The Plant Cell, Vol. 20: 1152–1168, April 2008, www.plantcell.org © 2008 American Society of Plant Biologists

Identification and Regulation of TPS04/GES, an *Arabidopsis* Geranyllinalool Synthase Catalyzing the First Step in the Formation of the Insect-Induced Volatile C_{16}-Homoterpene TMTT [W]

Marco Herde,[a] Katrin Gärtner,[a] Tobias G. Köllner,[b] Benjamin Fode,[a] Wilhelm Boland,[c] Jonathan Gershenzon,[b] Christiane Gatz,[a,1] and Dorothea Tholl[d]

[a] Albrecht-von-Haller-Institute for Plant Sciences, Georg-August-University Göttingen, D-37073 Göttingen, Germany
[b] Department of Biochemistry, Max-Planck-Institute for Chemical Ecology, D-07745 Jena, Germany
[c] Department of Bioorganic Chemistry, Max-Planck-Institute for Chemical Ecology, D-07745 Jena, Germany
[d] Department of Biological Sciences, Virginia Polytechnic and State University, Blacksburg, Virginia 24061

Volatile secondary metabolites emitted by plants contribute to plant–plant, plant–fungus, and plant–insect interactions. The C_{16}-homoterpene TMTT (for 4,8,12-trimethyltrideca-1,3,7,11-tetraene) is emitted after herbivore attack by a wide variety of plant species, including *Arabidopsis thaliana*, and is assumed to play a role in attracting predators or parasitoids of herbivores. TMTT has been suggested to be formed as a degradation product of the diterpene alcohol (*E,E*)-geranyllinalool. Here, we report the identification of Terpene Synthase 04 (*TPS04*; *At1g61120*) as a geranyllinalool synthase (GES). Recombinant TPS04/GES protein expressed in *Escherichia coli* catalyzes the formation of (*E,E*)-geranyllinalool from the substrate geranylgeranyl diphosphate. Transgenic *Arabidopsis* lines carrying T-DNA insertions in the *TPS04* locus are deficient in (*E,E*)-geranyllinalool and TMTT synthesis, a phenotype that can be complemented by expressing the *GES* gene under the control of a heterologous promoter. *GES* transcription is upregulated under conditions that induce (*E,E*)-geranyllinalool and TMTT synthesis, including infestation of plants with larvae of the moth *Plutella xylostella* and treatment with the fungal peptide alamethicin or the octadecanoid mimic coronalon. Induction requires jasmonic acid but is independent from salicylic acid or ethylene. This study paves the ground to address the contribution of TMTT in ecological interactions and to elucidate the signaling network that regulates TMTT synthesis.

INTRODUCTION

The majority of the millions of insect species on Earth feed on plants to obtain their nutrients. Consequently, plants have evolved efficient direct and indirect defense mechanisms to protect themselves against herbivorous insects (Baldwin and Preston, 1999; Sabelis et al., 2001; Ament et al., 2004). In direct defense responses, herbivore performance is affected by toxic or antinutritional plant metabolites. In indirect defense responses, plants attract natural enemies of herbivores, most commonly by the release of volatiles upon herbivore damage. These tritrophic interactions increase the plant's fitness and therefore are evolutionarily advantageous (Dicke and Sabelis, 1989; Van Loon et al., 2000; Fritzsche-Hoballah and Turlings, 2001; Kessler and Baldwin, 2001).

The C_{16}-homoterpene TMTT (for 4,8,12-trimethyltrideca-1,3,7,11-tetraene) is an herbivore-induced volatile that is emitted from a number of plants, including maize (*Zea mays*), lima bean (*Phaseolus lunatus*), and tomato (*Solanum lycopersicum*) (Hopke et al., 1994; Ament et al., 2004; Williams et al., 2005). Several studies suggest an ecological role of TMTT with respect to the attraction of herbivore enemies. For instance, carnivorous predatory mites (*Phytoseiulus persimilis*) preferred the odor source of lima bean plants attacked by spider mites (*Tetranychus urticae*) to the odor of plants damaged by beet armyworm (*Spodoptera exigua*) larvae (de Boer et al., 2004). Two pieces of evidence suggest that TMTT influenced the foraging behavior of the predatory mites. First, volatiles released by the spider mite–infested plants contained larger amounts of TMTT than volatiles from plants damaged by beet armyworm larvae. Second, when TMTT was added to the odor of beet armyworm–attacked plants, predatory mites preferred this odor to that of spider mite–infested plants. Likewise, tomato plants released larger amounts of TMTT after spider mite infestation than control plants (Kant et al., 2004). This increase in volatile production coincided with the increased olfactory preference of predatory mites for infested plants, suggesting that TMTT emission in tomato is likely to play a similar role in the attraction of predatory mites as in lima bean. In addition, TMTT induces the expression of defense genes in lima bean, indicating that it also might play a role in plant–plant interactions (Arimura et al., 2000).

[1] Address correspondence to cgatz@gwdg.de.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plancell.org) is: Christiane Gatz (cgatz@gwdg.de).
[W] Online version contains Web-only data.
www.plantcell.org/cgi/doi/10.1105/tpc.106.049478

In recent years, *Arabidopsis thaliana* was shown to be a suitable model plant for investigating tritrophic interactions between plants, herbivores, and their enemies. For example, the feeding of caterpillars of the crucifer pest *Pieris rapae* resulted in the emission of TMTT and other volatiles by *Arabidopsis* that attracted the parasitoid wasp *Cotesia rubecula* (Van Poecke et al., 2001). The parasitization of *P. rapae* caterpillars by *C. rubecula* resulted in an increase in plant fitness in terms of seed production (Van Loon et al., 2000). Olfactometer experiments with transgenic *Arabidopsis* constitutively emitting the C_{11}-homoterpene 4,8-dimethyl-1,3,7-nonatriene (DMNT), a TMTT-related compound, showed attraction of the predatory mite *P. persimilis* (Kappers et al., 2005).

Previous studies by Boland and coworkers (1998) have shown that the C_{16}-homoterpene TMTT is derived from the diterpene (C_{20}) alcohol (*E,E*)-geranyllinalool. Feeding of deuterium-labeled (*E*,*E*)-geranyllinalool to lima bean leaves resulted in the conversion of this precursor to TMTT, suggesting a sequential enzymatic oxidative degradation of (*E*,*E*)-geranyllinalool to TMTT (Figure 1) (Gäbler et al., 1991). Recently, Ament et al. (2006) reported a stringent correlation between the synthesis of (*E,E*)-geranyllinalool and TMTT in tomato leaves in response to treatment with jasmonic acid (JA). It can be proposed that (*E,E*)-geranyllinalool is formed from geranylgeranyl diphosphate (GGPP), the central prenyl diphosphate intermediate in diterpene synthesis, via the activity of a diterpene synthase (geranyllinalool synthase [GES]), which catalyzes the hydrolysis of the diphosphate moiety and allylic rearrangement to give a tertiary alcohol with a terminal olefin (Figure 1). Terpene synthase (TPS) reactions analogous to the formation of (*E,E*)-geranyllinalool are mediated by linalool synthase (LIS) from *Clarkia breweri* (Pichersky et al., 1994, 1995) and (*E*)-nerolidol synthases (NES) from maize (Degenhardt and Gershenzon, 2000), cucumber (*Cucumis sativus*), and lima bean (Bouwmeester et al., 1999). Rather than converting the GES substrate, GGPP, these enzymes carry out the analogous conversion of the C_{10}- and the C_{15}-prenyl diphosphate intermediates, geranyl diphosphate (GPP) and farnesyl diphosphate (FPP), into linalool and (*E*)-nerolidol, respectively, of which nerolidol is further degraded into DMNT. Whereas several sequences for LISs and NESs are available from different species (Dudareva et al., 1996; Schnee et al., 2002; Aharoni et al., 2004; Martin et al., 2004), a gene encoding GES has not yet been identified.

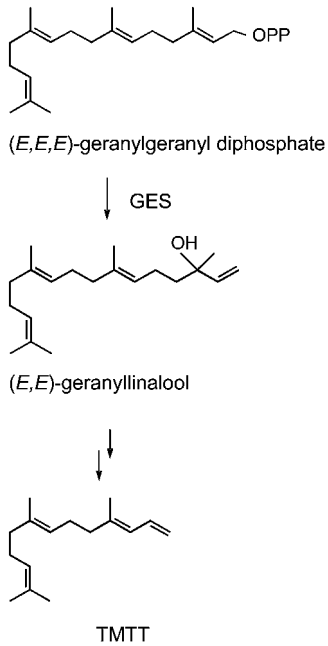

Figure 1. Proposed Biosynthesis of the Volatile C_{16}-Homoterpene TMTT.

GES is proposed to catalyze the formation of (*E,E*)-geranyllinalool from the substrate all-*trans*-GGPP, the central precursor in diterpene biosynthesis. (*E,E*)-Geranyllinalool is further converted into TMTT by uncharacterized sequential steps of oxidative degradation (Boland et al., 1998).

Here, we present biochemical and genetic evidence that *At1g61120* encodes a *GES* gene in *Arabidopsis*, thus specifying an important genetic component of herbivore-induced indirect defense systems. Regulation of *GES* transcription relies on the octadecanoid-dependent signaling pathway and is not modified by salicylic acid (SA) or ethylene.

RESULTS

TMTT Synthesis after Treatment of *Arabidopsis* Plants with Alamethicin

Our strategy to identify the *GES* gene from *Arabidopsis* was to determine conditions that would yield relatively high levels of TMTT and to screen the 32 potential *TPS* genes annotated in the *Arabidopsis* genome (Aubourg et al., 2002) for inducible expression. In lima bean, TMTT synthesis can be induced efficiently by alamethicin, a peptide mixture from the fungus *Trichoderma viride* that forms ion channels in membranes (Engelberth et al., 2001). To test whether alamethicin induces a similar response in *Arabidopsis* leaves, the 12 most expanded rosette leaves were detached from 5- to 6-week-old plants and incubated for 30 h with their petioles submerged in an aqueous solution of alamethicin. Volatiles were continuously collected during the first and second light periods (0 to 9 h and 21 to 30 h after the beginning of alamethicin treatment, respectively) using a closed-loop stripping method as described previously (Boland et al., 1984; Donath and Boland, 1995; Tholl et al., 2005). Three major induced volatile constituents were detected by gas chromatography–mass spectrometry (GC-MS) in both light periods: methyl salicylate (MeSA), the sesquiterpene (*E,E*)-α-farnesene, and the C_{16}-homoterpene TMTT (Figure 2; see Supplemental Figure 1 online). Also, minor amounts of the presumed TMTT precursor (*E*,*E*)-geranyllinalool were emitted from induced leaves, as confirmed by mass spectrometry using a synthetic (*E*,*E*)-geranyllinalool standard (see Supplemental Figure 2A online).

Identification of Inducible TPS Transcripts

To identify candidate *GES* genes, RT-PCR analysis was performed on RNA of plants induced for 30 h with alamethicin using primers against 32 of the putative *TPS* cDNAs of the *Arabidopsis*

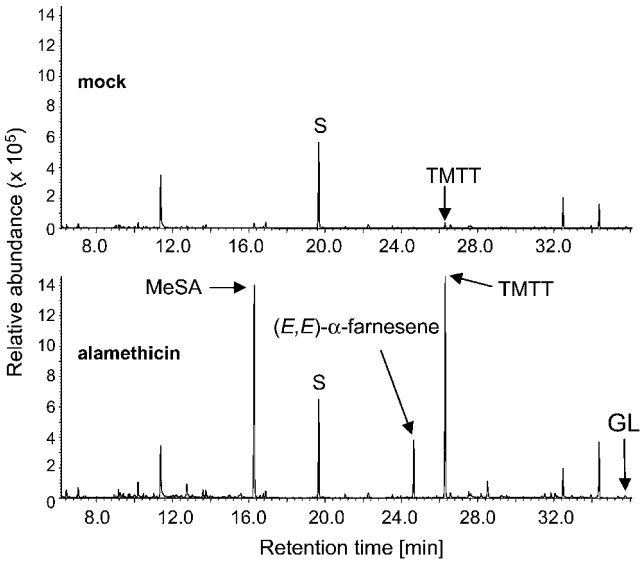

Figure 2. Induced Emission of (*E,E*)-Geranyllinalool and TMTT from *Arabidopsis* Leaves in Response to Treatment with the Fungal Elicitor Alamethicin.

Total ion GC-MS chromatograms of volatiles emitted from mock-treated (0.1% ethanol; top panel) and alamethicin-treated (in 0.1% ethanol; bottom panel) *Arabidopsis* leaves. Volatiles were collected from detached rosette leaves during 0 to 9 h and 21 to 30 h of treatment by a closed-loop stripping procedure. Results are shown for the second interval of volatile collection. Mock-treated leaves emitted trace amounts of TMTT but no MeSA, (*E,E*)-α-farnesene, or (*E,E*)-geranyllinalool (GL). S, nonyl acetate standard.

genome (Chen et al., 2003). As described previously, most of the *TPS* genes were not transcribed in leaves (Ro et al., 2006). Four transcripts were inducible upon alamethicin treatment: *At2g24210*, *At4g16740*, *At4g16730*, and *At1g61120* (see Supplemental Figure 3 online). *At2g24210* has already been characterized as a myrcene/ocimene synthase and *At4g16740* as an ocimene synthase (Bohlmann et al., 2000; Fäldt et al., 2003). Both genes and the closely related gene *At4g16730* cluster in the *TPS*-b subfamily, of which all gene members characterized to date have been identified as monoterpene synthases (Aubourg et al., 2002; Chen et al., 2003, 2004). Thus, only *At1g61120*, which was annotated previously as *TPS04* (Aubourg et al., 2002), remained as a potential candidate for encoding the diterpene synthase GES. TPS04 clusters along with the two characterized *Arabidopsis* diterpene synthases involved in gibberellin biosynthesis and thus is likely to convert the 20-carbon substrate GGPP. In addition, it is 43% similar to *Clarkia*-type linalool synthases (Aubourg et al., 2002) that catalyze a similar reaction as GES but convert the 10-carbon substrate GPP.

Our assumption that *At1g61120* might encode a GES was corroborated by the observed correlation of *At1g61120* transcription with the emission of TMTT and (*E,E*)-geranyllinalool under different stress conditions (Figure 3). When treating intact plants with the octadecanoid mimic coronalon or when infesting them with larvae of the moth *Plutella xylostella*, both (*E,E*)-geranyllinalool and TMTT as well as *At1g61120* transcripts accumulated to higher levels compared with control plants. By contrast, the sesquiterpene α-farnesene was not emitted after *P. xylostella* feeding. Mechanical wounding by scratching the leaf surface with a razor blade and pinching leaves with forceps did not induce any of the four volatiles, either between 0 and 8 h after wounding or between 21 and 31 h. Consistently, no *At1g61120* mRNA was detectable by RNA gel blot analysis after mechanical wounding (data not shown).

Functional Characterization of *At1g61120* as GES

To confirm the catalytic activity of the *At1g61120*-encoded enzyme, the complete open reading frame was cloned into the *Escherichia coli* expression vector pASK-IBA7. In the presence of the affinity-purified recombinant enzyme, GGPP was converted into a single product (Figure 4A), which was identified as (*E,E*)-geranyllinalool by comparison of its retention time and mass spectrum with those of an authentic standard (see Supplemental Figure 2B online). No product was obtained after incubation of the enzyme with GPP and FPP as potential substrates. Based on this biochemical evidence, we call the *At1g61120*-encoded gene product GES from here on. Determination of GES activity in the presence of three different concentrations of Mg^{2+} and Mn^{2+} ions revealed the highest activity at 10 mM Mg^{2+} (Figure 4B), which is consistent with the ion preference of other plant TPSs (Davis and Croteau, 2000).

Analysis and Complementation of *GES* Loss-of-Function Plants

To prove the in planta function of the *GES* gene, corresponding knockout lines were analyzed. In lines *salk_039864* and *salk_078187*, the T-DNAs are located in intron 6 and exon 8, respectively (Figure 5A). In contrast with wild-type plants, neither mutant line accumulated any detectable *GES* mRNA after coronalon treatment (Figure 5B). Consistently, no (*E,E*)-geranyllinalool and TMTT were detected in the head space of the mutants (Figure 5C). Although reduced emission of MeSA and (*E,E*)-α-farnesene upon coronalon treatment was observed in the mutants in this experiment, their appearance indicated that the treatment was effective.

To complement the phenotype, the *Arabidopsis* knockout line *salk_039864* was transformed with the *GES* gene under the control of heterologous promoters. To avoid possible secondary effects caused by constitutive (*E,E*)-geranyllinalool or TMTT production, we first expressed the *GES* gene under the control of the alcohol-inducible *AlcA* promoter (*ProAlcA*; Figure 6A). For reasons yet unknown, a 6-kb genomic fragment starting with the presumed transcriptional start site had to be used rather than the cDNA to obtain stable mRNA accumulation. As shown in Figure 6B, knockout mutants expressing the *GES* gene under the *AlcA* promoter emitted TMTT and (*E,E*)-geranyllinalool only after ethanol induction, which correlates with *GES* transcript accumulation under these conditions (Figure 6C).

Transgenic plants expressing the *GES* genomic fragment under the control of the constitutive cauliflower mosaic virus (*CaMV*) *35S* promoter (*Pro35S*) showed constitutive emission of TMTT and (*E,E*)-geranyllinalool (Figures 7A and 7C). These plants

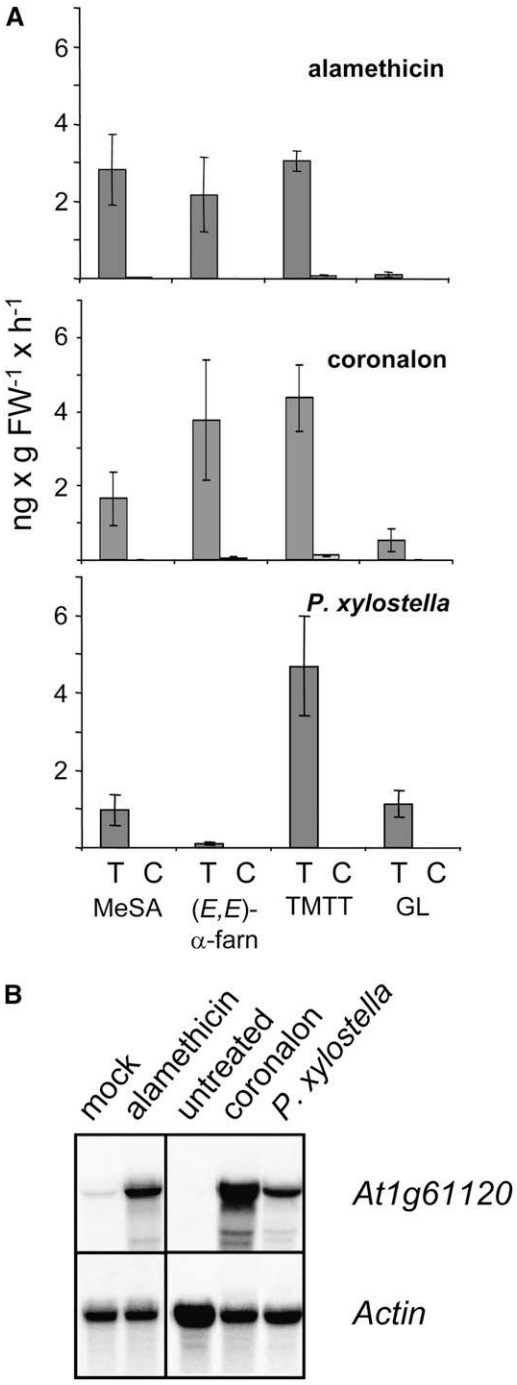

Figure 3. Comparison of Volatile Emissions and Expression of *At1g61120* in *Arabidopsis* Leaves upon Elicitor Treatment and Herbivore Challenge.

(A) Quantitative analysis of the four major volatiles MeSA, (*E*,*E*)-α-farnesene, TMTT, and (*E,E*)-geranyllinalool (GL). As highest emission rates in response to most treatments were observed in the second light phase, only results obtained during this period (21 to 30 h of treatment) are reported. Alamethicin (5 μg/mL) was applied to detached leaves, and coronalon (100 μM) was added to the medium of hydroponically grown plants. Continuous insect-feeding experiments were conducted by the application of two *P. xylostella* larvae in the third to fourth instar on each medium-sized and fully expanded rosette leaf. The results represent means ± SE of three replicates. Experiments were repeated at least once with similar results. C, control; FW, fresh weight; T, treatment.

(B) RNA gel blot analysis of *At1g61120* transcription in *Arabidopsis* leaves after alamethicin treatment (5 μg/mL) applied through petioles of cut leaves, coronalon treatment (100 μM; applied to roots of intact plants for 31 h), and *P. xylostella* feeding for 31 h. Control RNA was collected from leaves treated with 0.1% ethanol through petioles (mock) or from intact hydroponically grown plants. The blot was rehybridized with a probe for *Actin2* to document equal loading.

also showed reduced growth and the appearance of lesions on the cotyledons, depending on the degree of the expression of the transgene (Figures 7B and 7D). However, this phenotype, which was expressed only under long-day conditions, vanished when plants matured.

Quantitative analysis (Figure 7E) of volatiles from detached leaves of 7-week-old *Pro35S:GES* plants revealed that TMTT is emitted at levels (means of 2.6 ng·g^{-1} fresh weight^{-1}) comparable to those of wild-type plants after alamethicin treatment (Figures 3A [means of 3.1 ng·g^{-1} fresh weight·h^{-1}] and 7E [means of 2.1 ng·g^{-1} fresh weight·h^{-1}]). Emission rates of (*E,E*)-geranyllinalool (means of 0.47 ng·g^{-1} fresh weight·h^{-1}) were similar to those of wild-type plants induced with coronalon or by *P. xylostella* (Figures 3A and 7E).

The ratios of TMTT to (*E,E*)-geranyllinalool emitted from 3-week-old plantlets of the alcohol-inducible and constitutive *GES*-expressing lines were in the range of 1 to 1.5, respectively (Figures 6C and 7C), which were lower than those observed in wild-type plants treated with alamethicin (~30:1), coronalon (~9:1), or *P. xylostella* (~4:1) (Figure 3), indicating less efficient oxidative degradation of (*E,E*)-geranyllinalool. However, in mock-treated or untreated leaves of 7-week-old *Pro35S:GES* plants (Figure 7E), the TMTT:(*E,E*)-geranyllinalool ratio was higher (3 to 5.5:1) than in the younger transgenic plants, indicating that the steps leading to oxidative degradation might already be induced under these conditions. Levels of TMTT and *(E,E)*-geranyllinalool in *Pro35S:GES* plants did not change significantly after induction with alamethicin (Figure 7E), indicating that the amounts of precursor metabolites and/or the activity of the enzymes catalyzing the oxidative degradation could not be enhanced further in this experiment.

Subcellular Localization of GES

To determine the subcellular localization of GES, transgenic *GES* knockout plants expressing a GES-YFP (for yellow fluorescent protein) fusion under the control of the *CaMV 35S* promoter were established. Analysis of leaf epidermal cells from five *Pro35S:GES-YFP* plant lines revealed yellow fluorescence outside the plastids (Figure 8). Volatile analysis of the transgenic plants documented the emission of (*E,E*)-geranyllinalool, confirming the functionality of the expressed protein as GES (see Supplemental Figure 4 online). *Pro35S:GES-YFP* plants had no visual phenotype, which might be due to lower TMTT and/or (*E,E*)-geranyllinalool synthesis compared with *Pro35S:GES* plant lines.

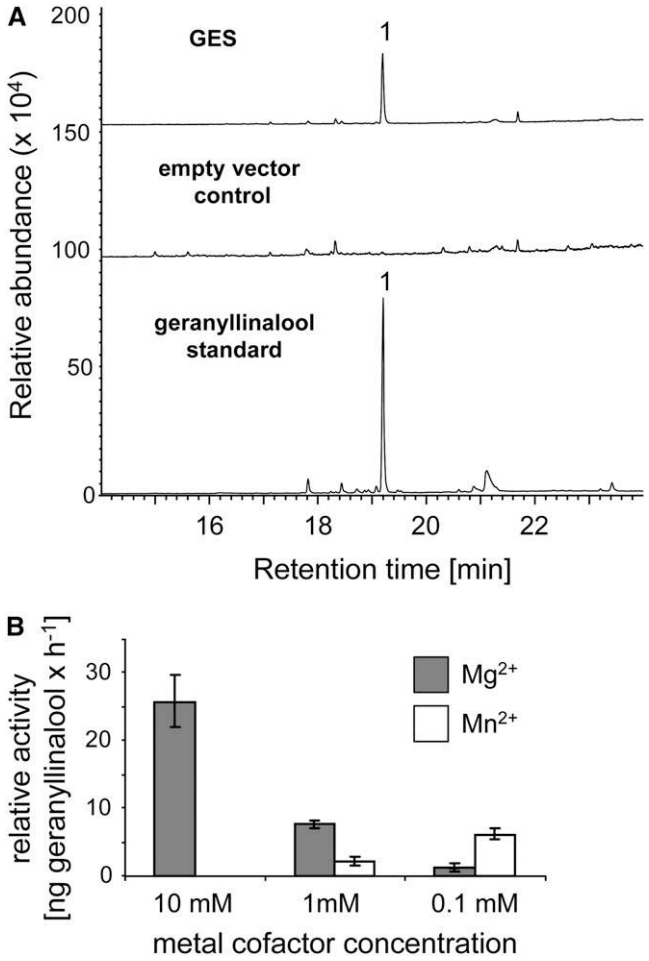

Figure 4. GC-MS Analysis of Products Formed from GGPP by Recombinant GES Enzyme.

GES was expressed in *E. coli*, extracted, purified, and incubated with the substrate all-*trans*-GGPP. The resulting terpene products were separated by GC-MS.

(A) MS detector traces are shown for the products obtained from assays with the purified GES enzyme. An extract from *E. coli* carrying the empty expression vector was subjected to the same purification procedure and served as a negative control. The major peak was identified as (*E,E*)-geranyllinalool (1) via comparison with an authentic standard, as shown in the bottom chromatogram.

(B) The catalytic activity of the partially purified enzyme was measured in the presence of the divalent metal ions Mg^{2+} and Mn^{2+} at different concentrations. Means ± SE of triplicate assays are shown.

Induction of *GES* Transcription and TMTT Synthesis in Mutants Deficient in Stress-Related Signaling Pathways

Next, we tested the possible roles of major stress-related signaling pathways for the induction of *GES* transcription and TMTT synthesis. Respective *Arabidopsis* mutants were treated with alamethicin and RNA was extracted for RNA gel blot analysis (Figure 9A). The *dde2-2* mutant (von Malek et al., 2002), which is defective in the biosynthesis of the major octadecanoid phytohormones 12-oxo-phytodienoic acid (OPDA) and JA (Park et al., 2002), as well as the *acx1/5* double mutant, which is unable to convert OPDA into JA (Schilmiller et al., 2007), did not respond to alamethicin. Thus, JA or its derivatives are essential for induction by alamethicin. Also, the F-box protein COI1, which is a central regulator of most JA-mediated responses (Xie et al., 1998; Devoto et al., 2005), is required for *GES* transcription. JA signaling can be influenced by two other stress hormones: ethylene (Penninckx et al., 1998) and SA (Spoel et al., 2003). Ethylene-insensitive *ein2* (Guzman and Ecker, 1990), SA-degrading *nahG* (Gaffney et al., 1993), and SA synthesis–deficient *sid2* (Nawrath and Metraux, 1999; Wildermuth et al., 2001) plants were equally efficient in *GES* induction as wild-type plants. Plants compromised in a major branch of the SA signal transduction network (*npr1*; Cao et al., 1994) also did not show an altered response with regard to *GES* induction.

Consistent with the results at the *GES* transcript level, no significant differences in the amount of (*E,E*)-geranyllinalool and TMTT synthesis were detected when comparing the volatile profiles of selected SA- and ethylene-deficient/insensitive mutants with the profile of wild-type plants (Figure 9B). As expected, *nahG* plants failed to emit MeSA. The *npr1* mutant, which lacks

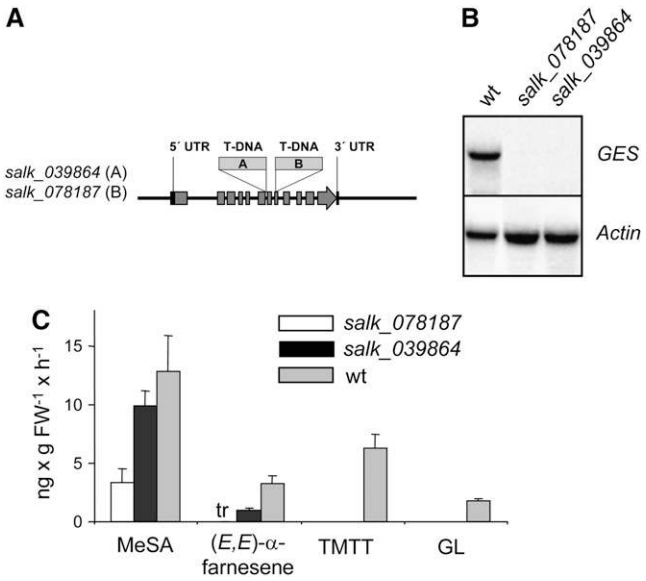

Figure 5. Coronalon-Induced Volatile Emission and *GES* Expression in Leaves of *Arabidopsis* Wild-Type and *GES* T-DNA Insertion Lines.

(A) Positions of T-DNA insertions in *At1g61120* (*GES*). The exons are represented by the gray boxes, and flanking regions and introns are represented by the black line. Black boxes symbolize untranslated regions (UTR) of the first and last exons. The two independent T-DNA insertions are indicated as boxes A and B.

(B) RNA gel blot analysis of *GES* transcript levels in leaves of wild-type and T-DNA insertion lines after application of 100 μM coronalon for 30 h to roots of hydroponically grown plants. The blot was rehybridized with a probe for *Actin2* to document equal loading.

(C) Quantitative analysis of volatiles emitted between 21 and 30 h after the beginning of coronalon treatment of single hydroponically grown wild-type and mutant plants. The results represent means + SE of three replicates. FW, fresh weight; tr, trace.

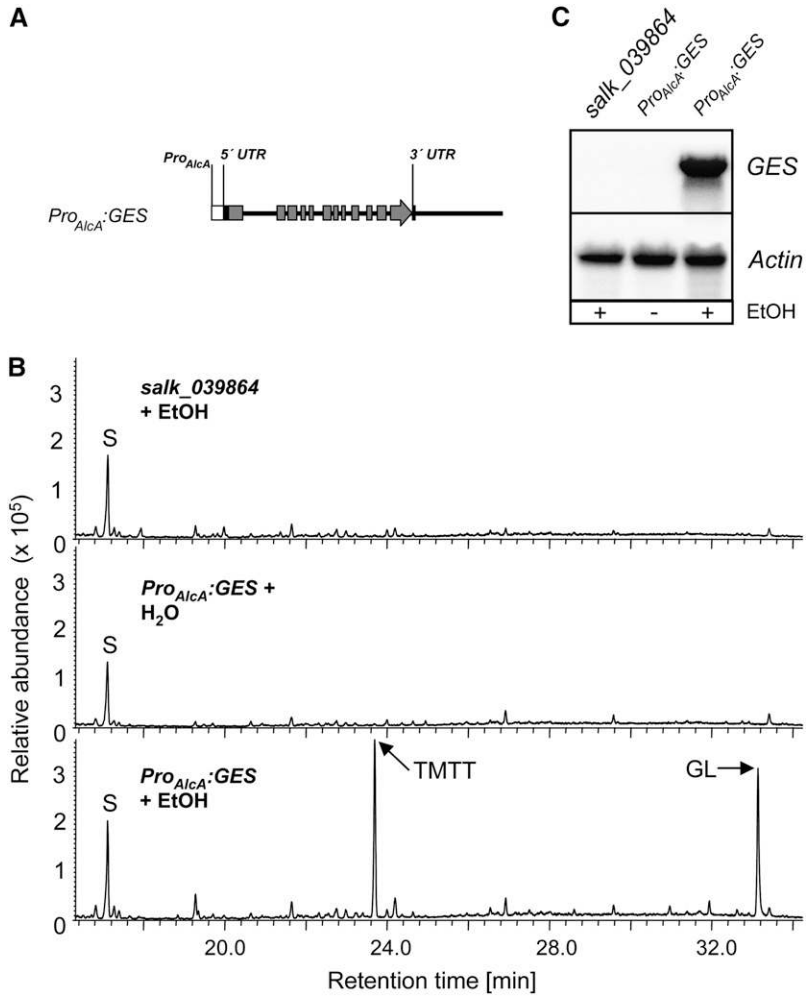

Figure 6. Complementation Analysis of the *GES* Insertion Line *salk_039864*.

(A) Schematic drawing of the chimeric gene used to complement the *Arabidopsis* insertion line *salk_039864*. The transgene (*ProAlcA:GES*) consists of a 6-kb genomic fragment starting with the presumed transcriptional start site (as inferred from the cDNA) under the control of the alcohol-inducible *AlcA* promoter. The exons are represented by the gray boxes, and flanking regions and introns are represented by the black line. UTR, untranslated region.
(B) GC-MS analysis of volatiles emitted from leaves of untransformed *salk_039864* and of *ProAlcA:GES* transformants. Twenty 3-week-old plants grown on soil under long-day conditions were sprayed with 4.7% ethanol prior to continuous volatile collection for 31 h (control plants were sprayed with water only). Chromatograms selected for 69 *m/z* are shown. GL, (*E,E*)-geranyllinalool; S, nonyl acetate standard.
(C) *GES* transcript analysis in leaves of mutant lines. Twenty 3-week-old plants grown on soil under long-day conditions were sprayed with 4.7% ethanol (EtOH; control plants were sprayed with water only). Material for RNA analysis was harvested after 31 h. The blot was rehybridized with a probe for *Actin2* to document equal loading.

the autoregulatory negative feedback loop for SA synthesis (Cao et al., 1994), emitted ~10-fold higher amounts of MeSA than wild-type plants. Emission of all the volatiles, including TMTT and (*E,E*)-geranyllinalool, depended on COI1.

Expression Profile of the *GES* Promoter

To assess whether the steady state levels of the *GES* mRNA are determined by the activity of the *GES* promoter, a 1.62-kb fragment upstream of the annotated transcriptional start site was inserted 5′ to the β-*glucuronidase* (*GUS*) reporter gene, and the resulting chimeric gene (*ProGES:GUS*) was stably transformed into *Arabidopsis* plants (Figure 10A). RNA gel blot analysis revealed *GUS* transcription upon alamethicin treatment (Figure 10B). However, in contrast with the endogenous *GES* mRNA, some residual *GUS* transcript levels were detected in the uninduced state, indicating that the *GES* promoter might retain some activity in the absence of inducing conditions.

In developing or mature flowers, GUS activity was detected in the stigma, anthers, filaments, and sepals but not in petals (Figure 10C). GUS activity occurred also in the abscission zone of floral organs (Figure 10C). In leaves, GUS activity was detected at wound sites (Figure 10C), which was somewhat unexpected given that no *GES* mRNA was detected by RNA gel blot analysis,

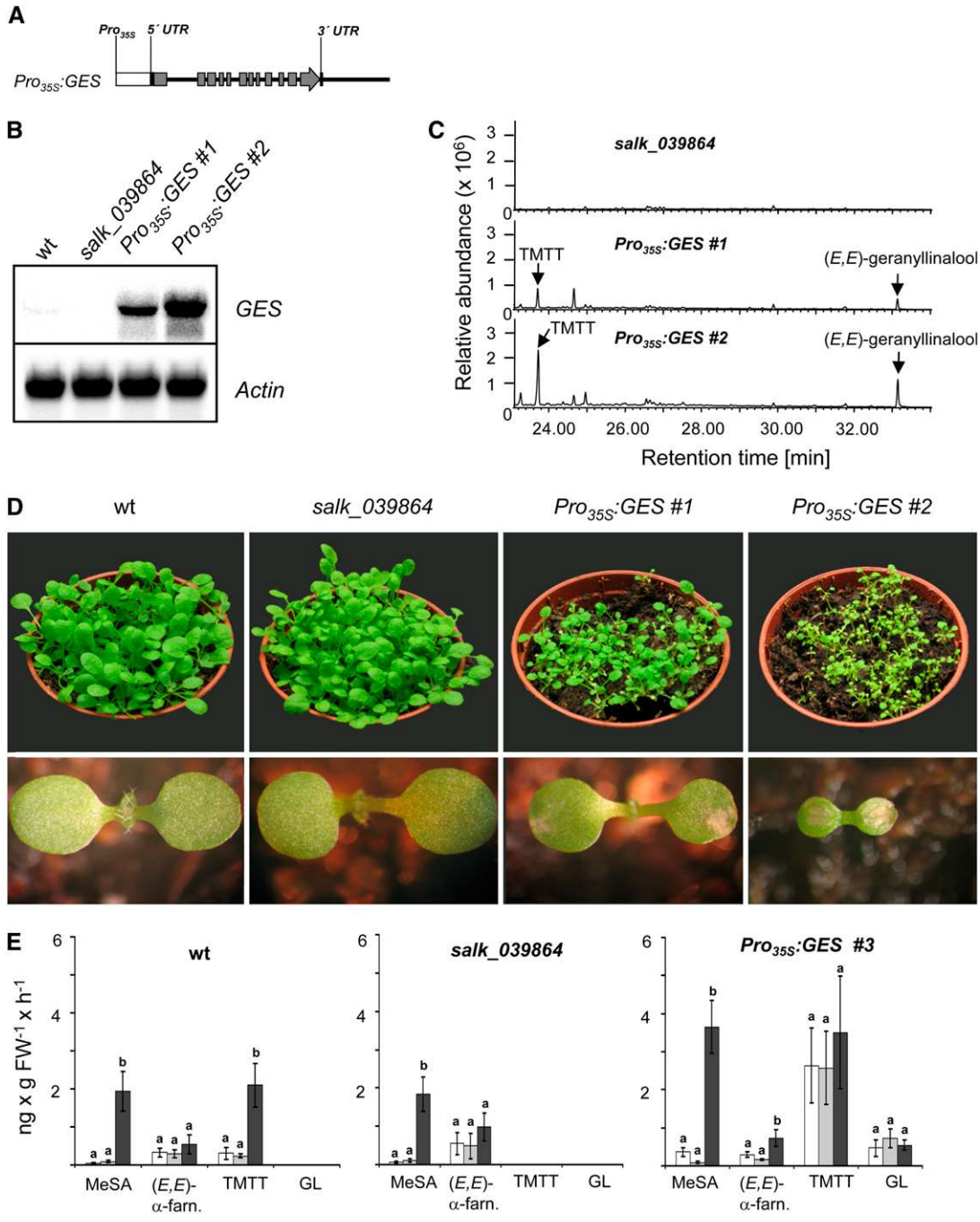

Figure 7. Phenotype and Volatile Analysis of Seedlings with Constitutive *GES* Expression.

(A) Schematic drawing of the chimeric gene used to complement the *Arabidopsis* insertion line *salk_039864*. The transgene consists of a 6-kb genomic fragment starting with the presumed transcriptional start site (as inferred from the cDNA) under the control of the constitutive *CaMV 35S* promoter. The exons are represented by the gray boxes, and flanking regions and introns are represented by the black line. UTR, untranslated region.

(B) RNA gel blot analysis of *GES* transcripts in leaves of two *Pro35S:GES* lines (1 and 2) compared with the wild type and the knockout line *salk_039864*. Leaf material was harvested from 20 3-week-old untreated plants grown under long-day conditions. The blot was rehybridized with a probe for *Actin2* to document equal loading.

(C) GC-MS analysis of volatiles emitted from leaves of *salk_039864* transformed with the *Pro35S:GES* gene. Volatiles were collected continuously for 31 h from 20 plants grown as described in **(B)**. Total ion chromatograms are shown.

(D) Top row, phenotype of 3-week-old plants grown under long-day conditions. Bottom row, phenotype of 10-d-old seedlings. The phenotype is representative for at least six independent lines showing expression of the *GES* transcript.

at least at 31 h after wounding (see above). This inconsistency was resolved by a time course analysis of transcript levels in leaf material adjacent to the wound site (2 to 3 mm). Real-time RT-PCR revealed that *GES* transcripts accumulated transiently up to 2 h after wounding, albeit at approximately threefold lower levels than after treatment with alamethicin (Figure 10D). Transcript levels were down to background levels at 4 h. The observed GUS staining is likely to result from this early and transient increase of promoter activity. However, this increase does not seem to be sufficient for sustained TMTT emission after wounding (see above). The wound-inducible *VSP2* gene showed a longer period of transcript accumulation, indicating that different mechanisms of wound-induced activation act on the two genes analyzed here.

DISCUSSION

Herbivore-induced release of volatiles is a common plant defense mechanism that serves to attract predators or parasitoids of the attacking insects. The composition of the herbivore blend can vary depending on the type of the insect pest, raising questions concerning the roles of the individual components and the mechanisms that regulate their synthesis. A prerequisite for this analysis is the knowledge of the corresponding biosynthetic genes. Here, we report the identification of a TPS that is required for the synthesis of the TMTT precursor (*E*,*E*)-geranyllinalool in *Arabidopsis*. Transgenic/mutant plants with reduced or increased *GES* expression and promoter:reporter gene fusions provide valuable tools for elucidating the function, biosynthesis, and regulated synthesis of TMTT in *Arabidopsis*.

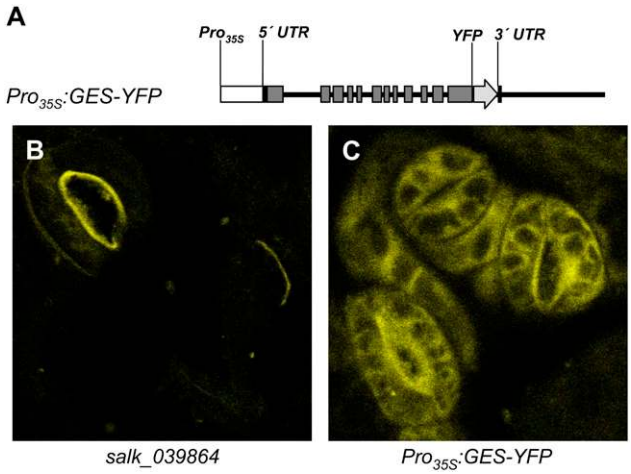

Figure 8. Subcellular Localization of GES-YFP Fusion Proteins in *Arabidopsis*.

(A) Schematic drawing of the *GES-YFP* fusion gene used to transform the *Arabidopsis* insertion line *salk_039864*. The transgene consists of a 6-kb genomic fragment starting with the presumed transcriptional start site (as inferred from the cDNA) under the control of the constitutive *CaMV 35S* promoter. The YFP tag was integrated upstream of the GES stop codon. The exons are represented by gray boxes, and flanking regions and introns are represented by the black line. UTR, untranslated region. **(B)** and **(C)** Epidermal peels from true leaves of 10-d-old plants of the *salk_039864* line **(B)** and the same knockout line transformed with *Pro35S:GES-YFP* **(C)** were used for fluorescence microscopy.

The Primary Structure of GES Is Consistent with its Presumed Function as a Diterpene Synthase

Compared with known annotated TPSs from *Arabidopsis* and other species, GES shares highest similarity (43%) to LISs from *C. breweri* and *Clarkia concinna* (Cseke et al., 1998; Aubourg et al., 2002) (Figure 11). As the formation of (*E*,*E*)-geranyllinalool from the C_{20} substrate GGPP is analogous to the synthesis of the C_{10}-monoterpene alcohol linalool from the precursor GPP, the functional characterization of At GES is consistent with its phylogenetic position close to Cb LIS in the TPS-f subfamily (Aubourg et al., 2002). *Clarkia*-type LISs form an odd group within the TPS superfamily, as they are the only known monoterpene synthases of angiosperm origin that contain a conserved N-terminal 200–amino acid domain of unknown function and that exhibit a gene structure of 12 exons, which has been suggested to be similar to that of the presumed progenitor gene of all plant *TPS*s (Bohlmann et al., 1998; Trapp and Croteau, 2001; Aubourg et al., 2002). All of the other known monoterpene synthases of angiosperm origin, for instance the *Arabidopsis* LIS, consist of only seven exons and lack the conserved 200–amino acid domain. It seems attractive to explain the unusual position of *Clarkia*-type LISs by assuming that they have evolved from a GES-type diterpene synthase by mutations limiting the size of the substrate binding pocket at the catalytically active site of the enzyme. Analysis of the volatile bouquet of *Clarkia* leaves after treatment with one of the elicitors of TMTT emission could clarify whether *Clarkia* expresses a GES.

Compared with the other annotated TPSs of the *Arabidopsis* genome (Aubourg et al., 2002), GES is most similar to the two characterized diterpene synthases copalyl diphosphate synthase

Figure 7. (continued).

(E) Emission rates of MeSA, (*E*,*E*)-α-farnesene, TMTT, and (*E*,*E*)-geranyllinalool (GL) from leaves of wild-type, *salk_039864*, and *Pro35S:GES* lines. Plants were first grown for 3 weeks under long-day conditions, which allows the selection of strong expressors (deduced from the phenotype; see **[D]**). Subsequently, they were cultivated for 4 weeks under short-day conditions. Volatiles were collected from 16 to 20 detached leaves during 0 to 7 h of treatment in 20 mL of tap water (white bars), 0.1% ethanol (mock; gray bars), and alamethicin (5 μg/mL 0.1% ethanol; black bars). Volatile collection and analysis were performed as described in Methods. The results represent means ± SE of six replicates (wild type and *Pro35S:GES*) or four replicates (*salk_039864*; $n = 3$ for mock treatment). Statistical analysis (one-way analysis of variance) was performed for each compound on log-transformed data. Letters above each bar indicate significant differences among each set of volatiles after Tukey's test ($P < 0.05$). For statistical values, see Supplemental Table 1 online. FW, fresh weight.

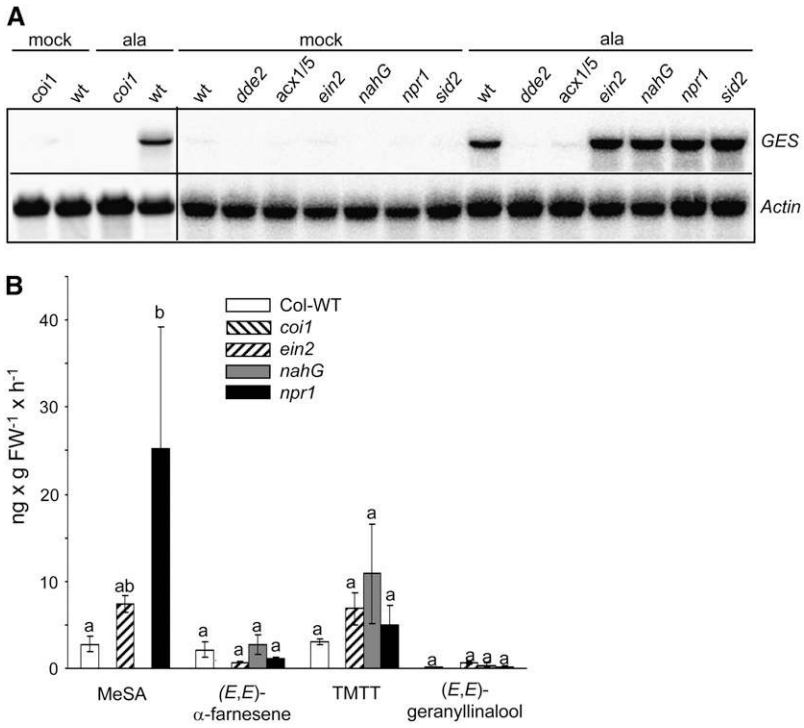

Figure 9. *GES* Transcript Analysis and Leaf Volatile Emission in Different Genotypes Impaired in SA, JA, and Ethylene Signaling/Biosynthesis after Induction with Alamethicin.

(A) RNA gel blot analysis of *GES* transcript levels in detached leaves of wild-type plants and mutants deficient in SA, JA, and ethylene abundance or signal transduction. Alamethicin (ala; 5 μg/mL) was applied through petioles of cut leaves; for mock treatment, a 0.1% ethanol solution was used. RNA was harvested after 24 h. The blots were rehybridized with a probe for *Actin2* to document equal loading. Induction of the *coi1* mutant was done in intact hydroponically grown plants as described in Methods. The experiment was repeated twice with similar results.

(B) Volatiles were collected during 21 to 30 h after the beginning of alamethicin treatment from detached leaves of wild-type plants and mutants deficient in SA, JA, and ethylene abundance or signal transduction. Treatments and volatile analysis were conducted as described in Methods. The results represent means ± SE of three replicates. Letters above each bar indicate significant differences for each set of volatiles after Tukey's test ($P < 0.05$). For statistical values, see Supplemental Table 2 online. FW, fresh weight.

(CPPS) and *ent*-kaurene synthase (KS) (Figure 11). Thus, the primary structure of GES is consistent with its function as a diterpene synthase. CPPS, KS, and GES differ from the other *Arabidopsis* TPSs by the N-terminal conserved 200–amino acid motif and the ancestral exon/intron structure (12 to 15 exons) mentioned above. Like KS, GES contains the highly conserved amino acid sequence motif DDXXD that is required for the ionization-initiated reaction mechanism of most TPSs (Davis and Croteau, 2000; Tholl, 2006).

Biotic stress–induced TMTT emissions have been described in several plant species (Hopke et al., 1994; Ament et al., 2004; Williams et al., 2005), which suggested that this defense reaction might have widespread importance. Therefore, it is likely that genes encoding GES enzymes are present in many other plant species. An herbivore-inducible GES activity that correlated with induced TMTT emission was found in tomato (Ament et al., 2006), but database searches do not provide evidence for the existence of *Arabidopsis*-type *GES* genes in the tomato genome or other genomes. This might be due to variations in the nucleotide sequence hampering a straightforward identification of the gene by sequence similarity. Alternatively, *GES* genes might have arisen independently in other species as a product of convergent gene evolution. Comprehensive phylogenetic analysis of plant TPSs, including angiosperm and gymnosperm LIS, showed that convergent evolution is a typical feature of the *TPS* gene family (Martin et al., 2004).

GES Is Not Localized to the Plastids

While diterpene synthases are primarily located in the plastid, no obvious plastid transit peptide sequence could be identified for GES with the TargetP and ChloroP algorithms. Indeed, compared with the other ancestral *TPS* genes *CPPS* and *KS*, *GES* has lost three N-terminal exons that are still present in the plastid-localized proteins CPPS and KS (Trapp and Croteau, 2001). Protein-YFP fusion studies confirmed that the GES enzyme is not targeted to the chloroplast (Figure 8) and indicated that it resides in the cytosol or the endoplasmic reticulum It is likely that the GES substrate GGPP is present in these compartments, since previous GFP fusion studies of *Arabidopsis* GGPP synthases identified two enzymes with a localization pattern similar to that observed for GES (Okada et al., 2000).

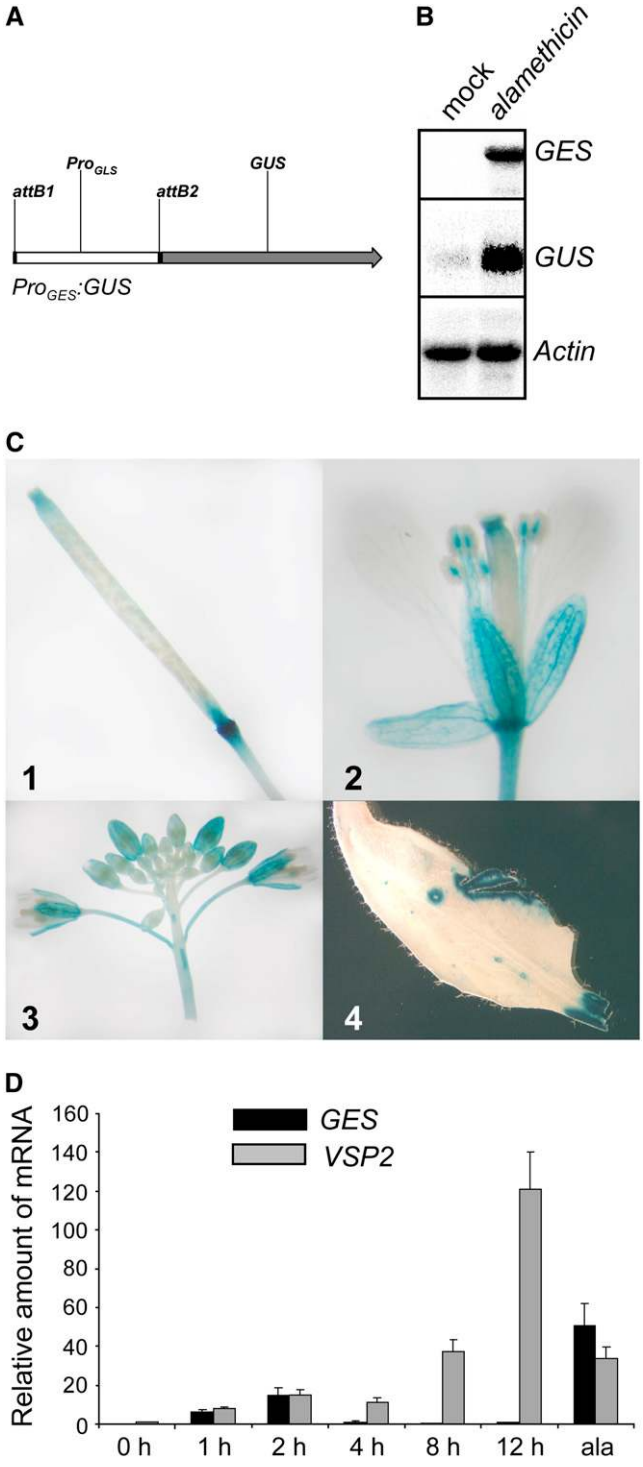

Figure 10. *GUS* Transcript Levels and GUS Activity in *ProGES:GUS* Plants.

(A) Schematic drawing of the *ProGES:GUS* construct. The *GES* promoter (−1162 to +3) was cloned upstream of the *GUS* reporter gene using Gateway technology. The *attB2* site is part of the 5′ untranslated region of the *GUS* gene.

(B) RNA gel blot analysis of *GUS* and *GES* transcript levels in hydroponically grown transgenic *ProGES:GUS* plants after induction with alamethicin (5 μg/mL) or ethanol (0.1%) for 31 h.

(C) Histochemical GUS staining of a silique (1), a flower (2), an inflorescence (3), and a wounded leaf (4) from *ProGES:GUS* plants. The results are representative for at least 10 independent transgenic lines.

(D) *GES* expression in wild-type plants in response to wounding (1 to 12 h) and treatment with alamethicin (ala; 24 h). Six-week-old hydroponically grown plants were wounded with forceps. Leaf material within ~3 mm adjacent to the wound marks was harvested. Material from five leaves was combined before extraction. Relative levels of *GES* and *VSP2* (a wound-inducible control gene) were determined by real-time RT-PCR using SYBR Green I chemistry. Values were normalized to the expression of *At1g13320* (protein phosphatase type 2). As a control, plants were treated with 5 μg/mL alamethicin through the roots, and RNA was harvested after 24 h. The results represent means + SE of three technical replicates. The experiment was repeated with similar results (see Supplemental Figure 5 online).

Expression of *GES* under Heterologous Promoters Allows the Uncoupling of (*E,E*)-Geranyllinalool and TMTT Synthesis from the Emission of Other Volatiles

In plants expressing *GES* under the promoters *ProAlcA* and *Pro35S*, emission of (*E,E*)-geranyllinalool and TMTT was detected, indicating that *GES* expression is sufficient to generate the products of the pathway (Figures 6 and 7). TMTT and (*E,E*)-geranyllinalool levels in these transgenic plants did not exceed the values observed after *P. xylostella* infestation and could not be further elevated by alamethicin. This could be due to either the limited availability of precursor metabolites or the negative feedback mechanisms of the products TMTT and (*E,E*)-geranyllinalool on the respective biosynthetic enzymes.

In this work, we noticed lower TMTT:(*E,E*)-geranyllinalool ratios (1- to 1.5-fold; Figures 6C and 7C) when volatiles were measured from 3-week-old intact transgenic plantlets compared with measurements performed with detached leaves of 7-week-old plants (3.7- to 5.5-fold; Figure 7E). In addition, TMTT emission levels of younger untreated transgenic plants (Figure 6C) were ~10-fold lower compared with those of detached leaves of older plants. As the background levels of α-farnesene and TMTT were exceptionally high in the experiments shown in Figure 7E, we suspect that precursor synthesis and oxidative degradation might have been preinduced. Further work on the regulation of these potentially rate-limiting steps of TMTT synthesis is needed to understand the pathway comprehensively. The relatively high levels of (*E,E*)-geranyllinalool in alamethicin-treated *Pro35S:GES* plants (Figure 7E) compared with alamethicin-treated wild-type plants suggest that under conditions of high GES activity, the oxidative degradation can become rate-limiting.

The *Arabidopsis* plants ectopically expressing At *GES* can be compared with plants expressing strawberry (*Fragaria* sp) NES targeted to mitochondria (Kappers et al., 2005). Analogous to the oxidative degradation of TMTT to (*E,E*)-geranyllinalool, nerolidol is converted to the C_{11}-homoterpene DMNT, presumably by the same enzymes. The conversion of nerolidol to DMNT in uninduced plants was variable and could be stimulated by JA. However, as these processes might depend on the environmental conditions, experiments with plants encoding both transgenes,

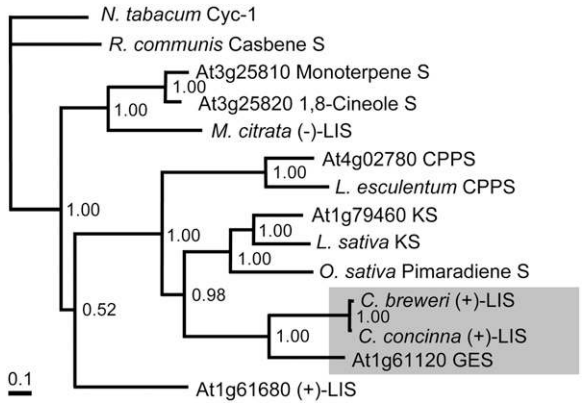

Figure 11. Sequence Comparison of *Arabidopsis* GES with Additional *Arabidopsis* and Other Plant TPSs.

Bayesian tree generated from an alignment of 14 TPS proteins, including *Arabidopsis* GES (*At1g61120*), *C. breweri* LIS, *C. concinna* LIS, and selected *Arabidopsis* and other angiosperm monoterpene and diterpene synthases of primary and secondary metabolism. *Arabidopsis* genes used for the analysis are as follows: *GES* (*At1g61120*), *LIS* (*At1g61680*), *myrcene/(E)-β-ocimene synthase* (*At3g25810*), *1,8-cineole synthase* (*At3g25820*), *CPPS* (*At4g02780*), and *KS* (*At1g79460*). Other monoterpene and diterpene synthase genes come from *Nicotiana tabacum*, *Rhizinus communis*, *Mentha citrata*, *Solanum lycopersicum, Lactuca sativa*, and *Oryza sativa*. Numbers above nodes represent Bayesian posterior probabilities.

Pro35S:GES and *Pro35S:FaNES*, would have to be performed to determine whether the efficiency of oxidative degradation of nerolidol and (*E,E*)-geranyllinalool follows the same pattern.

At the seedling stage, constitutive *GES* expression leads to growth retardation, which was also observed for DMNT-emitting plants (Kappers et al., 2005). Moreover, plants expressing *GES* developed lesions on the cotyledons (Figure 7D). One potential explanation is that the GGPP pool in the cytosol/endoplasmic reticulum compartment, which is needed for protein prenylation, is depleted by the constitutive activity of GES. Constitutive expression of the plastidic diterpene synthase taxadiene synthase leads to a pale bleached phenotype because of the depletion of precursors for chlorophyll biosynthesis (Besumbes et al., 2004). The different phenotypes—lesions versus bleaching—support our finding that GES is not localized in the plastid (Figure 8). Apart from metabolic disturbances caused by constitutive GES activity, the lesion phenotype could be elicited by TMTT and/or (*E,E*)-geranyllinalool acting as plant signals. This notion is supported by the findings of Arimura et al. (2000), who reported TMTT-induced transcription of defense genes in lima bean.

GES Transcription Requires a Functional Octadecanoid Pathway and Is Not Affected by SA and Ethylene

Mutant analysis proved that JA or its derivatives and the F-box protein COI1 are necessary for alamethicin-induced *GES* transcription and the emission of (*E,E*)-geranyllinalool and TMTT (Figure 9). In addition, *GES* transcription and the emission of (*E,E*)-geranyllinalool and TMTT were induced by the octadecanoid mimic coronalon (Figure 3). Coronalon has structural resemblance to the phytotoxin coronatine and the JA-Ile conjugate, which was recently shown to interact with COI1 (Thines et al., 2007). Therefore, we postulate that JA-Ile or a structurally related conjugate represents the signal that induces *GES* transcription.

Many JA-dependent responses are modulated by other phytohormones like SA, ethylene, and abscisic acid, allowing plants to integrate different external stimuli into their defense responses. The induced emission of MeSA in response to herbivore feeding and alamethicin treatment (Figures 2 and 3) suggested that SA might be a positively modulating hormone, as described for lima bean and tomato (Koch et al., 1999; Ament et al., 2004). However, *GES* transcript levels and emission of (*E,E*)-geranyllinalool and TMTT were not altered in *nahG* plants, which are characterized by reduced SA levels (Figure 9). In lima bean, SA directly affects JA biosynthesis by blocking the conversion of OPDA to JA. This mechanism leads to a different composition of the volatile blend depending on the internal SA levels: in the absence of SA, JA-inducible monoterpenes and sesquiterpenes are synthesized; in the presence of SA, OPDA or other octadacenaoids, which accumulate as a result of the SA-mediated inhibition of the last steps of JA biosynthesis, induce mostly the homoterpenes DMNT and TMTT (Koch et al., 1999). In tomato, JA is sufficient to induce TMTT release, but SA is required after spider mite infection, which indicates that SA also plays a role in modulating octadecanoid regulation in this species (Ament et al., 2006). At the same time, our results show that *GES* transcription and (*E,E*)-geranyllinalool/TMTT synthesis are not subject to the antagonistic effect of SA that was demonstrated for *PDF1.2* and other JA-inducible genes (Glazebrook et al., 2003; Spoel et al., 2003). As revealed by the *ein2* mutant (Figure 9), ethylene also does not influence the response.

Our notion that compounds derived from the octadecanoid pathway are necessary, if not sufficient, for the induction of *GES* transcription is supported by public databases (http://www.genevestigator.ethz.ch/at [Zimmermann et al., 2004] and http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) that report increased *GES* transcript levels after treatments that activate octadecanoid biosynthesis, like infection with *Botrytis cinerea* or *Alternaria brassicicola* (van Wees et al., 2003), and treatment with ozone. Furthermore, expression is induced by infection with the coronatine-synthesizing bacterium *Pseudomonas syringae macilicola* in wild-type, *nahG*, *sid2*, *npr1*, and *ein2* genotypes (Glazebrook et al., 2003), confirming our results that SA and ethylene do not modify the response (Figure 9).

The *GES* Promoter Is Constitutively Active in Flowers and Reveals Local and Transient Activation upon Wounding

In general, steady state mRNA levels are determined by the activity of the promoter and the stability of the mRNA. To investigate the contribution of the *GES* promoter to the regulation of *GES* expression, transgenic plants carrying a chimeric *ProGES:GUS* gene were generated. The *GUS* transgene was inducible by alamethicin, indicating that regulatory *trans* factors

bind to the 1162 promoter fragment. Therefore, the chimeric *ProGES:GUS* gene can be used to dissect the regulatory events that determine the activity of the promoter. However, elevated transcript levels of the reporter gene in the uninduced state were detected, hinting at a lower stability of the *GES* transcript compared with the *GUS* transcript.

GUS staining was observed in floral organs (specifically, the stigma, anthers, and sepals) (Figure 10), which correlates with the detection of trace amounts of TMTT from flowers (Tholl et al., 2005). Moreover, there is a strong correlation between genes expressed in sepals and MeJA-induced genes (www.genevestigator.ethz.ch/at; Zimmermann et al., 2004), supporting our notion that metabolites of the octadecanoid pathway play an important role in the regulation of *GES* transcription. The expression pattern of *GES* in the flower resembles those of the recently characterized *Arabidopsis* caryophyllene, linalool, and multiproduct monoterpene synthases. However, these genes are expressed exclusively in flowers (Tholl et al., 2005) and cannot be induced in leaves by treatment with alamethicin.

Real-time RT PCR analysis of plant material adjacent to wound sites indicated that *GES* mRNA (Figure 10D) accumulated transiently with kinetics that resemble the kinetics of JA accumulation (Stintzi et al., 2001). Apparently, this induction leads to strong local GUS staining at the wound sites (Figure 10C) but is not sufficient to yield TMTT levels that are detectable by the experimental setup used in this study. Transcript levels of the wound-inducible *VSP2* gene were induced for a longer period. Whether the maintenance of *GES* transcription upon *P. xylostella* feeding is a consequence of continuous wounding or whether it is induced by specific insect-derived elicitors remains to be investigated.

In conclusion, the identification of GES provides a useful tool to address the contribution of TMTT in plant–plant, plant–fungus, and plant–insect interactions. Moreover, the signaling pathway that regulates TMTT synthesis can be further dissected using the *GES* gene.

METHODS

Growth Conditions and Plant Material

The Columbia (Col-0) ecotype of *Arabidopsis thaliana* was used as the wild type (obtained from Lehle Seeds). Wild-type (Col-0) and mutant/transgenic plants were grown either in soil in a growth chamber (22°C and ~140 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR) or in a nonsterile hydroponic culture (Gibeaut et al., 1997) on a rockwool support. If not indicated otherwise in the figure legends, plants were grown for 5 to 6 weeks under short-day conditions (8-h-light/16-h-dark photoperiod). Plants were used in the prebolting rosette stage with a total biomass of ~5 g. Mutants were obtained from the following sources: *coi1-1* (Xie et al., 1998) from J. Turner (University of East Anglia, Norwich, UK); *dde2-2* (Park et al., 2002) from B. von Malek and B. Keller (University of Zurich, Switzerland); *acx1/5* (Schilmiller et al., 2007) from G. Howe (Michigan State University, East Lansing); *ein2-1* (Guzman and Ecker, 1990) and *npr1-1* (Cao et al., 1994) from the Nottingham Arabidopsis Stock Center (Nottingham, UK); *nahG* (Gaffney et al., 1993; Lawton et al., 1995) from L. Friedrich (Syngenta Biotechnology, Research Triangle Park NC); and *sid2-2* Nawrath and Metraux, 1999; Wildermuth et al., 2001) from F. Ausubel (Harvard University, Cambridge, MA). *coi1-1* mutant plants, which are male-sterile, were screened from F2 seed pools as described (Reymond et al., 2000) and afterward transferred to hydroponic culture for further analysis.

Plant Treatments

For treatments with coronalon (or alamethicin for wild-type and *coi1* plants [Figure 9]), hydroponically grown plants were transferred with their supports into individual falcon tubes (for gene expression analysis) or glass beakers (for volatile collection) containing 25 and 30 mL of hydroponic medium, respectively. Either alamethicin (Sigma-Aldrich) or coronalon (W. Boland, Max-Planck-Institute for Chemical Ecology) was added to a final concentration of 5 μg/mL, 0.1% ethanol or 33 μg/mL (100 μM), 0.1% ethanol, respectively. Control plants were treated with 0.1% ethanol only (mock). For alamethicin treatment of detached leaves, plants were grown on soil for 4 to 6 weeks as described above. If not stated otherwise in the figure legends, 14 rosette leaves were cut off from the base of the petiole and transferred into a small glass beaker filled with 10 mL of alamethicin solution (5 μg/mL, 0.1% ethanol) so that only the petiole of each leaf was submerged. For feeding experiments with *Plutella xylostella* (kindly provided by T. Mitchell-Olds, Duke University, Durham, NC), 6-week-old soil-grown rosette plants with their root balls wrapped in aluminum foil were placed in 3-liter bell jars. Two third- or fourth-instar larvae were applied on each medium-sized and fully expanded rosette leaf and allowed to feed for 30 h, leading to an estimated consumption of total leaf mass of ~35%. For induction of the alcohol-inducible *AlcA* promoter, 20 3-week-old plants grown on soil were sprayed with 5 mL of 4.7% ethanol prior to volatile collection. As a control treatment, plants were sprayed with 5 mL of water only.

Volatile Collection and Analysis

For volatile collection in response to treatment with alamethicin and coronalon, detached rosette leaves or single intact hydroponically grown plants were placed in glass beakers containing the elicitor solution as described above and transferred into a 1-liter bell jar. If not indicated otherwise in the figure legends, emitted volatiles were collected during 0 to 9 h and 21 to 30 h from the beginning of the treatment period in a controlled-climate chamber (23°C, 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR) by a closed-loop stripping method (Boland et al., 1984; Donath and Boland, 1995) as described previously (Chen et al., 2003; Tholl et al., 2005). Collection periods were interrupted by a 12-h dark period, and bell jars were vented between collection intervals. Volatiles from 3-week old soil-grown transgenic *GES*-expressing lines were sampled continuously for 31 h. For the *P. xylostella* treatment, plants were placed in 3-liter bell jars as described above and volatiles were collected during 0 to 9 h and 21 to 30 h from the start of larval feeding. Volatiles were routinely collected on Super-Q (25 mg) traps, but charcoal traps were used for volatile collection from intact soil-grown plants of the *Pro35S:GES* lines and from plants in response to mechanical wounding or insect feeding. Volatile compounds were eluted with 100 μL (Super-Q traps) or 40 μL (charcoal traps) of CH_2Cl_2, and 120 ng of nonyl acetate in 6 μL was added as a standard. No major differences in the volatile profiles were observed between the different trapping materials. Samples were analyzed on a Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett-Packard 5973 quadrupole mass selective detector. Separation was performed on a 5% phenyl-methylpolysiloxane (DB5) column (J&W Scientific) of 30 m × 0.25 mm i.d. × 0.25 μm film thickness. Helium was the carrier gas (flow rate, 2 mL/min), a splitless injection (injection volume, 2 μL) was used, and a temperature gradient of 5°C/min from 40°C (3-min hold) to 220°C was applied.

The identities of (*E,E*)-α-farnesene, MeSA, TMTT, and (*E,E*)-geranyllinalool were determined by comparison of retention time and mass spectra with those of authentic standards and with mass spectra in the National Institute of Standards and Technology and Wiley libraries. For identification of (*E,E*)-geranyllinalool, a commercial standard (Acros

Organics) and (*E,E*)-geranyllinalool derived from acid hydrolysis of all-*trans*-GGPP were used. Treatment of all-*trans*-GGPP with 3 M HCl leads to the formation of (*E,E*)-geranyllinalool as the primary product via allylic rearrangement and all-*trans*-geranylgeraniol as a minor product (Hefner et al., 1998). For quantification, representative selected ion peaks of each compound were integrated (single ion method) and the amounts were calculated in relation to the response of nonyl acetate at *m/z* 69. Response curves for the quantified compounds relative to nonyl acetate were generated by injecting a mixture of equal amounts of authentic standards and nonyl acetate.

Heterologous Expression of GES in *Escherichia coli*

For expression in *E. coli*, the open reading frame of *GES* was amplified with the primer pair PI/PII and cloned as a *Bsa*I fragment into the expression vector pASK-IBA7 (IBA). The construct was introduced into the *E. coli* strain TOP10 (Invitrogen). Sequence analysis confirmed that no errors were introduced by DNA amplification. Liquid cultures of the bacteria harboring the expression construct were grown at 18°C for 8 h. Expression was induced by the addition of anhydrotetracycline (IBA) to a final concentration of 200 μg/L. After 20 h of incubation at 18°C, the cells were collected by centrifugation and disrupted by a 4 × 30-s treatment with a sonicator (Bandelin UW2070) in chilled extraction buffer (50 mM MOPSO, pH 7.0, 5 mM $MgCl_2$, 5 mM sodium ascorbate, 0.5 mM phenylmethylsulfonyl fluoride 5 mM DTT, and 10% [v/v] glycerol). The cell fragments were removed by centrifugation at 14,000*g*, and the supernatant was desalted into assay buffer (10 mM MOPSO, pH 7.0, 1 mM DTT, and 10% [v/v] glycerol) by passage through an Econopac 10DG column (Bio-Rad), yielding the partially purified enzyme analyzed in Figure 4B. The *Strep*-tagged enzyme was further purified on a *Strep*-Tactine affinity column (IBA) according to the manufacturer's instructions.

To determine the catalytic activity of the recombinant protein, enzyme assays containing 89 μL of the protein extract, 10 μL of GGPP (440 ng/μL), and 1 μL of $MgCl_2$ (1, 0.1, and 0.01 M, respectively) or 1 μL of $MnCl_2$ (1, 0.1, and 0.01 M, respectively) were performed in a Teflon-sealed, screw-capped 1-mL GC glass vial. The assays were overlaid with 80 μL of hexane containing 10 ng/μL nonyl acetate as an internal standard and incubated for 2 h at 30°C. To extract the enzyme products, the assays were mixed for 60 s. The organic phase then was removed and analyzed by GC-MS as described above using a temperature program from 60°C (2-min hold) to 280°C at 8°C/min.

Genotyping of Plant Material

Mutants carrying a T-DNA insertion in the *GES* gene were identified in the insertional mutant population distributed by the Nottingham Arabidopsis Stock Center (ecotype Col-0) (Sessions et al., 2002). Genomic DNA was isolated using the method described (Fulton et al., 1995). The T-DNA insertion event in *At1g61120* was confirmed by PCR and sequencing of the right and left border PCR products.

Complementation Analysis

The BAC clone JATY50A19 (pYLTAC17 with an ~40-kb insert) containing the *At1g61120* locus was identified from the *Arabidopsis thaliana* Integrated Database (http://atidb.org/cgi-perl/gbrowse/atibrowse) and obtained from the John Innes Centre Genome Laboratory (http://dev.jicgenomelab.co.uk/jgl/). JATY50A19 was cut with *Aat*II, and resulting fragments were separated on a 0.7% agarose gel. A 13-kb fragment that contained the *At1g61120* locus was gel-purified and cloned in the *Aat*II site of pGEM-T (Promega), resulting in the plasmid pGEM-T/GES. The *CaMV 35S* promoter or the *AlcA* promoter (Caddick et al., 1998; Roslan et al., 2001) was fused to the 5′ untranslated region of *GES* as annotated by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) by recombinant PCR. For first-round PCR, the *CaMV 35S* promoter (−1011 to +3) was amplified from pB2GW7 (Karimi et al., 2005) using the primers P1 and P3 (see Supplemental Table 3 online for primer sequences). The *AlcA* promoter (−298 to +3) including 77 bp of vector sequences was amplified from pCAMBIA3300 (a kind gift of Andreas Hiltbrunner, Syngenta, Jealott's Hill International Research Centre) using the primers P2 and P3. P3 was extended by the first 36 bp of the *GES* 5′ untranslated region. The PCR products were gel-purified and used together with pGEM-T/GES in a second round of PCR using P1 or P2 and the primer P4 of the *GES* genomic fragment. The resulting PCR products contained the 5′ untranslated region and part of the first exon (up to +302) of *GES* downstream of the *CaMV 35S* or *AlcA* promoter. After subcloning in pSK (Stratagene) and sequencing, the *GES* promoter in pGEM-T/GES was replaced by the *CaMV 35S* or *AlcA* promoter using a *Bsp*120I/*Esp*3I restriction digest. The *Pro35S:GES* construct was cut with *Aat*II/*Sac*I and ligated in the *Aat*II/*Sac*I-cut binary vector pB2GW7 (Karimi et al., 2005). To combine the *ProAlcA:GES* gene with the *Pro35S:AlcR* construct (Caddick et al., 1998) in a binary vector, a synthetic *Hin*dIII double-stranded oligonucleotide (annealed P5 and P6) including a *Sda*I and a *Xma*JI site was first cloned in *Hin*dIII-cut pCAMBIA3300. The *ProAlcA:GES* construct was cut with *Nhe*I/*Pst*I and cloned in the *Xma*JI/*Sda*I sites of the modified pCAMBIA3300.

For the generation of transgenic plants, binary plasmids were electroporated (GenePulser II; Bio-Rad) into *Agrobacterium tumefaciens* strain GV3101 (pMP90). The resulting agrobacteria were used to transform Col-0 plants by the floral dipping method (Clough and Bent, 1998). Transgenic seeds containing the *Pro35S:GES* or the *ProAlcA:GES* construct were selected by spraying a solution of BASTA (glufosinate) herbicide according to the manufacturer's instructions (AgrEvo).

C-Terminal Fusion of GES with YFP

Recombinant PCR was performed to integrate the restriction sites *Cfr*9I and *Asi*SI upstream of the *GES* stop codon. The PCR fragment was generated using pGEM-T/Pro35S:GES as template and the oligonucleotides P7, P8, and P9 as primers (see Supplemental Table 3 online for primer sequences). The PCR product was cut with *Bsh*TI and cloned in *Bsh*TI-cut pGEM-T/Pro35S:GES. The *YFP* gene was amplified using primers P10 and P11, which introduced *Cfr*9I and *Asi*SI sites at the two ends. The *YFP* PCR fragment was cut with *Cfr*9I and *Asi*SI and cloned in the *Cfr*9I/*Asi*SI sites of the construct created before. The transfer of the *Pro35S:GES-YFP* construct in the binary vector pB2GW7 (Karimi et al., 2005) and the subsequent generation of transgenic plants was done as described above. Transformants expressing the transgene were selected by RNA gel blot analysis. The functionality of the fusion protein was verified by volatile collection. For fluorescence imaging, 2-week-old plants grown on Murashige and Skoog medium, 2% sucrose, 0.64% agar were analyzed by laser scanning microscopy.

RNA Gel Blot Analysis

Total RNA was extracted using the Trizol method (Invitrogen) and analyzed by RNA gel blot analysis as described (Heinekamp et al., 2002). An 846-bp *GES*-specific probe was amplified by PCR using the primer combination P12 and P13 (see Supplemental Table 3 online for primer sequences). Generation of the *Actin2* probe was done as described (An et al., 1996).

Construction and Analysis of the *ProGES:GUS* Reporter Gene Fusion

A 1.2-kb *GES* promoter fragment was amplified by PCR from genomic DNA using primers P14 and P15 (see Supplemental Table 3 online for primer sequences) that carried the recombination sequences for the

Gateway technology. The *GES* promoter sequences were recombined into pDONR201 (Invitrogen) by a BP recombination reaction according to the manufacturer's instructions. The *GES* promoter fragment was subsequently recombined into the binary vector pGateGUS (kindly provided by S. Kushnir, Ghent University) by an LR recombination reaction. Transgenic plants were generated as described above. Histochemical GUS assays were done as described (Jefferson et al., 1987).

Quantification of *GES* Transcript Levels with Real-Time RT-PCR

RNA extraction of plant leaf material was performed as described above. cDNA synthesis was performed with 1 μg of total RNA, 20 pmol of oligo(dT) (18 dT), and 200 pmol of random nonamer oligonucleotides. Water was added to a final reaction volume of 12.5 μL. The mixture was heated to 70°C for 10 min, 20 nmol deoxynucleoside triphosphates (dNTPs), 4 μL of 5× reaction buffer (Fermentas), and 30 units of ribonuclease inhibitor (Eppendorf) were added (final volume, 20 μL), and the mixture was incubated at 37°C for 10 min. One hundred units of RevertAid H Minus M-MuLV reverse transcriptase (Fermentas) was added (final volume, 20 μL), and the mixture was incubated at 42°C for 70 min, then heated to 70°C for 10 min. The iCycler system (Bio-Rad) was used for the amplification and quantification of cDNA using primers for *GES* (P16 and P17), *VSP2* (P18 and P19), and *At1g13320* (protein phosphatase type 2; P20 and P21) as references (see Supplemental Table 3 online for primer sequences) (Czechowski et al., 2005). The amplification mix consisted of 1× NH_4 reaction buffer (Bioline), 2 mM $MgCl_2$, 100 μM dNTPs, 0.4 μM of primers, 0.25 units of BIOTaq DNA polymerase (Bioline), 10 nM fluorescein (Bio-Rad), 100,000× diluted SYBR Green I solution (Cambrex), 1 μL of a 1:10 dilution of cDNA as template, and double distilled water to a total volume of 25 μL. PCR consisted of a 3-min denaturation step at 95°C followed by 40 cycles of 20 s at 95°C, 20 s at 55°C, and 40 s at 72°C. Three technical replicates were done on the same cDNA during one run in the iCycler.

Determination of *TPS* Gene Transcription by RT-PCR

For expression analysis of the *TPS* genes *At2g24210*, *At4g16730*, *At4g16740*, and *At1g61120* in detached rosette leaves after 30 h of treatment with alamethicin, semiquantitative RT-PCR was performed with primers P22 and P23 (*At2g24210*), P24 and P25 (*At4g16730*), P26 and P27 (*At4g16740*), and P28 and P29 (*At1g61120*) as listed in Supplemental Table 3 online. RNA extraction of plant leaf material was performed as described above. Two micrograms of RNA were reverse-transcribed into cDNA in a 20-μL reaction with 0.5 μg of poly(dT) primer, 0.2 mM of each dNTP, and 200 units of SuperScript II reverse transcriptase (Invitrogen). PCR was performed in 30 cycles with 0.2 μM of each primer, 0.2 mM of each dNTP, and 0.25 units of Platinum Taq polymerase (Invitrogen). Reactions with primers P30 and P31 for *Actin8* (see Supplemental Table 3 online) were performed to judge the equality of cDNA template concentrations.

Phylogenetic Analysis

Amino acid sequence alignment (see Supplemental Data Set 1 online) was produced with ClustalX (http://bips.u-strasbg.fr/fr/Documentation/ClustalX) and exported as a Nexus file. A Bayesian tree was generated in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), which uses the Markov chain Monte Carlo method. The program was run for 300,000 generations with sample frequency of 10, and the SD of split frequencies was stabilized at ~0.002. When summarizing the substitution model parameters and trees, 7500 samples (i.e., the first 25% of all samples) were used for burn-in. Model jumping was used to estimate the appropriate amino acid fixed-rate model. The Wag model was selected with >99% posterior probability. Two independent runs converged to the same tree. Statistical support for nodes was assessed by Bayesian posterior probability. Treeview (Page, 1996) was used to visualize the tree. Bayesian analysis based on an alignment of truncated proteins lacking putative transit peptide sequences resulted in an almost identical phylogenetic tree with minor changes in Bayesian posterior probabilities.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *Arabidopsis* 1,8-cineole synthase (At3g25820), AY691947; *Arabidopsis ent*-copalyl diphosphate synthase (At4g02780), U11034; *Arabidopsis* kaurene synthase (At1g79460), AF034774; *Arabidopsis* (+)-linalool synthase (At1g61680), AF497485; *Arabidopsis* multiproduct monoterpene synthase (At3g25810), AF497484; *Clarkia breweri* (+)-linalool synthase, U58314; *Clarkia concinna* (+)-linalool synthase, AF067602; *Solanum lycopersicum* copalyl diphosphate synthase, AB015675; *Lactuca sativa* KS, AB031205; *Mentha citrata* (−)-linalool synthase, AY083653; *Nicotiana tabacum Cyc-1* diterpene synthase, AY049090; *Oryza sativa* 9β-pimara-7,15-dienesynthase, AB126934; *Rhizinus communis* casbene synthase, L32134.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Time Course of Volatile Emissions from *Arabidopsis* Rosette Leaves in Response to Treatment with Alamethicin.

Supplemental Figure 2. Mass Spectral Analysis of (*E,E*)-Geranyllinalool Emitted from *Arabidopsis* Leaves and Synthesized by the Recombinant GES Enzyme.

Supplemental Figure 3. Induction of *TPS* Gene Transcripts in *Arabidopsis* Rosette Leaves upon Treatment with Alamethicin.

Supplemental Figure 4. GC-MS Analysis of Volatiles Emitted from Leaves of *Pro35S:GES-YFP* and *Pro35S:GES* Transformants.

Supplemental Figure 5. Biological Replicate of Results Shown in Figure 10D: Wound-Induced Gene Expression of *Arabidopsis GES* as Determined by Real-Time RT-PCR.

Supplemental Table 1. Statistical Values for One-Way Analysis of Variance Performed for the Data Analysis Shown in Figure 7E.

Supplemental Table 2. Statistical Values for One-Way Analysis of Variance Performed for the Data Analysis Shown in Figure 9B.

Supplemental Table 3. List of Primers.

Supplemental Data Set 1. Protein Sequence Alignment of *Arabidopsis* GES with Other *Arabidopsis* and Plant TPSs Corresponding to Figure 11.

ACKNOWLEDGMENTS

We thank Bettina Raguschke, Katrin Heisse, Anna Hermann, Annette Gunkel, and Roland Scholz for excellent technical assistance and G. Howe for providing the *acx1 acx5* double mutant prior to publication. We thank Grit Kunert for help with the statistical analysis. We thank Shuangchun (Jemery) Yan and Boris Vinatzer for support with the molecular phylogenetic analysis. This work was supported by the Max Planck Society (D.T., J.G., and W.B.), by funds from Virginia

Tech (D.T.), and by grants from the Alfried-Krupp-von-Bohlen-and-Halbach Foundation (M.H.) and the Deutsche Forschungsgemeinschaft (Grant GA330-16; K.G.).

Received December 6, 2006; revised February 2, 2008; accepted March 20, 2008; published April 8, 2008.

REFERENCES

Aharoni, A., Giri, A.P., Verstappen, F.W., Bertea, C.M., Sevenier, R., Sun, Z., Jongsma, M.A., Schwab, W., and Bouwmeester, H.J. (2004). Gain and loss of fruit flavor compounds produced by wild and cultivated strawberry species. Plant Cell **16:** 3110–3131.

Ament, K., Kant, M.R., Sabelis, M.W., Haring, M.A., and Schuurink, R.C. (2004). Jasmonic acid is a key regulator of spider mite-induced volatile terpenoid and methyl salicylate emission in tomato. Plant Physiol. **135:** 2025–2037.

Ament, K., Van Schie, C.C., Bouwmeester, H.J., Haring, M.A., and Schuurink, R.C. (2006). Induction of a leaf specific geranylgeranyl pyrophosphate synthase and emission of (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene in tomato are dependent on both jasmonic acid and salicylic acid signaling pathways. Planta **224:** 1197–1208.

An, Y.Q., McDowell, J.M., Huang, S., McKinney, E.C., Chambliss, S., and Meagher, R.B. (1996). Strong, constitutive expression of the *Arabidopsis ACT2/ACT8* actin subclass in vegetative tissues. Plant J. **10:** 107–121.

Arimura, G., Ozawa, R., Shimoda, T., Nishioka, T., Boland, W., and Takabayashi, J. (2000). Herbivory-induced volatiles elicit defence genes in lima bean leaves. Nature **406:** 512–515.

Aubourg, S., Lecharny, A., and Bohlmann, J. (2002). Genomic analysis of the terpenoid synthase (AtTPS) gene family of *Arabidopsis thaliana.* Mol. Genet. Genomics **267:** 730–745.

Baldwin, I.T., and Preston, C.A. (1999). The eco-physiological complexity of plant responses to insect herbivores. Planta **208:** 137–145.

Besumbes, O., Sauret-Gueto, S., Phillips, M.A., Imperial, S., Rodriguez-Concepcion, M., and Boronat, A. (2004). Metabolic engineering of isoprenoid biosynthesis in *Arabidopsis* for the production of taxadiene, the first committed precursor of Taxol. Biotechnol. Bioeng. **88:** 168–175.

Bohlmann, J., Martin, D., Oldham, N.J., and Gershenzon, J. (2000). Terpenoid secondary metabolism in *Arabidopsis thaliana*: cDNA cloning, characterization, and functional expression of a myrcene/(*E*)-beta-ocimene synthase. Arch. Biochem. Biophys. **375:** 261–269.

Bohlmann, J., Meyer-Gauen, G., and Croteau, R. (1998). Plant terpenoid synthases: Molecular biology and phylogenetic analysis. Proc. Natl. Acad. Sci. USA **95:** 4126–4133.

Boland, W., Gäbler, A., Gilbert, M., and Feng, Z. (1998). Biosynthesis of C11 and C16 homoterpenes in higher plants: Stereochemistry of the C–C-bond cleavage reaction. Tetrahedron **54:** 14725–14736.

Boland, W., Ney, P., Jaenicke, L., and Gassmann, G. (1984). A 'closed-loop-stripping' technique as a versatile tool for metabolic studies of volatiles. In Analysis of Volatiles, P. Schreier, ed (Berlin: Walter de Gruyter), pp. 371–373.

Bouwmeester, H.J., Verstappen, F.W., Posthumus, M.A., and Dicke, M. (1999). Spider mite-induced (3*S*)-(*E*)-nerolidol synthase activity in cucumber and lima bean. The first dedicated step in acyclic C11-homoterpene biosynthesis. Plant Physiol. **121:** 173–180.

Caddick, M.X., Greenland, A.J., Jepson, I., Krause, K.P., Qu, N., Riddell, K.V., Salter, M.G., Schuch, W., Sonnewald, U., and Tomsett, A.B. (1998). An ethanol inducible gene switch for plants used to manipulate carbon metabolism. Nat. Biotechnol. **16:** 177–180.

Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X. (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6:** 1583–1592.

Chen, F., Ro, D.K., Petri, J., Gershenzon, J., Bohlmann, J., Pichersky, E., and Tholl, D. (2004). Characterization of a root-specific *Arabidopsis* terpene synthase responsible for the formation of the volatile monoterpene 1,8-cineole. Plant Physiol. **135:** 1956–1966.

Chen, F., Tholl, D., D'Auria, J.C., Farooq, A., Pichersky, E., and Gershenzon, J. (2003). Biosynthesis and emission of terpenoid volatiles from *Arabidopsis* flowers. Plant Cell **15:** 481–494.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana.* Plant J. **16:** 735–743.

Cseke, L., Dudareva, N., and Pichersky, E. (1998). Structure and evolution of linalool synthase. Mol. Biol. Evol. **15:** 1491–1498.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., and Scheible, W.R. (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol. **139:** 5–17.

Davis, E.M., and Croteau, R. (2000). Cyclization enzymes in the biosynthesis of monoterpenes, sesquiterpenes, and diterpenes. In Topics in Current Chemistry: Biosynthesis—Aromatic Polyketides, Isoprenoids, Alkaloids, F.J. Leeper and J.C. Vederas, eds (Heidelberg, Germany: Springer-Verlag), pp. 53–95.

de Boer, J.G., Posthumus, M.A., an d Dicke, M. (2004). Identification of volatiles that are used in discrimination between plants infested with prey or nonprey herbivores by a predatory mite. J. Chem. Ecol. **30:** 2215–2230.

Degenhardt, J., and Gershenzon, J. (2000). Demonstration and characterization of (*E*)-nerolidol synthase from maize: A herbivore-inducible terpene synthase participating in (3*E*)-4,8-dimethyl-1,3,7-nonatriene biosynthesis. Planta **210:** 815–822.

Devoto, A., Ellis, C., Magusin, A., Chang, H.S., Chilcott, C., Zhu, T., and Turner, J.G. (2005). Expression profiling reveals COI1 to be a key regulator of genes involved in wound- and methyl jasmonate-induced secondary metabolism, defence, and hormone interactions. Plant Mol. Biol. **58:** 497–513.

Dicke, M., and Sabelis, M.W. (1989). Does it pay plants to advertise for bodyguards? In Causes and Consequences of Variation in Growth Rate and Productivity of Higher Plants, H. Lambers, M.L. Cambridge, H. Konings, and T.L. Pons, eds (The Hague, The Netherlands: SPB), pp. 341–358.

Donath, J., and Boland, W. (1995). Biosynthesis of acyclic homoterpenes: Enzyme selectivity and absolute configuration of the nerolidol percursor. Phytochemistry **39:** 785–790.

Dudareva, N., Cseke, L., Blanc, V.M., and Pichersky, E. (1996). Evolution of floral scent in *Clarkia*: Novel patterns of *S*-linalool synthase gene expression in the *C. breweri* flower. Plant Cell **8:** 1137–1148.

Engelberth, J., Koch, T., Schuler, G., Bachmann, N., Rechtenbach, J., and Boland, W. (2001). Ion channel-forming alamethicin is a potent elicitor of volatile biosynthesis and tendril coiling. Cross talk between jasmonate and salicylate signaling in lima bean. Plant Physiol. **125:** 369–377.

Fäldt, J., Arimura, G., Gershenzon, J., Takabayashi, J., and Bohlmann, J. (2003). Functional identification of AtTPS03 as (*E*)-beta-ocimene synthase: A monoterpene synthase catalyzing jasmonate- and wound-induced volatile formation in *Arabidopsis thaliana.* Planta **216:** 745–751.

Fritzsche-Hoballah, M.E., and Turlings, T.C.J. (2001). Experimental evidence that plants under caterpillar attack may benefit from attracting parasitoids. Evol. Ecol. Res. **3:** 553–565.

Fulton, T.M., Chunwongse, J., and Tanksley, S.D. (1995). Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Mol. Biol. Rep. **13:** 207–209.

Gäbler, A., Boland, W., Preiss, H., and Simon, H. (1991). Stereochemical studies on homoterpene biosynthesis in higher plants: Mechanistic, phylogenetic, and ecological aspects. Helv. Chim. Acta **74:** 1773–1789.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. Science **262:** 754–756.

Gibeaut, D.M., Hulett, J., Cramer, G.R., and Seemann, J.R. (1997). Maximal biomass of *Arabidopsis thaliana* using a simple, low-maintenance hydroponic method and favorable environmental conditions. Plant Physiol. **115:** 317–319.

Glazebrook, J., Chen, W., Estes, B., Chang, H.S., Nawrath, C., Metraux, J.P., Zhu, T., and Katagiri, F. (2003). Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. Plant J. **34:** 217–228.

Guzman, P., and Ecker, J.R. (1990). Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. Plant Cell **2:** 513–523.

Hefner, J., Ketchum, R.E., and Croteau, R. (1998). Cloning and functional expression of a cDNA encoding geranylgeranyl diphosphate synthase from *Taxus canadensis* and assessment of the role of this prenyltransferase in cells induced for taxol production. Arch. Biochem. Biophys. **360:** 62–74.

Heinekamp, T., Kuhlmann, M., Lenk, A., Strathmann, A., and Droge-Laser, W. (2002). The tobacco bZIP transcription factor BZI-1 binds to G-box elements in the promoters of phenylpropanoid pathway genes in vitro, but it is not involved in their regulation in vivo. Mol. Genet. Genomics **267:** 16–26.

Hopke, J., Donath, J., Blechert, S., and Boland, W. (1994). Herbivore-induced volatiles: The emission of acyclic homoterpenes from leaves of *Phaseolus lunatus* and *Zea mays* can be triggered by a beta-glucosidase and jasmonic acid. FEBS Lett. **352:** 146–150.

Huelsenbeck, J., and Ronquist, F. (2001). MrBayes: Bayesian inference of phylogeny. Bioinformatics **17:** 754–755.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6:** 3901–3907.

Kant, M.R., Ament, K., Sabelis, M.W., Haring, M.A., and Schuurink, R.C. (2004). Differential timing of spider mite-induced direct and indirect defenses in tomato plants. Plant Physiol. **135:** 483–495.

Kappers, I.F., Aharoni, A., van Herpen, T.W., Luckerhoff, L.L., Dicke, M., and Bouwmeester, H.J. (2005). Genetic engineering of terpenoid metabolism attracts bodyguards to *Arabidopsis.* Science **309:** 2070–2072.

Karimi, M., De Meyer, B., and Hilson, P. (2005). Modular cloning in plant cells. Trends Plant Sci. **10:** 103–105.

Kessler, A., and Baldwin, I.T. (2001). Defensive function of herbivore-induced plant volatile emissions in nature. Science **291:** 2141–2144.

Koch, T., Krumm, T., Jung, V., Engelberth, J., and Boland, W. (1999). Differential induction of plant volatile biosynthesis in the lima bean by early and late intermediates of the octadecanoid-signaling pathway. Plant Physiol. **121:** 153–162.

Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S., and Ryals, J. (1995). Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. Mol. Plant Microbe Interact. **8:** 863–870.

Martin, D.M., Fäldt, J., and Bohlmann, J. (2004). Functional characterization of nine Norway spruce TPS genes and evolution of gymnosperm terpene synthases of the TPS-d subfamily. Plant Physiol. **135:** 1908–1927.

Nawrath, C., and Metraux, J.P. (1999). Salicylic acid induction-deficient mutants of *Arabidopsis* express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. Plant Cell **11:** 1393–1404.

Okada, K., Saito, T., Nakagawa, T., Kawamukai, M., and Kamiya, Y. (2000). Five geranylgeranyl diphosphate synthases expressed in different organs are localized into three subcellular compartments in Arabidopsis. Plant Physiol. **122:** 1045–1056.

Page, R.D.M. (1996). TREEVIEW: An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. **12:** 357–358.

Park, J.H., Halitschke, R., Kim, H.B., Baldwin, I.T., Feldmann, K.A., and Feyereisen, R. (2002). A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in *Arabidopsis* due to a block in jasmonic acid biosynthesis. Plant J. **31:** 1–12.

Penninckx, I.A., Thomma, B.P., Buchala, A., Metraux, J.P., and Broekaert, W.F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis.* Plant Cell **10:** 2103–2113.

Pichersky, E., Lewinsohn, E., and Croteau, R. (1995). Purification and characterization of *S*-linalool synthase, an enzyme involved in the production of floral scent in *Clarkia breweri.* Arch. Biochem. Biophys. **316:** 803–807.

Pichersky, E., Raguso, R.A., Lewinsohn, E., and Croteau, R. (1994). Floral scent production in *Clarkia* (Onagraceae) (I. Localization and developmental modulation of monoterpene emission and linalool synthase activity). Plant Physiol. **106:** 1533–1540.

Reymond, P., Weber, H., Damond, M., and Farmer, E.E. (2000). Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis.* Plant Cell **12:** 707–720.

Ro, D.K., Ehlting, J., Keeling, C.I., Lin, R., Mattheus, N., and Bohlmann, J. (2006). Microarray expression profiling and functional characterization of AtTPS genes: Duplicated *Arabidopsis thaliana* sesquiterpene synthase genes At4g13280 and At4g13300 encode root-specific and wound-inducible (*Z*)-gamma-bisabolene synthases. Arch. Biochem. Biophys. **448:** 104–116.

Ronquist, F., and Huelsenbeck, J.P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19:** 1572–1574.

Roslan, H.A., Salter, M.G., Wood, C.D., White, M.R., Croft, K.P., Robson, F., Coupland, G., Doonan, J., Laufs, P., Tomsett, A.B., and Caddick, M.X. (2001). Characterization of the ethanol-inducible alc gene-expression system in *Arabidopsis thaliana.* Plant J. **28:** 225–235.

Sabelis, M.W., Janssen, A., and Kant, M.R. (2001). Ecology. The enemy of my enemy is my ally. Science **291:** 2104–2105.

Schilmiller, A.L., Koo, A.J., and Howe, G.A. (2007). Functional diversification of acyl-coenzyme A oxidases in jasmonic acid biosynthesis and action. Plant Physiol. **143:** 812–824.

Schnee, C., Kollner, T.G., Gershenzon, J., and Degenhardt, J. (2002). The maize gene terpene synthase 1 encodes a sesquiterpene synthase catalyzing the formation of (*E*)-beta-farnesene, (*E*)-nerolidol, and (*E*,*E*)-farnesol after herbivore damage. Plant Physiol. **130:** 2049–2060.

Sessions, A., et al. (2002). A high-throughput *Arabidopsis* reverse genetics system. Plant Cell **14:** 2985–2994.

Spoel, S.H., et al. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell **15:** 760–770.

Stintzi, A., Weber, H., Reymond, P., Browse, J., and Farmer, E.E. (2001). Plant defense in the absence of jasmonic acid: The role of cyclopentenones. Proc. Natl. Acad. Sci. USA **98:** 12837–12842.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A., and Browse, J. (2007). JAZ

repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature **448:** 661–665.

Tholl, D. (2006). Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. Curr. Opin. Plant Biol. **9:** 297–304.

Tholl, D., Chen, F., Petri, J., Gershenzon, J., and Pichersky, E. (2005). Two sesquiterpene synthases are responsible for the complex mixture of sesquiterpenes emitted from *Arabidopsis* flowers. Plant J. **42:** 757–771.

Trapp, S.C., and Croteau, R.B. (2001). Genomic organization of plant terpene synthases and molecular evolutionary implications. Genetics **158:** 811–832.

Van Loon, J.J.A., De Boer, J.G., and Dicke, M. (2000). Parasitoid-plant mutualism: Parasitoid attack of herbivore increases plant reproduction. Entomol. Exp. Appl. **97:** 219–227.

Van Poecke, R.M., Posthumus, M.A., and Dicke, M. (2001). Herbivore-induced volatile production by *Arabidopsis thaliana* leads to attraction of the parasitoid *Cotesia rubecula*: Chemical, behavioral, and gene-expression analysis. J. Chem. Ecol. **27:** 1911–1928.

van Wees, S.C., Chang, H.S., Zhu, T., and Glazebrook, J. (2003). Characterization of the early response of *Arabidopsis* to *Alternaria brassicicola* infection using expression profiling. Plant Physiol. **132:** 606–617.

von Malek, B., van der Graaff, E., Schneitz, K., and Keller, B. (2002). The *Arabidopsis* male-sterile mutant *dde2-2* is defective in the ALLENE OXIDE SYNTHASE gene encoding one of the key enzymes of the jasmonic acid biosynthesis pathway. Planta **216:** 187–192.

Wildermuth, M.C., Dewdney, J., Wu, G., and Ausubel, F.M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature **414:** 562–565.

Williams, L., III, Rodriguez-Saona, C., Pare, P.W., and Crafts-Brandner, S.J. (2005). The piercing-sucking herbivores *Lygus hesperus* and *Nezara viridula* induce volatile emissions in plants. Arch. Insect Biochem. Physiol. **58:** 84–96.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G. (1998). COI1: An *Arabidopsis* gene required for jasmonate-regulated defense and fertility. Science **280:** 1091–1094.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., and Gruissem, W. (2004). GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol. **136:** 2621–2632.