PLANT SCIENCE

Emission of volatile organic compounds from petunia flowers is facilitated by an ABC transporter

Funmilayo Adebesin,[1] Joshua R. Widhalm,[1,2]* Benoît Boachon,[1] François Lefèvre,[3] Baptiste Pierman,[3] Joseph H. Lynch,[1] Iftekhar Alam,[3]† Bruna Junqueira,[3] Ryan Benke,[1] Shaunak Ray,[4] Justin A. Porter,[5] Makoto Yanagisawa,[6] Hazel Y. Wetzstein,[5] John A. Morgan,[1,4] Marc Boutry,[3] Robert C. Schuurink,[7] Natalia Dudareva[1,2,5]‡

Plants synthesize a diversity of volatile molecules that are important for reproduction and defense, serve as practical products for humans, and influence atmospheric chemistry and climate. Despite progress in deciphering plant volatile biosynthesis, their release from the cell has been poorly understood. The default assumption has been that volatiles passively diffuse out of cells. By characterization of a *Petunia hybrida* adenosine triphosphate–binding cassette (ABC) transporter, PhABCG1, we demonstrate that passage of volatiles across the plasma membrane relies on active transport. *PhABCG1* down-regulation by RNA interference results in decreased emission of volatiles, which accumulate to toxic levels in the plasma membrane. This study provides direct proof of a biologically mediated mechanism of volatile emission.

Plants can direct up to 10% of photosynthetically fixed carbon toward biosynthesis of volatile organic compounds (VOCs) (*1*), which are low-molecular-weight lipophilic molecules (~100 to 200 Da) with high vapor pressures at ambient temperature. Plant VOCs are chemically diverse and mainly represented by terpenoids, fatty acid derivatives, benzenoids, and phenylpropanoids. VOCs play essential roles in pollinator and seed-disperser attraction, above- and belowground defense against herbivores and pathogens, plant-plant signaling, and allelopathy (*2*, *3*). They also provide protection against abiotic factors such as high light, temperature, and oxidative stress (*4*). Moreover, emitted plant VOCs contribute to the production of secondary organic aerosols that act as cloud condensation nuclei, thus affecting atmospheric chemistry and climate (*5*, *6*). Global annual emission of plant benzenoids alone is estimated to be millions of tons, leading to the formation of up to 10 Tg y^{-1} of aerosols (*6*). Plant VOCs are also of value to the floriculture, flavor, cosmetic, and fragrance industries.

In flowers and roots, VOCs are primarily produced in epidermal cells, from which they are released into the atmosphere (*7*, *8*) and rhizosphere (*9*), respectively. In vegetative organs, VOCs are synthesized in glandular trichomes (e.g., in tomato, basil, and peppermint) (*10*) and reach the atmosphere through mechanical disruption, or they are made in the leaf mesophyll (*11*) and exit through the stomata (*12*) or cuticle (*13*). Irrespective of the biosynthetic site and emission route, at the subcellular level, VOCs must traverse the cytosol and cross the plasma membrane, hydrophilic cell wall, and sometimes the cuticle to exit the cell. It has been an open question how VOCs cross these barriers in intact cells to reach the environment or intercellular air spaces connected to stomata.

The default assumption that VOCs simply diffuse out of cells (*14*–*17*) may be true for small VOCs such as isoprene. However, modeling has predicted that emission of most VOCs driven solely by diffusion would lead to toxic VOC accumulation in membranes as a result of preferential partitioning of these compounds into lipid bilayers (*17*). Such accumulation would cause reorganization of cellular membranes that could lead to leakage of organellar or cellular content (*18*). This raises the hypothesis that VOC emission requires active transport (*17*).

We used *Petunia hybrida* flowers, which emit benzenoid and phenylpropanoid volatiles in a temporally regulated manner, to investigate whether VOC emission relies on protein-mediated export. Because certain nonvolatile hydrophobic compounds, such as waxes and diterpenes, are exported across the plasma membrane by adenosine triphosphate–binding cassette (ABC) transporters (*19*, *20*), we searched petunia petal RNA sequencing (RNA-seq) data sets (*21*) for ABC transporter transcript sequences. We found one highly expressed candidate that was up-regulated 103-fold in petunia flowers between day −1 (bud stage) and day 2 after opening (postanthesis), the developmental stages with the lowest and highest VOC emission, respectively (*22*) (fig. S1). This gene encodes *Petunia hybrida* ABC subfamily G member 1, PhABCG1, a predicted plasma-membrane transporter of unknown function that is expressed almost exclusively in petals of open flowers and regulated by the ODORANT1 transcription factor (*23*), which controls VOC biosynthesis in petunia flowers (*24*).

To determine whether PhABCG1 is involved in VOC emission, we generated transgenic petunia RNA interference (RNAi) lines under control of the petal-specific linalool synthase promoter (*25*) to decrease *PhABCG1* expression (fig. S2). Three independent lines with *PhABCG1* transcript levels reduced by 70 to 80% (Fig. 1A) exhibited 52 to 62% decreases in total VOC emission (Fig. 1B), with a concomitant 101 to 157% increase in total internal VOC pools (Fig. 1B). In *PhABCG1*-RNAi flowers, emission of each individual VOC was reduced to a different extent (fig. S3A), and their corresponding internal pools increased (fig. S3B). Amounts of alcohol glycosides, representing 13% of the total VOC internal pool, were ~2.5 times as high in transgenic as in wild-type flowers (fig. S3C).

To directly test for transport activity, we expressed His-StrepII–tagged PhABCG1 (fig. S4) in *Nicotiana tabacum* Bright Yellow 2 (BY-2) suspension cells. Of 50 screened transformants, the majority gave a positive signal close to the expected molecular mass (75.2 kDa), as detected with antibodies against the His tag (fig. S5). Plasma membranes prepared from microsomal fractions were enriched in both PhABCG1 and the plasma-membrane marker H^+-dependent adenosine triphosphatase (fig. S6). As a half-size ABC transporter, PhABCG1 possesses a single membrane domain and a single nucleotide-binding domain and may function as a homo- or heterodimer (*19*). Because no other half-size ABCG genes were up-regulated in flowers as much as *PhABCG1* (fig. S1), we hypothesized that PhABCG1 functions as a homodimer. When subjected to size-exclusion chromatography, PhABCG1 (fig. S7) elutes before the 158-kDa marker (fig. S8), consistent with the predicted homodimer size (2 × 75.2 kDa) in a detergent micelle (~70 kDa). To test for PhABCG1 transport activity, we chose methylbenzoate and benzyl alcohol as substrates because they are major VOC constituents emitted by petunia flowers (*22*). On incubation of control and transgenic BY-2 lines expressing *PhABCG1* with ^{14}C-labeled substrates, we measured cell-associated radioactivity corresponding to the difference between passive diffusion into and active transport out of the cells. The observed decrease in cell-associated radioactivity in transgenic BY-2 cells relative to control cells demonstrates that PhABCG1 transports methylbenzoate and benzyl alcohol out of cells (Fig. 2, A and C). Two additional transgenic BY-2

[1]Department of Biochemistry, Purdue University, West Lafayette, IN 47907, USA. [2]Purdue Center for Plant Biology, Purdue University, West Lafayette, IN 47907, USA. [3]Institut des Sciences de la Vie, Université catholique de Louvain, Croix du Sud 4-5, Box L7-04-14, 1348 Louvain-la-Neuve, Belgium. [4]School of Chemical Engineering, Purdue University, 480 Stadium Mall Drive, West Lafayette, IN 47907-2100, USA. [5]Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907, USA. [6]Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907-2054, USA. [7]Department of Plant Physiology, University of Amsterdam, Swammerdam Institute for Life Sciences, Science Park 904, 1098 XH Amsterdam, Netherlands.
*Present address: Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907, USA.
†Present address: Plant Biotechnology Division, National Institute of Biotechnology, Ganakbari, Savar, Dhaka 1349, Bangladesh.
‡Corresponding author. Email: dudareva@purdue.edu

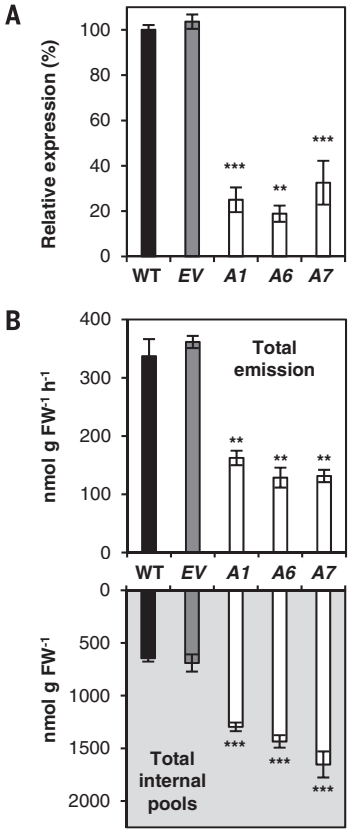

Fig. 1. Effect of *PhABCG1* down-regulation on VOC emission and internal pools in petunia flowers. (**A**) *PhABCG1* mRNA levels determined by quantitative reverse transcription polymerase chain reaction on day 2 postanthesis in wild-type (WT), empty vector control (EV), and three independent *PhABCG1*-RNAi lines (A1, A6, and A7). (**B**) Total VOC emission rate and internal pools from WT, EV, and *PhABCG1*-RNAi flowers on day 2 postanthesis. Volatile emission was collected from 18:00 to 22:00 hours (local time). Internal pools were measured at 22:00 hours. All data are means ± SEM (n = 3). $^{**}P < 0.01$; $^{***}P < 0.001$ (Student's t test). FW, fresh weight; h, hours.

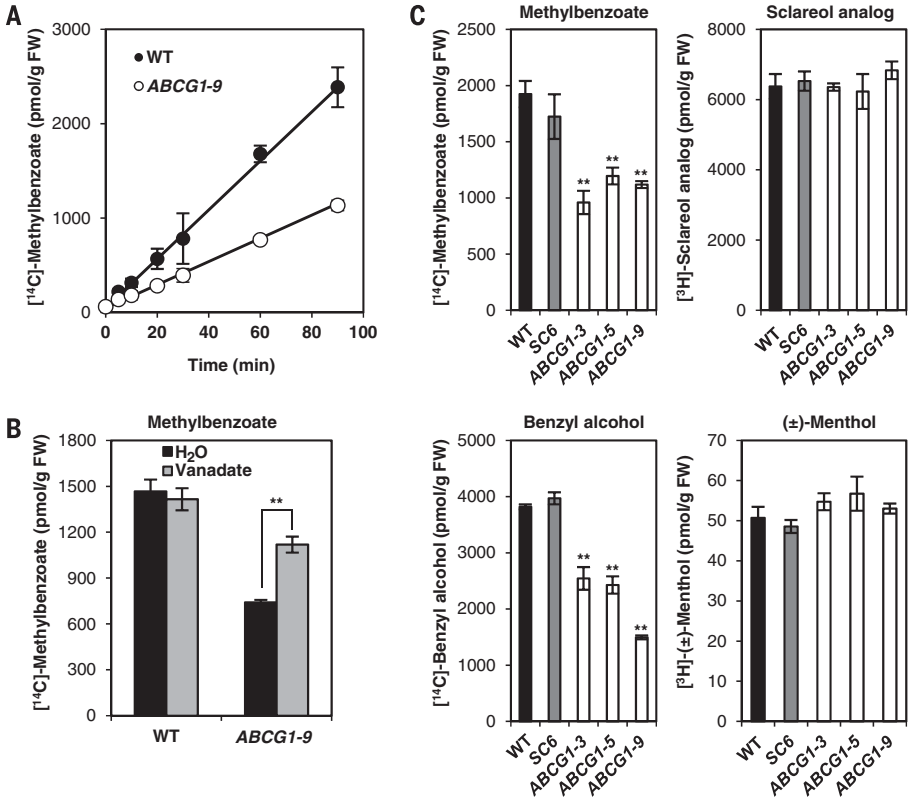

Fig. 2. Transport assays of *PhABCG1* expressed in *N. tabacum* BY-2 suspension cells. (**A**) WT and *PhABCG1*-expressing (ABCG1-9) BY-2 cells were incubated with [^{14}C]methylbenzoate. After the indicated time, cells were harvested and radioactivity was measured. (**B**) WT and ABCG1-9 BY-2 cells were incubated for 60 min with [^{14}C]methylbenzoate in the presence or absence of vanadate. (**C**) Transport assays with cells from the WT line, a line expressing an antibody (SC6) as a negative control, and three *PhABCG1*-expressing lines (ABCG1-3, ABCG1-5, and ABCG1-9) with radiolabeled methylbenzoate, benzyl alcohol, menthol, and a sclareol analog. Incubation time was 60 min for each compound. Values are means ± SEM (n = 3). $^{**}P < 0.01$ (Student's t test).

lines exhibited similar results (Fig. 2C). The antibody-expressing BY-2 cell line SC6 (*26*), used as a negative control, behaved like wild-type cells. Vanadate, a known inhibitor of ABC transporters, increased methylbenzoate retention by 51% in a *PhABCG1*-expressing BY-2 line, confirming the active nature of transport (Fig. 2B). Transport assays with labeled monoterpene menthol and an analog of the diterpene sclareol [^{3}H-decahydro-2-hydroxy-2,5,5,8a-tetramethyl-1-naphthalene ethanol, a substrate of the *N. tabacum* NtPDR1 transporter (*20*)] revealed no difference between *PhABCG1*-expressing and wild-type BY-2 cells (Fig. 2C). We conclude that PhABCG1 transports phenylpropanoid and benzenoid compounds.

We identified 16 additional ABCG transporter candidates in our RNA-seq data sets (fig. S1). All had lower transcript levels than *PhABCG1* on day 2 postanthesis, and only two candidates (*Ph4681* and *Ph5139*) exhibited up-regulation on day 2 versus day −1, (fig. S1, A and B). Whereas expression of *Ph4681* was found to be 0.5% that of *PhABCG1*, expression of *Ph5139* was 17% that of *PhABCG1*. However, transient RNAi down-regulation of *Ph5139* had no effects on VOC emission or internal pools (fig. S9).

Phylogenetic analysis of all petunia flower ABCG transporters identified by RNA-seq and half-size *Arabidopsis thaliana* transporters with known function revealed PhABCG1 to have one closely related petunia protein, Ph19708 (65% amino acid identity), and no *Arabidopsis* ortholog (fig. S10). *Ph19708* expression was 0.2% that of *PhABCG1* (fig. S1), indicating that it has little, if any, contribution to VOC emission. Two additional PhABCG1 homologs (Ph13519 and Ph9795, sharing ~45% amino acid identity with PhABCG1) clustered with three *Arabidopsis* ABCG transporters known for transporting wax precursors (AtABCG11, AtABCG12, and AtABCG13) (*27*) and clustered separately from PhABCG1 and Ph19708 (fig. S10). To check whether PhABCG1 contributes to wax precursor transport, we analyzed wax in wild-type and *PhABCG1*-RNAi flowers (fig. S11). We found no statistically significant differences in wax composition, quantity, and thickness, indicating that PhABCG1 is not involved in export of wax constituents.

To test our model prediction that accumulated VOCs have detrimental effects on membrane integrity (*17*), we stained petunia petals of 2-day-old wild-type and *PhABCG1*-RNAi flowers with two compounds having different modes of action. The first, propidium iodide, diffuses into cells and stains nucleic acids only if the plasma membrane is disrupted (*28*). The second, fluorescein diacetate, passes through intact cell membranes and remains colorless until the acetate moiety is removed by intracellular esterases. The deacetylated fluorescein is fluorescent and unable to pass through intact cellular membranes (*29*). We found that in *PhABCG1*-RNAi petals, propidium iodide stained nuclei, consistent with damage to the plasma membrane by accumulated VOCs (Fig. 3A). Mimicking this effect, wild-type flowers exhibited similar

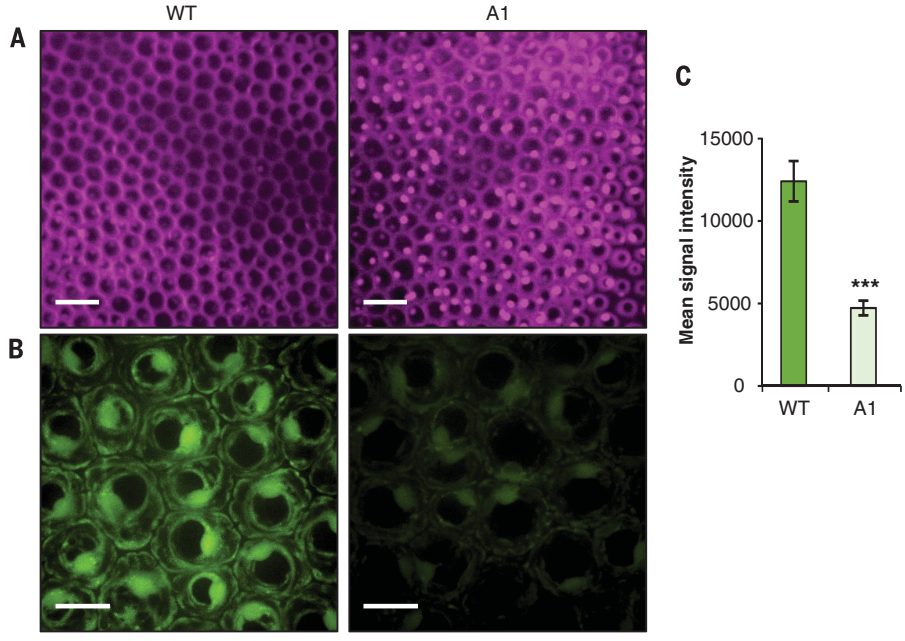

Fig. 3. Effect of *PhABCG1* down-regulation on cell membrane integrity in petunia flowers. (**A**) Confocal microscopy images of 2-day-old WT and *PhABCG1* transgenic flowers stained with propidium iodide (scale bars, 50 μM). (**B**) Confocal microscopy images of 2-day-old WT and *PhABCG1* transgenic flowers stained with fluorescein diacetate (scale bars, 20 μM). (**C**) Quantification of fluorescence intensity of fluorescein in WT and PhABCG1 A1 lines stained with fluorescein diacetate. Values are means ± SE (n = 19). ***P < 0.001 (Student's t test).

staining when fed with high concentrations (15 to 30 mM) of benzaldehyde (fig. S12). We also found less fluorescein staining in *PhABCG1*-RNAi petals (Fig. 3B), providing independent evidence for disruption of the cell membrane. Flowers from *PhABCG1*-RNAi lines were smaller and had reduced fresh weight relative to controls (fig. S13). However, scanning electron microscopy of the epidermal conical cells of transgenic and wild-type petals revealed no difference in basal diameter of cells or cell shape (fig. S14). This suggests that differentiation and expansion of these cells take place before VOC accumulation and thus are unaffected.

Here we show that VOC emission from petunia flowers depends not on passive diffusion but rather on protein-mediated transport across the plasma membrane. Transporters may regulate emission of VOCs in other plants and in organisms throughout other kingdoms of life, where VOCs play important roles in chemical communication.

REFERENCES AND NOTES

1. J. Peñuelas, J. Llusià, *Trends Ecol. Evol.* **19**, 402–404 (2004).
2. N. Dudareva, F. Negre, D. A. Nagegowda, I. Orlova, *Crit. Rev. Plant Sci.* **25**, 417–440 (2006).
3. E. Pichersky, J. Gershenzon, *Curr. Opin. Plant Biol.* **5**, 237–243 (2002).
4. F. Loreto, J. P. Schnitzler, *Trends Plant Sci.* **15**, 154–166 (2010).
5. D. F. Zhao *et al.*, *Nat. Commun.* **8**, 14067 (2017).
6. P. K. Misztal *et al.*, *Sci. Rep.* **5**, 12064 (2015).
7. N. Kolosova, D. Sherman, D. Karlson, N. Dudareva, *Plant Physiol.* **126**, 956–964 (2001).
8. G. Scalliet *et al.*, *Plant Physiol.* **140**, 18–29 (2006).
9. F. Chen *et al.*, *Plant Physiol.* **135**, 1956–1966 (2004).
10. J. J. Glas *et al.*, *Int. J. Mol. Sci.* **13**, 17077–17103 (2012).
11. G. Cinege, S. Louis, R. Hänsch, J. P. Schnitzler, *Plant Mol. Biol.* **69**, 593–604 (2009).
12. U. Niinemets, F. Loreto, M. Reichstein, *Trends Plant Sci.* **9**, 180–186 (2004).
13. U. Niinemets, M. Reichstein, M. Staudt, G. Seufert, J. D. Tenhunen, *Plant Physiol.* **130**, 1371–1385 (2002).
14. E. Pichersky, J. P. Noel, N. Dudareva, *Science* **311**, 808–811 (2006).
15. M. Borghi, A. R. Fernie, F. P. Schiestl, H. J. Bouwmeester, *Trends Plant Sci.* **22**, 338–350 (2017).
16. M. C. Schuman, H. A. Valim, Y. Joo, in *Deciphering Chemical Language of Plant Communication*, J. D. Blande, R. Glinwood, Eds. (Springer, 2016), pp. 3–34.
17. J. R. Widhalm, R. Jaini, J. A. Morgan, N. Dudareva, *Trends Plant Sci.* **20**, 545–550 (2015).
18. S. A. Mendanha, A. Alonso, *Biophys. Chem.* **198**, 45–54 (2015).
19. H. E. McFarlane, J. J. H. Shin, D. A. Bird, A. L. Samuels, *Plant Cell* **22**, 3066–3075 (2010).
20. J. Crouzet *et al.*, *Plant Mol. Biol.* **82**, 181–192 (2013).
21. J. R. Widhalm *et al.*, *Nat. Commun.* **6**, 8142 (2015).
22. J. C. Verdonk *et al.*, *Phytochemistry* **62**, 997–1008 (2003).
23. A. Van Moerkercke, C. S. Galván-Ampudia, J. C. Verdonk, M. A. Haring, R. C. Schuurink, *J. Exp. Bot.* **63**, 3157–3171 (2012).
24. J. C. Verdonk, M. A. Haring, A. J. van Tunen, R. C. Schuurink, *Plant Cell* **17**, 1612–1624 (2005).
25. L. Cseke, N. Dudareva, E. Pichersky, *Mol. Biol. Evol.* **15**, 1491–1498 (1998).
26. S. Mercx, J. Tollet, B. Magy, C. Navarre, M. Boutry, *Front. Plant Sci.* **7**, 40 (2016).
27. J. Kang *et al.*, *Arabidopsis Book* **9**, e0153 (2011).
28. N. Rolny, L. Costa, C. Carrión, J. J. Guiamet, *Plant Physiol. Biochem.* **49**, 1220–1227 (2011).
29. M. Krasnow, M. Matthews, K. Shackel, *J. Exp. Bot.* **59**, 849–859 (2008).

ACKNOWLEDGMENTS

This work was supported by grant IOS-1655438 from the National Science Foundation to J.A.M. and N.D. and in part by grants from the Belgian National Fund for Scientific Research and the Interuniversity Poles of Attraction Program (Belgium). M.Y. was supported by NSF grant MCB-1121893 to D. B. Szymanski. We thank J. Nader for excellent technical help and A. Withrow for assisting with transmission electron microscopy. The supplementary materials contain additional data.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/356/6345/1386/suppl/DC1
Materials and Methods
Figs. S1 to S15
References (*30*–*50*)

1 March 2017; accepted 22 May 2017
10.1126/science.aan0826

Emission of volatile organic compounds from petunia flowers is facilitated by an ABC transporter

Funmilayo Adebesin, Joshua R. Widhalm, Benoît Boachon, François Lefèvre, Baptiste Pierman, Joseph H. Lynch, Iftekhar Alam, Bruna Junqueira, Ryan Benke, Shaunak Ray, Justin A. Porter, Makoto Yanagisawa, Hazel Y. Wetzstein, John A. Morgan, Marc Boutry, Robert C. Schuurink and Natalia Dudareva

Science **356** (6345), 1386-1388.
DOI: 10.1126/science.aan0826

Active transport of aromas

Volatile organic compounds (VOCs) serve as invisible lines of communication among host plants, pathogens, commensals, community groups, and, with flowers, their pollinators. Studying petunia flowers, Adebesin *et al.* show that VOCs do not passively diffuse out of the cells but are actively shuttled across the plasma membrane by an ABC (ATP-binding cassette) transporter (see the Perspective by Eberl and Gershenzon). Disabling the transporter results in damage to the cell's membranes by intracellular accumulation of VOCs.

Science, this issue p. 1386; see also p. 1334