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# Regulation of floral scent production in petunia revealed by targeted metabolomics

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**Abstract**

*Petunia hybrida* line W115 (Mitchell) has large white flowers that produce a pleasant fragrance. By applying solid phase micro extraction (SPME) techniques coupled to GC–MS analysis, volatile emission was monitored in vivo using a targeted metabolomics approach. Mature flowers released predominantly benzenoid compounds of which benzaldehyde, phenylacetaldehyde, methylbenzoate, phenylethylalcohol, iso-eugenol and benzylbenzoate were most abundant. This emission had a circadian rhythm reaching its maximum at dusk. During petal limb expansion two sesquiterpenes were emitted by the petunia flowers, tentatively identified as germacrene D and cadina-3,9-diene. In vitro analysis showed that the petal limbs and stigma were the main producers of the benzenoids and sesquiterpenes, respectively. Moreover, comparison of in vivo and in vitro analysis indicated that volatiles were not stored during periods of low emission but rather were synthesized de novo. DNA-microarray analysis revealed that genes of the pathways leading to the production of volatile benzenoids were upregulated late during the day, preceding the increase of volatile emission. RNA-gel blot analyses confirmed that the levels of phenylalanine ammonia lyase (PAL) and *S*-adenosyl methionine (SAM) synthase transcripts increased towards the evening. Our results suggest that the circadian production of volatile benzenoids in petunia W115 is, at least partly, regulated at the transcript level.


*Keywords:* *Petunia hybrida*; Headspace; Floral scent; Solid phase micro extraction; Volatile benzenoids; Flower development; Microarray; Phenylalanine ammonia lyase

## 1. Introduction

Sweet floral fragrances are characteristic for many insect-pollinated flowers and are involved in the attraction and guidance of pollinators to the reproductive organs (Dobson, 1994; Dobson and Bergström, 2000). These floral fragrances consist of complex bouquets of volatiles of which the composition and levels determine the character of the fragrance and the attractiveness for bee and moth pollinators (Brown, 2002). Typically, fragrances consist of terpenoids, fatty acid derivatives, indole compounds and benzenoids (Knudsen and Tollsten, 1993). Natural floral fragrances are used as ingredients for perfumes and knowledge about specific components in floral fragrances can be used for the production of synthetic perfumes.

Plants naturally control the timing of floral fragrance emission to guard the cost-benefit balance. Nocturnally pollinated plants predominantly emit their volatiles during the night and this circadian rhythm is regulated through an internal clock (Loughrin et al., 1991). It was recently shown that this internal clock is also present in *Antirrhinum majus* (snapdragon) and *Rosa hybrida*, which emit volatiles during the day (Helsper et al., 1998; Kolosova et al., 2001a). The diurnally emitting plants *Brassica napus* and *Trifolium repens* L. lack this circadian clock. In these plants, the rhythm of volatile emission is

*Abbreviations:* PDMS, polydimethylsiloxane; SPME, solid phase micro extraction; PAL, phenylalanine ammonia lyase; SAM, *S*-adenosyl methionine; DOXP, 1-deoxy-D-xylulose-5-phosphate.

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directly controlled by light (Jakobsen and Olsen, 1994; Jakobsen et al., 1994).

The biochemical pathways leading to the precursors of the various volatiles have been well studied in leaves and plant cell cultures. Sesquiterpenes are derived from the mevalonate-pathway in the cytosolic compartment, while monoterpenes are synthesised in plastids via the DOXP-pathway; fatty acid derivatives such as C6-aldehydes and alcohols are mainly produced from intermediates of the lipoxygenase-hydroperoxide lyase pathways; indole is an alkaloid synthesised from tryptophan and benzenoids have a common precursor in phenylalanine (for a review see Pichersky and Gershenzon, 2002). The biochemical reactions leading from these precursors to the actual volatile compounds and the molecular and genetic control of the pathways, however, remain largely unsolved. Progress in elucidating these reactions has been made in *Clarkia breweri* and snapdragon. *C. breweri* flowers produce 8–12 different volatiles, among which linalool, an acyclic monoterpene, and the benzenoids benzylacetate, methylsalicylate, methyleugenol and methylisoeugenol. The enzymes catalysing the last step in the biosynthesis of these volatiles have been purified and characterised while their corresponding cDNAs have been cloned (Dudareva and Pichersky, 2000). In *C. breweri* the production of linalool, benzylacetate, methylsalicylate and methyl(*iso*)eugenol during flower development seems to be controlled at transcript level, with the increase in transcripts occurring 1–2 days prior to the increase in enzyme activity and emission of the corresponding compound (Dudareva and Pichersky, 2000).

The snapdragon benzoic acid methyl transferase (BAMT), which is involved in the production of methylbenzoate, and its cDNA have been characterized (Dudareva et al., 2000). This enzyme is predominantly present in the conical cells of the inner epidermal layer of the petal lobes (Kolosova et al., 2001b). The transcript level of BAMT correlates with the amount of methylbenzoate produced during flower development (Kolosova et al., 2001a). However, the oscillating factor over a 24-h time-span is benzoic acid, the precursor of methylbenzoate. Transcriptional and translational control mechanisms of BAMT appear to be less important than the presence of benzoic acid. In petunia Mitchell flowers, the activity of BAMT oscillates with methylbenzoate emission. However, BAMT activity remains high during the day when little methylbenzoate is produced. The regulation here seems to be partly on the level of the precursor, benzoic acid, which levels are very low during the day (Kolosova et al., 2001a).

We are using *Petunia hybrida* W115 (Mitchell) as a model system for elucidating the biosynthesis of various volatile compounds by flowers and the regulation thereof. The large white flowers produce a musty scent only in the evening and night, suggesting a circadian regulation. *P. hybrida* is genetically well characterised and line W115 is easy to transform (Lucker et al., 2001). This paper reports on the identification of the dominant volatile compounds emitted by petunia flowers in vivo using an SPME-targeted metabolomics approach. Moreover, the developmental, temporal and spatial characteristics of volatile release are described in detail as well as the contribution of the various flower organs to the volatile signature. In addition, DNA-microarray techniques in combination with RNA gel-blot analyses provide evidence that the genes in the pathways leading to the precursors of floral volatiles are regulated at the transcript level.

## 2. Results

### 2.1. Identification of petunia flower volatiles using SPME and GC–MS

To analyze the volatile compounds produced by petunia flowers in vivo, and to be able to follow volatile release during flower development of single flowers, we set up a rapid targeted metabolomics sampling method. A Solid Phase Micro Extraction (SPME) device was placed in the floral headspace to sample the volatiles (Fig. 1), which were subsequently analysed by GC–MS. The major volatiles detected are listed in Table 1. The GC–MS chromatogram (Fig. 2A) showed four major peaks representing the benzenoid molecules benzylaldehyde, methylbenzoate, iso-eugenol, and benzylbenzoate. The four minor peaks represented the benzenoids phenylacetaldehyde and phenylethylalcohol, and the aliphatic aldehydes decanal and dodecanal. The validity of this SPME-method, using an 100 μm PDMS coated fiber, was confirmed by GC–MS analysis of conventionally

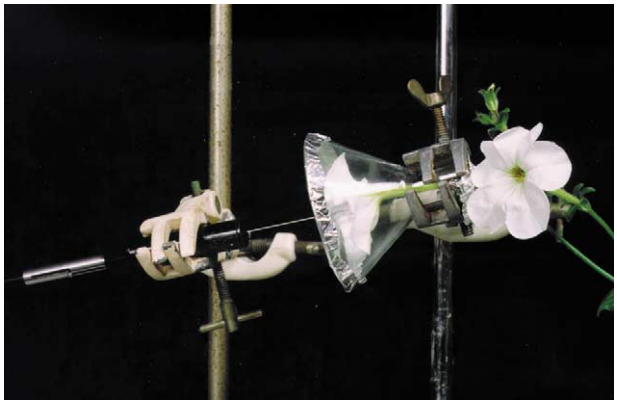

Fig. 1. Experimental design for headspace-SPME of *Petunia hybrida* W115 flowers in vivo. A glass funnel was placed around a developing flower thereby minimally interfering with its development. Aluminium foil was placed over both sides of the funnel to limit air movement. The SPME device was fixed in front of the glass funnel, with the hollow SPME needle stuck through the aluminium foil, exposing the PDMS fiber to the volatile compounds present in the floral headspace.

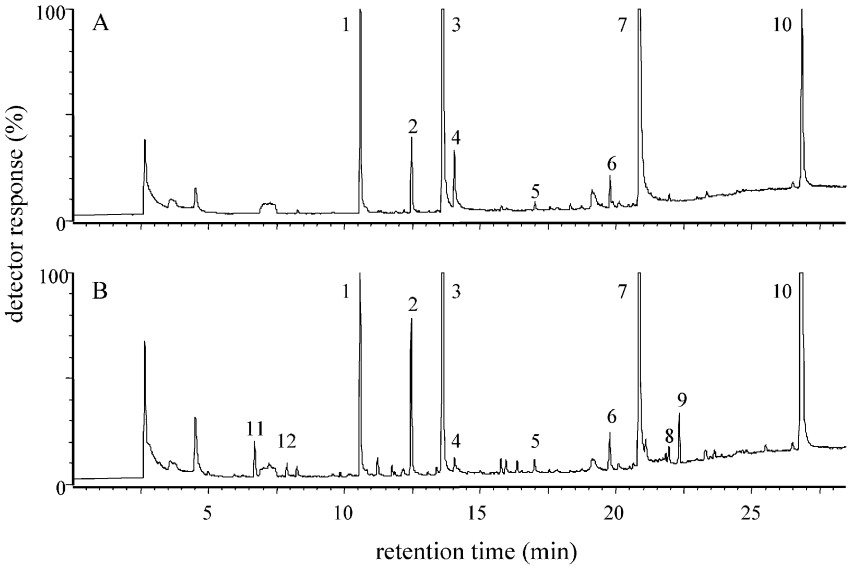


Fig. 2. Comparison of in vivo and in vitro measurements of petunia floral headspace. GC–MS chromatograms (100% scale = $2\times10^6$ total ion counts) obtained from headspace-SPME of petunia W115 flowers in vivo (A) and $CaCl_2$-extracts (B). Flowers were analyzed after 15 h in the light period. The major peaks are numbered as in Table 1.

(with organic solvents) extracted flowers (performed by Quest, Naarden, The Netherlands). The GC–MS chromatograms matched qualitatively (data not shown). Mass spectrum analysis revealed the presence of at least 50 other minor compounds, among which mainly other benzenoids (e.g. benzylalcohol, methylsalicylate and phenyl, methyl, and ethyl esters), while monoterpenes were not found.

To determine the contribution of the various flower organs to the volatile emission, we set-up a method that enabled us to measure volatile production in vitro. For this, intact flowers were submerged in liquid nitrogen and ground in a saturated $CaCl_2$ solution in which enzymatic reactions should be inhibited (Buttery et al., 1987). Upon subsequent heating and stirring of the extract, the volatiles evaporated from the solution into the headspace, enabling the SPME fiber to adsorb them. The validity of this in vitro method was first tested on whole flowers by comparison of the GC–MS chromatogram with that obtained by using the in vivo SPME method (Fig. 2A vs. 2B). Compared to the in vivo headspace chromatograms (Fig. 2A), the in vitro chromatograms (Fig. 2B) showed several additional peaks. The mass spectra of peaks 8 and 9 corresponded with that of two sesquiterpenes tentatively identified as germacrene D and cadina-3,9-diene, respectively. The presence of the fatty acid breakdown products 3-hexanal and 2-hexanal (peaks 11 and 12) from the lipoxygenase (LOX) pathway (Feussner and Wasternack, 2002) indicated that some lipid peroxidation still occurred during sample preparation. Though minor differences were found for the in vitro method compared to the in vivo method, it was clear that the in vitro method was suitable for detecting all compounds that were detected in vivo.

### 2.2. Temporal emission of petunia W115 volatiles

To investigate whether volatile emission was constant or under circadian control, the headspace of a single flower was sampled by SPME in vivo at different time points (Fig. 3). Volatile release was lowest early into the light period (Figs. 3A and B) and increased afterwards, reaching maximum levels after 15 h of light (Fig. 3C) and 3 h into the dark period (Fig. 3D). Methylbenzoate

Table 1
GC and MS data of some major volatiles detected in the floral headspace of *Petunia hybrida* W115[a]

| Peak | Relative retention time (min) | Main *m/z* fragments (in order of abundance) | Compound names |
|---|---|---|---|
| *Benzenoids* | | | |
| 1 | 10.58 | 105, 106, 77 | Benzaldehyde |
| 2 | 12.45 | 91, 92, 65, 120 | Phenylacetaldehyde |
| 3 | 13.63 | 105, 77, 136, 51 | Methylbenzoate |
| 4 | 14.05 | 91, 92, 122, 65 | Phenylethylalcohol |
| 7 | 20.86 | 164, 149, 77, 91, 103 | Isoeugenol* |
| 10 | 26.85 | 105, 91, 77, 51 | Benzylbenzoate |
| *Aliphatic aldehydes* | | | |
| 5 | 15.70 | 43, 41, 57, 55, 70, 82 | Decanal |
| 6 | 19.71 | 43, 57, 41, 55, 44, 82 | Dodecanal |
| *Sesquiterpenes* | | | |
| 8 | 21.75 | 161, 91, 105, 79, 204 | Germacrene-D* |
| 9 | 22.30 | 161, 119, 105, 134, 204 | Cadina-3,9-diene* |
| *Fatty acid derivatives* | | | |
| 11 | 6.69 | 41, 55, 69, 83 | 3-Hexenal |
| 12 | 7.88 | 41, 55, 69, 83 | 2-Hexenal |

[a] Volatiles were identified by screening the NIST library for comparable MS-spectra and, except for compounds marked with *, by subsequent comparison with authentic reference compounds.

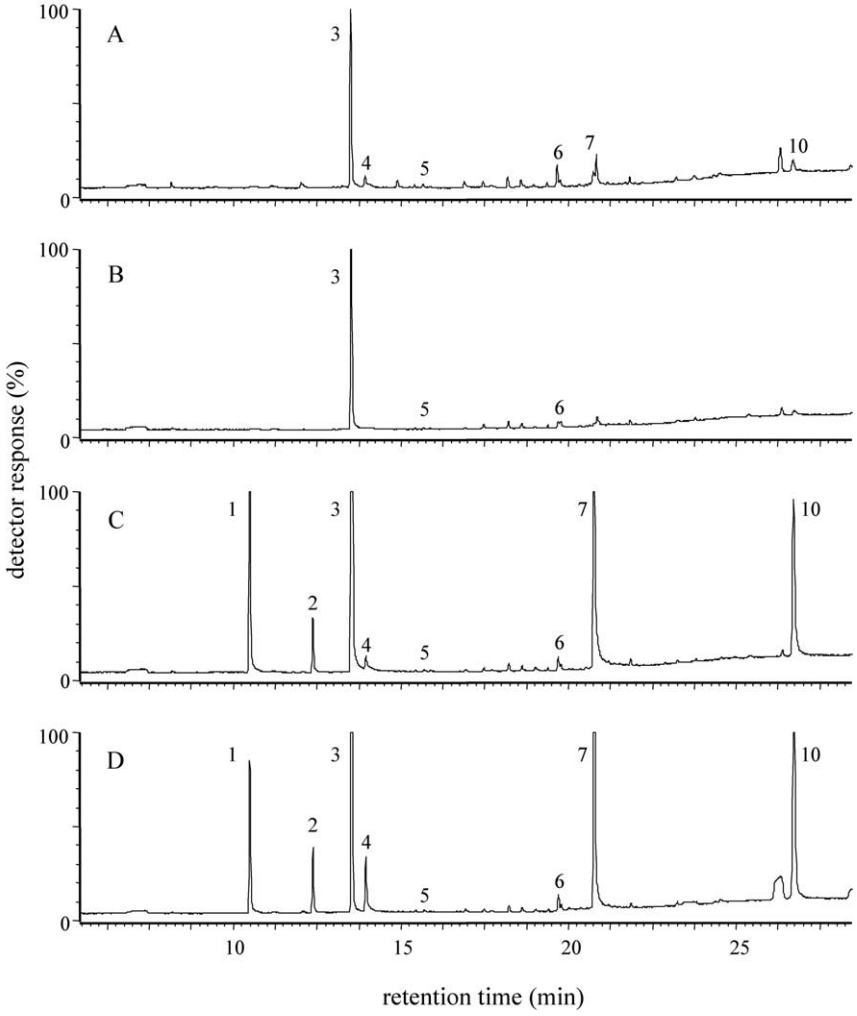


Fig. 3. Temporal volatile emission from petunia flowers. GC–MS chromatograms (100% scale = $2\times10^6$ total ion counts) show the volatile components released by a single open flower in vivo. Headspace-SPME was performed after (A) 7 h in the light; (B) 11 h in the light; (C) 15 h in the light; (D) 3 h in the dark. The experiments were carried out with a freshly (1-day) opened flower. Light/dark conditions were 16 h/8 h, the light period starting at 02:00 h.

was prominently present in all the flower headspace samples, while other benzenoids appeared at the end of the light period. In Fig. 4, the emission of the four major benzenoids over a period of 48 h, is depicted together with the light regime. Evidently, benzenoid emission by the flowers followed a circadian rhythm, with maximum levels at the end of the light period. This rhythm in emission continued until senescence of the flowers (data not shown), which usuallly occurred 6 or 7 days after opening of the flower.

Using the in vitro method, we analyzed the volatiles of flowers harvested at the same time points as in Fig. 3. The volatile levels were lowest early into the light period and highest at the end, similar to that found for the natural emission of flower volatiles. This indicates that volatiles were not stored during periods of low emission but rather were continuously synthesised de novo during emission.

### 2.3. Volatile release during flower development

In order to study volatile emission in relation to flower development, small flower buds (length about 2 cm) were enclosed in glass funnels and the headspace volatiles were sampled in vivo during 24 hours of development (Fig. 5). During the growth of the buds (Figs. 5A and B), a small peak of methylbenzoate was detected in the headspace. The aldehydes decanal and dodecanal became detectable in the headspace of full-grown flower buds (Fig. 5B) and the levels released did not change significantly during further development. Upon expansion of the corolla limbs (Fig. 5C) and opening of the flower (Fig. 5D), the peaks of methylbenzoate and of the two sesquiterpenes (germacrene D and cadina-3,9-diene) became the most obvious peaks in the chromatogram of the floral headspace. When the flower developed further, the emission of these sesqui-

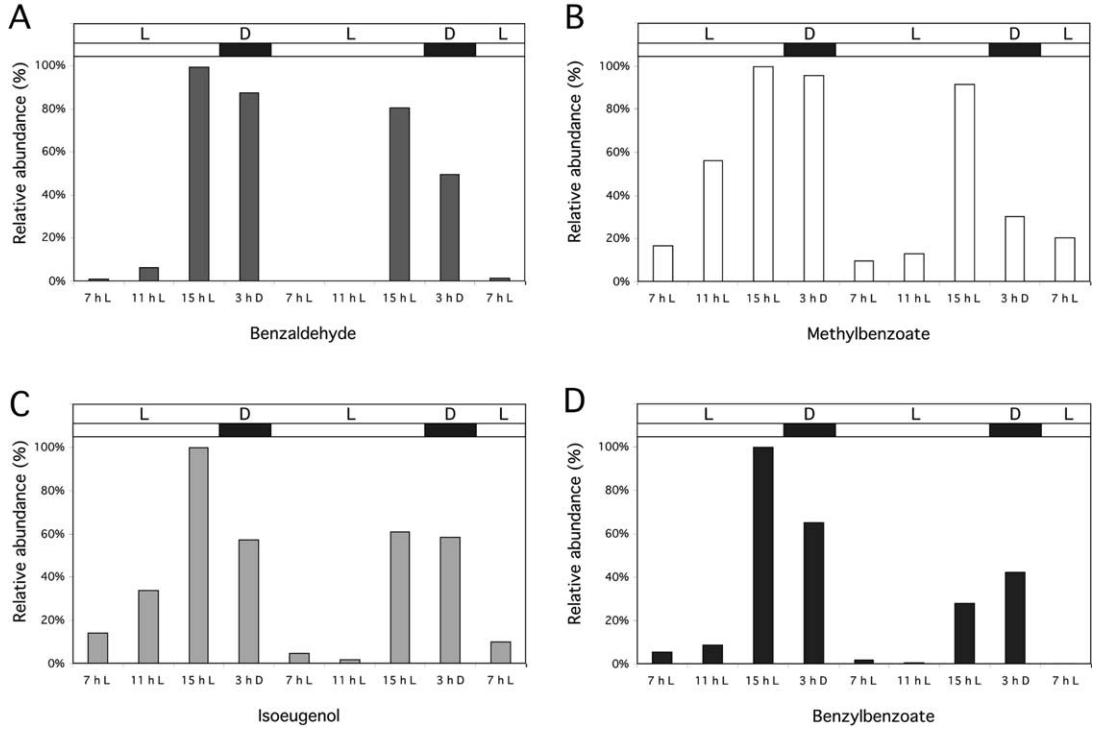


Fig. 4. Rhythmic emission of four major volatile benzenoids. After flower opening, flower volatiles were analyzed four times a day, over a 48-h period, using in vivo headspace-SPME. The four major benzenoids are shown in separate graphs; (A) benzaldehyde, (B) methylbenzoate, (C) isoeugenol, and (D) benzylbenzoate. 7 h L: 7 h in the light; 11 h L: 11 h in the light; 15 h L: 15 h in the light; 3 h D: 3 h in the dark. Plants were grown under 16 h/8 h regime. Top white bars indicate light periods (L); top black bars indicate dark period (D). Each component is plotted as a percentage of its maximum value.

terpenes decreased while that of benzenoids increased (Figs. 5E and F). The marked increase in the levels of the benzenoids corresponded with the start of the first day/night cycle of the freshly opened flowers. The onset of flower senescence was characterized by a low level of volatile emission, which was especially obvious at the end of the light period (data not shown).

### 2.4. Petunia flower petals are the richest source of volatiles

Since the localization of volatile production within the petunia flower tissues was unknown, we compared the volatile release from the different flower organs of one single flower. The volatiles produced by each organ were analyzed using the in vitro assay. The GC–MS chromatograms of the volatiles indicated that methylbenzoate was produced by all flower organs (Fig. 6). The highest levels of methylbenzoate and other benzenoids were observed with extracts derived from petal tissues: the corolla tube (Fig. 6E) and particularly the petal limbs (Fig. 6F). Acetic acid 2-phenylethylester ($t_R = 17.0$ min; $m/z$ 104, 43, 91, 105, 65) was only detected in the petal limb extracts. Cadina-3,9-diene was mainly detected in stigma extracts. A small peak of germacrene D was present in chromatogram of the stigma, and absent in the chromatograms of the other flower organs. The sepal and the style produced mainly methylbenzoate while the stamen (anthers with pollen exposed) released benzaldehyde additionally.

### 2.5. Transcriptional regulation of floral scent

In order to identify cDNAs encoding proteins involved in the regulation and biosynthesis of floral scent, a cDNA library was constructed from petal limbs that were harvested at the onset of volatile emission. From this library, 837 cDNAs were randomly picked, PCR amplified and spotted onto glass slides to generate a cDNA microarray. For the microarray experiments, we created highly standardized growth conditions. Plants were grown in growth chambers, under a 16 h light regime (21 °C, 70% relative humidity). A comparison of volatile emission from greenhouse-grown petunia flowers with those from the growth chambers revealed that the rhythm of emission of volatile benzenoids was similar, but that the peak of emission occurred two hours earlier in flowers from the growth chambers (data not shown).

Differential mRNA expression was studied with cDNA probes generated from flowers four hours into the light period, when there was very little volatile emission, and flowers ten hours in the light, approximately two hours before the start of volatile emission. In flowers from plants ten hours in the light period, 39

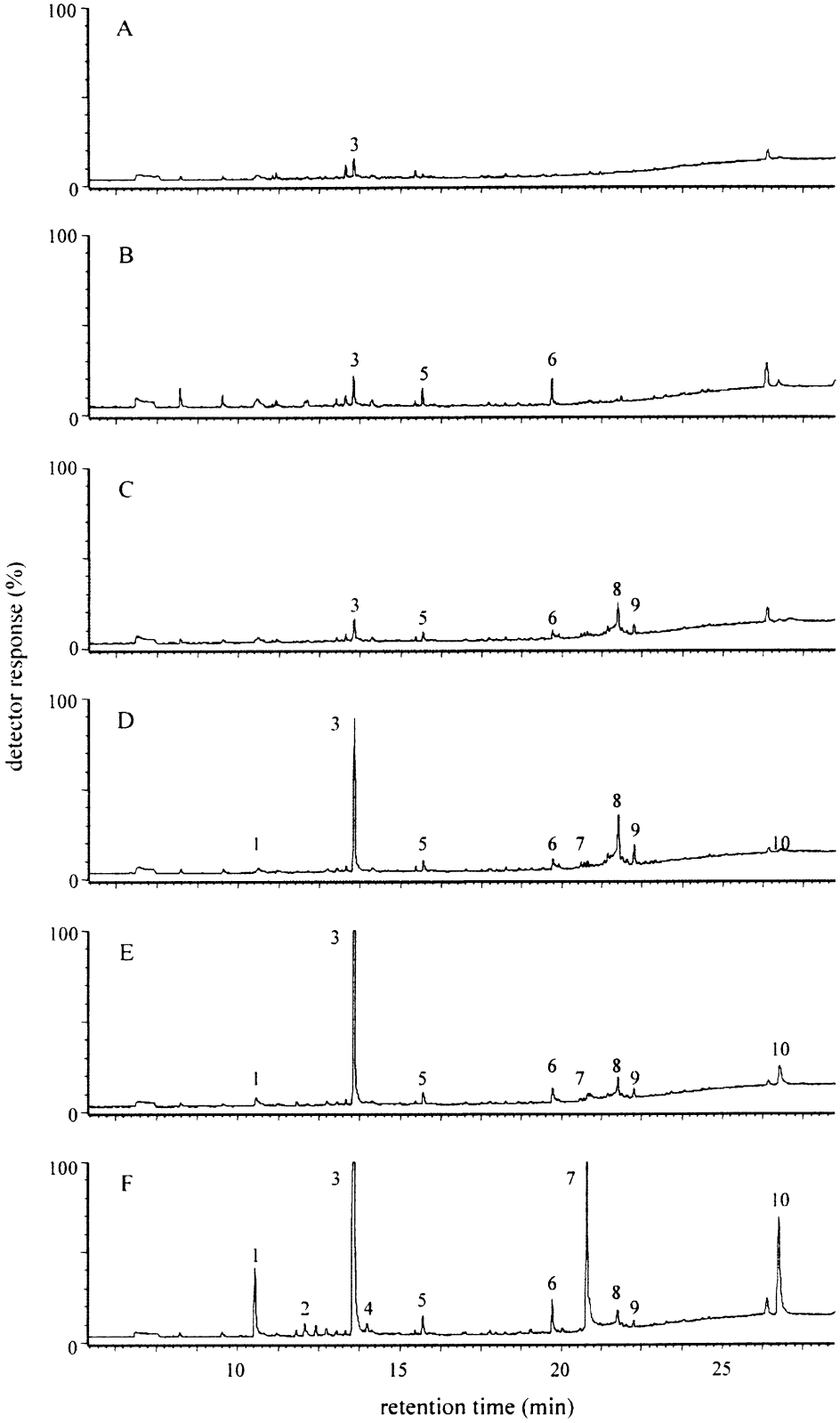


Fig. 5. Volatile emission during petunia flower development. GC–MS chromatograms (100% scale = $2\times10^6$ total ion counts) of volatiles present in the floral headspace, sampled by SPME in vivo, during the development of one single W115 flower from bud stage to fully-opened flower, are shown. At each sampling time, the length of the flower bud or the diameter of the petal limbs, once the flower had opened, was determined as a measure of flower development. (A) 14 h in the light, bud length 4 cm; (B) the next day 6 h in the light, bud length 5 cm; (C) 9 h 15 min in the light, flower starts to open, diameter 2 cm; (D) 11 h in the light, diameter 4 cm; (E) 12 h 10 min in the light, diameter 4 cm; (F) 14 h in the light, flower fully opened, diameter 4.5 cm.

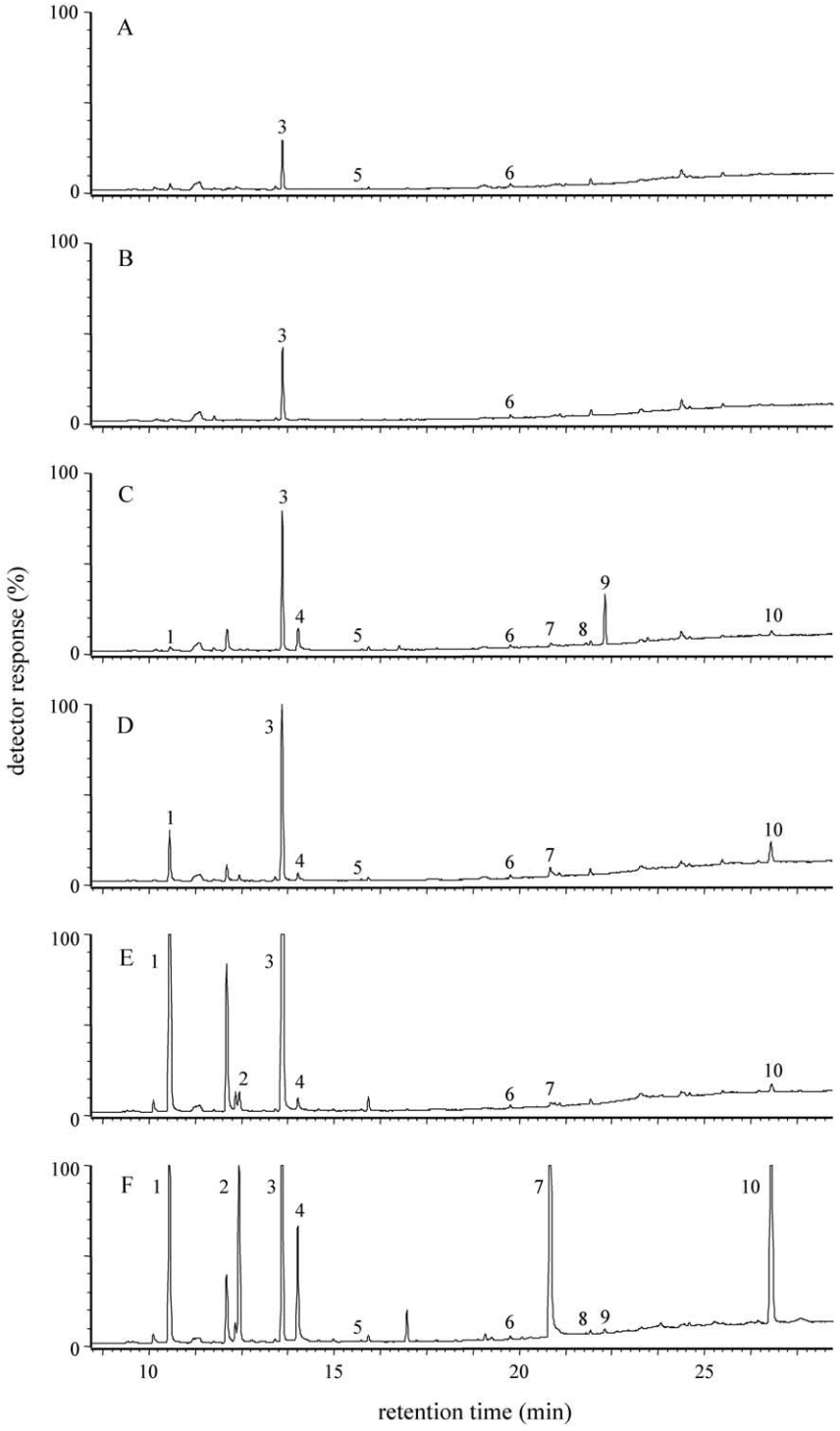


Fig. 6. Volatile production by different petunia flower organs. Tissues were separated from whole flowers (harvested at 17:00 h; 15 h L) and sampled by headspace-SPME of $CaCl_2$-extracts. (A) sepals; (B) style; (C) stigma; (D) stamen plus ripe pollen; (E) corolla tube (=petals minus limbs); (F) petal limbs (100% scale = $2\times10^6$ total ion counts).

cDNAs were identified of which the transcript levels showed more than a two-fold increase and 34 cDNAs of which the transcript levels showed at least a two-fold decrease (a detailed analysis of micro-array data will be published elsewhere). Eight of the 39 cDNAs were members of the PAL gene family. The transcript levels corresponding to all eight PAL cDNAs increased with a longer light period (average fold-increase of 6.08, S.D. = 1.42). Since PAL is a key enzyme in the production of benzenoids (Jarvis et al., 2000), we investigated the transcript levels of one of the PAL cDNAs in more detail by RNA-gel blot analysis. PAL mRNA levels

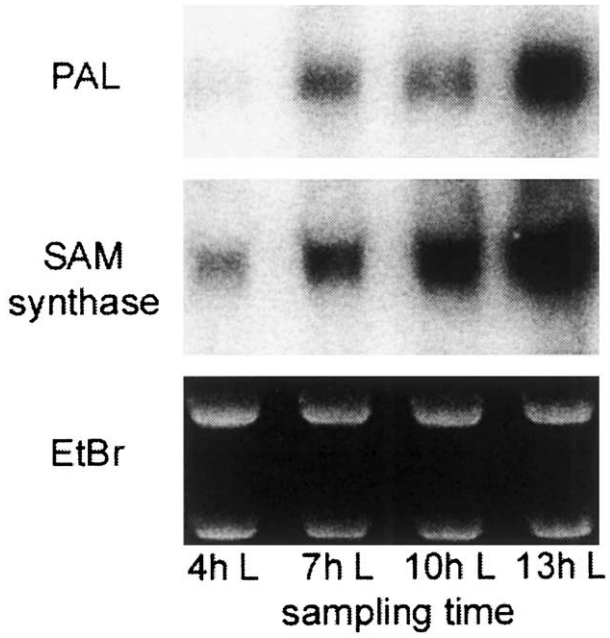


Fig. 7. Temporal gene expression of PAL and SAM synthase. Petunia W115 corollas were harvested from plants from growth chambers (16 h light regime) with the light period stating at 6:00 h. From these corollas, total RNA was isolated after 4 h of light (4 h L), 7 h of light (7 h L), 10 h of light (10 h L), and 13 h of light (13 h L) and subjected to gel electrophoresis (5 μg per lane), blotted onto nylon membranes and hybridised with a PAL probe and a SAM synthase probe. Lower panel: ethidium bromide staining of RNA gel.

were indeed low early into the light period but increased throughout the light period (Fig. 7). We also monitored the transcript levels of PAL by RNA-gel blot analysis during a 48 h period. These experiments showed that the high levels of PAL mRNA late in the light period were reset to low levels early into the light period the next day (data not shown). The microarray analysis revealed that several other cDNAs encoding for proteins involved in the biosynthesis of precursors of phenylalanine were found to have higher transcript levels in flowers toward the end of the light period, similar to PAL. These cDNAs encode for 3-deoxy-D-*arabino*-heptolosonate-7-phosphate (DAHP) synthase and 5-*enol*-pyruvylshikimate-3-phosphate (EPSP) synthase, which both participate in the shikimate pathway, and for chorismate synthase and prephenate dehydratase, which act just downstream of the shikimate pathway. All transcript levels corresponding to these cDNAs were 4–7 times higher at 10 h into the light period.

Sequence analysis revealed that *S*-adenosyl-methionine (SAM) synthase was represented 10 times and that all 10 cDNAs were identical. Transcript levels for these cDNAs increased with an average of 4.8-fold (S.D. = 2.07) at 10 h into the light period compared with the earlier time point. RNA-gel blot analysis confirmed that SAM synthase mRNA levels increased with the length of the light period (Fig. 7), similar to the pattern of PAL mRNA increase. Microarray data showed that methionine synthase, which could be responsible for the supply of substrate for SAM synthase was upregulated 7-fold at 10 h into the light period and that *S*-adenosyl-L-homocystein (SAHC) hydrolase, involved in the regeneration of SAM, was upregulated approximately 6-fold.

## 3. Discussion

*Petunia hybrida* has been used as a model system for studying anthocyanin- and flavonoid biosynthesis, and flower development (Quattrocchio, 1994; Quattrocchio et al., 1999; Souer et al., 1996; Tobena-Santamaria et al., 2002; Van der Krol, 1989). This study clearly indicates that *P. hybrida* can also be used as a model system for elucidating volatile benzenoid production by flowers. The scent of the flowers of line W115 (Mitchell) consists almost exclusively of benzenoids, which are predominantly produced by the petals at the beginning of the dark period. We used a targeted metabolomics approach, employing the rapid and sensitive SPME method to determine the composition and temporal production of the floral scent in vivo. DNA micro array analysis showed that PAL, a key enzyme in benzenoid synthesis, was transcriptionally induced prior to the onset of volatile emission. Moreover, SAM synthase, which produces *S*-adenosyl methionine, the methyl-donor of enzymes involved in the production volatile methylated benzenoids, was also upregulated prior to volatile emission.

The advantage of SPME analysis over the use of headspace trapping with solid adsorbents such as Tenax lies in its speed and simplicity. Moreover, the SPME is the method of choice for short sampling periods during which adsorbents such as Tenax might not provide the desired sensitivity. A drawback of the use of SPME is that quantitative analysis is more difficult (Matich et al., 1996). Nevertheless, similar SPME methods have been successfully applied to analyze flavour compounds from a variety of plants and fruits. The 100 μm PDMS fiber is considered to be the best choice to identify benzenoids, terpenes and fatty acid derivatives (Flamini et al., 2002; Matich et al., 1996; Verhoeven et al., 1997).

Using headspace-SPME coupled to GC-MS, we were able to analyze the volatiles released in vivo by a single flower twice an hour with a 30 min GC run. The SPME fiber was always located exactly at the same position in front of the flower to ensure consistency in the measurements. For detection of volatile production by flowers organs and tissues, we developed an in vitro assay based on SPME analysis of volatiles evaporated from heated extracts (Buttery et al., 1987). This in vitro method would also be very useful for automated high-throughput screenings of volatiles from plant tissues.

The timing of benzenoid volatile release probably relates to the activity of the natural moth-pollinators of one of the progenitors of line W115, *Petunia axillaris* (Bradley et al., 1998; Quattrocchio et al., 1999). Other moth-pollinated species, like *Nicotiana sylvestris*, have comparable emission patterns (Loughrin et al., 1990, 1991). It has been suggested that the presence of "white floral" odour components, such as methylbenzoate, benzylalcohol, phenylethylalcohol, and isoeugenol, is linked to the pollination by moths (Knudsen and Tollsten, 1993; Levin et al., 2001; Loughrin et al., 1991). The morphology of the W115 flower is also typical for moth-pollinated plant species. Plants of this type usually produce long tubular white flowers that are considered vesperescent, that is, they are more fragrant in the evening (Loughrin et al., 1990).

Emission of benzenoids starts when a flower opens (Fig. 5), although a small amount of methylbenzoate is already produced when a flower is still closed. This might be emitted from the white part of the bud. It is clear that the petals are the main source of the volatiles (Fig. 6). This observation is similar to the situation in *C. breweri*, where the release of linalool was mainly from the petals (Pichersky et al., 1994), and in *Antirrhinum majus*, where the emission of methylbenzoate is mostly contributed by the petals (Dudareva et al., 2000).

Two sesquiterpenes were detected in the petunia stigma extract and were most abundant directly after flower opening (Figs. 4 and 5). The latter situation can be explained by the fact that these stigma-produced sesquiterpenes had accumulated in the closed flower since they were not detected at such high levels at later stages of the flower life span. A transgenic line that produced more stigma-like structures had an increased level of these two sesquiterpenes (data not shown). The ratio of the two sesquiterpenes was different when measured in vitro or in vivo. A possible explanation is a difference in volatility of the two compounds; germacrene D could be more volatile than cadina-3,9-diene and levels were therefore higher in the in vivo measurement, while cadina-3,9-diene remained more in the tissue and was therefore higher in the in vitro measurement. These sesquiterpenes could either act as phytoalexins involved in defensive mechanisms against pathogens (Dixon, 2001), or they might have a function in guiding the pollinators to the stigma once they have reached the flower. This latter function has been suggested in buttercups (*Ranunculus acris*), where isoprenoids constitute the scent from all flower parts, except for the pollen, which mostly emit sesquiterpenes (Bergström et al., 1995). The aliphatic aldehydes decanal and dodecanal (peaks 5 and 6) were emitted continuously. The variation in release patterns of compounds from different chemical classes led us to conclude that the biosynthetic pathways of benzenoids, sesquiterpenes, and acyl lipids derivatives in petunia W115 flowers are under different regulatory control mechanisms.

The pattern of volatile emission, with low levels early during the light period and high levels late during the light period, was similar for all benzenoids: benzaldehyde, phenylactetaldehyde, methylbenzoate, phenylethylalcohol, isoeugenol, and benzylbenzoate. It matches the previously described pattern for methylbenzoate emission by petunia Mitchell flowers (Kolosova et al., 2001a). We found that the emission of methylbenzoate 3 h into the dark period was several fold higher than the emission in the middle of the light period (after 7 h of light; Fig. 3). This observation corresponds with the finding of Kolosova et al. (2001a), that the lowest emission levels of methylbenzoate during the day were about 20% of its maximum. The same authors also showed that PAL enzyme activity was at its lowest level in the middle of the light period. This is in accordance with our data regarding PAL mRNA levels, which are also low during the light period and increase just before the beginning of the dark period (Fig. 7). The low levels of PAL mRNA early into in the light period might explain low PAL activity and low benzoic acid levels (Kolosova et al., 2001a), and methylbenzoate emission around that time (Fig. 4). Though the pathway for biosynthesis of benzoid acid is not elucidated in flowers, it is generally accepted that the first commenced step is the conversion of L-phenylalanine to *trans*-cinnamic acid by PAL (E.C. 4.3.15). In snapdragon, a diurnal emitter of methylbenzoate, PAL mRNA levels decrease during the day and increase during the night, opposite to what occurs in petunia, which emits methylbenzoate predominantly nocturnally (Fig. 4; Kolosova et al., 2001a). Other PAL genes might be present in petunia that have a different kind of transcriptional regulation in flowers. The fact that, besides PAL, cDNAs encoding four important enzymes in the shikimate pathway and directly downstream of it were found to be upregulated, suggests that parts of the routes leading to the biosynthesis of phenylalanine and the subsequent benzenoid pathway are co-ordinately regulated in petunia flowers.

SAM synthase (EC 2.5.1.6) produces *S*-adenosyl methionine (SAM), the methyldonor for at least three enzymes known to methylate benzenoids in *C. breweri* and snapdragon. SAM synthase levels in petunia are low four hours into the light period but increase throughout the light period (Fig. 7), and are reset to low levels the next day (data not shown). This suggests that SAM synthase is involved in providing *S*-adenosyl-methionine to the methyltransferases that produce the methylated benzenoids. SAM synthase is also important in the methionine cycle (Yang cycle), which has a role in ethylene production and in the biosynthesis of polyamines. Both polyamines (Serafini-Fracassini et al., 2002) and ethylene (Muller et al., 2002)

are involved in senescence processes in flowers. However, as we used freshly opened flowers, harvested within a single day, and because petunia flowers can last for up to 7 days, it is not likely that the increase in SAM synthase is related to senescence in these flowers. Because methionine synthase and SAHC hydrolase were upregulated as well, it is possible that the synthesis towards *S*-adenosyl methionine (SAM) increases at the end of the light period, indicating an orchestrated control.

In conclusion, we have demonstrated that SPME coupled to GC–MS is an outstanding tool to measure volatile production by petunia Mitchell flowers in vivo. These volatiles are predominantly benzenoids of which the production appears, at least partly, to be regulated at the transcriptional level. We propose that petunia Mitchell is an excellent model system to elucidate the biosynthesis and regulation of benzenoid synthesis and that the DNA-microarrays provide an useful tool to gain further knowledge of the genes involved.

## 4. Experimental

### 4.1. Plant material and growth

Experiments were carried out with homozygous lines of *P. hybrida* W115, which is also called Mitchell [*P. axillaris*×(*P. axillaris*×*P. hybrida*)], and produces large scenting white flowers. Plants were grown in a greenhouse with day/night temperatures of 22/17 °C and 16/8 h light/dark (02:00–18:00 h; artificial light from 02:00 h to 08:00 h). Plants bearing at least three mature flowers were used in all experiments. In the growth chambers, plants were grown at 21 °C and 70% humidity and 300 μE $m^{-2}$ $s^{-1}$, under a 16/8 h light/dark regime, with the light period starting at 06:00 h.

### 4.2. Collection of headspace samples

#### 4.2.1. Solid-phase micro extraction of flower volatiles in vivo

A glass funnel with a diameter of 8–10 cm was cautiously placed over a young flower with its petal limbs still closed, positioning the flower tube in the narrow part of the funnel and the petals in the widening part. At the start of volatile sampling, a floral headspace was created by wrapping aluminium foil over both ends of the funnel. During the in vivo volatile measurements, the light an temperature conditions inside the funnel were maintained nearly identical to the normal growing conditions by placing the funnel horizontally thereby preventing inference of the aluminium foil with the light source. The volatiles released were subsequently sampled using solid-phase micro extraction (SPME). The SPME-device was fixed in front of the glass funnel with the hollow needle stuck through the aluminium foil for about 0.5 cm (Fig. 1). The flower volatiles were adsorbed by exposing the fused silica fiber coated with 100 μm polydimethylsiloxane (PDMS) (Supelco Inc., Bellefonte, PA) to the floral headspace for 30 min. After each sampling period, the aluminium foil was removed to regain air movement around the flower. In order to study volatile emission during flower development, small flower buds (length about 2 cm) were enclosed in glass funnels and the headspace volatiles were sampled during subsequent development. Full expansion and opening of the flower from a full-grown bud took approximately 5 h.

#### 4.2.2. Sampling of volatiles from flower extracts

Entire flowers were immediately frozen in liquid nitrogen and ground in 1.5 ml of saturated $CaCl_2$ (5M) using a pestle and mortar. After grinding, 0.75 ml of the extracts was transferred into 1.8 ml glass vials containing a small magnetic stirring bar. The vial was placed in a water bath of 50 °C, and after 5 min of temperature equilibration the headspace of the stirred $CaCl_2$-extracts was sampled for 30 min by SPME with a 100 μm PDMS-fiber. For analysis of volatiles from various flower parts, the tissues were first separated from the frozen whole flower. Each flower part was then ground in 1.5 ml of saturated $CaCl_2$, and sampled as described above.

### 4.3. GC–MS analysis of volatile compounds

Capillary gas chromatography–mass spectrometry (GC–MS) analysis was performed using a Fisons 8060 gas chromatograph directly coupled to a MD 800 mass spectrometer (Fisons, Mainz-Kastel, Germany) as described by Verhoeven et al. (1997). Compounds were thermally desorbed from the SPME-fiber in the injector port for 1 min at 250 °C, with the port in splitless injection mode. Compounds were separated on a capillary HP-5 column (50 m×0.32 mm, film thickness 1.05 μm; Hewlett Packard) with He (37 kPa) as a carrier gas. The GC oven was programmed for 2 min at 80 °C, followed by an increase of 8 °C $min^{-1}$ to 250 °C, at which temperature it was left for 5 min. Mass spectra of eluting compounds were generated at 70 eV and recorded each second ($m/z$ 35–400). Volatile compounds were identified by screening the NIST library for comparable mass spectra and by comparison with authentic reference compounds. Chromatograms visualised in the Figs. 2, 3, 5 and 6 are examples of three independent experiments, with similar results. The peak at $t_R$ 12.0 min that was sometimes present in the chromatograms (see e.g. Fig. 6E) was a PDMS-fiber related compound.

### 4.4. RNA isolation and gel blotting

Total RNA from W115 corollas was isolated using the TriZOL™-LS reagent (Gibco BRL) method. RNA-

gel blotting, hybridization, and probe preparation was performed as previously described (Laxalt et al., 2001). After hybridization, the membranes were washed twice with 2×SSC, 0.1% SDS for 20 min and 0.2×SSC, 0.1% SDS for 20 min at 65 °C. Hybridizing bands were visualised by autoradiography and by phosphoimaging (Molecular Dynamics, Storm 840).

### 4.5. cDNA library construction, PCR amplification, and microarray construction

Open corolla tissue from scenting petunia W115 flowers was used to construct a cDNA library in the UNI-XR vector (Stratagene). A total of 837 petunia cDNAs from single plaques were amplified by polymerase chain reaction (PCR) using the universal T3 and T7 primers. PCR products were purified with Multiscreen plates (MAFBNOB50 Millipore Intertech, Bedford, MA) according to the manufacturers protocol. Similarly, 179 petunia cDNAs from the collection of Dr. R. Koes and Dr. F. Quattrocchio (Free University, Amsterdam) were amplified and purified. The 1016 products were transferred to a 384-format plate using a Packard multiprobe II EX robotic liquid handling system AMPII/EI (Perkin Elmer, Wellesley, MA, USA) prior to application on microscope slides.

### 4.6. Micro array construction, probe labelling and analysis

#### 4.6.1. Arraying, slide processing, hybridization, scanning, and data acquisition

Amplified cDNA sequences were applied onto CMT-CAPS™ coated slides (Corning Inc., Corning, NY) with a 12-pinned arraying robot (Molecular Dynamics, Sunnyvale, USA). The cDNAs were arrayed in duplicate within a 12 $cm^2$ area. Each probe contained approximately 0.25 ng of PCR product. The slides were left overnight to dry at 55% relative humidity, and UV-cross linked by applying 150 mJ for 2 min. Slides were hybridized in an automated slide processor (Amersham Biosciences) as follows: after prehybridisation in 2×SSPE; 0.2% SDS at 55 °C for 2 h, the probe solution (200 μl) was injected and the slides were incubated at 42°C for 16 h. The chamber was then heated to 50 °C and the slides were washed with 1×SSC; 0.2% SDS for 10 min, followed by 0.1×SSC; 0.2% SDS, once for 10 min and once for 4 min. Slides were flushed with 0.1×SSC followed by isopropanol, and air dried for 200 s. The arrays were scanned for fluorescence emission with a Molecular Dynamics scanner. A separate scan was conducted for each of the two florescent dyes used for hybridisation. Using Array-vision software (Imaging Research Inc., Ontario, Canada), the integrated signal intensity was measured for each element on the array from which the local background intensity was subtracted. Using Spotfire software (Spotfire Inc., Somerville, MA, USA), the intensity of the elements was then normalized. The output was given as the logarithm of Cy5/Cy3 intensity. The average output of four independent elements was calculated, and data that had a standard deviation larger than 0.1 was discarded.

#### 4.6.2. Fluorescent targets and probes

Total RNA was further purified with QIAgen RNeasy columns. First strand cDNA was prepared a follows: oligo(dT) 25mer (1.2 μM final concentration) was added to 20 μg of the purified RNA and the mixture was heated to 70 °C for 10 min before placing it on ice. Reverse transcription was performed for 2.5 h at 42 °C in 20 μl, using 200 units Superscript II RNAse $H^-$ reverse transcriptase (Life Technologies), 10 mM DTT, 0.1 mM of dATP, dGTP, and dTTP; 0.05 mM of dCTP; 0.05 mM cyanine 3 (Cy3)-dCTP or Cy5-dCTP (Amersham Biosciences AP) in the buffer supplied. The samples were subsequently placed on ice for five minutes before degradation of the RNA through the addition of 2 μl 2.5 M NaOH for 15 min at 37 °C. The reaction was stopped by neutralization with 10 μl 2 M MOPS before purification with GFX columns (Amersham Biosciences). The incorporation of Cy3 and Cy5 was quantified spectrophotometrically, by measuring the absorption of Cy3 (at 550 nm) and Cy5 (at 649 nm), and the amount of cDNA (at 260 nm). For blockage of the poly-dT tail of the cDNA, 40 ng oligonucleotide $A_{80}$ was added and the mixture was heated for 2 min by 95 °C, followed by incubation at 70 °C for 30 min. The Cy3 and Cy5 samples were mixed before addition of hybridization buffer (Amersham Biosciences) and one volume formamide.

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