The floral volatile phenylpropanoid/benzenoid pathway in petunia

Van Moerkercke, A.

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CHAPTER 4

A plasma membrane-localised ABC-transporter associated with floral fragrance in petunia petals

Alex Van Moerkercke, Carlos S. Galván-Ampudia, Michel A. Haring and Robert C. Schuurink

Department of Plant Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands.

for submission

SUMMARY

Recent progress in our understanding of the volatile benzenoid/phenylpropanoid pathway indicates compartmentalisation and transport for the production of these compounds. How trafficking towards the plasma membrane after synthesis occurs and how these compounds are emitted remains largely unknown. We have identified a gene encoding a putative ABC-transporter (*PhABCG1*) in *Petunia hybrida* cv. Mitchell. *PhABCG1* expression peaks after anthesis in petals and transcripts accumulate rhythmically during the night and day, coinciding with the developmental and rhythmic emission of volatiles. Because *PhABCG1* transcripts accumulate after anthesis, we can exclude a role in pigmentation. Like the non-fragrant cv. R27, some fragrant petunias such as *P. axillaris axillaris* N. (PaaN) do not express *PhABCG1*. The *PhABCG1* promoter of Mitchell is active in fragrant, but not in non-fragrant petals. A regulator of the volatile benzenoid/phenylpropanoid pathway in petunia, the R2R3-MYB transcription factor ODORANT1 (ODO1), can *trans*-activate the *PhABCG1* promoter in *Nicotiana benthamiana* leaves and petals of R27 that normally do not express *ODO1*. Moreover, transient overexpression of *ODO1* in petals of the fragrant cv. Mitchell increases transcript abundance of *PhABCG1*. Therefore, a role associated with the floral volatile benzenoid/phenylpropanoid pathway is proposed for *PhABCG1*. Because PhABCG1 localises to the plasma membrane, we can exclude a role in intracellular trafficking of compounds. Silencing *PhABCG1* in Mitchell plants did not result in a significant difference in volatile emission under the conditions tested. However, when ectopically expressed in PaaN petals, internal volatile pools of benzylbenzoate accumulated, but this did not result in a significant change in emission of these compounds.

INTRODUCTION

Both biosynthesis and emission of floral volatiles in petunia occurs primarily in petals. The floral volatile headspace of petunia is dominated by benzenoids and phenylpropanoids, which are derived from *L*-Phe via a series of branched pathways, involving multiple organelles. Several genes encoding enzymes catalysing steps in *L*-Phe production (Colquhoun *et al.*, 2010a; Maeda *et al.*, 2010), and the branched pathways leading to the production of C_6-C_3 (Koeduka *et al.*, 2006; Dexter *et al.*, 2007), C_6-C_2 (Kaminaga *et al.*, 2006) and C_6-C_1 (Negre *et al.*, 2003; Boatright *et al.*, 2004; Van Moerkercke *et al.*, 2009) compounds, have been identified and the corresponding enzymes have been characterised in petunia. In roses, enzymes catalysing final steps are found primarily in the epidermal cell layers (Scalliet *et al.*, 2006; Bergougnoux *et al.*, 2007) and in snapdragon, the enzymes catalysing the final two steps in the production of the volatile benzenoid compound methylbenzoate (MeBA), are localised to the conical epidermal cells (Kolosova *et al.*, 2001b; Long *et al.*, 2009). Whether the entire volatile phenylpropanoid/benzenoid production is limited to epidermal cells is not known. Petal mesophyll cells were reported to contain high amounts of starch, which are likely needed to provide the carbon source and energy for the biosynthesis of scent compounds (Sood *et al.*, 2006). These carbon sources, via phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P), feed the shikimate and aromatic amino acid pathways resulting in the production of *L*-Phe, (Tzin and Galili, 2010) the precursor of floral volatiles phenylpropanoid/benzenoids. The involvement of both plastids in *L*-Phe production (Colquhoun *et al.*, 2010a; Maeda *et al.*, 2010) and of peroxisomes in the β-oxidative production of volatile C_6-C_1 benzenoids (Van Moerkercke *et al.*, 2009) has been reported in petunia. Trafficking of metabolites across plastidial and peroxisomal membranes may require specialised transport proteins (e.g. Weber *et al.*, 2005; Visser *et al.*, 2007). For instance, the ATP-binding cassette (ABC) transporter COMATOSE (AtABCD1) is required for transport of acyl-CoAs into peroxisomes in Arabidopsis (Footitt *et al.*, 2002).

After biosynthesis the volatile compounds need further trafficking across the cell membrane, the cell wall, and the cuticle layer, before they can be emitted. Whether this trafficking involves non-facilitated diffusion, active transport or both

is uncertain (Jetter, 2006). ABC-transporters can facilitate transport of compounds across the plasma membrane. The Arabidopsis ABCG11 (WBC11/DSO/COF) (Bird *et al.*, 2007; Panikashvili *et al.*, 2007), ABCG12 (WBC12/CER5) (Pighin *et al.*, 2004) and ABCG13 (WBC13) (Panikashvili *et al.*, 2011) are involved in cutin and/or wax deposition at the surface of Arabidopsis plants. *Nicotiana plumbaginifolia* ABC1 (NpPDR1) actively secretes the terpenoid sclareol from sclareol-treated cells (Jasinski *et al.*, 2001). ATP-dependent transport of aglycone monolignols across plasma membrane vesicles was demonstrated recently (Miao and Liu, 2010). Although intrinsic, non-selective permeability of the membrane for these compounds was shown as well, the authors however conclude that passive diffusion of these compounds unlikely plays a major role (Miao and Liu, 2010). Importantly, some of the monolignols tested, notably coniferylalcohol and coniferaldehyde, are precursors for the C_6-C_3 volatile phenylpropanoids eugenol and isoeugenol (Koeduka *et al.*, 2006; Dexter *et al.*, 2007). Whether volatile benzenoid/phenylpropanoid emission requires active transport across plasma membranes is not known. During petal development in snapdragon, the rate of emission of MeBA is closely associated with internal pool size, which seems to control the emission rate (Goodwin *et al.*, 2003). The ratio between emission rate and internal pool size decreases with the molecular weight of the compounds in flowers of petunia (Oyama-Okubo *et al.*, 2005). For certain compounds in rose, notably 2-phenylethanol, benzylalcohol and 2-phenylethylacetate, this ratio is dependent on the developmental stage of the petals: whereas the internal pool size of these compounds remains constant during early and late phases of development, no emission is seen at late stages (Bergougnoux *et al.*, 2007). This could point to active secretion of these compounds by transporters or alternatively to changes in petal cuticle composition that specifically affects transport and/or diffusuin of these compounds. In snapdragon, no clear association between MeBA emission and wax properties during flower development was seen, suggesting the cuticle shows little diffusive resistance to volatile emission (Goodwin *et al.*, 2003). Despite the likely involvement of ABC-transporters at various stages in the export of lipophilic compounds to the surface of the plant, none have been identified for the volatile benzenoid/phenylpropanoid pathway.

The super-family of plant ABC proteins is one of the largest families of proteins in the plant kingdom consisting of 131 members in the Arabidopsis genome and a similar number in rice (Garcia *et al.*, 2004). ABC-transporters couple ATP-hydrolysis to the transport of various compounds across membranes. They can principally be categorized according to the order and number of two functional domains: an ATP-binding cassette or nuclear binding fold (NBF) and a hydrophobic trans-membrane domain (TMD), responsible for ATP binding and hydrolysis, and metabolite transport and substrate selectivity, respectively (Sanchez-Fernandez *et al.*, 2001). Both domains can be present once (half-size transporters) or twice (full-size transporters) in a single protein, in a forward (TMD-NBF) or reverse (NBF-TMD) order. Half-size transporters need to form homodimers or heterodimers to form a functional protein (Graf *et al*., 2003; McFarlane *et al.*, 2010). The ABCG subfamily consists of the white-brown complex homologues (WBC) and pleiotropic drug resistant homologues (PDR), which are half-size molecules and full-size molecules, respectively, with the NBF and TMD organised in a reverse order (Verrier *et al.*, 2008). In Arabidopsis, the WBC subfamily comprises 29 members but only a functional role in cuticular lipid export (ABCG11, ABCG12 and ABCG13), kanamycin resistance (ABCG19), ABA export (ABCG25) and sporopollenin precursor transport (ABCG26) has been shown to date (Pighin *et al.*, 2004; Mentewab and Stewart, 2005; Bird *et al.*, 2007; Panikashvili *et al.*, 2007; Choi *et al.*, 2010; Kuromori *et al.*, 2010; Quilichini *et al.*, 2010; Panikashvili *et al.*, 2011), leaving the function of the other members of this group unknown.

Few ABC-transporters have been reported that are expressed in flower petals. The Arabidopsis ABCG13 is involved in the transport of petal and carpel cuticular lipids (Panikashvili *et al.*, 2011). The *Nicotiana plumbaginifolia PDR1* is expressed in the upper parts of the fragrant petal limbs, although not exclusively since it is also expressed in trichomes and roots, and upon pathogen infection in leaves (Stukkens *et al.*, 2005). A function for this cell membrane-localised ABCG-transporter in petals was not investigated, but a role in floral volatile secretion is possible (Stukkens *et al.*, 2005). In petunia, expression of genes involved in floral volatile benzenoid/phenylpropanoid biosynthesis is highly regulated during flower development and during the night and day, and is generally restricted to petals. A regulator of the pathway, the R2R3-MYB transcription factor *ODORANT1*

(*ODO1*), is co-expressed with several genes of the pathway (Verdonk *et al.*, 2005; Colquhoun *et al.*, 2010b), and was shown to activate the promoter of the shikimate gene *5-enolpyruvylshikimate-3-phospate synthase* (*EPSPS*), thereby controlling precursor supply (Verdonk *et al.*, 2005). We identified an ABCG-transporter, which is specifically expressed in petunia petals. Expression of *PhABCG1* is regulated by the R2R3-MYB TF ODORANT1 (ODO1), suggesting a link with floral fragrance in petunia. We show that PhABCG1 is localised to the plasma membrane, excluding a role in intracellular trafficking. Silencing or overexpression of *PhABCG1* in Mitchell does not seem to alter volatile emission significantly. However, ectopic expression in petals of a fragrant *P. axillaris* that normally does not express *PhABCG1* results in higher internal pools of the volatile compounds benzylbenzoate and phenylethylbenzoate compared to the control situation. If PhABCG1 is specific for these compounds and whether other compounds accumulate in these petals requires further investigation.

RESULTS

Identification of a gene encoding a putative ATP-binding cassette transporter

Microarray analysis conducted with *Petunia hybrida* cv. Mitchell petal tissue collected at two different time points during the day/night cycle (Verdonk *et al.*, 2003) revealed a cDNA encoding a putative ATP-binding cassette (ABC) protein that was highly upregulated 3h before the onset of the dark period compared to 9h before the dark period (26-fold induction, $P = 0.006$, $n = 3$). The transcript is between 1.8 and 3.2 bp, suggesting it encodes a half-size ABC-transporter (Figure 1a). The full-length transcript was obtained using rapid amplification of cDNA ends (5'-RACE). This cDNA is 2178 bp, with a 146 bp 5'-untranslated region (UTR) and 130 bp 3'-UTR. Isolation of the corresponding genomic region showed a gene structure with eight exons and seven introns (Figure 1b).

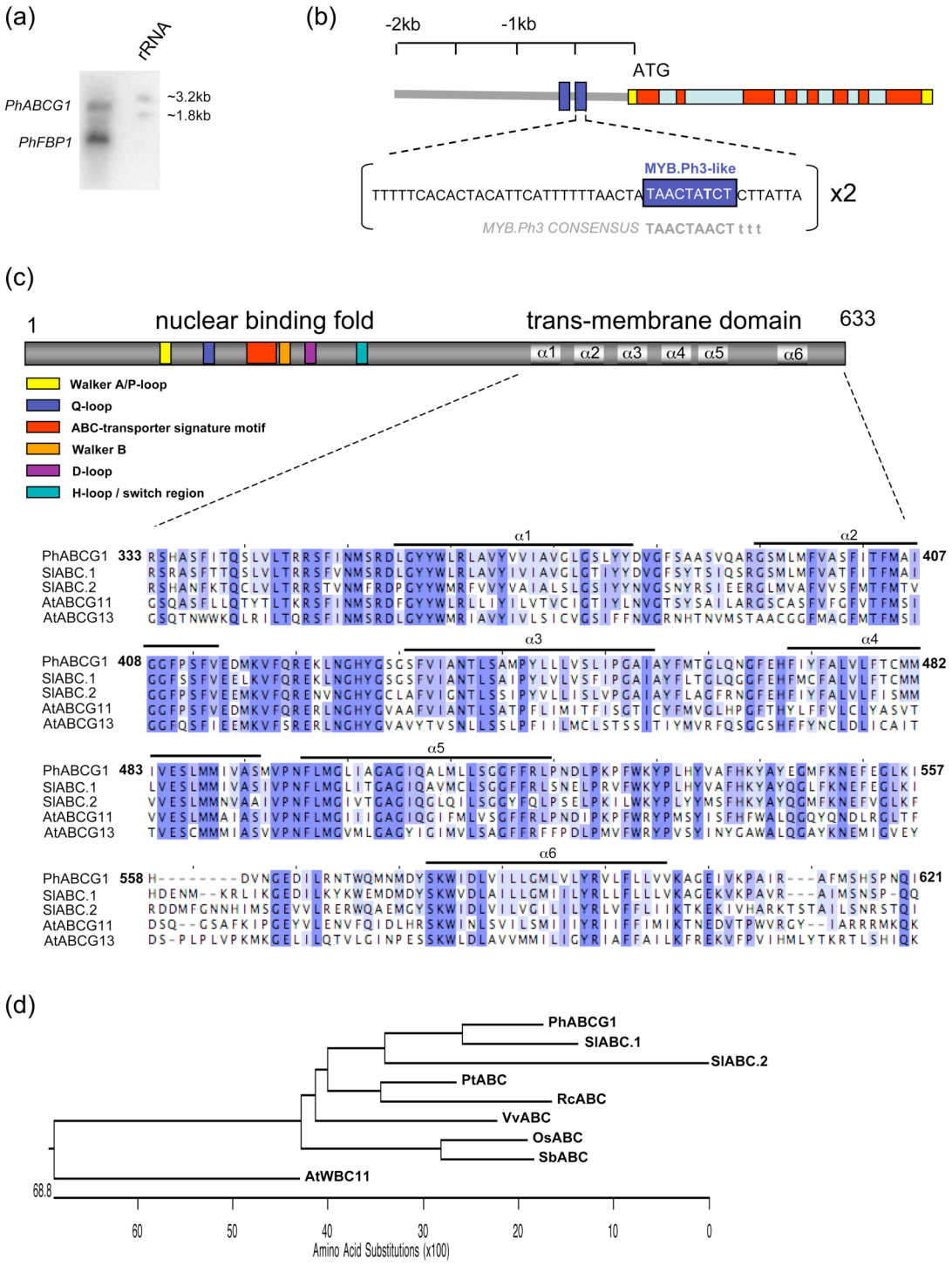

Figure 1. Structure of *PhABCG1*. (a) RNA gel-blot showing the size of the full size *PhABCG1* and *FLORAL BINDING PROTEIN 1* (*FBP1*) transcripts in Mitchell petals. The sizes of the ribosomal RNAs are indicated on the right. (b) The genomic region consists of 8 exons (red) and 7 introns (blue). The UTRs are in yellow. The 2kb promoter region contains a 39 bp repeat (indicated with x2) that has a MYB.Ph3-like binding site. (c) PhABCG1 encodes a half size ABC-transporter of 663 AAs. The nuclear binding fold and tans-membrane domain are located the N-terminus and C-

terminus, respectively. Characteristic features in the nuclear binding fold are indicated as boxes. An alignment of the putative trans-membrane domain with those of the closest homologs in *Solanum lycopersicon* (SlABC.1 and SlABC.2) and with those of Arabidopsis ABCG11 and G13 is shown. The six putative α-helices spanning the membrane are indicated above the amino acid sequence. (d) Phylogentic tree of the TMDs used for the alignment in (c), extended with those of the closest homologs in *Oriza sativa* (Os, NM_001069981), *Populus trichocarpa* (Pt, XM_002300152), *Ricinus communis* (Rc, XM_002525791), *Vitis vinifera* (Vv, XM_002270611), *Sorghum bicolor* (Sb, XM_002460339). The tree was made using MegAlign.

The cDNA putatively encodes a protein of 633 amino acids that we named PhABCG1. The protein contains the highly conserved Walker A and B boxes (Walker *et al.*, 1982), separated by the Q-loop and ABC signature (Higgins *et al.*, 1986) and flanked by the D-loop and H-loop/switch region, which together correspond to the nuclear binding fold (NBF; Figure 1c). A Kyte-Doolittle (Kyte and Doolittle, 1982) plot revealed 6 stretches with a high content of hydrophobic residues located at the C-terminus of the polypeptide, corresponding to six putative α-helices (α1-α6 in Figure 1c) spanning the membrane (transmembrane domain; TMD). In this order and number, the NBF-TMD structure is typical for proteins belonging to the ABCG/WBC subfamily of ABC proteins. We searched for homologous proteins using the BLAST tool of NCBI. The closest characterised protein is the Arabidopsis ABCG11/WBC11/COF1/DSO (Pighin *et al.*, 2004) with 47% AA identity between the TMDs. Homology with the floral-specific AtABCG13 (Panikashvili *et al.*, 2011) is 40%. The closest uncharacterised putative homologs belong to *Ricinus communis* (62% between the TMDs), *Populus trichocarpa* (61.5%), *Vitis vinifera* (62%), *Sorghum bicolor* (60%) and *Oryza sativa* (60%). We further looked for putative homologous ABC-transporters in the recently sequenced genome of tomato and found two proteins that show 78 % (SlABC.1) and 57 % (SlABC.2) AA identity in the TMDs with PhABCG1. An alignment of the TMDs of PhABCG1, AtABCG11 and G13, SlABC.1 and 2, along with a phylogenetic tree of the TMDs of the aforementioned proteins are shown in Figures 1c and 1d, respectively. The *PhABCG1* sequence could not be retrieved from a 454 Petunia database (*P.*

axillaris and *P. integrifolia*; www.petuniaplatform.net) and only short ESTs with low percentage of similarity were found.

We isolated a 2009 bp promoter fragment using genome walking. The sequence contains TATA motifs immediately upstream of the putative transcriptional start site (TTS). The promoter also contains a 39 bp perfect repeat (Figure 1b), located 719-676 and 675-642 nucleotides upstream of the ATG, in which a putative MYB binding domain resides (TAACTATCT at 696-688 and 658-650) that resembles a previously defined binding site (TAACTAACT) for MYB.Ph3 and AmMYB305 in the promoter of flavonoid genes (Solano *et al.*, 1995a; Solano *et al.*, 1995b).

The *PhABCG1* expression pattern suggests a role in floral scent production and is restricted to a subset of fragrant petunias

Expression analysis in Mitchell plants showed that this gene is highly expressed in the petal limbs and to a lesser extent in petal tubes of petunia flowers whereas a signal could barely be detected in any other tissue using RNA-blot analysis (Figure 2a). Three-hour interval sampling of petunia petals and subsequent RNA-blot analysis showed rhythmical transcript accumulation during the day and night in phase with *ODO1* (Figure 2b). Transcript abundance increased during flower development and peaked at anthesis (Figure 2c). This expression pattern of *PhABCG1* is similar to and specific for genes of the volatile benzenoid/phenylpropanoid pathway in petunia and is thus indicative for a role in floral scent production.

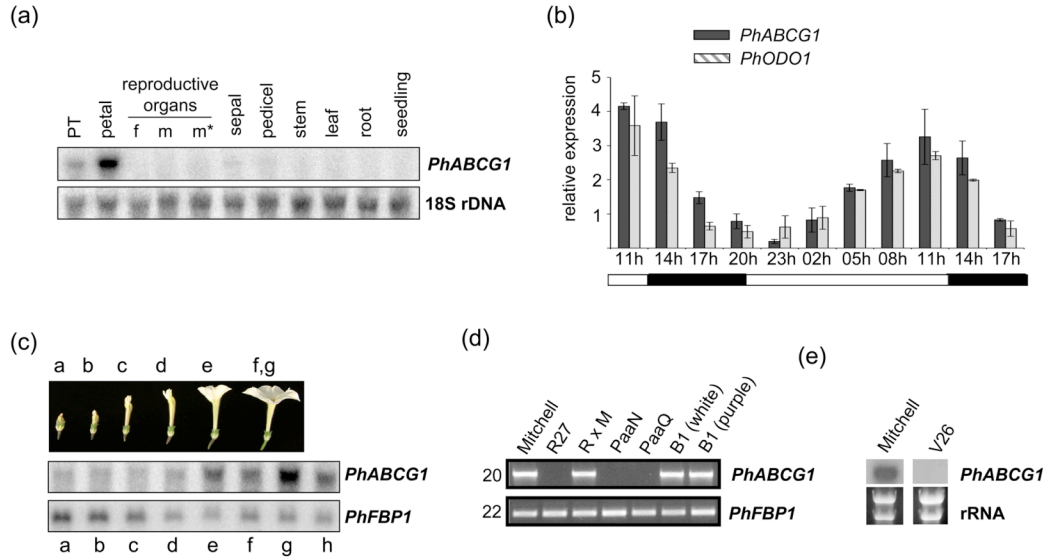

Figure 2. Accumulation of *PhABCG1* transcripts. RNA gel-blot and RT-PCR experiments showing *PhABCG1* transcript levels. For RNA-gel blot analyses, blots were hybridised with the indicated probes. Re-hybridisation with an *FLORAL BINDING PROTEIN 1* (*FBP1*) or an 18S rDNA probe was used to show loading of the gel or to enable normalisation. For RT-PCR, the numbers of cycles used is shown on the left and *FBP1* was used as a reference gene. Experiments were performed at least twice and representative blots and gels are shown. (a) Tissue-specific accumulation in Mitchell. (b) Rhythmic accumulation in Mitchell. Mean values are shown (n = 2). Error bars indicate maximum and minimum values. Black and striped bars indicate *PhABCG1* and *ODO1* transcript levels, respectively. Petal tissue was harvested at the times indicated. Black and white bars below the X-axis indicate dark and light conditions, respectively. (c) Developmentally regulated transcript accumulation in Mitchell petals. Developmental stages of the petals: a-e, as indicated; f and g indicate open flowers before and after anthesis, respectively; h indicates a senescent flower. (d, e) *PhABCG1* transcript levels in petals of different petunias. (d) RT-PCR on petal tissue of Mitchell (M), R27 (R), an R27 x Mitchell F1 cross (R x M), the fragrant *P. axillaris axillaris* N. and Q. (PaaN and PaaQ) and purple and white flowers of petunia line B1 (Spitzer *et al.*, 2007). (e) RNA gel-blot analysis using petal RNA from Mitchell and V26 flowers. Ethidium bromide was used to show loading of the gel.

To verify if *PhABCG1* expression is correlated with scent production, we analysed transcript levels in fragrant and non-fragrant petunias using RNA gel-blot analysis and semi-quantitative RT-PCR. *PhABCG1* is highly expressed in

Mitchell petals, but transcripts could not be detected for the non-fragrant R27 cultivar (Figure 2d). Surprisingly, *PhABCG1* expression could not be seen in the fragrant cv. V26 either (Figure 2e). The sequence corresponding to the full length CDS including introns could be PCR-amplified from V26 gDNA (data not shown), indicating a copy is present in its genome. Also the fragrant *P. axillaris axillaris* N. does not express *PhABCG1* at detectable levels (Figure 2d), However, expression is seen in petals of the fragrant *P. hybrida* line B1 (Figure 2d).

PhABCG1 promoter activity in fragrant and non-fragrant petunias

To further examine the expression pattern observed in Figure 2d, we analysed the activity of the *PhABCG1* promoter of Mitchell in fragrant *P. axillaris axillaris* N. (PaaN), non-fragrant R27 and Mitchell petals. For this, we fused a 2kb promoter region of Mitchell (including the 5'-UTR) to an intron-containing *β-glucuronidase* (*uidA*) CDS and assessed the activity in petals using agro-infiltration (Figure 3). As expected from the expression analyses, the Mitchell promoter was highly active in the Mitchell petals. Similarly high expression levels were seen in PaaN petals. Promoter activity in R27 flowers is ca. 7-fold lower compared to the Mitchell promoter but the promoter is more active in a R27 x Mitchell (R x M) F1 cross, although less compared to Mitchell (Figure 3).

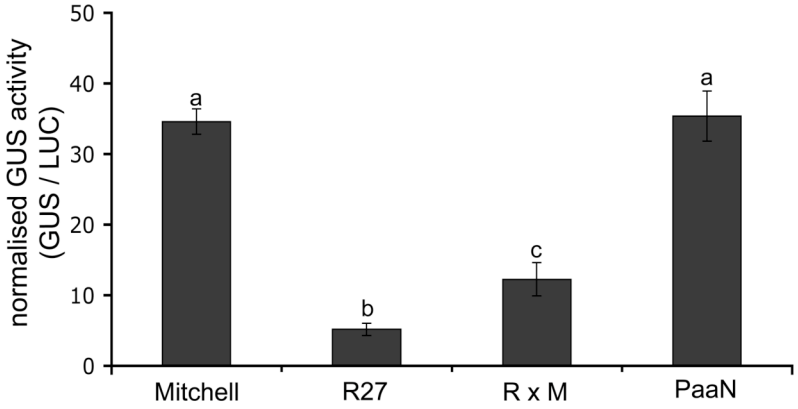

Figure 3. *PhABCG1* promoter activity in petals of fragrant and non-fragrant petunias. Mitchell *PhABCG1* promoter activity in petals of *P. hybrida* cvs. Mitchell,

R27, an F1 R27 x Mitchell cross (R x M) and *P. axillaris axillaris* N. (PaaN). Petals were co-infiltrated with *A. tumefaciens* harbouring ABCG1:GUS and 35S:LUC (AVERAGE ± SE, *n = 4*). The experiment was performed twice and the results of a representative experiment is shown. Letters indicate significant differences (ANOVA, $P < 0.05$ according to least significant differences *post-hoc* analysis).

ODORANT1 activates the *PhABCG1* promoter and direct its transcription

Flowers of the non-fragrant R27 cv. do not express *ODO1* at detectable levels whereas flowers of fragrant petunias do (Figure 4a). In addition, the promoter contains two putative MYB binding sites (Figure 1b). To test if ODO1 can *trans*-activate the *PhABCG1* promoter, we used a *trans*-activation assay in *Nicotiana benthamiana* leaves as described in **CHAPTER 3**. We first ectopically expressed a 2kb promoter reporter construct (ABCG1:GUS) together with *35S:ODO1* in *N. benthamiana* leaves. As a control, the same reporter construct was separately co-infiltrated with the R2R3-MYB TFs EOBII, ANTHOCYANIN 2 (AN2), or with RED FLUORESCENT PROTEIN (RFP) effector constructs (Figure 4b). Activity was induced up to 7-fold when co-infiltrated with *ODO1* compared to the control infiltrations (Figure 4c). These results indicate that ODO1 activates the *PhABCG1* promoter in *N. benthamiana* leaves. Since R27 flowers do not express *ODO1* and since the Mitchell promoter is not active in R27 petals, we ectopically co-expressed the ABCG1:GUS reporter construct and the 35S:ODO1 effector construct in R27 petals, and subsequently assessed the promoter activity. We used the 35S:RFP as a control as both *AN2* and *EOBII* are expressed in R27 flowers. Similarly as for leaves, the *PhABCG1* promoter could be activated by ectopically expressed *ODO1* in R27 petals (Figure 4d), confirming ODO1-dependent expression of *PhABCG1* in a non-fragrant flower background.

To further corroborate the molecular link between ODO1 and *PhABCG1* in Mitchell petals, we transiently overexpressed *ODO1* in Mitchell flowers and subsequently monitored *PhABCG1* expression levels at a time-point where *ODO1* and *PhABCG1* expression are normally low (Figure 2b). Figure 4e shows increased *ODO1* transcript levels as a result of *ODO1* overexpression with concomitant increased *PhABCG1* levels. As a control, flowers were infiltrated

with a 35S:LUC construct, which did not result in increased *ODO1* and *PhABCG1* transcript abundance (Figure 4e).

Subcellular localisation of PhABCG1

To verify the subcellular localisation of PhABCG1, we created an N-terminal translational fusion to mVenus (Nagai *et al.*, 2002) under control of a double CaMV 35S promoter and transiently expressed it in *P. hybrida* cv. Mitchell petals and *N. benthamiana* leaves using agro-infiltration (Verweij *et al.*, 2008; Van Moerkercke *et al.*, 2009). Initial attempts to clone the fusion construct with the *PhABCG1* CDS failed, possibly due to toxicity in *E. coli,* and therefore we used the full genomic region. Confocal imaging of the fusion protein showed co-localisation with the cell membrane marker FM4-64 (Bolte *et al.*, 2004) in the conical cells of the adaxial epidermis of petunia petals. Figure 5a and Figure 5d show the transmitted light through the adaxial conical cells and at the base of the cells, respectively. Single confocal sections show co-localisation of mVenus-ABCG1 with the FM6-46 marker in these cells (Figures 5b, e). A 3D-reconstruction of multiple confocal projection images of the same cells, represented in an angle of 45°, shows co-localisation at the entire surface (Figure 5g-i). Co-localisation with FM4-64 was also observed in epidermal cells of tobacco leaves (Figure 5c, f), confirming plasma membrane-localisation in non-petal tissue.

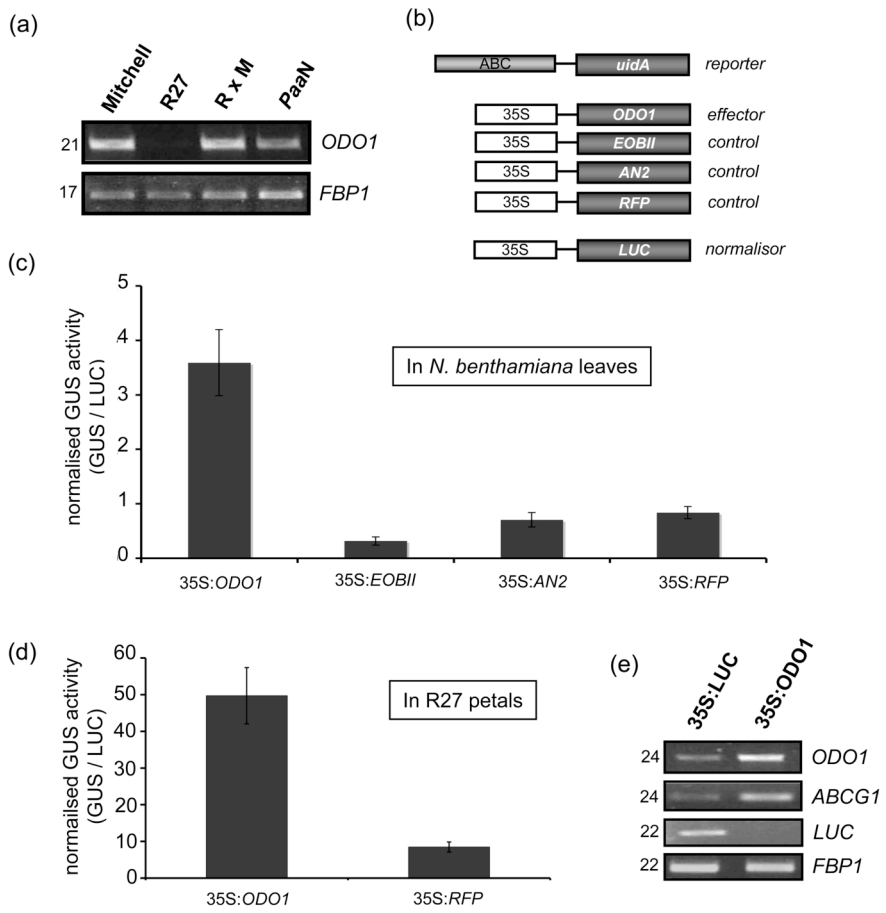

Figure 4. *PhABCG1* is activated by ODORANT1. (a) RT-PCR showing transcript levels in petals of Mitchell (M), R27 (R), an R27 x Mitchell F1 cross (R x M) and *P. axillaris axillaris* N. (PaaN). The numbers of cycles used is shown on the left. *FLORAL BINDING PROTEIN 1* (*FBP1*) was used as a reference gene. (b) Constructs used for the *trans*-activation assay. (c, d) *Trans*-activation assay with ectopically expressed effector and control constructs in leaves of *N. benthamiana* and petals of R27. Co-infiltration with 35S:LUC was used to normalize for infiltration and protein extraction efficiency. (e) RT-PCR analysis showing *ODO1* transcript levels in Mitchell petals transiently overexpressing *PhABCG1*, at a time during the day when *PhABCG1* and endogenous *ODO1* levels are low. Petals expressing *LUC* were used as a control. The numbers of cycles used is shown on the left. *FLORAL BINDING PROTEIN 1* (*FBP1*) was used as a reference gene. The experiment was performed three times and the result of one representative experiment is shown.

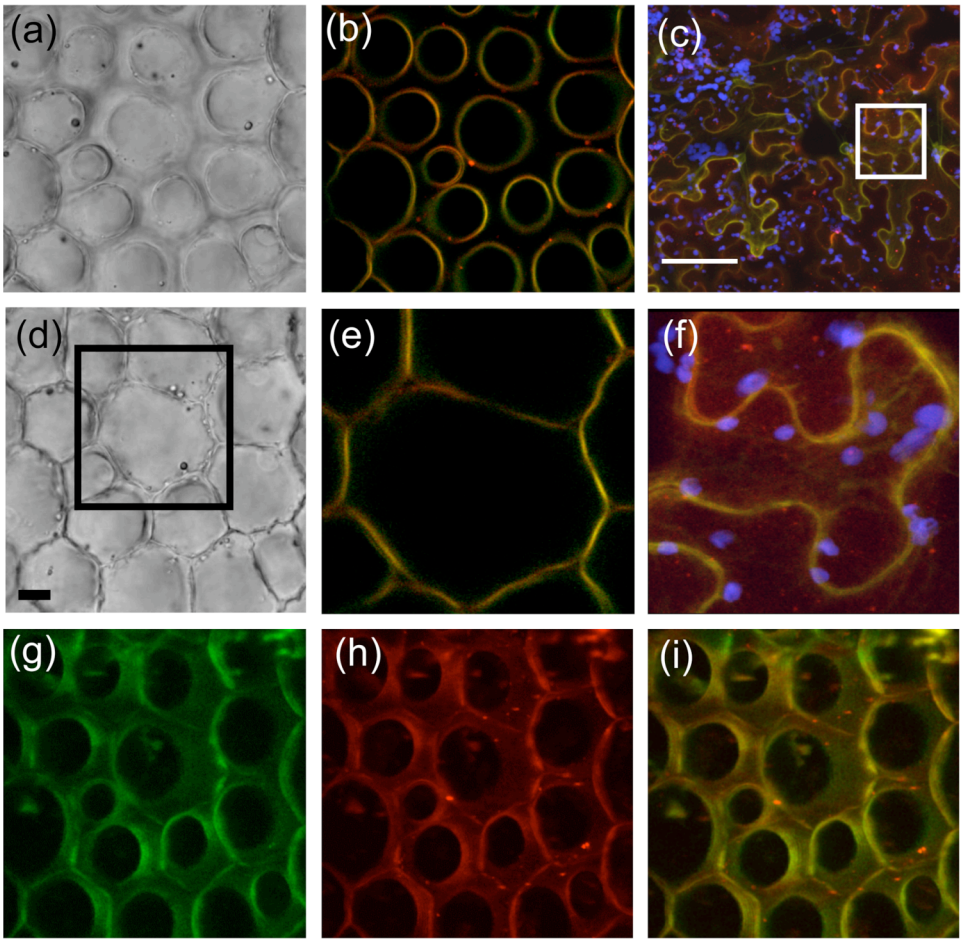

Figure 5. PhABCG1 localises to the plasma membrane. Transient expression in petunia petals and *Nicotiana benthamiana* leaves showing plasma membrane-localisation of 35S:mVenus-ABCG1. Transmitted light through the conical cells (a) and at the base of the conical cells (d) of the adaxial epidermal cell layer of petunia petals. (b) Merged image of a single confocal section of the image in (a). (e) Merged image of a single confocal section of the boxed area in image (d). The scale bar represents 10 μm. (e) Magnification taken from the boxed area in (d). (g, h, i) Confocal projection image of mVenus (g) and FM4-64 (h) showing co-localisation at the plasma membrane (i). The images are 3D-reconstruction of multiple confocal images, including those shown in (b) and (e), and shown in an angle of 45°. (c) Merged image of a single confocal section showing PhABCG1 (green), FM4-64 (red) and autofluorescence (blue) in epidermal cells of *N. benthamiana* leaves. The scale bare represents 100 μm. (f) Magnification taken from the boxed area in (c).

The effect of *PhABCG1* silencing on volatile emission levels

Because the expression pattern of *PhABCG1* suggested a role in floral scent production, we stably silenced *PhABCG1* in *Petunia hybrida* Mitchell plants and analysed the emission of volatile compounds. Two independent T_1 lines (T_0 selfings) with 50% (line 9) and 70% (line 10) reduced *PhABCG1* transcript levels and one line with only 10% (line 12) reduced transcript levels were obtained (Figure 6a, b) out of 12 transgenic lines. No significant difference in volatile emission was detected between lines 9 and 10 compared to both wild type and line 12 (Figure 7). However, a slight but non-significant, increase in emission in both lines could be observed for methylbenzoate, benzylbenzoate, benzylacetate, phenylethylbenzoate, isoeugenol and vanillin (Figure 7).

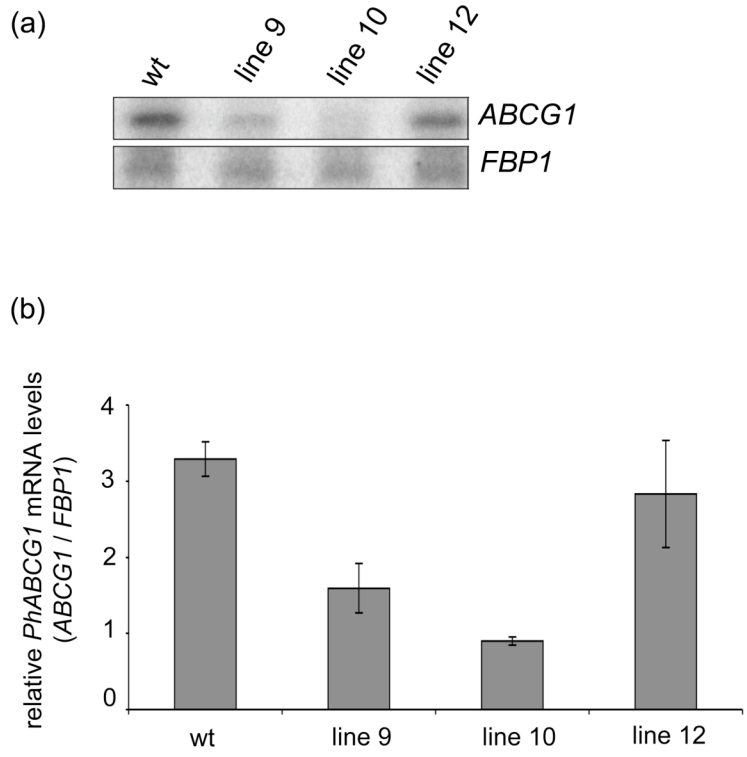

Figure 6. **Expression analysis of *PhABCG1*-silenced lines. (a)** *PhABCG1* mRNA levels in wild type (wt) petals and petals of three transgenic lines (9, 10 and 12). Total RNA was isolated from 2-day-old petals at the onset of the dark period. The numbers on top represent independent transgenic T_1 (T_0 selfings) lines. Sampling and RNA extraction were independently performed twice. Blots were hybridised with

a *PhABCG1*-specific probe. Re-hybridisation with a *FLORAL BINDING PROTEIN 1* (*FBP1*) probe was performed to show loading of the gel. (b) Quantified *PhABCG1* mRNA levels using RNA gel blot analysis. *FBP1* was used to normalise mRNA levels (mean and maximum levels are shown, *n* = *2*).

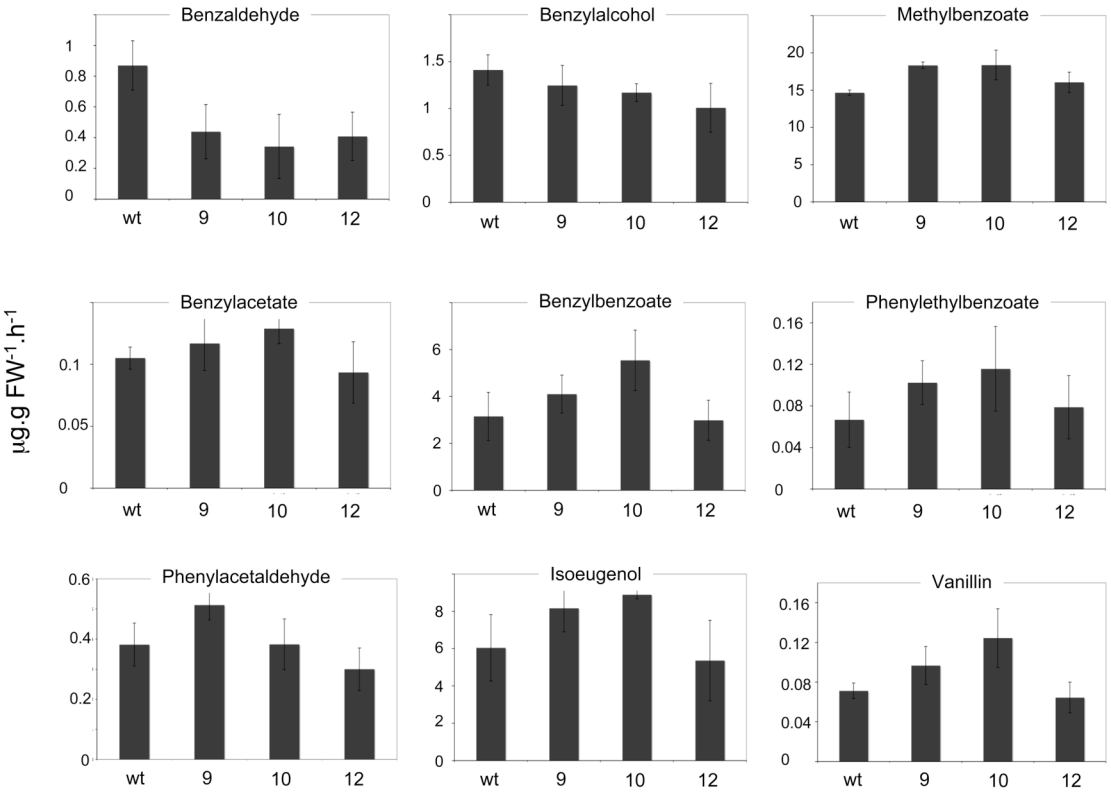

Figure 7. Volatile emission levels in *PhABCG1*-silenced lines. Emission of volatile phenylpropanoid/benzenoid compounds in wild type, two silenced (lines 9 and 10) and one non-silenced (line 12) T_1 line. For each line, two detached flowers per experiment were used (AVERAGE ± SE, *n* = *4*). No significant differences could be detected (*P* > 0.05).

Ectopic expression of *PhABCG1* in *Petunia axillaris* petals

A reduction in transcript abundance of up to 70% in line 10 (Figure 6) did not result in a significant change in volatile emission in Mitchell flowers (Figure 7). *Petunia axillaris axillaris* N. (PaaN) emits volatile benzenoids/phenylpropanoids,

notably benzylbenzoate, methylbenzoate, phenylethylbenzoate, isoeugenol, phenylacetaldehyde and benzaldehyde (Hoballah *et al.*, 2005), but does not express *PhABCG1* (Figure 2d). To test if PhABCG1 is involved in the specific volatile signature of Mitchell flowers, this petunia was chosen for transient expression of *PhABCG1* in its petals followed by headspace, internal volatile compound accumulation and GC-MS analyses. *PhABCG1* was expressed in *ABCG1*-infiltrated PaaN petals, but not in petals infiltrated with 35S:LUC (Figure 8c). We could not detect any significant change (n = 4, $P > 0.1$) in volatile emission compared with *LUC*-infiltrated control flowers (Figure 8a) but a slight decrease for benzylbenzoate, phenylacetaldehyde and methylbenzoate, and a slight increase for benzaldehyde was observed. The emission of isoeugenol could only be detected in non-infiltrated control flowers and one LUC-infiltrated flower (n = 4). Phenylethylbenzoate emission could not be detected.

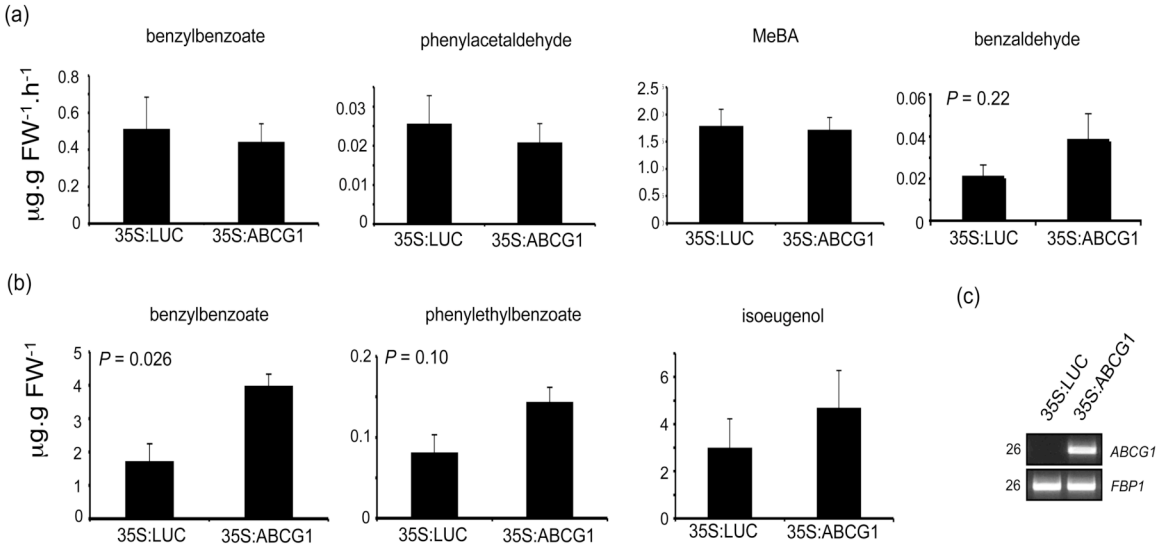

Figure 8. Transient expression of *PhABCG1* in petals of *Petunia axillaris*. Petals of *P. axillaris axillaris N.* were infiltrated with *A. tumefaciens* harbouring a 35S:LUC or 35S:ABCG1 construct. (a) Emission of benzylbenzoate, phenylacetaldehyde, methylbenzoate and benzaldehyde (AVERAGE ± SE, *n = 4*). No significant differences were seen for benzylbenzoate, phenylacetaldehyde and MeBA ($P > 0.5$). Difference for benzaldehyde is indicated (student t-test). (b) Internal levels of benzylbenzoate, phenylethylbenzoate and isoeugenol. Petal tissue was collected at the beginning of the dark period (AVERAGE ± SE, *n = 4*). Differences

are indicated (student t-test). (c) RT-PCR showing ectopic expression of *PhABCG1* in petals after agroinfiltration. The number of cycles is indicted left. *FLORAL BINDING PROTEIN 1* (*FBP1*) was used as a reference gene.

Interestingly, internal levels of benzylbenzoate were significantly higher in *ABCG1*-infiltrated petals compared to the control situation (n = 4, *P* = 0.02). Internal levels of phenylethylbenzoate (n = 4, *P* = 0.1) and isoeugenol (n = 4, *P* = 0.45) were not significantly higher in infiltrated petals (Figure 8b). Increased accumulation of benzylbenzoate was seen in two independent experiments. Accumulation of isoeugenol in both ABCG1-infiltrated petals and control-infiltrated petals could only be detected in one experiment (n = 4). Internal pools of benzaldehyde and phenylacetaldehyde could not be detected. Production of compounds that are absent in control-infiltrated petals was not observed. It should be noted that the internal levels of the compounds (as well as total emitted output), possibly as a result of the infiltration, was lower compared to non-infiltrated wild type flowers.

DISCUSSION

PhABCG1 is associated with fragrance biosynthesis in petunia petals

The expression pattern of a gene can be indicative for its function. Transporters are often co-expressed with enzymes that metabolise the substrates/products that need transport. As a consequence, it is likely they are under control of the same regulators. For instance, the *Zea mays* MRP3, encoding a full-size ABCC transporter, was shown to transport anthocyanins into the vacuole (Goodman *et al.*, 2004). Importantly, *ZmMPR3* is co-expressed with genes of the anthocyanin pathway and regulators of anthocyanin biosynthesis control *ZmMRP3* expression (Goodman *et al.*, 2004). Likewise, the Arabidopsis *ABCG26/WBC27*, required for exine formation during pollen development, is co-expressed with genes encoding enzymes involved in sporopollenin precursor synthesis (Choi *et al.*, 2010) and *ZmWBC11a*, involved in cuticular wax secretion, is regulated by the same regulator that activates genes of lipid metabolism (Javelle *et al.*, 2010).

PhABCG1 transcripts were predominantly detected in petal tissue (Figure 2a), which is in accordance with the site of volatile production and with the petal-specific expression of previously described genes of fragrance biosynthesis in petunia (Schuurink *et al*., 2006). Importantly, *PhABCG1* transcripts accumulate after anthesis, when floral volatile production starts (Figure 3c). This excludes a role for PhABCG1 in pigment transport/biosynthesis, since pigments are produced early in flower development (Martin and Gerats, 1993) and is in agreement with the developmental regulation of genes of the volatile benzenoid/phenylpropanoid pathway (Verdonk *et al*., 2003).

Like the emission of floral volatiles, transcription of volatile benzenoid/phenylpropanoid genes in petunia oscillates during the night and day (Colquhoun *et al.*, 2010b). Previously, micro-array analysis using Mitchell wildtype petals harvested 9h and 3h before the onset of the dark period (Verdonk *et al.*, 2003), revealed rhythmic transcript levels for genes encoding ODO1, 3-ketoacyl-Co thiolase 1 (KAT1), benzoyl-CoA:benzylaclohol/phenylethylalcohol benzoyl transfrerase (BPBT), phenyalanine ammonia lyase (PAL) 1/2, chorismate mutase (CM) and isoeugenol synthase (IGS), all of which have later been shown to be involved in floral fragrance biosynthesis (Negre *et al.*, 2003; Boatright *et al.*, 2004; Verdonk *et al.*, 2005; Koeduka *et al.*, 2006; Dexter *et al.*, 2007; Van Moerkercke *et al.*, 2009; Colquhoun *et al.*, 2010a). Importantly, *PhABCG1* transcript abundance oscillates in phase with that of *ODO1* (Figure 2b), which also cycles in phase with mRNA levels of *acetyl-CoA:coniferyl alcohol acetyltransferase* (*CFAT*) (Dexter *et al.*, 2007; Colquhoun *et al.*, 2010b), *PAL1* and *EPSPS* (Verdonk *et al.*, 2003). In flowers silenced for *ODO1*, *EPSPS* and *PAL1* mRNA levels were reduced (Verdonk *et al.*, 2005) and ODO1 is able to trans-activate the *EPSPS* promoter in petunia leaves (Verdonk *et al.*, 2005). The Mitchell *PhABCG1* promoter activity appears to be correlated with *ODO1* expression, since a transcriptional fusion construct is active in different *ODO1*-expressing petals, notably those of Mitchell and PaaN (Figure 3). The *PhABCG1* promoter cannot be activated in flowers of R27 (Figure 3) that do not express *ODO1* (Figure 4a). However, when *ODO1* is ectopically expressed in R27 petals, the Mitchell promoter can be activated (Figure 4d). In agreement with this, the promoter is active in a R27 x Mitchell F1 cross (Figure 3) and transcripts accumulate in petals of this line (Figure 2d), excluding a role for a dominant

negative regulator in R27 and suggesting that R27 petals lack an activator of *PhABCG1* expression. The link between *PhABCG1* expression and ODO1 was corroborated in two ways. First, *PhABCG1* transcripts accumulate in petals in which *ODO1* is over-expressed (Figure 4e), meaning that *PhABCG1* transcript levels depend, at least indirectly, on those of *ODO1*. Second, ectopically expressed *ODO1* can activate the *PhABCG1* promoter in tobacco leaves and petals of R27 (Figure 4c,d), showing the molecular link with *PhABCG1* regulation *in planta*.

The *PhABCG1* promoter contains a putative MYB binding site (TAACTA**T**CT) within a 39 bp perfect repeat (Figure 1a). This element differs in one nucleotide from that of the MYB.Ph3 binding site (TAACTA**A**CT) found in promoters of flavonoid genes (Solano *et al.*, 1995a). Mutating the adenosine to thymidin in this sequence (TAACTA**A**CT to TAACTA**T**CT) disrupts binding by MYB.Ph3 *in vitro* (Solano *et al.*, 1995a). Whether ODO1 binds to this element needs further investigation. Since the MYB.Ph3 site is also bound by the homolog of EOBII, AmMYB305 (Solano *et al.*, 1995a), we sought to investigate if EOBII could activate the *PhABCG1* promoter. Unlike ODO1, EOBII could not *trans*-activate the *PhABCG1* promoter in tobacco leaves (Figure 4c), which is in agreement with the lack of promoter activity in R27 petals, which express *EOBII* (**CHAPTER 3**). Why some of the *ODO1*-expressing, fragrant petunias, notably *P. axillaris axillaris* N. and the cv. V26, do not express *PhABCG1* is unclear. Attempts to PCR-isolate the V26 and PaaN promoters using different Mitchell-specific primers failed. The origin of the petunia line B1 is not known, but it may well have been derived from a Mitchell-relative. Hence, expression is seen in petals of this line (Figure 2d) as in the R27 x Mitchell cross.

Towards a function for the PhABCG1 protein

Most cell membrane-localised ABC-transporters in plants export compounds from the cytoplasm to the apoplast (Jasinski *et al.*, 2001; Rea, 2007), but importers of primary and secondary metabolites have been described in plants as well (Shitan *et al.*, 2003; Lee *et al.*, 2008; Kang *et al.*, 2010). The PhABCG1 protein localised to the plasma membrane of conical epidermal cells of petunia petals and epidermis cells of *N. benthamiana* leaves (Figure 5). This suggests

that PhABCG1 can form a homodimer but does not exclude heterodimerisation. For instance, the plasma membrane-localised AtABCG12 is retained in the ER in the absence of its obligate heterodimer partner AtABCG11, but G11 does not need G12 for plasma membrane-localisation (McFarlane *et al.*, 2010). Alternatively, a putative heterodimer partner of PhABCG1 is present in both cell types. This would mean that PhABCG1 is promiscuous to form heterodimers, a common feature of ABCG-proteins (Graf *et al*., 2003; McFarlane *et al.*, 2010), and that expression in the correct cell type is not a prerequisite for plasma membrane-localisation. In addition, the mVenus-ABCG1 fusion protein in conical cells was seen at the entire surface of the membrane (Figure 5g-i). Although experimentation showing localisation of floral fragrance biosynthesis is still fragmented, at least the final steps in volatile biosynthesis occur in epidermal cells of rose (Scalliet *et al.*, 2006; Bergougnoux *et al.*, 2007) and snapdragon (Kolosova *et al.*, 2001b; Long *et al.*, 2009). Therefore, PhABCG1 could be involved in volatile release from the epidermal surface in Mitchell. However, because we used a constitutive promoter to drive the mVenus-fusion in our localisation experiments, the cell layer in which *PhABCG1* is expressed remains unknown.

Petunia axillaris axillaris N. (PaaN) emits a subset of volatiles emitted by Mitchell (Verdonk *et al.*, 2003; Hoballah *et al.*, 2005) and does not express *PhABCG1* (Figure 2d). This led us initially to hypothesise the involvement of PhABCG1 in the emission of one or more Mitchell-specific volatile compounds. To investigate this, we stably silenced *PhABCG1* in Mitchell plants. Despites a slight increase for some compounds, we could not detect a significant change in emission of any compound in the silenced lines (Figure 7). Because the closest homolog in Arabidopsis is involved in culticle formation (Pighin *et al.*, 2004), we checked if the silenced flowers showed a deficit in their cuticles by means of a toluidine blue (TB)-test (Tanaka *et al.*, 2004). In both wild type and transgenic plants, the petals did not take up the TB, suggesting a functional cuticle in these petals (data not shown). Also, no visible morphological floral phenotype could be observed in the silenced lines. An in-depth study of the cutin and wax composition would, however, be needed to assess a small qualitative change in composition as a result of silencing. Goodwin *et al.* (2003) reported no clear association between MeBA emission and cuticle properties during flower

development in snapdragon. During petal development in snapdragon, the emission of MeBA is closely associated with internal pool size (Goodwin *et al.*, 2003), which determines the emission rate. This is also true for the rhythmic production and emission of this compound in snapdragon (Kolosova *et al.*, 2001a), but wax composition during the night and day was never investigated. If this can be extrapolated to petunia and generalised to other compounds is not known.

The only moderate silencing obtained by our approach (Figure 6) in addition to functional redundancy could explain the lack of a detectable emission phenotype. As an example, an *abcg13* null mutant in Arabidopsis shows floral organ fusion, but several lines displaying various degrees of *AtABCG13* downregulation did not display this phenotype (Panikashvili *et al.*, 2011). Likewise, stable silencing of *NpPDR2* in *N. plumbaginifolia* plants, did not result in a detectable phenotype under the tested conditions (Trombik *et al.*, 2008). Partial redundancy has been suggested for plant ABCG-transporters, including the full-size transporters AtABCG36/AtABCG37 (Ruzicka *et al.*, 2010) and the half-size transporters AtABCG11/AtABCG12 (Panikashvili *et al.*, 2007). Redundant ABC-transporters do not necessarily need to be related to each other. The human ABCB1 and ABCC1 transporters share 17% amino acid sequence identity but nevertheless show overlapping substrate specificity (Yazaki, 2006). In addition to active transport, trafficking of volatiles could also involve diffusion under specific conditions, which could partially compensate for the loss of active transport. For instance, the Arabidopsis cell membrane-localized ABCG40 transporter facilitates cellular uptake of ABA in addition to diffusion. The ratio between the two mechanisms is pH-dependent, with diffusion being the dominant mechanism under non-stress conditions, i.e. at physiological pH. This results in only 18% reduced ABA uptake in an *abcg40* knockout at pH 5.7, but up to 69% at pH 7 (Kang *et al.,* 2010). Related to this, a decrease in endogenous concentrations and an increase in emission of volatile compounds from flowers of petunia was reported for increasing temperatures from 20°C to 30°C (Sagae *et al.*, 2008). At lower temperatures, the ratio of the emitted and endogenous concentration between the compounds becomes more apparent. This means that at low temperatures, the emission of large compounds is more hampered than that of small compounds (Sagae *et al.*, 2008). The authors did not investigate

temperatures below 20°C, although in natural habitats of *Petunia* species, during the night when their pollinators are active, temperatures can drop far below 20°C. Lower temperatures would implicate reduced volatile biosynthesis due to lower enzymatic activities, but also reduced breakdown of products, and thus possibly reduced internal volatile pools, as well as lower evaporation rates and possible change in wax composition. It will be interesting to investigate floral emission at lower temperatures in both wild type and our transgenic lines.

Because of the above-mentioned observations, we set up to express *PhABCG1* in petals of PaaN. Interestingly, in these flowers, benzylbenzoate, and to a lesser extent phenylethylbenzoate and isoeugenol, accumulated more compared to control-infiltrated flowers (Figure 8b). Following infiltration, internal pools of other compounds could not be detected with our method used. Surprisingly, this increase did not result in an increase of these compounds in the headspace (Figure 8a). If any, internal levels showed a minor, though non-significant decrease, with exception of benzaldehyde that showed a non-significant increase. Accumulation of compounds could mean that *PhABCG1* is involved in the import of precursor compounds into epidermal cells. Alternatively, assuming promiscuity for PhABCG1 dimerisation, ectopic expression of *PhABCG1* could cause a dominant negative effect on volatile emission, thereby increasing the internal pools. In both cases it is unclear why the emission levels remained relatively unaffected. However, in lines silenced for *KAT1*, internal pools were much more affected then the concomitant emission levels as well (**CHAPTER 2**, Van Moerkercke *et al*., 2009).

In conclusion, *PhABCG1* is co-expressed with genes of the floral phenylpropanoid/benzenoid pathway suggesting a link with this pathway, but a function in a different process cannot be excluded from co-expression analysis alone (Kaneda *et al.*, 2011). However, we also show that a regulator of floral fragrance biosynthesis, ODO1, activates *PhABCG1*. Therefore, a function in this pathway is likely. Because ODO1 also regulates *EPSPS* expression, it is possible that PhABCG1 is involved in transport of precursors early in the pathway, but a role in volatile emission cannot be excluded. In addition, we cannot exclude a role for ODO1 that goes beyond volatile production. Cell-membrane localisation of PhABCG1 excludes a role in intracellular trafficking of compounds. Our results show that ectopically expressed *PhABCG1* influences

the internal concentration of the volatile benzenoid compound benzylbenzoate and putatively other compounds like phenyethylbenzoate and isoeugenol. This further corroborates the molecular link between *PhABCG1* and ODO1 and suggests its involvement in the volatile phenylpropanoid/benzenoid pathway. Direct transport assays in plant cell culture or yeast could be used to test PhABCG1 transport specificity towards these compounds. Once the cellular layer in which PhABCG1 acts is known, it will be of further interest to test if PhABCG1 acts as an importer or exporter. It will be needed to assess the internal compound accumulation in our silenced lines. Finally protein-protein interaction assays can identify putative dimer partners of PhABCG1.

EXPERIMENTAL PROCEDURES

Plant material and stable transformation

Petunia x hybrida cv. Mitchell (also referred to as cv. W115) wild type and transgenic plants, *P. hybrida* cultivars V26 and R27, *P. axillaris axillaris* N., petunia line B1 (Spitzer *et al*., 2007) and *Nicotiana benthamiana* were grown in standard greenhouse conditions (16h photoperiod, 300-400 $\mu mol.m^{-2}.s^{-1}$ light intensity, 60-65% humidity, and day/night temperatures of 22/17°C). Plants were moved to controlled growth chambers (16h photoperiod, 250-350 $\mu mol.m^{-2}.s^{-1}$ light intensity, 70% relative humidity and constant temperature of 21°C) at least 3 days prior to the experiments, except otherwise stated.

For stable transformation of Mitchell plants, *Agrobacterium tumefaciens* GV3101 (pMP90) was used (Koncz and Schell, 1986). Transgenic plants were obtained using a standard leaf disc transformation protocol (Horsch *et al*., 1985), with minor modifications as described in **CHAPTER 2**. Rooted T_0 plants were transferred to soil and screened for transgene integration by PCR using construct-specific primers. Next generation plants (T_1) were obtained by manual self-pollination and subsequent selection on half strength MS (pH 5.8) agarose (0.7%) plates containing 75 $mg.L^{-1}$ kanamycin.

Construct design

To create the *PhABCG1* hairpin-RNAi-construct (ir-*ABCG1*) a 565 bp fragment including the 3'-UTR of the cDNA, was PCR-amplified (forward primer: aaaaagcaggctgattgtagcaagcatggtc; reverse primer: agaaagctgggtggactcaacaaatatgcatc; underlined are the *PhABCG1*-specific nucleotides) to introduce the AttB sites in two rounds as described by the manufacterer (Invitrogen). A nested PCR was performed to introduce the full AttB sites using primers AttB1-F and AttB2-R (Invitrogen) and the resulted product was recombined into pDONR207 (Invitrogen).

The obtained plasmid was subsequently recombined with pK7GWIWG(I) (Karimi *et al.*, 2002), creating the hpRNAi construct. The PCR-amplified target region was sequenced before transformation to *A. tumefaciens* GV3101 (pMP90).

The promoter reporter construct was made using standard molecular cloning techniques. A 2005 bp fragment of the promoter including the 5`-UTR was PCR-amplified introducing an *Asc*I and a *Nco*I site, and cloned *Asc*I/*Nco*I upstream of an intron-containg *uidA* CDS (GUS) at the start codon. The p*ABCG1*:*GUS*-t*NOS* cassette was sequenced and subsequently cloned *Asc*I/*Ehe*I into pBINPLUS (van Engelen *et al*., 1994) pre-digested with *Asc*I and *Sma*I, creating the ABCG1:GUS reporter construct. The 35S:ODO1, 35S:EOBII, 35S:AN2 and 35:LUC have been described previously (Verdonk, 2006; **CHAPTER 3**) (Quattrocchio *et al.*, 1999).

The 35S-driven *PhABCG1* and mVenus-*ABCG1* constructs were created using multisite Gateway technology (Invitrogen). For both constructs, the *PhABCG1* genomic region including stop codon was PCR-amplified introducing the AttB recombination sites and subsequently recombined into pDONR201 (BP reaction; Invitrogen), creating ABCG1-pENTR. For the 35S:mVenus-ABCG1 fusion, ABCG1-pENTR was recombined into a destination vector (LR reaction), containing the CaMV 35S promoter, along with the entry clones pGEM Box1 mVenus (Nagai *et al.*, 2002) and pGEM Box3 tNos. For the 35S:ABCG1 construct, ABCG1-pENTR was recombined into a destination vector (LR reaction), containing the CaMV 35S promoter, along with the entry clone pGEM Box3 tNos.

Expression analyses

Two-day old flowers of *Petunia hybrida cv.* Mitchel wildtype and transgenic T_1 (T_0 selfings) plants were used for RNA gel-blot analyses. Independent sample collections for all experiments and transcript analyses were performed twice. For the time-course, 2-day-old wild-type petals were collected during a 30h period with 3h intervals. For the developmental course, wild-type petal tissue of flowers in various stages of development (1cm bud, 2 cm bud, 3 cm bud, 4 cm bud, flower opening, one-day-old open flower, three-day-old open flower and senescent flower) was collected 2h before the onset of the dark period. The different tissues were collected from wild-type plants 2h before the onset of the dark period. Two-day-old petals of transgenic and wild type plants were harvested at the onset of the dark period to assess transcript levels in the transgenic lines. Total RNA was extracted using TriZol (Invitrogen). Eight microgram was loaded on gel and the blots were hybridised using a *PhABCG1* specific probe (forward primer: 5'-ctcaattcatcaacctagtgttg-3' and reverse primer: 5'-ggtatttccaaaatggcttagg-3'). To visualise loading of the gel, the tissue blot was re-hybridised with an 18S probe (forward primer: 5'-agcaggctaaggtctcgt-3' and reverse primer: 5'- agcggatgttgcttttagga-3'). For the developmental course and time-course a petunia *floral binding protein 1* (*FBP1)* probe was used (forward primer: 5'-gtgttctttgtgatgctcgtg-3' and reverse primer: 5'-caacctctcctgcaaatttgg-3') to show loading of the gel.

For RT-PCR, wild-type petals of *P. axillaris, P. hybrida* cv. Mitchell, *P. hybrida* cv. R27, petunia line B1 and a R27 x Mitchell F1 cross were collected in winter in the greenhouse 1h before artificial illumination ceased. For the transient *ODO1*-overexpression experiment, petals of Mitchell were infiltrated with *A. tumefaciens* harbouring either a 35S:LUC or 35S:ODO1 construct and incubated for 36h and sampled 12h before the onset of the artificial dark period. Total RNA was extracted using the Qiagen Plant RNA extraction kit and 1 μg was used for cDNA synthesis in a 20 μL reaction volume after DNase (Invitrogen) treatment to remove contaminating gDNA. Semi-quantitative RT-PCR was performed on 10x diluted cDNA samples using a standard PCR program and Taq polymerase (DreamTaq, Fermentas). Twenty cycles were performed using forward primer 5'-ctcaattcatcaacctagtgttg-3' and reverse primer 5'-ggtatttccaaaatggcttagg-3' for *ABCG1,* primers 5'- gttggtggtagctgagagtcag-3' and 5'-gactctaagcaaatctaacttcc-3' for *ODO1* and primers 5'-gaaaggcccggcgccattc-3' and 5'-gaagggccacacccttaggt-3' for *LUC*. Amplification of *FBP1* (22 cycles) was used to show the amount of input cDNA, using forward primer 5'-gtgttctttgtgatgctcgtg-3' and reverse primer 5'-caacctctcctgcaaatttgg-3'.

Genomic DNA extraction and promoter isolation

A 2kbp promoter region of the *PhABCG1* gene was isolated as described for the *ODO1* promoter in **CHAPTER3** using *PhABCG1*-specific primers**.**

Promoter analyses and *trans*-activation assays using agroinfiltration

Greenhouse-grown *Petunia hybrida* cv. Mitchell and R27, *P. axillaris axillaris* N. and a R27 x Mitchell F1 cross were used for promoter analyses. *A. tumefaciens* harbouring the ABCG1:GUS construct were used and promoter analyses in petals were performed as described in **CHAPTER 3**.

For *trans*-activation assays, the same reporter construct was used. A 35S-driven ODO1 (Verdonk *et al*., 2006) was used as effector. As a control, 35S:RFP, 35S:AN2 (Quattrocchio *et al*., 1999) or 35S:EOBII (**CHAPTER 3**) were used. Trans-activation assays in *N. benthamiana* leaves were performed as described in **CHAPTER 3**. For *trans*-activation in R27 petals, *A. tumefaciens* were infiltrated at OD_{600} = 0.15. Infiltrated tissue was harvested for RT-PCR and GUS analyses.

Headspace and GC-MS analyses

Volatile sampling of wild type and transgenic flowers was performed as in **CHAPTER 2**. In short, flowers were detached and volatiles of three flowers per desiccator were trapped on Tenax for 24h (n = 3). Volatiles were eluted with a pentane:ether (4:1 v/v) mixture spiked with *p*-cymene. GC-MS analysis was performed as in **CHAPTER 2**.

Transient expression of petals

Petals of attached flowers of *P. axillaris axillaris* N. were infiltrated with *A. tumefaciens* harbouring a 35S:ABCG1 construct and incubated for 48h. As a control infiltration, flowers were infiltrated with 35S:LUC-containing *A. tumefaciens*. Infiltration of petals was performed as described in **CHAPTER 3**. Two detached infiltrated flowers per desiccator were used to trap volatiles for 24h, starting 2h before the onset of the dark period (n = 4). The experiment was performed only once. For internal compound analysis, infiltrated petals were frozen in liquid N_2 1h before the onset of the dark period. Homogenised tissue was extracted in hexane spiked with *p*-cymene and concentrated before analysis (n = 4). GC-MS analysis was performed as in **CHAPTER 2**. The experiment was performed twice.

Petals of Mitchell were infiltrated with *A. tumefaciens* harbouring a 35S:ODO1 or 35S:LUC construct and incubated for 48h before tissue collection at a time when *ODO1* and *PhABCG1* expression were low (3h in the light period).

Confocal microscopy

Petunia hybrida cv. Mitchell petals and *Nicotiana benthamiana* leaves were observed using a 20x water objective with a Nikon Eclipse T1 microscope coupled to an Nikon A1 confocal scanning head. mVENUS fluorescence was monitored with a 525-555nm band pass emission filter (488 nm excitation) and the FM4-64 (Molecular probes, Invitrogen) was visualized with a 570-620nm band pass emission filter (488 nm excitation). The images were processed by ImageJ (http://rsb.info.nih.gov/ij/) and assembled in MS Power Point. Petals were infiltrated and incubated 48h. Flowers were taken for analysis during the light period.

Toluidine blue (TB) test

The TB test for cuticle analysis was adopted from Tanaka *et al.* (2004). Wild type and transgenic flowers were detached and submerged in several aqueous solutions of TB ranging from 0.05 to 0.5% (w/v) for 5 min, after which excess of TB was removed with distilled water. Alternatively, flowers were vacuum infiltrated with an aqueous solution of 0.5% TB for 2 min.

Statistical analyses

Statistical analyses were performed using SPSS. Analysis of Variance (ANOVA) and least significant differences *post-hoc* analysis were used for all experiments unless otherwise stated.

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