

Interlinking showy traits: co-engineering of scent and colour biosynthesis in flowers

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Received 10 December 2007;

revised 23 January 2008;

accepted 31 January 2008.

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Summary

The phenylpropanoid pathway gives rise to metabolites that determine floral colour and fragrance. These metabolites are one of the main means used by plants to attract pollinators, thereby ensuring plant survival. A lack of knowledge about factors regulating scent production has prevented the successful enhancement of volatile phenylpropanoid production in flowers. In this study, the Production of Anthocyanin Pigment1 (*Pap1*) *Myb* transcription factor from *Arabidopsis thaliana*, known to regulate the production of non-volatile phenylpropanoids, including anthocyanins, was stably introduced into *Petunia hybrida*. In addition to an increase in pigmentation, *Pap1*-transgenic petunia flowers demonstrated an increase of up to tenfold in the production of volatile phenylpropanoid/benzenoid compounds. The dramatic increase in volatile production corresponded to the native nocturnal rhythms of volatile production in petunia. The application of phenylalanine to *Pap1*-transgenic flowers led to an increase in the otherwise negligible levels of volatiles emitted during the day to nocturnal levels. On the basis of gene expression profiling and the levels of pathway intermediates, it is proposed that both increased metabolic flux and transcriptional activation of scent and colour genes underlie the enhancement of petunia flower colour and scent production by *Pap1*. The co-ordinated regulation of metabolic steps within or between pathways involved in vital plant functions, as shown here for two showy traits determining plant–pollinator interactions, provides a clear advantage for plant survival. The use of a regulatory factor that activates scent production creates a new biotechnological strategy for the metabolic architecture of fragrance, leading to the creation of novel genetic variability for breeding purposes.

Keywords: flavonoids, fragrance, petunia, phenylpropanoids, Production of Anthocyanin Pigment1 (*Pap1*), volatiles.

Introduction

In nature, flower colour and fragrance are two of the main means adopted by plants to attract pollinators, thereby ensuring plant reproductive success (Hoballah *et al.*, 2007). These characteristics are also commercially important, in that they greatly influence not only the yield and quality of many crops, but also their commercial appeal. Flower scent is a composite character determined by a complex mixture of low-molecular-weight volatile molecules, classified by their

biosynthetic origin into terpenes, phenylpropanoids and fatty acid derivatives (Croteau and Karp, 1991; Chappell and Jones, 1995; Dudareva *et al.*, 2004). Several structural genes responsible for the formation of volatile compounds have been identified (van Schie *et al.*, 2006; Schuurink *et al.*, 2006), including genes responsible for the formation of volatile phenylpropanoid and benzenoid compounds (Boatright *et al.*, 2004; Kaminaga *et al.*, 2006; Tieman *et al.*, 2006; Dexter *et al.*, 2007; Spitzer *et al.*, 2007); however, the precise biochemical steps determining the pathway are still largely

unknown (Boatright *et al.*, 2004; Schuurink *et al.*, 2006). Although a moderate increase in volatile terpenoid production has been achieved in flowers and fruits via the ectopic expression of specific structural genes (Lucker *et al.*, 2006; Davidovich-Rikanati *et al.*, 2007), no enhancement of floral volatile phenylpropanoid emission has been reported. Limitations in the engineering of floral scent (Pichersky and Dudareva, 2007) include a shortage of substrate availability, which restricts metabolic flow (Schwab, 2003; Lucker *et al.*, 2006). Moreover, volatile production in plants often has a rhythmic pattern, and is thus limited to a certain period of the day. In *Petunia axillaris*, rhythms of volatile production are nocturnal, and have been reported to be both circadian and light controlled (Underwood *et al.*, 2005; Verdonk *et al.*, 2005; Schuurink *et al.*, 2006). One intriguing way to increase the metabolic flow is to use transcription factors that control multiple steps in various branches of the pathway (Mahmoud and Croteau, 2002; Koes *et al.*, 2005). However, knowledge about the regulation of flower scent biosynthesis is scarce (van Schie *et al.*, 2006; Schuurink *et al.*, 2006) and only a single transcription factor, *ODORANT1*, has been shown to be involved in the regulation of volatile biosynthesis in flowers. Furthermore, although the role of *ODORANT1* in the production of phenylpropanoid scent compounds in petunia was revealed following RNA interference (RNAi)-mediated suppression, its ability to boost metabolic flow towards scent production remains to be investigated (Verdonk *et al.*, 2005).

Another class of metabolites determining showy traits is the anthocyanin pigments, which derive from a well-defined branch of the phenylpropanoid pathway. Numerous structural and regulatory genes involved in anthocyanin biosynthesis have been extensively used for the genetic manipulation of flower colour (Winkel-Shirley, 2001; Chandler and Tanaka, 2007). The regulation of anthocyanin biosynthesis has been shown to occur primarily through the action of *Myb* transcription factors (Koes *et al.*, 2005). However, the activities of some of these regulators are not restricted to the anthocyanin shunt. For example, the Production of Anthocyanin Pigment1 (*Pap1*) *Myb* transcription factor from *Arabidopsis thaliana* regulates the production of various non-volatile compounds via the activation of several branches of the phenylpropanoid pathway (Borevitz *et al.*, 2000; Harmer *et al.*, 2000; Sharma and Dixon, 2005; Tohge *et al.*, 2005; Xie *et al.*, 2006).

A link between scent (volatile phenylpropanoids/benzenoids) and colour (anthocyanins) can be anticipated on the basis of their common biochemical origin, as well as their similar biological role, i.e. 'advertisement'. Indeed, diversion of metabolic flux from one branch of the phenylpropanoid

pathway to another has been demonstrated (Zuker *et al.*, 2002). In this study, by introducing *Pap1* transcription factor from *Arabidopsis* into petunia, the simultaneous enhancement of both branches of the phenylpropanoid pathway, i.e. those leading to the production of colour and scent in flowers, is demonstrated.

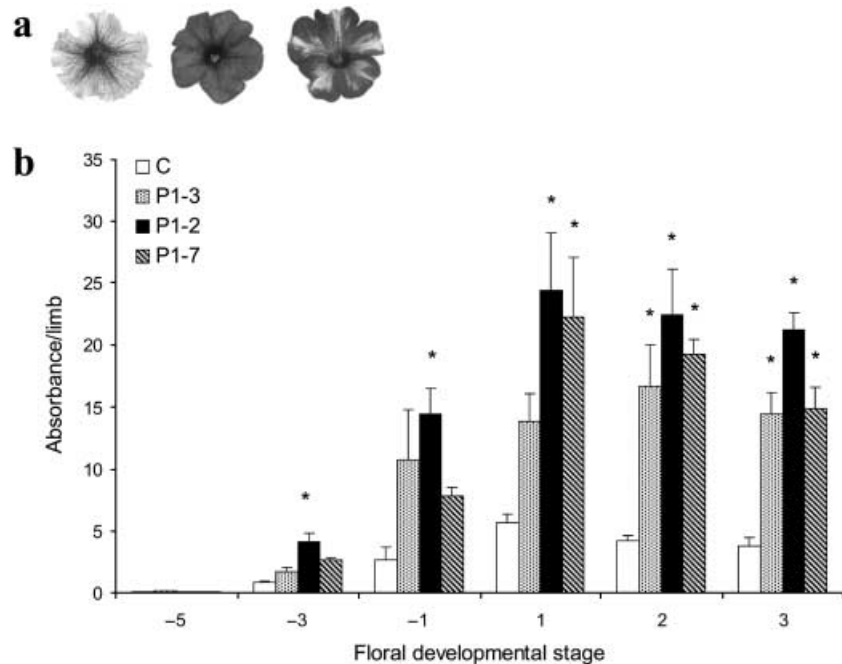
Results

Increased pigmentation and floral scent levels in *Pap1*-transgenic petunia

Petunia hybrida cv. 'Blue Spark' was used because its flowers are pigmented, by contrast with the commonly studied white-flowering cv. Mitchell (W115) (Verdonk *et al.*, 2005). Constitutive cauliflower mosaic virus (CaMV) 35S-driven expression of *Pap1* in petunia had no negative effect on plant development, and transgenic plants did not appear to be different from control plants with respect to growth rate, development and time to flowering. The kinetics of flower senescence were also comparable for control and *Pap1*-transgenic flowers (average of 12.5 ± 1.1 and 12.9 ± 0.2 days, respectively, for visible wilting of flowers detached at anthesis). Transgenic plants expressing *Pap1* exhibited increased levels of pigmentation (Figure 1a). Transformation of petunia with the identical vector carrying the *UidA* gene coding for the β -glucuronidase (*Gus*) reporter gene instead of *Pap1* yielded only transgenic plants with true-to-type flower colour (data not shown). Analyses of anthocyanin content in the corolla limbs of three independent transgenic lines revealed increased anthocyanin levels throughout flower development, up to ninefold relative to the limbs of control plants (Figure 1b). The limbs of both control and *Pap1*-transgenic lines demonstrated similar developmental patterns of anthocyanin accumulation, peaking at around anthesis. Anthocyanin levels in the leaves of *Pap1*-transgenic lines were also strongly increased (c. 60-fold) relative to those of control plants.

Transgenic petunia flowers exhibiting enhanced pigmentation (five independent transgenic lines) were noticeably more fragrant than control flowers. To compare quantitatively the floral scent profile of transgenic and control flowers, dynamic headspace analysis was conducted. Control flowers emit the benzenoid compounds benzaldehyde and methylbenzoate. The emission of benzaldehyde, a common petunia scent compound, was increased three- to fivefold in independent *Pap1*-transgenic lines relative to control flowers (Figure 2a, b). In contrast, methylbenzoate emission levels, as well as the expression levels of the gene responsible for its formation, were similar in *Pap1*-transgenic and control flowers (Figure S1,

Figure 1 Increased pigmentation in petunia flowers expressing cauliflower mosaic virus (CaMV) 35S-driven Production of Anthocyanin Pigment1 (*Pap1*). (a) Flowers of control and *Pap1*-transgenic petunia lines P1-2 and P1-7 (from left to right, respectively). (b) Anthocyanin content in corolla limbs of control (C) and *Pap1*-transgenic lines P1-3, P1-2 and P1-7. Limbs of flowers at different developmental stages [from 5 days before anthesis (–5) to 3 days post-anthesis (3)] were collected, and anthocyanin was extracted and estimated using the formula $\text{Absorbance} = A_{530} - 0.25A_{657}$. Columns represent the mean values of independent experiments ($n = 3$). Standard errors are indicated by vertical bars. The significance of the differences in anthocyanin content between control non-transgenic and *Pap1*-transgenic lines was calculated using Tukey's all pairwise multiple comparison procedure following one-way analysis of variance (ANOVA), conducted separately for each floral developmental stage. Asterisks indicate values significantly different ($P < 0.05$) from control flowers.



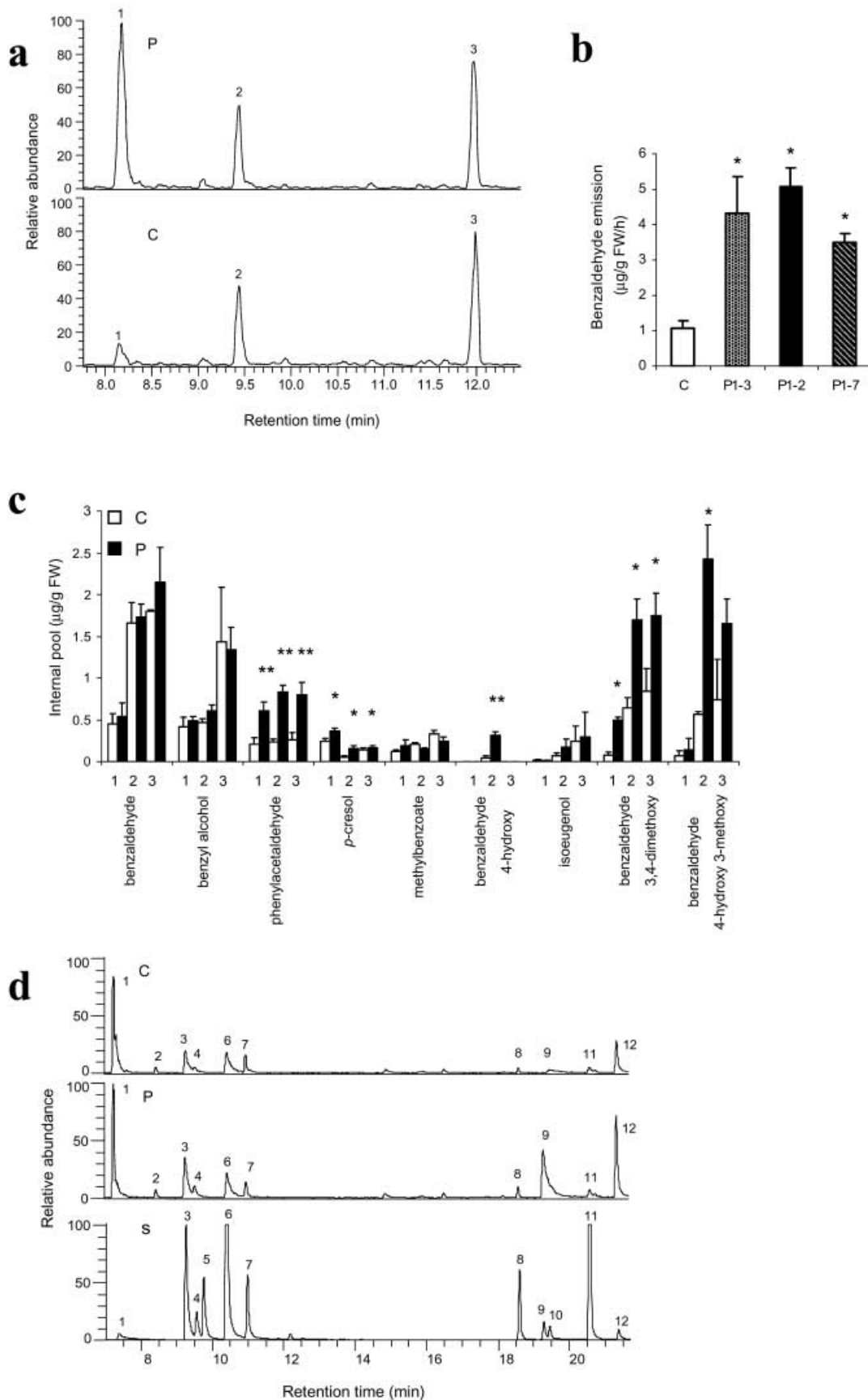
see 'Supplementary material'). The internal pool levels of benzaldehyde, as well as of benzyl alcohol, methylbenzoate and isoeugenol, were similar in transgenic flowers and in controls. In contrast, internal pool levels of phenylacetaldehyde, benzaldehyde 4-hydroxy, benzaldehyde 3,4-dimethoxy (vanillin methyl ether) and benzaldehyde 4-hydroxy 3-methoxy (vanillin) were up to 10-fold higher in transgenic relative to control flowers (Figure 2c,d). Patterns of volatile accumulation and emission in transgenic and control flowers were similar throughout flower development, as well as during the day/night cycle (Figures 2c, S2 and S3, see 'Supplementary material').

Increased phenylalanine (Phe) utilization and metabolic flux in *Pap1*-transgenic petunia

To gain an insight into the metabolic flux within the phenylpropanoid pathway in transgenic vs. control flowers, and to assess substrate availability for volatile production, the levels of pathway intermediates were analysed. The internal pool size of Phe oscillated greatly between day and night in control flowers, with peak accumulation occurring at night (Figure 3a). However, this nocturnal increase in Phe level was not observed in *Pap1*-transgenic flowers. Indeed, night-time Phe levels were five- to sevenfold lower in transgenic vs. control flowers, whereas daytime Phe levels were only slightly affected (Figure 3a). The levels of cinnamic and coumaric acids were similar in *Pap1*-transgenic and control flowers (Figure S4, see

'Supplementary material'). As L-phenylalanine ammonia lyase (PAL), via catabolism of Phe, catalyses a major branch point between primary and secondary metabolism, and hence can control flux in the phenylpropanoid pathway, the expression level of *Pal* was analysed in transgenic and wild-type flowers. RNA-blot analyses revealed elevated *Pal* mRNA levels in *Pap1* flowers relative to controls (Figure 3b). To further evaluate whether the night-time depletion of the Phe pool in *Pap1*-transgenic flowers (Figure 3a) was a result of increased utilization of Phe, an *in vivo* stable isotope-labelling approach was used to monitor the rate of $^2\text{H}_5$ -Phe conversion to benzaldehyde. $^2\text{H}_5$ -Phe was more rapidly converted to benzaldehyde in transgenic flowers, resulting in 57% labelling of total benzaldehyde compared with 30% in the limbs of control flowers (Figure 3c), and reflecting an 80% higher flux from Phe to benzaldehyde in transgenic flowers.

To examine the effect of *Pap1* on the expression of genes downstream from *Pal* in the phenylpropanoid/benzenoid pathway (namely *C4h* coding for cinnamic acid-4-hydroxylase, *Chs* coding for chalcone synthase, *Chi* coding for chalcone isomerase, *F3h* coding for flavanone-3-hydroxylase, *Dfr* coding for dihydroflavonol-4-reductase and *Paas* coding for phenylacetaldehyde synthase), semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was conducted using RNA isolated from transgenic and control flowers. Higher expression levels of *C4h*, involved in the formation of coumaric acid, were detected in *Pap1*-transgenic flowers, in addition to higher levels of *F3h* and *Dfr*, which are



involved in later biochemical steps of the anthocyanin pathway. The expression of early anthocyanin biosynthetic genes (*Chs* and *Chi*) remained unaffected in transgenic flowers (Figure 3d). The expression of *Paas*, a recently identified gene encoding the protein catalysing phenylacetaldehyde biosynthesis (Kaminaga *et al.*, 2006), was strongly up-regulated in *Pap1*-transgenic flowers relative to controls (Figure 3d). It should be noted that phenylacetaldehyde levels were also markedly increased in transgenic flowers (Figure 2c,d). The expression levels of 5-enolpyruvylshikimate 3-phosphate synthase (*Epsp synthase*), a well-characterized gene from the shikimate pathway involved in the formation of Phe (Schuurink *et al.*, 2006), were also determined. RNA levels of *Epsp synthase* were comparable in *Pap1*-transgenic and control petunia flowers (Figure 3d).

Abolishment of nocturnal rhythms of scent production through increased Phe availability on a *Pap1*-transgenic petunia background

To evaluate whether substrate availability limits volatile production in *Pap1*-transgenic flowers during the day, the levels of volatiles produced in *Pap1*-transgenic vs. control

Figure 2 Increased scent production in petunia flowers expressing Production of Anthocyanin Pigment1 (*Pap1*). (a) Representative chromatogram of volatiles emitted from control (C) and *Pap1*-transgenic line 2 (P) flowers, as determined using the dynamic headspace technique. Analysis was conducted for 12 h during the first night post-anthesis. 1, Benzaldehyde; 2, isobutylbenzene (internal standard); 3, methylbenzoate. (b) Levels of benzaldehyde emitted from flowers of control (C) and *Pap1*-transgenic lines (P1-3, P1-2 and P1-7). Columns represent the mean values of independent experiments ($n = 3$). Standard errors are indicated by vertical bars. FW, fresh weight. The means obtained from flowers of *Pap1*-transgenic lines were compared with those obtained from flowers of control non-transgenic petunia using Dunnett's method following one-way analysis of variance (ANOVA). Asterisks indicate values significantly different ($P < 0.05$) from control flowers. (c) Internal pools of volatile compounds accumulated in control (C) and *Pap1*-transgenic line 2 (P) limbs. Limbs of flowers at different developmental stages [from first to third day (1–3) post-anthesis] were analysed. Columns represent the mean values of three independent experiments ($n = 3$). Standard errors are indicated by vertical bars. The significance of the differences in internal pools of volatile compounds between *Pap1*-transgenic line 2 and control non-transgenic flowers, at different developmental stages, was calculated using Tukey's all pairwise multiple comparison procedure following two-way ANOVA, conducted separately for each compound. Values significantly different from control flowers are indicated by asterisks (* and ** for $P < 0.05$ and $P < 0.0001$, respectively). (d) Representative chromatogram of volatile compounds accumulating in limbs of *Pap1*-transgenic line 2 (P) and control (C) flowers. Chromatogram of authentic standards (S) of volatile compounds is shown. 1, Benzaldehyde; 2, isobutylbenzene (internal standard); 3, benzyl alcohol; 4, phenylacetaldehyde; 5, *o*-cresol; 6, *p*-cresol; 7, methylbenzoate; 8, benzaldehyde 4-hydroxy; 9, benzaldehyde 4-hydroxy 3-methoxy; 10, eugenol; 11, isoeugenol; 12, benzaldehyde 3,4-dimethoxy.

non-transgenic flowers were examined following feeding with Phe. Volatile accumulation and emission in control flowers were unaffected by Phe feeding, both during the day and at night (Figure 4). In *Pap1*-transgenic flowers, night-time volatile accumulation and emission were unaffected following Phe feeding (Figure 4), as were the levels of accumulated volatile compounds following Phe feeding during the day (not shown). However, increased volatile emission was detected in Phe-fed *Pap1*-transgenic flowers relative to their non-fed counterparts during the day (c. seven- and fivefold increase in benzaldehyde and methylbenzoate emission, respectively; Figure 4b).

Discussion

Since the isolation of the *Pap1* transcription factor from *Arabidopsis* (Borevitz *et al.*, 2000), extensive studies of its effect on the transcriptome have shown its involvement in the regulation of several dozens of genes belonging to the phenylpropanoid pathway (Harmer *et al.*, 2000; Tohge *et al.*, 2005). In *Arabidopsis*, the over-expression of *Pap1* results in the accumulation of lignin, hydroxycinnamic acid esters and flavonoids, including anthocyanins, which impart a prominent purple colour to plant organs (Borevitz *et al.*, 2000). Ectopic expression of *Pap1* in petunia, as described here and by Matousek *et al.* (2006), also leads to a strong increase in anthocyanin content. Remarkably, volatile phenylpropanoid metabolism is also strongly affected by the over-expression of *Pap1* in petunia flowers (Figure 2). Increased volatile production in *Pap1*-transgenic petunia occurred within the previously reported innate rhythmic pattern of nocturnal volatile production (Schuurink *et al.*, 2006) found in control flowers. The inability of *Pap1* to alter the native pattern of scent production may be explained by the lack of another factor(s), such as regulators of gene expression acting in concert with *Pap1* (Zimmermann *et al.*, 2004; Koes *et al.*, 2005), and/or a lack of substrate availability (Schwab, 2003). Indeed, although in both control and *Pap1*-transgenic petunia flowers volatile emission peaked during the night, feeding of *Pap1*-transgenic flowers with Phe led to increased volatile emission during the day, reaching night-time levels, and abolished the nocturnal rhythm.

Volatile compounds derive from several branches of the phenylpropanoid pathway. Benzaldehyde represents a key intermediate in the coenzyme A (CoA)-independent, non- β -oxidative pathway which contributes to the formation of benzoic acid from cinnamic acid (Boatright *et al.*, 2004). The biosynthesis of vanillin has also been proposed to occur through the non- β -oxidative route, with benzaldehyde 4-hydroxy as

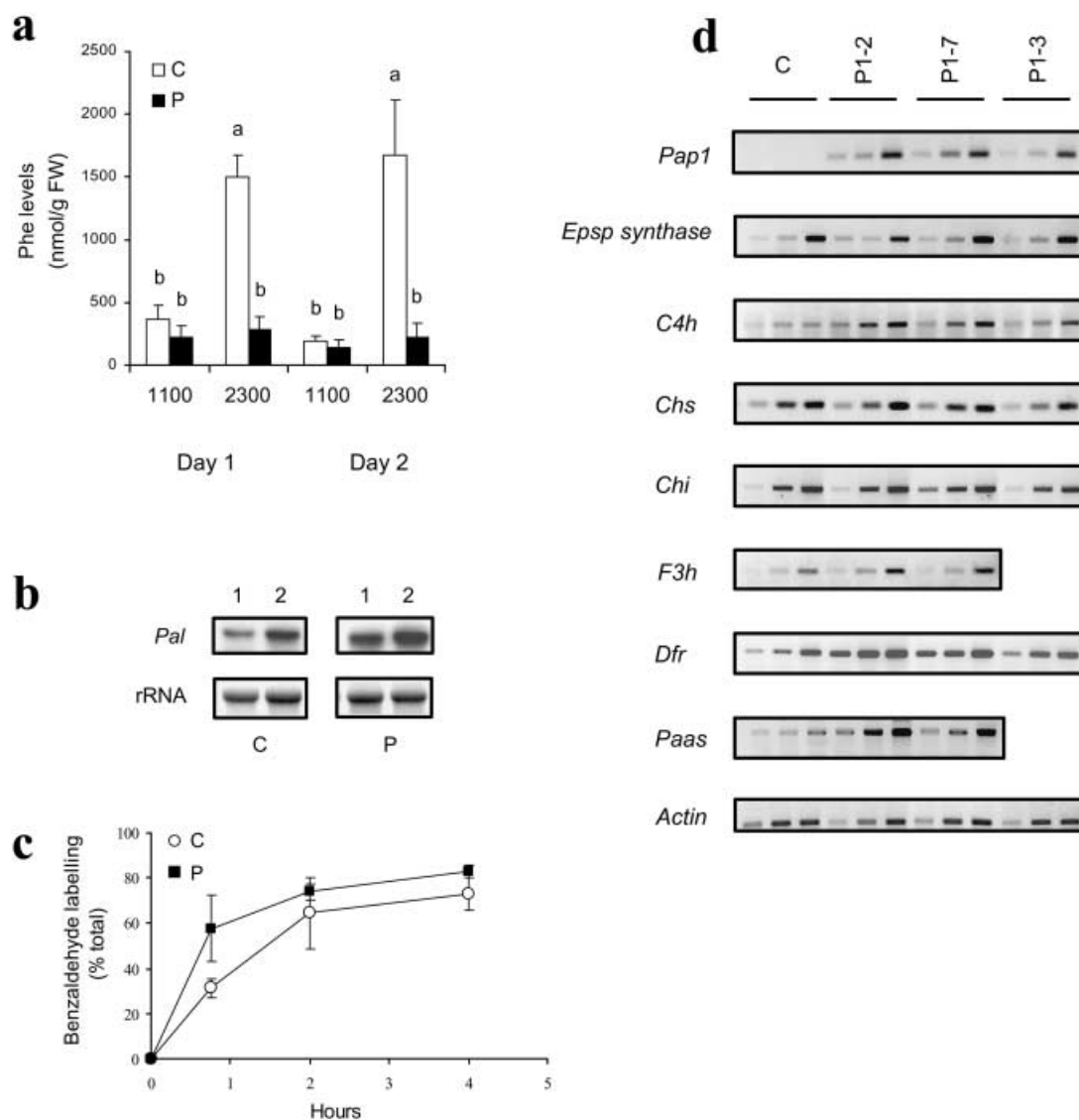


Figure 3 Increased activation of the phenylpropanoid pathway in petunia flowers expressing Production of Anthocyanin Pigment1 (*Pap1*). (a) Levels of phenylalanine (Phe) in limbs of control (C) and *Pap1*-transgenic line 2 (P) petunia were analysed during the day (11.00 h) and at night (23.00 h) on the first and second days post-anthesis. Columns represent the mean values of independent experiments ($n = 3$). Standard errors are indicated by vertical bars. The significance of the differences in Phe levels between *Pap1*-transgenic line 2 and control non-transgenic flowers at different developmental stages during a day/night cycle was calculated using Tukey's all pairwise multiple comparison procedure following multifactor analysis of variance (ANOVA). Values with different letters are significantly different ($P < 0.05$). FW, fresh weight. (b) RNA-blot analysis of phenylalanine ammonia lyase (*Pal*) levels in limbs [first (1) and second (2) days post-anthesis] of control (C) and *Pap1*-transgenic line 2 (P) flowers. (c) *In vivo* labelling kinetics of benzaldehyde in control (C) and *Pap1*-transgenic line 2 (P) petunia flowers. Volatiles, emitted from excised corolla limbs (collected at 23.00 h on the second day post-anthesis) fed with $^2\text{H}_5$ -Phe, were sampled for 0.75, 2 and 4 h, and the total amount and isotope abundance of benzaldehyde were analysed by gas chromatography-mass spectrometry (GC-MS). (d) Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showing the expression of genes from the phenylpropanoid pathway in *Pap1*-transgenic and control petunia flowers. cDNA prepared from total RNA isolated from limbs of control (C) and three *Pap1*-transgenic lines (P1-2, P1-7 and P1-3) was used for PCR amplification of specific genes (see text for gene definitions). For each sample, three amplification products (following 26, 28 and 30 cycles of PCR from left to right) are shown. Ethidium bromide-stained agarose gels show RT-PCR products.

an intermediate (Podstolski *et al.*, 2002). Phenylacetaldehyde biosynthesis, by contrast, has been shown to occur directly from Phe in a reaction catalysed by a single enzyme (Kaminaga *et al.*, 2006). Increased production of phenylacetaldehyde in transgenic petunia flowers could be explained by the

activation of *Paas*, as revealed by the increased levels of its transcript. The enhanced biosynthesis of other volatiles (benzaldehyde, vanillin and its derivatives), as well as anthocyanins, could be attributed to the activation of several genes, i.e. those involved in the skeleton (*Pal* and *C4h*) and

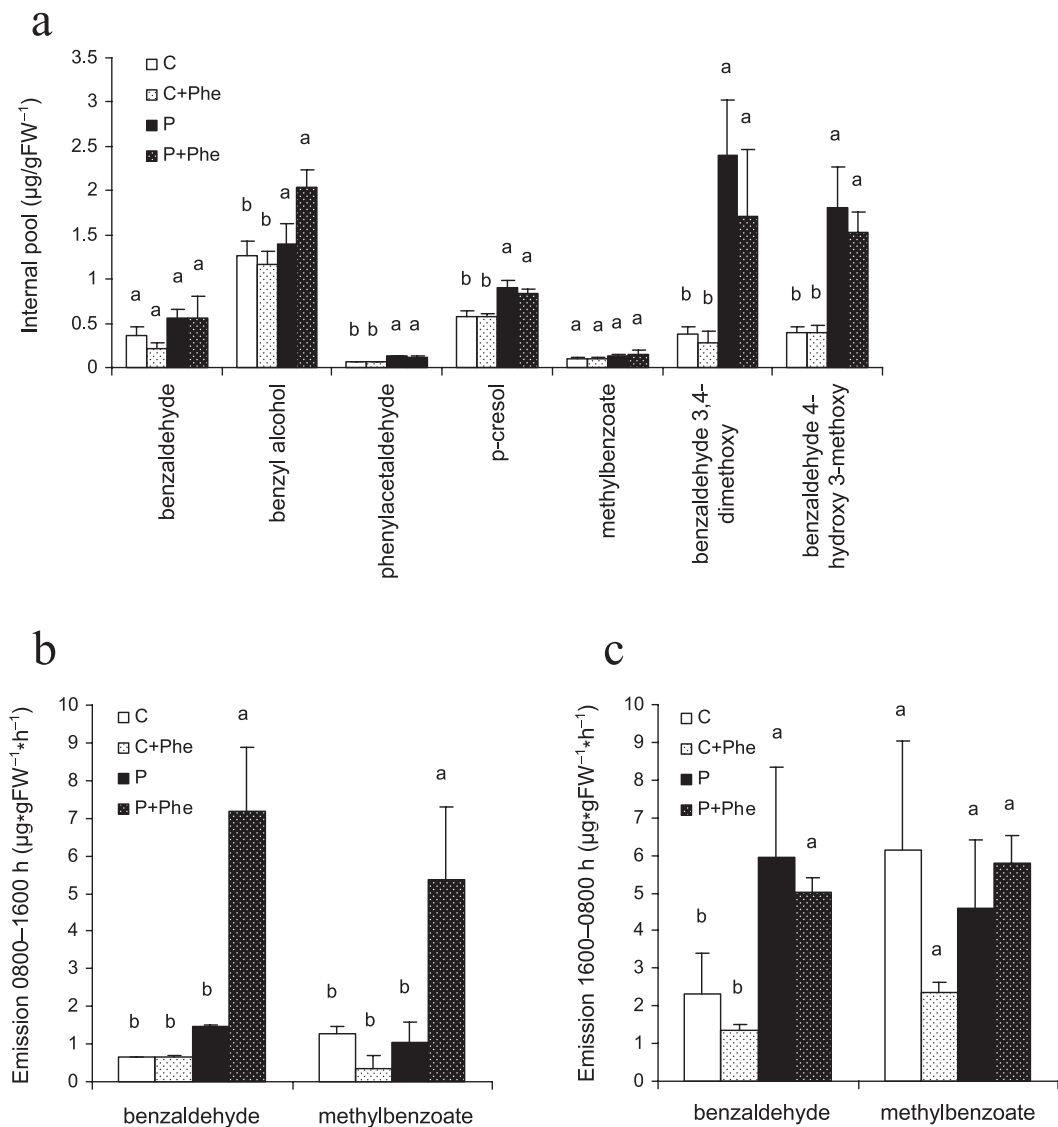


Figure 4 Effect of phenylalanine (Phe) feeding on volatile production in Production of Anthocyanin Pigment1 (*Pap1*)-transgenic and control flowers. (a) Internal pools of volatile compounds accumulated in control (C) and *Pap1*-transgenic line 2 (P) limbs with (+ Phe) or without feeding with Phe. FW, fresh weight. (b, c) Levels of benzaldehyde and methylbenzoate emitted from control (C) and *Pap1*-transgenic line 2 (P) flowers with (+ Phe) or without feeding with Phe. The headspace was collected during the day (08.00–16.00 h) (b) and night (16.00–08.00 h) (c). Columns represent the mean values of independent experiments ($n = 3$). Standard errors are indicated by vertical bars. The significance of the differences in the internal pools of volatile compounds (a) and in the emission levels of volatile compounds between *Pap1*-transgenic line 2 and control non-transgenic flowers with or without Phe feeding during the day (b) or night (c) was calculated using Tukey's all pairwise multiple comparison procedure following two-way analysis of variance (ANOVA), conducted separately for each compound. Values with different letters are significantly different ($P < 0.05$).

specific branches (*F3h* and *Dfr*) of the phenylpropanoid pathway (summarized in Figure 5).

The importance of *Pal* and *C4h* coupling for efficient allocation of carbon into the phenylpropanoid pathway has been demonstrated in bean and tobacco plants (Blount *et al.*, 2000; Schuurink *et al.*, 2006). These reports described a negative feedback loop of phenylpropanoid metabolism caused by *trans*-cinnamic acid, a phenomenon which has also

been documented in pharmacological assays (Blount *et al.*, 2000). Furthermore, the synergistic effect of poplar PAL, C4H and the C4H redox partner cytochrome p450 reductase on phenylpropanoid metabolic flow has been demonstrated in yeast (Ro and Douglas, 2004). The lack of enhancement of volatile emission in non-transgenic petunia flowers following Phe feeding also supports the possibility of a negative feedback loop caused by the accumulation of intermediates in the

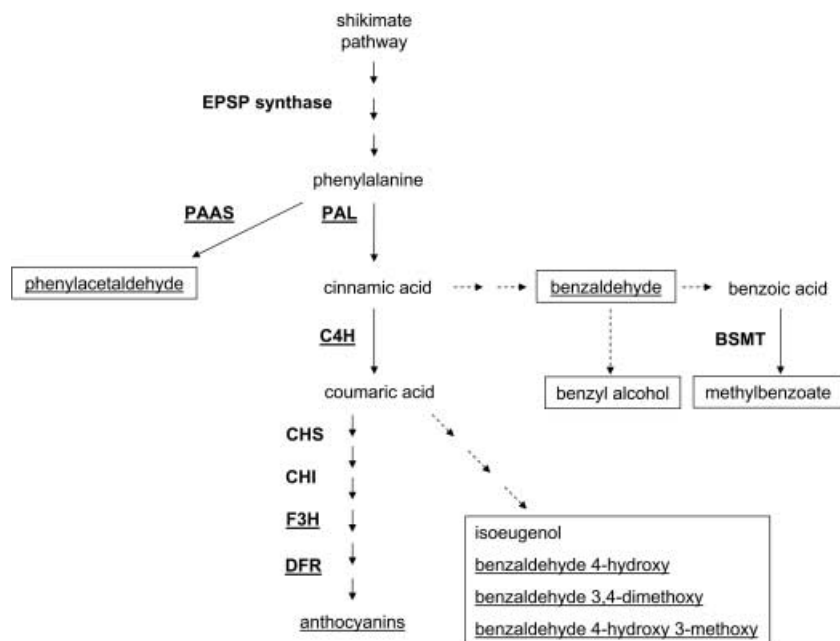


Figure 5 Schematic representation of the pathway leading to the production of some phenylpropanoids determining colour and scent profiles in petunia flowers. Single arrows indicate one-step reactions. Volatile compounds are boxed, and full and broken arrows indicate established and proposed biochemical reactions, respectively. Metabolites produced to a higher level and genes whose expression was up-regulated in Production of Anthocyanin Pigment1 (*Pap1*)-transgenic vs. control petunia flowers are underlined. BSMT, S-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase; C4H, cinnamic acid-4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol-4-reductase; EPSP synthase, 5-enolpyruvylshikimate 3-phosphate synthase; F3H, flavanone 3-hydroxylase; PAAS, phenylacetaldehyde synthase; PAL, phenylalanine ammonia lyase.

phenylpropanoid pathway. Furthermore, the co-ordinated activation of *Pal* and steps downstream of cinnamic acid (i.e. *C4h*, *F3h* and *Dfr*), revealed in *Pap1*-transgenic flowers, may reflect increased Phe utilization, which would account for both the reduced Phe pool and the increased flux without negative feedback within the phenylpropanoid pathway. As *Epsp synthase* transcript and Phe levels were similar in *Pap1*-transgenic and control flowers during the day, and as feeding with Phe led to increased volatile emission in *Pap1*-transgenic but not control flowers during the day, it can be suggested that Phe utilization, rather than production, is affected by *Pap1*.

The observation that *F3h* and *Dfr*, but not genes catalysing early steps in the pathway (*Chs* and *Chi*), were up-regulated in *Pap1*-transgenic relative to control flowers may suggest that early steps are not rate limiting for anthocyanin accumulation; for example, the native *Chs* and *Chi* levels/activities are sufficient to support the increased flux resulting from efficient Phe utilization. Indeed, increased anthocyanin accumulation as a result of the activation of late anthocyanin genes by transcriptional regulators has been reported in petunia (Ben-Meir *et al.*, 2002). Alternatively, increased accumulation of anthocyanins may result from a shift in the balance between the levels of flavones/flavonols and anthocyanins. For example, competition between DFR and flavonol synthase (FLS) enzymes for the substrate has been documented (Ben-Meir *et al.*, 2002), and the activation of *Dfr* and/or silencing of *Fls* has been used to increase the anthocyanin content in petunia (Davies *et al.*, 2003).

Although ectopic *Pap1* expression clearly resulted in the metabolic enhancement of floral scent, the biological role of this transcription factor in scent regulation has yet to be examined on the genetic background of plant systems producing volatile phenylpropanoids. *Arabidopsis* flowers do not produce volatile phenylpropanoids and are not optimal for the assessment of the involvement of *Pap1* in the regulation of the underlying processes (Aharoni *et al.*, 2003; Chen *et al.*, 2003). Moreover, no information is available on the involvement of *Pap1* homologues (Mathews *et al.*, 2003; Borovsky *et al.*, 2004; Hoballah *et al.*, 2007) in scent production in other plant systems. The petunia *Pap1* homologue Anthocyanin2 (*An2*) may not be a true orthologue, as it is responsible for the expression of late anthocyanin genes (Quattrocchio *et al.*, 1999), whereas *Pap1* in *Arabidopsis* also up-regulates early genes from the pathway (Borevitz *et al.*, 2000; Tohge *et al.*, 2005). Notwithstanding, the ectopic expression of *An2* in *Petunia axillaris* does not affect volatile phenylpropanoid production (Hoballah *et al.*, 2007).

The concomitant effect exerted by *Pap1* in petunia flowers on two showy traits, colour and scent, may reflect an endogenous regulatory link between these functionally complementary metabolic pathways. The co-ordinated regulation of metabolic branches within a pathway (e.g. flavonoids and sinapate esters), or even of distinct biochemical pathways (e.g. flavonoids and carotenoids), leading to the production of secondary metabolites that share a biological role, has been reported previously (Li *et al.*, 1993; Davuluri *et al.*, 2005; Lewinsohn *et al.*, 2005). Moreover, the co-regulation of

functionally linked processes (i.e. development and secondary metabolism) has been demonstrated in *Catharanthus roseus*, in which both cell differentiation towards root formation and the production of root-specific alkaloids were induced by ectopic expression of a single transcription factor from *Arabidopsis* (Montiel *et al.* 2007). The use of a regulatory factor activating scent production, as revealed here, creates a new biotechnological strategy for the metabolic architecture of fragrance. Moreover, the co-ordinated regulation of metabolic steps within or between pathways involved in vital plant functions, as shown here for two showy traits determining plant–pollinator interactions, provides a clear advantage for plant survival, and should aid in the creation of novel genetic variability for breeding purposes.

Experimental procedures

Plant material

Petunia hybrida cv. 'Blue Spark' (Danziger-'Dan' Flower Farm, Mishmar Hashiva, Israel) was used for the generation of transgenic plants. Plants were potted and grown in the glasshouse under 22 °C/16 °C day/night temperatures and a natural photoperiod (1500–1700 and 500–1200 $\mu\text{mol}/\text{m}^2/\text{s}$ in the summer and winter, respectively) for 2 years in Rehovot, Israel. Control non-transgenic plants were randomly intermixed with the transgenic lines.

Experimental design and statistical analysis

Unless otherwise indicated, petunia flowers were collected at 23.00 h on the first night post-anthesis. For developmental analysis, buds and flowers were analysed 120, 72 and 24 h pre-anthesis (stages –5, –3 and –1, respectively), and 24, 48 and 72 h post-anthesis (stages 1, 2 and 3, respectively). For day/night experiments determining the steady-state internal pool sizes of volatile and non-volatile phenylpropanoid compounds, tissue was collected at two time points: 11.00 and 23.00 h. Headspace collection for day/night experiments was conducted at 08.00–16.00 and 16.00–08.00 h, respectively.

For headspace analysis and determination of the internal pools of volatile and non-volatile phenylpropanoid compounds (including anthocyanin content), at least three independent experiments ($n = 3$), each with at least three replicate samples, were performed during the course of 2 years. Each sample consisted of three to four detached flowers collected from six 2-month-old petunia plants. Statistical analysis was conducted on the means of independent experiments. When either two-way or multifactor analysis of variance (ANOVA) was used, interactions between treatments were calculated. When interaction was non-significant ($P > 0.05$), the overall effect of the treatment is reported. When interaction was significant ($P < 0.05$), Tukey's all pairwise contrast tests (as detailed in the figure legends) were used to compare the effects of combined treatments. All statistical calculations were performed using JMP IN 5 (JMP IN Software, SAS Institute Inc., Cary, NC, USA).

Construction of chimeric gene and plant transformation

Pap1 (GENBANK accession no. AF325123) coding for *Myb* transcription factor was cloned from *Arabidopsis* 'Colombia' DNA using the PCR primers 5'-ATCTGCAGACTTATACCTTTACAATTTGTTTA-3' and 5'-TCAAAGTGCAGAACTAAGCCCA-3'. The amplified fragment was inserted into a pCd shuttle vector between the CaMV 35S promoter and octopine synthase (OCS) terminator, and the entire construct was then inserted into binary vector pCGN1559 containing the neomycin phosphotransferase II gene (*NptII*) (Guterman *et al.*, 2006). The construct was transferred via *Agrobacterium tumefaciens* to petunia cv. 'Blue Spark' using the standard leaf disc transformation method (Guterman *et al.*, 2006).

RNA analyses

Total RNA (10 μg) was isolated from control and transgenic plants (*S1*), fractionated through a 1% formaldehyde gel and transferred to a Hybond N+ membrane (Amersham-Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). ^{32}P -labelled *Pal* (GENBANK accession no. AY705976) (Rediprime; Amersham-Pharmacia Biotech) and *S*-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase (*Bsmt1*, GENBANK accession no. AY233465) PCR fragments served as probes. Blots were hybridized in buffer containing 0.263 M Na_2HPO_4 , 7% (w/v) sodium dodecylsulphate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA) and 1% (w/v) bovine serum albumin (BSA) at 60 °C, washed twice in $2 \times$ standard saline citrate (SSC), 0.1% SDS at 60 °C for 20 min each, and exposed to X-ray film (Fuji, Tokyo, Japan) with two intensifying screens at –70 °C. For semi-quantitative RT-PCR analyses, total RNA was treated with RNase-free DNase (Promega, Madison, WI, USA), and cDNA was generated using oligo(dT)₁₅ primer and M-MLV RT (both from Promega). Control samples were generated without the addition of RT to the reaction. The primers used for semi-quantitative RT-PCR experiments are shown in Table 1. cDNA amplification was conducted with an initial denaturation step of 94 °C for 3 min, followed by 26–30 cycles of 94 °C for 10 s, 62 °C for 10 s, 72 °C for 20 s, and a final elongation step of 72 °C for 10 min. Actin cDNA was used as a reference for the standardization of cDNA amounts in the reactions with different petunia lines. To confirm that the analysed samples were not contaminated with DNA, PCR amplification was also conducted with samples generated without RT.

Collection and extraction of volatile compounds

For headspace analysis (Guterman *et al.*, 2006), flowers were weighed and volatiles were collected using an adsorbent trap consisting of a glass tube containing 200 mg of polymer Porapak Type Q (80/100 mesh; Alltech, Deerfield, IL, USA) held in place with plugs of silanized glass wool. Trapped volatiles were eluted using 3 mL of hexane, and 2 μg of isobutylbenzene was added per sample as an internal standard for each.

To determine the pool sizes of volatile compounds in corolla limbs, tissue (0.5–1.5 g fresh weight) was ground in liquid nitrogen and extracted in hexane (4 mL/g of tissue) containing 0.5 $\mu\text{g}/\text{mL}$ isobutylbenzene as internal standard. Following overnight incubation with shaking at 150 r.p.m., the extract was centrifuged at 10 500 **g**

Table 1 Primers used for semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) experiments

Gene	GENBANK accession number	Primer sequence
<i>Pap1</i>	AF325123	5'-TTCCTACAACACCGGCACTAA-3' 5'-TTTCTGTTGTCGTCGCTTCA-3'
<i>Epsp synthase</i>	M21084	5'-TCATTACATGCTTGGTGCCTTG-3' 5'-TTGGCTGCTAATGGATCCAGA-3'
<i>C4h</i>	AF255013	5'-TTGTCAAATGTGGGAGCGTG-3' 5'-TGGGATCAAACCTGGCATC-3'
<i>Chs-A</i>	X14599	5'-GAGGAAGCATTTAAACCTTT-3' 5'-CACTCAAGCCCTCACCAG-3'
<i>Chi</i>	Y00852	5'-ATGAATTCGGGATGTTGTACAGGTC-3' 5'-ATGAATCTTTCTCGGCTCCGGTTT-3'
<i>F3h</i>	X60512	5'-ATGAGCTCCACACTGATCCAGGAACC-3' 5'-ATTCTAGATTACAACAGTAGGTAGGA-3'
<i>Dfr</i>	X15537	5'-ATGACGTCGTTGGTCCATTCACACA-3' 5'-ATTCTAGAATGGCAATGGCTTCG-3'
<i>Paas</i>	DQ243784	5'-ATCTCGAGCAACCCAGAATTTGATGGTCA-3' 5'-AGAATTCACACCACCACCACCA-3'
<i>Actin</i>	AA660796	5'-GGTTTTGCTGGGATGATGC-3' 5'-CATTGAATGTCTCAACATGATTGAGTC-3'

See text for gene definitions.

for 10 min and the supernatant was filtered through a 25-mL syringe with a 0.2- μ m sterile nylon filter. To assess whether the bound form of benzaldehyde accumulates in petunia limbs, tissue (0.6 g) was incubated with 9 mL of phosphate citrate buffer pH 4.5, with or without 15 units of glucosidase mixture Novarom G (Novo Nordisk Ferment Ltd., Bagsvaerd, Denmark), at 37 °C overnight, and then transferred to -20 °C to stop the enzymatic reactions. Hexane (10 mL) containing 0.5 μ g/mL of isobutylbenzene as internal standard was added to each sample and, following vortexing, the upper phase was recovered.

To evaluate the effect of Phe feeding on volatile production, control and *Pap1*-transgenic flowers were collected at 08.00 and 16.00 h, placed in a glass vial containing 10 mL of water, with or without 5 mg L-Phe (Duchefa, Haarlem, the Netherlands), and headspace was collected during the day (08.00–16.00 h) and night (16.00–08.00 h). Flowers were weighed prior to the collection of emitted volatiles from detached petunia flowers. To assess the internal pools of volatiles, control and *Pap1*-transgenic flowers collected at 16.00 h were placed in a glass vial containing 10 mL of water, with or without L-Phe, and, at 23.00 h, volatiles were extracted from the tissue (0.5–1.5 g fresh weight) as above.

Gas chromatography-mass spectrometry (GC-MS) analysis of volatile compounds

GC-MS analysis of volatile compounds collected and extracted as described above was performed using a device composed of a Pal autosampler (CTC Analytic, Zwingen, Switzerland), a TRACE GC 2000 equipped with an Rtx-5SIL MS (Restek; inside diameter, 0.25 μ m; 30 m \times 0.25 mm; Bellefonte, PA, USA) fused-silica capillary column, and a TRACE DSQ quadrupole mass spectrometer (ThermoFinnigan,

Hemel Hempstead, Hertfordshire, UK). Helium was used as the carrier gas at a flow rate of 0.9 mL/min. The injection temperature was set to 250 °C (splitless mode), the interface to 280 °C and the ion source adjusted to 200 °C. The analysis was performed under the following temperature programme: 5 min of isothermal heating at 50 °C, followed by a 5 °C/min oven temperature ramp to 260 °C, and a final increase from 250 to 280 °C at 10 °C/min. The transfer line temperature was 280 °C. The system was equilibrated for 1 min at 70 °C before injection of the next sample. Mass spectra were recorded at two scans per second, with a scanning range of 40–450 mass-to-charge ratio and an electron energy of 70 eV. Compounds were tentatively identified (> 95% match) based on the National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health (NIST/EPA/NIH) Mass Spectral Library (Data Version: NIST 05, Software Version 2.0d) using the XCALIBUR v1.3 program (ThermoFinnigan) library. Further identification of the compounds was based on a comparison of mass spectra and retention times with those of authentic standards (Sigma, Milwaukee, WI, USA) analysed under similar conditions.

Analytical methods for the identification and quantification of non-volatile phenylpropanoid compounds

For the quantification of Phe pool size, tissue (0.5–1.5 g fresh weight) was extracted with methanol, followed by phase separation with chloroform and water (10 mL methanol : 5 mL chloroform : 6 mL water). Amino acids in the aqueous phase were purified by Dowex-50-H⁺ ion-exchange chromatography. The pool size of Phe was quantified by GC-MS of *N*-(*O*,*S*)-heptafluorobutryl isobutyl amino acid derivatives, using α -amino-*n*-butyrate as an internal standard (Boatright *et al.*, 2004). To determine the labelling of the endogenous non-volatile intermediate metabolites, limbs of control and *Pap1*-transgenic petunia were extracted with methanol and analysed by liquid chromatography-mass spectrometry (LC-MS). Prior to injection into the mass spectrometer, the extracted compounds were separated using a Supelco Discovery HS C₁₈ column (15 cm \times 2.1 mm inside diameter) attached to a Waters 2690 separations module (Milford, MA, USA) with attached column oven. Compound elution was monitored at 210 and 280 nm with a Waters 996 UV/visible photodiode array detector. Complete baseline separation was achieved at a flow rate of 0.25 mL/min with the column incubated at a constant temperature of 40 °C. Solvent A was 0.05% formic acid in water; solvent B was 100% acetonitrile. The column was pre-equilibrated with 5% solvent B in solvent A. After injection of up to 25 μ L of aqueous sample, the column was washed with 0.5 mL of pre-equilibration solvent. Compounds were eluted from the column with a linear gradient of 5%–66% solvent B over 13.75 mL. The column was then washed by increasing solvent B to 100% (linear gradient in 0.75 mL) and holding at 100% solvent B for 0.75 mL. The column was then re-equilibrated by returning it to 5% solvent B (over 0.75 mL), followed by a 2.5-mL wash with this solvent. The total run time was 70 min (Boatright *et al.*, 2004).

In vivo isotope labelling of benzaldehyde

Stable isotope labelling (Boatright *et al.*, 2004) was conducted in a glass container by placing the cut surface of excised limbs of petunia

flowers (total of 10 limbs of 0.2 g each per experiment) on moist filter paper supplied with 75 mm unlabelled Phe combined with radio-labelled Phe (2 μ L of L-Phe-ring- $^2\text{H}_5$, Cambridge Isotope Laboratories, Andover, MA, USA). Volatile collection and headspace analysis were conducted as described above. Labelling experiments were started at 23.00 h, and were conducted on limbs from flowers 2 days post-anthesis, when volatile emission is highest and most constant, to eliminate the effect of rhythmicity. Glass tubes containing adsorbent trap were replaced and eluted at four time points: 45, 60, 120 and 240 min from $^2\text{H}_5$ -Phe feeding. Newly synthesized labelled benzaldehyde exhibited a 5-atomic mass unit (a.m.u.) mass shift. The percentage of labelling was determined at each time point as the intensity of the shifted representative molecular ion divided by the sum of the intensities for unshifted and shifted representative molecular ions.

Anthocyanin content

To determine the anthocyanin content (Zuker *et al.*, 2002), tissue (100 mg fresh weight) was extracted in 1 mL of methanol containing 1% (v/v) HCl. Following overnight incubation in the dark at -4°C with shaking at 150 r.p.m., the extract was centrifuged at 10 500 g for 10 min. The anthocyanin content in the supernatant was determined using the formula $A_{530} - 0.25A_{657}$, allowing for the subtraction of chlorophyll interference.

Acknowledgements

We thank Danziger-'Dan' Flower Farm for providing the plant material and Dr Hillary Voet for assistance in the statistical analyses. This work was funded by US-Israel Binational Agricultural Research and Development (BARD) research grant no. US-3437-03 to AV and ND, and Israel Science Foundation grant no. 505/05 to AV. AV is an incumbent of the Wolfson Chair in Floriculture.

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Supplementary material

The following supplementary material is available for this article:

Figure S1 Methylbenzoate production in flowers of control and Production of Anthocyanin Pigment1 (*Pap1*)-transgenic petunia lines. (a) Levels of methylbenzoate emitted from flowers of control (C) and *Pap1*-transgenic (P1-3, P1-2 and P1-7) lines were determined using the dynamic headspace technique. Analysis was conducted for 12 h during the first night post-anthesis. Columns represent the mean values of independent experiments ($n = 3$). Standard errors are indicated by vertical bars. Data were subjected to one-way analysis of variance (ANOVA), and no significant effect ($P < 0.05$) of genotype was revealed. (b) RNA-blot analysis of *S*-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase (*Bsmt*) levels in limbs [first (1) and second (2) days post-anthesis] of control (C) and *Pap1*-transgenic line 2 (P).

The blot was rehybridized with an 18S rRNA probe to ensure equal loading of samples.

Figure S2 Developmental profiles of benzaldehyde emission in control and Production of Anthocyanin Pigment1 (*Pap1*)-transgenic petunia flowers. Levels of benzaldehyde emission from control (C) and *Pap1*-transgenic line 2 (P) flowers at different developmental stages were determined using the dynamic headspace technique. Analysis was conducted for 12 h using flowers during the first, second and third night post-anthesis (1, 2 and 3, respectively). Each point represents the mean values of independent experiments ($n = 3$). Standard errors are indicated by vertical bars. The significance of the differences in benzaldehyde emission between *Pap1*-transgenic line 2 and control non-transgenic flowers, at different developmental stages, was calculated using Tukey's all pairwise multiple comparison procedure following two-way analysis of variance (ANOVA). Values significantly different from control flowers are indicated by asterisks ($P < 0.0001$).

Figure S3 Benzaldehyde production during a day/night cycle in control and Production of Anthocyanin Pigment1 (*Pap1*)-transgenic petunia flowers. (a) Levels of benzaldehyde emission from control (C) and *Pap1*-transgenic line 2 (P) flowers 2 days post-anthesis were determined using the dynamic headspace technique. Headspace collections were performed during the day for 12 h under light conditions (06.00–18.00 h) and during the night for 12 h under dark conditions (18.00–06.00 h). (b) Determination of internal pools of benzaldehyde. Free and total (following glucosidase treatment) pools of benzaldehyde were assayed in limbs collected during the day (11.00 h) and night (23.00 h) using

extraction with hexane. Columns represent the mean values of independent experiments ($n = 3$). Standard errors are indicated by vertical bars. The significance of the differences in the levels of benzaldehyde emission (a) and internal pool (b) between *Pap1*-transgenic line 2 and control non-transgenic flowers during the day/night cycle was calculated using Tukey's all pairwise multiple comparison procedure following two-way and multi-factor analysis of variance (ANOVA), respectively. Values with different letters are significantly different ($P < 0.05$ and $P < 0.0001$ in a and b, respectively).

Figure S4 Pools of cinnamic (a) and coumaric (b) acids in control and Production of Anthocyanin Pigment1 (*Pap1*)-transgenic petunia flowers. The levels of cinnamic and coumaric acids in the limbs of control (C) and *Pap1*-transgenic line 2 (P) petunia were analysed during the day (11.00 h) and at night (23.00 h) on the first day post-anthesis. Columns represent the mean values of independent experiments ($n = 3$). Standard errors are indicated by vertical bars. Data were subjected to two-way analysis of variance (ANOVA), and no significant effect ($P < 0.05$) of treatments was revealed.

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