Supporting Information

Martin et al. 10.1073/pnas.0901387106

SI Materials and Methods

Plant Material. Dormant *V. vinifera* L. Cabernet Sauvignon canes (clone 15 grafted on rootstock 101–14) containing 6 nodes were harvested from Bull Pine Estate Vineyards (Vincor), Osoyoos, BC in January 2007 and 2008 and kept at 4 °C before rooting. Plants were grown as described by Mullins and Rajasekaran (1) with the following modifications. Before rooting, the bottom internode was scored with a razor blade and then dipped in a solution of 0.5% indole-3-acetic acid. Canes were rooted in a peat–vermiculite mixture (1:1) and maintained at 25 °C, while the aerial portions were kept in darkness at 4 °C. After approximately 4 weeks, rooted canes were planted in peat–vermiculite (1:1) and kept in a greenhouse with supplemental lighting of 800 μM m^{-2} s^{-1} for 16 h. To facilitate introduction to the volatile collection system, the flower bud was allowed to develop from either the second or third node from the top of the plant, and leaves were allowed develop from the node located 2 nodes below the flower bud. Development of the flower bud was encouraged by selectively pruning vegetative growth, as described by Mullins and Rajasekaran (1). The lower bud was allowed to develop until 10 leaves had been produced, at which point the shoot apical meristem was removed and axillary buds were quelled. No other shoots were allowed to develop.

Volatile Collection. An automated volatile collection system (Analytical Research Systems) was used as described previously (2). Before the plants were introduced to the collection system, the flowers on each inflorescence were counted. Plants were allowed to acclimate to the chamber for at least 2 days before the start of volatile collections. Each 24-hour cycle included three 5-hour collections during the light period followed by one 9-hour collection during the dark period. Volatiles were collected on SuperQ absorbent material (Alltech) and eluted with 0.5 mL of pentane containing 2.5 μg/mL of isobutylbenzene as an internal standard.

Extraction of Flower and Pollen Volatiles. For each stage (V to bloom), 10 flowers were separated into anthers, caps, and pistils/pedicels, and the individual flower parts were immediately submerged in 0.5 mL of pentane containing 2.0 μg/mL of isobutylbenzene as an internal standard. Because stage IV flowers were too small (1–2 mm long) for dissection, 10 intact flowers were submersed in pentane and then crushed with forceps to allow extraction. Extraction was performed by gentle shaking for 16 h at 4 °C. After extraction, the pentane fraction was removed, and 2 μL was analyzed by GCMS.

The Dobson protocol for pollen extraction (3) was modified as follows. To release pollen from anther sacs and to extract any volatiles in the pollenkitt, anthers from stage V and VI flowers (30 flowers per analysis) were sonicated in a Branson digital sonifier for <20 s in 0.5 mL of pentane at 10% power. The pentane fraction was then immediately removed, and 0.5 mL of methanol:chloroform (1:2) was added to another 24-h extraction of compounds from within the pollen grain. Both the pentane and the methanol:chloroform contained isobutylbenzene as an internal standard. Methanol:chloroform extracts were filtered although glass wool before analysis by GCMS.

GCMS Analysis. GCMS analysis was performed as described previously (4). For each analysis, 1 μL of sample was injected. The temperature program was as follows: 40 °C followed by 7 °C/min up to 100 °C, 2 °C/min up to 180 °C, and 10 °C/min up to 250 °C, and held for 2 min at this temperature. Compounds were identified by a combination of matching retention indices with library matches (Wiley7Nist05; ref. 5) and authentic standards when available.

Recombinant Protein Enzyme Assays. *E. coli* cultures harboring the VvValCS pET28b plasmid were grown for 16 h before induction and 6 h after induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside at 22 °C. Recombinant proteins were purified using His SpinTrap columns (GE Biosciences) in accordance with the manufacturer's directions. For functional enzyme characterization, single-vial assays were used as described previously (6, 7). In brief, 50–100 μg of the purified protein was added to 450 μL of terpene synthase assay buffer [50 mM Hepes, 10 μM $MnCl_2$, 7.5 mM $MgCl_2$, 10% glycerol (vol/vol), and 5 mM DTT] containing 92 μM FPP and overlaid with 500 μL of pentane containing 2.5 μg/mL of isobutylbenzene as an internal standard. Assays were incubated at 30 °C for 2 h before vortexing and centrifuging. Products were analyzed by GCMS as described above.

Protein Extraction for Western Blot Analysis. For intact flowers and berries, 15 mg of powdered frozen plant tissue was added to 40 μL of 4× lithium dodecyl sulfate loading buffer (Invitrogen) containing 60 μL of 0.5 M DTT. Protein was extracted by vortexing and incubation at 90 °C for 5 min. After centrifugation (15,000 × *g* for 1 min), 15 μL of the extract was loaded onto 12.5% SDS/PAGE gel used for silver staining and Western blot analysis. For individual flower parts, 30 flowers for each of 3 stages (V, VI, and bloom) were dissected into anthers, caps, and pistils and pedicels while kept frozen on dry ice. Total protein was extracted as described by Holmes-Davis et al. (8). In brief, the samples were homogenized in 2-mL centrifuge tubes using a plastic tissue homogenizer or a mortar and pestle (pistils only). After the addition of 300 μL of extraction buffer [200 mM Tris-HCl (pH 8.8), 4% SDS, 20% glycerol, and 80 mM DTT], the samples were boiled for 5 min with periodic vortexing before being centrifuged at 21,000 × *g* for 20 min at 4 °C. The supernatant was transferred to a 1.5-mL tube, after which proteins were precipitated by adding 1.2 mL of acetone. Precipitated proteins were dried for 16 h in a vacuum evaporator. The pellet was then disrupted with a pipette and washed 4 times with 1.2 mL of 80% acetone, followed by centrifugation at 21,000 × *g* for 15 min at 4 °C. After drying, the pellets were resuspended in 30 μL of 4× lithium dodecyl sulfate loading buffer (Invitrogen) and boiled for 5 min. For each protein gel, 10 μL was loaded.

Immunoblot analysis. SDS/PAGE gels were run for 2 h at 100 V. The gels were incubated with transfer buffer (Tris, M glycine, 20% methanol, and 0.1% SDS) for 20 min with gentle agitation. PVDF membranes were wetted in methanol (10 s) and then equilibrated in transfer buffer. A semidry transfer cell (Fisher Scientific) was used to transfer proteins (3.5 h, 13 V). Transfer efficiency was monitored by Ponceau S staining before overnight blocking with 5% skim milk in Tris-buffered saline with Tween-20 (TBST) at 4 °C overnight. Rabbit polyclonal VvVal antibodies were made against a synthetic peptide (EDLKKEVKRKLTAAC) made by Sigma-Genosys (Sigma-Aldrich). This peptide was chosen because it is predicted to be highly antigenic, is located close to the N terminus of the protein, and does not overlap with any of the other TPS we have cloned

in-house. Antibodies were affinity-purified before being used for immunostaining and immunolocalization. Monoclonal plant anti-actin [clone 10-B3 (MAbGPa)] produced in mice was used as a control for Western blot analysis from plant extracts. Incubation with anti-VvVal (1/200 dilution in TBST and 5% skim milk) was done for 1 h at 4 °C. Membranes were washed 4 times for 15 min each time at room temperature with TBST and 5% skim milk before being incubated for 1 h at room temperature with either Alexa 488 goat anti-rabbit (Invitrogen) or phosphatase-conjugated goat anti-rabbit alkaline (Sigma-Aldrich), both at 1/10,000 dilution. After secondary antibody incubation, membranes were washed once for 15 min with 5% skim milk, followed by four 15-min washes with TBST at room temperature. Alexa secondary antibodies were visualized using a Pharox FX Plus molecular imager (Bio-Rad). Visualization of alkaline phosphatase–conjugated secondary antibodies was done with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NCT) (Calbiochem) in the dark for 15 min.

Immunohistochemistry and Fluorescence Microscopy. Flower tissues were fixed and embedded as described previously (9). In brief, flowers were submerged in fixation buffer overnight at 4 °C with gentle agitation. Samples were then dehydrated in an ethanol series before infiltration with L.R. White acrylic resin (EM Sciences) over a 4-day period. Resin was cured overnight under vacuum at 60 °C. Serial cross-sections (2.5 μm thick) were sectioned using a vibrotome (model UC6 EM; Leica Microsystems) fitted with a Diatome Histo diamond knife and transferred in water to wells on hydrophobic slides (EM Sciences). Water was evaporated from the slides with a heat block (60 °C).

Sections were blocked by incubation with TBST and 5% skim milk for 1 h at room temperature. One slide was used for immunolabeling with either VvVal antibody (1/30 in TBST and 5% skim milk) or preimmune serum (similar concentration as α-VvVal and 1/30 in TBST and 5% skim milk) for 1 h at room temperature. Slides were washed 4 times for 15 min in TBST, followed by a 1-h incubation in Alexa 488 goat anti-rabbit (Invitrogen) diluted 1/100 in TBST and 5% skim milk. Slides were then washed as before, incubated for 1 min with a 1/100 dilution of 332 Hoechst (Calbiochem), and then rinsed in H_2O. After drying, coverslips were mounted using Fluoromount-G (SouthernBiotech).

Quantitative RT-PCR. Flowers of prebloom stages I–VI and bloom were used for RNA isolation, cDNA synthesis, and comparative Ct RT-PCR analysis as described by Reid et al. (10). The primers used to amplify VvVal cDNA were (agaggagcagattgaggatctga/aacagcagcagccgttaatttc), and the primers for the stably expressed endogenous control, GAPDH, were as described previously (10). All reactions were performed in triplicate on 2 independent biological replicates using 5 ng of cDNA. Dissociation curves, gel electrophoresis analysis, and cloning of the reaction product demonstrated the specificity of the PCR (data not shown). No-template controls also were included in these analyses. Primer efficiencies were determined using LinRegPCR (11), and all were found to exceed 1.8. Relative gene expression was calculated using these efficiencies, the Ct values determined by SDS version 1.2.2 software (Applied Biosystems), and the equation described by Pfaffl (12). The lowest level of detectable gene expression (at bloom) was used as a calibrator for these experiments.

1. Mullins MG, Rajasekaran K (1981) Fruiting cuttings: Revised method for producing test-plants of grapevine cultivars. *Am J Enol Viticult* 32:35–40.
2. Arimura G, Huber DP, Bohlmann J (2004) Forest tent caterpillars (*Malacosoma disstria*) induce local and systemic diurnal emissions of terpenoid volatiles in hybrid poplar (*Populus trichocarpa x deltoides*). *Plant J* 37:603–616.
3. Dobson HEM, Bergstroem G, Groth I (1990) Differences in fragrance chemistry between flower parts of *Rosa rugosa* thunb (Rosaceae). *Isr J Bot* 39:143–156.
4. Martin DM, Gershenzon J, Bohlmann J (2003) Induction of volatile terpene biosynthesis and diurnal emission by methyl jasmonate in foliage of Norway spruce. *Plant Physiol* 132:1586–1599.
5. Adams RP (2007) *Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy* (Allured, Carol Stream, IL).
6. Keeling CI, Weisshaar S, Lin RP, Bohlmann J (2008) Functional plasticity of paralogous diterpene synthases involved in conifer defense. *Proc Natl Acad Sci U S A* 105:1085–1090.
7. O'Maille PE, Chappell J, Noel JP (2004) A single-vial analytical and quantitative gas chromatography–mass spectrometry assay for terpene synthases. *Anal Biochem* 335:210–217.
8. Holmes-Davis R, Tanaka CK, Vensel WH, Hurkman WJ, McCormick S (2005) Proteome mapping of mature pollen of *Arabidopsis thaliana*. *Proteomics* 5:4864–4884.
9. Hudgins JW, Ralph SG, Franceschi VR, Bohlmann J (2006) Ethylene in induced conifer defense. *Planta* 224:865–877.
10. Reid KE, Olsson N, Schlosser J, Peng F, Lund ST (2006) An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. *BMC Plant Biol* 6:27.
11. Ramakers C, Ruijter JM, Deprez RH, Moorman AF (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* 339:62–66.
12. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45.

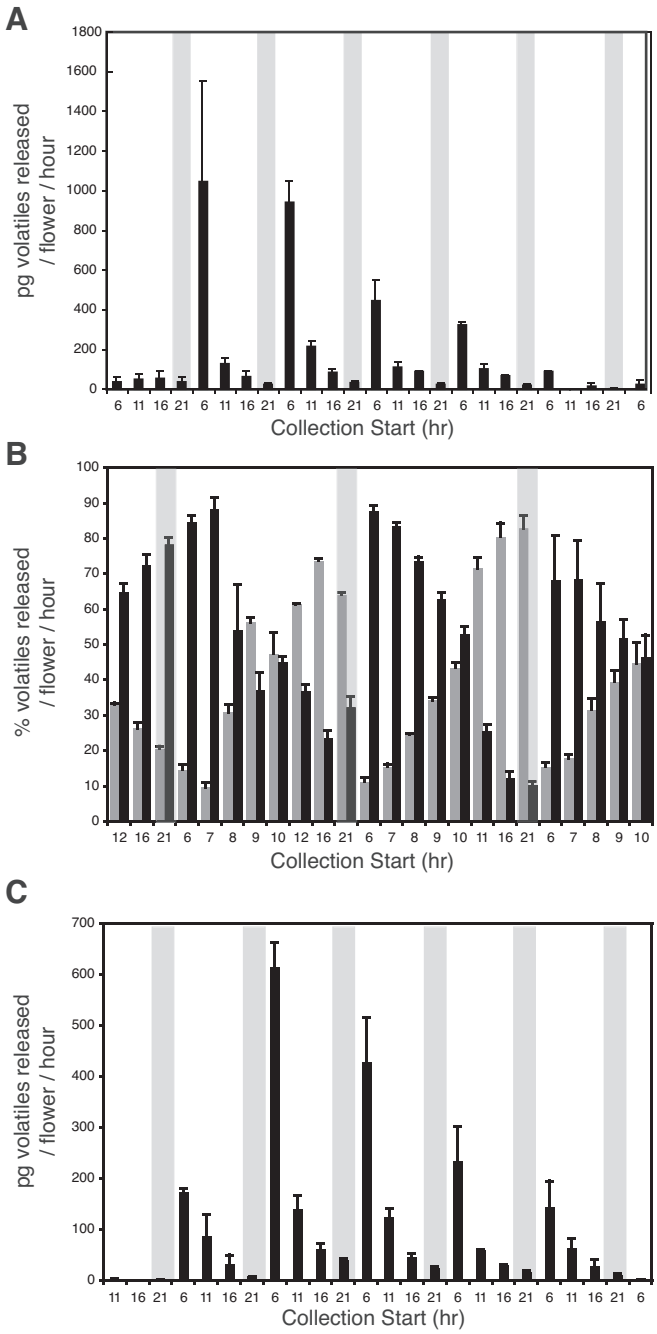

Fig. S1. Time courses of volatile emissions from grapevine flowers at bloom. Data represent the amount of volatiles detected by GCMS. Gray bars illustrate the period of darkness for each experiment. (*A*) The majority of volatiles were emitted over a 6-day period. Blooming began on the second day of the time course. Data points are the average of 5 replicates + SEM. (*B*) Relative abundance (percent) for the 2 major types of compounds emitted. Sesquiterpenes (black bars) constitute the majority of volatiles emitted during daylight hours, whereas aliphatics (gray bars) are more prevalent at night. Bars represent 2–4 replicates + SE + SEM. (*C*) Volatile emissions under constant temperature (22 °C) showing the same pattern as in (*A*).

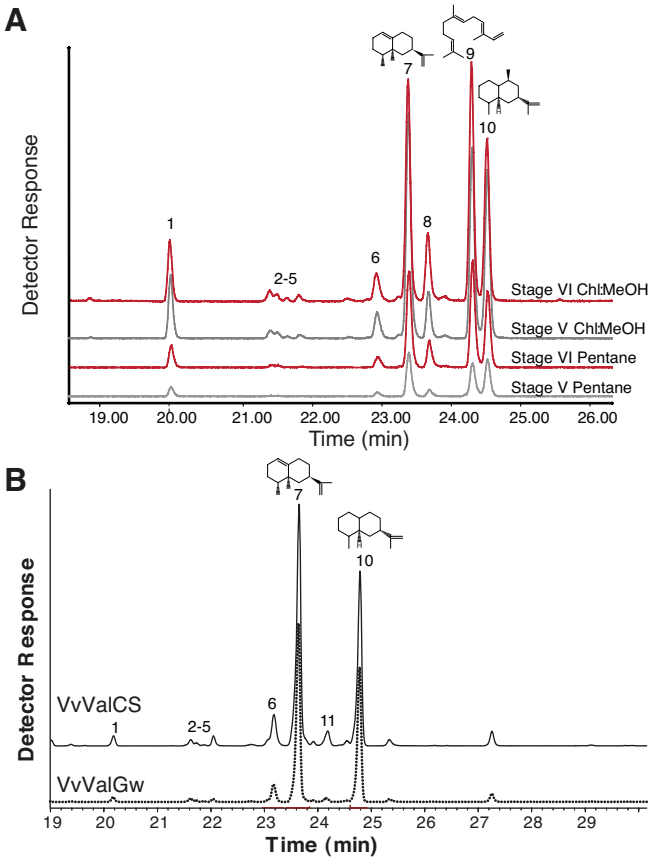

Fig. S2. GCMS analysis of volatile compounds from pollen extracts and VvVal enzyme assays. (*A*) Total ion chromatographs of volatile compounds extracted from stage V and VI pollen grains. Pentane extractions represent the volatiles accumulated in the pollenkitt. Chloroform/methanol extractions show the volatiles present within the pollen grain. Compounds are as follows: E-β-caryophyllene (1), spirolepechinene (2)*, α-humulene (3), unknown sesquiterpene (4), E-β-farnesene (5), selina-4,11-diene* (6), (+)-valencene (7), tridecanone/cis-α-bergomatene (8), E,E-α-farnesene (9), and (–)-7-epi-α-selinene (10). Tentative designations are labeled with an asterisk. Structures of major compounds shown from left to right include (+)-valencene, E,E-α-farnesene, and (–)-7-epi-α-selinene. (*B*) GCMS total ion chromatograph of products formed by VvValCS (solid line) or VvValGw (dashed line) when incubated with FPP . Compounds are as follows: E-β-caryophyllene (1), spirolepechinene (2)*, α-humulene (3), unknown sesquiterpene (4), E-β-farnesene (5), selina-4,11-diene* (6), (+)-valencene (7), (–)-7-epi-α-selinene (10), and Z-γ-bisabolene (11). Tentative designations are labeled with an asterisk. Structures of major compounds shown from left to right include (+)-valencene and (–)-7-epi-α-selinene.

Fig. S3. Amino acid alignment of VvVal from grapevine varieties Gewürztraminer (VvVal Gw) and Cabernet Sauvignon (VvValCS). The only 2 aa differences are shown in gray. The black highlighting indicates the sequence of the cysteine-conjugated peptide used for antibody production.

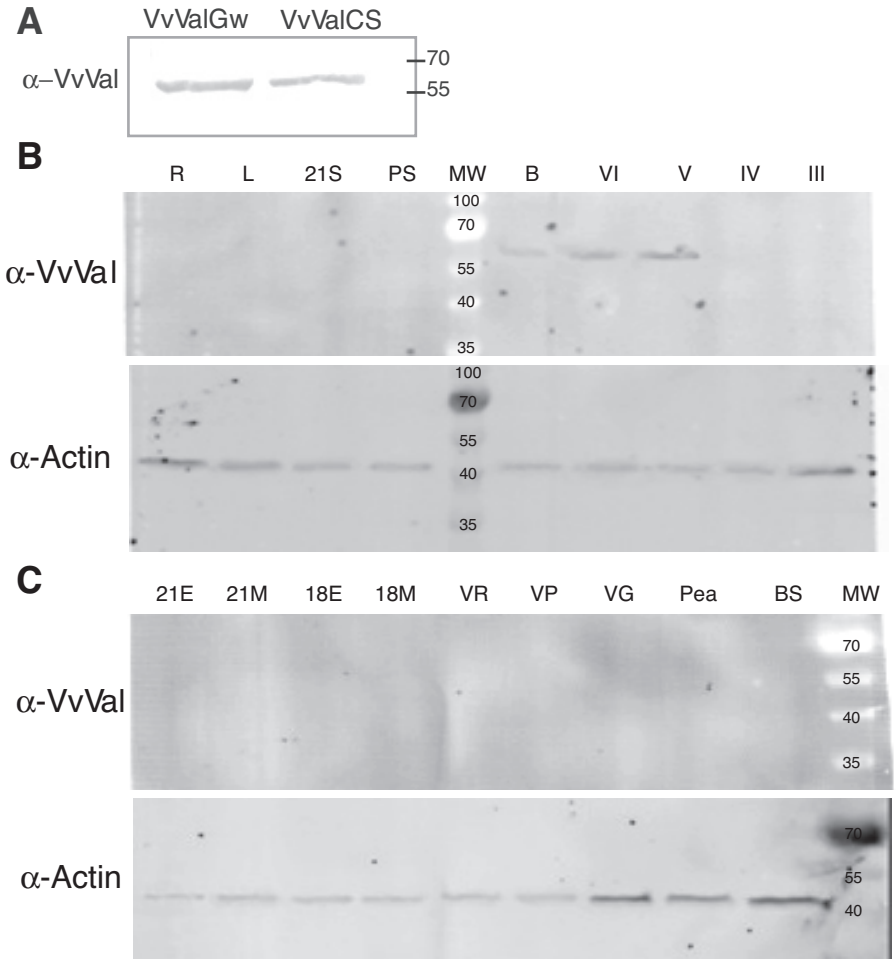

Fig. S4. Immunoblot analysis of VvVal protein expression. (*A*) Immunoblot specificity of anti-VvVal for recombinant VvVal and VvValCS. Protein gels were loaded equally with 2 μg of His spin column–purified expressed protein. (*B*) Immunoblot showing presence of VvVal in stages V, VI, and bloom of flower tissues. No detection of VvVal protein was seen in flowers of stages III and IV or in roots (R), leaves (L), seeds from pea-size berries (PS), or seeds from berries at 21 Brix (21S). Anti-actin controls are included to show equal loading. (*C*) No VvVal protein was detected in berries in the following stages: berry set (BS), pea size (Pea), veraison green (VG), veraison pink (VP), veraison red (VR), 18 Brix exocarp (18E), 18 Brix mesocarp (18M), 21 Brix exocarp (21E), and 21 Brix mesocarp (21M). Anti-actin controls are specified below.

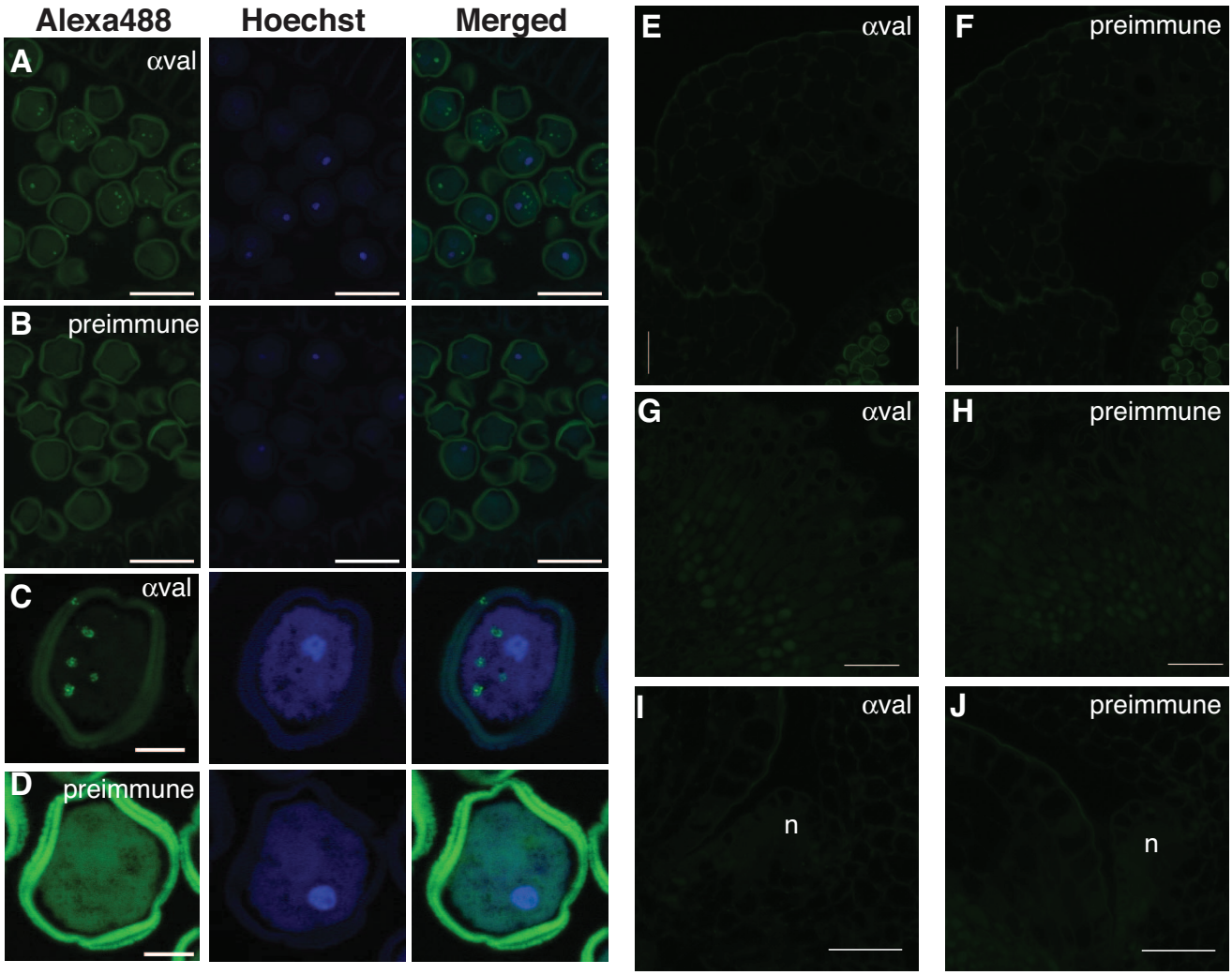

Fig. S5. Immunofluorescence localization of VvValCS protein to the pollen grains of stage VI flowers. (*A–D*) The first of the 3 images in a given row shows fluorescence from VvVal primary antibody with Alexa-488 secondary, the second image in the same row shows the same section with 332 Hoechst staining of nuclei, and the third image shows the 2 channels overlaid. VvVal is detected in the images with discrete green clusters. Diffuse autofluoresence is present for the blue and green channels for all treatments. (*A* and *B*) Images of a section of an anther locule containing pollen grains. VvVal is localized primary inside the stage VI pollen grains. Some label is found on the edges of the pollen grain in the intine/exine, but little label is found on the outside of the pollen grains or in other parts of the anther locule. (Scale bar = 50 μm.) (*C* and *D*) High-magnification images (63×) of representative pollen grains from stage VI. Most the label is present within the pollen grain, although some label is seen on the inner edges of the pollen grains as well. (Scale bar = 10 μm.) (*E–J*) Images of additional flower organs stained with either α-VvVal or preimmune. Similar autofluorescence is seen in both α-VvVal and preimmune images. (Scale bars = 50 μm.) (*E* and *F*) The cap. Distinct clusters of fluorescences are seen only in the pollen grains of the α-VvVal image. (*G* and *H*) Longitudinal section of the top of the stigma. (*I* and *J*) Longitudinal section of the lower flower, including the nectary (n).

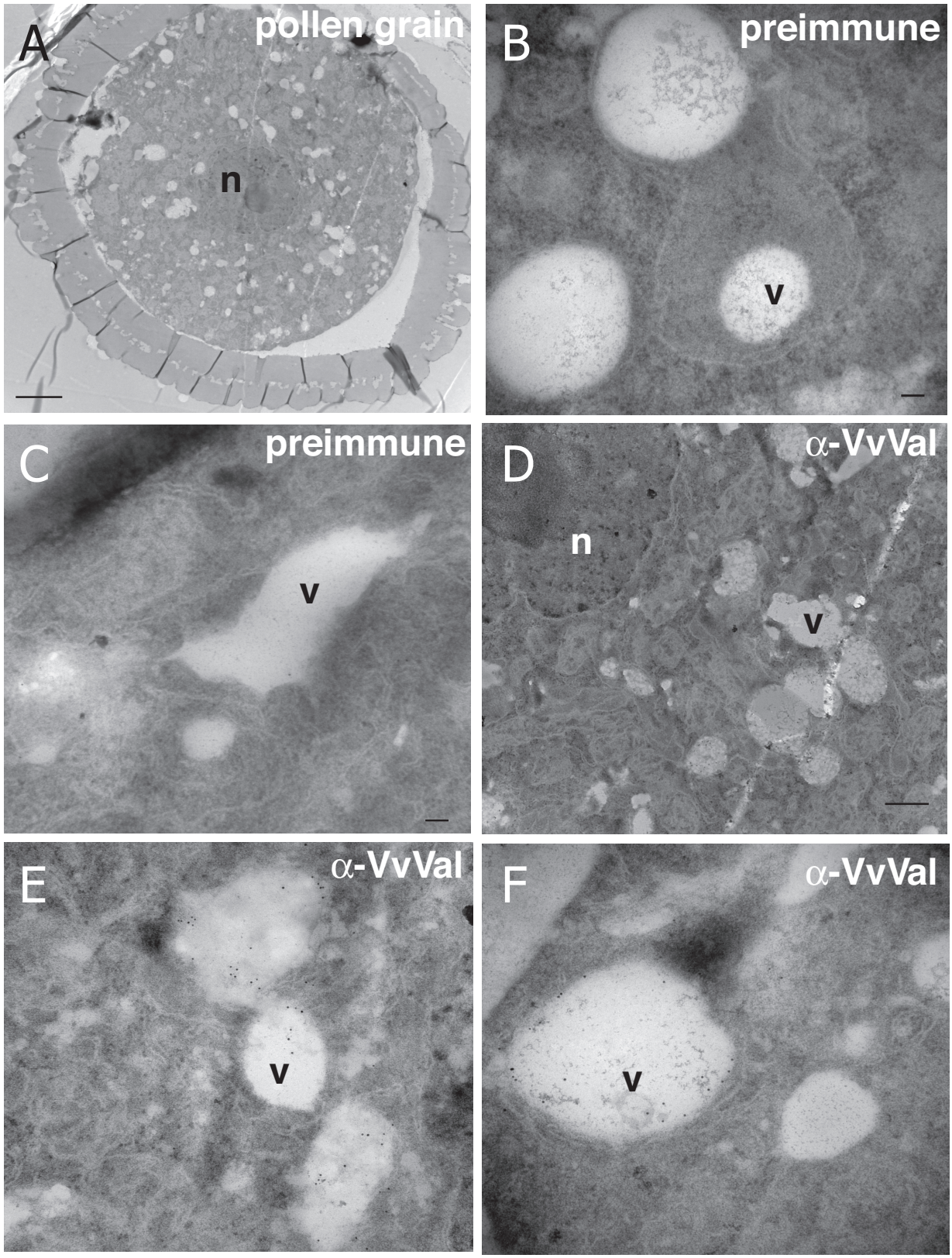

Fig. S6. (*A*) TEM showing a pollen grain with a single nucleus (n) visible. (Scale bar = 2 μm.) (*B–F*) Lipid vesicles (v) can be seen in all sections. In several areas, these structures seem to be fusing (arrows). Immunogold labeling of α-VvVal is seen as tiny round black dots. Most of the label is present on the outer edges of these lipid vesicles; no label is seen in the preimmune control. [Scale bar = 500 nm for (*B*), (*C*), (*E*), (*F*) and 100 nm for (*D*).]

Table S1. Percentage of each compound released between 0600 and 0700 hours, the maximum period of volatile release based on 2 individuals

Compound	Percentage	± SEM
7-Tetradecene	0.34	0.18
E-β-caryophyllene (1)	6.70	2.22
Spirolepechinene (2)*	1.35	0.29
α-Humulene (3)	0.88	0.20
Unknown sesquiterpene (4)	0.29	0.06
E-β-farnesene (5)	0.90	0.06
Spiro-(4,5)-decane*	0.27	
Gurjunene*	0.39	0.03
Selina-4,11-diene* (6)	4.22	0.55
E-β-ionone	0.50	0.00
(+)-Valencene (7)	23.00	2.11
Tridecanone/cis-a-bergomatene (8)	9.47	1.19
Pentadecane	3.29	0.08
E,E-α-farnesene (9)	22.17	1.60
(–)-7-epi-α-selinene (10)	15.55	0.93
Unknown sesquiterpene	0.06	0.06
Caryophyllene oxide*	0.22	0.05
6E,8E-hexadecadiene	0.24	0.24
1,3-Cyclooctadiene	0.10	0.10
Hexadecene	5.68	1.54
2-Pentadecanone	1.88	1.22
n-Hexadecanol	0.38	0.11
Nonadecane	0.77	0.33
Total	98.65	

*Tentative identifications.

Table S2. Blooming, determined by the number of caps that fell

Day	% Bloom	% Total volatiles/hour/flower
1	1.5	0.5
2	21	7.2
3	50	63.0
4	98	29.3

Numbers represent averages of 2 individuals.