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Monoterpenes Support Systemic Acquired Resistance within and between Plants

Marlies Riedlmeier,[a,1] Andrea Ghirardo,[b,1] Marion Wenig,[a,1] Claudia Knappe,[a] Kerstin Koch,[b] Elisabeth Georgii,[a] Sanjukta Dey,[a] Jane E. Parker,[c] Jörg-Peter Schnitzler,[b] and A. Corina Vlot[a,2]

[a] Helmholtz Zentrum Muenchen, Department of Environmental Sciences, Institute of Biochemical Plant Pathology, D-85764 Neuherberg, Germany

[b] Helmholtz Zentrum Muenchen, Institute of Biochemical Plant Pathology, Research Unit Environmental Simulation, D-85764 Neuherberg, Germany

[c] Max Planck Institute for Plant Breeding Research, Department of Plant-Microbe Interactions, D-50829 Cologne, Germany

ORCID IDs: 0000-0002-6376-1005 (M.R.); 0000-0002-7511-5510 (E.G.); 0000-0002-9825-867X (J.-P.S.); 0000-0002-8146-6018 (A.C.V.)

This study investigates the role of volatile organic compounds in systemic acquired resistance (SAR), a salicylic acid (SA)-associated, broad-spectrum immune response in systemic, healthy tissues of locally infected plants. Gas chromatography coupled to mass spectrometry analyses of SAR-related emissions of wild-type and non-SAR-signal-producing mutant plants associated SAR with monoterpene emissions. Headspace exposure of *Arabidopsis thaliana* to a mixture of the bicyclic monoterpenes α-pinene and β-pinene induced defense, accumulation of reactive oxygen species, and expression of SA- and SAR-related genes, including the SAR regulatory *AZELAIC ACID INDUCED1* (*AZI1*) gene and three of its paralogs. Pinene-induced resistance was dependent on SA biosynthesis and signaling and on *AZI1*. Arabidopsis *geranylgeranyl reductase1* mutants with reduced monoterpene biosynthesis were SAR-defective but mounted normal local resistance and methyl salicylate-induced defense responses, suggesting that monoterpenes act in parallel with SA. The volatile emissions from SAR signal-emitting plants induced defense in neighboring plants, and this was associated with the presence of α-pinene, β-pinene, and camphene in the emissions of the "sender" plants. Our data suggest that monoterpenes, particularly pinenes, promote SAR, acting through ROS and *AZI1*, and likely function as infochemicals in plant-to-plant signaling, thus allowing defense signal propagation between neighboring plants.

INTRODUCTION

As sessile organisms, plants are equipped with a sophisticated multilayered immune system including constitutive and inducible defenses (Spoel and Dong, 2012). Non-host resistance is the most robust and durable form of plant resistance to the majority of nonadapted microbes. If a host-adapted pathogen penetrates constitutive barriers of the plant's surface and cell wall, it encounters the extracellular space where, for example, pattern recognition receptors (PRRs) can recognize conserved microbial structures (elicitors or pathogen-associated molecular patterns [PAMPs]) (Macho and Zipfel, 2014). PRRs are located in the plasma membrane and contain an extracellular ligand recognition domain, often fused to an intracellular kinase signaling domain. Activation of PRRs induces a battery of host responses, including stomatal closure, a burst of reactive oxygen species (ROS), mitogen-activated protein kinase activation, salicylic acid (SA) production, and changes in host gene expression, known collectively as PAMP-triggered immunity (PTI) (Jones and Dangl, 2006; Spoel and Dong, 2012; Macho and Zipfel, 2014). Driven by natural selection, host-adapted pathogens have evolved effectors, many of which are secreted into the host cytoplasm to suppress PTI, resulting in effector-triggered susceptibility (Jones and Dangl, 2006). Effector-triggered susceptibility, in turn, can be counteracted by plant RESISTANCE proteins that recognize specific pathogen effectors and induce effector-triggered immunity (ETI) (Jones and Dangl, 2006; Spoel and Dong, 2012; Cui et al., 2015). Compared with PTI, ETI is generally a more robust form of plant defense, often culminating in a local hypersensitive response involving programmed cell death, which isolates the pathogen and protects remaining plant tissues from infection (Cui et al., 2015; Mur et al., 2008).

Both PTI and ETI trigger SA accumulation and expression of the SA marker gene *PATHOGENESIS-RELATED1* (*PR1*) in infected and also distal uninfected tissues of locally infected plants. The latter reaction is part of an inducible defense response known as systemic acquired resistance (SAR), a long-lasting SA-dependent immunity against a broad spectrum of (hemi-)biotrophic pathogens (Spoel and Dong, 2012; Fu and Dong, 2013; Shah et al., 2014). Putative long-distance signals that move from infected to distal tissues to induce SAR include the volatile methylated derivative of SA (methyl salicylate [MeSA]; Park et al., 2007), the dicarboxylic acid azelaic acid (AzA; Jung et al., 2009), the abietane diterpenoid dehydroabietinal (DA; Chaturvedi et al., 2012), a glycerol-3-phosphate (G3P) derivative (Chanda et al., 2011), the predicted lipid transfer proteins DEFECTIVE IN INDUCED RESISTANCE1 (DIR1) and DIR1-like (Maldonado et al., 2002;

[1] These authors contributed equally to this work.

[2] Address correspondence to corina.vlot@helmholtz-muenchen.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.plantcell.org) is: A. Corina Vlot (corina.vlot@helmholtz-muenchen.de).

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Champigny et al., 2013), and the non-protein amino acid pipecolic acid (Navarová et al., 2012). Most of these molecules are believed to act in parallel with SA in systemic but not local resistance responses. SAR-modulating interactions between these signals are increasingly recognized (Dempsey and Klessig, 2012; Shah et al., 2014; Gao et al., 2015). AzA, for example, appears to act synergistically with DA (Chaturvedi et al., 2012) and upstream of G3P, which promotes SAR in a positive feedback loop with DIR1 and AZELAIC ACID INDUCED1 (AZI1), possibly together with DIR1-like and one or more paralogs of AZI1, including EARLY ARABIDOPSIS ALUMINUM INDUCED1 (EARLI1) (Yu et al., 2013; Cecchini et al., 2015; Gao et al., 2015).

ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) is a nucleocytoplasmic, lipase-like protein that promotes the transcriptional reprogramming of parallel SA-dependent and SA-independent defense signaling pathways (Feys et al., 2001; Bartsch et al., 2006; García et al., 2010; Cui et al., 2017) in basal immunity against virulent pathogens and ETI conferred by intracellular nucleotide binding/leucine-rich repeat (NB-LRR) receptors (Aarts et al., 1998; Vlot et al., 2009; Cui et al., 2017). To mobilize these pathways, EDS1 forms heteromeric complexes with either one of its sequence-related partners, PHYTOALEXIN-DEFICIENT4 or SENESCENCE ASSOCIATED GENE101 (Feys et al., 2001, 2005; Vlot et al., 2009, Rietz et al., 2011, Wagner et al., 2013). EDS1 nuclear accumulation is essential for transcriptional defense reprogramming in basal immunity and ETI mediated by a major subclass of NB-LRR receptors called TIR-NB-LRRs (possessing a Toll-Interleukin 1-receptor-like N-terminal domain) (García et al., 2010; Bhattacharjee et al., 2011; Heidrich et al., 2011; Stuttmann et al., 2016).

During SAR, EDS1 is required both for generating the SAR signal in primary infected leaves and for perceiving the SAR signal in systemic uninfected tissues (Breitenbach et al., 2014). In *Arabidopsis thaliana*, pathogen infection with *Pseudomonas syringae* carrying the effector *AvrRpm1* activates ETI via the coiled-coil NB-LRR (CC-NB-LRR) receptor RESISTANCE TO PSEUDOMONAS SYRINGAE pathovar MACULICOLA1 (RPM1) (Dangl et al., 1992). Although RPM1-triggered local SA-dependent immune responses and programmed cell death genetically do not require *EDS1* (Aarts et al., 1998), both *eds1* and *pad4* mutant plants are SAR-defective after local RPM1 activation (Truman et al., 2007; Jing et al., 2011; Rietz et al., 2011; Breitenbach et al., 2014).

Previously, we exploited the SAR-specific phenotype of the *eds1* mutant in response to conditionally expressed *AvrRpm1* to identify proteins and metabolites that are associated with SAR (Breitenbach et al., 2014; Wittek et al., 2014, 2015). Here, we identify SAR-associated volatile organic compounds (VOCs) that are emitted from Arabidopsis rosettes in an *EDS1*-dependent manner in response to *AvrRpm1.* So far, VOCs in plant defense have been implicated mostly in direct and indirect responses to herbivory (Dicke, 2009; Ghirardo et al., 2012; Scala et al., 2013a; Pierik et al., 2014; Dong et al., 2016). However, there are indications that plant-, bacteria-, or fungus-derived VOCs can affect plant innate immunity (Yi et al., 2009; Dicke and Baldwin, 2010; Scala et al., 2013a, 2013b; Choi et al., 2014; Naznin et al., 2014; Song et al., 2015). Also, in addition to the putative role of the volatile benzenoid MeSA in SAR, further possible functions of VOCs in SAR have been discussed (Heil and Ton, 2008) but have remained largely uninvestigated to date.

In this study, we present a comprehensive analysis of Arabidopsis VOC emissions during SAR signaling. Using gas chromatography coupled to mass spectrometry (GC-MS), plant headspace exposure, and transcript profiling approaches as well as Arabidopsis mutants, we found that monoterpenoid VOCs are highly correlated with SAR competence. We further establish that monoterpenes play an essential role in plant-to-plant innate immune signaling, suggesting that monoterpenoid VOCs are part of an ecologically relevant mechanism to relay signals to other plants in the nearby environment.

RESULTS

Identification of SAR-Related VOCs

SAR signaling was induced by dexamethasone (DEX) treatment of 4- to 5-week-old transgenic plants expressing hemagglutinin (HA)-tagged *AvrRpm1* from a DEX-inducible transgene (*pDEX:AvrRpm1-HA*) in the Col-0 wild type and *eds1-2* genetic backgrounds of Arabidopsis (Breitenbach et al., 2014). Significant VOC emissions were observed 1 to 7 h after the onset of the treatment. VOCs were collected during two sampling periods (SPs): between 1 and 4 h (SP1) and 4 and 7 h (SP2) after the DEX treatment. Using GC-MS, we detected a total of 39 compounds in background-corrected emission profiles from DEX-treated Col-0 *DEX:AvrRpm1-HA* and *eds1-2 DEX:AvrRpm1-HA* plants. Chemical identification indicated the presence of five mono- and three sesquiterpenes, while the remaining compounds were classified as fatty esters, fatty aldehydes, alkanes, aromatics, and alcohols (Supplemental Table 1).

Multivariate data analysis (MDA) of the VOC emission rates from DEX-treated Col-0 *DEX:AvrRpm1-HA* and *eds1-2 DEX:AvrRpm1-HA* plants revealed a clear separation of Col-0 and *eds1-2* profiles during both sampling periods (Figures 1A, SP1, and 1B, SP2). Partial least square regression (PLSR) using the regression type OPLS (orthogonal PLS) of samples collected during SP1 (Figures 1A, 1C, and 1E) indicated that β-pinene, α-pinene, camphene, isopropyl palmitate, and sabinene were the strongest discriminant compounds between Col-0- and *eds-1-2*-derived samples ($P = 0.012$; cross-validated [CV]-ANOVA) and were emitted at statistically different emission rates ($P < 0.01$, Student's *t* test) (marked in red in Figures 1C and 1E; Supplemental Table 1). The same analysis of samples collected during SP2 (Figures 1B, 1D, and 1F) confirmed that release of these VOCs was significantly different ($P < 0.05$, CV-ANOVA) between the two genotypes, although to a lesser extent compared with SP1. Overall, five VOCs were negatively correlated with *eds1-2* in samples collected during SP1 (Figure 1E), and the emissions of α-pinene, β-pinene, and camphene (Figure 2A) were not detected from DEX-treated *eds1-2 DEX:AvrRpm1-HA* plants during both sampling periods (Figures 2B to 2D). Emissions of the monoterpene sabinene and of isopropyl palmitate, the ester of isopropylalcohol and palmitic acid (C_{19}), were reduced in *eds1-2* compared with wild-type plants, but remained detectable (Figures 2E and 2F). In DEX-treated Col-0 *DEX:AvrRpm1-HA* plants, the monoterpene emissions tended to

decrease from the early time point, SP1, to the later one, SP2, indicating that the weaker difference between Col-0 and *eds1-2* seen with MDA in SP2 was due to an overall decrease in emission rates.

Together, the data associate the SAR deficiency of *eds1* mutant plants with reduced emissions of at least five VOCs. Notably, four of these compounds are closely related monoterpenes: α-pinene, β-pinene, camphene (Figure 2A), and sabinene are biochemically produced from the same plastidic isoprenoid pathway (Tholl and Lee, 2011). Almost undetectable emissions of α-pinene, β-pinene, and camphene from *eds1-2* mutant plants (Figures 2B to 2D) prompted us to investigate whether these monoterpenes have a role in inducing SAR.

Monoterpenes Enhance Arabidopsis Resistance to *P. syringae* Bacteria

To assess the possible biological relevance of α-pinene, β-pinene, and camphene in plant immunity, an experimental setup was designed to treat plants with individual compounds in gas-tight glass desiccators. In these 5.5-liter compartments, eight 4.5-week-old Arabidopsis wild-type Col-0 plants were incubated over 3 d with different amounts of either camphene or a mixture of the structural isomers α- and β-pinene 1:1 (v:v). The pinene mixture contained both of the α-pinene enantiomers [(±)-α-pinene] and the naturally prevalent β-pinene enantiomer (−)-β-pinene (Figure 2A). Different monoterpenes, MeSA as a positive control, or the VOC solvent hexane as a negative control were applied onto a filter paper in the desiccators. Every 24 h, the supplemented air was replaced with fresh air from the inflow of the growth chamber. After changing the air, the appropriate monoterpene, or the MeSA or hexane treatments, were applied again. After 3 d, plants were removed from the desiccators and two fully expanded leaves were inoculated with *P. syringae* pv *tomato* strain DC3000 (*Pst*). *Pst* bacterial growth was then monitored by measuring in planta *Pst* titers at 4 d postinoculation (dpi).

Park et al. (2007) showed that treatment of tobacco (*Nicotiana tabacum*) leaves with MeSA enhanced the systemic resistance of the plants to tobacco mosaic virus. Pre-experiments with different concentrations of MeSA revealed that Arabidopsis required ~22-fold less MeSA for maximal defense induction compared with the concentrations applied to tobacco (Park et al., 2007). Arabidopsis wild-type plants that had been exposed to 1.6 μmole MeSA for 3 d supported ~10-fold less growth of *Pst* compared with plants that had been exposed to the hexane solvent only (negative control), indicating that resistance to *Pst* was induced by MeSA (Figure 3A), presumably due to its conversion to SA (Shulaev et al., 1997; Koo et al., 2007; Park et al., 2007). A similar reduction in *Pst* growth was observed after incubation of wild-type plants with the pinene mixture in a concentration-dependent manner, suggesting that α- and/or β-pinene enhanced Arabidopsis resistance to *Pst* (Figure 3A). Treatment of plants with 0.6 μmole of the pinene mixture, which amounts to a concentration in the headspace of ~2.6 ppmv (parts per million by volume), triggered the strongest defense response and was used in all subsequent head space exposure experiments. We next assessed if and how the length of the headspace exposure affects pinene-induced resistance. Pilot experiments showed that the pinene mixture did not enhance resistance to *Pst* or *PR1* transcript accumulation in the treated plants if the 3-d exposure time was reduced to 1 d (see lane 6 in Figure 8D) or 2 d, respectively. To exclude a possible effect of shorter pinene exposures if plants were left additional time to respond, we kept 1-d exposed plants in the growth chamber for two additional days (time point 1+2d) and 2-d exposed plants for one additional day (time point 2+1d) before inoculating them with *Pst*. Because *Pst* growth was significantly reduced after 3 d of headspace exposure to 0.6 μmole of the pinene mixture, but not after the 1+2d and 2+1d treatments (Figure 3B), we concluded that the pinene mixture must be applied for three consecutive days to induce defense. Once established, the pinene-induced defense response was relatively stable, reducing growth of a *Pst* inoculum that was applied 1 or 3 d after the completion of a 3-d headspace exposure (Supplemental Figure 1; time points 3+1d and 3+3d).

Similarly to the emissions of α- and β-pinene, camphene emissions from *AvrRpm1*-expressing *eds1-2* plants were reduced to levels below the detection limit (Figure 2D). Also, headspace exposure of plants to camphene reduced *Pst* growth in leaves to a similar extent as the pinene mixture (Figure 3C). Notably, camphene enhanced plant resistance to *Pst* when applied at a lower amount of 0.1 μmole, which amounted to a concentration in the headspace of ~0.4 ppmv. The application of higher camphene amounts (0.7 and 1.4 μmole; Figure 3C) did not enhance Arabidopsis resistance to *Pst* growth. This is comparable to our previous findings for SAR-inducing folates and the AzA precursor 9-oxo nonanoic acid, which trigger SAR in a concentration-dependent manner and lose their activity when applied at higher concentrations (Wittek et al., 2014, 2015). Together, the data suggest that SAR is sensitive to the concentration of (*EDS1*-dependent) SAR signaling components. Here, the resistance-inducing capacity of camphene was confirmed in combination with the pinene mixture. Exposing plants to 0.1 μmole of camphene and 0.6 μmole of the pinene mixture reduced *Pst* growth compared with each of the individual treatments (Figure 3 D), suggesting that camphene acts additively with α- and β-pinene in the induction of defense.

Because monoterpenes are believed to have antimicrobial activity (Tholl and Lee, 2011), we excluded a possible toxic effect of the pinene mixture and of camphene on *Pst* by propagating serial dilutions of *Pst* on plates that were either supplemented with 500 μM of the VOCs or incubated in desiccators and exposed to the VOC amounts that enhanced plant resistance to *Pst* (Supplemental Figure 2). Neither of these treatments compromised *Pst* growth compared with that of the untreated controls or of *Pst* propagated in the presence of MeSA or the hexane solvent amount corresponding to that in the pinene mixture and camphene. Because camphene and both pinenes are thus likely nontoxic to *Pst*, the data suggest that these compounds act as infochemicals that promote Arabidopsis resistance to *Pst* growth in planta.

In plants, both enantiomers of α-pinene, i.e., (+)-α-pinene and (−)-α-pinene, can co-occur, while β-pinene is generally found as the (−)-isoform (Finefield et al., 2012), which was included in the pinene mixture. Next, we sought to ascertain if (−)-β-pinene has an enantiomer-specific function in plant immunity. As shown above, the headspace exposure of plants with the pinene mixture reduced growth of subsequently applied *Pst* bacteria (Figure 3E).

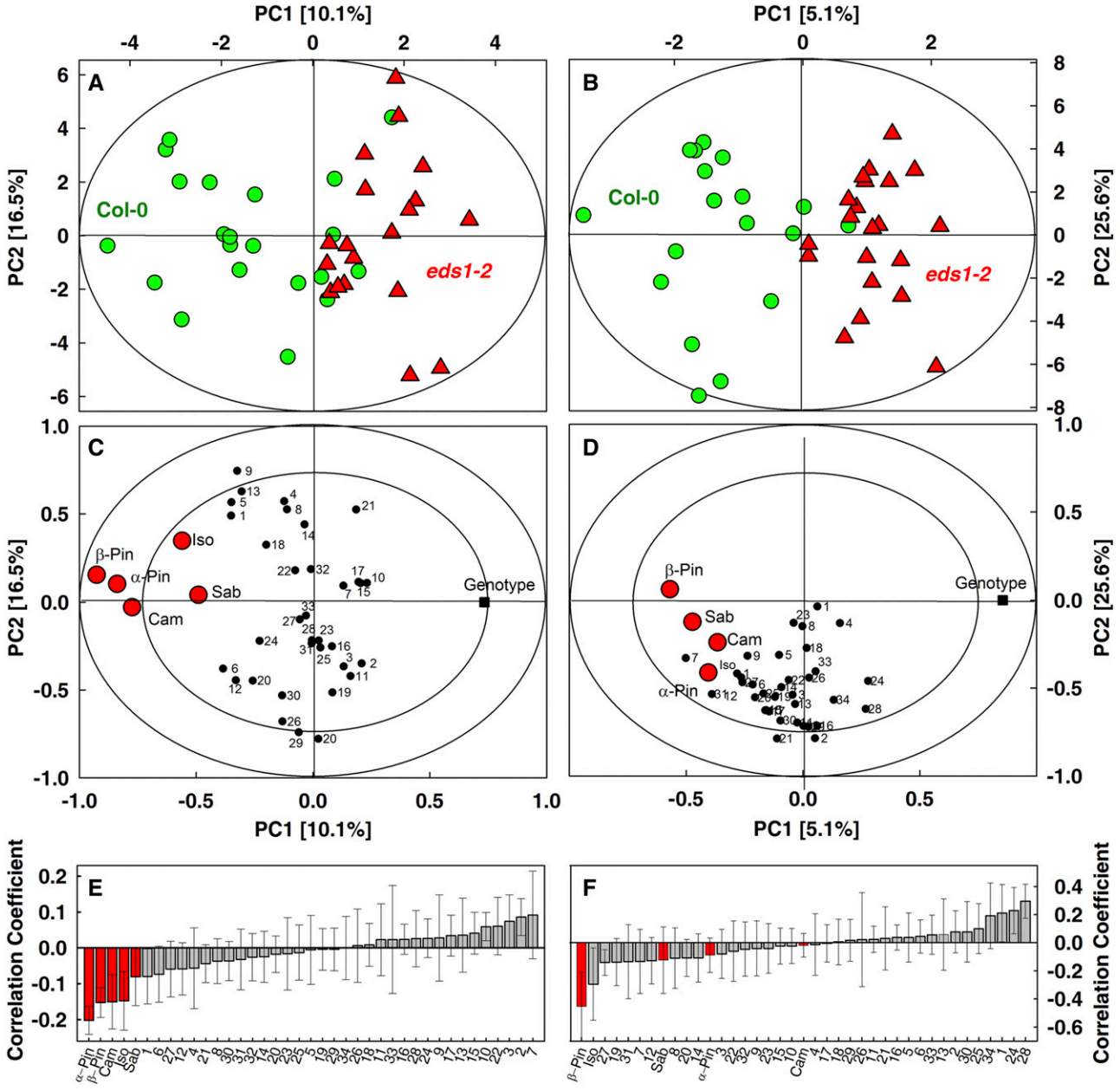

Figure 1. Correlation of VOCs to SAR.

The volatile emissions from DEX-treated Col-0 *DEX:AvrRpm1-HA* and *eds1-2 DEX:AvrRpm1-HA* plants were measured by GC-MS and analyzed by orthogonal partial least square regression (OPLS). The left panels (**[A]**, **[C]**, and **[E]**) represent sampling period 1 (SP1; VOCs collected 1–4 h after DEX treatment) and the right panels (**[B]**, **[D]**, and **[F]**) represent sampling point 2 (SP2; VOCs collected 4–7 h after DEX treatment).

(A) and **(B)** OPLS score plots (green circles, Col-0; red triangles, *eds1-2*). The ellipses indicate the model tolerance based on Hotelling's t^2 with a significance level of a = 0.05. Each circle represents an individual measurement of leaf volatiles, given as VOC emission rate (pmol m^{-2} s^{-1}).

(C) and **(D)** OPLS loading plots shown with the correlation scaled. The outer and inner ellipses indicate 100% and 75% of explained variance, respectively. Circles represent the X-loadings (VOCs) and squares depict the Y-loadings (plant genotype).

(E) and **(F)** Correlation coefficient plots of the VOC emission rates correlating VOC emissions with *eds1-2*. The correlation coefficients are scaled and centered, and the error bars are derived from the jackknife method. Bars represent the average ± SE of 18 to 21 replicates.

(A) to **(F)** OPLS model fitness for both the sampling periods (SP1/SP2): r^2 (x) = 61/54%, q^2 (cum) = 56/34% using 1 predictive component. RMSEE (root mean square error of estimation) = 0.35/0.31; RMSEcv (root mean square error of cross-validation) = 0.43/0.47; P = 0.012/<0.05 CV-ANOVA. Significant differences in VOC emissions between Col-0 and *eds1-2* in **(C)** to **(F)** are highlighted in red (Student's *t* test, P < 0.05). PC, principal component; α-Pin, α-pinene; β-Pin, β-pinene; Cam, camphene; Iso, isopropyl palmitate; Sab, sabinene. Numbers in **(B)** to **(F)** refer to tentatively identified VOCs (Supplemental Table 1).

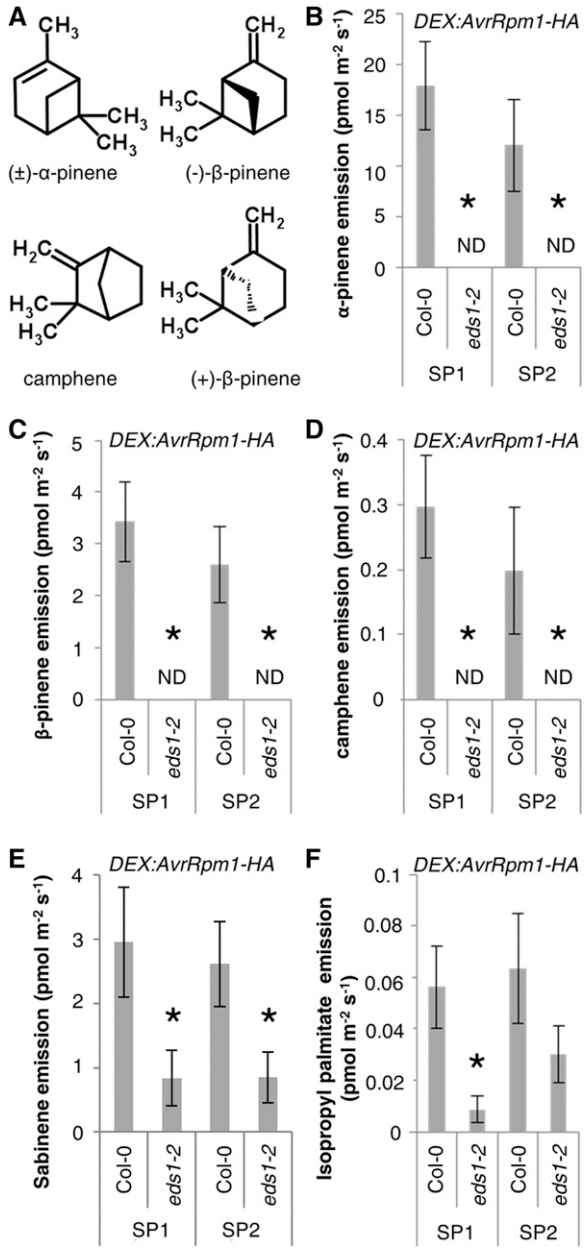

Figure 2. Structures and Emission Rates of VOCs Potentially Related to SAR.

(A) Chemical structures of the VOCs used in this study. Structures were taken or adapted from Chemspider.

(B) to **(F)** Emission rates of α-pinene **(B)**, β-pinene **(C)**, camphene **(D)**, sabinene **(E)**, and isopropyl palmitate **(F)** from DEX-treated Col-0 *DEX:AvrRpm1-HA* (Col-0) and *eds1-2 DEX:AvrRpm1-HA* (*eds1-2*) plants during sampling period 1 (SP1; 1–4 h after the DEX treatment) and SP2 (4–7 h after the DEX treatment). Emission rates are plotted relative to the projected rosette areas of the emitting plants. Bars represent the average of 18 to 21 biologically independent replicates as defined in the Methods ±SE. Asterisks indicate statistically significant differences from the Col-0 control (Student's *t* test, $P < 0.05$).

A similar reduction in *Pst* growth was observed in plants that had been treated with a mixture of (±)-α-pinene and (+)-β-pinene compared with the hexane-treated plants (Figure 3E), indicating that the bioactivity of β-pinene in Arabidopsis immunity is not enantiomer-specific. By contrast, treatment of plants with either (±)-α-pinene or (−)-β-pinene alone did not reduce *Pst* growth compared with that in hexane-treated plants (Figure 3F). Therefore, both α- and β-pinene appear to be required for pinene-induced immunity in Arabidopsis.

Because commercially available natural compounds are often not sold as pure chemicals, it was crucial to analyze the purity of the VOC standards by GC-MS analysis. Camphene was relatively pure and did not contain additional monoterpene compounds (Supplemental Figure 3). By contrast, the purity of the (±)-α-pinene and (−)-β-pinene standards that were used over the course of the experiments ranged from relatively pure (Supplemental Figure 3B) to contaminated with >10% of camphene and >5% of the monoterpene limonene (Supplemental Figure 3A). The monocyclic monoterpene limonene had previously been associated with plant immunity (Rodríguez et al., 2014) and herein also enhanced Arabidopsis resistance to *Pst* when applied in amounts ranging from 0.08 to 0.8 μmole (Supplemental Figure 4). Although 0.08 μmole of limonene approximately corresponded to the amount of limonene present in the pinene mixture that was used in the experiments summarized in Figures 3A and 3C, limonene-induced resistance was less robust than pinene-induced resistance, particularly in the lower concentration range, suggesting that limonene contamination was not (alone) causal for the observed pinene-induced resistance response. Also, later experiments with relatively pure compounds (Supplemental Figure 3B) showed that a mixture of (±)α- and either (−) or (+)β-pinene sufficed to enhance plant resistance to *Pst* (Figures 3B and 3D to 3F). In summary, headspace exposure of Arabidopsis to the monoterpenes pinene and camphene (Figure 3) enhances plant immunity to *Pst* in a manner that is dependent on the concentration and, in the case of pinene, on the presence of both structural isomers of the compound in the volatile blend.

Monoterpene-Induced Resistance Depends on SA and *AZI1*

In the following experiments, we analyzed the role of monoterpenes in Arabidopsis immunity and their relation to SA. First, we investigated whether *EDS1* is necessary for the plant defense response to headspace exposure with pinenes, besides its role upstream of monoterpene emission (Figures 1 and 2). We also studied effects of volatile pinenes on the *non-expressor of PR genes1-1* (*npr1-1*) mutant, which is defective in signaling downstream of SA (Cao et al., 1997; Vlot et al., 2009). As expected, treatment of wild-type plants with either MeSA or the pinene mixture reduced the growth of *Pst*, confirming that both classes of volatile compounds led to enhanced plant resistance to *Pst* (Figure 4A). In SA-mediated basal immunity, *EDS1* promotes SA production and *eds1* mutant plants display enhanced susceptibility to *Pst* (Feys et al., 2001). This was confirmed here by finding enhanced growth of *Pst* in mock-treated *eds1-2* compared with wild-type Col-0 plants (Figure 4A). Also, *eds1* mutant plants supported reduced *Pst* growth in response to SA (Feys et al., 2001) or its

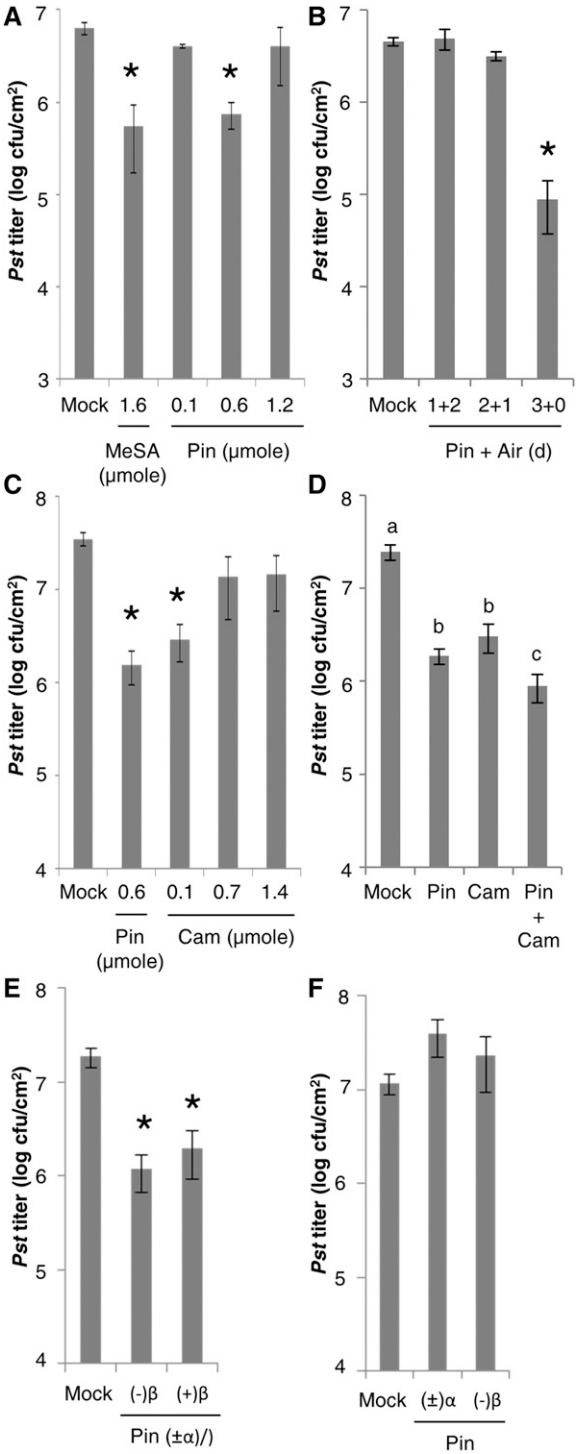

Figure 3. Monoterpene-Induced Resistance in Arabidopsis against *Pst*.

(A) and **(C)** Plants were exposed as described in the Methods to hexane (negative control; Mock), 1.6 μmole of MeSA (positive control), or different concentrations of a mixture (±)α-pinene and (−)β-pinene (1:1 v:v) (Pin; **[A]**) or camphene (Cam; **[C]**) as indicated below the panels.

volatile methylated derivative MeSA compared with the control-treated plants (Figure 4A). By contrast, treatment of *eds1-2* mutant plants with the pinene mixture did not enhance their resistance to *Pst* growth (Figure 4A), indicating that *EDS1* is required for the plant response to volatile pinenes. Similarly, the SA signaling mutant *npr1-1* did not respond with reduced *Pst* growth after headspace exposure to MeSA or pinenes (Figure 4A), indicating that SA signaling is required for pinene-induced resistance. The latter trends were confirmed in additional experiments using the SA biosynthesis mutant *sa induction deficient2-1* (*sid2-1*; Wildermuth et al., 2001). SID2 is also known as ISOCHORISMATE SYNTHASE1 (ICS1) and is part of the isochorismate biosynthetic pathway that is essential for pathogen-induced SA accumulation (Wildermuth et al., 2001; Vlot et al., 2009). In contrast to wild-type plants, which responded to exposure to volatile pinenes by reducing *Pst* growth, *sid2-1* mutant plants did not support pinene-induced resistance to *Pst* (Figure 4B). Together, the data suggest that downstream processing of pinene-derived signals requires SA biosynthesis and signaling for increased plant immunity.

To assess if pinenes induce SA signaling, we analyzed the transcript accumulation of the SA marker gene *PR1* in leaves of plants exposed for 3 d to MeSA or the pinene mixture compared with that in hexane-treated plants. With both types of volatile infochemicals, we observed an increase in *PR1* expression compared with the hexane control (Figure 4C), suggesting that the pinene mixture induces SA signaling. This observation was supported by microarray analysis in which the genome-wide transcriptional response of leaves after 3 d of exposure to volatile pinenes relative to the hexane-treated control plants was monitored (Table 1; Supplemental Data Set 1). Pinene-mediated induction of *PR1* gene expression displayed highest amplitude compared with other regulated genes. In total, the microarray analysis revealed 1214 genes with differential expression in pinene- and control-treated tissues (limma *t* test, $P < 0.05$, no fold-change cutoff; Supplemental Data Set 1). Of these, 132 genes were at least 2-fold upregulated and 133 genes were at least 2-fold downregulated in response to the headspace exposure with the

(B) Plants were exposed to hexane (Mock) for 3 d or to 0.6 μmole of Pin for 1, 2, or 3 d followed by 2, 1, or 0 d in the growth chamber (Air) as indicated below the panel.

(D) Plants were exposed to hexane or to 0.6 μmole of Pin, 0.1 μmole of Cam, or 0.6 μmole of Pin + 0.1 μmole of Cam (Pin + Cam).

(E) and **(F)** Plants were exposed to hexane or to 0.6 μmole of the following pinenes (Pin): (±)α-pinene and (−)β-pinene (1:1 v/v) [(±)α/(−)β], (±)α-pinene, and (+)β-pinene (1:1 v/v) [(±)α/(+)β], (±)α-pinene [(±)α], or (−)β-pinene [(−)β] as indicated below the panels.

(A) to **(F)** After 3 d of treatment, the plants were inoculated with *Pst*, and the resulting in planta *Pst* titers were determined at 4 dpi. Bars represent the average of three replicates ± SD, and asterisks (**[A]** to **[C]** and **[E]**) indicate significant differences from the mock controls (Student's *t* test, $P < 0.05$). Different letters above the bars in **(D)** indicate statistically significant differences (Student's *t* test, $P < 0.05$). These experiment were repeated two (**[B]** and **[D]**) to at least three times (**[A]**, **[C]**, **[E]**, and **[F]**) with comparable results.

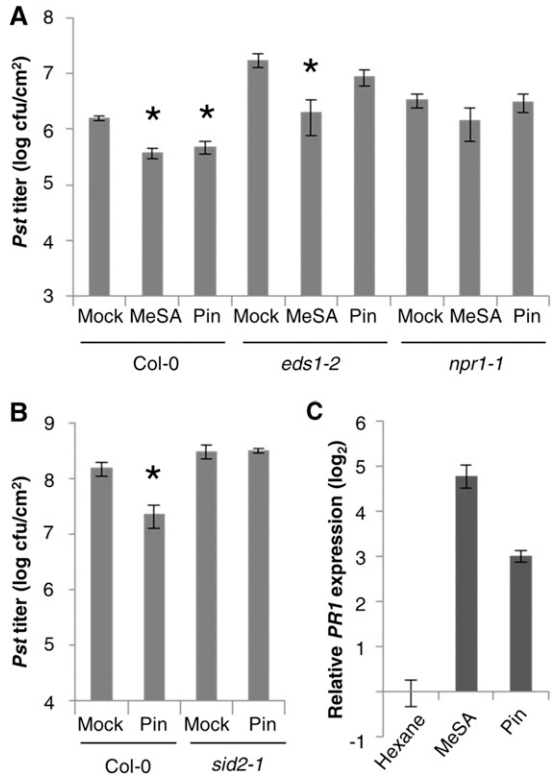

Figure 4. Monoterpene-Induced Resistance Related to SA Signaling.

Plants were exposed to hexane (negative control; Mock), 1.6 μmole of MeSA (positive control), or 0.6 μmole of (±)α-pinene:(−)β-pinene (1:1 v/v) (Pin) as described in Methods.

(A) and **(B)** After 3 d, the treated Col-0, *eds1-2*, *npr1-1* **(A)**, and *sid2-1* **(B)** plants were inoculated with *Pst*, and the resulting in planta *Pst* titers were determined at 4 dpi. Bars represent the average of three replicates ± SD, and asterisks indicate significant differences from the mock controls (Student's *t* test, $P < 0.05$).

(C) After 3 d of treatment, *PR1* transcript accumulation was determined relative to that of *UBIQUITIN* in Col-0 plants by RT-qPCR. Bars represent the average of three replicates ± SD. These experiments were repeated at least three times with comparable results.

pinene mixture compared with control-treated plants. Gene Ontology (GO) enrichment analysis of the upregulated genes revealed overrepresentation of defense-related processes with cell death, SAR, and SA signaling as well as transport representing the major components (Figure 5A; Supplemental Data Set 2). The six most significantly regulated genes (false discovery rate-adjusted P value [Q] < 0.05; Table 1; Supplemental Data Set 1) were consistently upregulated in all individual biological replicates (Figure 5B). They included the PTI-associated *FLG22-INDUCED RECEPTOR-LIKE KINASE1* (*FRK1*; Po-Wen et al., 2013) and the *AZI1* paralogs *AZI3* and *AT4G12500* (*AZI4* in Cecchini et al., 2015). Also, we found a consistent induction of the additional *AZI1* paralog *EARLI1*, which is essential for SAR (Cecchini et al., 2015). Similarly, expression of transcription factors supporting both SA biosynthesis (*CALMODULIN BINDING PROTEIN 60g* [*CBP60g*]; Wang et al., 2009) and SA signaling (*WRKY38*; Kim et al., 2008) was increased in the pinene treatment as well as that of *APOPLASTIC, EDS1-DEPENDENT15* (*AED15*), a chitinase (*CHI*) that we previously associated with SAR in Col-0 *DEX:AvrRpm1-HA* plants (Table 1, Figure 5B; Breitenbach et al., 2014). Taken together, the data suggest that headspace exposure of Arabidopsis plants to pinene induces defense-related gene expression that has significant overlap with SA and SAR-like responses.

With its paralogs *EARLI1*, *AZI3*, and *AZI4*, expression of *AZI1* was moderately and consistently induced by ~1.6-fold in the pinene- compared with control-treated plants analyzed on the microarrays (Table 1; Supplemental Data Set 1). *AZI1* is in part functionally redundant with *EARLI1* in SAR (Cecchini et al., 2015), and we sought to confirm pinene-induced transcript accumulation changes of *AZI1*, *EARLI1*, *AZI3*, and *AZI4* by RT-qPCR. Across seven biologically independent replicates, exposure of Arabidopsis to pinenes induced *AZI1* and *EARLI1* transcript accumulation by ~4-fold compared with that in mock-treated plants and *AZI4* and *AZI3* transcript accumulation by ~6- to 8-fold, respectively (Supplemental Figure 5). *AZI1* is thought to act in a positive feedback loop with G3P, the levels of which are reduced in *gly1* (G3P dehydrogenase) mutant plants (Chanda et al., 2011; Yu et al., 2013). To investigate if *AZI1* and/or G3P are involved in monoterpene-induced resistance, we exposed *azi1-2* and *gly1-3* mutant plants to the pinene mixture for 3 d, after which plants were infected with *Pst.* In contrast to wild-type control plants that responded to the pinene treatment by reducing *Pst* growth compared with that in hexane/control-treated plants, *azi1-2* mutants did not respond to the pinene treatment, indicating that *AZI1* is necessary for monoterpene-induced bacterial resistance (Figure 6). By contrast, *gly1-3* mutant plants responded to the pinene mixture with *Pst* growth reduction that was comparable to that of wild-type plants (Figure 6). This indicates that G3P is dispensable for monoterpene-induced resistance. Together, the data suggest that *AZI1* supports immunity downstream of monoterpenes and independently of G3P.

Monoterpenes Induce ROS Accumulation

Exposure of Arabidopsis to the volatile fungal sesquiterpene α-thujopsene stimulates production of superoxide anion radicals ($O_2^{\bullet-}$) in roots (Ditengou et al., 2015). Additionally, SAR depends on ROS, and the accumulation of AzA might be enhanced by $O_2^{\bullet-}$ (Wang et al., 2014). Therefore, we assessed whether monoterpenes induce $O_2^{\bullet-}$ in Arabidopsis. To this end, plants were exposed to the pinene mixture for 3 d, after which $O_2^{\bullet-}$ was visualized in the leaves after staining with nitroblue tetrazolium (NBT) (Ditengou et al., 2015). Similar to α-thujopsene, the pinene mixture enhanced the accumulation of $O_2^{\bullet-}$ (Figure 7), suggesting that ROS accumulate in response to treatment with the pinene mixture, as part of a relay mechanism through which these monoterpenes enhance defense.

Monoterpene Biosynthesis Is Essential for SAR

To further test our hypothesis that volatile monoterpenes play a role in SAR, we assessed the importance of monoterpene biosynthesis in plant defense and SAR. Arabidopsis *GERANYLGERANYL REDUCTASE* (*GGR*) encodes a type II small subunit of the heterodimeric GERANYL DIPHOSPHATE SYNTHASE (GPS) (Wang and Dixon, 2009; Tholl and Lee, 2011). GPS is responsible for the

Table 1. Summary of Pinene-Induced Transcriptional Changes Overlapping with SAR and SA-Dependent Biological Processes (GO Analysis; www.arabidopsis.org)

Gene	Locus	Full Name	Log_2 Fold Change[a]	P Value[a]	*Q* Value[a]	Biological Process
PR1	At2g14610	PATHOGENESIS-RELATED GENE1	2.81	0.011	NA	SAR, response to water deprivation, response to vitamin B1, defense response
AZI3	At4g12490	AZELAIC ACID INDUCED3	2.26	0.000	0.025	Defense response to fungus, lipid transport
AZI4	At4g12500	NA	2.19	0.000	0.016	Lipid transport
CHI /AED15	At2g43570	CHITINASE, putative/APOPLASTIC EDS1-DEPENDENT15	1.97	0.041	NA	SAR, response to virus, cell wall macromolecule catabolic process, other cellular and metabolic processes
WRKY38	At5g22570	WRKY DNA-BINDING PROTEIN38	1.88	0.002	NA	SA-mediated signaling pathway, defense response to bacterium, regulation of transcription
MLO12	At2g39200	MILDEW RESISTANCE LOCUS O12	1.80	0.000	0.016	Cell death, defense response, defense response to fungus, incompatible interaction, leaf senescence
FRK1	At2g19190	FLG22-induced receptor-like kinase 1	1.72	0.000	0.016	Defense response to bacterium, protein phosphorylation
EARLI1	At4g12480	EARLY ARABIDOPSIS ALUMINUM INDUCED1	1.64	0.000	NA	Induced systemic resistance, defense response to fungus, lipid transport, response to abscisic acid, response to cold, response to salt stress
BBE7	At1g26420	Berberine bridge enzyme 7	1.49	0.000	0.016	Oxidation-reduction process
NA	At3g46280	NA	1.48	0.000	0.018	Phosphorylation
CBP60g	At5g26920	CALMODULIN-BINDING PROTEIN 60G	1.21	0.003	NA	Regulation of SAR, regulation of SA biosynthetic process, abscisic acid-activated signaling pathway, other cellular and metabolic processes
AZI1	At4g12470	AZELAIC ACID INDUCED1	0.69	0.008	NA	SAR, induced systemic resistance, lipid transport, plant-type hypersensitive response, defense response to fungus, other response to cold

Q value (false discovery rate-adjusted P value of limma *t* test) of genes differentially regulated are shown only if <0.05.
[a]Data extracted from Supplemental Data Set 1.

biosynthesis of terpene precursors including geranyl diphosphate (GPP), the main precursor of all monoterpenes (Tholl and Lee, 2011). GGR is believed to function as an "accelerator" of GPS activities and as a "modifier" of chain length of the product of GPS from GGPP (C_{20}) to GPP (C_{10}), specifically supporting production of (C_{10}) monoterpenes (Wang and Dixon, 2009; Tholl and Lee, 2011; Yin et al., 2017). Before assessing the role of GGR in monoterpene-mediated SAR, we analyzed *GGR* transcript accumulation in two T-DNA insertion mutants, *ggr1-1* and *ggr1-2*, and in wild-type plants by RT-qPCR. Leaves of *ggr1-1* mutants contained very low (1.1% relative to the wild type) levels of *GGR* transcripts and in *ggr1-2* plants *GGR* expression was reduced by ~60% compared with the wild-type control (Figure 8A). We also compared growth of a SAR-inducing *Pst AvrRpm1* inoculum in wild-type, *ggr1-1*, and *ggr1-2* plants. Because *Pst AvrRpm1* grew to similar titers in the *ggr* mutants and wild-type plants at 2 and 4 dpi (Supplemental Figure 6), we concluded that these mutations do not affect local AvrRpm1-induced ETI leading to SAR.

To investigate SAR, we inoculated two lower leaves of wild-type, *ggr1-1*, and *ggr1-2* plants with *Pst AvrRpm1* and monitored SAR development using a challenge infection of the systemic leaves with *Pst*. At 4 dpi, *Pst* titers in systemic leaves of *Pst AvrRpm1* preinoculated wild-type plants was ~10-fold lower than in mock pretreated plants, indicating that SAR was induced by primary *Pst AvrRpm1* infection (Figure 8B). We did not observe a repression of *Pst* growth in systemic leaves of the *ggr1-1* and *ggr1-2* mutants, indicating that SAR induction was abolished in the *ggr* mutant background (Figure 8B). Similar to the local response of the mutants to *Pst AvrRpm1* (Supplemental Figure 6), the local response to *Pst* in both *ggr* mutants was similar to that of wild-type plants (compare *Pst* titers after the challenge infections of the mock-treated plants in Figure 8B). These data suggest that *GGR* is essential for the induction of SAR but not for local resistance responses.

Additionally, we tested whether reduced monoterpene levels due to compromised monoterpene precursor biosynthesis are causal for defective SAR in the *ggr* mutants. Using a similar experimental regime as above, we exposed wild-type, *ggr1-1*, and *ggr1-2* plants to MeSA or the pinene mixture. *Pst* growth was reduced in wild-type plants that had undergone 3 d of headspace incubation with either MeSA or pinenes compared with plants that had been treated with hexane prior to the *Pst* inoculation (Figure 8C). Both *ggr* mutants responded to volatile MeSA by reducing *Pst* growth compared with the hexane-treated plants, arguing that

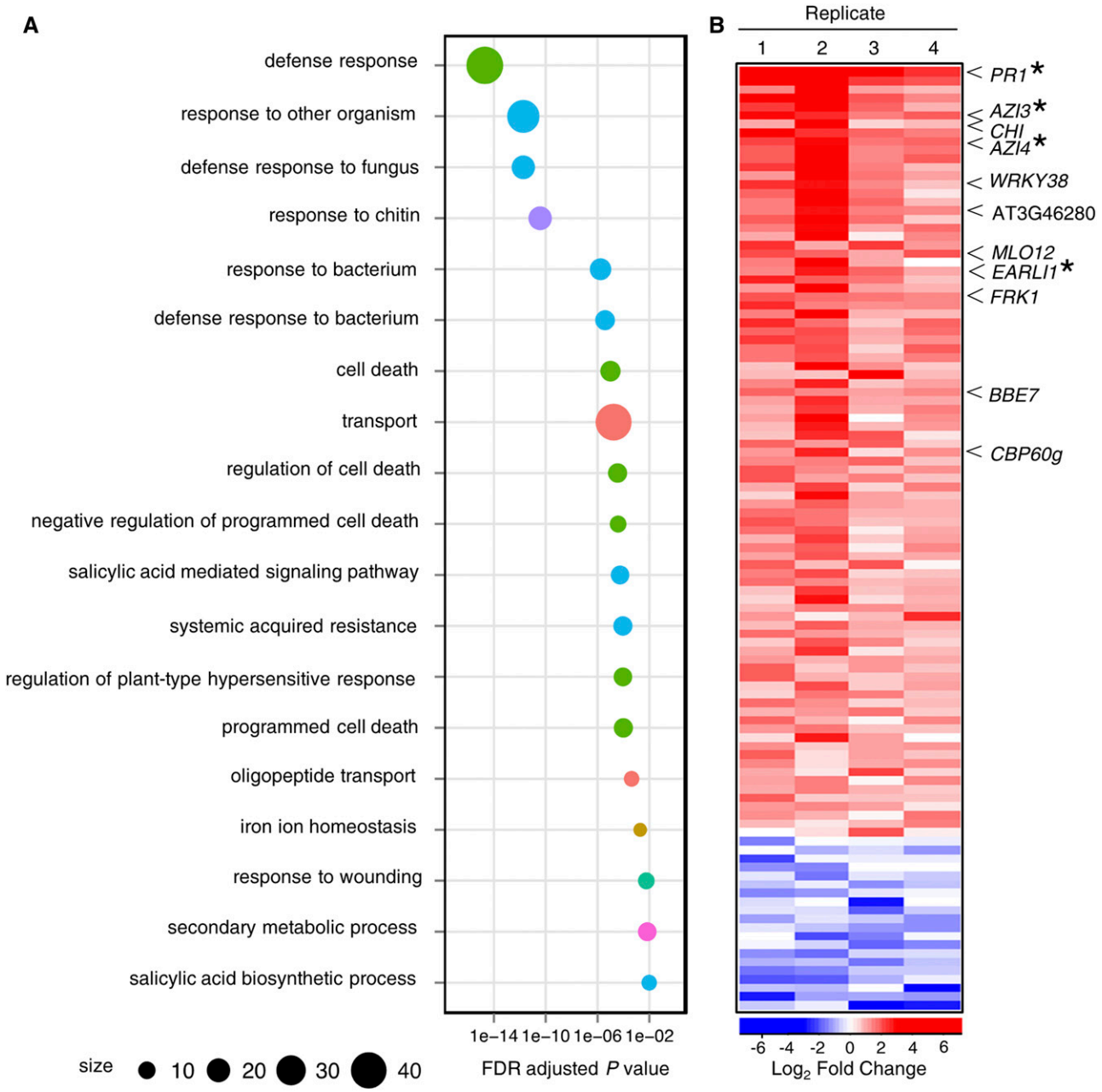

Figure 5. Pinene-Triggered Changes in the Arabidopsis Gene Expression Profile.

(A) Significantly enriched GO terms (TAIR) among the upregulated genes obtained from microarray data ($P < 0.05$ and $\log_2$ fold change >1). The circle size indicates the number of genes annotated with the respective term. Identical color indicates related GO terms originating from a specific ancestral node.
(B) Heat map of all regulated genes ($P < 0.05$ and absolute $\log_2$ fold change > 1) with consistent direction of change across the four biological replicates. Asterisks indicate genes, whose transcriptional regulation by the pinene mixture was confirmed by RT-qPCR (Supplemental Figure 5).

GGR might act upstream of SA in defense signaling (Figure 8C). More importantly, both *ggr* mutants responded normally to the pinene exposure by reducing *Pst* growth compared with the mock control (Figure 8C). These results suggest that an external application of pinenes compensates for reduced endogenous monoterpene biosynthesis in plant defense. To further assess if a failure in monoterpene production might underlie the SAR defect in *ggr* mutant plants, we aimed to chemically complement the SAR-deficient phenotype of *ggr1-1* plants with a pinene headspace exposure. To this end, wild-type and *ggr1-1* mutant plants were inoculated in the first two true leaves with *Pst AvrRpm1* to induce SAR signaling. After 3 d, a secondary *Pst* inoculum was applied to the systemic tissue. Because *Pst* growth was reduced in preinfected wild type, but not *ggr1-1* mutant plants, compared

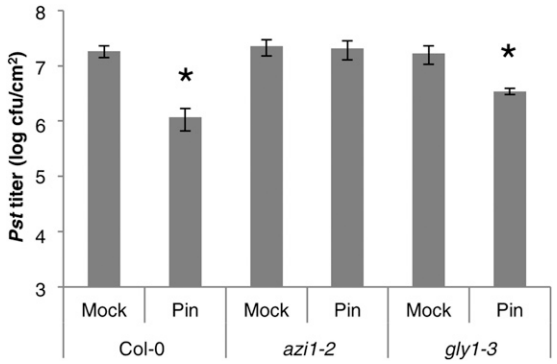

Figure 6. Monoterpene-Induced Resistance in Col-0 Wild-Type, *azi1-2*, and *gly1-3* Mutant Plants.

The plants were exposed to hexane (negative control/mock) or to 0.6 μmole of (±)α-pinene:(−)β-pinene (1:1 v/v) (Pin) as described in Methods. After 3 d, the plants were inoculated with *Pst*, and the resulting in planta *Pst* titers were determined at 4 dpi. Bars represent the average of three replicates ± SD, and asterisks indicate significant differences from the mock controls (Student's *t* test, $P < 0.05$). This experiment was repeated three times with comparable results.

with that in mock-pretreated control plants, SAR signaling was functional in wild-type but not *ggr1-1* mutant plants (Figure 8D, lanes 1, 2, 7, and 8). Chemical complementation was performed by exposing plants to 0.6 μmole of the pinene mixture for 1 d on either the first (T1), second (T2), or third (T3) day of the 3-d incubation that was used to establish SAR. As controls for the pinene treatment, naïve wild-type plants were incubated with the pinene mixture for 3 d (positive control) or for 1 d corresponding to either T1, T2, or T3. While in planta *Pst* growth was reduced after 3 d of headspace exposure of wild-type plants to the pinene mixture, *Pst* growth was unaffected in plants that had undergone either of the 1-d treatments (Figure 8D, lanes 3–6). However, incubation of *Pst AvrRpm1*-infected *ggr1-1* plants with the pinene mixture for 1 d restored the ability of the mutant plants to support SAR as evidenced by reduced *Pst* growth in the systemic tissue of *Pst AvrRpm1*-infected *ggr1-1* plants that were treated with the pinene mixture at T1, T2, or T3 (Figure 8D, lanes 10–12) compared with the corresponding hexane/mock control (Figure 8D, lane 9). Thus, pinene application complemented the SAR-defective phenotype of the *ggr1-1* mutant, and this was independent of the time point of the application (Figure 8D, lanes 10–12). Although the relationship between pinenes and SAR does not appear to be restricted to particular temporal events in the establishment of SAR, the data suggest that monoterpene production, emission, and/or recognition is essential for SAR.

Monoterpenes Contribute to Defense-Related Plant-to-Plant Communication

Because monoterpenes are highly volatile and not normally stored in Arabidopsis cells (Tholl and Lee, 2011), we reasoned that these compounds could also support defense-related plant-to-plant communication. To test this hypothesis, we incubated eight Col-0 wild-type Arabidopsis receiver plants in a vacuum desiccator together with 12 mock-treated or *Pst AvrRpm1*-infected sender plants. The incubations were performed as in the headspace exposure experiment described above. After 3 d, the receiver plants were inoculated with *Pst* and in planta *Pst* titers were determined at 4 dpi. Wild-type receiver plants responded to coincubation with *Pst AvrRpm1*-infected wild-type plants by reducing *Pst* growth compared with wild-type plants that had been coincubated with mock-treated wild-type plants (Figure 9). This indicated that wild type-to-wild type communication occurs in response to *Pst AvrRpm1*, resulting in increased resistance to *Pst* in the receiver plants. However, *Pst* growth was not reduced in wild-type receiver plants after their coincubation with *Pst AvrRpm1*-infected *eds1-2*, *ggr1-1*, or *ggr1-2* mutant plants compared with the corresponding mock controls (Figure 9; Supplemental Figure 7). Thus, emissions from both *eds1-2* and *ggr* plants lack VOCs that are essential for plant-to-plant propagation of defense signaling. We hypothesized that these VOCs are monoterpenes, including camphene and α- and β-pinene, which were absent from the emissions of *avrRpm1-HA*-expressing *eds1-2* mutant plants (Figures 2B to 2D). To verify this hypothesis, we measured camphene and α- and β-pinene in the emissions of *Pst AvrRpm1*-infected wild-type and *ggr1-1* mutant plants and compared them to the same emissions of mock-treated wild-type plants. *Pst AvrRpm1* progressively enhanced camphene and α- and β-pinene emissions from wild-type plants at 1 to 3 d after infection (Figure 10). By contrast, the emissions of camphene and α- and β-pinene remained at the basal (T0) level at 1 to 3 d after the mock treatment. The α-pinene and camphene

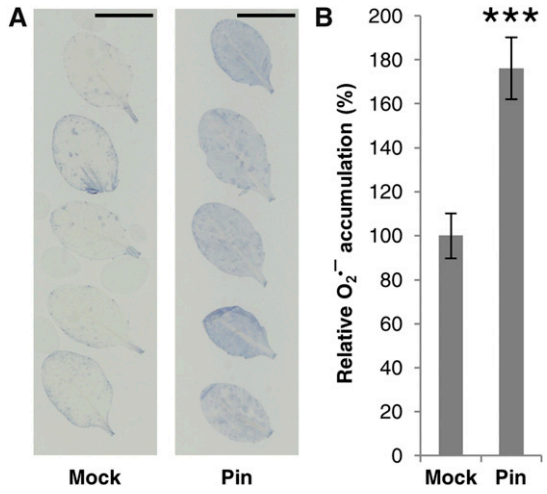

Figure 7. Superoxide Anion Radical Accumulation in Response to Pinene Treatment.

Col-0 wild-type plants were exposed to hexane (negative control; Mock) or to 0.6 μmole of (±)α-pinene:(−)β-pinene (1:1 v/v) (Pin) as described in Methods. After 3 d, the accumulation of superoxide anion radicals ($O_2^{\bullet-}$) was visualized with nitroblue tetrazolium **(A)**. The pixel intensity of the pinene-treated leaves was quantified relative to that of the mock-treated leaves, which was set at 100% **(B)**. Data in **(A)** and **(B)** stem from two out of four biologically independent replicate experiments with comparable results. Black bars in **(A)** indicate 1 cm. Bars in **(B)** represent the average of 10 replicates ± SE, and asterisks indicate a statistically significant difference from the Mock control (Student's *t* test, ***$P < 0.0001$).

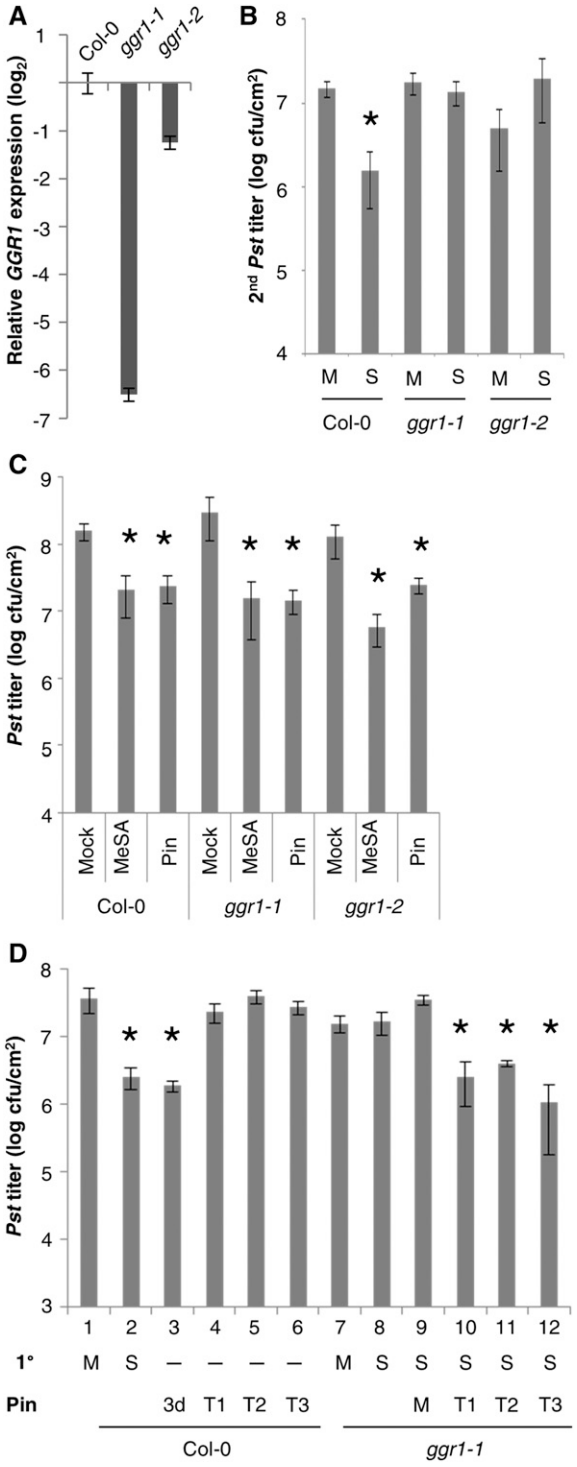

Figure 8. The Prenyltransferase GGR Is Essential for SAR.

(A) *GGR* transcript accumulation in wild-type, *ggr1-1*, and *ggr1-2* plants was determined relative to that of *UBIQUITIN* by RT-qPCR. Bars represent the average of three replicates ± SD.

(B) SAR in wild-type, *ggr1-1*, and *ggr1-2* plants. Plants were treated in two lower leaves with 10 mM $MgCl_2$ (Mock; M) or with *Pst AvrRpm1* (SAR; S). Three days later, the systemic (2nd) leaves were inoculated with *Pst*, and the resulting in planta *Pst* titers were determined at 4 dpi.

(C) Monoterpene-induced resistance in wild-type, *ggr1-1*, and *ggr1-2* plants. Plants were exposed to hexane (negative control; Mock), 1.6 μmole of MeSA (positive control), or 0.6 μmole of (±)α-pinene:(−)β-pinene (1:1 v/v) (Pin) as described in Methods. After 3 d, the plants were inoculated with *Pst*, and the resulting in planta *Pst* titers were determined at 4 dpi.

(D) Chemical complementation of the SAR-deficient phenotype of *ggr1-1* plants with pinene. As a primary treatment (1°), Col-0 wild-type and *ggr1-1* plants were either treated as in **(B)** in the lanes marked with M and S or left untreated (−). Simultaneously, plants were exposed to hexane (Mock; M) or Pin for 3 d (3d) as in **(C)** or to Pin for 1 d either on the first (T1), second (T2), or third (T3) day of the normal treatment. Subsequently, all plants were inoculated with *Pst*, and the resulting in planta *Pst* titers were determined at 4 dpi. Bars in **(B)** to **(D)** represent the average of three replicates ± SD, and asterisks indicate significant differences from the mock controls (Student's *t* test, $P < 0.05$). These experiments were repeated two (**[A]** and **[D]**) to at least three times (**[B]** and **[C]**) with comparable results.

emissions were significantly different between *Pst AvrRpm1*-infected and mock-treated wild-type plants at 3 dpi (Figures 10A and 10C). PCA analysis showed that all three monoterpenes positively correlated with the observed higher emission rates (Figures 10A to 10C) of *Pst AvrRpm1*-infected compared with mock-treated wild-type plants, in particular at 3 dpi (Figure 10D). Thus, *Pst AvrRpm1* enhanced emissions of the monoterpenes camphene and α- and β-pinene in Arabidopsis. Importantly, camphene and α- and β-pinene could not be detected in the basal and *Pst AvrRpm1*-induced emissions of *ggr1-1* mutant plants (Figure 10). Because both *eds1-2* and *ggr1-1* plants thus showed reduced camphene and α- and β-pinene emissions (Figures 2 and 10), the ability of sender plants to emit these monoterpenes was strongly associated with plant-to-plant defense propagation (Figure 9; Supplemental Figure 7). In contrast to monoterpenes, MeSA emissions in all samples remained below our detection limit (Supplemental Figure 8), which approximated a MeSA emission rate of ~3.2 pmol m^{-2} s^{-1}. Taken together, our data suggest that monoterpenes, in particular camphene and α- and β-pinene, have a prominent role in SAR and SAR-like signaling between plants.

DISCUSSION

Here, we show that monoterpene emissions are essential for Arabidopsis SAR but not for local SA-mediated immunity. Additionally, monoterpenes appear to act in plant-to-plant signaling in a manner reminiscent of SAR. *AvrRpm1-HA*-expressing Arabidopsis plants emitted four monoterpenes and a fatty acid derivative in an *EDS1*-dependent manner, thus linking these emissions to SAR competence (Figures 1 and 2). Headspace exposure of wild-type Arabidopsis to a mixture of α-pinene and β-pinene induced SA-mediated immunity against *Pst* (Figures 3 to 5). Reciprocally, suppression of monoterpene biosynthesis and emission in *ggr* mutant plants abolished SAR and the ability of *Pst AvrRpm1*-infected plants to trigger SAR-like immunity in neighboring wild-type plants (Figures 8 to 10). Our transcript profiling, biochemical, and genetic analysis using *azi1* and *gly1* mutants

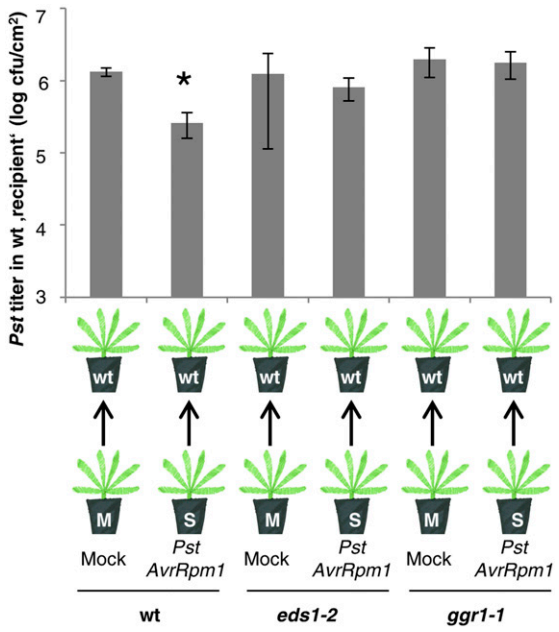

Figure 9. Monoterpenes Are Important for Plant-to-Plant SAR Signaling.

Eight wild-type receiver plants were incubated in gas-tight desiccators together with 12 mock-treated (M) or *Pst AvrRpm1*-infected (S) Col-0 wild-type, *eds1-2*, or *ggr1-1* sender plants as indicated below the panel. After 3 d, the receiver plants (recipients) were inoculated with *Pst*, and the resulting in planta *Pst* titers were determined at 4 dpi. Bars represent the average of four replicates ± SD, and the asterisk indicates a significant difference from the mock control (Student's *t* test, $P < 0.005$). This experiment was repeated three times with comparable results.

further connected pinene-induced immunity with the SAR-associated putative lipid transfer proteins AZI1, EARLI1, and their paralogs AZI3 and AZI4, and with the accumulation of $O_2^{\bullet-}$ (Figures 5 to 7). This work defines monoterpenes, in particular pinenes and camphene, as volatile signaling intermediates in SAR and plant-to-plant SAR-like signal relay in Arabidopsis.

Monoterpenes and *EDS1*-SA Signaling

Early studies of the role of *EDS1* in SA-mediated resistance showed that *EDS1* promotes pathogen-induced SA accumulation and signaling (Feys et al., 2001). SA or its functional analog benzothiadiazole induces *PR1* transcript accumulation in *eds1* mutant plants, consistent with *EDS1* acting upstream of SA (reviewed in Vlot et al., 2009). SA, in turn, enhances *EDS1* transcript accumulation, together fortifying immunity in a positive feedback loop (Figure 11). Besides boosting SA resistance, EDS1 can partially compensate for disabled SA signaling, thus further strengthening basal immunity and ETI (Cui et al., 2017). Our analysis here shows that monoterpene emissions also depend on *EDS1.* However, in contrast to SA, the pinene mixture did not significantly enhance *EDS1* expression (Supplemental Data Set 1). Nevertheless, the mixture of α- and β-pinene enhanced immunity in an *EDS1*- and SA-dependent manner (Figure 4). Previously, we showed that *EDS1* is necessary for the systemic perception of SAR signals, likely propagating immune signaling via the *EDS1*-SA positive feedback loop (Breitenbach et al., 2014). Similarly, *EDS1* action in the plant's response to pinenes might be associated with an EDS1 role in fortifying SA signaling (Figure 11). Notably, the pinene mixture enhanced expression of the *EDS1*-dependent *CBP60g* gene encoding a transcription factor that regulates expression of *ICS1* and accumulation of SA (Wang et al., 2009; Cui et al., 2017; Table 1). Together, our data suggest that pinenes promote SA signaling via an *EDS1*-SA positive feedback loop involving *CBP60g*.

EDS1-Monoterpene-AzA Signaling in SAR

We documented a SAR-specific role in plant immunity for the monoterpene biosynthesis-associated gene *GGR* (Figure 8). Monoterpene biosynthesis depends on the chloroplastic methylerythritol phosphate (MEP) pathway (Tholl and Lee, 2011). Also in the chloroplast, the putative phloem-mobile SAR signal AzA accumulates via peroxidation of C_{18} membrane lipids (Zoeller et al., 2012). We show that plant exposure to pinenes triggers the accumulation of $O_2^{\bullet-}$ in wild-type Arabidopsis leaves (Figure 7), while $O_2^{\bullet-}$ is one of several ROS species that stimulate the peroxidation of C_{18} unsaturated fatty acids (Zoeller et al., 2012; Wang et al., 2014). AzA is believed to promote SAR in a pathway that acts locally (in SAR signal biosynthesis or transmission) and in parallel with SA (Jung et al., 2009; Wang et al., 2014; Cecchini et al., 2015). Strikingly, SA-independent EDS1 actions upstream of AzA are also important for SAR (Breitenbach et al., 2014; Wittek et al., 2014).

Arabidopsis exposure to the pinene mixture induced expression of the SAR-associated genes *AZI1* and *EARLI1* as well as their paralogs *AZI3* and *AZI4* (Table 1, Figure 5). EARLI1 and AZI3 share with AZI1 a subcellular localization to the endoplasmic reticulum (ER), plasma membrane, and the outer chloroplast membrane, in particular accumulating at ER/chloroplast contact sites (Cecchini et al., 2015). Because AZI4 shares AZI3's structural features determining its subcellular localization to ER/chloroplast contact sites (Cecchini et al., 2015), AZI4 might colocalize with AZI1, EARLI1, and AZI3. As putative lipid-transfer proteins, AZI1 and EARLI1 have been hypothesized to facilitate the intracellular transport of SAR-associated signals from their chloroplastic site of biosynthesis via the ER to plasma membrane-associated apoplast or phloem loading sites for long-distance movement (Cecchini et al., 2015). In that putative function, AZI1 and EARLI1 are each required for local SAR signal emission and appear to stimulate the systemic movement of AzA (Cecchini et al., 2015). Thus, the induction of *AZI1* and its paralogs downstream of pinenes might promote immunity mediated by AzA.

Together, the regulation of AzA downstream of monoterpene biosynthesis or emission and subsequent induction of $O_2^{\bullet-}$ might be central to an *EDS1*-regulated, monoterpene-dependent, local SA-independent, and SAR-specific signaling pathway (Figure 11). In parallel, this pathway relies on intact SA biosynthesis and signaling (Figure 4; Jung et al., 2009), which likely is important in the systemic tissue for an effective monoterpene- and AzA-induced immune response. Although exposure of *Pst AvrRpm1*-infected *ggr1-1* plants to the pinene mixture complemented the normally SAR-deficient phenotype of these plants (Figure 8), we

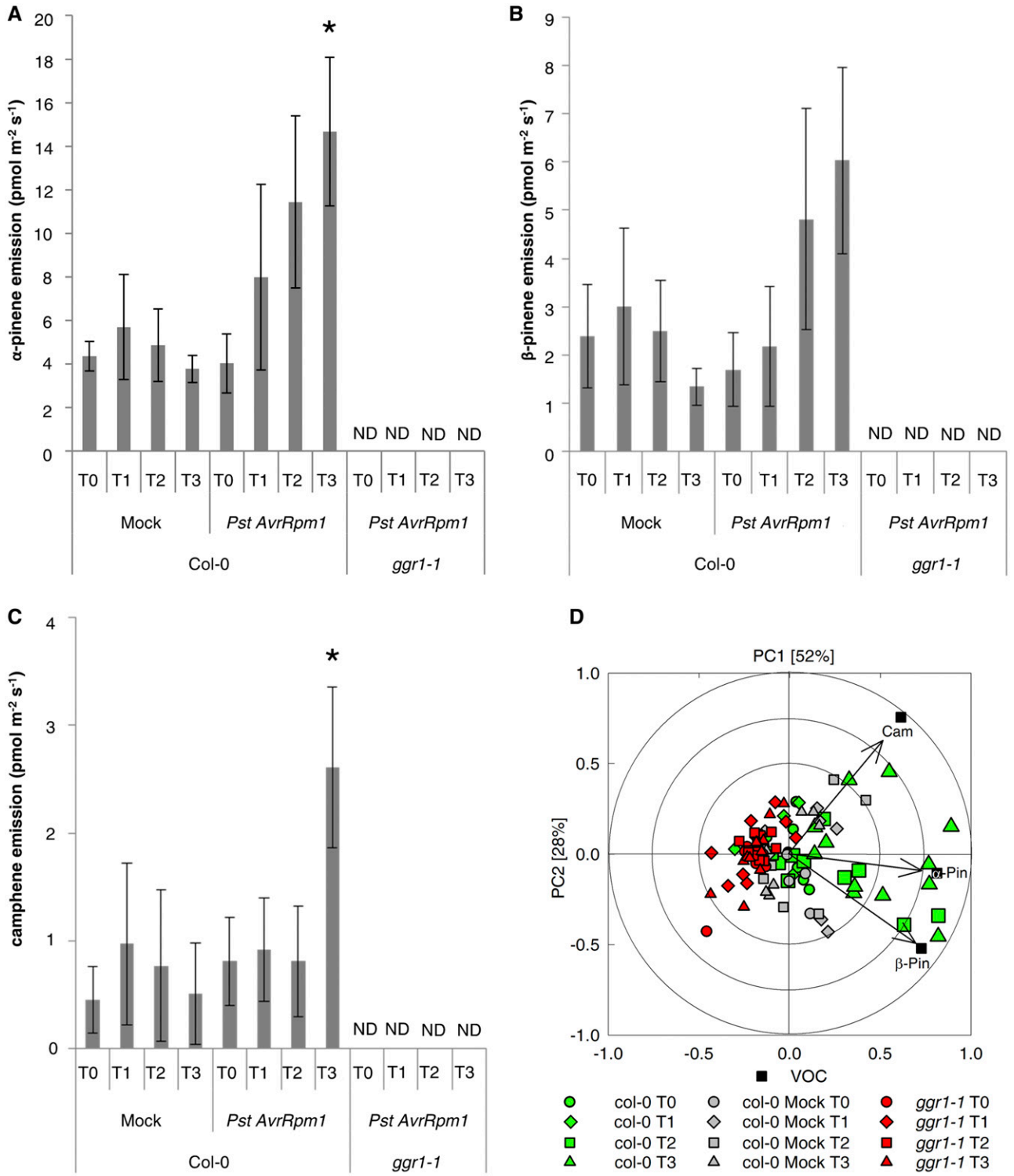

Figure 10. Arabidopsis Monoterpene Emissions Are Induced by *Pst AvrRpm1* and Dependent on *GGR*.

(A) to **(C)** Emission rates of α-pinene **(A)**, β-pinene **(B)**, and camphene **(C)** from Col-0 wild-type and *ggr1-1* mutant plants 1 d before (T0) and during the first (T1), second (T2), and third (T3) day after spray inoculation of the plants with *Pst AvrRpm1* or a corresponding mock treatment. VOCs were collected during the day for 8 h per sampling period. Emission rates are plotted relative to the projected rosette areas of the emitting plants. Bars represent the average of 6 to

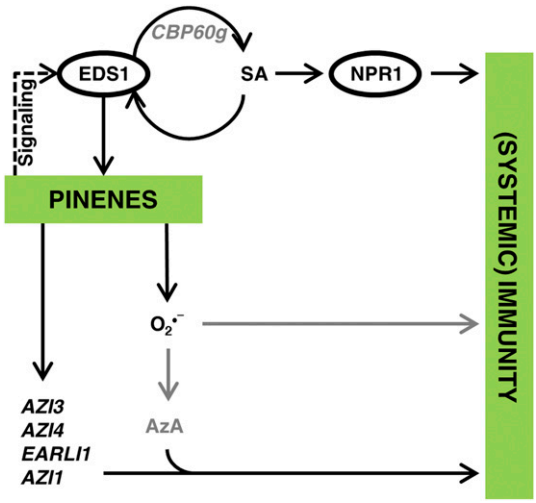

Figure 11. Working Model of the Role of Pinenes in Plant Immunity.

Pinenes accumulate downstream of EDS1 and trigger immunity via EDS1, SA biosynthesis (possibly via CBP60g), and NPR1-mediated SA signaling. Also, pinenes trigger the accumulation of superoxide anion radicals ($O_2^{\bullet-}$) that might themselves induce immunity or induce the accumulation of AzA promoting SAR together with AZI1 and EARLI1. Finally, pinenes induce the expression of *AZI1* and its paralogs *EARLI1*, *AZI3*, and *AZI4* and act through AZI1 to enhance immunity. Established interactions are depicted in black, and hypothetical interactions are depicted in gray. Solid arrows indicate induction or activation, broken arrows indicate signaling, and the rounded arrows indicate the EDS1-SA positive feedback loop. Proteins are circled. Genes are in italics, and compounds are in plain lettering.

cannot exclude that monoterpenes affect SAR through the air in parallel with known intraplant SAR signaling cascades. Nevertheless, it is known that ROS promote SAR upstream of AzA and AZI1 in a signaling cascade that acts in parallel with SA (Yu et al., 2013; Wang et al., 2014; Gao et al., 2015), and it is tempting to speculate that monoterpenes act farther upstream in this SAR-intrinsic pathway (Figure 11).

The MEP Pathway in SAR

The C_{10} monoterpene precursor GPP is produced in the chloroplast as a result of the MEP pathway (Tholl and Lee, 2011). Blocking the MEP pathway by fosmidomycin, a chemical inhibitor of the second enzyme of the pathway, 2C-METHYL-D-ERYTHRITOL 4-PHOSPHATE SYNTHASE, compromised SA-mediated immunity (Gil et al., 2005). By contrast, reducing the enzyme that catalyzes the penultimate reaction in the same pathway (1-HYDROXY-2-METHYL-2-BUTENYL 4-DIPHOSPHATE SYNTHASE) enhanced SA-mediated immunity as well as jasmonic acid-associated defense responses (Gil et al., 2005; Lemos et al., 2016). This was attributed to an elevated accumulation of the upstream nonvolatile intermediate MEcPP (2C-methyl-D-erythritol 2,4-cyclodiphosphate) rather than to possible volatile monoterpene products of the pathway (Gil et al., 2005). Translocated from the chloroplast to the nucleus, MEcPP acts as retrograde signal modulating the expression of stress-related genes (Xiao et al., 2012). Notably, a possible differential accumulation of the retrograde signal MEcPP cannot explain the plant-to-plant signaling phenotype documented here. Together, the data suggest a dual role of MEP pathway intermediates and products regulating innate immunity via MecPP and specifically promoting SAR through monoterpenes.

Monoterpene Perception in SAR and Plant-to-Plant SAR-Like Signaling

The volatile emissions from infected Arabidopsis plants enhanced resistance in surrounding plants to a subsequent *Pst* infection (Figure 9; Supplemental Figure 7). This plant-to-plant SAR-like effect has been observed before in Arabidopsis and *Nicotiana tabacum* (tobacco) but was associated with MeSA emissions only (Shulaev et al., 1997; Koo et al., 2007). Here, we observed a tight association between plant-to-plant SAR-like signaling and monoterpene emissions. Reduced α-pinene, β-pinene and camphene contents in emissions from *eds1-2* and *ggr1-1* mutant plants compromised induction of SAR-like immunity in neighboring plants, suggesting that monoterpenes are essential for this response. Other VOCs that trigger SA-mediated immunity include MeSA and the C_9 aldehyde nonanal (Shulaev et al., 1997; Park et al., 2007; Koo et al., 2007; Yi et al., 2009). In our experiments, MeSA emissions remained below the detection limit (Supplemental Figure 8). Nonanal, the other known infochemical, was detected here and emitted at similar rates from *AvrRpm1-HA*-expressing wild-type and *eds1-2* mutant plants (Supplemental Table 1). *Pst AvrRpm1* progressively enhanced α-pinene, β-pinene, and camphene emissions from Arabidopsis over the course of 3 d (Figure 10), which equals the time necessary for the pinene mixture to enhance Arabidopsis resistance to *Pst* (Figure 3). The pinene and camphene amounts needed to trigger resistance responses in the headspace exposure experiments were in the ppmv range, exceeding by ~1000-fold the concentrations recorded herein (Figures 2 and 10) and in naturally occurring forest canopies (Fuentes et al., 2007; Noe et al., 2012). However, relatively low ambient monoterpene levels do not exclude that higher concentrations can occur in the immediate vicinity of plant leaves at the forest floor or in dense canopies. Moreover, the relatively low Arabidopsis monoterpene emissions (Figures 2 and 10) sufficed to support plant-to-plant SAR signaling (Figure 9), suggesting that monoterpenes, in particular pinenes and camphene, provide essential reinforcement to other signals, which might include

7 (Col-0 Mock) to 9 (Col-0 *Pst AvrRpm1*) or 10 (*ggr1-1 Pst AvrRpm1*) biologically independent replicates as defined in the Methods ±SE. Asterisks indicate statistically significant differences to the corresponding mock controls (Student's *t* test, $P < 0.05$). ND, not detectable.

(D) PCA biplot of α-pinene, β-pinene, and camphene emission rates (pmol m^{-2} s^{-1}) from *Pst AvrRpm1*-infected Col-0 (green) and *ggr1-1* (red) plants and from mock-treated Col-0 plants (gray) at T0 (circles), T1 (diamonds), T2 (squares), and T3 (triangles). The ellipses denote 100, 75, and 50% (outer to inner, respectively) explained variance. The arrows were added to indicate the directions of the VOC variables (in black squares) projected into the 2-d plane of the biplot. The variances explained by principal components (PC) 1 and 2 are given in parentheses.

nonanal and MeSA, in the volatile infochemical emission pattern of *Pst AvrRpm1*-infected plants.

The chemical properties of (mono)terpenes allow these molecules to diffuse through the apolar, waxy cuticle of plant leaves (Schmid et al., 1992). Depending on the atmospheric concentration, monoterpenes can accumulate in other plants (Spielmann et al. 2016). For example, terpenes emitted by *Rhododendron tomentosum* were found in the leaf cuticles of neighboring birch (*Betula* spp) trees (Himanen et al., 2010). It is thus possible that volatile monoterpenes emitted from local infected leaves are absorbed by the cuticles of systemic leaves of the same or neighboring plants. It is interesting to note that SAR is abolished in *acyl carrier protein4* (*acp4*) mutant plants, which exhibit reduced cuticular wax and cutin formation (Xia et al., 2009). *ACP4* supports systemic SAR signal perception and thus might be important for the absorption of volatile signals, including monoterpenes. After their uptake via the cuticle (or stomata), monoterpenes (likely as components of natural plant-emitted VOC blends) trigger defense signaling at least in part via the induction of $O_2^{\bullet-}$ (Figures 7 and 11). Additionally, AZI1 and its paralogs might contribute to the early perception of monoterpenes (Figures 6 and 11). In addition to the well-established role of AZI1 in the regulation of local SAR signal emission (Jung et al., 2009; Wang et al., 2014; Cecchini et al., 2015), Lim et al. (2016) recently reported evidence for an additional function of AZI1 in systemic SAR signal perception. It is possible that monoterpenes act upstream of this systemic function of AZI1 in SAR signal perception within and between plants.

Plant VOCs have long been recognized as plant-to-plant signaling molecules, allowing plants undergoing a particular stress to warn their neighbors. Until now, plant-to-plant signaling was mostly studied with respect to plant-insect interactions and abiotic stress responses (Scala et al., 2013a; Pierik et al., 2014; Dong et al., 2016). However, an increasing number of plant-derived and other (microbial/fungal) biogenic VOCs has been associated with SA signaling over the past 10 years (Yi et al., 2009; Junker and Tholl, 2013; Choi et al., 2014; Naznin et al., 2014; Song et al., 2015), suggesting an ecological importance of VOC-mediated communication between organisms in plant innate immunity. The diterpene DA likely acts as a systemic mobile nonvolatile signal in Arabidopsis SAR (Chaturvedi et al., 2012), while another diterpene compound has been associated with SA-mediated resistance responses in tobacco (Seo et al., 2003). A connection between monoterpene accumulation and SA-mediated defenses was documented in bean and orange (Arimura et al., 2000; Rodríguez et al., 2014). In lima beans (*Phaseolus lunatus*), exogenously applied ocimene induced SA-associated defense genes (Arimura et al. 2000). In orange (*Citrus* × *sinensis*), monoterpene downregulation enhanced jasmonic acid-associated defense responses, while SA was induced in response to *Penicillium digitatum* in orange fruits normally accumulating the monoterpene limonene (Rodríguez et al., 2014). Similar to MeSA, the emissions of α-pinene and sabinene from Arabidopsis are induced by methyl jasmonate (Kegge et al., 2013). However, a physiological role of bicyclic monoterpenes, such as α- or β-pinene, in plant responses to stress has not been reported so far. This work provides the evidence linking bicyclic monoterpenes to plant immunity. More specifically, these volatile signaling molecules are important for SAR and might function as infochemicals mediating SAR-like responses between plants. In an ecological context, monoterpenes might allow plants to recognize signals from plants of the same or other species (Dicke, 2009; Dicke and Baldwin, 2010; Pierik et al., 2014; Pickett and Khan, 2016) leading to SAR. Preliminary data suggest that the monoterpene emissions from Norway spruce (*Picea abies*) needles (Ghirardo et al., 2010) enhance defense in Arabidopsis against *P. syringae* (Supplemental Figure 9). Hence, plant-to-plant SAR-like signaling, in which monoterpenes play a role, will be subject to future studies in homogeneous and mixed plant settings.

METHODS

Plant Materials and Growth Conditions

All experiments were performed in the *Arabidopsis thaliana* ecotype Columbia-0 (Col-0). Mutants *eds1-2*, *npr1-1*, *sid2-1*, and *azi1-2* as well as Col-0 *pDEX:AvrRpm1-HA* and *eds1-2 pDEX:AvrRpm1-HA* plants were previously described (Cao et al., 1997; Wildermuth et al., 2001; Mackey et al., 2002; Bartsch et al., 2006; Jung et al., 2009; Breitenbach et al., 2014). The T-DNA insertion lines SALK_208952C (*ggr1-1*) and SALK_210207C (*ggr1-2*) were obtained from the Nottingham Arabidopsis Stock Centre (Scholl et al., 2000). Seeds of selected homozygous plants were used for the experiments. Plants were grown as described previously (Breitenbach et al., 2014). Two weeks after germination, plants were watered once with Biomükk (Bio-Farming-Systems) according to the manufacturer's instructions. Four- to five-week-old plants were used for all experiments. For VOC application and plant-to-plant communication experiments, plants were grown in stainless steel pots (Rotilabo-Messbecher high-grad steel, diameter of 30 mm; Roth).

Pathogens and Infections

Pst and *Pst* carrying the bacterial effector AvrRpm1 (*Pst AvrRpm1*) were maintained as described (Breitenbach et al., 2014). SAR was induced with a primary infection of the first two true leaves with *Pst AvrRpm1* as described (Breitenbach et al., 2014). For challenge infections, two upper leaves of control-treated, SAR-induced, or VOC-treated plants were syringe-infiltrated with 10^5 colony-forming units (cfu) per mL of *Pst*. *Pst* growth was monitored at 4 dpi as described earlier (Breitenbach et al., 2014). Similarly, the in planta *Pst AvrRpm1* growth was monitored after inoculation of fully expanded leaves with 10^5 cfu per mL of the bacteria. Finally, spray inoculations were performed with 10^8 cfu/mL of *Pst AvrRpm1* in 0.01% Tween 20 (v:v) and compared with mock treatments with 0.01% Tween 20 (v:v).

Chemical Treatments

All chemicals were purchased from Sigma-Aldrich and Roth. For VOC measurements, Col-0 and *eds1-2* plants carrying *pDEX:AvrRpm1-HA* were sprayed until drop-off with 30 μM DEX in 0.01% (v:v) Tween 20. VOC treatments were performed in 5.5-liter gas-tight glass desiccators (Rotilabo-Glas-Exsikkatoren; Roth). The desiccators were filled with eight pots/plants, a filter paper for VOC application, and fresh air from the inflow of the growth chamber. For the treatments, 200 μL of hexane was supplemented with different amounts of VOCs (ranging from ~0.1 to ~1.4 μmole, maximally evaporating to form concentrations in the air ranging from ~350 parts per billion by volume to ~6 ppmv in the desiccator). All VOC solutions were prepared freshly in 300 μL gas-tight glass (HPLC) vials and applied to a filter paper in the desiccators using an HPLC syringe and the desiccator gas tap. As a negative control, 200 μL of hexane alone was used. The plants were incubated for 3 d, during which the supplemented

air, including the applied treatment, was replaced every 24 h. After 3 d, the plants were removed from the desiccators and two fully expanded leaves were either harvested for further analysis or challenged with *Pst* as described above.

Plant-to-Plant Communication

Plant-to-plant communication was analyzed in 5.5-liter gas-tight glass desiccators (Rotilabo-Glas-Exsikkatoren; Roth). Stainless steel pots containing three plants each were sprayed with 10^8 cfu/mL of *Pst AvrRpm1* in 0.01% Tween 20 (v/v) and allowed to dry for 1 h. A corresponding mock treatment was performed with 0.01% Tween 20 (v:v). Subsequently, four treated pots were enclosed per dessicator together with four pots containing two wild-type "receiver" plants each. The plants were incubated for 3 d, during which the dessicators were opened every 24 h to let in fresh air. Subsequently, the receiver plants were challenged with *Pst* as described above.

VOC Collection and Analysis

VOCs that were emitted from DEX-treated plants were collected in two sampling intervals from 1 to 4 h and from 4 to 7 h after DEX treatment in eight cuvettes (~4.2 liters) that were run in parallel. The plants were acclimatized to the cuvettes overnight prior to the DEX treatment and VOC collection. The cuvettes were made of glass except for their base, which was made of Plexiglas. The cuvettes were 13 cm in height and had the shape of a frustum with upper and lower base diameters of 13 and 26.9 cm, respectively. Twelve plants were enclosed in each cuvette, and the cuvettes were continuously flushed with 0.2 liters min^{-1} of VOC-free synthetic air (79% N_2 and 21% O_2) mixed with pure CO_2 to a final concentration of 400 μmole CO_2 $mole^{-1}$ synthetic air. Inside the cuvettes, temperature, light intensity, and relative humidity (night/day) were 22.3 ± 0.3/23.7 ± 0.7°C, 0 ± 2/135 ± 15 μmole photons m^{-2} s^{-1} photosynthetic active radiation (10-h photoperiod), and 92 ± 2/50 ± 15% relative humidity, respectively. A part of the air exiting the cuvettes was diverted using Teflon t-pieces and the VOCs were collected at a flow rate of 0.1 liters min^{-1} for 180 min (i.e., 18-liter air sample) into glass cartridges filled with polydimethylsiloxane-foam (Gerstel) and 50 mg Carbopack B (mesh size 20/40; Sigma-Aldrich) adsorbents and 250 pmol δ-2-carene as internal standard. The inlet airflows were controlled using needle valves and measured before and after each measurement using a calibrated mass flow meter (ADM 3000; Agilent Technologies). Background measurements were performed twice, at the beginning and at the end of the experiments. For this, DEX-treated plants were removed from the soil immediately before enclosing the pots in the cuvettes. The final analysis included 16 background replicates and 18 to 21 VOC replicates per plant genotype. Biological replicates were collected in separate cuvettes and no more than four cuvettes were used at the same time per plant genotype. In doing so, the replicates were performed across five to six separate plant batches.

VOCs from *Pst AvrRpm1*-infected and mock-treated plants were collected as described above except that VOCs were collected for 8 h per sampling time point, which included 1 d before and three consecutive days after the treatments of 40 plants per cuvette. The final analysis of this experiment included eight background replicates and 7 to 10 VOC (biological) replicates from two independent plant batches per treatment and genotype.

The VOC samples were analyzed with a thermo-desorption unit (TDU; Gerstel) coupled to a GC-MS instrument (GC type, 7890A; MS type, 5975C; Agilent Technologies). The TDU-GC-MS measurements and analyses, including the chemical identifications corresponding to the GC-MS peaks, followed established procedures (Ghirardo et al., 2012, 2016; Weikl et al., 2016). Only the GC-MS temperature program was slightly modified: 40°C for 0 min, followed by ramping at 10°C min^{-1} to 130°C and holding for 5 min, followed by ramping at 80°C min^{-1} to 175°C and holding for 0 min, followed by ramping at 2°C min^{-1} to 200°C and holding for 0 min, followed by ramping at 4°C min^{-1} to 220°C and holding for 0 min, followed by ramping at 100°C min^{-1} to 300°C and holding for 6 min.

Analysis of GC-MS Data

VOCs were quantified and identified as described previously (Ghirardo et al., 2012, 2016). For background correction, all quantified GC-MS peaks observed in plant-derived samples were subtracted by the mean values of the corresponding peaks originating from the background measurements. After background correction, emission rates of VOCs were calculated on a projected rosette area basis (pmol m^{-2} s^{-1}). For rosette area determination, all plants had been photographed with a fixed installed camera system prior to the treatments, and the projected rosette areas were quantified using picture pixels (www.gimp.org).

The VOC data were statistically analyzed by MDA using PCA and PLSR as previously described (Ghirardo et al., 2016; Jud et al., 2016; Weikl et al., 2016) using the software package SIMCA-P version 13.0.0.0 (Umetrics). Before MDA, data were preprocessed by log transformation [X = log(X+min)], mean centered, and scaled to unit variance. Cross-validation was used to validate the number of significant PCA and PLSR components (Eriksson et al., 2006) using a 99% confidence level on parameters and seven cross-validation groups.

For the analysis of the data derived from DEX-treated plants, PCA was used for the initial exploration of the data set and the detection of outliers (Ghirardo et al., 2005), ensuring an objective and unsupervised analysis. Subsequently, OPLS was performed using as X-variables all non-background corrected (but normalized to internal standard) data, including VOCs collected from both plant and background measurements. The Y-variables described in a binary mode the sample collected from plants (Y = 1) and those from background (Y = 0). Of the initial 137 peaks, 39 peaks were statistically and positively correlated to plant emissions. The remaining 98 peaks were associated with background and therefore discarded in the following analyses. For further analyses, background-corrected VOC emission rates of the 39 plant-derived peaks from each sample were used as X-variables. OPLS was performed using samples from SP1 and SP2 separately, aiming to correlate VOCs to the two different genotypes, Col-0 (Y = 0) and *eds1-2* (Y = 1), independent of the sampling periods.

The OPLS results were validated using analysis of variance testing of cross-validated predictive residuals (CV-ANOVA; Eriksson et al., 2008). The overall analysis aimed to identify if and at which degree the emission potentials of VOCs positively or negatively correlated to the plant genotype *eds1-2*. A volatile compound was classified discriminant when both (1) importance in the projection was higher than 1 (VIP > 1) and (2) the uncertainty bar computed by jackknife method (Efron and Gong, 1983) was smaller than its respective VIP value. Finally, the emission rates of the VOCs that were discriminant for plant genotype were additionally challenged by Student's *t* test.

NBT Staining

Leaves of pinene- and control-treated plants were stained with NBT essentially as described (Ditengou et al., 2015) with the following modifications: NBT was kept in the leaves for 20 min prior to destaining. After destaining, the leaves were cleared in 100% ethanol for 15 min and subsequently in 80% ethanol for 30 min, each at 80°C. Finally, the leaves were kept in 30% glycerol and photographed with a fixed camera system. The pixels were quantified with ImageJ.

RNA Isolation and RT-qPCR Analysis

RNA isolation, cDNA synthesis, and SYBR Green-based qPCR using primers for *PR1* and *UBIQUITIN* (*UBI*) were performed as described (Breitenbach et al., 2014). The nucleotide sequences of the other qPCR primers used in this study are listed in Supplemental Table 2.

Microarray Analysis

For each plant treatment, RNA from four biologically independent samples, each containing material from four plants, was analyzed on 8×60K custom Arabidopsis microarrays (design ID 29132; Agilent Technologies; A-GEOD-16892) that were used for one-color gene expression analysis (Low Input Quick Amp Labeling; Agilent Technologies). All procedures were performed strictly according to the manufacturer's instructions. The data were extracted using Agilent Feature Extraction Software (version 9) with template GE1_1010_Sep10. The data were analyzed with R, version 3.0.3, using the Bioconductor package limma, version 3.18.13 (Smyth, 2004; Ritchie et al., 2015). After background correction, quantile normalization, and $\log_2$ transformation, expression values of probes were averaged according to TAIR10 annotation, resulting in expression information for 24,606 genes. Differential expression analysis was performed using a linear model with the plant treatment as fixed effect and the experimental block as a random effect. For GO enrichment analysis, the function fisher.test was applied and false discovery rate-based multiple testing correction was done with p.adjust. The GO annotation and description of genes was taken from the org.At.tair.db package, version 2.10.1. In addition, GO annotation at TAIR based on literature and INTERPRO domain matches (Berardini et al., 2004) was downloaded from https://www.arabidopsis.org/download_files/GO_and_PO_Annotations/Gene_Ontology_Annotations/ATH_GO_GOSLIM.txt on April 26, 2016 and used to annotate the list of differentially expressed genes with terms from the domain "Biological Process." Gene aliases were taken from https://www.arabidopsis.org/download_files/Genes/gene_aliases_20130831.txt (version 2015-01-29 downloaded on August 31, 2016). The visualization of nonredundant significantly enriched GO terms was performed with ggplot2 (Wickham, 2009). The shown GO terms were restricted to those annotated by TAIR, and the color scheme was derived from inclusion relationships between the annotated gene lists of each term: Nodes have the same color as the most specific ancestor node that includes them; the number of colors corresponds to the number of nodes not included in any other node.

Accession Numbers

The sequence of *GGR* can be found under accession number NM_120007 (NCBI) or At4g38460 (TAIR). Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5475.

Supplemental Data

Supplemental Figure 1. Pinene-induced resistance to *Pst* remains stable for 1 to 3 d after treatment.

Supplemental Figure 2. Methyl salicylate, a mixture of (±)α- and (−)β-pinene (v/v = 1:1), and camphene are not toxic to *Pseudomonas syringae* pathovar *tomato.*

Supplemental Figure 3. Chemical composition of the methyl salicylate, (±)α-pinene, (−)β-pinene, (+)β-pinene, and camphene standards.

Supplemental Figure 4. Limonene headspace exposure enhances Arabidopsis resistance to *Pst* growth.

Supplemental Figure 5. Pinene-induced *AZI1*, *EARLI1*, *AZI3*, and *AZI4* transcript accumulation in Arabidopsis leaves.

Supplemental Figure 6. *Pst AvrRpm1* growth in wild-type, *ggr1-1*, and *ggr1-2* plants.

Supplemental Figure 7. Monoterpenes are important for plant-to-plant SAR signaling

Supplemental Figure 8. MeSA emissions from *Pst AvrRpm1*-infected Col-0 wild-type and *ggr1-1* mutant plants remained below the limit of detection.

Supplemental Figure 9. Spruce needle emissions enhance Arabidopsis resistance to *Pst* growth.

Supplemental Table 1. VOCs in the emissions of DEX-treated Col-0 *DEX:AvrRpm1-HA* and *eds1-2 DEX:AvrRpm1-HA* plants.

Supplemental Table 2. Oligonucleotides used in this study.

Supplemental Data Set 1. Summary of microarray analysis: differentially expressed genes in pinene-treated compared with control-treated samples.

Supplemental Data Set 2. Gene Ontology terms enriched within the pinene-upregulated genes.

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AUTHOR CONTRIBUTIONS

J.E.P., J.-P.S., and A.C.V. conceived the research. M.R., A.G., M.W., J.-P.S., and A.C.V. designed the research. M.R., A.G., M.W., C.K., and K.K. performed the research. E.G. customized the computational analysis of microarray data. M.R., A.G., M.W., E.G., S.D., and A.C.V. analyzed the data. A.C.V. wrote the manuscript. M.R., A.G., S.D., E.G., J.E.P., and J.-P.S. contributed to the first draft. All authors contributed to the final version of this work.

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