

A plant thiolase involved in benzoic acid biosynthesis and volatile benzenoid production

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SUMMARY

The exact biosynthetic pathways leading to benzoic acid (BA) formation in plants are not known, but labeling experiments indicate the contribution of both β -oxidative and non- β -oxidative pathways. In *Petunia hybrida* BA is a key precursor for the production of volatile benzenoids by its flowers. Using functional genomics, we identified a 3-ketoacyl-CoA thiolase, *PhKAT1*, which is involved in the benzenoid biosynthetic pathway and the production of BA. *PhKAT1* is localised in the peroxisomes, where it is important for the formation of benzoyl-CoA-related compounds. Silencing of *PhKAT1* resulted in a major reduction in BA and benzenoid formation, leaving the production of other phenylpropanoid-related volatiles unaffected. During the night, when volatile benzenoid production is highest, it is largely the β -oxidative pathway that contributes to the formation of BA and benzenoids. Our studies add the benzenoid biosynthetic pathway to the list of pathways in which 3-ketoacyl-CoA thiolases are involved in plants.

Keywords: benzoic acid, 3-ketoacyl-CoA thiolase, β -oxidation, benzenoids, floral volatiles, petunia.

INTRODUCTION

In many plants, floral volatiles play a role in attracting pollinators (Stuurman *et al.*, 2004). The production and emission of these volatiles are therefore tightly regulated, not only spatially and diurnally but also developmentally. Flowers of petunia (*Petunia hybrida* cv. Mitchell) emit benzenoid and phenylpropanoid-related volatiles (Verdonk *et al.*, 2003), which are derived from the common precursor Phe (Boatright *et al.*, 2004). *Petunia hybrida* cv. Mitchell has emerged as a model of choice to study the biosynthesis, emission and regulation of these classes of volatiles.

Based on their carbon skeleton, Phe-derived volatiles are categorised as C₆-C₁, C₆-C₂ and C₆-C₃ compounds. For the C₆-C₁ compounds, the benzenoids, the propyl side chain of Phe needs to be shortened by two carbon atoms. It has been reported that this shortening can occur either via a β -oxidative (Ribnicky *et al.*, 1998; Jarvis *et al.*, 2000; Hertweck *et al.*, 2001) or a non- β -oxidative pathway with benzoyl-CoA and benzaldehyde as precursors for benzoic acid (BA), respectively (Figure 1). Whereas the β -oxidative pathway needs activation by coenzyme A (CoA), the non- β -oxidative pathway occurs both CoA dependently (Abd El-Mawla and

Beerhues, 2002; Boatright *et al.*, 2004) and CoA independently (Schnitzler *et al.*, 1992; Boatright *et al.*, 2004).

Despite the importance of the C₆-C₁ compound BA in plant secondary metabolism (Wildermuth, 2006), only a few enzymatic steps leading to its synthesis have been characterised, whereas the corresponding genes remain elusive. For the non- β -oxidative CoA-dependent pathway the enzymatic activities of cinnamate:CoA ligase, cinnamoyl:CoA hydratase/lyase and benzaldehyde dehydrogenase, whose successive actions starting from *t*-cinnamic acid lead to the formation of BA, have been detected in *Hypericum androsaemum* L. cell cultures (Abd El-Mawla and Beerhues, 2002). Recently, the involvement of a snapdragon benzaldehyde dehydrogenase in the production of BA was shown by transient expression in petunia flowers (Long *et al.*, 2009). Enzymatic activities for the other two pathways have not been described yet, although many labelling studies with precursors have suggested their presence in plants (Ribnicky *et al.*, 1998; Boatright *et al.*, 2004; Orlova *et al.*, 2006).

In petunia, BA is a key intermediate in volatile benzenoid production. It serves as an immediate precursor for benzoic

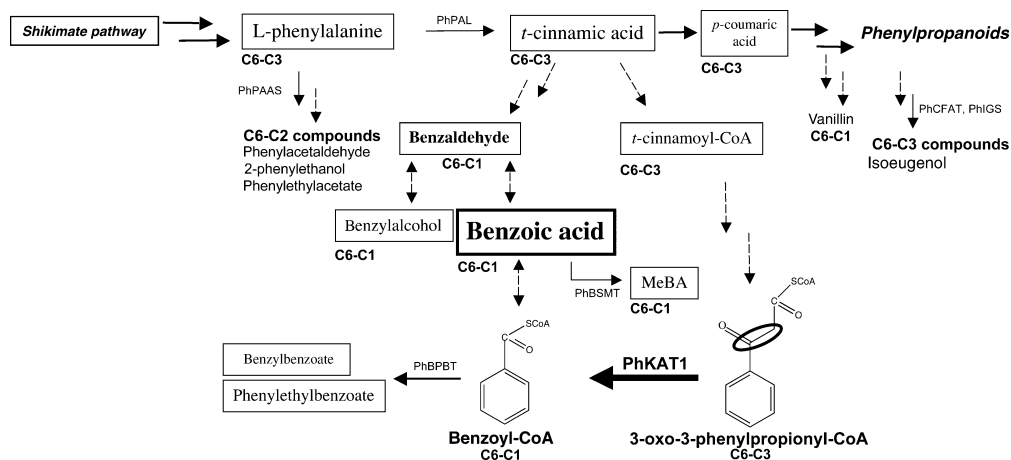


Figure 1. Schematic representation of the biosynthetic pathways leading to C₆-C₁, C₆-C₂ and C₆-C₃ compounds in petunia petals and the proposed biochemical reaction catalysed by PhKAT1.

The common precursor for all compounds is L-Phe. The C₆-C₁ compounds are derived from *t*-cinnamic acid and the propyl side chain can be shortened by two carbon atoms via either a non- β -oxidative or a β -oxidative pathway, with benzaldehyde and benzoyl-CoA as intermediates for benzoic acid (BA) production, respectively. The β -oxidative pathway is dependent on CoA activation of *t*-cinnamic acid and requires the action of a 3-ketoacyl-CoA thiolase (PhKAT1) for the production of benzoyl-CoA. Benzoyl-CoA is subsequently used to produce benzylbenzoate and phenylethylbenzoate using benzylalcohol and 2-phenylethanol as co-substrates, respectively. Benzoyl-CoA can also be converted to BA directly by the action of a thioesterase or indirectly via benzylbenzoate (not depicted; Boatright *et al.*, 2004). Alternatively, BA can be produced via oxidation of benzaldehyde. Dashed arrows indicate unknown genes in petunia. See text for details. Abbreviations: BPBT, benzoyl-CoA:benzyl alcohol/phenylethanol benzoyltransferase; BSMT, benzoic acid/salicylic acid methyl transferase; CFAT, acetyl-CoA:coniferyl alcohol acetyltransferase; IGS, isoeugenol synthase; KAT1, 3-ketoacyl-CoA thiolase 1; PAAS, phenylacetaldehyde synthase; PAL, phenylalanine ammonia lyase.

acid/salicylic acid methyl transferase (PhBSMT) leading to the production of methylbenzoate (MeBA) (Negre *et al.*, 2003), the most abundant volatile emitted by petunia flowers (Kolosova *et al.*, 2001a; Verdonk *et al.*, 2005). BA is also a precursor of salicylic acid (SA), which can be methylated by PhBSMT to methylsalicylate (MeSA), one of the minor components in the floral headspace, but SA might also be formed from (iso)chorismate as in *Arabidopsis* (Wildermuth, 2001). In addition, BA can be converted to benzoyl-CoA, one of the substrates of benzoyl-CoA:benzyl alcohol/phenylethanol benzoyltransferase (PhBPBT), which in combination with benzylalcohol or 2-phenylethanol produces benzylbenzoate and phenylethylbenzoate in petunia, respectively. Although a benzoyl-CoA ligase has not yet been identified in petunia, its activity has been detected in *Clarkia breweri* flowers (Beuerle and Pichersky, 2002). Labelling studies in petunia showed that only the non- β -oxidative CoA-independent and β -oxidative CoA-dependent pathways are active (Boatright *et al.*, 2004) and that the flux through these pathways differs in the light and dark period (Orlova *et al.*, 2006). Furthermore, *in vivo* stable isotope labelling indicated that benzylbenzoate is also an intermediate between Phe and BA (Boatright *et al.*, 2004).

In this study, we describe the identification and characterization of a petunia 3-ketoacyl-CoA thiolase (PhKAT1), which plays an important role in the β -oxidative pathway leading to the production of benzoyl-CoA from 3-oxo-3-phenylpropionyl-CoA (benzoylacyl-CoA). To determine

the role of *PhKAT1* in floral volatile benzenoid production, we generated transgenic plants suppressing *PhKAT1* expression (*ir-PhKAT1*). Analysis of these plants indicate that the β -oxidative pathway plays a major role in the production of BA and thus of precursors for C₆-C₁ volatiles. This 3-ketoacyl-CoA thiolase is present in the peroxisomes of petunia petals, adding the involvement of this organelle in volatile benzenoid biosynthesis.

RESULTS

PhKAT1 expression is characteristic for a floral benzenoid-related gene

In order to elucidate steps in the β -oxidative pathway, we used our microarray data (Verdonk *et al.*, 2005, 2006) to select genes whose expression correlated strongly with genes involved in floral scent production. One of these had high similarity (88%) to *Arabidopsis thaliana* 3-ketoacyl-CoA thiolase 5 (AT5G48880) and was tentatively named *PhKAT1*. Microarray analysis showed that expression of *PhKAT1* was 3.6-fold ($P = 0.0003$) higher in petals when scent emission started, 3 h before the dark period, than 9 h before the dark period, when scent emission was very low.

Figure 2(a) shows that *PhKAT1* is predominantly expressed in petal limbs and petal tubes and that expression is hardly detectable in other tissues, including whole seedlings. *PhKAT1* expression shows a diurnal rhythm with a

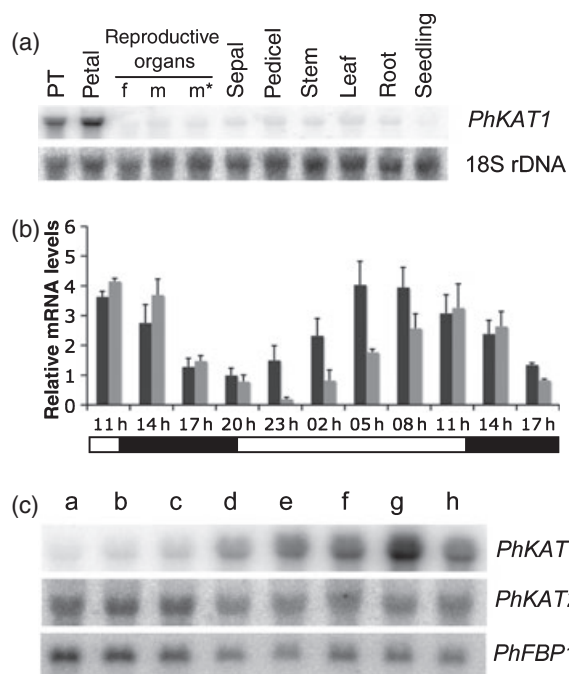


Figure 2. Petal-specific, rhythmic and developmentally regulated expression of *PhKAT1*.

(a) Tissue-specific expression. *Petunia hybrida* cv. Mitchell plants were used to collect material from petal tubes (PT), 2-day-old flower limbs (petal), reproductive organs (f: pistils, m: stamens, m*: stamens with dehisced anthers), sepals, pedicels, stems, leaves, roots and whole seedlings. All tissues were collected 2 h before the onset of the dark period. For RNA gel-blot analysis, 8 μ g total RNA was loaded per lane and the blot was hybridised with the *PhKAT1* coding region. The blot was re-hybridised with an 18S rDNA probe to illustrate loading of the gels. Two independent experiments were performed and a representative blot is shown.

(b) Rhythmic expression in petal limbs. Petal limbs of 2-day-old flowers were collected with 3-h intervals for a 30-h period. The dark period in the growth chamber started at 12.00 h and ended at 20.00 h. A representative RNA gel-blot is shown as Supplementary Figure S1, but tissue collection and gel-blot analysis were performed twice (means and maximum values are shown). RNA gel-blot analysis was performed as in (a). Blots were hybridised with the *PhKAT1* coding region and a *PhODO1* 3'-fragment. The blots were re-hybridised with *PhFBP1* to determine relative mRNA levels. Black bars represent relative *PhKAT1* and grey bars relative *PhODO1* transcript levels in petunia petals.

(c) Developmentally regulated expression in petal limbs. Wild type flowers at different stages during flower development were used to collect petal tissue 2 h before the onset of the dark period. Two-day-old petal limbs, excluding petal tube tissue, of at least three flowers were pooled per sample. RNA gel-blot analysis was performed as in (a). The blot was re-hybridised with a *PhFBP1* to illustrate loading of the gel. The same blot was re-hybridised with a *PhKAT2* probe to show its distinct developmental expression pattern from *PhKAT1*. Letters indicate different developmental stages of the flower (a: 1 cm, b: 3 cm, c: 4 cm, d: flower almost open, e: flower opening (1 cm diameter), f: open flower (day 1), g: open flower with dehisced anthers and h: senescent flower). Two independent experiments were performed and a representative blot is shown. Abbreviations: FBP1: floral binding protein 1; KAT1 and 2: 3-ketoacyl-CoA thiolase 1 and 2; ODO1: ODORANT1.

peak before the dark period, which precedes the peak of *ODORANT1* (*PhODO1*; Verdonk *et al.*, 2005) expression by approximately 3–6 h (Figure 2b). This finding is consistent with the nocturnal emission of volatile compounds and

rhythmic production of its precursors (Kolosova *et al.*, 2001a; Verdonk *et al.*, 2003). At the onset of volatile emission, *PhKAT1* expression is highest when the flower is fully open after the anthers have dehisced (Figure 2c), correlating with the developmental pattern of volatile emission in petunia flowers (Verdonk *et al.*, 2003).

By blasting the publicly available SGN (Solanaceae Genomics Network, <http://sgn.cornell.edu/>) EST collection with the full length *PhKAT1* cDNA sequence, we identified an EST with high similarity to *PhKAT1*. We obtained its full-length cDNA sequence (75% identity at the nucleotide level and 78% identity at the amino acid level) and named it *PhKAT2*. We analysed the expression pattern using RNA-blot analysis and unlike for *PhKAT1*, transcript levels of *PhKAT2* did not change during petal development (Figure 2c) and did not show a rhythmic pattern (Figure S1). Although this finding does not exclude a role for *PhKAT2* in benzenoid biosynthesis, it strongly suggests no association with floral scent biosynthesis.

The petunia 3-ketoacyl-CoA thiolase protein is localised to peroxisomes *in planta*

PhKAT1 is predicted to encode a protein of 462 amino acids containing three conserved thiolase signatures (Figure S2). Typically for thiolases, *PhKAT1* contains a peroxisomal targeting signal 2 (Gietl, 1990; Johnson and Olsen, 2003; Reumann, 2004; Carrie *et al.*, 2007) at the N-terminus (Figure S2). To verify whether *PhKAT1* is localised to the peroxisomes *in planta*, we constructed a *PhKAT1*-GFP fusion protein and expressed it in petunia petals by means of *Agrobacterium*-mediated transient transformation (Shang *et al.*, 2007). Figure 3 shows that *PhKAT1*-GFP co-localises with the mCherry-peroxisomal marker px-rk (Nelson *et al.*, 2007), indicating peroxisomal targeting of *PhKAT1 in planta*, in accordance with the localization of Arabidopsis 3-ketoacyl-CoA thiolases (Carrie *et al.*, 2007).

Silencing of *PhKAT1* reduces C₆-C₁ benzenoid volatile emission

To investigate the role of *PhKAT1* in floral scent production, we generated stable transgenic lines suppressing *PhKAT1* expression by post-transcriptional gene silencing. For this experiment, an inverted repeat of 174 bp containing parts of the 3'-end of the cDNA (Figure S2) that has low similarity with *PhKAT2*, was constitutively expressed under control of the CaMV 35S-promoter in *P. hybrida* cv. Mitchell plants. Initial screening using RNA gel-blot and headspace analyses identified four independent silenced T₀ lines with reduced *PhKAT1* expression and volatile benzenoid emission (data not shown). Three lines were chosen for further analysis in the T₁ generation. In this generation, one line seemed to have lost its phenotype and was discarded for further analysis, resulting in two independent silenced T₁ lines (*ir-PhKAT1* lines 4 and 7) and one transgenic non-silenced T₁

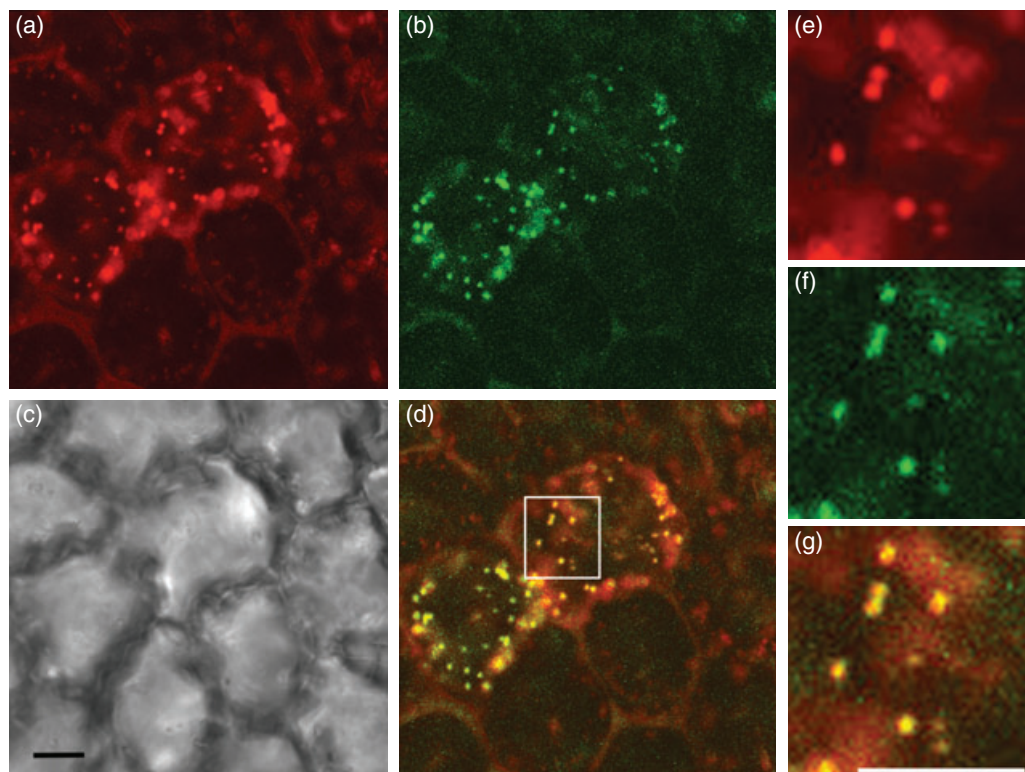


Figure 3. *In vivo* peroxisomal targeting of PhKAT1.

The mCherry peroxisomal marker px-rk (a) and 35S:PhKAT1-GFP (b) were transiently co-expressed in Mitchell flowers. The merged and bright field images are shown in (d) and (c), respectively.

(e, f, g) Magnification taken from the boxed area shown in (d). Confocal images were obtained with a Zeiss LSM 510 confocal laser scanning microscope. Co-localisation was seen several times in independent experiments. The scale bars represent 10 μm .

line (*ir-PhKAT1* line 6). For both silenced lines, expression levels were ca. 25% of wild type levels (Figure 4a,b). *PhKAT2* levels remained unaltered in these lines, confirming gene-specific suppression of *PhKAT1* (Figure 4a). Because we hypothesised that *PhKAT1* produces benzoyl-CoA from 3-oxo-3-phenylpropionyl-CoA, we initially tried to specifically measure 3-oxo-3-phenylpropionyl-CoA thiolase activity, but attempts to do so were unsuccessful. Most thiolase activities, however, are measured with the general substrate, acetoacetyl-CoA, which we used. Overall thiolase activity in total protein extracts from petals harvested 1 h in the dark period, as measured by the decrease in acetoacetyl-CoA, was 50% lower in the *ir-PhKAT1* line 7 (Figure 5), indicating a correlation with lower *PhKAT1* transcript levels.

To examine the effect of reduced *PhKAT1* expression on volatile emission, these T₁ lines were used for headspace collections and subsequent GC-MS analyses. Emission levels of the C₆-C₂ compounds phenylacetaldehyde (Figure 6a), phenylethylacetate and 2-phenylethanol (Figure S3) were not reduced in the *ir-PhKAT1* lines. Phenylethylbenzoate, which is produced from 2-phenylethanol and benzoyl-CoA, had on average a 3.2-fold reduced emission (Figure 6a), suggesting reduced benzoyl-CoA level in these lines. The

emission of benzylbenzoate, which is also directly dependent on benzoyl-CoA as co-substrate, was on average 3.5-fold reduced (Figure 6a). Also, emission of MeBA (Figure 6a) whose production depends on BA, was lower in the *ir-PhKAT1* lines (2.7-fold on average). Emission levels of the other C₆-C₁ compounds, benzaldehyde (Figure 6a), benzylalcohol and benzylacetate (Figure S3), were significantly less (2-fold, 5.4-fold and 4.5-fold on average, respectively) and the emission of MeSA was significantly lower only in line 7 (Figure S3). The C₆-C₃ compound isoeugenol had slightly higher emission in both silenced lines compared with the wild type, but not compared with the non-silenced transgenic line 6 (Figure 6a). Vanillin, which was shown to be synthesised from *p*-coumaric acid (Figure 1) in *Vanilla planifolia* (Podstolski *et al.*, 2002), unlike the other C₆-C₁ compounds, remained unaltered in the silenced lines (Figure S3).

To exclude that the decrease in emission of volatiles was due to reduced transcript levels of relevant benzenoid biosynthetic genes, we analysed their transcripts levels. Analysis of the transcript levels of *PhBPBT*, *PhBSMT1* and petunia phenylacetaldehyde synthase (*PhPAAS*), which synthesise benzylbenzoate and phenylethylbenzoate, MeBA

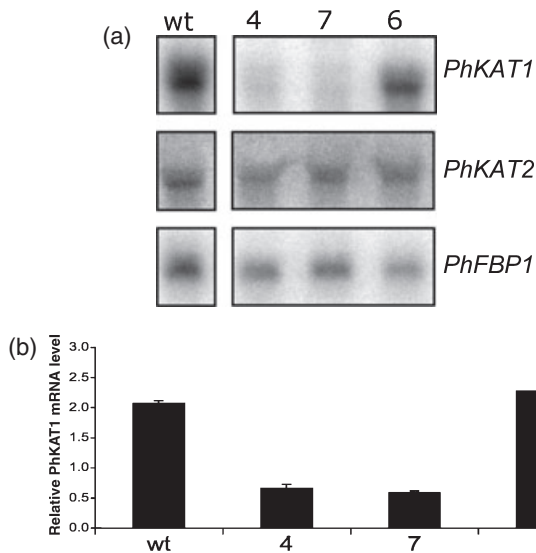


Figure 4. RNA gel-blot analysis of wild type and *ir-PhKAT1* plants. (a) *PhKAT1* mRNA levels in petals of *ir-PhKAT1* T₁ and wild type plants. Total RNA was extracted from 2-day-old flowers (three per sample) 30 min before the onset of the dark period. The numbers on top represent independent transgenic T₁ lines. A fragment containing the *PhKAT1* full-length coding region was used as a probe. The blot was re-hybridised with *PhKAT2* showing sequence-specific silencing of *PhKAT1*. Probing with *PhFBP1* enabled quantification of *PhKAT1* transcript levels. Representative blots are shown. (b) Relative *PhKAT1* mRNA levels in petals of *ir-PhKAT1* and wild type plants (means and maximum levels are shown, $n = 2$). Abbreviations: FBP1: floral binding protein 1; KAT1 and 2: 3-ketoacyl-CoA thiolase 1 and 2.

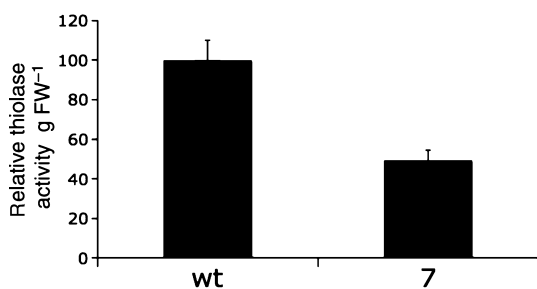


Figure 5. Thiolase activity is reduced in *ir-PhKAT1* plants. Crude protein extracts were prepared from 2-day-old wild-type flowers and flowers of *ir-PhKAT1* line 7, harvested 1 h in the dark period. Thiolase activity was determined by measuring the decay of acetoacetyl-CoA. Products were separated and detected on HPLC as described in Burns *et al.* (2005). The mean values and standard deviations of four independent experiments are shown.

and phenylacetaldehyde respectively, showed that *PhKAT1* silencing did not affect transcript levels of these genes (Figure S4).

Silencing of *PhKAT1* influences benzoic acid levels and internal C₆-C₁ compound pools

Because MeBA emission levels were reduced (Figure 6a) and *PhBSMT* transcript levels unaffected (Figure S4) in *ir-PhKAT1* lines, we hypothesised that the level of the precursor of MeBA, BA, would be lower in these lines (Figure 1). To

test this hypothesis, we measured BA levels in wild type flowers and flowers of line 7 at a time point during the dark period, when volatile emission is high (4 h in the dark period). In wild type flowers, BA levels were approximately four times higher than in line 7 (86 ± 8 and $21 \pm 6 \mu\text{g g FW}^{-1}$, respectively), illustrating the contribution of *PhKAT1* and thus the β -oxidative pathway, to BA biosynthesis (Figure 6b).

As it was recently shown that for most compounds internal volatile pools are highest during the dark period (Orlova *et al.*, 2006), we reasoned that the effect of *PhKAT1* silencing on internal volatile pools would be most pronounced during the night, when emission peaks as well. Therefore, we decided to analyse internal C₆-C₁ volatile pools after 4 h in the dark period in petals of line 7 and wild type. Internal pools of benzylbenzoate, phenylethylbenzoate and methylbenzoate were indeed significantly reduced in line 7 (30-fold, 36-fold and 18-fold, respectively) and benzaldehyde internal pools were undetectable in the silenced line (Figure 6b).

DISCUSSION

Petunia 3-ketoacyl-CoA thiolase 1, a peroxisomal protein, is involved in the central C₆-C₁ floral volatile biosynthetic pathway and contributes to benzoic acid formation in petunia flowers

Petunia has been used extensively as a model system to study the biosynthesis of flavonoids, particularly those involved in floral pigmentation (Koes *et al.*, 2005). Recently, it has emerged as a model system to study the biosynthesis of benzenoid and phenylpropanoid-related floral volatiles (Schuurink *et al.*, 2006; Pichersky and Dudareva, 2007). Previous work, however, has concentrated on identifying genes and enzymes involved in the final 1–2 steps resulting in the synthesis of the volatiles themselves (Negre *et al.*, 2003; Boatright *et al.*, 2004; Kaminaga *et al.*, 2006; Koeduka *et al.*, 2006; Dexter *et al.*, 2007). In this study, we used functional genomics and targeted metabolomics to identify and probe the function of *PhKAT1* that encodes an enzyme potentially centrally positioned in the pathway leading to the biosynthesis of BA (Figure 1). The homology of *PhKAT1* to known thiolases led to the hypothesis that it could be involved in the β -oxidative shortening of the propyl side-chain of 3-oxo-3-phenylpropionyl-CoA, leading to the production of benzoyl-CoA, which is used as a co-substrate for the production of benzylbenzoate and phenylethylbenzoate (Boatright *et al.*, 2004). This reaction would be analogous to the shortening by two carbons of the 3-ketoacyl-CoA substrates in fatty acid degradation, catalysed by *bona fide* thiolases (Germain *et al.*, 2001). Furthermore, its expression levels and patterns suggested involvement in scent biosynthesis. To test this hypothesis, we stably silenced *PhKAT1* in petunia plants.

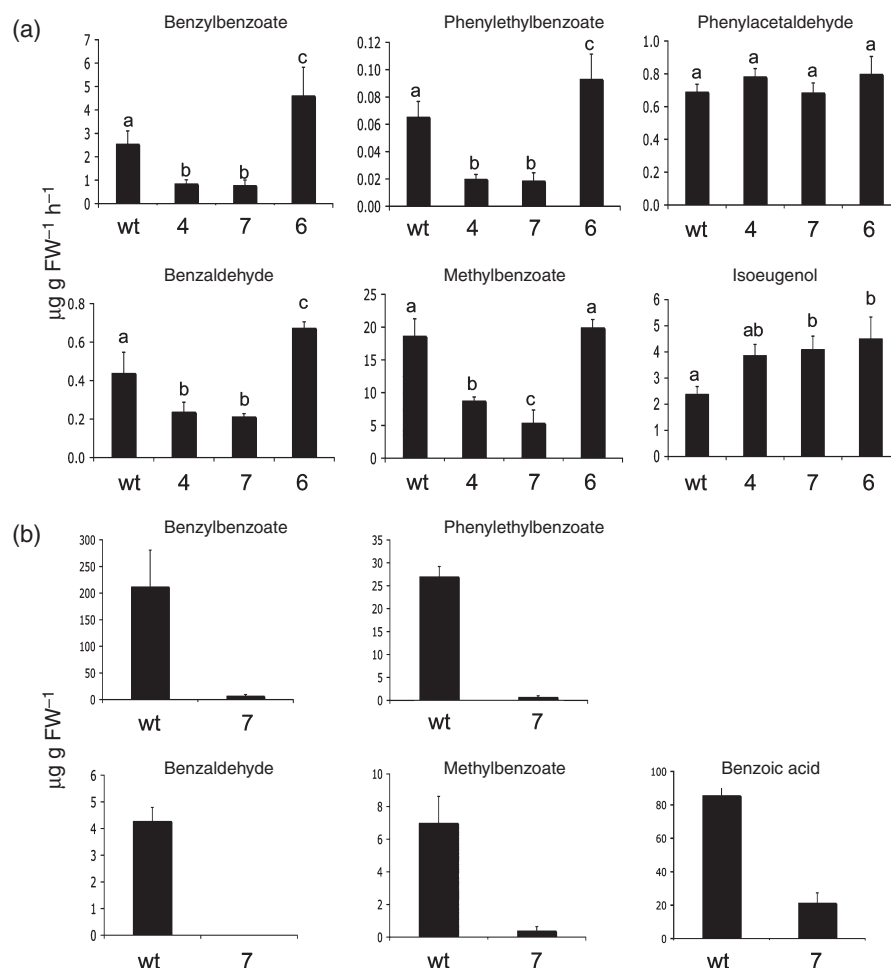


Figure 6. Effect of *PhKAT1* silencing on the emission levels of selected compounds representing the C₆-C₃, C₆-C₂ and C₆-C₁ compounds and on internal benzoic acid (BA) and C₆-C₁ volatile pools.

(a) Quantified emission of volatile benzenoids/phenylpropanoid compounds in wild type and *ir-PhKAT1* plants. Flowers of two independent silenced T₁ lines (4 and 7), one Mitchell control line (wt) and one transgenic non-silenced T₁ line (6) were used for headspace analyses. Volatiles were collected for 23 h and analysed using GC-MS. Emission levels are given per hour, given a constant emission rate, per gram fresh weight and standardised using *p*-cymene as internal standard. For each line, the average of seven experiments was taken, using three detached flowers per experiment. Standard errors are shown. Letters indicate significant differences among lines (ANOVA, $P < 0.05$ according to least significant difference *post-hoc* analysis). The emission levels of the other volatile compounds are shown in Figure S3.

(b) Quantified internal levels of BA and C₆-C₁ volatile compounds. For each measurement, three flowers of *ir-PhKAT1* line 7 and a wild type plant were collected 4 h after the onset of the dark period (mean \pm SD, $n = 3$). Internal BA levels were quantified using added ¹³C-BA and values are given in $\mu\text{g g FW}^{-1}$ (mean \pm SE, $n = 6$).

In these transgenic plants, *PhKAT1* transcript levels in the petals at the peak of volatile production were decreased by as much as 75% (Figure 4b) and overall thiolase activity was reduced by 50% (Figure 5). On the metabolite levels, we observed significant decreases in internal pools of BA, methylbenzoate, benzylbenzoate, phenylethylbenzoate and benzylaldehyde (Figure 6b) and significant decrease in emission of methylbenzoate, benzylbenzoate, phenylethylbenzoate and benzylaldehyde (Figure 6a), but not of the C₆-C₃ compound isoeugenol, the C₆-C₂ compounds phenylacetaldehyde (Figure 6a), 2-phenylethanol and phenylethylacetate (Figure S3) and the *p*-coumaric-derived C₆-C₁ compound vanillin (Figure S3). Interestingly, internal pool

levels of the C₆-C₁ compounds were more reduced than the emission levels, which can reflect a constant emission rate of these compounds, depleting the internal pools even further. Although MeSA can be produced from BA via SA, the moderate reduction in MeSA emission levels suggests that SA is also produced via (iso)chorismate, as has been shown in *Arabidopsis* (Wildermuth, 2001). Similar results were seen in *ir-PhODO1* plants, which show dramatically reduced BA levels but only slightly reduced MeSA emission levels (Verdonk *et al.*, 2005). The existence of this route leading to SA and MeSA has yet to be shown in *petunia*.

The decreased levels of compounds that are derived from BA or benzoyl-CoA, unlike those that are not derived from

these two precursors (the C₆-C₂ and C₆-C₃ compounds), strongly suggest that PhKAT1 is involved in BA biosynthesis, probably through the conversion of 3-oxo-3-phenylpropionyl-CoA to benzoyl-CoA. Efforts to develop an enzymatic assay to measure 3-oxo-3-phenylpropionyl-CoA thiolase activity were not successful. Because most thiolases have activity towards acetoacetyl-CoA, measuring acetoacetyl-CoA thiolase activity in a cell represents the activity of most if not all thiolases present. Therefore, because transcript levels were reduced with 75%, the residual thiolase activity of 50% indicates that either other thiolases are still active or the PhKAT1 protein levels were less reduced than its transcripts levels. Nevertheless, this result shows that reduction of *PhKAT1* transcript levels reduces overall thiolase activity in petunia petals.

Silencing of *PhKAT1* did not alter 2-phenylethanol levels in these lines (Figure S3). Phenylethylbenzoate emission levels, however, were significantly reduced (Figure 6a). As phenylethylbenzoate is produced from 2-phenylethanol and benzoyl-CoA by PhBPBT (Boatright *et al.*, 2004; Dexter *et al.*, 2007), it is likely that reduced benzoyl-CoA levels in *ir-PhKAT1* lines are the cause for lower phenylethylbenzoate emission, supporting the hypothesis that benzoyl-CoA is the product of PhKAT1. Similarly, in *Streptomyces maritimus*, the thiolase *encJ* knock-out strain KJ produces less benzoyl-CoA-derived enterocin (Xiang and Moore, 2003). Our results also showed a peroxisomal localization for PhKAT1 and thus synthesis of benzoyl-CoA in this organelle. This finding is consistent with the recent report that a mutation in a gene encoding the peroxisomal protein benzoyl-CoA ligase (AT1G65880) eliminates benzoyl-CoA (or BA) biosynthesis in Arabidopsis seeds (Kliebenstein *et al.*, 2007).

In plants, β -oxidation is not only essential for fatty acid catabolism (Germain *et al.*, 2001), but also for the production of indole acetic acid (Zolman *et al.*, 2001), jasmonic acid (Afithhile *et al.*, 2005) and valine (Lange *et al.*, 2004). In *Arabidopsis*, there are three *3-ketoacyl-CoA thiolase (KAT)* genes, but only for *AtKAT2* (AT2G33150) it is known that it is important for fatty acid β -oxidation, jasmonic acid biosynthesis and indole acetic acid production (Hayashi *et al.*, 1998; Germain *et al.*, 2001). *In silico* co-expression analysis groups *AtKAT5* with genes of the flavonoid pathway, suggesting it has a role different from that in general fatty acid β -oxidation (Carrie *et al.*, 2007). Because *PhKAT1* is not expressed in seedlings (Figure 2a), it is not likely to be involved in fatty acid β -oxidation. In addition, it has been hypothesised that in some plant species biosynthesis of vanillin, which is a minor volatile in petunia, occurs by a process that mirrors fatty acid β -oxidation (Loscher and Heide, 1994; Podstolski *et al.*, 2002). However, the silencing of *PhKAT1* did not affect the emission of vanillin (Figure S3), excluding a role for *PhKAT1* in vanillin biosynthesis in petunia flowers. It is likely that *PhKAT2*, which is already expressed early during flower development (Figure 2c), when no volatile benzenoids are

produced (Verdonk *et al.*, 2003) and that lacks rhythmic expression in petals (Figure S1), is involved in fatty acid degradation or the synthesis of other metabolites, but not in the synthesis of floral scent compounds.

What is the proportional contribution of β -oxidation to the synthesis of benzenoids in petunia flowers?

While elucidating the specific biosynthetic pathways leading to BA in plants has been a long and difficult process that is still incomplete, feeding experiments previously suggested the contribution of both the β -oxidative and the non- β -oxidative pathway with benzoyl-CoA and benzaldehyde as intermediates, respectively (Ribnicky *et al.*, 1998; Abd El-Mawla and Beerhues, 2002; Boatright *et al.*, 2004). Silencing of *PhBPBT* and subsequent metabolic flux analysis following feeding of labelled Phe to flowers of these transgenic plants indicated that both β -oxidative and the non- β -oxidative pathways contribute to the synthesis of benzenoid compounds in petunia (Boatright *et al.*, 2004). Flux analysis of *ir-PhBPBT* transgenic flowers suggested that benzylbenzoate is a precursor to some of the free BA found in the petals (Orlova *et al.*, 2006). Our results strongly support a major role for a peroxisomal thiolase in the synthesis of benzoyl-CoA and consequently for the synthesis of benzenoids. From our results it is clear that mainly the β -oxidative pathway contributes to BA formation in petunia flowers during the night (Figure 6b) although isotope labelling and modelling studies suggested a greater flux through the non- β -oxidative pathway (Orlova *et al.*, 2006). The reduced internal BA pool levels (four-fold reduction) correlate perfectly with the reduced *PhKAT1* transcript levels (four-fold reduction) in the dark period, when BA levels (Kolosova *et al.*, 2001a), *PhKAT1* transcript levels (Figure 2b) and volatile emission (Verdonk *et al.*, 2005) are high. Our results however, do not exclude the contribution of additional routes leading to the biosynthesis of BA (Boatright *et al.*, 2004; Orlova *et al.*, 2006). Related to this outcome, an aldehyde oxidase that converts benzaldehyde to BA was identified in Arabidopsis recently (Ibdah *et al.*, 2009).

Although benzylbenzoate, phenylethylbenzoate and BA levels are reduced in both the *ir-PhBPBT* and our *ir-PhKAT1* plants, there are also several differences, not in the least because PhBPBT concerns an enzyme in a final step, i.e. making the volatiles benzylbenzoate and phenylethylbenzoate. The most striking difference is that silencing of *PhBPBT* increased benzaldehyde emission (Orlova *et al.*, 2006; Dexter *et al.*, 2008) and internal pools (Orlova *et al.*, 2006) whereas benzaldehyde emission and internal pools decreased as a consequence of *PhKAT1* silencing (Figure 6a,b). The immediate precursor of benzylbenzoate, benzylalcohol, was reduced in *ir-PhKAT1* plants as well but increased in *ir-PhBPBT* plants. Labelling studies indicated that benzylalcohol can be produced from both benzaldehyde and benzylbenzoate in petunia flowers (Boatright *et al.*, 2004).

Because the exact nature of the enzymes that are involved in benzaldehyde and benzylalcohol production in plants is not known, it is difficult to explain these different results. One interpretation is that accumulation of intermediates upstream of benzoyl-CoA in the *ir-PhKAT1* plants could inhibit the activity of benzylalcohol and/or benzaldehyde-producing enzymes, as is the case for PhBPBT inhibition by coniferyl aldehyde (Dexter *et al.*, 2007). Alternatively, accumulated intermediates could inhibit transcription of biosynthetic genes, as is the case for *PhBSMT* by Phe (Boatright *et al.*, 2004). Finally, reduced benzoyl-CoA and/or BA levels in *ir-PhKAT1* plants could enhance the formation of BA from benzaldehyde through the non- β -oxidative pathway in the dark period (Figure 1) as suggested for *ir-PhBPBT* plants in the light period (Orlova *et al.*, 2006). This enhancement would deplete the benzaldehyde internal pool and consequently reduce benzylalcohol internal pool and emission levels. These *ir-PhKAT1* lines can be important tools for future labelling studies to investigate the proportional contributions of the β -oxidative and non- β -oxidative pathway in more detail.

Regulation of floral scent production and the role of compartmentalization

In this study, we have shown that the expression pattern of *PhKAT1* (Figure 2) is characteristic for a floral benzenoid-related gene (Kolossova *et al.*, 2001a; Negre *et al.*, 2003; Boatright *et al.*, 2004; Underwood *et al.*, 2005; Verdonk *et al.*, 2005; Kaminaga *et al.*, 2006; Koeduka *et al.*, 2006; Dexter *et al.*, 2007). We have further shown that expression of *PhKAT1* peaks approximately 3–6 h earlier in petals than *PhODO1* during the day/night cycle (Figure 2b), which precedes peak volatile emission by approximately 2–3 h (Verdonk *et al.*, 2005). Reanalysis of our microarray experiments with *ir-PhODO1* plants (Verdonk *et al.*, 2005) showed that *PhKAT1* expression is not influenced by *PhODO1* silencing (ratio 1.04; $P = 0.44$). Apparently, the expression of *PhODO1* and *PhKAT1* are under different transcriptional control mechanisms.

Volatile benzenoid/phenylpropanoid production and emission are spatially, developmentally and diurnally regulated. This fact makes sense as these volatiles have Phe as precursor in common with many other primary and secondary metabolites. Also methyl donors, supplied by the SAM cycle (Verdonk *et al.*, 2003, 2005), are shared with other pathways (Negre *et al.*, 2003; Schuurink *et al.*, 2006). It is therefore expected that channelling and the existence of different substrate pools are part of the regulatory machinery of the benzenoid pathway. When fluxes are perturbed, for instance by the accumulation of intermediates as a consequence of *petunia* acetyl-CoA:coniferyl alcohol acetyltransferase (*PhCFAT*) silencing, this outcome has a direct effect on PhBSMT and PhBPBT enzymatic activity (Dexter *et al.*, 2007). PhBSMT is likely to be localised in the cytosol in

analogy to the snapdragon S-adenosyl-L-methionine:BA carboxyl methyltransferase (AmBAMT) (Kolossova *et al.*, 2001b). However, it was modelled by Boatright *et al.* (2004) that a second, large pool of MeBA is stored in the vacuole. Here we show that PhKAT1 localises to the peroxisome, adding another layer of regulation. In analogy with fatty acid β -oxidation, CoA-activated compounds like benzoyl-CoA could be transported across the peroxisomal membrane by ABC-transporters (Footitt *et al.*, 2002). Both in the cytosol and the peroxisomes, benzoyl-CoA can be converted to BA by the action of thioesterases (Figure 1), which would implicate the existence of different BA and benzoyl-CoA pools in the cell. Tilton *et al.* (2004) identified a peroxisomal acyl-CoA thioesterase that is likely not involved in fatty acid β -oxidation, but in another process in plants. The substrate for this thioesterase has not been identified yet and it remains to be seen whether multiple thioesterases that can act on benzoyl-CoA exist in *petunia* petals. Finally, the snapdragon benzaldehyde dehydrogenase, involved in the non- β -oxidative pathway, was recently shown to be located in the mitochondria (Long *et al.*, 2009). Our finding that PhKAT1 and thus the β -oxidative pathway localises to the peroxisomes means that not only distinct routes with different enzymes are involved in the production of BA and volatile benzenoids, but that these enzymes are active in different cellular compartments.

EXPERIMENTAL PROCEDURES

Plant material, growth conditions and transformations

Petunia hybrida cv. Mitchell [*P. axillaris* \times (*P. axillaris* \times *P. hybrida* cv. Rose of Heaven)] wild type and transgenic plants were grown in a greenhouse (16 h photoperiod, 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, 60–65% humidity and day/night temperatures of 22/17°C) during winter (T_0 lines) and summer (T_1 lines). For sample collection and volatile headspace analysis, plants were moved to controlled growth chambers (16 h photoperiod, 250–350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, 70% relative humidity and constant temperature of 21°C) at least 3 days prior to the experiments.

Transgenic plants were obtained using a standard leaf disc transformation protocol (Horsch *et al.*, 1985), with minor modifications. The shoot-inducing medium contained 500 mg L^{-1} carbenicillin, 300 mg L^{-1} kanamycin and 20 mg L^{-1} nystatin. The root-inducing medium contained 500 mg L^{-1} carbenicillin, 50 mg L^{-1} kanamycin and 20 mg L^{-1} nystatin. Media contained 3% sucrose (w/v) as the sole carbon source. Rooted plants were transferred to soil and screened for transgene integration by PCR using construct-specific primers. Next generation plants were obtained by manual self-pollination and subsequent selection on half strength MS (pH 5.8) agarose (0.7%) plates containing 80 mg L^{-1} kanamycin.

Constructs design

Constructs were produced using standard molecular biological methods. To create the *PhKAT1* hairpin construct, a 174 bp fragment was amplified with forward primer 5'-**aaaaagcaggctcgatc**ttgcaatgctcgga-3' and reverse primer 5'-**agaagctgggtcaagttcac**taaatcctgct-3' that included *AttB* adapters (in bold; GATEWAY system, Invitrogen, <http://www.invitrogen.com>). This fragment was

cloned in the pDONR207 entry vector (Invitrogen) and subsequently recombined with pK7GWIWG2(I) (Karimi *et al.*, 2002), generating a *PhKAT1* hairpin-RNAi construct under control of the CaMV 35S promoter. To create the CaMV 35S-driven C-terminal GFP fusion construct (35S:KAT1-GFP), the *PhKAT1* CDS was amplified with the introduction of an *NcoI* site at the ATG start codon and an Ala-Gly-linker followed by an *XbaI* site excluding the stop codon (fw primer: 5'-atgcatggagaaagcaattcaaagg-3' and rev primer: 5'-cctctagaacctgctttcgcatccttgataag-3'; restriction sites are underlined; the Ala-Gly-linker is in bold). The GFP CDS was amplified from plasmid SGFP2 (Kremers *et al.*, 2007), introducing an *XbaI* site and Ala-Gly-linker adjacent to the ATG start codon and a *SacI* site beyond the stop codon (fw primer 5'-ggctagagcaggtATGgtgagcaagggcgag-3' and rev primer 5'-cgagctTTActgtacagctcgccatgccgag-3'; restriction sites are underlined; the Ala-Gly-linker is in bold; start and stop codon are in upper case). Using a three-point ligation, both fragments were ligated *NcoI-SacI* in a shuttle vector between the CaMV 35S promoter and the nopalyn synthase terminator (*tnos*). The 35S:PhKAT1-GFP:tnos cassette was excised using *HindIII* and *SfoI* and transferred to pBINplus (van Engelen *et al.*, 1995) between *HindIII* and *SmaI*. The 35S:PhKAT1-GFP:tnos cassette was sequenced and the plasmid was transferred to *Agrobacterium tumefaciens* GV3101 (pMP90).

Volatile sampling, GC-MS analysis and benzoic acid measurements

For volatile sampling (headspace analysis), T₁ transgenic lines (T₀ selfings) and wild type plants were used. Headspace analyses were performed essentially as described by Verdonk *et al.* (2005), except that three detached flowers per desiccator were measured for a 23 h period, with seven replicas per line. GC-MS analysis was performed as described by Verdonk *et al.* (2005), but here 5 ng *p*-cymene was used as an internal standard. A synthetic mix with known amounts of volatile compounds was analysed using GC-MS, enabling quantification and identification of compounds.

For internal volatiles and BA measurements, one T₁ transgenic line (line 7) and wild type plants were used. Flower limbs – three flowers per sample – were harvested 4 h into the dark period, pooled and frozen in liquid nitrogen and stored at –80°C. Internal volatiles were extracted using hexane as described by Boartright *et al.* (2004). Three independent replicates were taken for each plant. BA measurements were performed as described by Zhang and Zuo (2004) with a few modifications. Homogenised flower petals were extracted twice with ethyl acetate, after addition of 2N HCl and ¹³C-BA as the internal standard. Hexane-extracted and derivatised samples were analysed by means of GC-MS. For each plant, six measurements were performed.

RNA isolation and gel-blot analysis

T₁ transgenic lines and wild type petunia plants were used for RNA gel-blot analyses. Independent sample collections for all gel-blot experiments were performed twice. With the exception of the developmental course, 2-day-old flowers were used in all experiments. All tissues except those for the time course were sampled two h before the onset of the dark period. For the time course, petal limbs of three flowers were taken at 3-h intervals for a 30-h period. During the experiment, the dark period in the growth chamber started at 12.00 h and ended at 20.00 h. For the developmental course, petal limbs (without petal tube) were harvested when the flower was 1, 3 or 4 cm long, opening (1 cm diameter), open (day 1), open with dehiscent anthers or senescing.

Total RNA was extracted using Trizol reagent (Invitrogen). For RNA gel-blot analysis, 8 µg of total RNA per sample was loaded

on gel. In order to normalise the RNA gel-bots, blots were re-hybridised with the petunia floral binding protein 1 (*PhFBP1*) (Angenent *et al.*, 1992) probe for the time course and the developmental blot and with a 18S rDNA probe (fw primer: 5'-agcaggctaagtctcgt-3'; rev primer: 5'-agcggatgttgccttagga-3') for the tissue blot. For all cDNA probes, fragments containing the full coding sequence were used except for *PhKAT2* (fw primer: 5'-tgcctctgtatctgacttg-3'; rev primer: 5'-ctactatgtgtggcttctc-3') and *PhFBP1* (fw primer: gttctttgtgatgctcg; rev primer: ctctctcgcaatttgg). For the petunia *ODORANT1* (*PhODO1*) probe, forward primer 5'-gtcacagcggcagcagctac-3' and reverse primer 5'-ctaactctagtagttccagac-3' were used, generating a 518 bp fragment, which does not include the R2R3-MYB domain.

Thiolase assay

For the thiolase assay, wild type flowers and flowers of line 7 (T₂ generation) were used. Crude protein extracts were prepared from 2-day-old flowers, harvested 1 h in the dark period and used immediately. The extraction buffer consisted of 100 mM Tris/HCl pH 8.0, 14 mM 2-mercaptoethanol, 5 mM Na₂S₂O₅, 10% glycerol, 5% PVPP and 0.2 mM PMSF. Thiolase activity was determined by measuring the decay of acetoacetyl-CoA. The final volume of the assay was 100 µl and conditions were as follows: 100 mM Tris pH 8.0, 50 mM KCl, 25 mM MgCl₂, 50 µM CoA and 50 µM acetoacetyl-CoA. Products were separated and detected on HPLC as described in Burns *et al.* (2005). *n* = 4; standard deviations are shown.

Agrobacterium-mediated transient transformation and confocal microscopy

Agroinfiltration of petunia petals was performed as described by Verweij *et al.* (2008). To enable co-localization, two separate *A. tumefaciens* GV3101 cultures harbouring the KAT1-GFP fusion construct or the peroxisomal marker px-rk, which contains mCherry with a C-terminal peroxisomal targeting signal 1 (Nelson *et al.*, 2007), respectively, were mixed 1:1 (v/v) prior to infiltration. For confocal analysis, infiltrated petals were embedded in 80% glycerol to reduce light scattering of the conical epidermal cells. GFP and mCherry were imaged using a Zeiss LSM 510 confocal laser scanning microscope (Vermeer *et al.*, 2008).

Isolation of the full-length cDNA of *PhKAT2*

Total RNA was extracted from petals using Trizol reagent as described above. One microgram of total RNA was used to synthesise cDNA using M-MuLV reverse transcriptase (Fermentas, <http://www.fermentas.com>). The full-length cDNA was obtained using a 5'-RACE kit (Invitrogen) and confirmed by nucleotide sequencing.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Expression of *PhKAT2* during the day/night cycle.

Figure S2. Nucleotide and amino acid sequence of *PhKAT1*.

Figure S3. Effect of *PhKAT1* silencing on volatile emission.

Figure S4. Effect of *PhKAT1* silencing on transcript abundance of some other benzenoid/phenylpropanoid-related biosynthetic genes.

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Accession numbers: The *PhKAT1* and *PhKAT2* cDNA sequence data can be found in the GenBank database under the accession numbers FJ657663 and FJ657664, respectively.