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

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CHAPTER 1

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

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

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GENERAL INTRODUCTION

Plants are sessile organisms that need specific mechanisms to survive and reproduce. To be able to adapt to environmental changes, plants have evolved to produce a wide array of so-called specialized metabolites. A small proportion of plant specialized metabolites is volatile and belong to the class of terpenoids, benzenoids /phenylpropanoids and fatty acid derivatives (Dudareva *et al.*, 2013). These volatile organic compounds (VOC) are involved in (a)-biotic stress responses and in communication between plants and their antagonists and mutualists (for instance pollinators). More than 1700 VOCs have been identified from at least 90 plant families (Knudsen *et al.*, 2006).

VOCs are lipophilic molecules with low molecular weight and high vapour pressure at ambient temperature (Pichersky *et al.*, 2006). Biosynthesis of VOCs occurs in all plant organs: roots, stems, leaves, fruits, seeds and flowers. Plants release VOCs, especially green life volatiles and terpenoids as part of plant defense responses, either to deter herbivores from feeding on plants or to attract natural enemies of the herbivores (Schnee *et al.*, 2006, Scala *et al.*, 2013). Apart from their importance for plant defense, VOCs released from flowers mainly perform as signal molecules to attract animal pollinators. Volatile bouquets from flowers can function as sex pheromones to bees, or a guide for hawkmoth and bees to food (nectar), and as a consequence, these pollinators transfer pollen from plant to plant (Ayasse *et al.*, 2003, Klahre *et al.*, 2011, Klatt *et al.*, 2013).

The role of floral scents in pollination syndromes has been studied in several plant species. These studies are facilitated by the fact that floral volatiles can be collected using dynamic headspace sampling from flowers, and the VOCs can be separated and identified using gas chromatography-mass spectrometry (GC-MS). Floral scent can be very specific in targeting specific pollinators. Bat pollinated flowers emit sulphur-containing volatile compounds at night (Muchhala and Serrano, 2015). Snapdragon flowers specifically attract bees by emitting methylbenzoate during day (Negre *et al.*, 2003). Terpenoid compounds are important for bumblebee attraction to *Mimulus lewisii* (Byers *et al.*, 2014). Volatile emission from a flower does not only serve as pollinator attractant, but also as deterrent against florivores, as has been described for Petunia flowers (Klahre *et al.*, 2011, Kessler *et al.*, 2013).

P.hybrida cv. Mitchell has been used to study floral volatile benzenoids and phenylpropanoids (FVBP) biosynthesis. *P.hybrida* cv. Mitchell has a large white flowers, that emit a large amounts of benzenoid/phenylpropanoid compounds in the late evening (Verdonk *et al.*, 2003). The production and emission of FVBP are regulated in a rhythmic fashion and mostly produced by the petals (Verdonk *et al.*, 2003, Colquhoun *et al.*, 2010). This petunia cultivar emerged as a choice for a model system because it is an inbred colchidiploid that has a

short life cycle (approximately 4 months) from seed to seed. It is easy to generate stable transgenic plants using leaf-disk transformation and also suitable for Agrobacterium infiltration because of its large flowers and leaves (Gerats and Vandenbussche, 2005, Vandenbussche *et al.*, 2016). Moreover, transformation of protoplasts from petunia petals is also feasible (Faraco *et al.*, 2011). Petunia flowers are easily to cross or self-pollinate, and produce a high number of seeds and plants can also be propagated by cuttings. *P.hybrida* cv. Mitchell can be manipulated for biochemical analysis using antisense, co-suppression or RNAi-mediated silencing and by over-expression. Recently, petunia genome sequencing data have been released (https://solgenomics.net/) that will propel the petunia as model system in plant genetics and breeding research further (Bombarely *et al.*, 2016).

The composition and quantity of petunia volatiles change during i) flower development, ii)with the endogenous circadian rhythm, iii) upon pollination, iv) in response to hormonal regulation and v) with different environmental conditions (Verdonk *et al.*, 2003, Colquhoun *et al.*, 2010, Cna'ani *et al.*, 2015, Fenske *et al.*, 2015). The focus of this chapter is to describe the current knowledge of the regulation and production of volatile benzenoids/phenylpropanoids in *P.hybrida* cv. Mitchell flowers. Furthermore, we will discuss the role of petunia VOCs in plant reproduction.

1. Biochemistry of petunia volatiles

Volatile benzenoids and phenylpropanoids dominate the headspace of petunia flowers (Verdonk *et al.*, 2005). Petals of *P.hybrida* cv. Mitchell produce phenylacetaldehyde, 2-phenylethylethanol, phenylethylbenzoate, benzaldehyde, methylbenzoate, benzylalcohol, benzylbenzoate, methylsalicylate, benzylacetate, isoeugenol, eugenol, vanillin and phenylethylacetate. The floral volatile benzenoid/phenylpropanoid (FVBP) pathway initiates from the shikimate biosynthesis. Production and emission of FVBPs are regulated by transcriptional control of shikimate and FVBP biosynthesis genes, substrate availability and activity of scent biosynthetic enzymes. The volatiles are exported across the plasma membrane by an active transporter, adenosine triphosphate-binding cassette 1 (ABCG1)(Adebesin *et al.*, 2017).

Benzenoid/phenylpropanoid biosynthesis

The FVBP compounds are all derived from phenylalanine (Phe), which is produced via the shikimate and arogenate pathways. The shikimate biosynthesis starts from phosphoenolpyruvate (PEP) and erythrose-4-P (E4P), and involves seven enzymatic reactions to produce chorismate plants, including 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHPS) and 5-enolpyruvylshikimate-3-phosphate (EPSPS), which have been characterized in *Petunia hybrida* (Steinrucken *et al.*, 1986, Langer *et al.*, 2014)(Fig. 1). The involvement of *EPSPS* in petunia volatile biosynthesis was illustrated by the activation of its promoter by ODORANT1 (ODO1), a key regulator of floral scent biosynthesis (Verdonk *et al.*, 2005). The production of Phe is initiated from chorismate via arogenate pathway, with chorismate mutase (CM) catalyzing the conversion of chorismate to prephenate. The conversion of prephenate to Phe is catalyzed by prephenate amino transferase (PPA-AT) and arogenase dehyratase (ADT) via prephenate and arogenate respectively (Maeda *et al.*, 2010, Maeda *et al.*, 2011). Alternatively, Phe can also be synthesized via tyrosine and phenylpyruvate by phenylpyruvate aminotransferase (PPY-AT) in petunia petals (Yoo *et al.*, 2013, Oliva *et al.*, 2017) (Fig. 1).

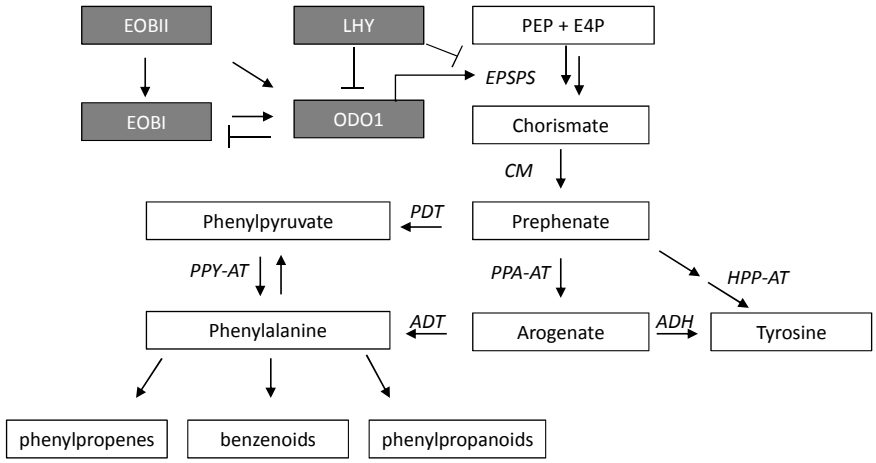

Figure 1: A diagram representing the interaction between transcription factors of phenylpropanoid/benzenoid and shikimate/aromatic amino acid pathway. The transcription factors; EOBI, EOBII, LHY and ODO1 are shown as grey boxes. Transcriptional activation or inhibition are represented by black arrows and T-bars, respectively. Shikimate and aromatic amino acid pathway are shown in white boxes. Phenylalanine is a precursor for phenylpropanoid/benzenoid biosynthesis. Arrows indicate established biochemical steps. Multiple arrows represent several biochemical reactions. Enzymes are marked in italic. Abbreviations: ADH: arogenate dehydrogenase; ADT: arogenate dehydrotase; CM: chorismate mutase; E4P: D-erythrose 4-phosphate; EOB: EMISSION OF BENZENOID; EPSPS: 5-enolpyruvylshikimate-3-phosphate; HPP-AT: 4-hydroxyphenylpyruvate aminotransferase; LHY: LATE ELONGATED HYPOCOTYL; ODO1: ODORANT1; PDT: prephenate dehyratase; PEP: Phosphoenolpyruvate; PPA-AT: prephenate aminotransferase ; PPY-AT: phenylpyruvate

The first committed step in C_6-C_1 and C_6-C_3 biosynthesis of petunia is deamination of Phe to *trans*-cinnamic acid, *t*-CA via phenylalanine ammonia lyase (PAL) (Fig. 1). Formation of benzenoids (C_6-C_1) from *t*-CA requires shortening of the propyl side chain by 2 carbons via β-oxidative pathway, non-β-oxidative pathway or combination of these pathways (Boatright *et al.*, 2004). The β-oxidative pathway begins with conversion of *t*-CA to cinnamoyl-CoA, which is catalyzed by cinnamate CoA-ligase (CNL) (Colquhoun *et al.*, 2012, Klempien *et al.*, 2012). Cinnamoyl-CoA hydratase-dehydrogenase (CHD) is a bifunctional enzyme that is responsible for conversion of cinnamoyl-CoA to 3-oxo-3-phenylpropanoyl-CoA (3O3PP-CoA) (Qualley *et al.*, 2012) (Fig. 2). The last step of this pathway, shortening of the propyl side chain of 3O3PP-CoA, occurs by 3-ketoacyl-CoA thiolase 1 (KAT1), resulting in the formation of benzoyl-CoA in peroxisomes (Van Moerkercke *et al.*, 2009). In combination with benzylalcohol and phenylethylethanol, benzoyl-CoA is converted to benzylbenzoate and phenylethylbenzoate, catalyzed by benzoyl-CoA:benzylalcohol/2-phenylethanol benzoyltransferase (BPBT) (Orlova *et al.*, 2006). Benzoyl-CoA is hydrolyzed to benzoic acid (BA) as well, and BA methylation results in methylbenzoate, the largest emitted compounds by *P.hybrida* cv. Mitchell petals (Negre *et al.*, 2003, Verdonk *et al.*, 2003).

Despite the fully elucidated β-oxidative pathway, the enzymatic reactions in the non-β-oxidative pathway in petunia are still unknown. As proposed, the alternative non-β-oxidative pathway proceeds via benzaldehyde, both with CoA-dependent and CoA-independent routes. The difference between CoA-independent and CoA-dependent pathway is that the CoA-dependent route starts from CoA esters instead of free acids (Fig 2). The conversion of CoA-ester substrate to 3-hydroxy-3-phenylpropanoyl-CoA (3H3PP-CoA) intermediates by

hydratase/lyase activity to form benzaldehyde, was detected in *Hypericum androsaemum* cell culture (Abd El-Mawla and Beerhues, 2002). CoA-independent route begins with the activation of *t*-CA to form benzaldehyde via 3-hydroxy-3-phenylpropanoic as intermediate (Fig.2). The final step of non-β-oxidative pathway is the conversion of benzaldehyde to BA by aldehyde oxidase or aldehyde dehydrogenase (Fig.2). Arabidopsis contains an aldehyde oxidase with broad substrates specificity, that can convert benzaldehyde to BA (Ibdah *et al.*, 2009). In snapdragon flowers, benzaldehyde dehydrogenase (BALDH) is used to convert benzaldehyde to BA (Long *et al.*, 2009). The biosynthetic gene for benzylacetate production in petunia has never been reported, but benzylacetate is predicted to be formed from benzyl alcohol as substrate through the action of alcohol acetyltransferase (Guterman *et al.*, 2006).

The relative contribution of β-oxidative and non-β-oxidative pathway towards BA biosynthesis in petunia is still unclear. Flux analysis indicated that a greater part of the flux goes through the non- β-oxidative pathway than through the β-oxidative pathway (Boatright *et al.*, 2004), but downregulation of *KAT1* showed that the β-oxidative pathway is the major route for BA biosynthesis (Van Moerkercke *et al.*, 2009). Recently, the discovery that *CNL1* underlies the scent QTL on chromosome 2, suggests that volatile benzenoids production are occurs via β-oxidative pathway in peroxisomes (Amrad *et al.*, 2016). Since 3H3PP-CoA is a potential substrate to produce benzaldehyde, most probably benzaldehyde is synthesized via CoA-dependent, non-β-oxidative route (Chapter 4).

The C_6-C_2 biosynthesis is initiated by phenyacetaldehyde synthase (PAAS), which converts Phe to phenylacetaldehyde and competes with PAL for the same substrate (Kaminaga *et al.*, 2006) (Fig 2). The enzyme involved in the production of phenylethanol in petunia is still unknown. In tomato fruit, phenylacetaldehyde reductase catalyzes the formation of phenylethanol from phenylacetaldehyde (Tieman *et al.*, 2007). Phenylethanol and benzylbenzoate are used by BPBT to form phenylethylbenzoate (Orlova *et al.*, 2006). An alcohol acetyltransferase presumably uses phenylethanol to produce phenylethylacetate (Guterman *et al.*, 2006), although the enzyme has never been characterized in petunia flowers.

The C_6-C_3 (phenylpropenes) pathway is also not fully elucidated. The production of volatiles isoeugenol and eugenol have been proposed to share the initial biosynthetic steps with the lignin pathway, up to the formation of coniferyl alcohol (Dexter *et al.*, 2007, Muhlemann *et al.*, 2014) (Fig 2). The volatile phenylpropene pathway begins when *t*-CA is hydroxylated to 4-coumaric acid by cinnamate 4-hydroxylase (C4H) (Colquhoun *et al.*, 2011a). 4-coumaric acid is then converted to 4-coumaryl-CoA by 4-coumarate CoA ligase (4CL), the intermediates for the phenylpropenes and anthocyanin biosynthesis (Klempien *et al.*, 2012). Recently, we characterized caffeoyl-CoA O-methyltrasferase (CCoAOMT) that methylates caffeoyl-CoA to feruloyl-CoA in *P.hybrida* cv. Mitchell (Chapter 2,3). Feruloyl-CoA is used by cinnamoyl CoA reductase (CCR1) to form coniferylaldehyde (Muhlemann *et al.*, 2014), which is probably converted to coniferyl alcohol by the cinnamyl alcohol dehydrogenase (CAD). Feruloyl-CoA has also been proposed as an intermediate for vanillin biosynthesis in petunia (Muhlemann *et al.*, 2014). Coniferylalcohol acetyl-transferase (CFAT) acetylates coniferyl alcohol to produce coniferyl acetate (Dexter *et al.*, 2007), which is then used as substrate to produce iso-(eugenol). The production of volatiles isoeugenol and eugenol are catalyzed by isoeugenol synthase (IGS) and eugenol synthase (EGS), respectively (Koeduka *et al.*, 2008).

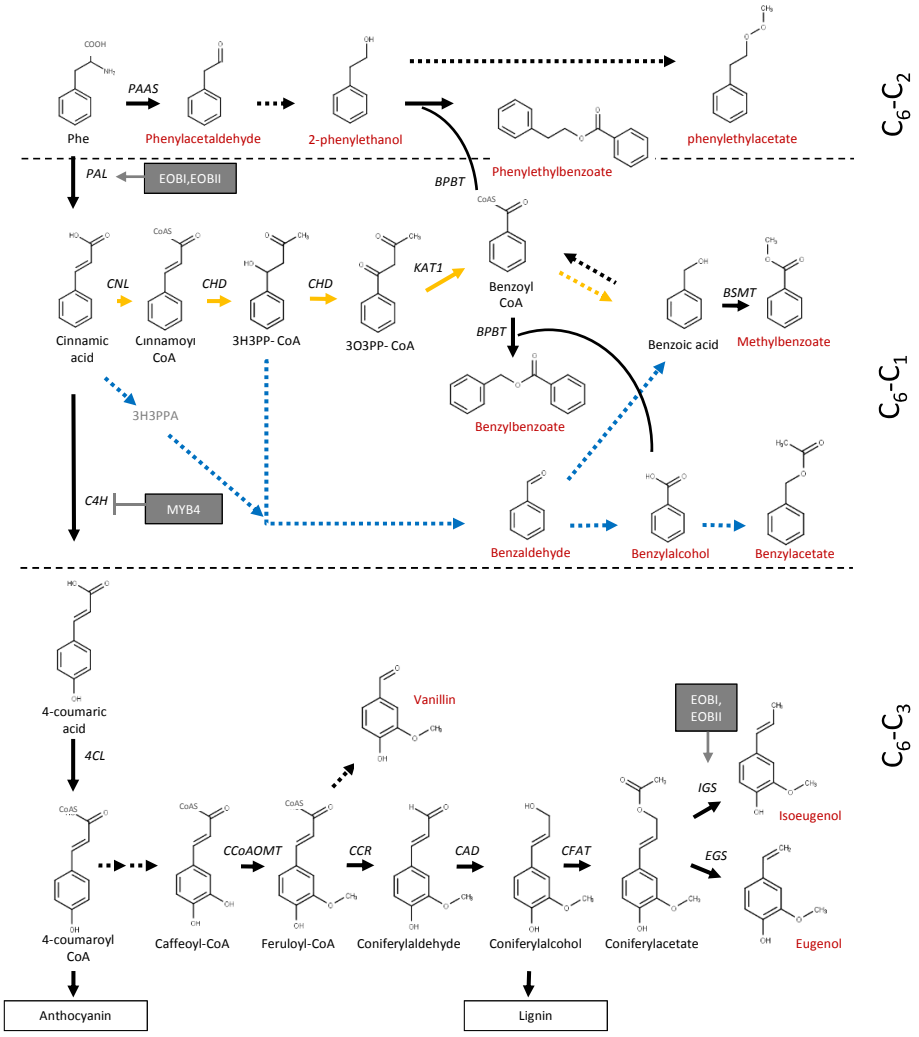

Figure 2. Metabolic pathways leading to volatile C_6-C_1, C_6-C_2 and C_6-C_3 compounds in petunia petals. Volatile compounds emitted by P.hybrida cv. Mitchell are in red. Yellow and blue arrows indicate the enzymatic reactions involve in β-oxidative and non β-oxidative pathway, respectively. Solid arrows and enzymes (italic) indicate established biochemical steps, while broken arrows represent hypothetical steps. Stacked arrows illustrate of involvement of multiple enzymatic reactions. EOBI and EOBII activate *IGS* and *PAL* promoters, whereas MYB4 inhibits the *C4H* expression, are shown in grey boxes. Abbreviations: 3H3PPA: 3-hydroxy-3-phenylpropanic acid; 3H3PP-CoA: 3-hydroxy-3-phenylpropanoyl-CoA; 3O3PP-CoA: 3-oxo-3-phenylpropanoyl-CoA; 4CL: 4-coumarate CoA ligase; BPBT: benzoyl-CoA:benzyl alcohol/phenylethanol benzoyltransferase ; BSMT: S-adenosyl-L-methionine:benzoic acid/salicylic acid methyltransferase; C4H: cinnamate-4-hydrolase; CAD: cinnamyl alcohol dehydrogenase; CCoAOMT: caffeoyl CoA O-methyltransferase; CCR: cinnamoyl CoA reductase; CFAT: coniferylalcohol acetyltransferase ; CNL: cinnamate CoA-ligase; CHD: Cinnamoyl-CoA hydratase-dehydrogenase; DFR: dihydroflavonol reductase; EGS: eugenol synthase; EOB: EMISSION OF BENZENOID; IGS: isoeugenol synthase; KAT1: 3-ketoacyl-CoA thiolase 1; PAAS: phenyacetaldehyde synthase; PAL: phenylalanine ammonia lyase.

2. Regulation of petunia floral volatile production

Developmental regulation and emission in flowers

Volatile benzenoid/phenylpropanoid production is tightly regulated in petunia spatially and temporally. In fragrant *P.hybrida* cv. Mitchell, benzenoids and phenylpropanoids are presumably emitted from epidermal cells of petal limbs. The highest transcript levels of FVBP genes are reached in petal limbs (Verdonk *et al.*, 2003, Colquhoun *et al.*, 2010). However, gene expression can also be detected in vegetative tissues at low levels (Verdonk *et al.*, 2003, Colquhoun *et al.*, 2010). Another hallmark of scent-related genes in petunia is that FVBP gene expression is developmentally regulated throughout flower lifecycle. FVBP transcript levels and emission are low in flower buds, and increased consistently throughout the developmental stages until anthesis. Both transcript levels and volatile emission remain high through the open flower stages, until they reach senescence (Verdonk *et al.*, 2003, Colquhoun *et al.*, 2010). FVBP production and emission coincide with anthesis, thus maximizing the pollination process of petunia.

In *P.hybrida* cv. Mitchell, ethylene is synthesized by the stigma/style shortly after pollination and by ovaries after fertilization. This is later followed by autocatalytic ethylene synthesis in petal limbs 24 to 36 hours after pollination, to accelerate petal senescence and permanently decrease volatile emission (Negre *et al.*, 2003, Underwood *et al.*, 2005). Role of ethylene in FVBP synthesis has been described in series of experiments using pollinated *P.hybrida* cv. Mitchell and ethylene-insensitive 44568 plants. FVBP gene expression and enzyme activities were reduced after 10 hours of ethylene treatment, and subsequently emission of benzenoids/phenylpropanoids was eliminated in *P.hybrida* cv. Mitchell, but not in 44658 (Negre *et al.*, 2003, Underwood *et al.*, 2005, Colquhoun *et al.*, 2010). This indicates that ethylene production is a signal to the corolla, that successful fertilization has taken place and floral volatile is no longer required for pollinator attraction.

Rhythmic emission of petunia volatiles

Emission of benzenoids/phenylpropanoids in *P.hybrida* cv. Mitchell flowers oscillates during day and night cycle and peaks at night (Verdonk *et al.*, 2003). However, robust oscillation of emission has not been observed in continuous light, but it displays a circadian rhythm in continuous dark although the emission levels were lower than in light/dark grown plants (Fenske *et al.*, 2015). Since *P.hybrida* cv. Mitchell is a hybrid of *P.axillaris* and *P.integrifolia*, the diurnal rhythm pattern might be partially attributed from both parents (Fenske *et al.*, 2015). *P.axillaris* maintains rhythmic emission of volatiles in continuous light, whereas *P.integrifolia* emission does not (Hoballah *et al.*, 2005). Still, *P.hybrida* cv. Mitchell is the most suitable model to study on rhythmic emission and regulation of benzenoid and phenylpropanoid volatiles as the many transcriptional regulators and most FVBP biosynthetic genes have been characterized from this cultivar, and benzenoid/phenylpropanoid metabolic pathway is almost fully elucidated (Gübitz *et al.*, 2009, Colquhoun and Clark, 2011).

Transcript levels of FVBP genes have daily rhythmic pattern, but the circadian clock does not have influence on the timing of biosynthetic gene expressions (Fenske *et al.*, 2015). For instance, *BSMT* expression diurnally oscillates in light/dark cycles and peaks in the afternoon, but the oscillation is not sustained in continuous light and weak in continuous dark (Kolosova *et al.*, 2001, Fenske *et al.*, 2015). In contrast, the precursor genes in the upstream

metabolic pathway, *PAL* and *EPSPS* transcript levels show diurnal rhythm in light/dark cycles as well as in both continuous light and dark (Fenske *et al.*, 2015, Cheng *et al.*, 2016). PAL activities also displayed daily oscillation pattern in petunia and snapdragon, but peaked at different times (Kolosova *et al.*, 2001). The gene expression and enzymatic activities correlate with the emission of volatiles by petunia and snapdragon flowers, which peak at night and day respectively (Kolosova *et al.*, 2001). The transcript levels of *PAL* are in a similar rhythm as BA levels, suggesting that the rhythmicity petunia emission is mainly regulated at precursor levels (Fenske *et al.*, 2015, Cheng *et al.*, 2016).

Transcription factors

Volatile benzenoid/phenylpropanoid genes are regulated by a few transcription factors. ODORANT1 (ODO1) is a main regulator of FVBP synthesis, and downregulation of *ODO1* reduced the expression levels of *EPSPS*, *PAL* and most of FVBP biosynthetic genes and resulted in a diminished volatiles emission (Verdonk *et al.*, 2005). ODO1 regulates the FVBP production by activating the *EPSPS* promoter (Verdonk *et al.*, 2005) (Fig. 1). *ODO1* expression has similar rhythmic pattern as *EPSPS* and *PAL* and their transcript levels peak three to four hours before the onset of dark periods to precede the emission of FVBP volatiles (Fenske *et al.*, 2015). Interestingly, the *ODO1* promoter of *P.hybrida* cv. Mitchell contains evening element (EE) motifs (Van Moerkercke *et al.*, 2011), which provide binding sites to circadian-clock transcription factors, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) (Michael and McClung, 2002). Recently, *LHY* has been identified in *P.hybrida* cv. Mitchell flowers and it binds to the EEs in *ODO1*, *EPSPS* and *IGS* promoters (Fenske *et al.*, 2015). Constitutive expression of *LHY* severely suppressed the expression of *ODO1*, *EPSPS* and *PAL*, and reduced most of FVBPs in petunia. On the other hand, downregulation of *LHY* led to the earlier expression of *ODO1* and precursor genes, as well as the emission of volatiles (Fenske *et al.*, 2015). The results indicate that LHY regulates the timing of FVBP emission in petunia, by the repression of *ODO1* and possibly of several FVBP genes in the morning (Fenske *et al.*, 2015)(Fig.1).

Major discoveries of transcription factors regulating the volatile benzenoid/phenylpropanoid pathway have been made in fragrant petunia. The expression patterns of these regulatory genes correlate with the emission of volatile benzenoid/phenylpropanoid spatially and temporally. First, the expression of regulatory genes is high in petal limbs. Second, the expression reaches maximal levels at anthesis, remains high in open flowers and decreases upon senescence. Third, the expression of transcription factors displays a rhythmic pattern and precedes the emission of volatiles.

The first regulator of petunia scent production, a R2R3-MYB transcription factor, ODORANT1 (ODO1) has been identified in *P.hybrida* cv. Mitchell flowers using a transcriptomic approach (Verdonk *et al.*, 2005). *ODO1*-silenced lines reduced the expression of *DAHPS*, *EPSPS*, *CM*, *PAL* and most FVBP biosynthetic genes (except *BPBT* and *BSMT*). Downregulation of *ODO1* also resulted in a severe reduction of volatile benzenoid/phenylpropanoid production in *P.hybrida* cv. Mitchell (Verdonk *et al.*, 2005). ODO1 was shown to activate *EPSPS* promoter, suggesting that ODO1 controls the precursor availability in shikimate pathway (Verdonk *et al.*, 2005) (Fig.1). *ODO1* is exclusively expressed in fragrant petunia flowers, as the expression was not detected in non-fragrant petunia (Van Moerkercke *et al.*, 2011). The absent of MYB binding sites (MBS) of *ODO1* promoter in non-fragrant cultivar R27 explained the low expression of *ODO1* in its flowers (Van Moerkercke *et*

al., 2011). In addition, ODO1 can activate the ABC transporter promoter (*ABCG1*), which facilitates the benzenoid/phenylpropanoid compounds crossing the plasma membrane (Van Moerkercke *et al.*, 2012, Adebesin *et al.*, 2017) .

An interaction between ODO1 and another R2R3 MYB transcription factor, EMISSION OF BENZENOID II (EOBII) has been demonstrated by trans-activation assay in *N.bentamiana* leaves (Van Moerkercke *et al.*, 2011). EOBII can activate the *ODO1* promoter by binding to the MBS within 1.2kbp enhancer region of the start codon in *ODO1* promoter (Van Moerkercke *et al.*, 2011). Another R2R3 MYB-like transcription factors, EOBI was also shown to interact with the *ODO1* promoter by yeast one-hybrid and electrophoretic mobility shift assay (EMSA) (Spitzer-Rimon *et al.*, 2012) (Fig. 1). Both EOBI and EOBII activate *ODO1* promoters, as well as *PAL* and *IGS* promoters, and EOBII is able to bind the *EOBI* promoter in petunia leaf protoplast (Spitzer-Rimon *et al.*, 2010, Spitzer-Rimon *et al.*, 2012) (Fig. 1, Fig. 2). On the other hand, *ODO1*-silenced lines had upregulated *EOB1* transcript levels, suggesting a negative feedback loop between EOBI and ODO1 (Spitzer-Rimon *et al.*, 2010). Downregulation of *EOBI* and *EOBII* reduced the expression levels of *ODO1* and FVBP biosynthetic genes, and also the emission of benzenoid/phenylpropanoid (Spitzer-Rimon *et al.*, 2010, Spitzer-Rimon *et al.*, 2012). However, overexpression of *EOBII* did not upregulate *ODO1* transcript level, suggesting other factors are required to activate *ODO1* in petunia. *EOBII* has also been demonstrated to be involved in flower opening, as the flower of *EOBII*-silenced lines failed to enter anthesis (Colquhoun *et al.*, 2011b).

Based on available petunia transcript sequences with homology to Arabidopsis R2R3-MYB TFs, a repressor of *C4H* transcription, namely PhMYB4 has been characterized (Colquhoun *et al.*, 2011a) (Fig.2). Suppression of *PhMYB4* resulted in increased *C4H* transcript levels, and increased emission of C_6-C_3 volatiles. The emission of C_6-C_2 was reduced in *PhMYB4* suppressed lines, but this could be explained by channeling of the flux to the C_6-C_3 pathway (Colquhoun *et al.*, 2011a).

3. Petunia volatiles and pollination syndromes

Most of the time, plants have to compete for pollinator attention. Discrimination between flower traits will favor the selection of pollinators by being visible and detectable to the animals. For example, strong scented flowers are correlated with specialization in plant pollination system. Nocturnal moth-pollinated flowers are typified by having light color of flowers and often pleasant and strong scents at night (Raguso, 2008, Haverkamp *et al.*, 2016), which are mostly dominated by benzenoids and terpenoids (Hoballah *et al.*, 2005, Cunningham *et al.*, 2006, Okamoto *et al.*, 2008, van der Niet *et al.*, 2015). By contrast, day-pollinated flowers display brightly colored flowers in order to attract pollinators, they produce little or no fragrance. For instance, different color of flowers evoke specific response by bees and birds, depending on UV reflectance spectrum (Papiorek *et al.*, 2016).

The study of petunia flower characteristics that associate with pollination syndromes has rapidly developed in recent years. Three closely related species, *P.integrifolia*, *P.axillaris* and *P.exserta* have distinct flower characteristic, and each represents different pollinator's preferences. *P.integrifolia* has characteristic bee-pollinated flowers; violet-reddish flowers with a short and wide corolla tube, emitting small amount of volatiles (Hoballah *et al.*, 2005). *P.axillaris* has white flowers with a long and narrow corolla tube and emits large amount of volatiles from dusk to midnight (Hoballah *et al.*, 2005, Oyama-Okubo *et al.*, 2005).Its flowers

are primarily pollinated by hawkmoths (Ando *et al.*, 2001). *P.exserta* displays a typical hummingbird pollination; red flower petals, long petal tubes, no scent and it contains a copious amount of nectar (Hermann and Kuhlemeier, 2011). Natural hybrids of *P.axillaris* and *P.integrifolia* have never been reported in a sympatric population, although hand pollination results in fertile hybrid offspring (Ando *et al.*, 2001). It is thought that pollination by different insect orders is the major reason of lacking natural hybrids. Therefore, the genus Petunia is a suitable model system to study the molecular evolution of pollination syndromes (Stuurman *et al.*, 2004).

The genetic difference of nectar volume has been studied in *P.integrifolia* and *P.axillaris*. In laboratory, *P.axillaris* contains a ten times higher nectar volume than *P.integrifolia* (Stuurman *et al.*, 2004). Four minor QTLs were identified in an inbred line population of *P.integrifolia* x *P.axillaris*. Low additive effects of these QTLs were detected, suggesting that nectar volume is a highly polygenic trait (Galliot *et al.*, 2006).

P.integrifolia is thought to be an ancestor of the genus *Petunia* (Dell'olivo *et al.*, 2011). Studies have demonstrated that the transition from purple *P.integrifolia* flowers to white *P.axillaris* flowers are caused by inactivation of ANTHOCYANIN2 (AN2), a MYB transcription factor that regulates the genes encoding enzyme in anthocyanin biosynthesis (Quattrocchio *et al.*, 1999, Hoballah *et al.*, 2007). Recent work by Sheehan et al. (2016) revealed that the transition of purple to white color petunia is also beneficial to hawkmoth pollination. The hawkmoth prefers UV-absorbing flower (*P.axillaris*) to UV-reflecting flowers (*P.integrifolia*). Thus, gain of UV-absorbance by the activation of MYB-FL, which regulates *flavonol synthase* (*FLS*) expression in *P.axillaris*, revealed that both color and flavonol contribute the evolutionary shift from bee to moth pollination (Sheehan *et al.*, 2016).

Genetic dissection of the pollinator shift from bee to hawkmoth and from hawkmoth to hummingbird in petunia has been intensively studied recently. Two main quantitative trait loci (QTLs) on chromosome II and VII have been identified as major contributors to the pollination shift from hawkmoth to hummingbird (Klahre *et al.*, 2011, Amrad *et al.*, 2016). Introgression of *P.exserta* in the genetic background of *P.axillaris* revealed that the causal gene underlying the QTL on chromosome II is *CNL*, which lost its function during the transition from moth to hummingbird pollination (Amrad *et al.*, 2016), whereas the QTL on chromosome VII contained ODORANT1 (ODO1) (Klahre *et al.*, 2011). Introgression of *P. exserta* chromosome VII into *P.axillaris* abolished most of petunia volatiles (Klahre *et al.*, 2011), which is probably caused by a mutation in *ODO1* promoter (Van Moerkercke *et al.*, 2011) . Loss of CNL function reduced the emission of benzenoid volatiles, the main compounds for hawkmoth attraction, but did not reduce the C_6-C_3 volatiles. However, inactivation of CNL and ODO1 are unlikely to be the major cause for attracting hummingbirds, but rather to reduce the plant metabolic cost (Amrad *et al.*, 2016). Loss of flavonol in flowers and gain of visible color are more relevant in gaining hummingbird attraction.

Both *P.integrifolia* and *P.axillaris* flowers release volatiles, but in different composition and at different times. *P.axillaris* produces a bouquet of volatiles; methylbenzoate, benzaldehyde, benzylbenzoate, benzylalcohol, phenylacetaldehyde and (iso)eugenol, and the emission is controlled by an endogenous circadian clock (Hoballah *et al.*, 2005, Kondo *et al.*, 2006). *P.integrifolia* flowers produce only benzaldehyde and in similar quantities during the day and night (Hoballah *et al.*, 2005). Both species express *CNL*, but *P.integrifolia* lacks expression of *BPBT* and *BSMT* (Amrad *et al.*, 2016). BPBT and BSMT are responsible for the synthesis of

benzylbenzoate/phenylethylbenzoate and methylbenzoate, respectively (Fig. 2). An increase in the complexity of benzenoid compounds has been reported in a *P.axillaris* x *P.integrifolia inflata* F_1 hybrid. Only the *P.axillaris BPBT* and *BSMT* alleles were expressed in the F_1 hybrid, indicating that *cis*-acting polymorphisms are involved instead of transcription factors (Amrad *et al.*, 2016). Interestingly, a cross between *P.exserta*, which lacks *CNL* expression, but has active *BPBT* and *BSMT*, with *P.inflata* that has active *CNL*, restored methylbenzoate and benzylbenzoate production, but produced ten times less benzaldehyde than *P.inflata* (Amrad *et al.*, 2016). This indicates that a maximum amount of volatile production in flowers can be reached.

4. Outline of the thesis

Chapter 2
We characterize a caffeoyl-CoA *O*-methyltransferase (CCoAOMT1) from fragrant *P.hybrida* cv. Mitchell petals that methylates caffeoyl-CoA to feruloyl-CoA. Downregulation of *CCoAOMT1* leads not only to a reduction in eugenol production, but also leads to activation the anthocyanin pathway, which results in anthocyanin accumulation in flowers and vegetative tissues. The metabolic connection between scent and color are discussed in this chapter.

Chapter 3
In this chapter, we investigate the role of *CCoAOMT3*, an isoform of *CCoAOMT1*, in white *P.hybrida* cv. Mitchell and violet *P.hybrida* cv. V26 flowers. Downregulation of *CCoAOMT3* results in strong reduction of internal levels and emission of (iso)eugenol in *P.hybrida* cv. V26. However, *CCoAOMT3* downregulation in *P.hybrida* cv. Mitchell neither reduces (iso)eugenol emission, nor accumulation of anthocyanins, as has been reported in *CCoAOMT1* silenced lines (Chapter 2). Our findings indicate that these homologous genes are associated with different physiological functions.

Chapter 4
In this study, we investigate the role of evening elements (EE) in the *ODO1* promoter of *P.hybrida* cv. Mitchell and *P.hybrida* cv. V26. The absence of one EE in *P.hybrida* cv. V26 *ODO1* promoter not only correlates with the earlier expression of *ODO1* and volatile emission, but also increases the emission of benzaldehyde. A similar phenotype is observed upon mutation of both EEs from *P.hybrida* cv. Mitchell *ODO1* promoter, indicating the contribution of EE to the regulation of ODO1 and benzenoid/phenylpropanoid emission. We also propose a hypothetical pathway for benzaldehyde production in petunia.

Chapter 5
In Chapter 5, we selected recombinant inbred lines (RILs) of *P.axillaris* x *P.integrifolia* that produce more benzylbenzoate in the morning. QTL analysis shows that one major locus for early benzylbenzoate production is located on chromosome II. This chapter addresses the possible candidate gene that underlies the QTL on chromosome II.

Chapter 6
Here the findings of the thesis will be discussed in a broader context.

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