The floral volatile phenylpropanoid/benzenoid pathway in petunia

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CHAPTER 1

Petunia as a model to study floral volatile benzenoid/phenylpropanoid biosynthesis, regulation and transport

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1. Plant secondary metabolism

At least 100,000 secondary metabolites have been identified from a relative small number of plants species, but a clear function for most of them remains unknown (Verpoorte, 1998). Secondary metabolites have established roles in plant defence against pathogens (Batz et al., 1998; Dixon, 2001; Huang et al., 2010a) and herbivores (Pare and Tumlinson, 1999), as animal attractants or repellents (Pichersky and Gershenzon, 2002), in plant-plant communication (Baldwin et al., 2002; Kessler et al., 2006; Heil and Silva Bueno, 2007; Mirabella et al., 2008), and in abiotic stress responses (Dixon and Paiva, 1995; Fritz et al., 2006; Huang et al., 2010a). Their production often depends on the physiological and developmental stage of the plant and is triggered by biotic or abiotic interactions. Therefore it is highly coordinated and regulated. In addition to their roles in plant biology and animal nutrition, they are also indisputably valuable as sources for chemical and biomedical applications (Li et al., 1995; Jang et al., 1997; Verpoorte et al., 2000; Forkmann and Martens, 2001; Oksman-Caldentey and Inze, 2004). The notion that secondary metabolites are functional in plant growth and development is becoming increasingly evident (Taylor and Grotewold, 2005). Therefore the term “secondary” in this thesis does not refer to a qualitative but rather to a structural feature, i.e. compounds produced by pathways starting from “primary” products. Indeed, secondary metabolites are generally produced from primary metabolism precursors such as amino acids, lipids and carbohydrates. The vast number of different secondary metabolites contrasts the relative small number of precursors they are derived from. In part this can be explained because biosynthetic enzymes belong to general classes, such as O-methyltransferases and acetyltransferases, which can be promiscuous towards substrates (Dixon et al., 2001; Parvathi et al., 2001; Zubieta et al., 2002; Beekwilder et al., 2004; Guterman et al., 2006; Long et al., 2009). In addition, compounds can be produced via separate routes within a pathway or via separate pathways (Wildermuth et al., 2001).

The different classes of plant secondary metabolites are generally grouped according to the pathway they are derived from. Most of these natural products originate from the alkaloid, isoprenoid, flavonoid or phenylpropanoid pathways. Alkaloids (De Luca and Laflamme, 2001) are N-containing compounds
with characteristic toxic and pharmacological properties, which are produced from various amino acids (Phe, Tyr, Trp, Lys and ornithine). Indole alkaloids are produced from Trp and terpenoids. Isoprenoids are common in all plants and have functions in both primary and secondary metabolism (Lange et al., 2000). They are produced from the C\textsubscript{5} precursor isopentenyl diphosphate (IPP) either via the cytosolic mevalonate (Goldstein and Brown, 1990; Chappell, 1995) or the plastidial 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway (Rohmer et al., 1993; Schwender et al., 1996; Lange et al., 1998), and include some photosynthetic pigments, plant hormones and terpenes.

Phenylpropanoids are produced from L-Phenylalanine (L-Phe), which is produced via the shikimate pathway and aromatic amino acid pathway (Herrmann, 1995a; b; Herrmann and Weaver, 1999; Tzin and Galili, 2010). The basic C\textsubscript{6}-C\textsubscript{3} carbon skeleton of L-Phe is modified in a series of complex branched pathways, leading to the production of diverse phenolic compounds including flavonoids, lignins, coumarins and benzenoids (Winkel-Shirley, 2001; Boerjan et al., 2003; Schuurink et al., 2006). Flavonoids are derived from malonyl-CoA and L-Phe and include flavones, flavonols, isoflavonoids, stilbenes, anthocyanins, condensed tannins and chalcones. They are involved in biotic and abiotic stress responses, and pigmentation in flowers, seeds and fruits (Winkel-Shirley, 2001; Koes et al., 2005). Phenylpropanoids are involved in development (Murphy et al., 2000; Taylor and Grotewold, 2005) and their production is highly regulated by both biotic and abiotic factors (Li et al., 1993; Dixon and Paiva, 1995; Jin et al., 2000; Huang et al., 2010b) and the developmental program (Martin and Gerats, 1993). Fruit ripening, as an example, has been shown to involve an extensive reprogramming of the expression of phenylpropanoid genes, comprising flavonoid, monolignol and benzenoid/phenylpropanoid metabolism (Boss et al., 1996; Chen et al., 2006). The benzenoids and C\textsubscript{6}-C\textsubscript{3} phenylpropanoid compounds that are found in fleshy fruits and contribute to their complex flavour are also found in floral bouquets (headspace).
2. Pollination syndromes and floral volatiles

Plants have developed several ways to attract specific pollinators, collectively referred to as pollination syndromes (Faegri and Van der Pijl, 1979) (Stuurman et al., 2004). In addition to colour, shape, surface structure and nectar reward, many flowers produce volatile compounds. Distinct pollination syndromes have evolved in the genus Petunia. The white P. axillaris ssp. and coloured P. integrifolia ssp. are pollinated by hawk moths and bees, respectively (Hoballah et al., 2005). In addition, many different Petunia x hybrida cultivars have been generated. This has resulted in a large collection of fragrant and non-fragrant cultivars displaying floral colours ranging from pale white to violet and red flowers. The fragrant cv. Mitchell (Ausubel et al., 1980), also referred to as W115 (white 115) from the Amsterdam collection (Figure 1a), originates from a complicated hybrid cross between P. axillaris (Figure 1b) and the cultivar ‘Rose of Heaven’ and is extensively used to study floral fragrance biosynthesis and regulation. Like P. axillaris, the floral volatile bouquets of P. hybrida cv. Mitchell and V26 (violet 26) (Figure 1) mainly consists of phenylpropanoid/benzenoid compounds, but the exact composition and ratio of the compounds between these flowers differs. The P. hybrida cv. W138 produces most of these compounds as well, but the overall output is lower (Stuurman et al., 2004). Contrary, the cv. R27 (red 27) does not emit detectable levels of floral volatiles (Van Moerkercke, unpublished results).

Like other moth-pollinated plants, P. axillaris (and P. hybrida cv. Mitchell) emits volatiles in a rhythmic fashion, with levels rising from dawn until midnight (Verdonk et al., 2003; Hoballah et al., 2005) for most, but not all compounds (Simkin et al., 2004). Day-pollinated plants, like Anthririnum majus and certain Rose cultivars emit floral volatiles rhythmically with peak emission during the day (Kolosova et al., 2001a; Hendel-Rahmanim et al., 2007), corresponding with the activity of their pollinators. Some coloured P. integrifolia integrifolia spp. continuously emit benzaldehyde as a sole compound at levels comparable to those of P. axillaris axillaris N. (Hoballah et al., 2005).
Figure 1. Flowers of the different petunias used in this thesis. Petunia hybrida cv. Mitchell (a), P. axillaris axillaris N. (b), P. hybrida cv. V26 (c) and P. hybrida cv. R27 (d). The flowers of R27 are non-fragrant.

Floral volatiles may mainly serve as cues to attract pollinators, they can also have a role in defence (Hoballah et al., 2005) or as repellents (Junker and Bluthgen, 2011). They are generally lipophilic compounds with low to medium molecular weight and high vapour pressure, and are wildly distributed across the plant kingdom (Knudsen, 2006). Aliphatic compounds, terpenoids and phenylpropanoids/benzenoids constitute the largest classes of volatile compounds (Dudareva and Pichersky, 2006). Some of the compounds produced and emitted by flowers are also emitted by vegetative tissue (Gang et al., 2001; Gang et al., 2002). In C. breweri, benzylbenzoate emission from leaves could be induced after damaging but under normal condition is restricted to flowers (D’Auria et al., 2002). Generally, however, volatiles released from vegetative tissues serve to repel herbivores and to attract enemies of the herbivores (Kessler and Baldwin, 2001; Sabelis et al., 2001; Pichersky and Gershenson, 2002).
3. Floral volatile phenylpropanoid/benzenoid biosynthesis

Phenylpropanoid/benzenoid compounds dominate the floral headspace of fragrant petunias (Verdonk et al., 2003; Hoballah et al., 2005). Over 300 volatile phenylpropanoid/benzenoid compounds have been identified from floral headspaces (Dudareva and Pichersky, 2006). Given the relative small number of emitted compounds by petunia flowers, they are a suitable system to study the biosynthesis and regulation of this pathway. Volatile phenylpropanoid/benzenoid compounds emitted by fragrant petunias include the C$_6$-C$_3$ compounds eugenol and isoeugenol, the C$_6$-C$_2$ compounds 2-phenylethanol, 2-phenylacetaldehyde, 2-phenylethylactate and phenylethylbenzoate, and the C$_6$-C$_1$ (benzenoid) compounds benzaldehyde, benzylalcohol, methylbenzoate (MeBA), benzylacetate, methylsalicylate (MeSA), benzylbenzoate and vanillin (Figure 2). In addition, petunia emits non-phenylpropanoid/benzenoid floral compounds, which might serve different function then attracting pollinators (Verdonk et al., 2003; Simkin et al., 2004; Hoballah et al., 2005). The production and emission of these compounds requires the concerted action of many enzymes acting on different branches of the volatile phenylpropanoid/benzenoid pathway (Figure 2), which ultimately starts from primary carbohydrate metabolism.

3.1 Precursor biosynthesis

Phenolic compounds are derived from L-Phe, which is produced via the shikimate pathway and the aromatic amino acid pathway. The shikimate pathway uses phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P), which are produced in the glycolysis and the non-oxidative part of oxidative pentose phosphate pathway, respectively. The production of volatile phenylpropanoids/benzenoids requires a significant flux of carbon through the shikimate pathway and the carbohydrates fixed during photosynthesis must be transported to non-photosynthetic tissue such as flowers. The shikimate pathway ends with the production of chorismate, involving seven reactions catalysed by six enzymes (Herrmann and Weaver, 1999). Expressed sequence tags for 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase
Figure 2. Metabolic pathways leading to the production of volatile phenylpropanoids/benzenoids in petunia petals. Volatile compounds emitted by *Petunia hybrida* cv. Mitchell are boxed in grey. Hypothetical enzymatic steps in petunia petals (single dashed arrows) were deduced from feeding studies and
radioactive labelling experiments (Boatright et al., 2004) and indicate as yet uncharacterised enzymatic steps. Solid arrows and enzymes shown in red indicate known steps in petunia. Enzymes in blue are characterised steps in other plant ssp. (At: Arabidopsis thaliana, Cb, Clarkia breweri, Am, Anthirrinum majus, Nt: Nicotiana tabacum, Sl: Solanum lycopersicon). Multiple dashed arrows indicate multiple known and/or unknown steps. Arrows in green indicate enzymatic steps involved in the CoA-dependent β-oxidative pathway.

Abbreviations: DAHPS: 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase; EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase; CS: chorismate synthase; ADT: arogenate dehydratase; CM: chorismate mutase; ICS: isochorismate synthase; PAL: phenylalanine ammonia lyase; C4H: cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA-ligase; PAAS: phenylacetaldehyde; PAR: phenylacetaldehyde reductase; AAE: acyl activating enzyme; KAT1: 3-ketoacyl-CoA thiolase; BADH: benzoic acid dehydrogenase; BPBT: benzoyl-CoA:benzylalcohol/2-phenylethanol benzoyltransferase; BSMT: S-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase; CFAT: coniferylalcohol by acetyl-CoA:coniferyl alcohol acetyltransferase; CCoAOMT: caffeoyl-CoA O-methyltransferase; IGS: isoeugenol synthase; EGS: eugenol synthase; BEAT: acetyl-CoA:benzylalcohol acetyltransferase; BZL: benzoate:CoA ligase.

(DAHPS), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and chorismate synthase (CS) (Figure 2) have been identified in petunia petals (Verdonk et al., 2003; Spitzer-Rimon et al., 2010) and the contribution of EPSPS in floral fragrance biosynthesis was shown by its activation by the R2R3-MYB TF ODORANT1 (ODO1), a regulator of floral fragrance biosynthesis (Verdonk et al., 2005). L-Phe is further synthesised via the aromatic amino acid pathway starting from chorismate (Tzin and Galili, 2010). Two flower-specific cDNAs, arogenate dehydratase (ADT) and chorismate mutase (CM), involved in L-Phe biosynthesis in petunia petals were identified and their products have been characterised recently (Colquhoun et al., 2010b; Maeda et al., 2010) (Figure 2). As L-Phe is used for protein synthesis and shared with other pathways, regulation of L-Phe utilisation is critical and many feedback loops in L-Phe synthesis have been shown in plants (Tzin and Galili, 2010).
3.2 The volatile phenylpropanoid/benzenoid pathway

Structurally, the volatile benzenoids/phenylpropanoid compounds are categorised according to the length of the carbon side-chain attached to the benzene ring: C₆-C₁ compounds, C₆-C₂ compounds and C₆-C₃ compounds (Figure 2). In petunia, the first step for the synthesis of C₆-C₂ compounds involves a two-step reaction catalysed by the bifunctional phenylacetaldehyde synthase (PAAS), which converts L-Phe to 2-phenylacetaldehyde (Kaminaga et al., 2006). In tomato, aromatic amino acid decarboxylase (SlAADC) first converts L-Phe to phenethylamine, which is subsequently deaminated to 2-phenylacetaldehyde (Tieman et al., 2006) followed by reduction to 2-phenylethanol by 2-phenylacetaldehyde reductase (SIPAR) (Tieman et al., 2007). In rose flowers, a third route for 2-phenylethanol was suggested, involving phenylpyruvate and phenyllactic acid as intermediates (Watanabe et al., 2002).

The gene for 2-phenylethanol production in petunia has not been identified. 2-phenylethylacetate is likely produced from 2-phenylethanol by an acyltransferase, but genetic or enzymatic evidence in petunia is lacking. However, ectopically expression of a rose geraniol acetyl transferase in petunia petals resulted in an increased production of 2-phenylethylacetate but unaltered emission of 2-phenylethanol (Guterman et al., 2006), illustrating the promiscuity of certain enzymes towards multiple substrates.

The first step in C₆-C₁ and C₆-C₃ biosynthesis is catalysed by phenylalanine ammonia lyase (PAL), which converts L-Phe to trans-cinnamic acid (t-CA) and competes with PAAS for the same substrate (Figure 2). The C₆-C₃ carbons structure of t-CA needs shortening by two carbon atoms to produce C₆-C₁ compounds. Two basic pathways for the production of benzoic acid (BA) have been proposed in plants (Ryals et al., 1996) and labelling studies and flux analysis in petunia petals confirmed the involvement of a β-oxidative pathway and a non-β-oxidative pathway (Boatright et al., 2004) (Figure 2). Whereas the β-oxidative pathway needs activation by CoA, the non-β-oxidative pathway in petunia petals may or may not involve CoA-activation (Boatright et al., 2004). Benzoyl-CoA and benzaldehyde are the principal intermediates for the β-oxidative and non-β-oxidative pathways leading to BA, respectively, and their
identification has been used to confirm the involvement of either pathway (Wildermuth, 2006).

The activity of some of the enzymes of the CoA-dependent non-β-oxidative pathway has previously been detected in cell culture suspensions and cell-free extracts (Yazaki et al., 1991; Schnitzler et al., 1992; Abd El-Mawla and Beerhues, 2002), but the corresponding genes have remained elusive. It was estimated that the flux through the non-β-oxidative pathway is twice as large as the flux through the β-oxidative pathway (Boatright et al., 2004) and suppression of the flux to benzylbenzoate enhanced the flux through the non-β-oxidative pathway in the light period (Orlova et al., 2006). Ectopically and transiently expressed benzaldehyde dehydrogenase (BADH) from snapdragon increased free BA pools 2-fold in petunia flowers and the recombinant enzyme was able to convert benzaldehyde to BA in vitro (Long et al., 2009). However, an increase in BA, and some products thereof, was only seen when the flowers were fed with benzaldehyde (Long et al., 2009). Feeding experiments in N. tabaccum with labelled benzaldehyde showed BA and subsequent salicylic acid (SA) production via benzaldehyde, suggesting non-β-oxidative production of BA (Ribnicky et al., 2007). However, when radiolabelled L-Phe was fed, t-CA, BA and SA, but not benzaldehyde, were labelled (Ribnicky et al., 1998). Therefore, it was concluded that (1) BA production in tobacco occurred via the β-oxidative pathway and (2) the results previously obtained might have been an artefact of benzaldehyde feeding (Jarvis et al., 2000). In agreement with this, intermediates of the β-oxidative pathway were detected in N. attenuata and Cucumis sativus L. plants and benzaldehyde in these plants is thus likely produced from BA and not the other way around (Jarvis et al., 2000). BA production in bacteria mirrors the β-oxidative degradation of fatty acid degradation (Graham and Eastmond, 2002) (Figure 3), and involves activation of t-CA and the production of benzoyl-CoA (Figure 3). Although in bacteria most corresponding genes have been cloned (Hertweck and Moore, 2000; Hertweck et al., 2001; Moore et al., 2002; Xiang and Moore, 2003), in plants they have remained uncharacterised. We identified and characterised a peroxisomal 3-ketoacyl-CoA thiolase (KAT1) (Van Moerkercke et al., 2009, CHAPTER 2) and a peroxisomal acyl activating enzyme (AAE) in petunia, which putatively activates t-CA (Figure 2 and 3) (Colquhoun and Clark,
personal communication). Together, these experiments identified the CoA-dependent β-oxidative production of benzenoids as the dominant pathway.

Figure 3. Benzoyl-CoA production in petunia petals mirrors fatty acid catabolism. The three consecutive enzymatic conversions starting from trans-cinnamic acid resulting in the production of benzoyl-CoA are similar to those of the general fatty acid catabolism and involve the action of a hydratase, a dehydrogenase and thiolase. The latter was identified in petunia petals.

The benzoyl-CoA moiety is further used to produce volatile benzylbenzoate/phenylethylbenzoate from benzylalcohol/2-phenylethanol, respectively, catalysed by benzoyl-CoA:benzylalcohol/2-phenylethanol benzoyltransferase (BPBT) (Boatright et al., 2004) (Figure 2). BA could also be produced directly from benzoyl-CoA by the action of an acyl-CoA-thioesterase (Barillas and Beerhues, 2000; Tilton et al., 2004) and the reverse reaction in Clarkia breweri is catalysed by benzoate:CoA ligase (BZL) (Beuerle and Pichersky, 2002). SA can be produced from BA by the action of 2-hydroxylase in tobacco (Leon et al., 1993; Yalpani et al., 1993; Leon et al., 1995; Sawada et al., 2006; Yu et al., 2010) and L-Phe-derived SA production was shown in tobacco
and Arabidopsis by several reports (Jarvis et al., 2000; Huang et al., 2010b). Alternatively, SA production via isochorismate, involving isochorismate synthase (ICS), was shown in Arabidopsis (Wildermuth et al., 2001) (Figure 2). Methylbenzoate (MeBA) is produced by methylation of BA by S-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase (BSMT) (Negre et al., 2002). This enzyme shows affinity for SA as well (Figure 2), but internal pools and emitted levels of MeSA are lower due to lower concentration of SA in petal cells (Boatright et al., 2004). Benzylacetate is produced from benzylalcohol by acetyl-CoA:benzylalcohol acetyltransferase (BEAT) (Figure 2) in Clarkia breweri (Dudareva et al., 1998). Similar as for the production of 2-phenylethylacetate from 2-phenylethanol, benzylacetate could be produced from benzylalcohol by the action of a rose alcohol acetyltransferase (Guterman et al., 2006).

The first steps for the production of volatile C_6-C_3 phenylpropanoids are shared with the general phenylpropanoid pathway and involve the successive actions of PAL, cinnamate 4-hydroxylase (C4H) and 4-coumarate CoA-ligase (4CL) (Figure 2). In petunia, PAL and CH4 seem to be encoded by small gene families comprising three and two members, respectively (Colon et al., 2010; Colquhoun et al., 2010a). As for other phenylpropanoid compounds, p-coumaric acid (Orlova et al., 2006) and possibly p-coumaroyl-CoA are intermediates for the volatile C_6-C_3 phenylpropanoids. Coniferylacetate is produced from coniferylalcohol by acetyl-CoA:coniferyl alcohol acetyltransferase (CFAT) (Dexter et al., 2007). Interestingly, accumulation of intermediates as a result of CFAT-silencing resulted in reduced enzyme activities of BSMT and BPBT (Dexter et al., 2007), illustrating the effect of perturbations in one branch of the pathway on another branch. Coniferylalcohol is also the precursor for the production of S-lignin and G-lignin (Boerjan et al., 2003). Coniferylacetate is used by eugenol synthase (EGS) and isoeugenol synthase (IGS) to form the volatiles eugenol and isoeugenol, respectively (Koeduka et al., 2006) (Figure 2). The genes involved in the production of coniferylalcohol in petunia petals are unidentified and the exact route of its production is unknown. It is possible that the same genes responsible for coniferyl alcohol production for lignin biosynthesis are involved in production of C_6-C_3 compounds (Boerjan et al., 2003). With respect to this, we recently cloned an ODORANT1-dependent cDNA from petunia, which is predominantly
expressed in mature petals, encoding a putative caffeoyl-CoA O-methyltransferase (CCoAOMT) that potentially methylates caffeoyl-CoA to feruloyl-CoA (Van Moerkercke, unpublished results). The pathway leading to vanillin, a minor component in the petunia headspace, is only partially characterised in plants. It is produced from p-coumaric acid (Podstolski et al., 2002) and ferulic acid (Negishi et al., 2009), potentially involving a β-oxidative chain shortening leading to vanillin (Negishi et al., 2009), but a non-oxidative pathway was also proposed (Podstolski et al., 2002).

4. Developmental, rhythmic and spatial production of volatiles

4.1 Tissue-specific expression and transcript levels during flower development

The phenylpropanoid pathway operates in roots, stems, leaves, seeds and flowers. In fragrant petunias, volatile compounds are produced and emitted from the flower petals. In which tissue the specific branches are expressed depends on the TFs that act tissue-specifically. As a result, all identified genes involved in floral volatile phenylpropanoid/benzenoid biosynthesis are predominantly expressed in petals, although transcripts have been detected in other tissues as well (Orlova et al., 2006; Dexter et al., 2008).

The production and emission of floral volatiles is tightly regulated during flower development (Dudareva et al., 2000; Verdonk et al., 2003). Fragrance biosynthesis in a developing flower would lead to precursor competition with the production of pigments, which are produced during petal development (Martin and Gerats, 1993). Indeed, transcripts of chalcone synthase (CHS) peak early in flower development whereas transcripts of floral phenylpropanoid/benzenoid genes accumulate after anthesis (Verdonk et al., 2005). This implies the integration of flower opening, anthesis and fragrance biosynthesis, and requires the involvement of higher-order TFs, which co-ordinately regulate these processes. TFs that have roles in both development and secondary metabolism have been described (Montiel et al., 2007; Re et al., 2010). The ornamental tobacco MYB305 regulates genes involved in floral nectary production and phenylpropanoid biosynthesis, including PAL, NECTARIN (NEC) 1 and NEC5.
(Liu et al., 2009). Whereas MYB305 is expressed in early and late phases of flower development, its direct target genes NEC1 and NEC5 are expressed after anthesis (Liu et al., 2009). Stable silencing of MYB305 in ornamental tobacco resulted in a failure of petal opening, in addition to nectary deficiencies (Liu et al., 2009), suggesting that in tobacco, MYB305 coordinates both flower opening and production of nectary. Recently, a homolog of MYB305 was identified in petunia (Spitzer-Rimon et al., 2010).

Cis-regulatory sequences or promoter regions that determine flower-specific expression in petunia have been identified. Generally they reside in the promoter along with cis-regulatory elements conferring expression in response to environmental and developmental cues and often, these elements confer expression to other (floral) tissues as well (Benfey et al., 1990; van der Meer et al., 1990; Solano et al., 1995a; Faktor et al., 1996; Hill et al., 1998; Hartmann et al., 2005; Geng et al., 2009). The factors that bind to these elements in some case have been identified. For instance, the MYB.Ph3 TF binds to a MYB binding site in the promoter of flavonoid genes (Solano et al., 1995a; Solano et al., 1995b; Solano et al., 1997). For the fragrance genes in petunia, these elements have not yet been identified.

4.2 Rhythmic expression and the circadian clock

The production and emission of floral volatiles is energetically demanding and is regulated with respect to the time of the day, such that maximal activity is adjusted to the activity of their pollinators. The co-ordinated expression of fragrance genes during the night and day can be facilitated by trans-factors that recognise the cis-elements in the promoters of these genes. Volatile emission in petunia and Anthirrinum peaks at night and during the day, respectively, and is dependent on an endogenous circadian clock. In addition, transcript levels of the genes involved in volatile fragrance biosynthesis accumulate rhythmically (Kolosova et al., 2001a; Verdonk et al., 2003; Colquhoun et al., 2010c). Rhythmic production and emission does not seem to result from rhythmic activity of the corresponding enzymes, but rather from the availability of substrates, which accumulate during the night in petunia and the day in Anthirrinum (Kolosova et al., 2001a; Maeda et al., 2010). Enzymatic activities of BSMT, PAAS, PAL and
CM are high even when no volatiles are produced (Kolosova et al., 2001a; Dexter et al., 2007; Colquhoun et al., 2010b; Maeda et al., 2010), indicating relative constant protein levels that “override” the rhythmic expression of the biosynthetic genes. Whether protein levels of the TFs that regulate precursor availability follow rhythmic expression is unknown. The significance of these transcriptional patterns and ultimately how this complex transcriptional network is organised at the molecular level remains to be resolved.

Genome-wide studies have shown that many genes of the phenylpropanoid pathway are under control of the circadian clock in Arabidopsis (Harmer et al., 2000), with peak expression predominantly in the evening and night. Analysis of the promoter region of these genes showed an enrichment of certain motifs in these promoters. One over-represented motif is the circadian clock associated 1 (CCA1)-binding site (CBS)-related evening element (EE: aAAATATCT), which in the right context (Michael and McClung, 2002) can confer evening-specific transcription (Carre and Kay, 1995; Wang et al., 1997; Harmer et al., 2000; Harmer and Kay, 2005). Evening elements can be found in one or multiple copies in promoters of rhythmically expressed genes (Harmer et al., 2000). Some rhythmically expressed genes however do not contain EEs and thus evening-phased expression can be the consequence of other elements as well (Harmer et al., 2000). EPSPS cycles in phase with the TF ODORANT1 (ODO1) and ODO1 can trans-activate the EPSPS promoter in leaves (Verdonk et al., 2005). The promoter sequence of EPSPS does not contain EEs. This means that (1) evening-phased expression of EPSPS is not directed by EEs, (2) the putative EEs are located upstream of the investigated promoter sequence or (3) evening-phased expression of EPSPS is a direct consequence of the evening-phased expression of ODO1, without the need for a direct link with a clock-gene. In the latter case, rhythmic expression of EPSPS could be linked with the clock via circadian expression of upstream regulators like ODO1.

4.3 Transcription factors of the volatile benzenoid/phenylpropanoid pathway

Where and when the specific branches of the phenylpropanoid pathway are active depends on the transcription factors (TFs) that activate the subset of genes specific for a branch (Stracke et al., 2007). Many R2R3-MYB TFs that
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regulate the phenylpropanoid pathway have been identified (Dubos et al., 2010). For the production of flavonoids, these MYBs may interact with basic helix-loop-helix (bHLH), basic region-leucine zipper (bZIP) and WD-repeat proteins (Hartmann et al., 2005; Koes et al., 2005; Hichri et al., 2011), in a tissue-specific, developmental and light-responsive manner (Hartmann et al., 2005). Specific subsets of MYB TFs may activate a specific branch of the phenylpropanoid pathway. For instance, the myb11/myb12/myb111 triple mutant is affected in flavonol, but not anthocyanin biosynthesis, and all three TFs activate flavonol genes in a tissue-specific manner in Arabidopsis wild type seedlings (Stracke et al., 2007).

TFs that specifically regulate floral fragrance biosynthesis are predominantly expressed in petals (Verdonk et al., 2005; Colquhoun et al., 2010a; Spitzer-Rimon et al., 2010), correlating with the tissue-specific expression of their target genes and emission of volatiles in petunia. TFs often co-ordinately regulate multiple genes of the same pathway (Hartmann et al., 2005) and TFs that regulate other TFs are of particular interest because they operate as master regulators of a pathway. Co-regulation of genes of different pathways, involved in the same process, can be facilitated by higher-order TFs. For instance, the simultaneous production and emission of floral phenylpropanoid and terpenoids volatiles by Anthirrinum or Rose would need coordinated transcription of genes of both pathways. The effect of ectopic expression or silencing of these higher-order TFs are likely more pleiotropic than for TFs lower in the hierarchy. This hierarchical organisation of TFs is a common feature in transcriptional regulation and the identification of TFs and their target genes is thus crucial for understanding complex biological processes such as secondary metabolism.

Regulation of the volatile phenylpropanoid/benzenoid pathway is ill-defined (Schuurink et al., 2006). Recent efforts of several groups identified the first TFs of floral fragrance biosynthesis and some of their target genes (Verdonk et al., 2005; Colquhoun et al., 2010a; Spitzer-Rimon et al., 2010). Silencing of the petunia R2R3-MYB TF ODO1 resulted in a severe reduction in emission levels of most volatile benzenoids and phenylpropanoids (Verdonk et al., 2005). ODO1 can trans-activate the EPSPS promoter in petunia leaves, confirming its importance for substrate supply via the shikimate pathway (Verdonk et al., 2005). Silencing of ODO1 did not affect the production of flavonoids, since ODO1
transcripts accumulate only after flavonoids have been produced (Verdonk et al., 2005). The trans-activation of other promoters by ODO1 was not examined, but microarray experiments with ODO1-silenced lines have shown the indirect consequences on transcript abundance of many other genes involved in volatile biosynthesis in petunia flowers, notably EPSPS, DAHPS, PAL, CM, IGS, BSMT and BPBT (Verdonk et al., 2003; Verdonk et al., 2005).

Silencing of the petunia EMISSION OF BENZENOIDS II (EOBII) downregulates genes of the shikimate (CS and CM) and phenylpropanoid (PAL2) pathways, genes of the C₆-C₃ branch (CFAT, BPBT and IGS), and importantly, ODO1 (Spitzer-Rimon et al., 2010). Interestingly, some of the genes, notably EPSPS, DAHPS and PAL1, were affected in ODO1-silenced lines but not in EOBII-silenced lines (Spitzer-Rimon et al., 2010). Trans-activation was shown for IGS1 and the tobacco PALB promoters in Arabidopsis protoplasts. Importantly, transient overexpression of EBOII in petunia petals did not result in higher ODO1 transcript abundance at the peak of ODO1 expression, which led the authors to suggest that other factors might be needed for ODO1 activation in petunia (Spitzer-Rimon et al., 2010).

Finally, the petunia MYB4 was identified as a repressor of C4H expression (Colquhoun et al., 2010a). Although direct interaction was not shown, C4H1/2 transcript levels were upregulated in inverted repeat (ir)-MYB4 lines, with concomitantly increased C₆-C₃ volatile emission levels (Figure 2). In addition, transcript levels of PAL1/2, CM and ODO1 in these lines were higher, unaltered and slightly reduced, respectively (Colquhoun et al., 2010a). The former could be explained by depletion of the t-CA pool as a consequence of enhanced C4H activity, which normally feedback-inhibits PAL transcription (Blount et al., 2000) and could explain why emission of C₆-C₁ compounds, which also derive from t-CA, was unaffected. Contrary, the emission of most C₆-C₂ compounds was reduced (Colquhoun et al., 2010a). This could be due to depleted L-Phe pools as a consequence of increased feeding and possible channeling of substrates into the C₆-C₃ branch, directed by C4H.
5. Management of compounds within the cell

The large number of metabolites derived from L-Phe, which are often toxic and unstable, and the broad substrate specificity of certain modifying enzymes in vitro, are indicative of subcellular organisation of enzymes and their substrates (Winkel, 2004). Regulating the flux of metabolites within or between cells needs mechanisms that go beyond the level of transcriptional regulation and likely involves channeling, compartmentation and metabolite trafficking. Topics that received little attention so far are the cellular and subcellular localisation of floral volatile benzenoid/phenylpropanoid biosynthesis and the mechanism(s) of volatile emission from epidermal cells.

Few studies have focused on the cellular localisation of floral fragrance enzymes. The rose Orcinol O-methyltransferase (OOMT) and C. breweri S-adenosyl-L-methionin:(iso)eugenol O-methyltransferase (IEMT) were localised to the adaxial and abaxial epidermal cell layers (Dudareva and Pichersky, 2000; Scalliet et al., 2006; Bergougoux et al., 2007). The snapdragon S-adenosyl-L-methionin:benzoic acid carboxyl methyltransferase (AmBAMT) and AmBADH were localised to epidermal cells (Kolosova et al., 2001b; Long et al., 2009), showing that at least the final two steps in MeBA biosynthesis in snapdragon occur in epidermal cells, where also emission takes place. In petunia, these enzymes have not been localised at the cellular level, but it can be anticipated that at least the final steps occur in the epidermis as well. However, whether the entire pathway takes place in these cells is unknown. Production of indole alkaloids in Cantharanthus roseus has been shown to involve different cell-types (St-Pierre et al., 1999). In petals of of P. hybrida cv. W80 buds, the EPSPS promoter showed activity exclusively in the epidermal cell layer. In contrast, activity of the EPSPS promoter in mature flowers was seen in all cell layers of the petal (Benfey et al., 1990), but whether this result corresponds to the in situ mRNA localisation was not investigated. Also long-distance trafficking of flavonoids, putatively involving ABC-transporters for transport to the conductive tissue, has been shown (Buer et al., 2007)

Several compartments have been shown to be involved in the volatile phenylpropanoid/benzenoid pathway. Two enzymes involved in L-Phe biosynthesis in petunia petals, ADT and CM, have been localised to plastids
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using Arabidopsis protoplasts and a chloroplast import assay, respectively (Colquhoun et al., 2010b; Maeda et al., 2010). Only recently it was shown that also the last committed step in L-Phe biosynthesis occurs in plastids in Arabidopsis (Rippert et al., 2009). How L-Phe subsequently enters the cytosol is unknown. In tobacco, the cellular localisation of two representatives of the PAL family differs and depends on CH4 (Achnine et al., 2004). These differentially localised PAL isoforms and different pools of t-CA likely reflect channeling into the different branches of the phenylpropanoid pathway (Achnine et al., 2004). In mature petunia petals, three PAL and two C4H transcripts are detected, but it is not known where the encoded proteins are localised and in which specific pathway branch they are involved. However, only PAL2 transcript levels are regulated by EOBII (Spitzer-Rimon et al., 2010). Thus a connection of PAL2 with the volatile C₆-C₃ phenylpropanoids in petunia is likely. Contrary, MYB4 regulates transcription of both C4H1 and C4H2 (Colquhoun et al., 2010a).

In Rose, OOMT was localised in the cytosol, but a fraction became increasingly associated with membranes during development, suggesting a link with the secretory pathway (Scalliet et al., 2006). Whether this applies for all enzymes catalysing final steps is unknown. Certain released volatiles, like benzaldehyde and 2-phenylacetaldehyde, are precursors for other volatile compounds as well. In addition, some volatile intermediates are not found in the headspace. Together, this indicates channeling and/or a regulated process of volatile emission. The localisation of KAT1 and AAE12 in peroxisomes implies peroxisomal localisation of benzoyl-CoA and metabolite transport across the peroxisomal membrane (Van Moerkercke et al., 2009) (Colquhoun et al., in preparation). The snapdragon benzaldehyde dehydrogenase was localized to the mitochondria (Long et al., 2009), implicating a mitochondrial pool of BA that needs to be trafficked to the cytosol, where it can be methylated by BAMT (Kolosova et al., 2001b). Together, this means floral fragrance biosynthesis is highly compartmented and suggests the involvement of transporters.
CHAPTER 1

6. Outline of the thesis

CHAPTER 1 aims to give an introduction to the biochemistry, transcriptional regulation and cell biology of the floral volatile phenylpropanoid/benzenoid pathway in petunia.

In CHAPTER 2, we describe the identification and involvement of a new enzyme, a 3-ketoacyl-CoA thiolase, in the benzenoid pathway. Thiolases are best known for their involvement in the general fatty-acid catabolism. The involvement of a thiolase in the production of benzoic acid (BA) is known in prokaryotes but was never shown in plants.

CHAPTER 3 describes the functional dissection of the promoter of ODORANT1, a key regulator of volatile benzenoid/phenylpropanoids in petunia flowers. Furthermore, a molecular link between ODO1 and a second TF, Emission Of Benzenoids II (EOBII), is shown in this chapter, identifying EOBII as direct activator of ODO1 transcription.

CHAPTER 4 describes the identification of a petunia ABC-transporter of the G-subfamily and evidence for its involvement in production of floral fragrance in petunia. This chapter addresses some important issues regarding the cell biology of the volatile phenylpropanoid/benzenoid pathway.

In CHAPTER 5, the results described in this thesis are discussed.

REFERENCES


trichomes and leaves is controlled by the activities of specific acyltransferases and hydroxylases. *Plant Physiol*, **130**, 1536-1544.


