhythm of floral scent

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   Does the EE in wild petunia contribute to late emission of FVBP?

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GENERAL DISCUSSION

Different species of plants advertise unique floral features which specialize in attracting particular animals or group of animals. A suite of floral traits such as color, scent, shape and nectar volume have evolved to maximize pollination by animals. Ensembles of these floral traits demonstrate the adaptive value in pollination syndromes (Fenster *et al.*, 2004).

Among the traits related to a pollination syndrome, floral scent is known as an honest signal, that associates with the quality and quantity of nectar (Wright and Schiestl, 2009). Floral volatiles serve as signals to specific pollinators through emission at a specific time of the day when these pollinators are active. In petunia, floral scent production is temporally regulated by a few transcription factors. Although many studies have reported the rhythmic emission of floral volatiles in plants (Verdonk *et al.*, 2003, Effmert *et al.*, 2005, Kong *et al.*, 2012, Whitehead and Peakall, 2014), only for a few species the contribution of circadian clock in floral scent has been demonstrated (Kolosova *et al.*, 2001a, Fenske *et al.*, 2015, Yon *et al.*, 2016). In recent work, a clock gene, *LATE ELONGATED HYPOCOTYL* (*LHY*) has been shown to involve in regulating the timing of emission in petunia and *Nicotiana attenuata* flowers (Fenske et al., 2015, Yon et al., 2016).

1. Circadian rhythm of floral scent

The circadian clock has been shown to be involved in regulating volatile emission in petunia flowers (*P.hybrida* cv. Mitchell, *P.axillaris*) and snapdragon (*Antirrhinum majus*) (Kolosova *et al.*, 2001a, Oyama-Okubo *et al.*, 2005, Fenske *et al.*, 2015), but molecular components of this "scent" circadian clock have only been discovered recently (Fenske *et al.*, 2015). The emission of *P.hybrida* cv. Mitchell floral volatile benzenoids /phenylpropanoids (FVBP) is rhythmic and peaks in the dark (Verdonk *et al.*, 2003), whereas snapdragon flowers rhythmically emit methylbenzoate with its maximum peak during the day (Kolosova *et al.*, 2001a). The rhythmic emission by these two plant species during night and day period coincides with foraging activities of hawkmoths and bees, respectively. The question remains: how is the rhythm of floral scent emission achieved?

FVBP emission in *P.hybrida* cv. Mitchell shows a circadian oscillation in continuous dark, but not in continuous light, suggesting that the timing of FVBP genes expression is influenced by the circadian clock (Fenske *et al.*, 2015). In general, the expression of circadian clock genes maintain rhythmicity within periods of 24h in continuous light or darkness (McClung, 2006). This is the case for snapdragon flowers, for which the emission of methylbenozoate remains rhythmic in continuous light and dark, indicating that the circadian clock is more pronounced in these flowers (Kolosova *et al.*, 2001a). *Phenyacetaldehyde synthase* (*PAAS*), S-*adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase* (*BSMT*), *benzoyl-CoA:benzylalcohol/2-phenylethanol benzoyltransferase* (*BPBT*) and *isoeugenol synthase* (*IGS*) are biosynthetic genes encoding the enzymes in the final steps for the production of phenylacetaldehyde, methylbenzoate, benzylbenzoate and isoeugenol respectively in *P.hybrida* cv. Mitchell (Negre *et al.*, 2003, Boatright *et al.*, 2004, Kaminaga *et al.*, 2006, Koeduka *et al.*, 2008). The expression of these genes oscillate in the light and dark (L/D) cycle, but *BSMT*, *IGS* and *PAAS* do not sustain this oscillation in continuous dark (Fenske *et al.*, 2015, Cheng *et al.*, 2016). In snapdragon flowers, BAMT activity does not oscillate during the L/D cycle (Kolosova *et al.*, 2001b), indicating these biosynthetic genes are not an oscillation-determining factors. Interestingly, the levels of benzoic acid (BA), the precursor of

methylbenzoate change rhythmically in L/D cycle in *P.hybrida* cv. Mitchell and snapdragon flowers, with maximum levels at night and day, respectively. The BA rhythmicity is sustained in continuous dark, correlating with the emission of methylbenzoate (Kolosova *et al.*, 2001a). The level of L-phenylalanine (Phe), the precursor from which all FVBPs are derived, in the *P.hybrida* cv. Mitchell and snapdragon flowers also oscillates in L/D cycle with maximum peaks at night and day, respectively, concurrent with the timing of FVBP emission (Kolosova *et al.*, 2001a, Maeda *et al.*, 2010). The Phe level also peaks at night in *Nicotiana attenuata* flowers, correlating with the night emission of benzyl acetone (Kim *et al.*, 2011). Together, this suggests that the "scent" circadian clock influences the precursor availability and additionally the expression of FVBP genes in the final step of benzenoid/phenylpropanoid pathway.

The genes encoding enzymes in the upstream pathways and the regulators that control the precursor genes may control the precursor levels at night in *P.hybrida* cv. Mitchell. The transcript levels of the shikimate/arogenate pathway genes and upstream genes of FVBP biosynthesis: 5-enolpyruvylshikimate-3-phosphate (*EPSPS*), 3-deoxy-D-arabino-heptulosonate-7-phosphate (*DAHPS*) and arogenase dehyratase (*ADT*), and phenylalanine ammonia lyase (*PAL*), cinnamate 4-hydroxylase (*C4H*), 4-coumarate CoA ligase (*4CL*) display oscillatory patterns in L/D cycle, in which the highest transcript levels are 1 to 3 hour before the onset of dark period. Importantly, these genes sustain their rhythmic expression in continuous dark, suggesting the circadian emission of FVBP at night is mainly regulated in the upstream pathway (Fenske *et al.*, 2015, Cheng *et al.*, 2016). For a few of the precursor genes, the transcription factors that regulate them have been identified. The R2R3-MYB ODORANT1 (ODO1) regulates the shikimate pathway in *P.hybrida* cv. Mitchell, by activating the *EPSPS* promoter (Verdonk *et al.*, 2005) (Fig. 1). Although a direct interaction was not shown in flowers, ODO1 is able to activate the *EPSPS* promoter in petunia leaves (Verdonk *et al.*, 2005). Down-regulation of *ODO1* reduced the expression of shikimate pathway genes and emission of FVBP, as well as benzoic acid pools (Verdonk *et al.*, 2005). The role of *ODO1* in regulating petunia volatiles has been further supported by the identification of this gene as a major Quantitative Trait Loci (QTL) in scent production (Klahre *et al.*, 2011). Two MYB binding sites (MBS) in the *ODO1* promoter are necessary for high promoter activity in fragrant petunia (Van Moerkercke *et al.*, 2011). In addition, *P.hybrida* cv. Mitchell promoter contains two evening elements (EEs), which are located directly downstream of each MBS (Chapter 4). The EEs are potential binding sites for the circadian clock transcription factor, LATE ELONGATED HYPOCOTYL (LHY) in *P.hybrida* cv. Mitchell flower (Fenske *et al.*, 2015). Reduced expression of *LHY* leads to early production of scent, while constitutive expression of *LHY* leads to a strong reduction of scent production (Fenske *et al.*, 2015). Chapter 4 shows that transgenic Mitchell plants with an additional copy of ODO1, driven by its own promoter, but with the EEs deleted, expressed *ODO1* earlier and emitted FVBP earlier, but the expression of most biosynthetic genes was not affected. Furthermore, these transgenic flowers emitted more benzaldehyde and less benzylbenzoate and isoeugenol (Chapter 4). This phenotype has not been observed in suppression lines of *LHY*. Reduced expression of *LHY* not only shifted the peak expression of *ODO1*, but other FVBP genes as well (*EPSPS*, *chorismate mutase 1*(*CM1*), *ADT*, *PAL*, *3-ketoacyl-CoA ketothiolase* (*KAT1*) and *BPBT*) toward the morning (Fenske *et al.*, 2015). LHY binds to the EE sites in other promoters (*EPSPS* and *IGS*) as well, and possibly can bind to other promoters of genes in FVBP pathway, suggesting that LHY is likely setting the phase of *ODO1*, *EPSPS* and other FVBP genes for evening expression (Fenske *et al.*, 2015). Chapter 4 shows that lack of EEs did not lead to arrhythmic expression of *ODO1*, suggesting that other circadian clock factors than LHY are also involved regulating the rhythmic expression of

ODO1. Two clock homologs in *P.hybrida* cv. Mitchell, GIGANTEA (GI) and PSEUDO RESPONSE REGULATOR 5 (PRR5) showed same expression pattern as *ODO1,* peaking in the evening, suggesting LHY and these circadian clock genes may work in concert to regulate rhythmic FVBP production in *P.hybrida* cv. Mitchell (Fenske *et al.*, 2015). The R2R3-MYBs *EOBI* and *EOBII* that activate the *ODO1* promoter (Van Moerkercke *et al.*, 2011, Spitzer-Rimon *et al.*, 2012) do not oscillate in continuous dark (Fenske *et al.*, 2015), hence, we exclude their contribution. It seems that the interaction of LHY with the EEs in the *ODO1* promoter, determines the timing of *ODO1* expression and the downstream precursor genes in the hours preceding production of FVBPs.

How does the earlier expression ODO1 affect the composition of the FVBP?

As described above, benzoic acid (BA) levels in snapdragon and *P.hybrida* cv. Mitchell flowers correspond with the rhythmic emission of methylbenzoate during the day and night, respectively (Kolosova *et al.*, 2001a). However, it is still a question which pathways contribute to BA biosynthesis. Genetic evidence showed that BA production in *P.hybrida* cv. Mitchell flowers dominantly occurs via the β-oxidative pathway; cinnamoyl-CoA → 3-hydroxy-3-phenylpropanoyl-CoA (3H3PP-CoA) → 3-oxo-3-phenylpropanoyl-CoA (3O3PP-CoA) → Benzoyl-CoA → BA in peroxisome during the night (Orlova *et al.*, 2006, Van Moerkercke *et al.*, 2009, Colquhoun *et al.*, 2012, Qualley *et al.*, 2012) (Fig. 2-Chapter 1). However, BA can also be synthesized via non- β-oxidative pathway, with benzaldehyde as intermediate, which has been reported in snapdragon (Long *et al.*, 2009). Although there is an argument that conversion of exogenous supplied of benzaldehyde to BA cannot be taken as *in planta* evidence (Van Moerkercke *et al.*, 2009), different mechanism to synthesize BA during day and night might exist. Moreover, flux analysis showed that BA can be produced via both β-oxidative and non- β-oxidative pathway in *P.hybrida* cv. Mitchell flowers and can vary over L/D cycle (Boatright *et al.*, 2004, Orlova *et al.*, 2006).

In chapter 4, I showed that earlier expression of *ODO1* not only resulted in earlier emission of FVBP, but also elevated the emission levels of benzaldehyde in *P.hybrida* cv. Mitchell (Fig. 1). The question is what makes the change in flux to benzaldehyde production? I will discuss the possible mechanisms in this section.

Early expression of *ODO1* resulted in earlier expression of *EPSPS*, which thus may lead to the early synthesis of Phe in petals (not measured). Of note is the fact that *P.hybrida* cv. Mitchell is derived from *P.axillaris*, and that benzenoid/phenylpropanoid composition and timing of emission are similar to that of *P.axillaris* (Verdonk *et al.*, 2003). Rhythmic FVBP emission by *P.axillaris* also occurs in continuous light (Hoballah *et al.*, 2005), suggesting that circadian clock regulates the FVBP is more robust in *P.axillaris* than *P.hybrida* cv. Mitchell. The clock phenotype can be different among Petunia species. For instance, *P.integrifolia* flowers emits solely benzaldehyde during the day (PhD thesis M. Boersma, unpublished) which is light dependent (Hoballah *et al.*, 2005). In *P.hybrida* cv. Mitchell, a combination of both circadian and environmental factors may regulate the rhythmic emission of FVBP.

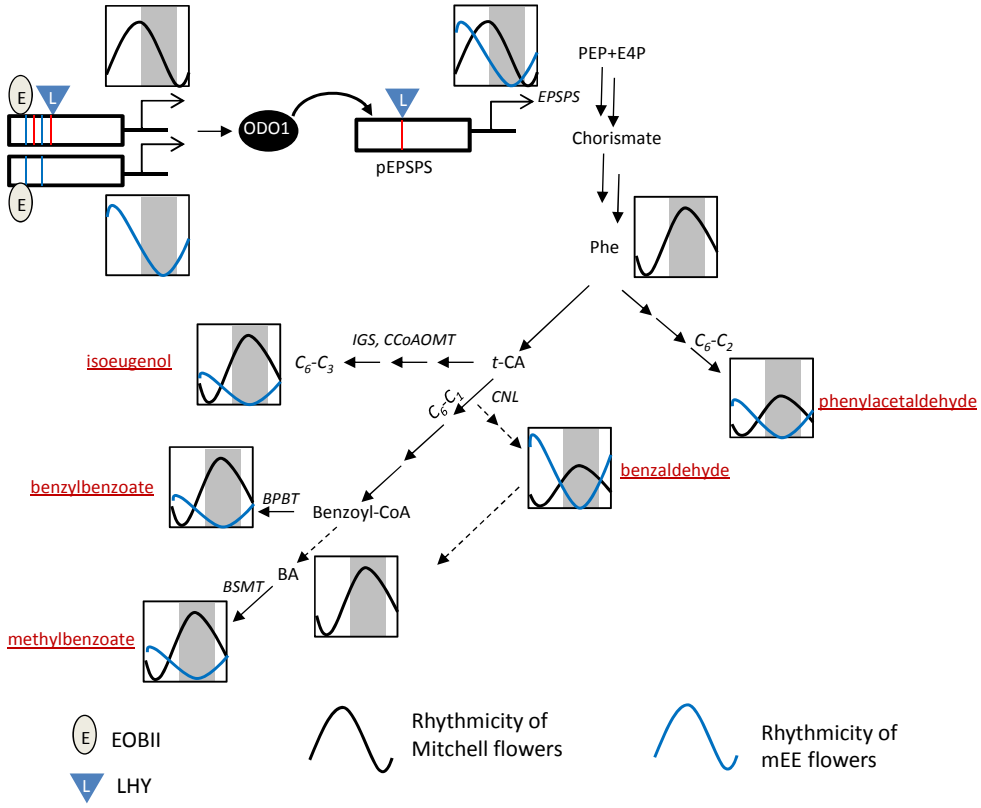

Figure 1: Differences in rhythmic pattern and composition of benzenoid/phenylpropanoid in *P.hybrida* cv. Mitchell and transgenic evening elements mutated (mEE) Mitchell lines. Transcript levels of *ODO1* and *EPSPS* are evening-phased in *P.hybrida* cv. Mitchell and midday-phased in mEE lines. Phe and BA pools in *P.hybrida* cv. Mitchell are night-phased, preceding the emission of volatile compounds (red letters-underlined). Blue and red vertical lines indicate MYB binding sites (MBS) and EEs motifs in promoter, respectively. White and grey boxes represent light and dark period, respectively. Abbreviation: *EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase*; BA: Benzoic acid; *BPBT: benzoyl-CoA:benzylalcohol/2-phenylethanol benzoyltransferase; BSMT:* S-*adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase; CCoAOMT: Caffeoyl-CoA O-methyltransferase; CNL: Cinnamate-CoA ligase;* EOBII: EMISSION OF BENZENOID II; *IGS: Isoeugenol synthase;* LHY: LATE ELONGATED HYPOCOTYL; *ODO1: ODORANT1*; Phe: Phenylalanine; *t*-CA: trans- cinnamic

It has been shown that light and temperature also contribute to rhythmic emission of *P.hybrida* cv. Mitchell (Cheng *et al.*, 2016). As Phe accumulates during the day, the FVBP genes are also expressed early in the light as shown in Chapter 4, including *PAL* in the *P.hybrida* cv. V26 line. High temperature and light exposure during the day have a negative effect on *PAL* transcript levels and enzymatic activity (Lo Piero *et al.*, 2005, Boo *et al.*, 2011). High temperatures lead to lower anthocyanin levels due to the lower PAL activity in plants (Shaked-Sachray *et al.*, 2002, Lo Piero *et al.*, 2005). A similar result has been reported for volatile biosynthesis in *P.hybrida* cv. Mitchell. For instance, increasing the ambient temperature from night to day can reduce the emission of floral volatiles produced by petunia (Cna'ani *et al.*, 2015a). Interestingly, the

negative effect of temperature was also robust at endogenous levels of volatiles during the day, indicating the expression of FVBP genes is also influenced by temperature (Ben Zvi *et al.*, 2008, Sagae *et al.*, 2008). PAL is the first committed enzyme that catalyzes the conversion of Phe to *t*-cinnamic acid (*t*-CA) (Ritter and Schulz, 2004) and *t*-CA must be in enough supply for benzenoid production in petunia petals via β-oxidative pathway. It seems that, during the day, light and temperature may take part in regulation of FVBP emission by controlling the flux distribution at benzenoid/phenylpropanoid pathway. Instead of importing the precursor into peroxisome, *t*-CA is redirected to the non-β-oxidative pathway and resulting in more benzaldehyde and benzylalcohol production in the cytosol during the day, since formation of *t*-CA from Phe is a one-step conversion (Coquoz *et al.*, 1998). Moreover, the flux contribution from benzaldehyde to BA appeared to be higher in the presence of light than dark in *BPBT* silencing lines, supporting that the benzenoid/phenylpropanoid branche is also light dependent (Orlova *et al.*, 2006). Thus, Phe levels and flux distribution analysis during day and night should be performed to fully understand the molecular mechanisms of FVBP production in peroxisomal β-oxidative and non- β-oxidative pathway in the cytosol. The internal volatiles are yet to be quantified in continuous light and dark, albeit the rhythm of both emission and endogenous pools were same in L/D cycle (Fenske *et al.*, 2015).

Does the EE in wild petunia contribute to late emission of FVBP?

A polymorphism in a single gene of a flower trait has been reported result in the attraction of different pollinators. QTL analysis in petunia suggests a shift in pollination syndromes can be explained by polymorphisms of transcription factors involved in floral color, UV absorbance or scent production (Hoballah *et al.*, 2007, Klahre *et al.*, 2011, Sheehan *et al.*, 2016). The mutation in the coding region of a R2R3-MYB transcription factor ANTHOCYANIN2 (AN2) of *P.axillaris* leads to a shift from purple to white flowers (Quattrocchio *et al.*, 1999, Hoballah *et al.*, 2007). In recent work, mutations in the *MYB-FL* promoter enhanced the expression of flavonol synthase (FLS), and in turn lead to enhanced UV absorbance in *P.axillaris* (Sheehan *et al.*, 2016). Another R2R3-MYB transcription factor, *ODO1*, localizes to a QTL on chromosome VII, which is responsible for scent production in petunia flowers (Klahre *et al.*, 2011). These studies demonstrated the strong influence of mutations in flower traits on the preferences of pollinators.

The absence of fragrance in *P.exserta* is caused by a polymorphism in *cis*-regulatory elements of *ODO1* (Klahre *et al.*, 2011), which is likely caused by mutation of MBS within the 1.2 kbp *ODO1* promoter (Van Moerkercke *et al.*, 2011). Presence of EEs in the same region can be another factor that control night production and emission of volatiles, as well as volatile composition in *P.axillaris*. Therefore, we compared the 1.2 kbp of promoter sequences of *ODO1* among wild and cultivars of petunia (Fig. 2) and summarized the number of EEs in Table 1. The numbers of EEs varied among fragrant petunia and the differences cannot be correlated with day and night emission of volatiles (Table 1). For instance, *P.axillaris* N and *P.integrifolia inflata* S6 have one EE although the flowers emit volatiles during night and day respectively (Hoballah *et al.*, 2005). *ODO1* transcript levels in both petunias are high at noon (PhD thesis M. Boersma, unpublished) similar to those of *P.hybrida* cv. V26 (Chapter 4). This suggests that *ODO1* transcription is not the primary factor that regulates night emission in *P.axillaris*. EEs can also be found in *EPSPS* and *IGS* promoters (Fenske *et al.*, 2015), and possibly in other promoter of FVBP genes as well. LHY may repress the morning expression of the upstream precursor genes (e.g *EPSPS*) which directly controls the availability of precursor at night in *P.axillaris*. Furthermore, *P.axillaris* and *P.inflata* have 2 and 3 copies of the clock gene,

gigantea (*GI*), respectively in their genome (Bombarely *et al.*, 2016). *GI* transcripts and proteins are modulated by circadian rhythm, light input and temperature response (Fowler *et al.*, 1999, David *et al.*, 2006, Gould *et al.*, 2006), suggesting that the extra copy of GI in *P.inflata* may have a new biological role. It would interesting to investigate the role of GI in regulating the rhythm of volatile emission of *P.axillaris* and *P.inflata*.

Although ODO1 seems to be one of the molecular players controlling total scent production of benzenoid/phenylpropanoid by *P.hybrida* cv. Mitchell flowers (Verdonk *et al.*, 2005), it may not directly determine the composition of the FVBP bouquet. In wild petunias, *P.axillaris* and *P.integrifolia*, the different levels of benzenoid compounds are unlikely caused by ODO1 regulation. In Chapter 5, we showed that the higher levels of benzylbenzoate in early during the day are likely caused by a gene on chromosome II. Although we could not correlate *BPBT1* and *BPBT2* expression levels in *P.axillaris* and the selected RILs with high, early benzylbenzoate production, we cannot exclude that these genes are causally related to the QTL. Interestingly, the overall benzaldehyde levels were higher in RILs, but methylbenzoate levels were lower than in *P.axillaris* (Chapter 5). *P.integrifolia* lacks *BSMT* expression, but has an active *ODO1* and *cinnamate-CoA ligase* (*CNL*) (Amrad *et al.*, 2016), thus loss of active BSMT copy on chromosome V in the RILs may lead to the absence of methylbenzoate. The methylbenzoate production can be restored in an F_1 of *P.integrifolia* x *P.exserta*, which has an active copy of *BSMT*, but lacks *ODO1* and *CNL* expression, which also causes a strong reduction of benzaldehyde production (Amrad *et al.*, 2016). This suggests that activation of *BSMT* (or/and *BPBT*) could redirect metabolic flux away from benzaldehyde toward methylbenzoate production (Amrad *et al.*, 2016). If no BSMT activity is present the default pathway leads to benzaldehyde production. Interestingly, tight genetic linkage between loci on chromosome II that specify scent production, UV absorbance and reproductive organs morphology have been identified, suggests that clustered floral traits create a "speciation island" able to respond to specific pollinators (Hermann *et al.*, 2013). Many FVBP genes are on chromosome II (*phenylacetaldehyde synthase* (*PAAS*)*, CNL*, *BPBT*, *PAL1* and *IGS*), indicating that these genes may have strong selection on pollination syndrome traits to keep separating two closely related species in a sympatric population (Hermann *et al.*, 2013). For example: loss of functional CNL is very important for transition from hawkmoth to hummingbird pollination (Amrad *et al.*, 2016), and a QTL on chromosome II (Chapter 5) may have contributed to the pollinator shift from bee to hawkmoth.

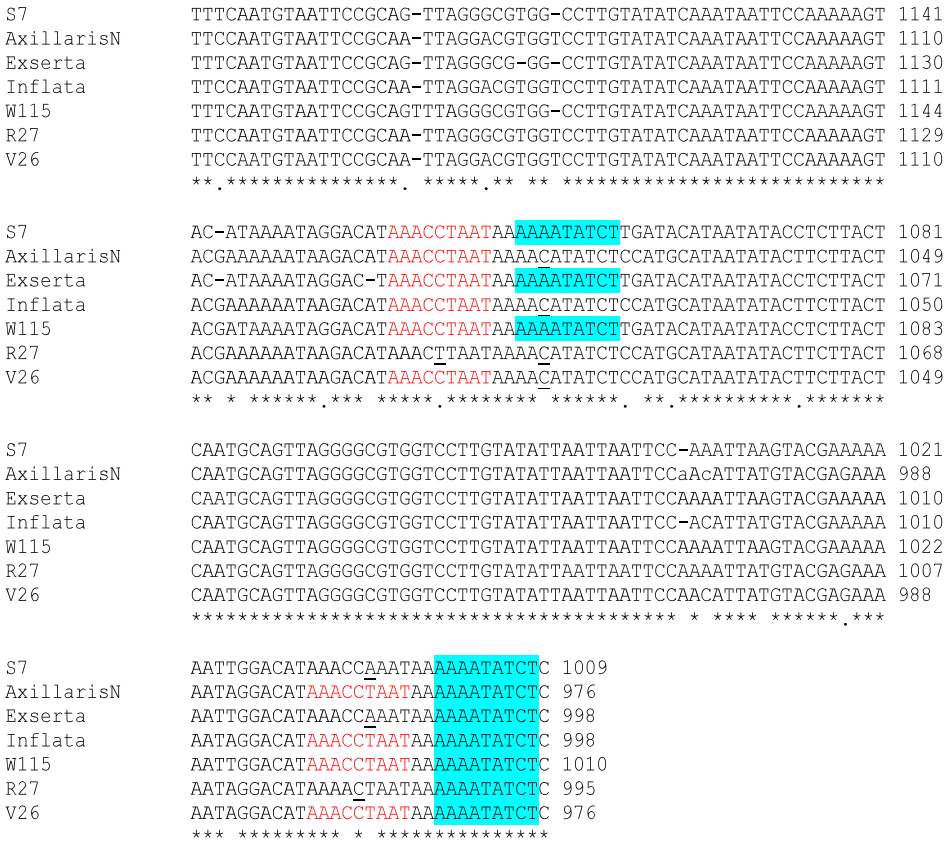

Figure 2: Sequence alignment of part of the ODO1 promoter from wild petunias (*P.axillaris parodii* S7, *P.axillaris* N, *P.integrifolia inflata* S6, *P.exserta*) and cultivars (*P.hybrida* cv. Mitchell, *P.hybrida* cv. V26, *P.hybrida* cv. R27). The alignment was generated using ClustalW (http://www.genome.jp/tools-bin/clustalw). The sequences upstream of the ATG, approximately between 1204 and 995, are shown. Red letters indicate the perfect sequence of MYB binding site (MBS) and blue boxes indicate the perfect sequence of evening elements (EEs). Abbreviations: *P.axillaris parodii* S7 (S7); *P.axillaris* N (AxillarisN); *P.exserta* (Exserta); *P.integrifolia inflata* S6 (Inflata); *P.hybrida* cv. Mitchell (W115); *P.hybrida* cv. V26 (V26); *P.hybrida* cv. R27 (R27).

Table 1: Summary of *ODO1* promoter sequences from 1204 bp to 995 bp upstream of ATG

Species/cultivars	MBS	EE	ODO1 expression	Peak of expression	Fragrance
***P.axillaris parodii* S7**	1	2	++	evening	Fragrant
***P.axillaris* N**	2	1	++	evening	Fragrant
P.exserta	1	2	-	-	Non-fragrant
***P. integrifolia inflata* S6**	2	1	++	midday	Fragrant
***P.hybrida* cv. W115**	2	2	++	evening	Fragrant
***P.hybrida* cv. R27**	1	1	-	-	Non-fragrant
***P.hybrida* cv. V26**	2	1	++	midday	Fragrant

2. The metabolic connection between scent and color biosynthesis

Flower color and scent are essential for the attraction of pollinators and hence for high pollination efficiency. In petunia flowers, both floral volatile and anthocyanin biosynthesis have been intensively studied in last few decades, including the characterization of transcription factors and biosynthetic genes (Schuurink *et al.*, 2006, Albert *et al.*, 2014, Muhlemann *et al.*, 2014). Benzenoid/phenylpropanoid and anthocyanin biosynthesis are derived from phenylpropanoid pathway and shared common precursors, Phe and 4-coumaryl CoA.

Anthocyanin is a class of flavonoid, which is synthesized in cytoplasm and stored in vacuoles (Mueller *et al.*, 2000). In petunia flowers, anthocyanins are produced at an early stage of flower development and the synthesis is ceased upon flower opening. The R2R3 MYB transcription factors AN2 and AN4 are exclusively expressed in petals and anthers, respectively, and form a complex with basic-helix-loop-helix, AN1, and WD-repeat protein, AN11 to regulate the anthocyanin pathway in petunia (de Vetten *et al.*, 1997, Quattrocchio *et al.*, 1999, Spelt *et al.*, 2000). Unlike the anthocyanin biosynthesis, FVBP production only begins during anthesis when flower are fully developed and reproductive organs are mature, hence ready for pollination (Verdonk *et al.*, 2003). This temporal separation could be a mechanism to prevent metabolic competition between these two pathways in flowers (Verdonk *et al.*, 2005). More arguments against metabolic competition have been reported between anthocyanin and benzenoid/phenylpropanoid production in petunia flowers. Silencing of EOBII in *P.hybrida* line P720 did not affect the anthocyanin levels, although FVBP production and emission were lower than controls (Spitzer-Rimon *et al.*, 2010).

A crosstalk between benzenoid/phenylpropanoid volatiles and anthocyanin can be anticipated on the basis of their common biochemical pathway. Manipulation of the R2R3 MYB transcription factor PH4 and phenylpropanoid biosynthetic genes indeed redirected the metabolic flux from one branch to another. Final petal color is regulated by PH4 that interacts with AN1 and AN11 to control acidification of the vacuole (Quattrocchio *et al.*, 2006). In contrast to AN2, PH4 plays role in floral pigmentation and volatile emission (Cna'ani *et al.*, 2015b). It was suggested that PH4 is involved in flower pigmentation via vacuolar acidification during bud development and shift towards scent emission following anthesis of flowers (Cna'ani *et al.*, 2015b). Like the other FVBP transcription factors, *PH4* transcript levels is developmentally regulated, the transcript in purple petunia, *P.hybrida* cv. V30 increased

throughout the developmental stages and reached the highest levels after anthesis, preceding the peak volatile emission of petunia (Cna'ani *et al.*, 2015b). PH4 plays no role in activation of structural anthocyanin genes, thus functional *AN2* is a "must" requirement to allow anthocyanin synthesis in petunia petals (Quattrocchio *et al.*, 2006).

In last decade, suppression of *flavanone-3-hydrolase* (*F3H*) in carnation has already suggested a metabolic connection between anthocyanin and benzenoid biosynthesis (Zuker *et al.*, 2002). Suppression lines of *F3H* showed a loss of anthocyanin pigments in carnation flowers and more methylbenzoate emission than in control lines (Zuker *et al.*, 2002). Silencing of *chalcone synthase* (*CHS*), a gene encoding the first committed enzyme in flavonoid biosynthesis, increased the production of (iso)eugenol in cultivated strawberry (Hoffmann *et al.*, 2011). However, pigmentation of petals was severely affected upon suppression of *F3H* and *CHS* in petunia flowers but FVBP levels were not increased (Tsuda *et al.*, 2004, Spitzer *et al.*, 2007), suggesting phenotypic effects may depend on the genes targeted gene. In Chapter 2 we showed that downregulation of one phenylpropene gene, *caffeoyl-CoA O-methyltransferase 1* (*CCoAOMT1*) in *P.hybrida* cv. Mitchell can lead to the production of pink flowers and purple leaves and stems. Flowers of these plants produce normal amounts of volatiles, although slightly less eugenol is produced. Although *P.hybrida* cv. Mitchell flowers lack a functional *AN2*, *DFR* might have been activated by PURPLE HAZE (PHZ), a R2R3 MYB transcription factor that regulates anthocyanin pathway in flowers and vegetative tissues (Albert *et al.*, 2011). Most likely the accumulation of a metabolite resulted in PHZ activation. The transcript levels of *CCoAOMT1* can be detected in buds (Chapter 2), thus we predict that the anthocyanin has been synthesized early during flower development and ceased when FVBP production started. Interestingly, introduction of *AN2* into *P.axillaris* had no effect on scent production although the anthocyanin biosynthesis was activated in the flowers (Hoballah *et al.*, 2007). The metabolic connection between flower color and scent in the phenylpropanoid pathway is during development, thus metabolic losses in both pathways can be minimized and on the other hand, plant pollination success can be maximized.

3. Outlook

There has been a boost in floral volatile research and petunia has become a perfect model to study the regulation of phenylpropanoid/benzenoid biosynthesis. In recent work, the clock gene *LHY* has been identified as a main regulator in controlling the timing of FVBP regulation in flowers (Fenske *et al.*, 2015, Yon *et al.*, 2016). However, more research efforts are needed to discover how *LHY* and other clock genes interact with other transcription factors and FVBP structural genes and how the changes in the floral clock affect the pollinator choice in field. The role of other clock components in regulation of temporal production of phenylpropanoid/benzenoid remains to be investigated (Bombarely *et al.*, 2016).

Color and scent variations can be part of pollination syndromes that contribute to reproductive isolation between closely related species (Hoballah *et al.*, 2007, Klahre *et al.*, 2011, Byers *et al.*, 2014, Bischoff *et al.*, 2015). Both structural and regulatory genes has been shown to be involved in the natural variation in pollination syndromes (Hoballah *et al.*, 2007, Klahre *et al.*, 2011, Amrad *et al.*, 2016). Mutations in the coding region of *AN2* or suppression of *ODO1* do not have an effect on volatile phenylpropanoids and anthocyanin production, respectively, in spite of the fact that these compounds are derived from a common precursor (Verdonk *et al.*, 2005, Hoballah *et al.*, 2007). Tight regulation, both temporally and developmentally, may contribute to the metabolic separation between these pathways. Although

manipulation of *PH4* and structural genes (*CCoAOMT1*) have shown a metabolic connection between color and scent, more studies on metabolic channeling in the phenylpropanoid pathway should be performed. Moreover, it would be of great interest to investigate how the biochemical and genetic changes affect the pollinator perception to provide more evidence on co-evolution between plant and pollinators.

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