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Isoprene and β-caryophyllene confer plant resistance via different plant internal signalling pathways

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Abstract

Isoprene and other terpenoids are important biogenic volatile organic compounds in terms of atmospheric chemistry. Isoprene can aid plant performance under abiotic stresses, but the fundamental biological reasons for the high emissions are not completely understood. Here, we provide evidence of a previously unrecognized ecological function for isoprene and for the sesquiterpene, ß-caryophyllene. We show that isoprene and ß-caryophyllene act as core components of plant signalling networks, inducing resistance against microbial pathogens in neighbouring plants. We challenged *Arabidopsis thaliana* with *Pseudomonas syringae*, after exposure to pure volatile terpenoids or to volatile emissions of transformed poplar or *Arabidopsis* plants. The data suggest that isoprene induces a defence response in receiver plants that is similar to that elicited by monoterpenes and depended on salicylic acid (SA) signalling. In contrast, the sesquiterpene, ß-caryophyllene, induced resistance via jasmonic acid (JA)-signalling. The experiments in an open environment show that natural biological emissions are enough to induce resistance in neighbouring *Arabidopsis*. Our results show that both isoprene and ß-caryophyllene function as allelochemical components in complex plant signalling networks. Knowledge of this system may be used to boost plant immunity against microbial pathogens in various crop management schemes.

KEYWORDS
Arabidopsis thaliana, ß-caryophyllene, isoprene, jasmonic acid, plant resistance, *Pseudomonas syringae*, salicylic acid, terpenes, volatile organic compounds

1 | INTRODUCTION

Isoprene, 2-methylbuta-1,3-diene, is the simplest terpene and most abundant biogenic volatile organic compound (VOC) on earth, providing an important role in atmospheric chemistry at regional and global scales (Claeys et al., 2004; Fuentes et al., 2000). More complex volatile terpenes, such as mono- and sesquiterpenes, also contribute to atmospheric reactions, enhancing, for example, secondary aerosol formation (Joutsensaari et al., 2005). Plants and other organisms emit a high diversity of various mono- and sesquiterpenes, compounds that are well known to function as signals in inter-specific interactions (Ninkovic, Rensing, Dahlin, & Markovic, 2019; Sharifi & Ryu, 2020). Isoprene, on the other hand, can protect plants from thermal and oxidative stresses, though the fundamental reasons for the large investments in isoprene emissions by several plant species are not yet completely elucidated (Monson, Weraduwage, Rosenkranz, Schnitzler,

Lena Frank and Marion Wenig contributed equally to this work.

& Sharkey, 2021; Sharkey & Monson, 2017). In addition to protection against abiotic stresses, isoprene has also been shown to affect flowering time (Terry, Stokes, Hewitt, & Mansfield, 1995), to speed-up plant growth (Loivamäki et al., 2007; Zuo et al., 2019) and to interfere with plant–insect interactions (Laothawornkitkul et al., 2008; Loivamäki, Mumm, Dicke, & Schnitzler, 2008).

The multiple functions of isoprene in plants became clearer in transgenic plants, which were engineered to suppress isoprene emissions (Behnke et al., 2007), and exhibited far-reaching adjustments in plant metabolism (Behnke et al., 2010). New information on a potential function of isoprene as a signalling molecule was provided by Harvey and Sharkey (2016), who detected the induced expression of defence-related genes after fumigating naturally non-isoprene emitting *Arabidopsis* plants with external isoprene. Similar abiotic stress-related, protective functions for isoprene-exposed non-isoprene emitting plants were shown by Ormeño et al. (2020). Recently, employing the model species *Arabidopsis thaliana*, isoprene was assigned a possible role as a signalling compound altering plant gene expression and resistance to abiotic stresses (Zuo et al., 2019). Altogether modification of isoprene emission capacity seems to cause a cascade of cellular adjustments that include known signalling pathways. It is suggested that these adjustments can interact to remodel adaptive growth–defence trade-offs (Monson et al., 2021). However, to which extent these results apply to natural isoprene emitters remains to be elucidated.

It has been previously described that plants are able to detect exogenous mono- and sesquiterpenes (Ditengou et al., 2015; Huang et al., 2011; Riedlmeier et al., 2017; Wenig et al., 2019). Presence of the gaseous monoterpenes α/β-pinene and camphene triggered salicylic acid (SA)-associated innate immunity in *Arabidopsis* (Riedlmeier et al., 2017), while sesquiterpenes such as (-)-thujopsene and β-caryophyllene promoted lateral root formation and induced plant resistance to microbes (Ditengou et al., 2015; Huang et al., 2011; Yamagiwa et al., 2011). Recently, it was shown that the sesquiterpene β-caryophyllene binds to the transcriptional co-repressor TOPLESS (TPL) complex, and thereby modulates jasmonic acid (JA)-mediated signalling (Nagashima et al., 2019). Moreover, exposure to some other volatiles, such as green leaf volatiles (GLVs) or indole has also been shown to alter plant internal signalling and defence responses (Arimura, Ozawa, Horiuchi, Nishioka, & Takabayashi, 2001; Erb, 2018; Erb et al., 2015; Frost et al., 2008). Yi, Heil, Adame-Alvarez, Ballhorn, and Ryu (2009), showed induced *PATHOGENESIS-RELATED PROTEIN2* (*PR2*) expression upon exposure to nonanal or methyl salicylate. Naznin et al. (2014), moreover, revealed alterations in both JA- and SA-signalling in *Arabidopsis* upon exposure to 3-methylphenol.

Plant resistance against pathogens may be induced through the activation of distinct internal signalling routes. Jasmonic acid has been associated with induced systemic resistance (ISR), a state of increased immunity triggered by beneficial microorganisms in the rhizosphere (Van Loon, Bakker, & Pieterse, 1998). In several studies, different volatile compounds were also shown to be involved in JA-associated plant defence responses (Arimura et al., 2001; Erb et al., 2015; Frost et al., 2008; Helms et al., 2017). JA-signalling has been shown to depend on JASMONATE RESISTANCE 1 (JAR1) protein (Nie et al., 2017). Systemic acquired resistance (SAR) is a systemic immune response in healthy tissues induced by local pathogen infection. The induction of SAR depends on SA-signalling via NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1; Cao, Glazebrook, Clarke, Volko, & Dong, 1997; Vlot, Dempsey, & Klessig, 2009) and on AGD2-LIKE DEFENSE RESPONSE PROTEIN 1 (ALD1; Song, Lu, McDowell, & Greenberg, 2004). ALD1 is an enzyme that produces the SAR-associated molecule pipecolic acid (Pip) and is required for SAR (Návarová, Bernsdorff, Döring, & Zeier, 2012; Song et al., 2004). We recently associated SAR with monoterpene emissions acting as cues for defence, both in systemic tissues during SAR and in neighbouring plants (Riedlmeier et al., 2017). The gene coding for LEGUME LECTIN LIKE PROTEIN 1 (LLP1) was, moreover, shown to be involved in SAR and monoterpene-induced resistance, acting downstream of both Pip and monoterpenes in the establishment of immunity (Breitenbach et al., 2014; Wenig et al., 2019).

Here, we tested the potential function of isoprene and the sesquiterpenes β-caryophyllene and (-)-thujopsene as volatile cues in inter-organismic signalling. We hypothesized that (a) exogenous isoprene and the sesquiterpenes β-caryophyllene and (-)-thujopsene can activate different signalling routes in *Arabidopsis thaliana* plants and (b) that this activation leads into differentially induced resistance against the hemibiotrophic pathogen *Pseudomonas syringae*. We aimed to first elucidate the functions of these volatiles in experiments employing pure terpenes and various *Arabidopsis* mutants defective either in JA-related (*jar1* mutant) or SA-related (*npr1*, and *ald1* mutants) signalling, or associated to these signalling pathways (*llp1* mutant). In the second step, the function of the terpenes was tested in closed and open set-ups using biological terpene sources and wild type and mutant *Arabidopsis* "receiver" plants. Based on our results, we suggest isoprene has a function in activating the SA-associated plant defence system, whereas β-caryophyllene, but not (-)-thujopsene, triggered plant resistance via JA-associated signalling.

2 | MATERIALS AND METHODS

2.1 | Plant and microbial materials and growth conditions

Wild-type *Arabidopsis thaliana* plants (ecotype Columbia-0 (Col-0), transgenic *Terpene synthase 21* (*TPS21*)-gene (At5g23960) overexpressing *Arabidopsis* Col-0 (TPS21OE) and the mutant lines *non-expressor of PR genes 1* (*npr1*) (Cao et al., 1997; Vlot et al., 2009), *jasmonate resistant 1* (*jar1*) (Nie et al., 2017), *legume lectin like protein 1* (*llp1*) (Breitenbach et al., 2014) and *agd2-like defence response protein 1* (*ald1*) (Song et al., 2004) were used in the experiments. For construction of the TPS21OE line, the *Arabidopsis* gene At5g23960 ([*E*]-β-caryophyllene synthase, EC 4.2.3.57), was cloned as described previously (Ting et al., 2015). The pBINplus vector containing the gene was transfected in Agl0 *Agrobacterium tumefaciens* and the transformed *Agrobacterium* strain was used for stable transformation of *Arabidopsis* Col-0 through floral dipping (Liu et al., 2011)). TPS21OE

plants were selected in vitro on plates containing half-strength MS 1% (wt:vol) agar supplemented with 50 μg/ml kanamycin and selected transformed lines were made homozygous.

The *Arabidopsis* plants were grown in growth chambers in normal potting soil mixed with silica sand 5:1 and kept at 20/22°C (night/day), 70% relative humidity and an incident photosynthetically active quantum flux density (PPFD) of 100 μmol photons m^{-2}/s with a 10 hr photoperiod. The plants used for the experiments were 4.5 weeks old. Plant cultivation procedures have been described previously (Breitenbach et al., 2014; Riedlmeier et al., 2017; Vlot et al., 2008).

Four different genotypes of grey poplar (*Populus* × *canescens* [Aiton] Sm. INRA clone 7,171-B4) were used in the experiments: Two isoprene-emitting (IE) genotypes (WT and K12 (empty vector [pBinAR] line)) and two well-characterized isoprene non-emitting (NE), transgenic genotypes (35S::PcISPS-RNAi lines RA1 and RA2; see Behnke et al. (2007)). Plantlets were amplified by micropropagation under sterile conditions (Leplé, Brasileiro, Michel, Delmotte, & Jouanin, 1992) and rooted plantlets (approx. Plant height 5 cm) were cultivated in the greenhouse in 2.2 L pots on a sandy soil (1:1 [vol:vol] silica sand and Frühstorfer Einheitserde). For optimum fertilization, the soil was initially complemented with a mixture of slow release fertilizers (Triabon (Compo, Münster, Germany) and Osmocote (Scotts Miracle-Gro, Marysville, USA); 1:1, 10 g/L of soil). Furthermore, liquid fertilizer was applied every 2 weeks (0.1% [wt/vol] Hakaphos® Grün, Compo, Münster, Germany). Climate conditions in the greenhouse were maintained at 22/20°C (day/night) and 50% RH) with a 16/8 hr photoperiod with supplemental lighting (200–240 μmol photons m^{-2}/s PPFD at canopy level).

The *Laccaria bicolor* strain S238N (Maire P.D. Orton) was cultivated on Pachlewski medium (P5) as described previously (Ditengou et al., 2015). To expose *Arabidopsis* to *L. bicolor* volatiles, two pieces of young mycelium (5 mm diameter) were transferred to the middle of a new Petri dish and cultivated at 20–25°C in the darkness on P5 medium for 12 days.

Pseudomonas syringae pv. *tomato* (*Pst*) DC3000 was used for infection assays and was maintained as described previously (Breitenbach et al., 2014). *Pst* carrying the bacterial type III secretion system effector protein AvrRpm1 (*Pst AvrRpm1*) was used to induce volatile emission from *Arabidopsis* in the plant-to-plant communication assay (Breitenbach et al., 2014; Riedlmeier et al., 2017).

2.2 | Fumigations of *Arabidopsis* with pure terpenes

For the treatment of wild-type *Arabidopsis* plants with different pure VOCs, four pots with three plants each were placed into 5.5 L gas-tight glass desiccators (Carl Roth GmbH, Germany) (Figure 1a). During exposure experiments with *Arabidopsis* mutants, 2–3 pots containing three Col-0 plants each and 2–3 pots with three mutant plants each were placed in the same desiccator. The plants were exposed for 72 hr to individual volatile compounds (Sigma–Aldrich) dissolved in hexane as previously described in detail (Riedlmeier et al., 2017). We used solutions of isoprene, α/β-pinene, β-caryophyllene and (-)-thujopsene, which were always freshly prepared before application. If the applied solutions evaporated in the headspace equally and no deposition took place, then following final concentrations could be reached: isoprene (43.6, 87.1, 435.6, 871.1 ppm), β-caryophyllene (0.2, 1.2, 1.7, 2.3 ppm), (-)-thujopsene (0.2, 1.2, 1.7, 2.3 ppm). A mixture of α/β-pinene (2.7 ppm) was used as a positive control, as this treatment has been shown to be highly efficient in inducing immunity in our previous work (Riedlmeier et al., 2017; Wenig et al., 2019). Hexane served as a negative control (Riedlmeier et al., 2017).

2.3 | Infection with the microbial pathogen *Pseudomonas syringae*

To verify whether the treatment with various VOCs induced resistance, two upper leaves of the *Arabidopsis* plants were infiltrated with 10^5 colony forming units (cfu) ml^{-1} (OD600 set to 0.0002) of *Pst*. Bacterial growth in the plant was monitored as described before (Wenig et al., 2019). To determine the duration of the induced resistance after the terpene exposure, plants were infiltrated with *Pst* on days 4, 7 and 10 (counted from the start of volatile exposure). The leaf discs were harvested 4 days after the infiltration (following the protocol in Riedlmeier et al., 2017).

2.4 | Toxicity assay

Hexane solution (200 μl) containing resistance-inducing concentrations of isoprene, α/β-pinene, β-caryophyllene or (-)-thujopsene were applied to *Pst* medium (NYGA: 5 g/L bactopeptone, 3 g/L yeast extract, 20 ml/L glycerol, 15 g/L agar). As a negative control, 200 μl pure hexane was used. Serial dilutions of a *Pst* suspension (10^6 cfu/ml, OD600nm set to 0.002) were spotted (20 μl per spot) on plates without or with 200 μl terpene/hexane solution. *Pst* colonies were counted after 2 days of cultivation at 25–28°C. Bacterial growth on the treated plates was monitored as described before (Riedlmeier et al., 2017).

2.5 | Exposure of *Arabidopsis* to biological VOC sources in desiccators

To expose *Arabidopsis* to poplar volatiles in glass desiccators, mature poplar leaves (leaf # 7–12 below the apex) were cut from the greenhouse grown trees. The leaves were kept with the petioles submersed in tap water in glass beakers outside of the desiccators for 30 min to prevent interference with wounding related stress signals (Brilli et al., 2011). For the exposure of *Arabidopsis*, the poplar leaves in the beakers were placed in the middle of the desiccators next to the *Arabidopsis* "receiver" plants (Figure 1a). The experiments were conducted in the growth chambers with the same settings as described above. Isoprene solution of 200 μl (87.1 ppm in the headspace) and

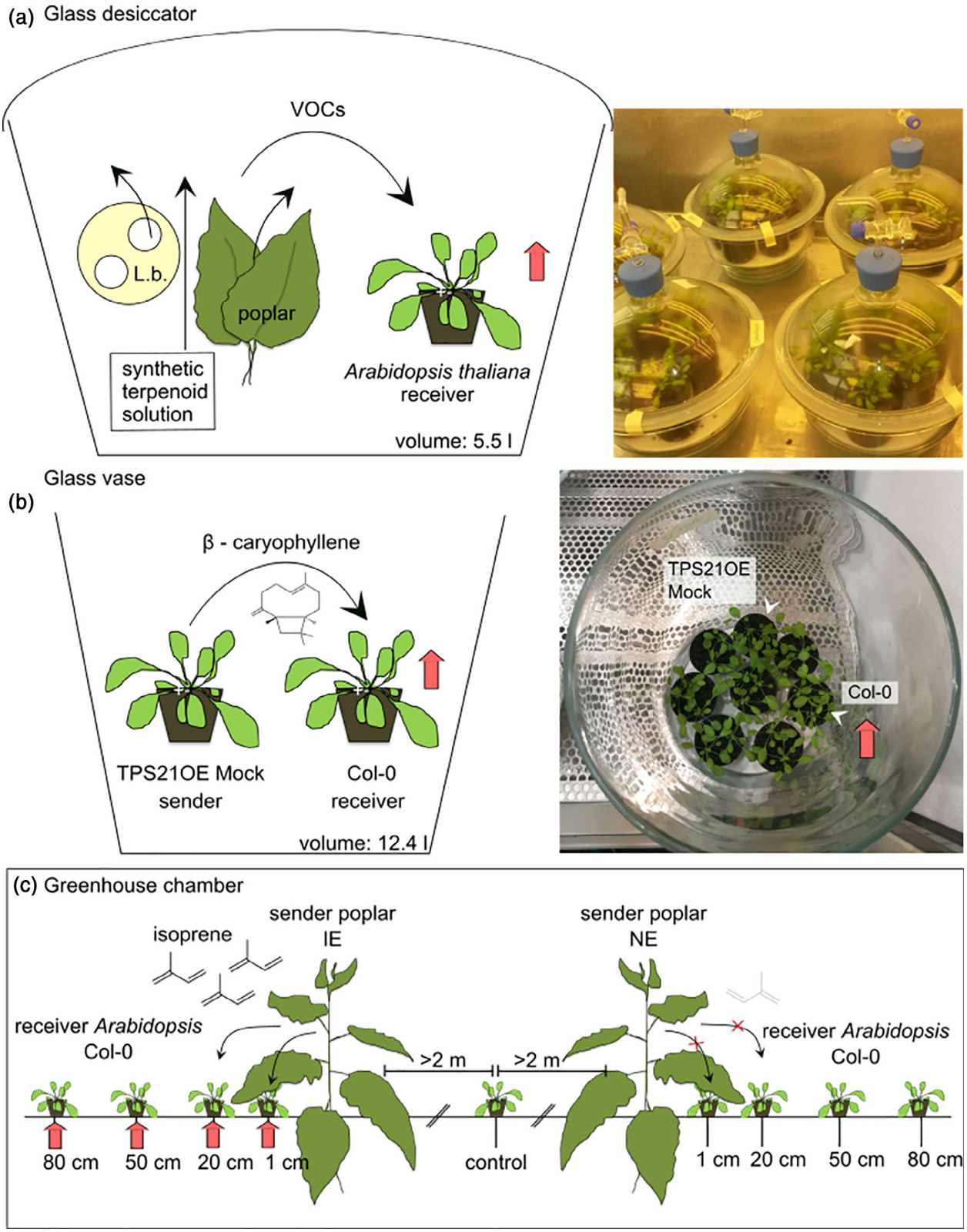

FIGURE 1 Different experimental set-ups used to expose *Arabidopsis thaliana* to VOCs. (a) *Arabidopsis* receiver plants were co-cultivated in a closed glass desiccator (5.5 L volume) with a filter paper containing different terpene solutions or a biological VOC source, such as *Laccaria bicolor* colonies or leaves of poplar (*Populus* × *canescens*). The desiccators were placed in a cultivation chamber for the exposure period (3 days). (b) *Arabidopsis* Col-0 receiver plants were co-cultivated with β-caryophyllene emitting transgenic *Arabidopsis* line (TPS21OE) in an open glass vase. (c) In an open space in a green house chamber *Arabidopsis* receiver plants were co-cultivated with increasing distance (1, 20, 50 and 80 cm) to isoprene-emitting (IE) and non-emitting (NE) poplar trees (details of the lines can be found in Behnke et al., 2007). IE and NE poplars were separated by 4–5 m. Col-0 plants placed at a 2 m distance between the poplar lines served as a room control to verify the specificity of the poplar-derived signal. Enhanced resistance to *Pseudomonas syringae* pv. *tomato* in the receiver plants is indicated by red arrows

200 μl pure hexane served as positive and negative controls, respectively.

To expose *Arabidopsis* to *L. bicolor* volatiles, Petri dishes containing 12-days old *L. bicolor* colonies were placed in the desiccators for 3 days. β-caryophyllene solution of 200 μl (0.2 ppm in the headspace) and 200 μl pure hexane served as positive and negative controls, respectively.

When living organisms were used as VOC sources, the desiccators were opened on the third day of co-cultivation to avoid CO_2 depletion and high humidity.

2.6 | Exposure of *Arabidopsis* to biological VOC sources in open systems

Plant-to-plant communication between TPS21OE and Col-0 was analysed in open glass vases without lid (Figure 1b). Four pots containing three Col-0 sender plants each were sprayed with 0.01% Tween-20 (vol:vol) solution containing either 10^8 cfu/ml of *Pst AvrRpm1* (to induce defence response) or 10 mM $MgCl_2$ (mock control treatment) and left to dry for 30 min. TPS21OE sender plants were also mock treated. Four treated pots with three sender plants and four pots containing two Col-0 receiver plants each were placed in each vase. The plants were incubated for 3 days after which the receiver plants were challenged with *Pst* as described above.

To investigate isoprene signalling between poplar genotypes and *Arabidopsis* in an open space, communication experiments were carried out in a separate greenhouse cabin (22/20°C (day/night), 16/8 photoperiod at a PPFD of 500 μmol photons m^{-2}/s; 40% RH). Wild-type *Arabidopsis* plants (4.5 weeks old) were fixed underneath a mature poplar leaf (1 cm) or placed in 20, 50 or 80 cm distance from the trees (Figure 1c). Care was taken that there was no shading on any of the *Arabidopsis* plants due to the poplar. Isoprene-emitting (IE; WT and K12) and non-emitting plants (NE; RA1 and RA2) were placed on opposite sides of the room in 4.5 m distance from each other to prevent cross communication. Control *Arabidopsis* plants were placed in the middle of the cabin in ca. 2 m distance from IE and from NE plants (Figure 1c). After 3 days of co-cultivation, *Arabidopsis* plants were challenged with *Pst* as described above.

2.7 | VOC analyses

For isoprene measurements over the whole poplar tree, leaf disks were cut by a hole punch (diameter 13 mm) from all the mature leaves of three poplar plants grown in green house. From each leaf, three disks were cut and used as technical replicates. After 30 min of stabilization time, each leaf disk was incubated in 200 μl of carbonated mineral water in 2 ml transparent glass vials for 2 hr under 32°C and light intensity of 1,000 μmol m^{-2} s^{-1} PPFD, similar to as described earlier in Loivamäki et al. (2007). Isoprene was measured from the headspace of the vial by proton transfer reaction-time of flight-mass spectrometer (PTR-ToF-MS, Ionicon Analytic, Innsbruck, Austria). The headspace of the samples was injected into the PTR-ToF-MS by using N_2 as carrier gas at a flow rate of 100 ml/min. As standard, 10.9 ppm isoprene in N_2 was used (BASI Schöberl GmbH, Germany).

To quantify isoprene concentration from poplar leaves in the desiccators, an aliquot of the headspace was sampled after incubating one leaf per desiccator for 6 hr in the growth chamber (22°C, 70% relative humidity, PPFD of 50 μmol photons m^{-2}/s inside of the desiccators). Volatiles were trapped on glass tubes filled with adsorbents (Tiiva et al., 2009) at an airflow rate of 20 ml/min for 5 min.

To quantify VOC emission of the TPS21OE lines, dynamic headspace sampling was carried out in glass cuvettes as described in Riedlmeier et al. (2017). Inside the cuvettes, the temperature was 23.7 ± 0.7°C and light intensity 135 ± 15 μmol photons m^{-2}/s photosynthetic active radiation. VOCs were collected at a flow rate of 0.1 L/min for 120 min from TPS21OE plants and 180 min from wild-type plants into glass cartridges filled with adsorbents (for details please see Riedlmeier et al., 2017).

The VOCs were analysed with gas chromatography–mass spectrometer (GC–MS) following established procedures (Ghirardo et al., 2020; Ghirardo, Heller, Fladung, Schnitzler, & Schröder, 2012).

2.8 | Statistics and reproducibility

For the statistical analyses, IBM SPSS Statistics version 21 (Armonk, NY, USA) was used. All data were tested using Kruskal–Wallis test followed by Mann–Whitney *U* test. Each statistical test is specified within the legend of a corresponding figure. Exact *p* values are given in Table S1. The raw data are given in Table S2.

3 | RESULTS

3.1 | Exogenous isoprene and β-caryophyllene improve plant resistance

Arabidopsis plants were exposed to selected terpenes in the surrounding gas phase to test if these compounds affect the "receiver" plant's resistance to the bacterial pathogen *Pseudomonas syringae* (for experimental set-up please see Figures 1a and 2a). When *Arabidopsis* was exposed to isoprene, the resistance to *Pseudomonas syringae* pv. *tomato* (*Pst*) was induced in a manner similar to that for a mixture of the monoterpenes α/β-pinene serving as the positive control (Figure 2b; Riedlmeier et al., 2017). The sesquiterpene β-caryophyllene also induced plant resistance, while the other sesquiterpene (-)-thujopsene was ineffective at the test concentration (Figure 2b). The effect of the single volatile terpenes was dependent on the applied concentration (Figures S1 and 2b). We observed that the effective dose decreased in concentration according to an increase in the structural complexity of the terpenes; from the simple C_5 hemiterpene isoprene to C_{10} monoterpenes to the C_{15} sesquiterpene β-caryophyllene (Figure S1).

A 3-day exposure of *Arabidopsis* to gaseous isoprene, α/β-pinene or β-caryophyllene led to persistent state of priming, lasting up to 4 days post-exposure. Isoprene-induced resistance lasted even up to 6 days

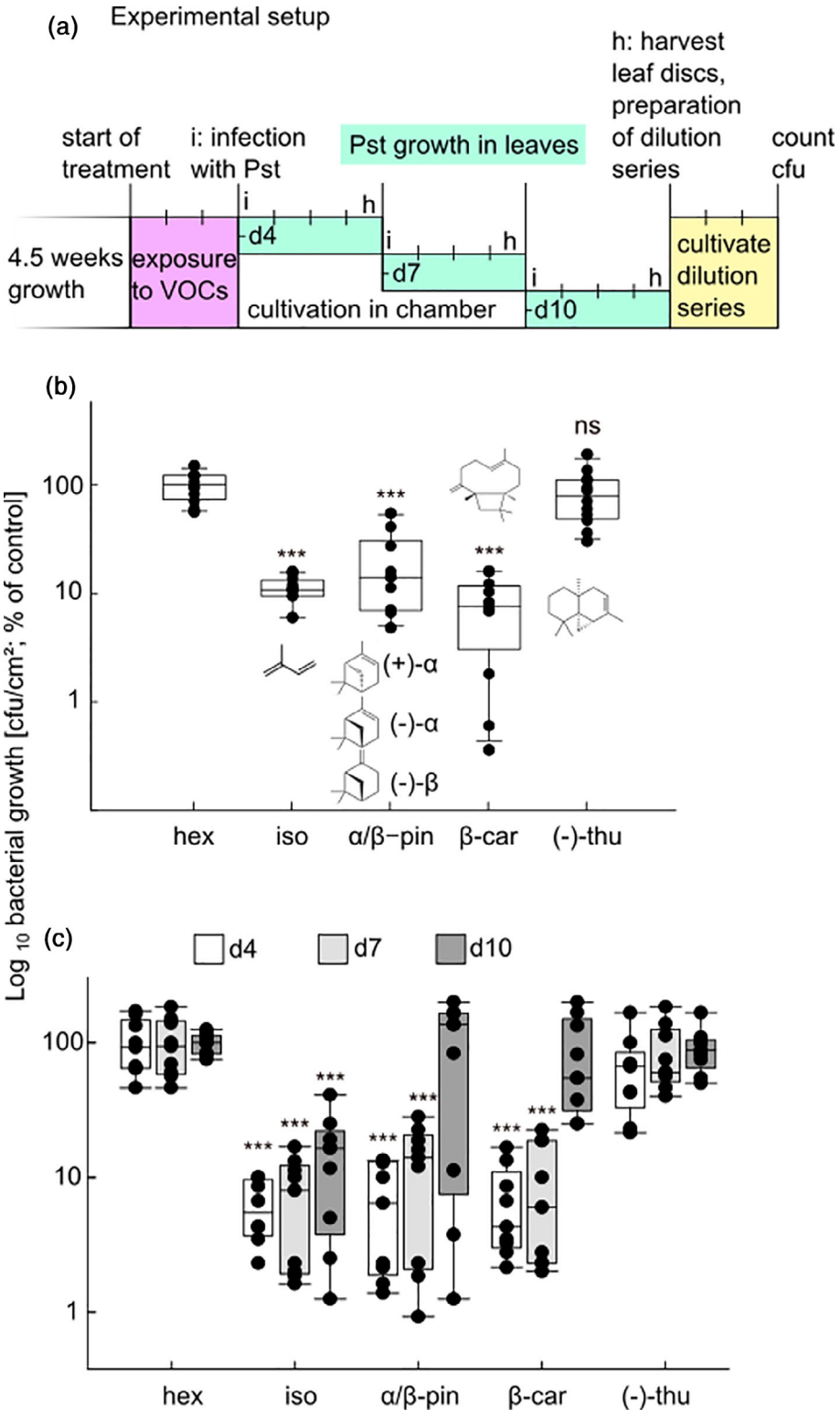

FIGURE 2 Isoprene, α/β-pinene and β-caryophyllene induce resistance to *Pseudomonas syringae* pv. *tomato* (*Pst*) in *Arabidopsis thaliana*. (a) Schematic overview of the experimental design. The days after the start of VOC exposure are indicated by tick marks. Infection with *Pst* was performed on day (d) 4, 7, 10 after VOC exposure. (b) Induction of resistance to *Pst* in *Arabidopsis* Col-0 4 days after the exposure to isoprene (iso) (87.1 ppm), α/β-pinene (α/β-pin) (2.7 ppm), β-caryophyllene (β-car) (0.2 ppm) and (-)-thujopsene ((-)-thu) (0.2 ppm). (c) To test the persistence of the resistance, *Arabidopsis* Col-0 was challenged with *Pst* in time series, that is, 4, 7 and 10 days after the start of the VOC exposure. All the experiments were conducted in glass desiccators (for details, see Figure 1a). Data shown are normalized to the mean of the hexane (hex; negative control) and dots indicate results obtained from four (b) or three (c) independent desiccator experiments, each experiment included three replicates. The median is represented by the horizontal line in the box blot. The top and the bottom of the box represent 75th and 25th percentile, and the boxes together with whiskers represent the 90th and 10th percentile of the data, respectively. Asterisks indicate the statistical differences between terpene treatments and hexane controls (Kruskal–Wallis and Mann–Whitney *U* test; $^{***}p \leq .001$) [Colour figure can be viewed at wileyonlinelibrary.com]

post-exposure (Figure 2c). The sesquiterpene (-)-thujopsene was ineffective at the concentration of 0.2 ppm at all tested time points (Figure 2c). This compound, however, slightly induced the resistance at the concentration of 1.2 ppm, whereas no effect was observed when higher concentrations were tested (Figure S1). Control experiments showed that the applied concentrations of terpenes had no direct antibiotic effect and did not directly influence the growth of *Pst* (Figure S2).

3.2 | Isoprene induces resistance through SA, β-caryophyllene through JA

To decipher the activated plant internal signalling routes upon perceiving a volatile terpene, we employed various *Arabidopsis* mutants that were deficient either in JA or SA signalling. Our results indicate a role of SA in the isoprene-triggered resistance: In SAR-deficient *npr1*

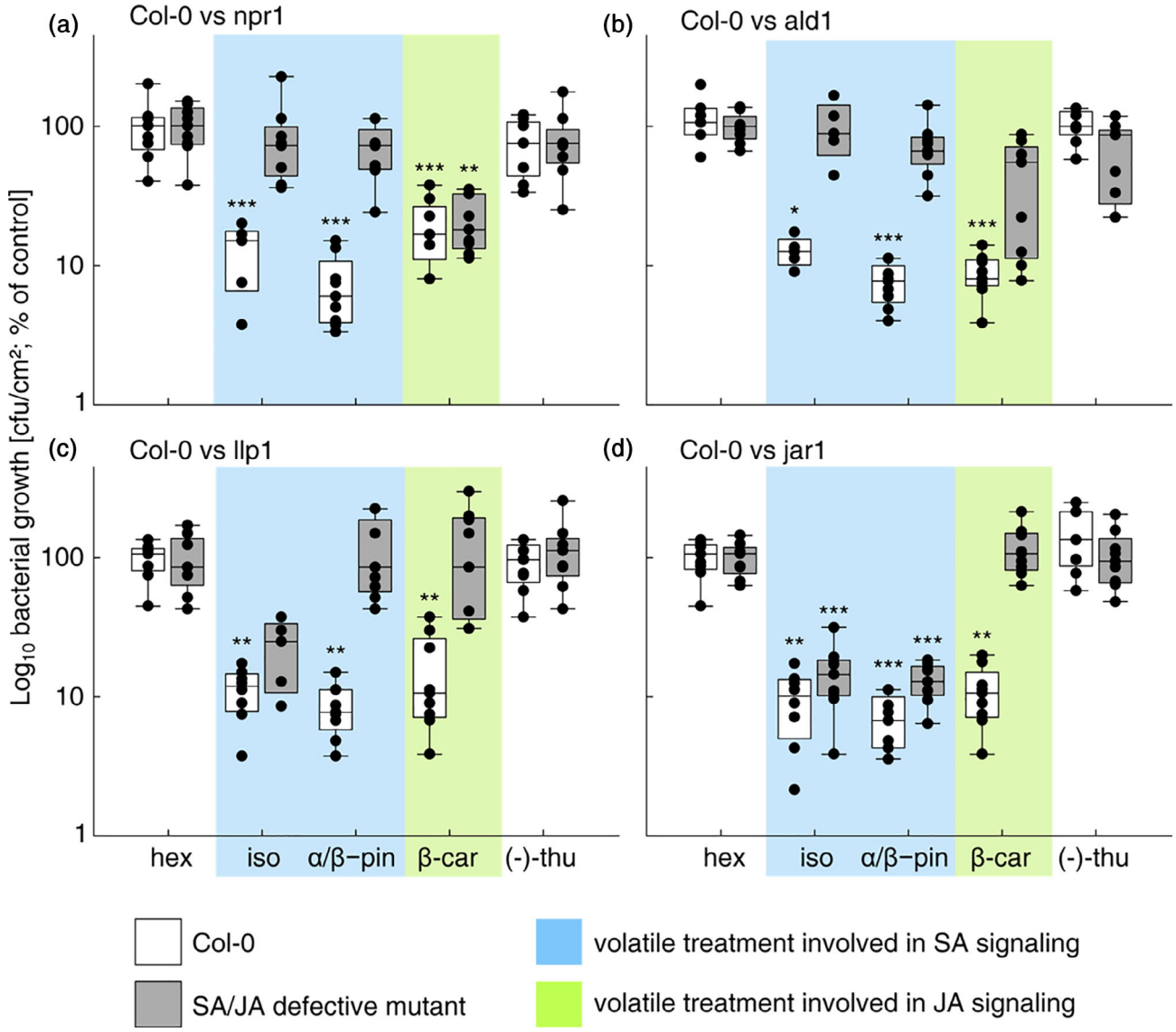

FIGURE 3 Isoprene (iso) and α/β-pinene (α/β-pin) induce resistance in an SA-dependent manner, whereas β-caryophyllene (β-car) might initiate resistance via JA in *Arabidopsis thaliana* against *Pseudomonas syringae* pv. *tomato* (*Pst*). Induction of resistance to *Pst* in wild-type Col-0 plants (white) and in SAR/ISR-defective *Arabidopsis* mutants (grey): (*non-expresser of PR genes 1* (*npr1*) (a), *agd2-like defence response protein1* (*ald1*) (b), *legume lectin like protein 1* (*llp1*) (c) and *jasmonate resistant 1* (*jar1*) (d)) after 3 days of exposure to the selected terpenes. Data shown are normalized to the mean of the hexane (hex) control of each line. Dots indicate the results obtained from three independent desiccator experiments, each experiment containing three replicates. The median is represented by the horizontal line in the box blot. The top and the bottom of the box represent 75th and 25th percentile, and the boxes together with whiskers represent the 90th and 10th percentile of the data, respectively. Asterisks indicate the statistical differences between treatments and controls (Kruskal–Wallis and Mann–Whitney U-test (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$). Blue and green colours indicate the involved phytohormone salicylic acid (SA) or jasmonic acid (JA), respectively. (-)-thu: (-)-thujopsene [Colour figure can be viewed at wileyonlinelibrary.com]

Arabidopsis mutants, gaseous isoprene or α/β-pinene did not induce resistance against *Pst* (Figure 3a). In contrast, β-caryophyllene induced resistance in *npr1* mutants, suggesting that this sesquiterpene functions independently of the SA-related *npr1*-gene in *Arabidopsis* (Figure 3a).

The function of ALD1, an enzyme that produces the SAR-associated molecule Pip and is required for SAR (Návarová et al., 2012; Song et al., 2004), was tested using *ald1* mutants. The mutant was insensitive to isoprene or α/β-pinene, resulting in no change in plant resistance (Figure 3b). *LLP1* acts downstream of both Pip and monoterpenes in the establishment of plant immunity (Breitenbach et al., 2014; Wenig et al., 2019). In the present study, the exposure of the *Arabidopsis llp1* mutant to α/β-pinene did not lead to an increased resistance (Figure 3c). The presence of the hemiterpene isoprene in the headspace of the *llp1* mutants, however, triggered resistance to *Pst* (Figure 3c and 4).

Knowing that the JA-signalling pathway depends on *JASMONATE-RESISTANT 1* (*JAR1*), we also tested the effect of volatile

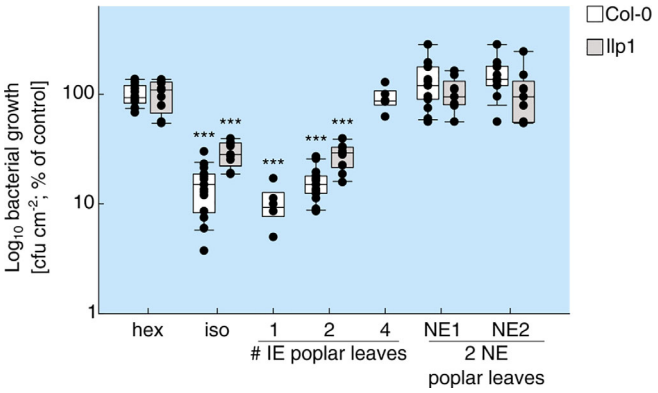

FIGURE 4 Induction of resistance to *Pst* in wild-type and mutant *Arabidopsis* plants after exposure to natural isoprene emitters. *Pst* growth is shown in Col-0 and *legume lectin like protein 1* (*llp1*) *Arabidopsis* plants after 3-days exposure to isoprene-emitting (IE) and non-emitting (NE; lines NE1 and NE2 (Behnke et al., 2007)) gravy poplar (*Populus* × *canescens*) leaves or pure isoprene (iso; 87.1 ppm). All the experiments were conducted in glass desiccators (for details see Figure 1a). Data shown are normalized to the mean of the hexane (hex) control. Dots indicate the results obtained from two-to-six independent desiccator experiments, each experiment containing three replicates. The median is represented by the horizontal line in the box blot. The top and the bottom of the box represent 75th and 25th percentile, and the boxes together with whiskers represent the 90th and 10th percentile of the data, respectively. Asterisks indicate the significant differences between treatments and controls (Kruskal–Wallis and Mann–Whitney *U* test; ***$p \leq .001$). Blue colour indicates the involved phytohormone salicylic acid (SA) (to be in line with the schema shown in Figure 8) [Colour figure can be viewed at wileyonlinelibrary.com]

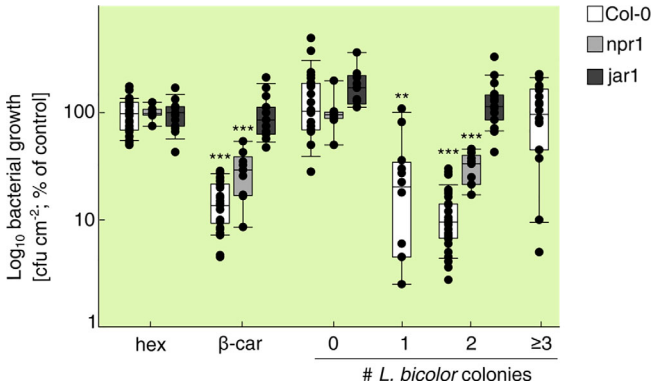

FIGURE 5 Induction of resistance to *Pst* in wild-type and mutant *Arabidopsis* plants after exposure to *Laccaria bicolor* volatiles. *Pst* growth is shown in *Arabidopsis* Col-0, *non-expressor of PR genes 1* (*npr1*) and *jasmonate resistant 1* (*jar1*)) after exposure to *Laccaria bicolor* volatiles or to pure β-caryophyllene (β-car; 0.2 ppm). All the experiments were conducted in glass desiccators (for details see Figure 1a). Data shown are normalized to the mean of the hexane (hex) control. Dots indicate the results obtained from 3 to 12 independent desiccator experiments, each experiment containing three replicates. The median is represented by the horizontal line in the box blot. The top and the bottom of the box represent 75th and 25th percentile, and the boxes together with whiskers represent the 90th and 10th percentile of the data, respectively. Asterisks indicate the significant differences between treatments and controls (Kruskal–Wallis and Mann–Whitney *U* test; **$p \leq .01$, ***$p \leq .001$). Green colours indicate the involved phytohormone jasmonic acid (JA) (to be in line with the schema shown in Figure 8) [Colour figure can be viewed at wileyonlinelibrary.com]

terpenes on the *jar1* mutant (Nie et al., 2017; Staswick, Su, & Howell, 1992). Both isoprene and α/β-pinene were observed to induce resistance in *jar1* plants, whereas the exposure of *jar1* mutants to β-caryophyllene showed no change in resistance to *Pst* (Figure 3d).

3.3 | Biological terpene emitters activate specific signalling routes in receiver plants

To investigate the ecological relevance of our findings, we asked whether a natural isoprene emitter can induce a similar response as volatilized pure terpene compounds. To this end, we tested the resistance-inducing ability of natural terpene emitters, such as the ectomycorrhizal fungus *Laccaria bicolor* (Müller et al., 2013) or isoprene-emitting (IE) and transgenic, isoprene non-emitting (NE) gravy poplar trees (*Populus* × *canescens*) (Behnke et al., 2007, Figure S3).

In a first step, we exposed *Arabidopsis* plants to the emissions of IE and NE poplar leaves (Figure S3). The results demonstrate that IE poplar leaves triggered resistance to *Pst*, whereas NE leaves were not able to induce plant resistance (Figure 4). The induction of defence was similar to that obtained with non-biogenic isoprene fumigation (Figure 3a–d) and was dependent on gas-phase concentration: one or two leaves of IE plants sharing the headspace with *Arabidopsis* plants triggered resistance, whereas a higher number of leaves did not. Young leaves of poplar emit monoterpenes, which in elderly mature leaves become replaced by isoprene emission (Brilli et al., 2009). Even in mature leaves, however, some monoterpenes are released, and this also occurs from NE poplars that have suppressed isoprene emission (Figure S3). The specificity of the isoprene effect, compared to the naturally co-occurring monoterpene emissions of poplar leaves, was therefore tested with *llp1* mutants, in which mono- and sesquiterpene-induced resistance was compromised (Figure 3c). Resistance induced by exposure of plants to IE poplar leaves developed normally in the *llp1* mutant (Figure 4), consistent with the results obtained with synthetic isoprene (Figure 3c).

The relevance of sesquiterpenes from a natural source for initiation of resistance was tested by cultivating *Arabidopsis* plants in a common headspace with axenic cultures of *L. bicolor*, an ectomycorrhizal fungus, which is known to emit sesquiterpenes such as β-caryophyllene, α-muurolene, β/γ-selinene and γ-cadinene (Ditengou et al., 2015). The exposure of *Arabidopsis* to the *L. bicolor* volatile blend led to an induction of plant resistance (Figure 5) comparable to that induced by exposure to synthetic β-caryophyllene (Figure 3a–d). We observed that one to two fungal colonies were most effective in inducing the resistance. Similar to the exposure to pure compounds (Figure 3d), the resistance was not induced by fungal colonies in the *jar1* mutant, which is deficient in JA-signalling. Comparable to the results with pure β-caryophyllene (Figure 3a), the *L. bicolor*

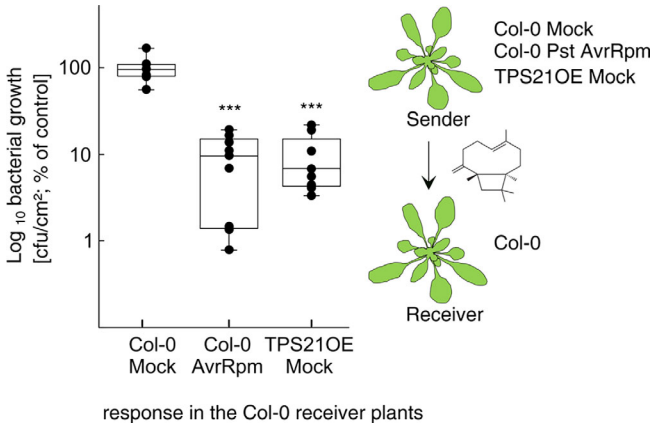

FIGURE 6 Volatile cues from *Arabidopsis thaliana* line TPS21OE induce resistance against *Pseudomonas syringae* (*Pst*) in open set-up. *Pst* growth is shown in the *Arabidopsis* Col-0 "receiver" -plants after exposure to the β-caryophyllene emitting *Arabidopsis* line TPS21OE in an open-top chamber system (See Figure 1b for the chamber set-up). Col-0 (mock treated) and Col-0 (treated with *PstAvrRpm1* (to induce monoterpene emission (Riedlmeier et al., 2017)) served as negative and positive control, respectively. Data shown are normalized mean of Col-0 (mock). Dots indicate the results obtained from three independent experiments, each experiment containing three replicates. The median is represented by the horizontal line in the box blot. The top and the bottom of the box represent 75th and 25th percentile, and the boxes together with whiskers represent the 90th and 10th percentile of the data, respectively. Asterisks indicate significant differences between treatments and controls (Kruskal–Wallis and Mann–Whitney *U* test; $^{***}p \leq .001$) [Colour figure can be viewed at wileyonlinelibrary.com]

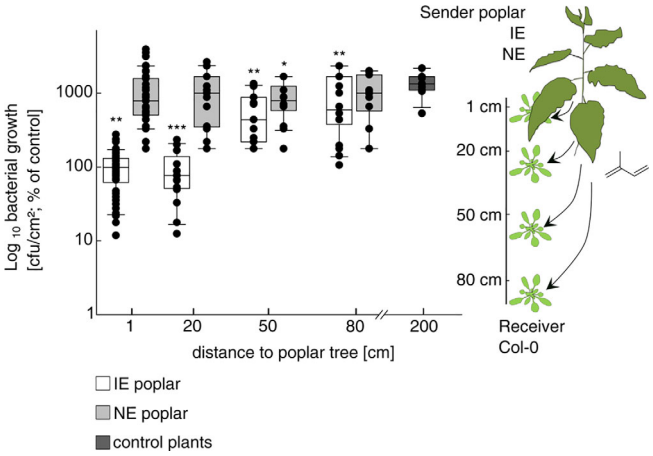

FIGURE 7 Volatile cues from wild-type *Populus* × *canescens* trees induce resistance against *Pseudomonas syringae* (*Pst*) in an open set-up. Induction of resistance to *Pst* in the *Arabidopsis* Col-0 "receiver"-plants after exposure to isoprene-emitting (IE) and non-emitting (NE) (details of the lines can be found in Behnke et al., 2007) *P.* × *canescens* "sender" plants in the open set-up. The *Arabidopsis* "receivers" were placed in 1, 20, 50 or 80 cm distance intervals to the poplar trees in a greenhouse chamber for 3 days. *Arabidopsis* plants placed in 200 cm distance between IE and NE plants served as control (for details Figure 1c). Data shown are normalized mean of [IE 1 cm distance]. Dots indicate the results obtained from three-to-nine independent experiments, each experiment containing three-to-six replicates. The median is represented by the horizontal line in the box blot. The top and the bottom of the box represent 75th and 25th percentile, and the boxes together with whiskers represent the 90th and 10th percentile of the data, respectively. Asterisks indicate significant differences between treatments and controls (Kruskal–Wallis and Mann–Whitney *U* test; $^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$) [Colour figure can be viewed at wileyonlinelibrary.com]

volatiles were able to induce resistance also in the *npr1* mutant (Figure 5), which is deficient in SA-signalling.

3.4 | Isoprene and β-caryophyllene emitters induce resistance under ambient air

To test the function of terpenes as volatile cues in plant-to-plant communication under natural, ambient air conditions, we conducted two assays using open-air experimental systems. First, we co-cultivated wild-type *Arabidopsis* "receiver" plants together with β-caryophyllene synthase overexpressing *Arabidopsis* "sender" plants (TPS21OE, Figure S4, Alquézar et al., 2017) in open-top glass containers (Figure 1b, the same set-up was previously used by Wenig et al., 2019). TPS21OE-plants constitutively emit β-caryophyllene and two minor sesquiterpenes, *cis*-caryophyllene and α-humulene (2 and 21% of the three most abundant, novel sesquiterpenes, respectively, Figure S4). These compounds were not detected in the emission profiles of wild-type *Arabidopsis* plants. Similar to the application of synthetic β-caryophyllene using closed desiccators, the "receiver" plants in the open system showed induced defence responses after exposure to constitutive β-caryophyllene emissions from nearby TPS21OE "sender" plants (Figures 1b and 6).

Proximity to IE poplar trees also induced resistance to *Pst* in *Arabidopsis* when positioned close together in the greenhouse. *Arabidopsis* "receiver" plants perceived isoprene from IE poplar trees across distances up to 80 cm and developed improved resistance against *Pst* (Figures 1c and 7 and). The induction of resistance was stronger in plants growing next to the emission source, compared to plants growing farther away (Figure 7). *Arabidopsis* plants placed 200 cm from the IE poplars were not affected, whereas exposure to NE plants lead only into very slightly induced resistance that might be due to emission of other volatile compounds, such as other terpenes (Figures 7 and S3).

4 | DISCUSSION

4.1 | Isoprene confers plant resistance similar to monoterpenes, via salicylic acid signalling

Monoterpenes and sesquiterpenes have various signalling functions in inter-specific interactions (Ninkovic et al., 2019; Sharifi & Ryu, 2020). High biogenic isoprene emissions by the leaves of some plant species (Sharkey & Monson, 2017), has, however, been proposed to serve as protection against abiotic stresses, such as high temperatures, drought or excessive light intensities (Behnke et al., 2007; Loreto et al., 2001;

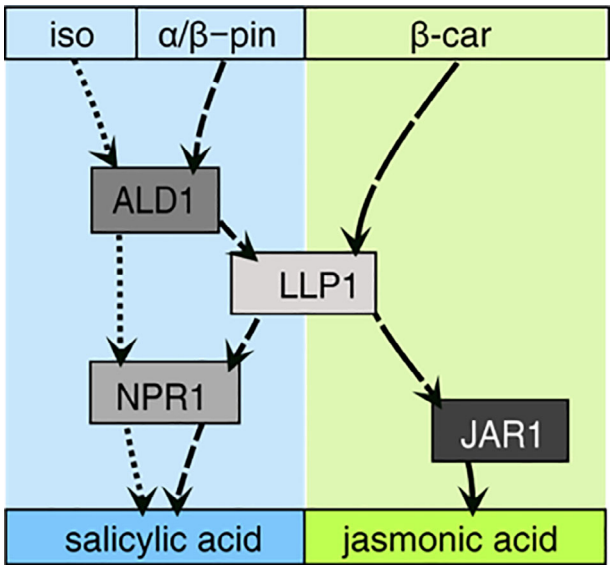

FIGURE 8 Schematic representation of potential plant internal terpene-mediated signal transduction. Isoprene needs ALD1 and NPR1 to induce resistance, α/β-pinene-induced defence additionally depends on LLP1. β-Caryophyllene induces NPR1-independent resistance through LLP1 and JAR1. Blue and green colours indicate the involved phytohormone salicylic acid (SA) or jasmonic acid (JA), respectively [Colour figure can be viewed at wileyonlinelibrary.com]

Loreto & Schnitzler, 2010; Loreto & Velikova, 2001). Our results reveal a novel, ecologically relevant function for isoprene within the context of a "volatile language" of plants. Our data show that *Arabidopsis* plants react to the presence of isoprene in the ambient air by inducing resistance to the microbial pathogen, *Pseudomonas syringae*. Employing different mutant genotypes, we show that pathogen resistance is coupled to signal transduction networks that are activated upon volatile terpene perception. For isoprene, our data show that an isoprene cue is processed in an SA-dependent manner in *Arabidopsis*. The isoprene-induced defence response was dependent on the presence of two proteins, ALD1 and NPR1, but independent of the protein, JAR1, in the downstream signal cascade. These combined results demonstrate the involvement of SA-, but not JA-signals in isoprene-induced defence response. Recently, it was shown that the monoterpenes, α- and β-pinene, induce accumulation of SA and SAR-related gene transcripts in *Arabidopsis* (Riedlmeier et al., 2017; Wenig et al., 2019). In contrast to monoterpene-induced resistance, which depends on LLP1 (Breitenbach et al., 2014; Wenig et al., 2019), isoprene-induced resistance was normal in the *Arabidopsis llp1* mutant, suggesting that the modes of action of isoprene and α/β-pinene differ at a certain point in the signal processing networks.

4.2 | The sesquiterpene, β-caryophyllene, induced plant resistance via jasmonic acid signalling

Previously, volatile-triggered changes in JA-associated signalling networks have been shown more often than those triggering SA networks. For example, GLVs (Arimura et al., 2001; Frost et al., 2008), indole (Erb et al., 2015) or the rare volatile (*E*,*S*)-conophthorin (Helms et al., 2017) were all shown to induce or adjust JA-dependent signalling. Moreover, in the present study, the sesquiterpene β-caryophyllene induced resistance through LLP1 and JAR1, but acted independently of NPR1, suggesting that the sesquiterpene cue was processed in a JA-dependent manner. This result is consistent with the recent finding that β-caryophyllene can bind to the TPL-like protein, co-repressor of JA-mediated signalling (Nagashima et al., 2019). TPL, together with adaptor proteins, blocks default activity of the transcription factor MYC2 in the absence of the JA-derivative, jasmonoyl-isoleucine (JA-ile [Perez & Goossens, 2013; Li, Uhrig, Thurow, Huang, & Gatz, 2019]). This function of β-caryophyllene is similar to the action of plant phytohormones, such as JA or auxin, which also function through transcription co-repressors (Nagashima et al., 2019; Perez & Goossens, 2013). Release of TPL may enable activation of MYC2 target genes, which play a central role in JA-mediated signalling (Nagashima et al., 2019). Our data thus stress the importance of β-caryophyllene in inter-specific interactions. It does not only function as a signal emitted by several plant and microbial species to interact with their environment (Huang et al., 2011; Hung, Lee, & Bennett, 2013; Rasmann et al., 2005), but this specific sesquiterpene can also be perceived by plants leading to changes in JA-signalling and induced resistance. In this context, the high number of isomeric sesquiterpenes is very interesting, if they can transmit distinct inter-specific signals. Moreover, not only plants, but also microbes emit a high variety of different terpenoids (Lemfack et al., 2017) that may be perceived by plants. When exploring other possible VOC signals, we found that (-)-thujopsene, a sesquiterpene released among others by the ectomycorrhizal fungi *L. bicolor* (Ditengou et al., 2015), did not induce plant resistance aboveground when applied in similar concentration as β-caryophyllene. This compound might, however, be effective at other concentrations: Our results suggest that at a specific concentration also (-)-thujopsene may be able to induce resistance. On the other hand, (-)-thujopsene can alter plant performance by other means than by inducing resistance. Interestingly, (-)-thujopsene was previously shown to impact *Arabidopsis* root architecture belowground, while β-caryophyllene did not (Ditengou et al., 2015). Together, these observations indicate that the response of (-)-thujopsene and β-caryophyllene may be tissue-specific and they reveal the possibility that distinct functions and activities occur among different isomers in the family of sesquiterpenes.

4.3 | Distinct signalling cues and specific plant responses?

We propose a scheme whereby the interactions of genes and phytohormones are required for volatile terpene-induced resistance (Figure 8). Isoprene requires two SA-signalling related proteins, ALD1 and NPR1 to induce resistance, while monoterpene-induced resistance additionally depends on LLP1. β-Caryophyllene induces resistance through LLP1 and JAR1, but acts independently of NPR1, suggesting involvement of JA-signalling. The activation of specific signalling routes suggests that natural selection has favoured the

capability for distinct volatile cues to explicitly target plant responses, thus being able to distinguish distinct information between or within organisms. Knowing that the JA- and SA-associated signalling routes can cross talk (Erb, 2018; Thaler, Humphrey, & Whiteman, 2012), a delicate adjustment of the plant response to specific volatile cues may be possible.

The effective deployment of specific signalling cues has been suggested to rather depend on the use of rare volatile compounds, such as β-caryophyllene, that display species and stress response specificity (Caparrotta et al., 2018), than for more ubiquitously released molecules, such as isoprene, or the common monoterpenes α- and β-pinene. Erb (2018) suggested recently, however, that such rather common volatile cues from different sources, such as microbes and neighbouring plants, might be integrated in a receiver plant for a more specific defence response. Integration of various cues, together with plant internal signalling cross talk, might create a complex network of signals that plants use for reliable resolution of responses to external biotic and abiotic stresses (Erb, 2018; Junker et al., 2018).

Similar to other volatile signals, which are often more powerful as a bouquet than as individual molecules (Šimpraga, Takabayashi, & Holopainen, 2016), isoprene, although a relatively abundant VOC at least in tree canopies of isoprene emitters, may be part of a more complex volatile blend forming specific signals in different ratios with other compounds. Often isoprene-emitting trees facing biotic stress induce the emission of mono- and sesquiterpenes, which appears to compensate for reductions in the constitutive emission of isoprene (Brilli et al., 2009). Thus, reduced rather than increased isoprene concentrations in the surrounding atmosphere might, along with increases in induced VOCs, be associated with the likelihood of increased stress.

4.4 | Distance and VOC concentration matter

According to previous research, the concentration of the volatile compound seems to play an important role in perceiving and recognizing the cue (Baldwin, Halitschke, Paschold, von Dahl, & Preston, 2006; Riedlmeier et al., 2017). Although the applied concentrations of pure solutions were relatively high in the present study, it is likely that these, as point source applied solutions, in fact resulted (due to possible deposition on surfaces and/or chemical reactions) in only short transient concentration peaks in the headspace of the exposure chamber. Thus, the actual effective concentration of individual terpenes cannot be identified from these "proof-of-principle"-data. The biological effectiveness of isoprene emitted from young poplars in a free atmosphere and the perception of the low β-caryophyllene emission from transgenic *Arabidopsis* plants in open systems, however, imply that low rather than very high volatile concentrations may be effective cues. Ultimately, concentration gradients in the atmosphere regulate the VOC exposure of a receiver plant (Baldwin et al., 2006). The present data also show that isoprene-induced resistance depends on the physical distance of the receiver from the sender in an open space. The concentration and spatial distribution of volatile terpenes within plant canopies can indeed vary greatly, for example, due to their high volatility, rapid atmospheric dilution and chemical reactivity (Holopainen & Blande, 2013; Pinto, Blande, Souza, Nerg, & Holopainen, 2010; Mofikoya et al., 2019). Within the blend of volatiles, isoprene is unique; in that, it possesses an extremely low boiling point (Mofikoya et al., 2019) and diffusivity, causing it to disperse rapidly by passive diffusion (Baldwin et al., 2006). Previously, however, also other highly volatile compounds, such as methacrolein, were shown to be an important part of VOC signalling between sagebrush (*Artemisia tridentate*) and tobacco (*Nicotiana attenuata*) plants within 15 cm distance (Kessler, Halitschke, Diezel, & Baldwin, 2006). This is a distance that would also permit isoprene to effectively transmit information (present study).

In addition, microbes can also emit isoprene (Kuzma, Nemecek-Marshall, Pollock, & Fall, 1995), which can be sensed by plants. Because microbes often grow in close association with plants, the signalling distance may be shorter than in the plant–plant interactions described here. Recently, it has been shown that root-internal isoprene can lead to changes in root architecture and redox status in certain root cells (Miloradovic van Doorn et al., 2020). For example, if epiphytic or endophytic bacteria release isoprene, the compound could lead to sensitive adjustments in plant redox balance and alter plant performance. The potential ecological significance of isoprene in microbe–plant interactions should be elucidated in the future.

4.5 | Volatile terpenes as signalling cues in plant communities

A potential function between isoprene emitters and non-emitters was recently proposed for complex plant communities (Ormeño et al., 2020). Taken the relatively short distance of the effective cue, isoprene might function especially in dense ecosystems, or even as a rapid, within-plant signalling cue. Baldwin et al. (2006) have suggested that for highly volatile substances, signalling may be most beneficial for protecting the foliage of the emitter tree, and other neighbouring trees with intertwined canopies. In natural populations of plants, there is a high likelihood that neighbours are genetically related, and therefore, that they share genetic contributions to fitness that are carried forward to future generations. Isoprene is emitted, for example, by bryophytes (Hanson, Swanson, Graham, & Sharkey, 1999) and by many arctic species (Ghirardo et al., 2020; Tiiva et al., 2007; Tiiva et al., 2009), which grow in small scale and species-rich, dense ecosystems. The present data stress the need to investigate whether isoprene plays a role in such ecosystems, for example, in the Arctic and/or in the tropics, where "eavesdropping" to isoprene-emitting neighbours may be advantageous for boosting plant immunity.

In a past commentary, Lerdau (2002) made the suggestion that while it is clear that plants emit complex mixtures of VOCs to the atmosphere, it is not clear that they possess mechanisms to respond to those compounds when received in a gaseous state. We have shown that there do appear to be mechanisms that allow plants to "listen" to the messages sent by their neighbouring organisms. Some

of the most commonly emitted VOCs, such as isoprene and several monoterpenes and sesquiterpenes, appear to sensitize complex signalling pathways in plant cells that, in turn, trigger cellular responses that enhance plant fitness. Understanding the communication traits, and their effects to harden plants against the detrimental influences of microbial infections and abiotic stresses, will yield new strategies for the effective management of agriculture and forestry.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Jörg-Peter Schnitzler conceived and designed the experiments with Lena Frank, Maaria Rosenkranz and A. Corina Vlot. Lena Frank and Maaria Rosenkranz prepared the manuscript with contributions from Jörg-Peter Schnitzler and A. Corina Vlot. Lena Frank and Marion Wenig performed VOC exposure assays and analysed data. Lena Frank, Maaria Rosenkranz, Andrea Ghirardo and Alexander van der Krol performed VOC analyses. Alexander van der Krol provided TPS21OE plants. A. Corina Vlot provided *Arabidopsis* mutants and edited the manuscript. Jörg-Peter Schnitzler and Maaria Rosenkranz supervised the study and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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