## Supplementary Note 8

# Extreme variation in volatile production in wild petunias: what can we learn from their genomes?

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**ABSTRACT**
The genus *Petunia* displays wide variation in the production and emission of floral benzenoid and phenylpropanoid (FBP) volatiles. Variation in volatile production is part of coordinated changes in multiple floral traits that lead to shifts in pollinator visitation. The molecular nature of the genetic changes is mostly unknown and might involve mutations in structural genes, *cis*-acting regulatory elements as well as transcription factors. Although the variation in FBP emission in some *Petunia* species and hybrids can be explained by the mutation of the regulatory gene *ODORANT1*, a general mechanism underlying shifts in FBP emission remains unknown. The sequencing of the genomes of two closely related *Petunia* species, *P.axillaris axillaris N* and *P. integrifolia inflata S6*, provides the tools to study the genomic mechanisms underlying shifts in FBP emission: *P.axillaris axillaris N* emits an abundant blend of FBP whereas *P. integrifolia inflata S6* only emits benzaldehyde. Here we present an annotated catalogue of all known structural and regulatory genes involved in FBP production in these two species. We show that dissimilarity in volatile emission cannot be explained by differences in the coding sequences of a single FBP biosynthetic, *L*-phenylalanine biosynthetic or regulatory gene. We conclude that the major increase in the amount, complexity and regulation of volatile emission during the evolution of *P. axillaris* has involved either hitherto unknown genes or *cis*-acting regulatory mutations in known genes. Interestingly, the genes involved in phenylacetaldehyde and (iso)eugenol production have no close homologs in other *Solanaceae*.

**INTRODUCTION**
Over 1700 volatile compounds have been identified from the floral headspace of 90 plant families, and the differences in floral volatiles at the species level is a promising tool for evolutionary studies in angiosperms (Knudsen, 2006). Pollination of many insect-pollinated plants is dependent on floral scent (Dudareva et al., 2004; Kessler et al., 2008; Klahre et al., 2011) and the abundance, ratio and composition of the volatiles in the floral headspace are suggested to be involved in reproductive isolation of closely related species (Dudareva et al., 2013). *Petunia* floral scent is mainly composed of phenylpropanoids and benzenoids (Verdonk et al., 2003), which are the second most abundant and widespread class of plant volatiles (Knudsen, 2006) (Figure 1).

Floral phenylpropanoids and benzenoids (FBPs) are derived from the precursor *L*-phenylalanine (*L*-Phe), which originates from the shikimate and predominantly arogenate pathway (Maeda et al., 2010; Tzin and Galili, 2010; Yoo et al., 2013). The phenylpropanoid related ($C_6$-$C_2$) compounds 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylethylacetate are synthesized via an unusual pathway in *Petunia* in which 2-phenylacetaldehyde is directly produced from *L*-Phe by phenylacetaldehyde synthase (PAAS) (Kaminaga et al., 2006). The biosynthesis of both benzenoids ($C_6$-$C_1$) and phenylpropanoids ($C_6$-$C_3$) starts with the deamination of *L*-Phe to *trans*-cinnamic acid (CA) by *L*-phenylalanine ammonia lyase (PAL). The $C_6$-$C_1$ pathway proceeds through the β-oxidative and non-β-oxidative pathway and yields benzaldehyde, benzylalcohol, methylbenzoate (MeBA), benzylacetate, methylsalicylate (MeSA) and benzylbenzoate (Boatright et al., 2004; Orlova et al., 2006; Van Moerkercke et al., 2009; Klempien et al., 2012; Qualley et al., 2012). The biosynthetic pathway leading to the $C_6$-$C_3$ volatiles isoeugenol and eugenol shares the precursor monolignol coniferyl alcohol with the lignin pathway (Muhlemann et al., 2014b),

whose oxygen group is eliminated during isoeugenol and eugenol formation (Koeduka et al., 2006). The exact biosynthetic route leading to vanillin is still unknown (Figure 1).

FBP emission and biosynthesis in *Petunia* is tissue-specific and can exhibit diurnal and developmental variation. It is intricately regulated by an array of MYB transcription factors that control different branches of the FBP biosynthetic pathway. The R2R3-MYB transcription factors ODORANT1 (ODO1), EMISION OF BENZENOIDSI/II/V (EOBI/II/V) and MYB4 are known to play a role in regulating FBPs biosynthesis (Verdonk et al., 2005; Spitzer-Rimon et al., 2010; Colquhoun et al., 2011; Van Moerkercke et al., 2011a; Spitzer-Rimon et al., 2012). The key regulator ODO1 regulates precursor supply to the FBP pathway and has been suggested to be in a feedback loop with EOBI and EOBII (Verdonk et al., 2005; Van Moerkercke et al., 2011a; Spitzer-Rimon et al., 2012; Van Moerkercke et al., 2012). MYB4 regulates *cinnamate-4-hydroxylase* (C4H) and thereby eugenol and isoeugenol biosynthesis (Colquhoun et al., 2011). The exact role of EOBV is not yet clear (Spitzer-Rimon et al., 2012), although a role as a negative regulator has recently been suggested (Cna'ani et al., 2015b). The clock gene LATE ELONGATED HYPOCOTYL (PhLHY) seems to directly determine the diurnal rhythm of FBP production by regulating *ODO1* expression (Fenske et al., 2015). Emission has recently been shown to be regulated by the R2R3-MYB transcription factor PH4 (Cna'ani et al., 2015a), which is predominantly known for its role in vacuolar acidification in relation to anthocyanin biosynthesis (Quattrocchio et al., 2006).

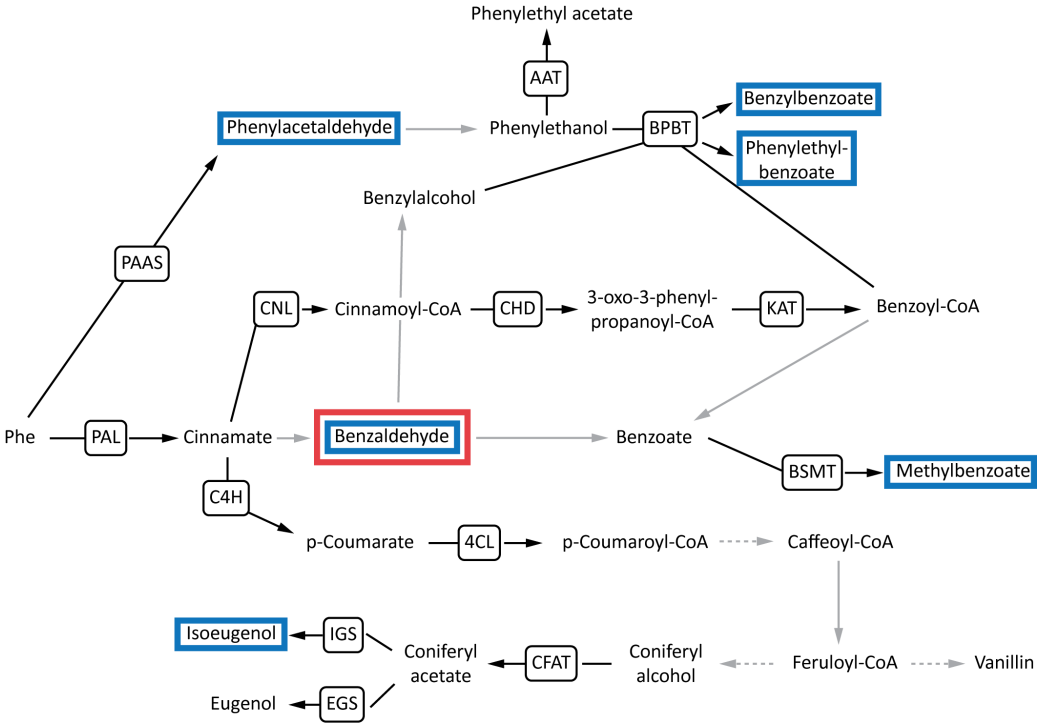


**Figure 1. Biosynthetic pathway leading to the formation of floral phenylpropanoids and benzenoids in *Petunia*.** Volatiles emitted by *PinfS6* are indicated by a red box and *PaxiN* volatiles by blue boxes. Grey arrows represent biochemical steps for which enzymes/genes have not been published in *Petunia* and dashed arrows represent multiple biochemical steps between metabolites. The precursor Phe is derived from the shikimate and arogenate pathway. Abbreviations: Phe: *L*-Phenylalanine; PAL: *L*-phenylalanine ammonia lyase; CNL: cinnamate-CoA ligase; CHD: cinnamoyl-CoA hydratase-dehydrogenase; C4H: cinnamate 4-hydoxylase; 4CL, 4-coumarate CoA-ligase; PAAS: phenylacetaldehyde synthase; KAT: 3-ketoacyl-CoA thiolase; BPBT: benzoyl-CoA:benzylalcohol/2-phenylethanol benzoyltransferase; BSMT: S-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase; CFAT: coniferylalcohol acetyl-CoA:coniferyl alcohol acetyltransferase; IGS: isoeugenol synthase; EGS: eugenol synthase; AAT: alcohol acetyltransferase.

Ultimately, the composition of the floral headspace is shaped by the selective pressures exerted by pollinators and florivores (Muhlemann et al., 2014a). Plants have evolved elaborate strategies to attract pollinators while preventing visitation by florivores. Within the *Petunia* floral headspace, certain FBPs were shown to attract pollinators, while others function in deterring of florivores (Kessler et al., 2013). Although little is known about the function of individual volatiles and specific volatiles mixes on insect behavior, *Petunia* species display typical moth or bee pollinated phenotypes. *P. axillaris* flowers are white and scented which is characteristic for nocturnal hawkmoth pollination (Ando et al., 2001; Klahre et al., 2011) whereas *P. inflata* has purple flowers which make few volatiles and are pollinated by bees (Stehmann, 1987; Ando et al., 2001). Scent is an important cue for hawkmoth visitation of *Petunia* flowers (Klahre et al., 2011), and the emission of volatiles is timed with pollinator activity (Hoballah et al., 2005). Different *P. axillaris* lines emit mixes of FBPs in which MeBA is dominant (Kondo et al., 2006; Koeduka et al., 2009). The *P. axillaris* line whose genome is sequenced in this study, *P.axillaris axillaris N* (*PaxiN*), emits benzylbenzoate, 2-phenylacetaldehyde, MeBA, benzaldehyde, phenylethylbenzoate and isoeugenol (Hoballah et al., 2005; Van Moerkercke et al., 2011b). In contrast, *P. inflata S6* (*PinfS6*) only emits benzaldehyde (Stuurman et al., 2004; Hoballah et al., 2005; Hoballah et al., 2007) (Figure 1).

A recent phylogenetic analysis of the genus robustly puts *P. inflata* and related bee-pollinated species into the ancestral short-tube clade, from which long-tubed species such *P. axillaris* are derived (Reck-Kortmann et al., 2014). Therefore, the evolution of a hawkmoth-pollination syndrome from a bee-pollinated ancestor has involved major changes in the amounts, complexity and diurnal regulation of FBPs. The availability of high quality genome sequences of representatives of the two clades provides new tools to study the genetic and genomic basis of this evolutionary transition.

Quantitative trait locus (QTL) analysis of a segregating population derived from crosses *PinfS6* or *P.axillaris* ssp *parodii* with a non-fragrant *P. hybrida* has shown that in, both populations, two loci can explain a large part of the differences in volatile production between the parents. This may suggest that only a few genes are responsible for the difference in the volatile profiles of *PaxiN* and *PinfS6* (Stuurman et al., 2004). The single-gene hypothesis can explain even faster adaptations: the rapid adaption of floral traits can be controlled by mutation of a single regulator (Dell'Olivo and Kuhlemeier, 2013). For instance, corolla color is under control of such a single regulator. The loss of color in *Petunia* is explained by mutations in the R2R3 MYB transcription factor ANTHOCYANIN2 (AN2) (Quattrocchio et al., 1999). It has also been shown that the mutation of two MYB binding sites in the promoter of *ODO1* is responsible for the loss of FBPs emission in the non-fragrant hybrid R27 (Van Moerkercke et al., 2011a). Furthermore, *ODO1* was identified as one of the two major QTLs that explain the difference in FBPs emission between *PaxiN* and *P. exserta* (Klahre et al., 2011). This points to a function for ODO1 as a single regulator of fragrance, similar to the function of AN2 in color biosynthesis.

The difference in floral scent production between closely related species of *Petunia* makes this an excellent model system to study evolutionary changes underlying shifts in FBP biosynthesis (Muhlemann et al., 2014a). Here, we show that the shift in volatile emission observed in *PaxiN* and *PinfS6* cannot be explained by the accumulation of mutations in the

coding regions of biosynthetic or regulatory genes. The shift in volatile emission is likely caused by the mutation of *cis*-acting regulatory elements or the mutation of a yet unknown transcriptional regulator, *L*-phenylalanine biosynthetic gene or transporter.

**RESULTS**

**38 FBP genes have been annotated in the *PaxiN* and *PinfS6* genome**

To annotate FBP genes in the *PaxiN* and *PinfS6* genome, *Petunia* sequences of five transcription factors, three *L*-phenylalanine biosynthetic genes and twelve biosynthetic genes acting downstream of *L*-Phe were obtained from Genbank (Table 1). These sequences were blasted against the *PaxiN* genome and the obtained sequences of *PaxiN* were subsequently used to identify the homologous genes in *PinfS6.* In total 38 gene models have been annotated for *PaxiN* and *PinfS6*. The transcription factors –but EOBI - and the FBP biosynthetic genes 3-ketoacyl-CoA thiolase 1 (*KAT1*), coniferylalcohol acetyl-CoA:coniferyl alcohol acetyltransferase (*CFAT*) and eugenol synthase (*EGS*) are encoded by single genes in both genomes. The *PinfS6* genome harbors an additional gene model of EOBI. The remaining biosynthetic genes have two (cinnamoyl-CoA hydratase-dehydrogenase (*CHD*), *C4H*, isoeugenol synthase (*IGS*)*, PAAS*) three (*PAL*, benzoyl-CoA:benzylalcohol/2-phenylethanol benzoyltransferase (*BPBT*), 4-coumarate CoA-ligase (*4CL*), cinnamate-CoA ligase (*CNL*)) or four (S-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase (*BSMT*)) copies. In addition, the *PinfS6* genome harbors a third gene model for *C4H*, whereas it has two gene models in *PaxiN.* Conversely, the *PaxiN* genome harbors a fifth and sixth gene model for *BSMT* and a third gene model for *PAAS*. *PAL*, *C4H* and *4CL* are relevant for both the FBP pathway and the anthocyanin pathway, and these genes have been discussed in (Supplementary Note 7). Thus we conclude that the complexity of the gene families is very similar between the two species.

**Defective FBP or *L*- Phe biosynthetic genes cannot explain the difference in floral volatiles emitted by *PaxiN* and *PinfS6***

To investigate whether divergence of genes underlies the difference between benzaldehyde emission by *PinfS6* and emission of many FBPs by *PaxiN* (Figure 1), the coding sequences (CDS) of FBP genes were compared after translation (Table 2). Interestingly, the similarity between putative orthologs of *PaxiN* and *PinfS6* was higher than between their paralogs. For example, *PaxiN*PAAS1 was more similar to *PinfS6*PAAS1 than to the other *PaxiN*PAAS genes (Figure 3a). However, there are some exceptions. For example, the similarity between the BPBT1 and 2 paralogs is higher than between their putative orthologs.

It is well known that an (early) stop codon or a frameshift in the CDS of a biosynthetic gene can cause the loss of a volatile from the floral headspace, as was observed for IGS and isoeugenol synthesis in *Petunia axillaris subsp. parodii* (Koeduka et al., 2009). Thus we first investigated all known biosynthetic genes, i.e. those involved in benzylbenzoate, 2-phenylacetaldehyde, MeBA, phenylethylbenzoate and isoeugenol biosynthesis (Figure 1). Overall, these genes are 95% similar at the protein level in *PaxiN* and *PinfS6* (Table 2). However, none of the known *PinfS6* FBP biosynthetic genes have additional stop codons or frameshifts. Moreover, we found mostly conservative amino acid changes suggesting that the massive differences in volatile emission between the two species are unlikely to be due to functional differences at the protein level of biosynthetic enzymes.

Another explanation for the very limited FBPs biosynthesis by *PinfS6* could be the mutation of one or a few *L*-phenylalanine biosynthetic genes (Stuurman et al., 2004). The functional mutation of a shikimate or arogenate pathway biosynthetic gene could prevent the precursor supply to the FBP pathway. 3-Deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) and chorismate mutase (CM) are the only two enzymes from the shikimate pathway of which the roles in FBP biosynthesis have been characterized (Colquhoun et al., 2010; Langer et al., 2014). *DAHP1* and *2* and *CM1* and *2* are all expressed in *Petunia* petals, but only DAHP1 and CM1 are shown to be involved in *L*-Phe biosynthesis for FBPs. The biosynthesis of *L*-Phe from shikimate in petunia can proceed through two alternative pathways, still silencing of an arogenate dehydratase (*ADT1*) leads to a great decrease in FBP emission (Maeda et al., 2010; Yoo et al., 2013). *PinfS6* and *PaxiN* have no frame shifts or additional stop codons in the CDS of the *L*-phenylalanine biosynthetic genes *CM1*, *DAPH1* or *ADT1*. *PinfS6* has one to three non-conserved amino acids changes in these proteins compared to *PaxiN* (Table 2), meaning that these proteins are likely functional in *PinfS6*. Interestingly, the *PinfS6* genome harbors a second gene model of *ADT1* separated by 4 kbp, *ADT1b*. The predicted amino acid sequence of ADT1b is identical to that of ADT1a, however unlike the intronless *ADT1a* it contains a small intron.

**_PaxiN_ and _PinfS6_ have only minor differences in currently known FBP regulators**

Because no functional mutations were found in the protein coding regions of genes responsible for FBP biosynthesis, the subsequent comparison focused on transcriptional regulators since, in principle, a lack of transcriptional regulators can shut down FBP biosynthesis (Verdonk et al., 2005; Langer et al., 2014). However, although functional mutations in transcriptional regulators could explain the loss of FBP biosynthesis it cannot explain why *PinfS6* emits benzaldehyde. Perhaps benzaldehyde emission could in this case be explained by precursor (cinnamic acid, Figure 1) outflow from other pathways (e.g. flavonoid, lignin) that is still converted to benzaldehyde as if this was by default, or the activity of a yet unknown transcription factor that regulates only this part of the FBP pathway.

Since the R2R3 MYB ODO1, a key regulator of FBP biosynthesis, can have a major role in shifting FBP biosynthesis (Klahre et al., 2011), this gene was studied in more detail. The protein sequences of ODO1 of *PaxiN* and *PinfS6* are identical in the R2R3 domain but differ by seven amino acids downstream and contains no frame shifts or stop codons. Two of these seven amino acids are identical in *PinfS6* and the fragrant *P. hybrida cv*. W115 and thus cannot contribute to changes in ODO1 functionality (Figure 2). A165G, S243N and T232I are the only non-conservative amino acid alterations in *PinfS6* compared to *PaxiN* and *P. hybrida cv. W115*. It remains to be tested if these mutations affect ODO1 functionality. *ODO1* is expressed at a lower level in *PinfS6* than *PaxiN* (Klahre et al., 2011), however this might lead to an overall reduction of FBP emission, and cannot explain a complete loss of FBP biosynthesis. It should also be noted here that mutation in two MYB-binding sites (MBS) in the promoter of *ODO1* leads to loss of FBP biosynthesis in *P. hybrida* (Van Moerkercke et al., 2011a), but these MBSs are present and intact in the promoters of *PaxiN* and *PinfS6*. In addition, the synteny between the corresponding regions (10kbp) of *PaxiN* and *PinfS6* around *ODO1* remained intact.

**Table 1. Original Genbank numbers and geneID of *PaxiN* and *PinfS6* FBP genes**

| Gene | Genbank | *PaxiN* geneID | *P. Inf6* geneID |
|---|---|---|---|
| **Biosynthetic genes** | | | |
| PAAS1 | DQ243784.1 | Peaxi162Scf00561g02002 | Peinf101Scf00985g02002 |
| PAAS2 | DQ243784.1 | Peaxi162Scf00152g02037 | |
| PAAS3 | DQ243784.1 | Peaxi162Scf00152g02036 | Peinf101Scf01065g00017 |
| BPBT1 | AY611496.1 | Peaxi162Scf00007g00012 | Peinf101Scf01180g01014 |
| BPBT2 | AY611496.1 | Peaxi162Scf00007g00011 | Peinf101Scf01180g01013 |
| BPBT3 | AY563157.1 | Peaxi162Scf00169g00922 | Peinf101Scf00276g03003 |
| BSMT1 | AY233465.1/AY233466/DQ494491 | Peaxi162Scf00047g11023 | Peinf101Scf00686g02008 |
| BSMT2 | AY233465.1/AY233466/DQ494491 | Peaxi162Scf00047g11028 | |
| BSMT3 | AY233465.1/AY233466/DQ494491 | Peaxi162Scf00047g11029 | |
| BSMT4 | AY233465.1/AY233466/DQ494491 | Peaxi162Scf03967g00005 | Peinf101Scf00437g04005 |
| BSMT5 | AY233465.1/AY233466/DQ494491 | Peaxi162Scf00423g00021 | Peinf101Scf00437g02026 |
| BSMT6 | AY233465.1/AY233466/DQ494491 | Peaxi162Scf00423g00119 | Peinf101Scf00437g02028 |
| CNL1 | JN120848 | Peaxi162Scf00294g04015 | Peinf101Scf00099g03010 |
| CNL2 | JN120848 | Peaxi162Scf00784g00010 | Peinf101Scf00099g04016 |
| CNL3 | JN120848 | Peaxi162Scf00294g00032 | Peinf101Scf00123g10004 |
| CHD1 | JX142126.1 | Peaxi162Scf00231g00330 | Peinf101Scf00961g08042 |
| CHD2 | JX142126.1 | Peaxi162Scf01363g00003 | Peinf101Scf05015g00020 |
| KAT1 | FJ657663.1 | Peaxi162Scf00052g08016 | Peinf101Scf00191g27016 |
| CFAT | DQ767969.1 | Peaxi162Scf00474g02020 | Peinf101Scf00221g12012 |
| EGS | EF467241.1 | Peaxi162Scf00020g17016 | Peinf101Scf00318g05021 |
| IGS1 | DQ372813.1 | Peaxi162Scf00889g02031 | Peinf101Scf01349g08001 |
| IGS2 | DQ372813.1 | Peaxi162Scf00060g00024 | Peinf101Scf00001g26039 |
| ***L*-phenylalanine biosynthetic genes** | | | |
| CM1 | EU751616 | Peaxi162Scf00166g09045 | Peinf101Scf01969g05044 |
| DAHP1 | JQ955569 | Peaxi162Scf00030g17016 | Peinf101Scf00007g07016 |
| ADT1a | FJ790412 | Peaxi162Scf00114g00001 | Peinf101Scf00392g01004 |
| ADT1b | | | Peinf101Scf00392g01021 |
| **Transcription factors** | | | |
| EOBIa | KC182628.1 | Peaxi162Scf00129g12034 | Peinf101Scf02365g00039 |
| EOBIb | | | Peinf101Scf05157g00020 |
| EOBII | EU360893.1 | Peaxi162Scf00080g06005 | Peinf101Scf00394g13005 |
| EOBV | GQ449250.1 | Peaxi162Scf00362g00831 | Peinf101Scf01468g01015 |
| MYB4 | HM447143.1 | Peaxi162Scf01221g00035 | Peinf101Scf00661gX |
| ODO1 | AY705977.1 | Peaxi162Scf00002g03010 | Peinf101Scf00284g00012 |

* PAL, C4H and 4CL have been described in Supplementary Note 7, (for abbreviations, see Figure 1; CM1: chorismate mutase 1 ; DAHP1: 3-deoxy-D-arabino-heptulosonate-7-phosphate; ADT1: arogenate dehydratase, EOB: EMISSION OF BENZENOIDS; MYB: myeloblastosis; ODO1; ODORANT1 )

Since the R2R3 MYB ODO1, a key regulator of FBP biosynthesis, can have a major role in shifting FBP biosynthesis (Klahre et al., 2011), this gene was studied in more detail. The protein sequences of ODO1 of *PaxiN* and *PinfS6* are identical in the R2R3 domain but differ by seven amino acids downstream and contains no frame shifts or stop codons. Two of these seven amino acids are identical in *PinfS6* and the fragrant *P. hybrida cv*. W115 and thus cannot contribute to changes in ODO1 functionality (Figure 2). A165G, S243N and T232I are the only non-conservative amino acid alterations in *PinfS6* compared to *PaxiN* and *P. hybrida cv. W115*. It remains to be tested if these mutations affect ODO1 functionality. *ODO1* is expressed at a lower level in *PinfS6* than *PaxiN* (Klahre et al., 2011), however this might lead to an overall reduction of FBP emission, and cannot explain a complete loss of FBP biosynthesis. It should also be noted here that mutation in two MYB-binding sites (MBS) in the promoter of *ODO1* leads to loss of FBP biosynthesis in *P. hybrida* (Van Moerkercke et al., 2011a), but these MBSs are present and intact in the promoters of *PaxiN* and *PinfS6*. In addition, the synteny between the corresponding regions (10kbp) of *PaxiN* and *PinfS6* around *ODO1* remained intact.

Two other R2R3 MYBs that play a role in FBPs biosynthesis, EOBI and EOBII, have only one amino acid change in *PinfS6* compared to *PaxiN*, A144V and V165M, respectively. In contrast to *PaxiN, PinfS6* harbors an additional *EOBI, EOBIb.* The predicted amino acid sequence of EOBIb has in comparison to EOBIa one amino acid less and eight amino acid changes, of which three conserved (PAM 250 >250). Similar as with ODO1, these amino acid changes are located outside the R2R3 domain of EOBI and EOBII. The amino acid changes in EOBII are conservative and moreover it is known that *EOBII* is expressed in both *PinfS6* and *PaxiN* (data not shown). The amino acid changes in EOBI are only weakly similar (PAM 250 =<0.5), however, it is unlikely that this leads to changes in the functionality of EOBI because the fragrant *Petunia cv.* P720 has the same amino acid substitution as *PinfS6*. Expression data for *EOBI* in *PinfS6* and *PaxiN* are unavailable yet.

Besides the loss of function of positive regulators, the gain of function of negative regulators could explain loss of FBP biosynthesis. The known negative regulators of FBP biosynthesis, MYB4 and EOBV (Colquhoun et al., 2011; Spitzer-Rimon et al., 2012), both have four amino acid changes in *PinfS6* compared to *PaxiN*, two of which are conservative. Interestingly, EOBV has a deletion of six amino acids in *PaxiN* towards the C-terminus. Whether these substitutions and deletions in EOBV lead to its reduced activity in *PaxiN* and increased FBP biosynthesis is unknown and requires further investigations, as well as *EOBV* transcript levels. Remarkably, *PaxiN* EOBV protein is completely identical to EOBV from the fragrant hybrid cv. P720.

**Table 2. Comparison of *PaxiN* and *PinfS6* FBP genes**

| **Biosynthetic genes** | *PaxiN* amino acids | *P.infS6 amino acids* | changes | of which conserved | similarity |
|---|---|---|---|---|---|
| PAAS1 | 506 | 506 | 24 | 16 | 95.26 |
| PAAS2 | 506 | | | | |
| PAAS3 | 506 | 506 | 30 | 17 | 94.07 |
| BPBT1 | 460 | 460 | 9 | 4 | 98.04 |
| BPBT2 | 460 | 460 | 7 | 4 | 98.48 |
| BPBT3 | 456 | 456 | 9 | 8 | 98.03 |
| BSMT1 | 357 | 357 | 29 | 18 | 91.88 |
| BSMT2 | 357 | | | | |
| BSMT3 | 336 | | | | |
| BSMT4 | 360 | 360 | 9 | 7 | 97.5 |
| BSMT5 | 358 | 358 | 13 | 5 | 96.38 |
| BSMT6 | 358 | 356 | 10 | 6 | 97.19 |
| CNL1 | 570 | 570 | 16 | 8 | 97.19 |
| CNL2 | 528 | 570 | 16 | 8 | 96.59 |
| CNL3 | 196 | 587 | 57 | 23 | 68.37 |
| CHD1 | 724 | 724 | 8 | 6 | 98.9 |
| CHD2 | 724 | 713 | 8 | 2 | 98.88 |
| KAT1 | 462 | 462 | 25 | 15 | 94.59 |
| CFAT | 454 | 458 | 23 | 10 | 93.61 |
| EGS | 308 | 308 | 6 | 2 | 98.05 |
| IGS1 | 323 | 323 | 9 | 4 | 97.21 |
| IGS2 | 319 | 319 | 17 | 10 | 94.67 |
| ***L*-phenylalanine precursor genes** | *PaxiN* amino acids | *P.infS6 amino acids* | changes | of which conserved | similarity |
| CM1 | 324 | 324 | 5 | 3 | 98.46 |
| DAHP1 | 533 | 533 | 3 | 1 | 99.44 |
| ADT1a | 424 | 424 | 5 | 2 | 98.82 |
| ADT1b | | 424 | 5 | 2 | 98.82 |
| **Transcription factors** | *PaxiN* amino acids | *P.infS6 amino acids* | changes | of which conserved | similarity |
| EOBIa | 202 | 202 | 1 | 0 | 99.5 |
| EOBIb | | 201 | 7 | 5 | 96.52 |
| EOBII | 197 | 197 | 1 | 1 | 99.49 |
| EOBV | 267 | 273 | 4 | 2 | 98.5 |
| MYB4 | 258 | 258 | 4 | 2 | 98.45 |
| ODO1 | 294 | 294 | 7 | 4 | 97.62 |

***** For abbreviations, see Figure 1 and table 1. Amino acid sequences have been aligned with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Conserved is PAM 250 >250).

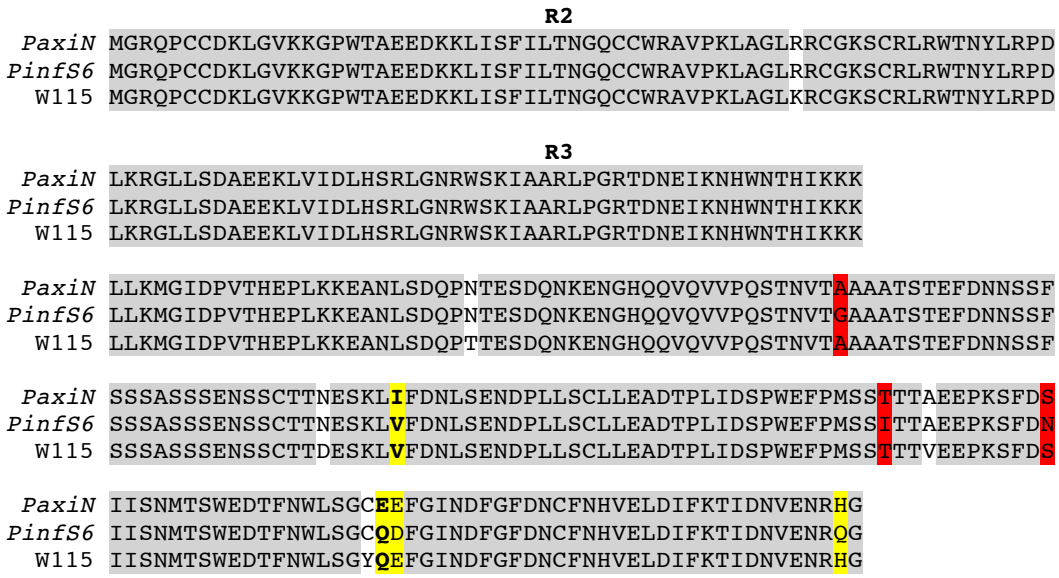


**Figure 2. Amino acid sequence alignment of Petunia ODO1.**

ODORANT1 (ODO1) from *P.axillaris axillaris N* (*PaxiN*), *P. integrifolia inflata S6* (*PinfS6*) and *P. hybrida cv.* Mitchell (W115) were aligned with ClustalW2. Yellow indicates amino acids that are conserved between *PaxiN* and *PinfS6* (Gonnet PAM 250 scoring >250), these amino acids are bold if they are similar between *PinfS6* and W115. Red indicates amino acids that are not conserved between *PaxiN* and *PinfS6*.

### PAAS, CFAT and IGS have no close homologs in other *Solanaceae*

Even though the phenylalanine pathway is highly conserved (Tohge et al., 2013a) there are differences between *Petunia* and other Solanaceae in biosynthesis of phenylpropanoids. For example, 2-phenylacetaldehyde is synthesized by PAAS in *Petunia* whereas tomato requires two enzymes for this conversion (Kaminaga et al., 2006; Tieman et al., 2006). We thus explored some differences in this regard between *Petunia* and other Solanaceae, using the genomes of *Nicotiana benthamiana*, *Solanum lycopersicum* and *Solanum tuberosum*. PAAS, CFAT and IGS, which are likely to be unique for *Petunia,* were selected for further analysis. The three *PaxiN* genes were used to blast the genomes. The protein sequences of the three best hits for PAAS, CFAT and IGS were obtained from *N. benthamiana*, *S. lycopersicum* and *S. tuberosum* and compared to *PaxiN* and *PinfS6*.

*P. hybrida* PAAS synthesizes 2-phenylacetaldehyde by oxidative decarboxylation and oxidation of *L*-Phe. PAAS also has been identified in rose but not in tomato (Kaminaga et al., 2006). In tomato two independent enzymes presumably catalyze the decarboxylation and oxidation of *L*-Phe to form 2-phenylacetaldehyde. A small family of decarboxylases in tomato catalyzes the first step in phenethylamine production. The second step is likely performed by a yet unidentified amine oxidase (Tieman et al., 2006). The most homologous gene model in tomato to *Petunia* PAAS is 76% similar (Solyc03g0455020.2.1 to *PaxiN*PAAS2). However, this putative tomato PAAS has an early stop codon that makes the tomato protein 124 amino acids shorter than *PaxiN*PAAS2. Additionally, all the Petunia PAAS copies are more similar to each other then to *N. benthamiana*, *S. lycopersicum* and *S. tuberosum* genes (Figure 3a).

Coniferyl acetate is synthesized by CFAT and is the direct substrate for IGS and EGS, which produce isoeugenol and eugenol, respectively. The two *Petunia* genes directly responsible for isoeugenol synthesis, CFAT and IGS, gave very poor blast hits in all the three

Solanaceae studied (Figure 3b and c). *Petunia* IGS is 47% identical to *Petunia* EGS (Koeduka et al., 2008) and the *Petunia* IGSs copies fall in a separate clade. In contrast, *Petunia* EGSs have higher similarity to putative reductases from *N. benthamiana*, *S. lycopersicum* and *S. tuberosum* (Figure 3b). Likewise, *Petunia* CFAT does not group together with genes from the other Solanaceae species. This implies that although these enzymes act in the same pathway, EGS is more conserved within the Solanaceae than IGS and CFAT.

**DISCUSSION**

*Petunia* has proven to be a suitable model system to study biosynthesis and regulation of the floral benzenoid phenylpropanoid (FBP) pathway (Verdonk et al., 2003; Muhlemann et al., 2014a). The sequence of the genomes from two closely related *Petunia* species, *PaxiN* and *PinfS6*, provides the scientific community with a valuable tool to study changes in FBP biosynthesis at the genomic level. *PaxiN* and *PinfS6* have distinct floral volatile profiles, and moreover, they are the ancestors of a comprehensive *P. hybrida* collection that display wide variety in FBP biosynthesis.

The analysis of both genomes shows that there are no frame shifts or stop codons present in *PinfS6* FBP biosynthetic genes compared to *PaxiN*. Moreover, *PinfS6* and *PaxiN* FBP biosynthetic proteins overall differ only 5% and most of those changes are silent or conservative. Small functional differences caused by changes in the protein sequences cannot be excluded, but it seems unlikely that these can account for the massive and complex increase in volatiles in *P. axillaris* (Table 2). Conversely, the loss of FBP biosynthesis in *PinfS6* could be the result of a mutation in upstream biosynthetic genes preventing precursor supply to the FBP pathway. *L*-Phe is an essential constituent of proteins as well as the precursor for many different secondary metabolites (Tohge et al., 2013b). Functional mutation of this pathway would probably lead to serious defects in plant, and this could explain the presence of multiple copies of shikimate and arogenate pathway and *PAL* genes in plants. Nevertheless, there are examples in which silencing of specific shikimate and arogenate pathway genes (*CM1, DAPH1* and *ADT1*) leads to reduced FBP emission in *Petunia* without causing severe phenotypes (Verdonk et al., 2005; Colquhoun et al., 2010; Maeda et al., 2010; Langer et al., 2014). Interestingly, the functionality of the known *L*-phenylalanine biosynthetic genes *CM1, DAPH1* and *ADT1*, is likely not altered in *PinfS6*. CM1, DAPH1 and ADT1a/ADT1b have only three, five and three amino acid changes compared to *PaxiN*, respectively (Table 2). Although *ADT1b* from PinfS6 is identical to *ADT1a* at the protein level, it contains an intron unlike *ADT1a*. It cannot be excluded that the changes in the protein sequences collectively underlie the phenotypic differences as polygenic trait as argued by Rockman (Rockman, 2012). However, the relatively simple genetic structure suggested by QTL analysis (Stuurman et al., 2004; Klahre et al., 2011) indicates the presence of major functional differences in as yet unidentified genes or in promoters of the genes analyzed here. Potential candidates also include transporters such as the proposed but unidentified plastidial *L*-Phe transporter, which delivers *L*-Phe from the plastid to the cytosol (Yoo et al., 2013).

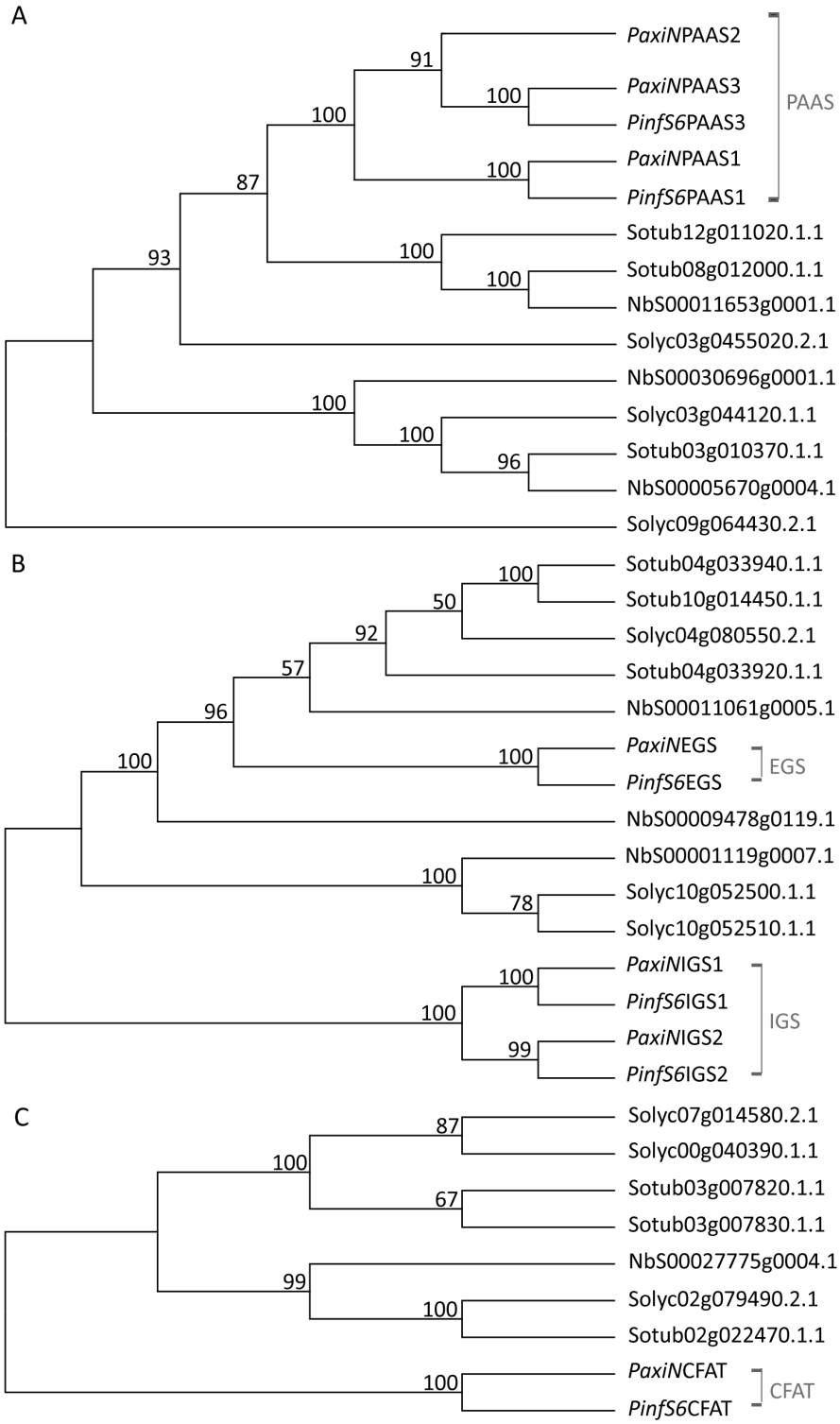


**Figure 3. Unrooted neighbor joining trees of PAAS (a), IGS and EGS (b) and CFAT (c).**
Unrooted neighbor joining trees from the protein sequence of different Solanaceae were created with MEGA6.06. Numbers represent Bootstrap values for 1000 replicates. Petunia identifiers are listed in table 1. Abbreviations: PAAS: phenylacetaldehyde synthase; CFAT: coniferylalcohol acetyl-CoA:coniferyl alcohol acetyltransferase; IGS: isoeugenol synthase; EGS: eugenol synthase; PaxiN: *Petunia axillaris axillaris N*; PinfS6: *Petunia integrifolia infalata S6*; Solyc: *Solanum lycopersicum*; Sotub: *Solanum tuberesum*; Nb: *Nicotiana benthamiana*.

We also investigated whether the difference in volatile emission by *PaxiN* and *PinfS6* can be explained by genetic variation in key transcriptional regulators of FBP emission. Prior evidence points to such a role for ODO1. Mutations in EOBII binding sites of the *ODO1* promoter are sufficient to lose the ability to synthesize FBPs (Van Moerkercke et al., 2011a). Moreover, two QTLs explain the difference in FBP biosynthesis in *PaxiN* and *P. exserta,* and *ODO1* was found on one of these two QTLs on chromosome VII (Klahre et al., 2011). Interestingly, *ODO1* is expressed in both *PaxiN* and *PinfS6,* although at a lower level in *PinfS6* (Klahre et al., 2011). It is expected that a lower level of *ODO1* expression would result in lower levels of all FBPs, which is not the case in *PinfS6*. Further analysis of ODO1 revealed that there are three non-conserved amino acid substitutions in *PinfS6* compared to the fragrant *PaxiN* and *P. hybrida cv.* W115 (Figure 2). It remains to be determined if these mutations have consequences for the activity of ODO1 in *PinfS6*, however, it's unlikely that these mutations alter ODO1 activity.

From our analysis of the genome it has become clear that *PinfS6* is likely carrying functional biosynthetic FBP genes. It has a dysfunctional fragrance system which can be restored by the replacement of chromosome III and a segment of chromosome IV with those from the non-fragrant hybrid W138 (Stuurman et al., 2004). Interestingly, W138 is non-fragrant since is lacks *ODO1* expression, thus ODO1 seems still functional in *PinfS6* (Verdonk et al., 2005). Replacement of chromosome II in *P. exserta* as well as in *PinfS6* with that of *PaxiN* restores FBP biosynthesis *(Stuurman et al., 2004; Klahre et al., 2011; Hermann et al., 2013).* Thus it seems that a major, unknown regulator is located on chromosome II that is absent in *PinfS6* (and *P. exserta*). One of the other regulators of FBP biosynthesis, EOBII, is located on chromosome II (Hermann et al., 2013). EOBII and EOBI are more conserved between *PinfS6* and *PaxiN* than ODO1 and both have only one amino acid substitution. This amino acid change is conservative in EOBII. In addition, the *PinfS6* genome harbors *EOBIb*, which has eight amino acid changes and one deletion compared to EOBIa. Altogether there seem no differences in ODO1, EOBI and EOBII between *PaxiN* and *PinfS6* that can explain the difference in FBP biosynthesis *(Sablowski et al., 1994; Tohge et al., 2013a)*.

The presence of a negative regulator in *PinfS6*, downregulating biosynthesis of benzylbenzoate, 2-phenylacetaldehyde, MeBA, phenylethylbenzoate and isoeugenol, could explain the differences between *PinfS6* and *PaxiN.* The only two known negative regulators of FBP biosynthesis to date are MYB4 and EOBV. MYB4 specifically regulates isoeugenol and eugenol biosynthesis and does not control the biosynthesis of the other volatiles (Colquhoun et al., 2011). The exact role of EOBV is still unknown, but silencing of EOBV leads to increased overall volatile emission, that is not further specified (Spitzer-Rimon et al., 2012). Recently a role as a wide-range transcriptional repressor of the pathway was suggested for EOBV (Cna'ani et al., 2015b). EOBV has four amino acid changes and a deletion of six amino acids in *PaxiN* compared to *PinfS6*. Since expression and activity data of EOBV are not available, the potential effect of mutations in EOBV on FBP biosynthesis remains elusive.

In order to study if the mutation of other *L*-phenylalanine biosynthetic genes could lead to diminutive FBP biosynthesis more understanding of this biosynthetic pathway is necessary. To target the search for mutations in *L*-phenylalanine biosynthetic genes causing the shift in FBP biosynthesis, expression data revealing which biosynthetic genes are differently expressed in *PaxiN* and *PinfS6* petals is essential or perhaps even protein levels and

enzymatic activities. It might also shed light on why *PinfS6* can still emit benzaldehyde. Its potential precursor, cinnamic acid, is also a precursor for anthocyanin and lignin biosynthesis and it is possible that cinnamic acid not utilized by these pathways could be converted to benzaldehyde by default. Interestingly, silencing of one of the FBP regulators, EOBI, in *P. hybrida* Mitchell (W115) does not alter benzaldehyde emission, whereas all other volatiles that are also emitted by *PaxiN* are affected by EOBI silencing (Spitzer-Rimon et al., 2012). It suggests that benzaldehyde emission could be regulated differently than emission of other FBPs.

Even though changes in the floral headspace are believed to be a promising tool for studying evolutionary mechanism at the species level (Knudsen, 2006), there are examples of studies using floral volatiles for studying evolution between species. A study comparing IGS and EGS between *Ocimum basilicum*, *Clarkia breweri* and *P. hybrida* led to the hypothesis that EGS and IGS have evolved independently at least twice in different plant lineages (Koeduka et al., 2008). Interestingly, *IGS* from *Petunia* seems to be more different from other Solanaceae genes than *Petunia* EGS. Tomato is not known to produce isoeugenol, and the lack of *IGS* genes in *N. benthamiana*, *S. lycopersicum* and *S. tuberosum* genomes implies that *IGS* has been lost or gained in different Solanaceae genera. Surprisingly, the *Petunia* biosynthetic gene directly upstream of IGS, CFAT, also falls in a separate clade than genes from *N. benthamiana*, *S. lycopersicum* and *S. tuberosum* (Figure 3c). Although *Petunia* EGS has homologous genes in other Solanaceae (Figure 3b), and tomato is reported to synthesize eugenol (Ortiz-Serrano and Gil, 2010), genera within the Solanaceae probably have a different acyltransferase responsible for the conversion of coniferyl alcohol. A similar situation appears to have occurred for 2-phenylacetaldehyde biosynthesis. *Petunia* 2-phenylacetaldehyde is synthesized by the enzyme PAAS, whereas tomato requires two enzymatic steps for the conversion of *L*-Phe (Kaminaga et al., 2006; Tieman et al., 2006). *Petunia* PAAS genes do clade separately from putative *N. benthamiana*, *S. lycopersicum* and *S. tuberosum* PAAS genes (Figure 3a). The tomato gene most homologous to *PaxiN*PAAS2, encodes a protein that is 124 amino acid shorter than its *Petunia* homolog. This might implicate that PAAS function has been lost in tomato and two other genes evolved in tomato to catalyze 2-phenylacetaldehyde formation. The presence of a limited number of FBP-producing species in the Solanaceae makes it difficult to unravel how benzenoid/phenylpropanoid biosynthesis has evolved in this family. However, our results clearly show that the Solanaceae have evolved multiply strategies for (iso)eugenol and 2-phenylacetaldehyde biosynthesis.

**METHODS**

FBP CDS were extracted from Genbank and annotated in WebApollo as described in the main article. Protein sequences were aligned with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Amino acids are considered conserved when the Gonnet PAM 250 scoring is >250. Unrooted neighbor joining trees were created with MEGA6.06 using ClustalW for the alignment and 1000 Bootstrap replicates for the phylogeny analysis (http://www.megasoftware.net/).

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