Regulation of pathogen-inducible volatile compounds in Arabidopsis and their role in plant defense

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
der Julius-Maximilians-Universität Würzburg

vorgelegt von

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aus dem Iran

Würzburg 2010

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Doktorurkunde ausgehändigt am: .....

This *thesis* is *dedicated* to my wonderful parents
And my always supportive brother

Acknowledments

First and foremost, I would like to express my deep and sincere gratitude to my supervisor PD. Dr. Jürgen Zeier for giving me the opportunity to work in his lab and I will be forever grateful for the guidance, ideas, encouragement; his excellent supervision over these years have been invaluable.

I highly appreciate Prof Dr. Werner Kaiser for his support during my work in Würzburg.

I wish to express my gratitude to the head of our department Prof. Dr. Markus Riederer.

I would like to thank Dr. Tatiana Zeier (Mishina) and Mr.Thomas Griebel as my colleagues for helpful assistance and contributing to the friendly atmosphere in our laboratory where make there a nice place to work in.

I wish to extend my warmest thanks to my colleagues in the Department of Botany II and the Julius-von-Sachs-Institute supporting me during my work.

I am very grateful to German Academic Exchange Service (DAAD) and the DFG (Graduiertenkolleg 1342) for financial support.

Finally, I owe special gratitude to my family for continuous and unconditional support: To my parents for the interest they showed in my studies and the motivation they gave me during those tiring times. Also, to my brother whose help and encourgenemt is always helping me. I would like to dedicate my thesis to my family.

List of Abbreviations

Avr	Avirulence
BSMT1	BENZOIC ACID/SALICYLIC ACID METHYLTRANSFERASE 1
BTH	benzo[1,2,3]thiadiazole-7carbothioic acid-S-methyl ester
$[Ca^{+2}]cyt$	cytosolic Ca^{2+}
coi1	*coronatine insensitive 1*
COR	coronatine
dde2	*delayed-dehiscence* 2
DMNT	(*E*)-4,8-dimethyl-1,3,7-nonatriene
EDS	enhanced disease susceptibility
ET	ethylene
Flg22	flagellin 22 (22-amino-acid, elicitor-active flagellin peptide)
FLS2	FLAGELLIN-SENSITIVE 2
GLV	green leaf volatile
H_2O_2	hydrogen peroxide
HR	Hypersensitive response
hrc	hypersensitive response conserved
hrp	hypersensitive response and pathogenicity
ICS1	ISOCHORISMATE SYNTHASE 1
INA	2, 6-dichloroisonicotinic acid
ISR	induced systemic resistance
JA	jasmonic acid
jar1	*jasmonic acid methylester resistant 1*
jin1	*jasmonate insensitive 1*
LPS	lipopolysaccharide
LRR	leucine-rich repeat
MAPK	mitogen-activated protein kinase
MeSA	methyl salicylate

NADPH	nicotinamide adenine dinucleotide phosphate
NB-LRR	nucleotide binding, leucine-rich repeat
NPR	non-expressor of pathogenesis-related genes
NO	nitric oxide
NDR	nonrace-specific disease resistance
O_2^-	superoxide
OPDA	12-oxophytodienoic acid
OPR3	OPDA-reductase 3
PAD	phytoalexin-deficient
PAMP	pathogen-associated molecular pattern
PR	pathogenesis-related
PRR	pattern recognition receptor
PCR	polymerase chain reaction
ROS	reactive oxygen species
SA	salicylic acid
SABP	SA binding protein
SAG	salicylic acid glucoside
SAR	systemic acquired resistance
SID	SA induction-deficient
T-DNA	Transposon-DNA
TIR	Toll and interleukin-1 receptor
TMTT	(E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene
TPS	terpene synthase
TTSS	type III secretion system
VOC	volatile organic compound

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1 INTRODUCTION

1.1 Plant-pathogen interactions

Above- and below-ground parts of plants continuously interact with various microorganisms, including bacteria, fungi, oomycetes, and viruses. Plant-microbe interactions can be beneficial for the plant. They might positively affect agricultural productivity and stimulate plant growth or the activation of natural plant defenses (Smith and Read, 1996). Nevertheless, many microbes are pathogenic and in principle able to cause disease on the plant. Plant pathogens can be broadly divided into two categories, such with a biotrophic and such with a necrotrophic lifestyle. Biotrophs require a living host to complete their life cycle, whereas necrotrophs kill host tissue and feed on the remains (Dangl and Jones, 2001; Glazebrook, 2005).

When a pathogen can overcome plant defense, the interaction is **compatible** (Jones and Dangl, 2006). However, plant disease is rare, and the majority of plant species are resistant to the attack of a potential pathogen. To combat disease, plants are armed with a variety of preformed and inducible defences. Such a multiple layered defense system is effective enough to resist to the attack of the majority of pathogens. Many pathogens are not able to overcome the first defense barriers that prevent colonization of the plant, and this form of resistance is termed **non-host resistance** (NHR, Thordal-Christensen, 2003; Mysore and Ryu, 2004). For instance, pathogenic microbes must access the plant interior and penetrate preformed mechanical barriers, such as the cuticle and the plant cell wall. Moreover, they must be able to detoxify antimicrobial compounds which are constitutively produced in the plant, so-called phytoanticipins (Agrios, 2005). Finally, when a pathogen has an avirulence (avr) gene, and a plant host has the corresponding disease resistance (R) gene, the plant can react to the pathogen by activating a battery of defense responses that interfere with its multiplication and prevent disease.This interaction becomes **incompatible,** and this type of resistance is called specific resistance (Kim et al., 2008).

Phytopathogenic Bacteria

In terms of the mode of pathogenesis, phytopathogens can be divided into three classes: **Necrotrophic pathogens** such as *Pectobacterium carotovorum* (previously *Erwinia carotovora*) or the fungus *Botrytis cinerea* kill host cells as the main strategy for obtaining nutrients, causing host cell death during early stages of the infection. By contrast, **biotrophic pathogens** obtain nutrients from living host cells withough causing host cell death (Mole et al., 2007; Melotto et al., 2008). Powdery mildews, downy mildews and rust fungi are known as biotrophic pathogen (Perferct and Green, 2001). Similarly, **hemibiotrophic pathogens** have the most aggressive phase of population increase in the absence of apparent host cell death. However, at later stages of pathogenesis, host cells die and infected tissues show extensive necrosis. *Pseudomonas syringae*, *Ralstonia solanacearum* and *Xanthomonas campestris* pv. *campestris* are best described as hemibiotrophic (Mole et al., 2007; Melotto et al., 2008).

Phytopathogenic bacteria often secrete proteins into the plant cell to alter host processes in a way that is favourable to the pathogen. The most important ones are effectors secreted through the type III secretion system (T3SS). T3SS components and virulence effectors are encoded by *hrp* (hypersensitive response and pathogenicity) genes, which are so named because they are required for bacteria to cause disease in susceptible plants and to elicit the hypersensitive responses (HR) in resistant plants (Lindgren et al., 1986; Cui et al., 2009). In fact, many T3SS effector genes in *P. syringae* were discovered based on their ability to trigger the HR in resistant plants, and have therefore been named avirulence (avr) genes (Leach and White, 1996). For example, the type III effector AvrPto was identified based on its avirulence activity in plants (Roland et al., 1992; Tang et al., 1999).

The T3SS is required to deliver effector proteins from the cytoplasm of gram-negative bacteria to the plant cell interior. This requires the transport of proteins across multiple physical barriers: the two bacterial membranes separated by a peptidoglycan layer and the plasma membrane of the plant cell, which is surrounded by a thick cell wall (Büttner and He, 2009). In addition to the T3SS, several pathovars of *Pseudomonas syringae* produce the phytotoxin coronatine (COR) which induces a wide array of effects in plants (Bender et al., 1999). COR leads to diffuse chlorosis of leaves, anthocyanin production, tendril coiling, root retardation (Feys et al., 1994;

Uppalapati et al., 2005), and promotes senescence in tobacco (Kenyon and Turner, 1990).

1.2 Plant defences

During the process of evolution, plants have evolved an elaborate defense system which is called plant immunity. Plant immunity relys on a combination of preformed defenses and induced responses. Preformed defenses are the basis of non-host resistance, whereas NHR, basal defense, and gene-for-gene resistance all include induced defense responses which are activated on perception of pathogen-derived elicitors.

The current view of plant defense can be presented as a four phased zigzag model proposed by Dangl and Jones (Fig. 1).

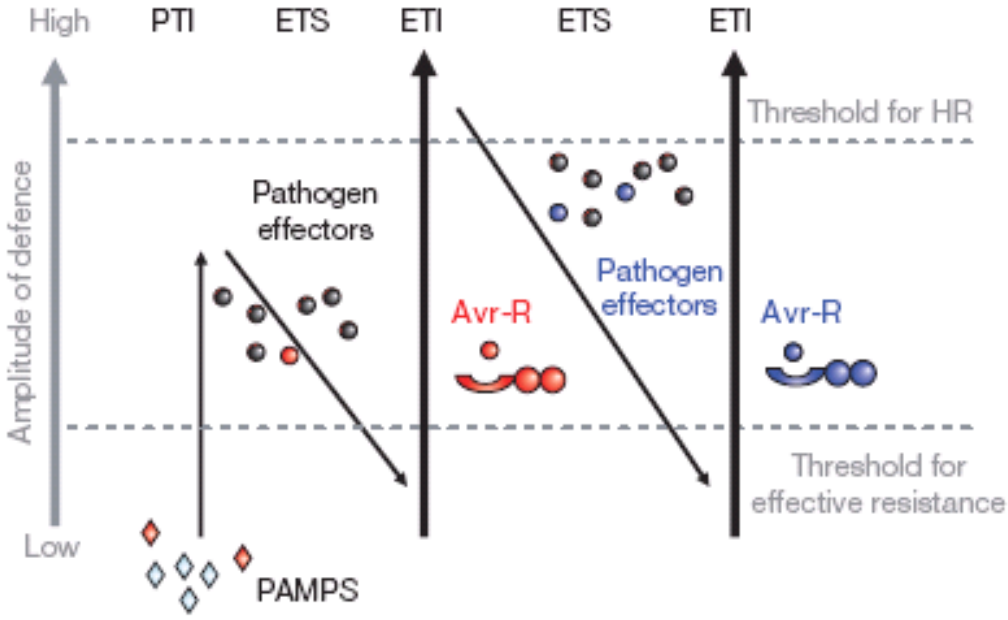

Figure1. Zigzag model (adapted from Dangl and Jones, 2006).

1.2.1 Preformed defense responses

Pre-formed defenses are the first obstacles a pathogen faces before invading a plant (Mysore and Ryu, 2004). They include mechanical defense barriers such as the waxy leaf cuticle and the root endodermis, and secondary metabolites as chemical barrier. Plants constitutively produce a

plethora of secondary metabolites, which can act as antimicrobial or toxic compounds during defense against pathogenic microorganisms and herbivorous insects. Low-molecular-weight compounds with antimicrobial activity are generally called phytoanticipins; they often play an important role in the expression of non-host resistance. Remarkable examples are saponins, which are produced consitutively in many plants but can also be induced as a result of pathogen infection (Morrisey and Osbourn, 1999). Recent evidence suggests that glucosinolates, amino acid-derived thioglucosides that are commonly synthesized and stored in cells of healthy crucifer plants, may be mobilized to pathogen challenge sites and subsequently degraded to mustard oils which act antimicrobially (Bednarek et al., 2009; Clay et al., 2009).

1.2.2 Induced defense responses

The second obstacle a pathogen has to face comprises inducible plant defense mechanisms. Activation of inducible defenses is triggered by specific recognition of general and specific elicitors, which is the basis of PAMP-triggered (PTI) and effector-triggered immunity (ETI), respectively. PTI is important to both non-host and basal resistance (Thordal-Christensen, 2003; Nürnberger et al., 2004; Oh and Collmer, 2005; Hou et al., 2009). ETI, an accelerated and amplified immune response to specific variants of a certain pathogen, is activated by plant intracellular resistance (R) proteins and usually results in the HR at infection sites (Boyes et al., 1998; Belkhadir et al., 2004).

1.2.2.1 PAMP-triggered immunity

PTI is activated upon the recognition of pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) that are not found in host cells (Shan et al., 2007). At the onset of PTI, PAMPs are recognized by pattern recognition receptors (PRRs) at the cell surface (Zipfel, 2008).

The recognition of M/PAMP by PRRs triggers different molecular, physical and pathogenesis-related changes to prevent entry and/or spread of pathogens. These events include ion fluxes across the plasma membrane, increased interacellular Ca^{+2}, an oxidative burst, MAP kinase (MAPK) activation, protein phosphorylation, receptor endocytosis, protein-protein

interactions, callose deposition to reinforce the cell wall, stomatal closure, induced expression of defence-related genes, the production of salicylic acid (SA), and accumulation of phytoalexins (Ligterink et al., 1997; Felix et al., 1999; Lee et al., 2001; Asai et al., 2002; Fellbrich et al., 2002; Navarro et al., 2004; Zeidler et al., 2004; Ramonell et al., 2005; Kaku et al., 2006; Melotto et al., 2006; Qutob et al., 2006; Mishina and Zeier, 2007a). Phytoalexins are low-molecular weight antimicrobial compounds that are synthetized *de novo* in response to pathogen attack. A well known phytoalexin in *Arabidopsis thaliana* is the indole derivative camalexin (Pedras et al., 2000). Camalexin is formed upon infection of Arabidopsis by a large variety of microorganisms, including bacteria, fungi, and oomycetes. Camalexin originates from tryptophan and its biosynthesis involves the cytochrome P450 enzymes CYP79B2 and CYP71B15 (PAD3; Glawischnig, 2007).

Well known examples of fungal PAMPs are chitin and ergosterol. Bacterial PAMPs include lipopolysaccarides (LPS), glycolipid components of outer membranes of Gram-negative bacteria, and flagellin, the major structural component of the bacterial flagellum which acts as a motility organ. LPS are perceived by a range of plant species as PAMPs, and it has been shown that the highly conserved lipid A part of LPS is sufficient to induce plant defense responses in Arabidopsis (Schwessinger and Zipfel, 2008). Moreover, LPS are vital for symbiotic signaling (Tellström et al., 2007) and play a role in induced plant defense responses including suppression of the hypersensitive response, expression of defense genes and induction of systemic acquired resistance (Desaki et al., 2006; Bittel and Robatzek, 2007; Mishina and Zeier, 2007b).

Flagellin is another extracellular PAMP which induces typical immune responses in various plant species as well as mammalian innate immunity (Zipfel and Felix, 2005; Underhill and Ozinsky, 2002). Flagellin contains a highly conserved N-terminal domain, and a peptide corresponding to a 22 amino acid long stretch of this domain, flg22, is sufficient for elicitor activity in several plants. In Arabidopsis, flg22 induces the formation of callose, accumulation of the defense protein **PR1**, and strong inhibition of seedling growth (Gomez-Gomez et al., 1999). Tomato is able to recognize a shorter version of the same epitope (flg15) and rice cannot recognize flg22 but can sense full-length flagellin (Takai et al., 2007; Wan et al., 2008). Notably, distinct plant families have developed recognition systems for additional microbial molecules. The bacterial cold shock protein (CSP) and the

translation elongation factor Tu (EF-Tu) are good examples. Although bacteria are rich in EF-Tu and CPS, the recognition of EF-Tu and CPS is restricted to the *Brassicacae* and *Solanaceae*, respectively (Zipfel and Felix, 2005). In addition to sense non-self molecules, plants and animals can also sense plant-derived molecules produced after infection, so-called danger-associated molecular patterns (DAMPs). For instance, plants are able to sense β-glucan oligomers, which are released from the plant cell wall by fungi and oomycetes (Fig. 2; Zipfel, 2009).

Most identified pattern recognition receptors are receptor-like kinases (**RLKs**) or receptor-like proteins (**RLPs**), except for the extracellular glucan-binding protein (GBP) that binds and hydrolyses heptaglucosides from *P. soja* (Schwessinger and Zipfel, 2008).

Flagellin sensing 2 (**FLS2**), a leucin-rich repeat receptor kinase (**LRR-RK**), is the PRR responsible for flagellin recognition in the model plant *Arabidopsis thaliana*. It is localized to the plasma membrane and was found to be internalized upon flg22 stimulation. Similarly, mammals use the Toll-like receptor **TLR5** to perceive bacterial flagellin (Boller and He, 2009). Functional Arabidopsis FLS2 orthologues have been recently identified in the *Solanaceae* plants *Nicotiana benthamiana* and tomato. Arabidopsis plants mutated in *FLS2* are hypersusceptible to infections with the pathogenic bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) after inoculation onto the leaf surface. This is not observed when bacteria are injected into the leaf tissue (Bittel and Robatzek, 2007). Also, *N.benthamiana* plants silenced for *NbFLS2* are more susceptible to a range of adapted and non-adapted bacteria (Hann and Rathjen, 2006). In Arabidopsis, pretreatment with flg22 restricts the growth of *Pst* DC3000, and *fls2* mutants are more susceptible to this bacterial pathogen (Zipfel et al., 2004).

In addition, a lack of flagellin recognition allows increased growth of the non-adapted bacteria *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *tabaci* (*Pta*; Li et al., 2005; de Torres et al., 2006). These data demonstrate the importance of flagellin perception in non-host resistance. Similarly, the LRR-RLK **EFR** (EF-TU RECEPTOR) which belongs to the LRR-RLK has been shown to be required for the perception of bacterial EF-Tu. Its extracellular domain consists of 21 LRR and interacts with the first 18 amino acids of the N-terminus of EF-Tu, the elf18 peptide (Altenbach and Robatzek, 2007). *Arabidopsis erf* mutants are susceptible to *Agrobacterium tumefaciens*

bacteria, explaining the essential role of EF-Tu perception in defense against bacteria. Recognition of this PAMP seems to be restricted to the *Brassicaceae* and is not found in other families, suggesting that EF-Tu perception is evolutionary young (Boller and He, 2009). However, both poplar and rice genomes contain numerous genes that encode LRR-RKs of very similar architecture, which suggests that they act as PRRs for as yet unidentified PAMPs (Fig. 2).

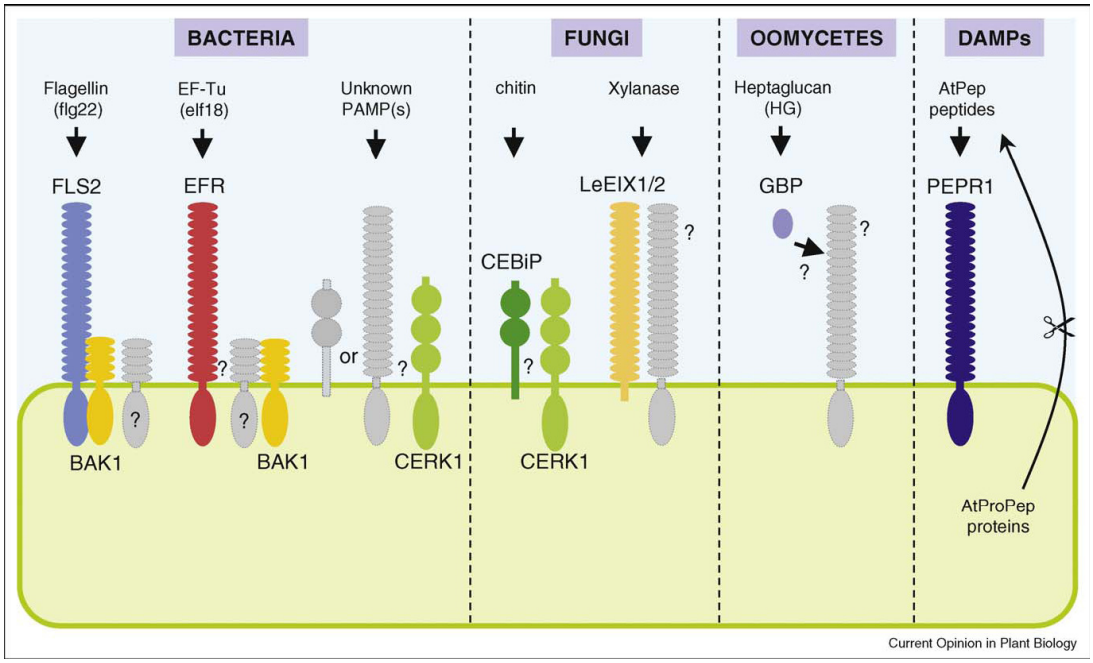

Figure 2. Plant PRRs and their signalling adapters (adapted from Zipfel, 2009).

To infect plants, pathogens need to attenuate PTI by directly targeting the PRRs and their associate protein by virulence effectors or downstream signaling components (Nicaise et al., 2009; phase 2, Fig. 1). Recently, Zhou and Chai (2008) have reviewed regulatory proteins in the PTI pathway that are directly targeted by TTSS effectors. For example, some studies demonstrate that AvrPto, AvrPtoB, AvrPto1 and AvrPtoB (HopAB2), which are unrelated effectors of Pst DC3000, directly interact with several receptor-like kinases, and/or PAMP receptors to interfere with their downstream signalling during infection. These receptors include the brassinolide-associated RLK BAK1 (*B*RI1 *a*ssociated *k*inase 1), the flagellin receptor FLS2, the Ef-Tu receptor EFR, and the chitin receptor CERK1 to block PAMP/MAMP-induced callose deposition and enhance bacterial virulence (Lewis et al., 2009; Zhou and Chai, 2008). AvrPto and AvrPtoB interact with

BAK1 in the split-ubiquitin yeast two-hybrid assay, by co-immunoprecipitation from protoplasts, and in *in vitro* pull-down assays (Gohre et al., 2008; Shan et al., 2008; Xiang et al., 2008). BAK1 contributes to innate immunity through its association with the flagellin receptor FLS2 *in vivo* (Chinchila et al., 2007; Heese et al., 2007; Kemmerling et al., 2007). AvrPto and AvrPtoB also inhibit PTI signalling through their direct interaction with the PAMP receptors FLS2, EFR and CERK1 (Gohre et al., 2008; Shan et al., 2008 ; Xiang et al., 2008; Gimenez-Ibanez et al., 2009).

HopAI1 is another example of a bacterial effector for which a specifc plant target is known. It belongs to a novel family of bacterial effectors highly conserved in animal pathogens such as *Salmonella ryphimurium*, *Shigella flexneri*, *Chromobacterium violaceum*, and the plant pathogenic bacterium *P. syringae*. HoPAI1 directly inactivates Arabidopsis MAP kinases by permanently dephosphorylating phosphothreonin of MAP kinases (MKP3 and MPK6) to block PAMP/MAMP-triggered responses (Zhang et al., 2007). Together, these discoveries illustrate an effective strategy employed by pathogens to suppress PTI by directly targeting PRRs and downstream signaling components.

1.2.2.2 Effector-triggered immunity

Plants have evolved resistance (R) proteins to directly or indirectly detect the effectors proteins. According to the gene-for-gene theory, these effector proteins were previously termed avirulence or Avr proteins. The gene-for-gene hypothesis was advanced by H.H. Flor, based on his work on the flax-rust fungus interaction in the 1940s and 1950s (Flor, 1971). This hypothesis states that when a pathogen has an *avr* gene, and a plant host has the corresponding *R* gene, the plant is resistant to the pathogen. When the plant is resistant, the pathogen is said to be avirulent and the interaction is said to be incompatible. By contrast, when the plant is susceptible, the pathogen is said to be virulent and the interaction is said to be compatible. The recognition of pathogen effectors initiates a final layer of the plant immune system, **effector-triggered immunity**.

In this case Avr proteins are recognized by nucleotide-binding site-leucine-rich repeat **(NBS-LRR**) R proteins that are structurally equivalent to animal ATERPILLER NOD NLR proteins (phase 3, Fig. 1). Plant NBS-LRR

proteins initiate a network of signaling pathways and induce a series of plant defense responses, such as an early and strong production of ROS (the so-called oxidative burst), calcium and ion fluxes, mitogen-associated protein kinase cascades, expression of pathogenesis-related genes, and the HR. Moreover, several small signaling molecules in the plant defense response, such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), are produced upon Avr protein recognition (Lamb and Dixon, 1997; Dong, 1998).

PATHOGENESIS-RELATED (PR) proteins (Tab. 2) are accumulating in the intercellular spaces as well as in vacuoles of various plant cells during interaction with pathogenic microorganisms (Neuhaus et al., 1991). In some cases, they can also be induced by abiotic stress, such as drought, salinity, wounding or heavy metals (Lawton and Lamb, 1987; Stintzi et al, 1993). A role of PR proteins in limiting pathogen activity, growth, and spread fits with the identification of the PR-2 family as β-1,3-endoglucanases and the PR-3, PR-4, PR-8, and PR-11 families as endochitinases, which potentially act against cell walls of fungi and bacteria. Notably, the prominent PR-1 proteins are often used as markers of the enhanced defensive state conferred by pathogen-induced systemic acquired resistance (SAR), but their biological activity has remained elusive (Van Loon et al., 2006).

Family Type	member	Properties	Arabidopsis gene (AT Number)
PR-1	tobacco PR-1a	Antifungal	AT2G14610
PR-2	tobacco PR-2	class I, II, and III endo-beta-1,3-glucanases, 25-35kD	AT3G57260
PR-3	tobacco P, Q	class I, II, IV, V, VI, and VII endochitinases, about 30kD	AT3G12500
PR-4	tobacco R	antifungal, *win*-like proteins, endochitinase activity, similar to prohevein C-terminal domain, 13-19kD	AT3G04720
PR-5	tobacco S	antifungal, thaumatin-like proteins, osmotins, zeamatins, permeatins, similar to alpha-amylase/trypsin inhibitors	AT1G75040
PR-6	tomato inhibitor I	protease inhibitors, 6-13kD	
PR-7	tomato P69	endoproteases	
PR-8	cucumber chitinase	class III chitinases, chitinase/lysozyme	
PR-9	lignin-forming peroxidase	peroxidases, peroxidase-like proteins	
PR-10	parsley PR-1	ribonucleases, Bet v 1-related proteins	
PR-11	tobacco class V chitinase	endochitinase activity	
PR-12	radish Ps-AFP3	plant defensins	AT1G19610
PR-13	*Arabidopsis* THI2.1	Thionins	AT1G12660
PR-14	barley LTP4	nonspecific lipid transfer proteins (ns-LTPs)	
PR-15	barley OxOa (germin)	oxalate oxidase	
PR-16	barley OxOLP	oxalate-oxidase-like proteins	
PR-17	tobacco PRp27	Unknown	

Table 2. Recommended Classification of Pathogenesis-Related Proteins (PRs) (Adapted from Sels et al., 2008)

Direct recognition

In some cases, a **direct interaction** between pathogen effectors and plant resistance proteins has been demonstrated. A direct physical association of the pathogen effector with the R immune receptor is the simplest form of direct recognition which is similar to a ligand binding to its receptor (Caplan et al., 2008). This model was first shown for the rice CC-NB-LRR Pi-ta that confers resistance to *M. grisea* Avr-Pita effector (Jia et al., 2000). It has been observed that both in yeast-two-hybrid (Y2H) and *in vitro* binding assays Avr-Pita specifically bind to the LRR domain of Pi-ta. Mutations in the LRR domain of the forms of Pi-ta occurring naturally eliminate both interaction in the Y2H assay and resistance in susceptible plants. Other examples of direct recognition which studied comprehensively are the flax TIR-NB-LRR L and M proteins that confer resistance to *Melampsora lini* fungal strains secreting AvrL567 and AvrM proteins. Both effectors are translocated during infection into host cells (Catanzariti et al., 2006; Dodds et al., 2006). Direct physical interactions, involving AvrL567/L and AvrM/M complexes have been found via Y2H assays (Dodds et al., 2006). The L/AvrL567 interaction comprises a series of different variation of R and Avr alleles, and notably, the specificity of the protein interactions which could be observed in yeast equals to the resistance responses induced *in planta*. Similarly, Arabidopsis TIR-NB-LRR RRS1-R was shown to physically interact with the *Ralstonia solanacearum* PopP2 effector (Deslandes et al., 2003).

Indirect recognition

Guarding is a strategy in which the virulence-promoting activity of effectors is detected, rather than the effector itself (Lewis et al., 2009). The **indirect recognition** mechanism proposed by the so-called Guard Model therefore supports the ability of a limited number of NB-LRR R proteins to recognize a multitude of pathogen effectors, by focussing on the more limited number of potential host protein targets and their modifications.

The Guard model was initially proposed to explain the role of Prf in AvrPto–Pto signaling. In this model, Pto is considered to be the virulence target of AvrPto, which is guarded by the 'real' R protein, Prf (Van der Hoorn et al., 2002). Another classical example that conforms to the Guard Hypothesis includes the Arabidopsis RIN4, RPM1 and RPS2 proteins. The guardee RIN4 (a negative regulator of plant defence) is targeted by three structurally unrelated *P. syringae* Avr proteins, AvrRpm1, AvrB and AvrRpt2, and

guarded by two CC-NB-LRR proteins, RPM1 and RPS2. RIN4 is either phosphorylated or cleaved upon interaction with AvrRpm1, AvrB or AvrRpt2, or this modification of RIN4 results in disruption of its inhibitory action on RPM1 or RPS2 and a subsequent activation of defense responses (Lewis et al., 2009; He and Sheen, 2007). Another Arabidopsis CC-NB-LRR protein, RPS5, recognizes the *P. syringae* effector protein AvrPphB by indirectly ‘‘sensing’’ its enzymatic activity (Shao et al., 2003). The RPS5 protein maintains extensive intramolecular and interdomain associations that assist PRS5 in a functionally inactive state (Ade et al., 2007). The current model for RPS5 activation suggests that, during infection, the bacterial AvrPphB effector specifically cleaves PBS1, leading to significant conformational changes in the associated RPS5. The NB domain is thereby relieved from LRR repression, and it is speculated that exchange of ADP for ATP at the NB domain results in an activated, ATP-bound form of RPS5 (Caplan et al., 2008). It is important to recognize that direct and guard type recognition most likely characterize the two ends of a spectrum (Rafiqi et al., 2009)

1.3 Defense signaling

The entrance of pathogens to the plant apoplastic space is generally associated with pathogen recognition events which subsequently trigger induction of post-invasion defense responses. There are at least three independent pathways leading to the transcriptional reprogramming associated with defense activation. Two of these pathways are defined by mutations either in the *EDS1*/*PAD4* (enhanced disease susceptibility) gene or the *NDR1* (non-race specific disease resistance) and *PBS2* gene. *EDS1* and *PAD4* affect the same spectrum of resistance genes, and both have homology to catalytic lipases. Recent studies showed that mutation in both genes can cause a defect in SA accumulation and expression of both genes can be induced by SA or pathogen infection. However, *NDR1* encodes a probable glycosylphosphatidylinositol (GIP)-anchored protein (Broocks, 2001; Nimchuk et al., 2003). The recent analysis shows that the downstream responses of different R-gene mediated signaling pathway in some cases need RAR1 and SGT1 (Austin et al., 2002; Fig. 3).

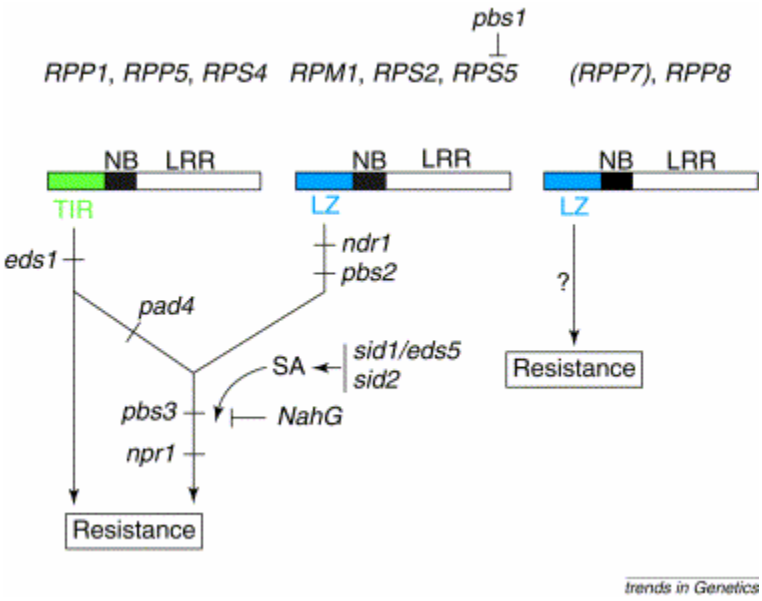

Figure 3. Dissection of *R* gene-mediated signaling pathways in *Arabidopsis* (Adapted from Feys and Parker, 2000).

The signal transduction network initiates after pathogen recognition involves two important components, SA-dependent and JA/ET-dependent signaling.

1.3.1 SA-dependent signaling

SA, a small phenolic compound, is a key signaling molecule for plant disease resistance. Its accumulation is associated with many immune responses in plants, such as systemic acquired resistance, basal resistance, gene-for-gene resistance, and even non-host resistance (Dangl and Jones, 2001; Mishina and Zeier, 2007a). SA triggers the induction of a number of genes (Maleck et al., 2000). The best characterized SA-inducible genes encode proteins with antimicrobial activity which are known as **PR**s (Van Loon and Van Strien, 1999; Tab. 2), and detoxifying or antioxidant enzymes such as glutathione S-transferases (GSTs) and glycosyltransferases (GTs) (Edwards et al., 2000; Li et al., 2001). Also, formation of HR is accompanied by the accumulation of a high level of SA to induce the production of defense proteins (Greenberg, 1997; Heath, 2000). Most of the SA produced *in planta* is converted into its two inactive forms: 2-SA-O-β-glucoside (SAG) and 2-methyl salicylate (MeSA) (Lee et al., 1995; Schuurik et al., 2006).

Two pathways of SA biosynthesis have been proposed that both require the primary metabolite chorismate (Wildermuth, 2006). Chorismate can be converted to SA via either phenyalanine or isochorismate. Biochemical studies using isotope feeding have suggested that plants synthesize SA from cinnamate produced by the activity of phenylalanine ammonia lyase (PAL). Silencing of PAL genes in tobacco or chemical inhibition of PAL activity in Arabidopsis, cucumber and potato reduces pathogen-induced SA accumulation. Genetic studies, on the other hand, indicate that the bulk of SA is produced from isochorismate. In bacteria, SA is synthesized from chorismate through two reactions catalyzed by isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL). Arabidopsis contains two ICS genes but has no gene encoding proteins similar to the bacterial IPL (Chen et al., 2009; Métraux, 2002). Expression of the Arabidopsis *SALICYLIC-ACID-INDUCTION DEFICIENT2* (*SID2*) gene, which encodes a chloroplast-localized ICS, is activated in tissues that are challenged by pathogen and in tissues exhibiting SAR (Shah, 2003). Application of SA complements the *sid2* defect, confirming the involment of *SID2* in SA synthesis.

PAD4 acts upstream of SA to promote SA accumulation and encodes a protein which is similar to the putative triacyl glycerol lipase EDS1. Although PAD4 and EDS1 have lipase motifs, it is not clear yet if they indeed act as lipases (Zhou et al., 1998; Falk et al., 1999; Jirag et al., 1999). Because lipases are hydrolytic enzymes that breakdown triacyglycerol into fatty acids any glycerols, it is predicted that *PAD4* may be involved in production of another defense molecule as well.

Different studies showed that mutation in *EDS1* or *PAD4* strongly reduce SA accumulation, suggesting that they act upstream of SA. This action is regulated by different EDS1 complexes, including nucleo-cytoplasmic EDS1-PAD4 hetrodimers and nuclear interactions between EDS1 and PAD4-related SENESENSE-ASSOCIATED GENE 101 (SAG101) protein (Feys et al., 2005). Additionally, Wildermuth et al. (2001) provided clear evidence that the main route of defense-induced SA production in Arabidopsis involves chloroplast–localized isochorismate synthase 1 (ICS1) encoded by *ICS1/EDS1/SID2*. Accumulation of SA is significantly reduced in *sid2* plants. However, there is some SA present in *sid2* mutants, which might suggest the involvement of the phenylalanine biosynthesis pathway. Alternatively, the low constitutive levels of SA in *sid2/ics1* might be derived from ICS2, the second

isochorismate synthase protein in Arabidopsis. The *ICS2* gene is not upregulated upon pathogen attack (Wildermuth et al., 2001). The order of events in SA signaling is complicated because the pathway includes several feedback loops.

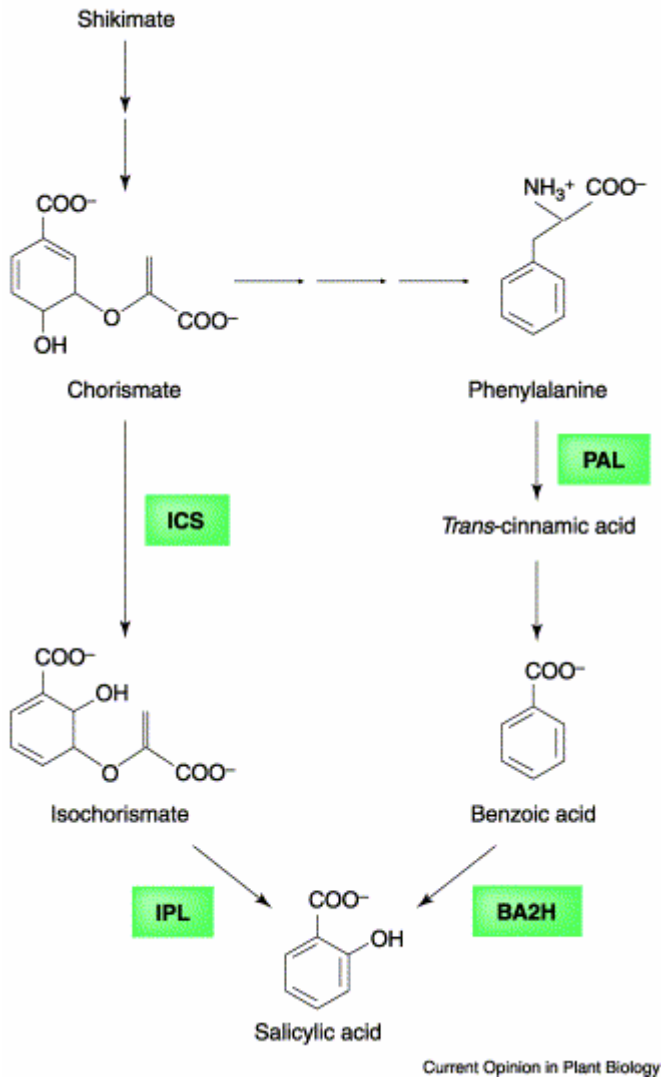

Figure 4. Proposed pathways for the biosynthesis of SA in plants (adapted from Shah, 2003).

Another protein with a key regulatory role in SA signaling is NON-EXPRESSOR OF PR1 (NPR1), also known as NIM1. *NPR1* acts downstream from SA which activates the expression of *PR-1* and plays a crucial role in systemic acquired resistance. NPR1 is normally present in the cytoplasm when the level of SA is low. When the SA level increases,

disulfide-connected NPR1 oligomers are reduced and separate into monomers. The monomers then have access to the nucleus where they interact with TGA-type transcription factors (Mou and Dong, 2003). TGA2, 5, and 6 are required for the activation of *PR-1* expression by SA (Zhang et al., 2003). Furthermore, some other evidences suggest that WRKY transcription factors are involved in SA-dependent defense responses, downstream of or associated with NPR1 (Loake and Grant, 2007).

1.3.2 JA/ET-dependent signaling

JA-dependent signaling proceeds through increased JA levels in response to pathogen attack. The JA pathway provides effective defense against attack of necrotrophic pathogens and (insect) herbivory.

Jasmonates including JA and its derivatives are oxygenated fatty acids or oxylipins, and are produced by the octadecanoid pathway. Linolenic acid is oxygenated by lipoxigenase (LOX) to form 13(S)-hydroxy linolenic acid (13-HPOT), which is then converted to 12-oxo-phytodienoic acid (OPDA) by allene oxide synthase (AOS) and allene oxide cyclase (AOC). JA is synthesized from OPDA with one step of reduction by OPR3 (OPDA-REDUCTASE 3) and three steps of β-oxidation (Berger, 2002; Cheong and Choi, 2007; Browse, 2009).

Downstream signaling components of the JA pathway include COI1, MYC2 and JAR1. Most if not all known activities of JA-defense signaling processes in Arabidopsis require the function of COI 1, which is an F-box protein that determines the target specificity of the E3 ubiquitin ligase SCF^{COI1} (where SCF indicates Skp/Cullin/F-box) (Feys et al., 1994; Li et al., 2004 and Glazebrook, 2005). A similar role in several species including tomato (*LeCOI1*), tobacco, and soybean for COI1 homologs has been reported. Interestingly, mutations in *LeCOI1* show that, at least in tomato, JA is involved in developmental processes, such as ovule development. Ovule development is not impaired in *coi1* mutants suggesting that other COI1-like proteins might regulate these processes in Arabidopsis (Li et al., 2004; Wang et al., 2005). It has been reported that RPD3b, a histone deacetylase, is a COI1 target (Devoto et al., 2002). Histon deacylation reduces the accessibility of chromatin to the transcription machinery (Lusser et al., 2001). Another putative target of COI1 is COS1. *COS1* encodes lumazine synthase, a key component of the riboflavin pathway.

The most well-described transcription factor in jasmonate signaling is the bHLHzip-type MYC2, which was identified in two independent genetic screens from the mutant *jin1* (Berger et al., 1996; Lorenzo et al., 2006). MYC2 regulates two branches of the JA pathway (Lorenzo et al., 2004; Boter et al., 2004; Dombrecht et al., 2007). It negatively regulates tryptophan metabolism and defense responses against necrotophic pathogens, but positively regulates other JA-dependent events such as anthocyanin and flavonoid biosynthesis, and inhibition of root growth. On the other hand, the ERF1 (Ethylene Response-Factor 1) transcription factor regulates JA and ET signaling in an opposite manner to MYC2. Therefore, there are two subpathways of JA-induced defense signaling. The first group of wound-responsive genes is up-regulated through the MYC2 transcription factor and this causes protection against herbivore attack. For activation of the second group, JA and ET act synergistically through ERF1 to activate defense against necrotrophic pathogens.

JA additionally regulates important physiological and developmental processes irrespective of defense, such as pollen viability. For example, the *opr3* mutant is defective in the gene encoding the OPDA reductase and deficient in JA biosynthesis, which results in male sterility (Stintzi and Browse, 2000; Berger, 2002). Furthermore, it has been shown that JA is involved in senescence-promotion, flower and fruit development, responses to wounding, root growth, and in limiting damage from abiotic agents (Farmer et al., 2003; Wasternack, 2007; Pauwels et al., 2008).

Ethylene (ET) production is regulated by development signals, and in response to biotic and abiotic stimuli (Wang et al., 2002). Components of the ET-signaling pathway include the nuclear-localized transcription factor EIN3, which activates ethylene response factor1 (ERF1). It binds to GCC box promoter elements to activate defense genes, such as *PDF1.2* and chitinase B (*CHI-B).* Moreover, its expression can be induced by ET or JA (Chao et al., 1997; Solano et al., 1998). Microarray analysis of plants over-expressing EFR1 has shown that ERF1 regulates the expression of both ET- and JA-responsive genes, indicating that EFR1 likely function downstream of the intersection between the ET- and JA-signaling pathways (Lorenzo et al., 2003).

In conclusion, it seems that JA-dependent responses are associated with large-scale reprogramming of gene expression such as *PDF1.2.* Some JA-

regulated genes are also regulated by ET. In the case of *PDF1.2,* both ET and JA are required to induce its expression. In Arabidopsis*, jar1* (*jasmonate resistance1*; allelic to *jin4)* mutant plants are defective in response to JA and have reduced JA-dependent gene expression (Staswick et al., 1992; Berger et al., 1996). In Arabidopsis, jasmonoyl-isoleucine (JA-Ile) is synthesized by an enzyme encoded by the *JAR1* gene (Staswick et al., 2002; Suza and Staswick, 2008).

1.3.3 Systemic acquired resistance

Systemic acquired resistance (SAR) is a state of heightened defense that provides long-lasting and broad spectrum resistance to microbial pathogens at the whole plant level. In many aspects, SAR resembles the immune response in animals. It was firstly reported by Ross (1961) that tobacco becomes resistant to infection after the HR triggered by an avirulent strain of tobacco mosaic virus. Later on, it has been reported that SAR is induced after an HR to other viruses, bacteria and fungi. However, Mishina and Zeier (2007b) demonstrated that induction of SAR is not limited to HR-inducing or necrozing pathogen but also occurs upon leaf contact with non-pathogenic microbes or after local treatment with bacterial PAMPs, such as flagellin or lipopolysaccharides. Apart from the initial stimuli which can establish SAR, signal transduction mechanisms underlying SAR is still being studied. Moreover, selected chemicals, including SA, 2,6-dichloroisonicotinic acid (INA) and benzo-(1,2,3)-thiodiazole-7-carbothionic acid S-methyl ester (BTH) can also induce resistance, either by activating the signalling pathway leading to SAR, or by functioning as intermediates in this same pathway (Jabs, 1999; Van Loon, 2000).

One essential component of SAR is the phenolic molecule salicylic acid (SA) which triggers expression of a subset of pathogenesis-related (*PR*) genes. Because of their antimicrobial activity, PR proteins are thought to directly contribute to pathogen resistance and SAR (Cao et al., 1994). Furthermore, it was initially assumed that SA has a role as a long distance signal associated with SAR. The necessity of SA signaling during SAR becomes obvious by the failure of the *Arabidopsis salicylic acid induction-deficient1* (*sid1*) and *sid2* mutants, which both are not capable to induce SA production locally and systemically, to establish SAR (Durrant and Dong , 2004).

The results of grafting experiments in tobacco plants expressing the bacterial *nahG* gene encoding salicylate hydroxylase, which degrades SA to catechol, showed that SA itself cannot be the mobile resistance inducing signal. Although these experiments suggest that accumulation of SA in distant leaves is critical for SAR establishment, they indicate that developing of SAR is not always correlated with elevated SA accumulation in the inoculated leaf (Vernooji et al., 1994). Therefore, other signal(s) should be involved in SAR induction and long-distance signalling. According to the hypothesis of Jung et al. (2009), any suitable long-distance signal should show elevated levels in petiole exudates of tissue treated with a SAR-inducing pathogen, be mobile in plants, and act in a manner that depends on SA.

SA derivatives such SAG and MeSA have therefore been suggested as candidates for SAR long-distance signals. SA can be modified to these two biologically inactive derivatives which result from glucosylation and methylation, respectively (Lee et al., 1995). SA-glucosyltransferase transfers a glucose moiety to either the phenolic hydroxyl group or to the carboxyl group of SA to yield SA 2-O-$\tilde{\beta}$-D glucose (SA glucoside [SAG] or SA glucose ester [SGE]). Two enzymes are involved in this process. The first, AtSAGT1 (designated UGT74F2 by Lim et al., 2002) forms SAG and SGE, whereas the second, AtSAGT2 (UGT74F1), only forms SGE (Lee and Raskin, 1999; Lim et al., 2002; Dean and Delaney, 2008). SAG is actively transported from the cytosol into the vacuole, and stored as an inactive from. The glucosylation of SA occurs primarily in the vicinity of the HR lesion, and a possible function of glucosylation of SA might be detoxification of SA and regulation of its level (Lee and Raskin, 1998). Although the physiological roles of such conjugates have not been fully elucidated, a role has been proposed for SAG in the establishment of SAR. Furthermore, since recently, there is a lively debate on the role of MeSA as a long distance signal for SAR (Park et al., 2007; Attaran et al., 2009).

Methyl salicylate (MeSA), a volatile ester, and its glucosylated derivate MeSAG are biologically inactive forms of SA. MeSA is normally absent in leaf tissue but it is produced locally in significant amounts after pathogen attack (Shulaev et al., 1997; Koo et al., 2007; Attaran et al., 2008; 2009). SA carboxyl methyltransferases (SAMTs), accountable for the formation of MeSA from SA, have been identified in several plants (Negre et al., 2002; Chen et al., 2003). In Arabidopsis, the *BSMT1* gene encodes a protein with both benzoic acid and SA methylation activities (Chen et al., 2003). *BSMT1*

is highly expressed in flowers and induced by treatment with alamethicin, a channel-forming peptide that mimics the effect of pathogen attack, by methyl jasmonate, and by herbivory. MeSA is also a constitutive component of floral scents from various plants, attracting pollinators or predators that capture herbivorous insects (Knudsen et al., 1993; Van Poecke et al., 2001). Additionally, it was suggested to act as an airborne signal that activates pre-immune disease resistance in the healthy tissues of the infected plants and in neighboring plants (Shulaev et al., 1997; Baldwin et al., 2006). Recently, Park et al. (2007) proposed a model for SAR signaling in tobacco, in which MeSA would act as a phloem-mobile SAR long-distance signal. In this model, the SA accumulating after tobacco mosaic virus (TMV) infection is converted to MeSA in inoculated tobacco leaves. Subsequently, MeSA can travel through the phloem from primary infected leaves to the distant tissue. By the esterase activity of SA-binding protein SAPB2 in systemic tissue, the methyl ester bond in MeSA can be cleaved to yield SA, and the concomittant rise in SA levels in the secondary leaves then would cause SAR (Forouhar et al., 2005; Park et al., 2007) [Fig. 5].

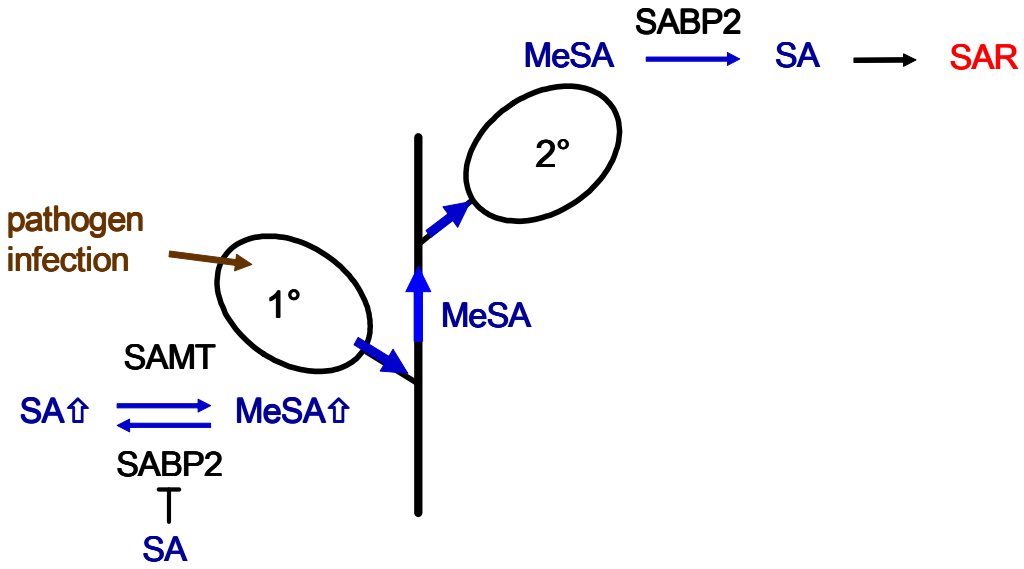

Figure 5. Proposed role of MeSA in establishment of SAR (according to Park et al., 2007; figure provided by J. Zeier)

Moreover, it has been reported that in Arabidopsis, 18 potentially functional methyl esterase genes exist, out of which five encode proteins with MeSA demethylase activity (Yang et al., 2008, Vlot et al., 2008b). Usage of T-DNA knock out and RNA interference technology resulted in generating of

transgenic plants partially lacking SA methylesterase expression. The failure of some of these lines to establish SAR was taken as an indication that MeSA was a universal mobile SAR signal in plants (Vlot et al., 2008a, 2008b).

Additionally, several studies implicate components biochemically different from SA might act as SAR long-distance signals. For instance, mutational analysis in Arabidopsis demonstrated that peptide and lipid derivatives take part in signal transduction from primary leaves to the distal tissue (Grant and Lamb, 2006; Chaturvedi et al., 2008). A mutation in *DEFECTIVE IN INDUCED RESISTANCE1* (*DIR1*) in Arabidopsis is not capable of generating or transmitting the SAR signal, but does not affect resistance in locally inoculated leaves. This acidic, apoplastically located protein which might act as a chaperon for a lipid signal is a member of the family of lipid transfer proteins and was detected in vascular fluids. Further, indication for a lipid-based signal molecule comes from the characterization of the *eds1* and *pad4* mutants, which are both defective in lipase-like proteins. The *eds1* (*enhanced disease susceptibility 1*) mutant was originally identified for its compromised local resistance to *Hyaloperonospora arabidopsidis* mediated by several resistance (*R*) genes, whereas *pad4* (*phytoalexin deficient 4*) was isolated in a screen for mutants with enhanced susceptibility to a virulent strain of *P. syringae* pv. *maculicola*. In *eds1* and *pad4* plants, even when a normal HR is elicited by pathogens that trigger the EDS1-independent pathway, SAR cannot be induced. Phloem exudates experiments indicate that EDS1 is required for both production of the mobile signal in the local tissue and perception of the signal in the systemic tissue (Durrant and Dong, 2004). Mutation of another gene, *SFD1*, which encodes a dihydroxyacetone phosphate reductase involved in glycerolipid synthesis, also compromises SAR and decreases SA accumulation and *PR-1* expression in systemic tissue after infection with an avirulent strain of *P. syringae* (Nandi et al., 2004). Recently, another lipid transferase family protein, AZI1 (AZELAIC ACID INDUCED 1) was proposed to modulate production and/or translocation of a mobile signal(s) during SAR (Jung et al., 2009). Jung and co-workers (2009) proposed that azealic acid could be a mobil signal to mount SAR by inducing AZI1 and by priming plant cells to establish faster or stronger defense responses. According to their experiments they proposed that AZI1 might either function downstream of the SFD1-dependent SAR signal or independent of it. Although many important questions still need to

be addressed, these data strongly suggest a role for lipid signaling in SAR. Furthermore, the oxylipin-derived defense hormone JA or a derivative thereof has been proposed as another potential lipid-derived SAR signal by Truman et al. (2007) in Arabidopsis.

The onset of SAR in Arabidopsis is controlled by *NPR1* also known as *NON-INDUCIBLE IMMUNITY1* (*NIM1*). Although *npr1* mutants are able to accumulate SA in pathogen-inoculated leaves, systemic leaves fail to elevate the levels of SA (Pieterse and Van Loon, 2004). Moreover, different studies indicate that in *npr1* mutant plants, the induction of *PR-1* is attenuated at the local and systemic level, suggesting that the NPR1 protein is a positive regulator of SAR required for transduction of the SA signal to activate downstream PR gene expression (Kinkema et al., 2000; Mou et al., 2003).

Mishina and Zeier (2006) introduced *FLAVIN-DEPENDENT MONOOXYGENASE1* (*FMO1)* as a new critical component of SAR in Arabidopsis. They have shown that the SAR response triggered by *Psm* or *Psm avrRpm1* is completely abolished in *fmo1* mutant plants. This is associated with a failure to accumulate SA and to express defense genes in distant leaves. In contrast to systemic responses, the *fmo1* mutation does not critically affect defense responses induced by *Psm avrRpm1* at the site of pathogen attack. At inoculation sites, *FMO1* expression is independent of SA accumulation and signaling through NPR1 and NDR1, but depends on the EDS1/PAD4 defense pathway. They also proposed the existence of an amplification loop operating in leaves distant from pathogen attack. According to this model, FMO1, ROS, salicylic acid and the defense regulators NPR1 and NDR1 cooperatively act in amplifying incoming signals in order to realize defense responses at the systemic level and SAR (Mishina and Zeier, 2006). Further studied showed that phytochrome regulation of SAR proceeds via the SAR regulator FMO1 (Griebel and Zeier, 2008).

1.3.4 Induced systemic resistance (ISR)

Similar to SAR, induced systemic resistance (ISR) is a form of systemic resistance that protects against a variety of fungal and bacterial pathogens. In contrast to SAR, ISR is induced in roots by plant growth promoting rhizobacterial strains and is independent of SA signaling. It has been identified in different plant species, such as bean, carnation, cucumber, radish, tobacco, tomato and in Arabidopsis (Van Loon et al. 1998; Pieterse et

al., 1998). ISR generally requires JA and ET signaling and, like SAR, is regulated by NPR1 (Grant and Lamb, 2007). A well-studied example is *Pseudomonas fluorescens* WCS417r-mediated ISR in Arabidopsis. *P. fluorescens* WCS417r applied on roots protects leaves from *Pst* and *Fusarium oxysporum f. sp. raphani* infection.

1.4 Volatile organic compounds (VOCs)

As a part of their interaction with other organisms, plants release a large variety of volatile organic compounds (VOCs) into the environment. The VOCs can be produced by plants as a consequence of insect herbivory or plant-microbe interactions. Subsequently, they are emitted into the atmosphere due to their volatility (Penuelas and Llusia 2003; 2004). A large number of reports exists about the biological function of VOCs, including indirect plant defense against insects, pollinator attraction, plant–plant communication, pathogen defense, removal of ROS, and other environmental stress adaptations (Kessler and Baldwin, 2001; Pichersky and Gershenzon, 2002; Dicke et al., 2003; Rasmann et al., 2005).

According to their structure and origin, VOCs are classified into three main groups: **phenylpropanoids** (e.g. MeSA), oxygenated fatty acid derivatives generated via oxylipin branch pathways (**green leaf volatiles; GLVs**), and **terpenoids** (Knudsen et al., 1993; Dudareva and Pichersky, 2000; Pichersky et al., 2006; Chehab et al., 2008). Generally, induced production of terpenoids follows the expression of biosynthetic genes such as terpene synthase (TPS) genes, so that the *de novo* production of induced volatile terpenoids takes at least a few hours. On the contrary, initiation of GLV emission is much faster (within seconds) from leaf and stem tissues after damage by the catalytic activity of hydroperoxide lyase (HPL) (Matusi, 2006; Choudhary et al., 2008; Arimura et al., 2009).

There are many reports demonstrating that emission of GLVs such as (*Z*)-3-hexenal, (*E*)-2-hexenal, and *n*-hexanal from damaged leaf tissue have several layers of activities, ranging from airborn signal activities to the possible involvement in induction of systemic plant responses (Mithöfer et al., 2005 ; Arimura et al., 2009). For instance, Paré et al. (2005) showed that emission of GLVs from leaf tissue triggers responses in neighboring plants. It is also reported that GLV possess antibacterial activities against both gram-negative and gram-positive bacteria (Matusi et al., 2006; Paré et al., 2006).

Moreover, it was shown that GLV has fungicidal activity, and their hydrophobic properties are essential for this activity (Kubo et al., 2003). In this context, Shiojiri et al. (2006) found that overexpression of HPL in Arabidopsis resulted in higher resistance against a necrotrophic fungal pathogen, *Botrytis cinerea*. Similarly, reduced emission of GLV caused by suppression of HPL results a higher susceptibility against the pathogen (Shiojiri et al., 2006b). The resistance enhancing effect of GLVs might be caused by a direct antimicrobial effect or by a signaling function of the C6-aldehydes leading to enhanced defense responses in Arabidopsis.

There is some evidence that GLVs are formed during the HR of beans upon *Pseudomonas* inoculation, and the amounts of GLVs formed are adequate to be toxic to the pathogenic bacteria (Noordermeer et al., 2001). Elevated expression of GLV biosynthesis genes accompanies ISR-like systemic resistance to *Colletotrichum graminicola* in maize plants induced by root colonizing *Trichoderma virens*. This might indicate the involvement of GLVs in priming of ISR responses after pathogen challenge (Djnovic et al., 2007; Shah, 2009).

Terpenoids (isoprenoids) constitute the largest group of VOCs and can be found in almost all plant species. They possess a broad range of functional roles in plants (Aubourg et al., 2002). Some terpenoids, i.e. gibberellins, are essential for plant growth, development and general metabolism (Croteau et al., 2000). Additionally, a large number of structurally diverse plant terpenoids are known or assumed to have specialized functions associated with interactions of sessile plants with other organisms in context with reproduction, defense or symbiosis (Gershenzon and Dudareva, 2007). The chemical diversity of plant terpenoids is probably a reflection of their many biological activities in nature. Terpenoids are synthesized from dimethylallyl diphosphate (DMADP) and isopentenyl diphophate (IDP) and are thus composed of a common five-carbon building block [isoprene unit] (Cane, 1999). There are two biosynthetic pathways for terpenoids: the mevalonate (MVA) pathway in the cytoplasm and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in plastids (Arimura et al., 2009; Fig. 6). The smallest plant terpenoids are the hemiterpenoids (C5). They can be formed directly from DMADP by TPS activity. Furthermore, assembly of two, three or four C_5 units by prenyl transferases (PT) yields geranyl diphoshate (GDP: C_{10}), farnesyl diphosphate (FDP; C_{15}) and geranylgeranyl diphoshate (GGDP, C_{20}) (Takahashi and Koyama, 2006). GDP, FDP and GGDP are the

substrates for TPS enzymes, and serve as the immediate precursors for the diverse group of all mono- (C_{10}), sesqui- (C_{15}) and diterpenes (C_{20}) (Bohlmann et al., 1998; Wise and Croteau, 1999; Christianson, 2006; Tholl, 2006; Bohlmann and Keeling, 2009). TPSs are often multiproduct enzymes, and thus even a single TPS can contribute significantly to the blend of terpenoids produced in response to herbivory (Köllner et al., 2004; Keeling and Bohlmann, 2006; Arimura et al., 2008a). These metabolites can be further functionalized by various cytochrome P450-dependent mono-oxygenases (P450), reductases, dehydrogenases or various classes of transferases (Bohlmann and Keeling, 2009).

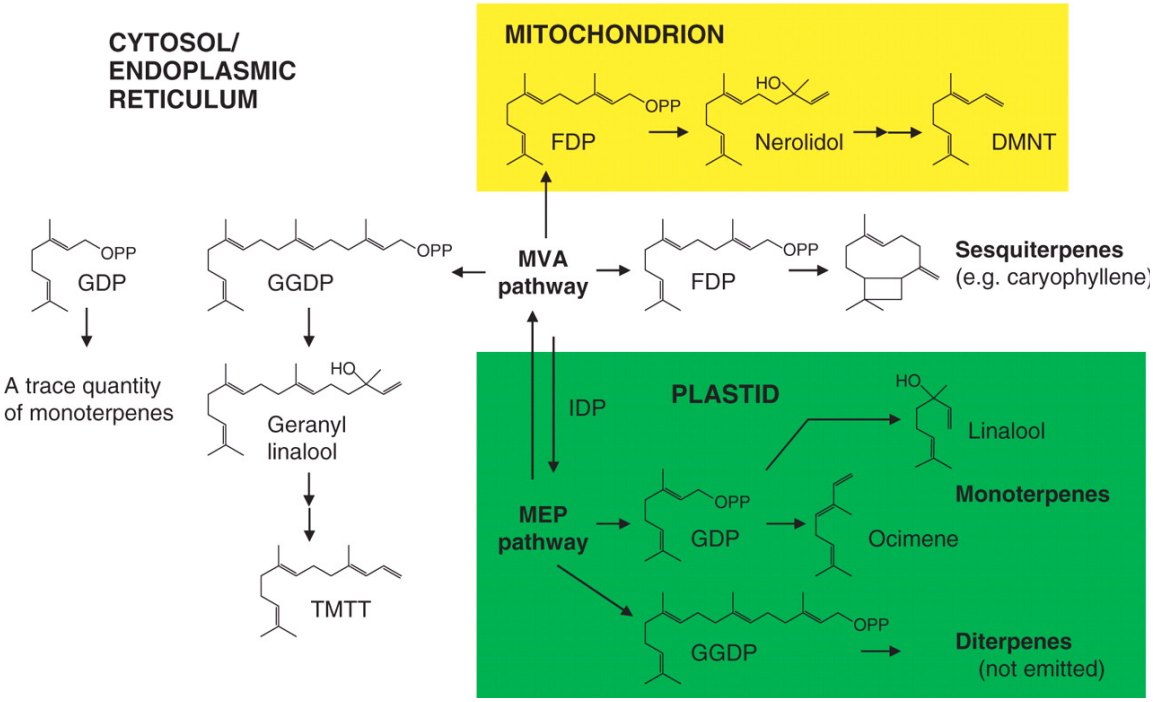

Figure 6. Schematic representation of the terpenoid biosynthetic pathway in plants (adapted from Arimura et al., 2009).

Terpenoid biosynthesis in plants can be spatially and temporally regulated during development and in response to biotic and abiotic factors, such as insect or pathogen damage, light intensity, temperature, humidity and nutrient availability (Van Poecke et al., 2001). Mono- and sesquiterpenoids are often emitted from specific floral tissues at particular times or developmental stages to attract pollinators (Dudevara et al., 2003). For

example, the monoterpene (*E*)-β-ocimene is a component of many floral scents (Knudsen et al., 1993) and represents one of the most common volatiles whose release is induced by herbivory (Paré and Tumlinson, 1999). Recently, it was reported that (*E*)-β-ocimene is an airborn signal inducing defense related genes in leaves of lima bean (Arimura et al., 2000a; 2000b; 2002). Additionally, apart from the monoterpene β-ocimene, the blend of VOCs from vegetative parts of Arabidopsis emitted in response to herbivores, comprises MeSA and the homoterpene TMTT (Tholl et al., 2009)

The C_{16}-homoterpene TMTT is a diterpene-derived volatile produced by numerous plants, including maize (*Zea mays*), lima bean (*Phaseolus lunatus*), and tomato (*Solanum lycopersicum*) in response to herbivory (Hopke et al., 1994; Ament et al., 2004; Williams et al., 2005). TMTT induces the expression of defense genes in lima bean, indicating that it also might play a role in plant–plant interactions (Arimura et al., 2000). Additionally, its release from O_3-damaged plants has been reported (Vuorinen et al., 2004). Although the role of inducible terpenoid volatiles in direct or indirect plant defense against herbivores is thoroughly studied, little is known about the role of terpenoids in defense against pathogenic microbes.

Kishimoto et al. (2006) reported that the monoterpene allo-ocimene induces resistance responses and primes defense reactions against the fungal pathogen *Botrytis cinerea*. Moreover, it was shown that VOCs including the monoterpenes (E)-β-ocimene and linalool, and the sesquiterpenes caryophyllene, β-elemene and α-farnesene are emitted from tobacco leaves as a response to *Pseudomonas syringae* infection (Huang et al., 2003). These studied raise this assumption that pathogen-induced terpenoids might function as typical phytoalexins, because many of those compounds possess direct antimicrobial properties *in vitro* (Hamilton-Kemp et al., 1995).

The class of volatile phenolic compounds comprises MeSA, which is produced via methylation of SA (Lee et al., 1995; Koo et al., 2007). The hydrophobic nature of MeSA might suggest a potential function as a diffusible intercellular signal. A recent study proposed that MeSA is a mobile signal for SAR in tobacco (Park et al., 2007; see 1.3.3). In addition to its involvement in SAR, MeSA has been implicated in a number of other biological and ecological processes. For instance, MeSA is often emitted as a volatile compound from plants that are being challenged by stress factors, such as insect feeding, elicitor treatment (Chen et al., 2003a), and virus

infection (Shulaev et al., 1997). It is also released from *Pseudomonas syringae*-infected tobacco and Arabidopsis (Huang et al., 2003; Attaran et al., 2008; 2009). The production of MeSA under these conditions suggests a biological function of MeSA in stress adaption of plants. For instance, volatilized MeSA after insect herbivory has been suggested to be involved in attaracting natural enemies of the feeding insects (Dicke et al., 1990). MeSA produced in tobacco after virus-infection has been suggested to function as an airborn signal that activates defense responses in neighboring plants (Shulaev et al., 1997).

1.5 The *Pseudomonas*-Arabidopsis interaction as a model system

The interaction between *A. thaliana* and *P. syringae* is an ideal system to investigate the relationship between pathogen growth, symptoms, defense responses and fitness effects because the three variables can be estimated independently (Kover and Schall, 2002). A significant milestone in the development of the Arabidopsis-*P.syringae* system was that this pathosystem can conform to the gene-for-gene relationship that underlies many well-known plant-pathogen interactions in nature (Keen, 1990). Besides incompatible interactions resulting in an HR, compatible interactions and non-host resistance can be studied by this pathosystem.

1.5.1 *Arabidopsis thaliana* as model plant

Arabidopsis thaliana (L.) Heynh. (Arabidopsis) is a small flowering plant that is widely used as a model organism in plant biology. Arabidopsis is a member of the mustard (*Brassicaceae*) family, which includes cultivated species such as cabbage and radish. Although it is not of major agronomic significance, it offers important advantages for basic research in genetics and molecular biology. *Arabidopsis thaliana* has a small genome (114.5 Mb/125 Mb total) that has been sequenced completely in 2000 by the Arabidopsis Genome Initiative (AGI). It has 5 chromosomes with extensive genetic and physical maps. Arabidopsis plants can be transformed efficiently using *Agrobacterium tumefaciens*. Therefore, a large collection of mutant lines generated by x-ray irradiation, chemical mutagenesis, and insertional mutagenesis with T-DNA and transposons extists. To identify the functions of genes involved in plant development or environmental responses,

researchers use either forward and/or reverse genetics approaches to screen large collection of mutant lines.

The length of the life-cycle of an Arabidopsis plant depends on growth condition and ecotype. Over 750 natural accessions of Arabidopsis have been collected from around the world. These accessions are quite variable in terms of form and development (e.g. leaf shape, hairiness) and physiology (e.g. flowering time, disease resistance) (Quiroga et al., 2000). The most popular Arabidopsis accessions are Columbia (Col-0), Landsberg *erecta* (La*er*) and Wassilewskija (Ws). These three ecotypes are widely used for both molecular and genetic studies, and are the chosen genetic background for the majority of Arabidopsis *T*-DNA insertion mutant lines (Passadari et al., 2007).

1.5.2 *Pseudomonas syringae* as a pathogen for Arabidopsis

Pseudomonas syringae is a gram-negative, rod-shaped bacterium with polar flagella (Agiros, 1997). The species *Pseudomonas syringae* is composed of strains that collectively infect hundreds of plant species and cause disease symptoms ranging from leaf spots to stem cankers. *P. syringae* can be best described as a hemi-biotrophic pathogen because its most aggressive phase of intercellular growth occurs in the absence of host cell death. All of the *P. syringae* strains examined contain a *hypersensitive response and pathogenicity hrp*/*hrc*-gene-encoded type III protein secretion system (T3SS), which is essential for bacteria to cause disease in susceptible plants and to trigger the HR, a rapid cell-death response at the site of pathogen infection in nonhost or resistant host plants (Jones and Dangl 2006; Goehre and Robatzek, 2008). Additonally, *P. syringae* strains are known to produce various phytotoxins like COR, which are necessary for the full virulence of individual *P. syringae* strains in their host plants (Katagiri et al., 2002; Nomura et al., 2005; Brooks et al., 2005). COR is a non-host-specific phytotoxin produced by several members of *P. syringae* group or pathovars, which consists of coronafacic acid (CFA), an analog of methyl jasmonate (MeJA), and coronamic acid (CMA), which resembles 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor to ethylene (Bender et al., 1999; Mitchel, 1982; Uppalapati et al., 2005).

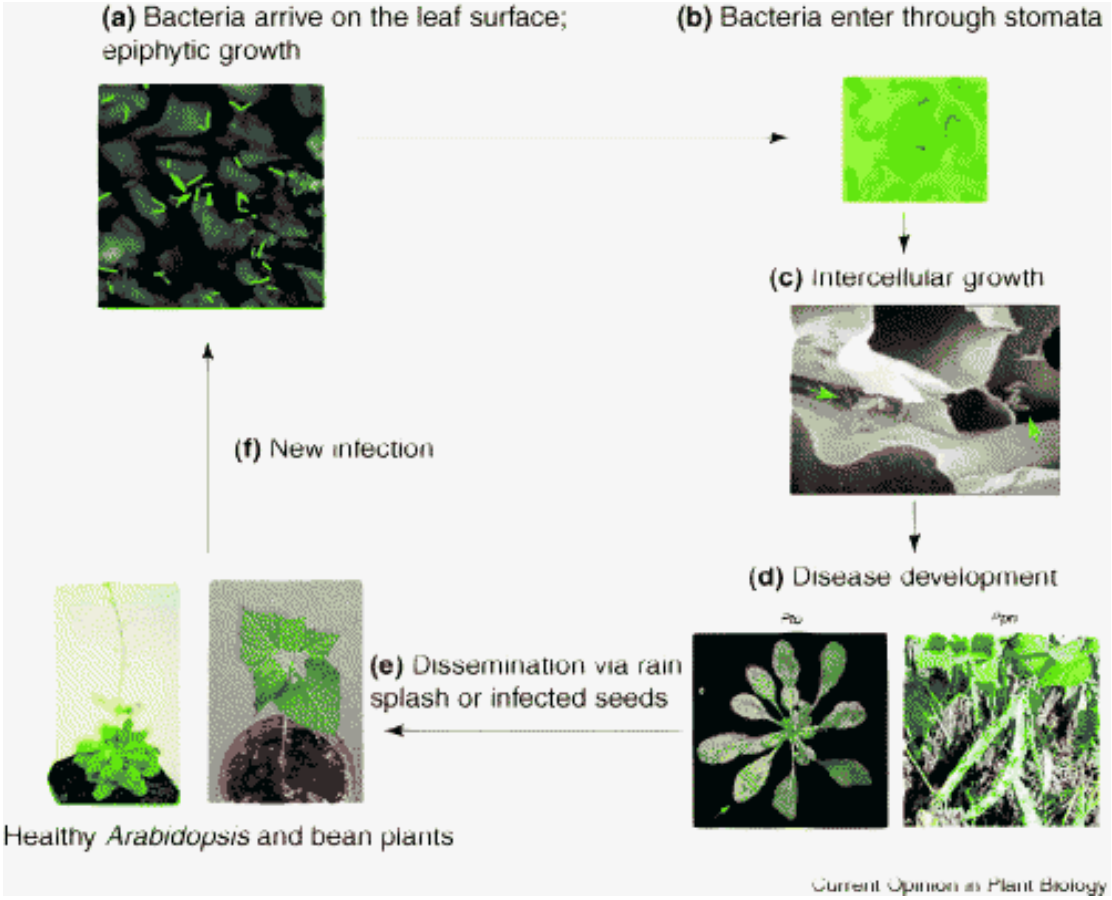

Figure 7. Infection cycle of *Pseudomonas syringae* (adapted from Nomura, Melotto and He, 2005).

In the late 1980s, several strains belonging to pathovars ***tomato***, ***maculicoa***, ***pisi***, and ***atropurpurea*** of ***Pseudomonas syringae*** were discovered to infect the model plant ***Arabidopsis thaliana***. The two virulent strains most commonly used today are ***P. syringae*** pv. ***tomato*** DC3000 and ***P.syringae*** pv. ***maculicola*** ES4326. The main reason for examining ***P. syringae*** strains as potential pathogens of Arabidopsis was because ***P. syringae*** had already been proven to be an excellent genetically tractable pathogen of soybean, tomato, and bean in the mid-1980s (Keen, 1990). The avirulence genes such as avrRpt2 and avrRpm1 were cloned to create avirulent pathogen strains, in order to study the HR and defense responses (Debrner et al., 1991; Dong et al., 1991; Whalen et al., 1991). It was also discovered that the above mentioned genes convert virulent *P. syringae* pathogens of pea, bean or soybean to avirulent ones on those host species, and that the soybean-associated avrB is recognized by Arabidopsis (Dangl et al., 1992; Innes et al., 1993; Whalen et al., 1991). Moreover, ***P. syringae*** pv. ***glycinea*** (*Psg*)

and ***P. syringae*** pv. ***phaseolicola*** (*Psp*), for which Arabidopsis is a non-host plant, are used to study non-host resistance (Lu et al., 2001; Mishina and Zeier, 2007; Ham et al., 2007).

2 AIMS OF THE WORK

The principal goal of the current work was to provide a better understanding of induced resistance responses in plants. Therefore, the interactions of the model plant Arabidopsis with different strains of the bacterial pathogen *Pseudomonas syringae* were studied at the molecular level. The particular focus of this work was to investigate the role of induced volatile organic compounds (VOCs) in pathogen defense of Arabidopsis, both by analytical-chemical and genetic means. In previous studies, VOCs were considered as possible phytoalexins due to their antimicrobial effects *in vitro,* or as signaling molecules which induce or prime plant defense responses. However, clear genetical evidence about the role of VOCs *in planta* was still missing.

The first aim of the present work was to identify and quantify the VOCs which are produced by Arabidopsis plants before and after infection with different *P. syringae* strains. After identifying the terpenoid TMTT and the phenolic compound methyl salicylate as the major VOCs synthesized by *P. syringae*-inoculated Arabidopis leaves, the defence signaling pathways that lead to the production of these compounds were characterized by analyzing pathogen-induced VOC production in Arabidopsis defence mutants.

The next goal was to study the functional role of TMTT and MeSA in pathogen defense. Therefore, *Arabidopsis* T-DNA insertion lines with defects in the terpene synthase gene *TPS4* and the methyl transferase gene *BSMT1* have been identified and their VOC profiles analyzed. Whereas mutants in *tps4* were totally defective in *P. syringae*-induced TMTT production, *bsmt1* mutants specifically failed to produce MeSA. This allowed to directly investigate the role of induced TMTT and MeSA production in pathogen resistance. Since MeSA was previously proposed as a critical mobile SAR signal in tobacco, a special focus of this work was to investigate the function of MeSA during systemic acquired resistance in Arabidopsis.

3 OWN RESEARCH

3.1 *Pseudomonas syringae* elicits emission of the terpenoid (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene in Arabidopsis leaves via jasmonate signaling and expression of the terpene synthase TPS4

Elham Attaran, Michael Rostás, and Jürgen Zeier. Molecular Plant-Microbe Interactions 21 (2008), 1482-1497.

Conception: 30 %
Experimental contribution: 95 %
Formulation of results: 30 %

3.2 Methyl salicylate production and jasmonate signaling are not essential for systemic acquired resistance in Arabidopsis

Elham Attaran, Tatiana E. Zeier, Thomas Griebel, and Jürgen Zeier.

The Plant Cell 21 (2009), 954-971.

Conception: 30 %
Experimental contribution: 80 %
Formulation of results: 30 %

PUBLICATION 1

Elham Attaran, Michael Rostás, and Jürgen Zeier

Pseudomonas syringae elicits emission of the terpenoid (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene in Arabidopsis leaves via jasmonate signaling and expression of the terpene synthase TPS4

Molecular Plant-Microbe Interactions (2008) 21: 1482-1497

Thesis author's contribution:

Conception: 30 %
Experimental contribution: 95 %
Formulation of results: 30 %

MPMI Vol. 21, No. 11, 2008, pp. 1482–1497. doi:10.1094/MPMI-21-11-1482. *e*-Xtra*

Pseudomonas syringae Elicits Emission of the Terpenoid (E,E)-4,8,12-Trimethyl-1,3,7,11-Tridecatetraene in *Arabidopsis* Leaves Via Jasmonate Signaling and Expression of the Terpene Synthase TPS4

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Submitted 9 April 2008. Accepted 9 July 2008.

Volatile, low–molecular weight terpenoids have been implicated in plant defenses, but their direct role in resistance against microbial pathogens is not clearly defined. We have examined a possible role of terpenoid metabolism in the induced defense of *Arabidopsis thaliana* plants against leaf infection with the bacterial pathogen *Pseudomonas syringae*. Inoculation of plants with virulent or avirulent *P. syringae* strains induces the emission of the terpenoids (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), β-ionone and α-farnesene. While the most abundant volatile, the C_{16}-homoterpene TMTT, is produced relatively early in compatible and incompatible interactions, emission of both β-ionone and α-farnesene only increases in later stages of the compatible interaction. Pathogen-induced synthesis of TMTT is controlled through jasmonic acid (JA)-dependent signaling but is independent of a functional salicylic acid (SA) pathway. We have identified *Arabidopsis* T-DNA insertion lines with defects in the terpene synthase gene *TPS4*, which is expressed in response to *P. syringae* inoculation. The *tps4* knockout mutant completely lacks induced emission of TMTT but is capable of β-ionone and α-farnesene production, demonstrating that TPS4 is specifically involved in TMTT formation. The *tps4* plants display at least wild type–like resistance against *P. syringae*, indicating that TMTT per se does not protect against the bacterial pathogen in *Arabidopsis* leaves. Similarly, the ability to mount SA-dependent defenses and systemic acquired resistance (SAR) is barely affected in *tps4*, which excludes a signaling function of TMTT during SAR. Besides *P. syringae* challenge, intoxication of *Arabidopsis* leaves with copper sulfate, a treatment that strongly activates JA biosynthesis, triggers production of TMTT, β-ionone, and α-farnesene. Taken together, our data suggest that induced TMTT production in *Arabidopsis* is a by-product of activated JA signaling, rather than an effective defense response that contributes to resistance against *P. syringae*.

Additional keywords: copper stress, disease resistance, terpene synthesis, terpene synthase4.

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*The *e*-Xtra logo stands for "electronic extra" and indicates that Figures 1 and 5 appear in color online.

Upon attempted infection with bacterial, fungal, or viral pathogens, plants induce a whole array of defense reactions that collectively contribute to counteract microbial invasion. Induced resistance responses often include cell-wall reinforcements, production of reactive oxygen species (ROS), the hypersensitive cell death response (HR), accumulation of antimicrobial pathogenesis-related (PR) proteins, synthesis of defense metabolites of lower molecular weight, and establishment of a primed state to allow a faster and more effective reaction towards subsequent pathogen encounter (Conrath et al. 2002; Dangl and Jones 2001).

In the model plant *Arabidopsis thaliana*, several metabolic pathways are activated in response to microbial pathogens to yield low–molecular weight defense substances. The oxylipin pathway produces jasmonic acid (JA), 12-oxophytodienic acid (OPDA), and phytoprostanes, which either act as signaling intermediates that trigger expression of specific sets of PR and other defense genes or mediate detoxification responses following stress exposure (Farmer et al. 2003; Mueller et al. 2008). Activation of the general phenylpropanoid pathway via phenylalanine ammonia lyase can lead to the synthesis of lignin precursors, which bear direct antimicrobial activity and, via oxidative polymerization to lignin-like phenolics, are thought to contribute to cell-wall reinforcements at infection sites (Lee et al. 2001; Mishina and Zeier 2007a). A related pathway yields pathogen-induced accumulation of the C6C1 phenolic salicylic acid (SA) and its derivatives via isochorismate synthase (Wildermuth et al. 2001). SA is a central defense signal ensuring basal and specific disease resistance towards many biotrophic and hemibiotrophic pathogens (Glazebrook 2005), and its accumulation is indispensable for the establishment of systemic acquired resistance (SAR) (Métraux 2002). SAR develops after a localized leaf contact with pathogenic or nonpathogenic microbes and provides broad-spectrum disease resistance against subsequent infections (Durrant and Dong 2004; Mishina and Zeier 2007b). In *Arabidopsis* leaf tissue facing attempted infection with biotrophic or necrotrophic pathogens, the tryptophan-associated branch of secondary metabolism is activated to yield the indole alkaloid camalexin (Glawischnig 2007). Camalexin is the characteristic phytoalexin of *Arabidopsis* and other crucifers that accumulates to high levels only in infected tissue and bears in vitro antimicrobial activity. In planta, it contributes to restrict leaf invasion by several necrotrophic but not biotrophic pathogens (Thomma et al. 1999; Zhou et al. 1999). Generally,

phytoalexins are a chemically heterogeneous group of nonpolar metabolites of lower molecular weight, which, depending on the plant species, can be derived from general phenylpropanoid, isoflavonoid, alkaloid, or terpenoid metabolism (Kuć 1995).

Chemical plant responses to microbial attack also include the induced emission of volatile organic compounds (VOC). For instance, emission of antimicrobial "green leaf volatiles" (GLV), which are C_6 aldehydes, alcohols, and their esters derived from the oxylipin pathway increases during bacterial infection in bean plants (Croft et al. 1993). Plant GLV release can prime neighboring plants to react more rapidly upon insect herbivore attack (Engelberth et al. 2004). In tobacco and *Arabidopsis* leaves, volatile methyl salicylate (MeSA) is produced via methylation of SA (Lee et al. 1995; Koo et al. 2007). MeSA has been implicated in both interplant communication as well as intraplant long-distance signaling culminating in increased whole-plant resistance against viral pathogens in tobacco (Park et al. 2007; Shulaev et al. 1997).

Terpenoids represent a major group of plant volatile compounds. The basic pathway of terpenoid biosynthesis involves the formation of the C_5 precursor units isopentenyl diphosphate and dimethylallyl diphosphate through either the plastid-localized methylerythritol phosphate or the cytosolic mevalonate pathway. Prenyltransferases catalyze the condensation of these C_5 precursors to yield C_{10}, C_{15}, or C_{20} prenyl diphosphates, which are converted to monoterpenes, sesquiterpenes, and diterpenes, respectively, by terpene synthases (TPS) (Tholl 2006). The primary terpene products can be further modified through oxidation, C-C-cleavage, and acylation reactions that yield terpenoids with altered physicochemical properties. The vapor pressure of the majority of terpenoids containing 5 to 20 carbon atoms is high enough to allow significant plant emission into the air (Dudareva et al. 2004). Upon feeding or egg deposition by herbivorous insects, vegetative plant tissue often produces and emits increased amounts of volatile terpenoids within hours (Turlings 1998; Wegener et al. 2001). Herbivore-induced terpenoids may function in indirect plant defense by attracting natural enemies of the herbivore or in direct defense against insects through repellent or toxic properties (Aharoni et al. 2003; Kessler and Baldwin 2001; van Poecke and Dicke 2004).

The role of terpenoids in defense against pathogenic microbes is less well understood. Tobacco plants challenged with *Pseudomonas syringae* bacteria increase the production of various VOC, including the monoterpenes (E)-β-ocimene and linalool and the sesquiterpenes caryophyllene, β-elemene, and α-farnesene (Huang et al. 2003). Pathogen-induced terpenoids might function as typical phytoalexins, because many of those compounds possess direct antimicrobial properties (Soković et al. 2006). Alternatively, they could participate in defense signaling. A recent study reporting that the monoterpene allo-ocimene induces resistance responses and primes defense reactions against the fungal pathogen *Botrytis cinerea* supports this view (Kishimoto et al. 2006). Deductions about the function of terpenoids in defense and resistance against microbial pathogens, however, either result from in vitro studies or from experiments in which plant responses have been triggered by external application of terpenoid compounds in nonphysiological amounts. Direct genetic evidence for a possible defensive role of terpenoids actually produced in planta is still missing.

In the present study, we have examined a possible involvement of terpenoids in inducible defense responses of the model plant *Arabidopsis thaliana* towards avirulent and virulent *Pseudomonas syringae* strains. We first show that emission of the C_{16}-homoterpene (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) is induced after inoculation with incompatible and compatible *P. syringae* strains. Induced TMTT production proceeds via upregulation of the TPS gene *TPS4* and is controlled through jasmonate signaling, because *tps4* T-DNA knockout plants as well as JA-pathway mutants either completely lack or display significantly reduced TMTT emission after pathogen contact. The use of *tps4* mutant plants devoid in TMTT production allowed us, for the first time, to assess the role of a terpenoid in defense and disease resistance under physiological conditions. We provide evidence that the failure to mount TMTT synthesis in *tps4* mutants does not critically affect local resistance or SAR, indicating that *P. syringae*–induced TMTT formation is not a decisive event for disease resistance towards the bacterial pathogen in *Arabidopsis*.

RESULTS

Arabidopsis terpenoid volatiles produced in response to *P. syringae* inoculation.

Leaf inoculation of *Arabidopsis* accession Col-0 with the compatible bacterial strain *Pseudomonas syringae* pv. *maculicola* ES4326 results in rapid bacterial multiplication in the leaf apoplast and development of yellowish, water-soaked disease lesions spreading in infected leaves (Mishina and Zeier 2007b). In comparison, ES4326 avrRpm1, a *P. syringae* pv. *maculicola* ES4326 strain expressing the AvrRpm1 avirulence protein, induces a HR at inoculation sites that restricts bacterial multiplication to a significant degree (Delledonne et al. 1998). The array of *Arabidopsis* defense reactions initiated in response to ES4326 or ES4326 avrRpm1 is well-studied and comprises accumulation of the defense metabolites SA, JA, and camalexin, as well as increased expression of various defense-related proteins. Induction of these defense reactions during the early interaction period generally occurs in a more pronounced manner in response to avirulent ES4326 avrRpm1 than in response to virulent ES4326 (Mishina et al. in press).

We investigated whether induced production of VOC in inoculated *Arabidopsis* leaves would be part of the plant defense arsenal against *P. syringae* attack. Pathogen-inoculated or control-infiltrated plants were therefore placed in airtight glass chambers, and whole-plant emission of volatile compounds was determined through collection on a trapping filter and subsequent analysis of filter eluates by gas chromatography–mass spectrometry (Rostás et al. 2006). Compared with $MgCl_2$-infiltrated control plants, Col-0 plants inoculated with ES4326 avrRpm1 emitted strongly elevated levels of MeSA and a substance at higher retention times with two dominant masses in its mass spectrum at m/z = 69 and m/z = 81 (Fig. 1A and B). Comparison of the mass spectrum with those of the National Institute of Standards and Technology (NIST 98) reference library, interpretation of mass spectral fragmentation patterns and coinjection with a standard substance identified the compound as (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) (Fig. 1C), a C_{16}-homoterpene produced by many plants in response to herbivory (Ament et al. 2006; Hopke et al. 1994; Paré and Tumlinson 1997). A detailed timecourse analysis revealed that TMTT is not produced in Col-0 leaves before 10 h postinoculation (hpi) with either avirulent or virulent *P. syringae* (Fig. 2A). Its emission was strongly increased between 10 and 24 hpi upon treatment with both ES4326 avrRpm1 and ES4326, and its production was maintained for two or at least three days in the compatible and incompatible interaction, respectively. Basal levels of emitted TMTT in untreated plants were virtually absent, and control infiltrations with $MgCl_2$ only caused traces of TMTT emission. Besides TMTT, two other terpenoid volatiles emitted in low amounts from *Arabidopsis* leaves, β-ionone and α-farnesene, were iden-

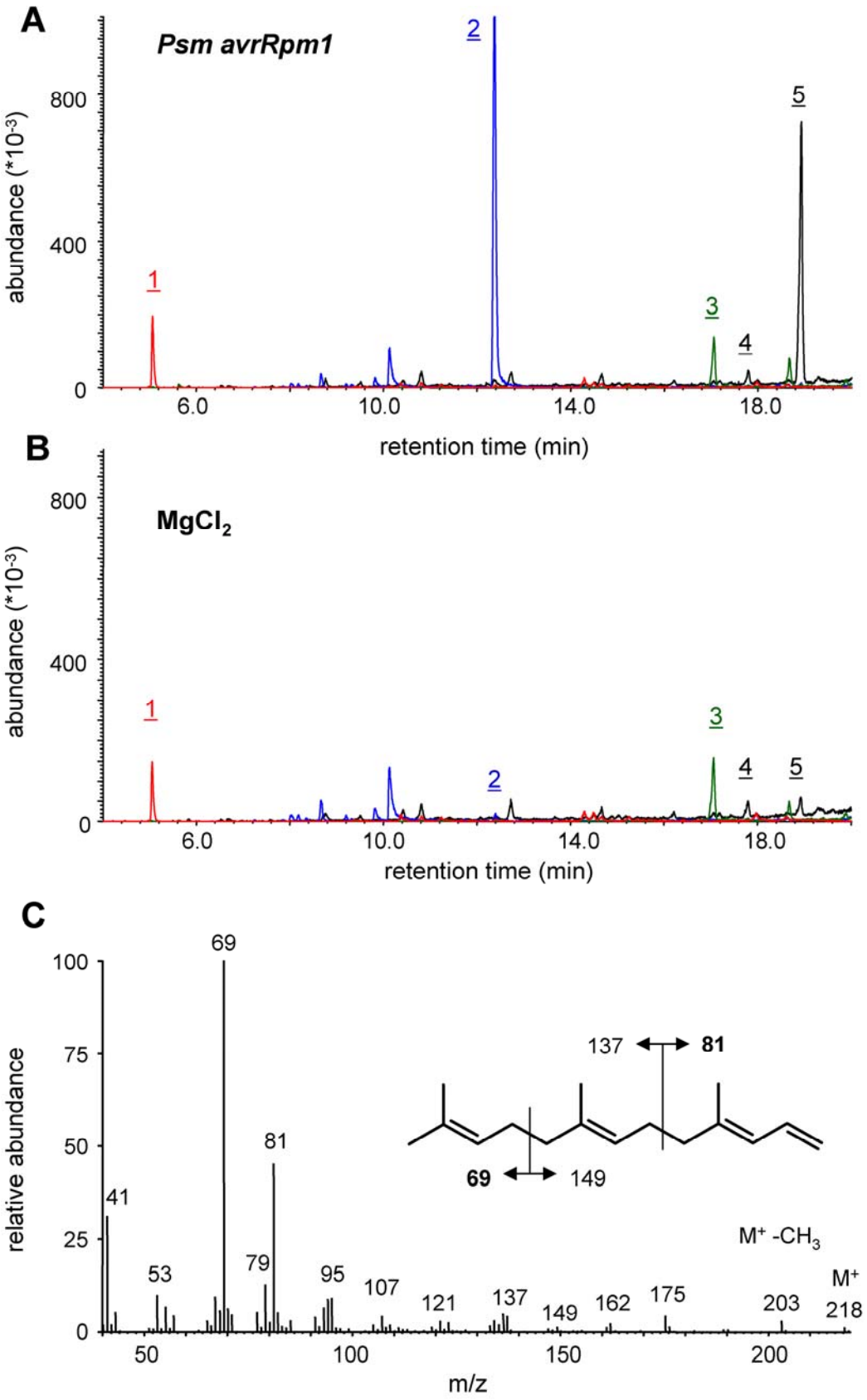

Fig. 1. Gas chromatography–mass spectrometry analyses of volatiles emitted from *Pseudomonas syringae*–treated *Arabidopsis* plants. **A** and **B,** Selected ion chromatograms (m/z 114, red; m/z 120, blue; m/z 177 green; m/z 81, black) illustrating profiles of released *Arabidopsis* volatiles. **A,** Volatiles were collected for 24 h after inoculation of leaves with *P. syringae* pv. *maculicola avrRpm1* (optical density = 0.01), and **B,** after infiltration with 10 mM $MgCl_2$ as a control treatment. The compounds represented by the peaks in the different ion chromatograms were identified as follows: 1, n-octane (internal standard; m/z 114); 2, methyl salicylate (m/z 120); 3, β-ionone (m/z = 177); 4, α-farnesene (m/z = 81); and 5, (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) (m/z 81). **C,** Mass spectrum of substance 5 (identified as TMTT), structural formula of TMTT, and proposed mass spectral fragmentation patterns.

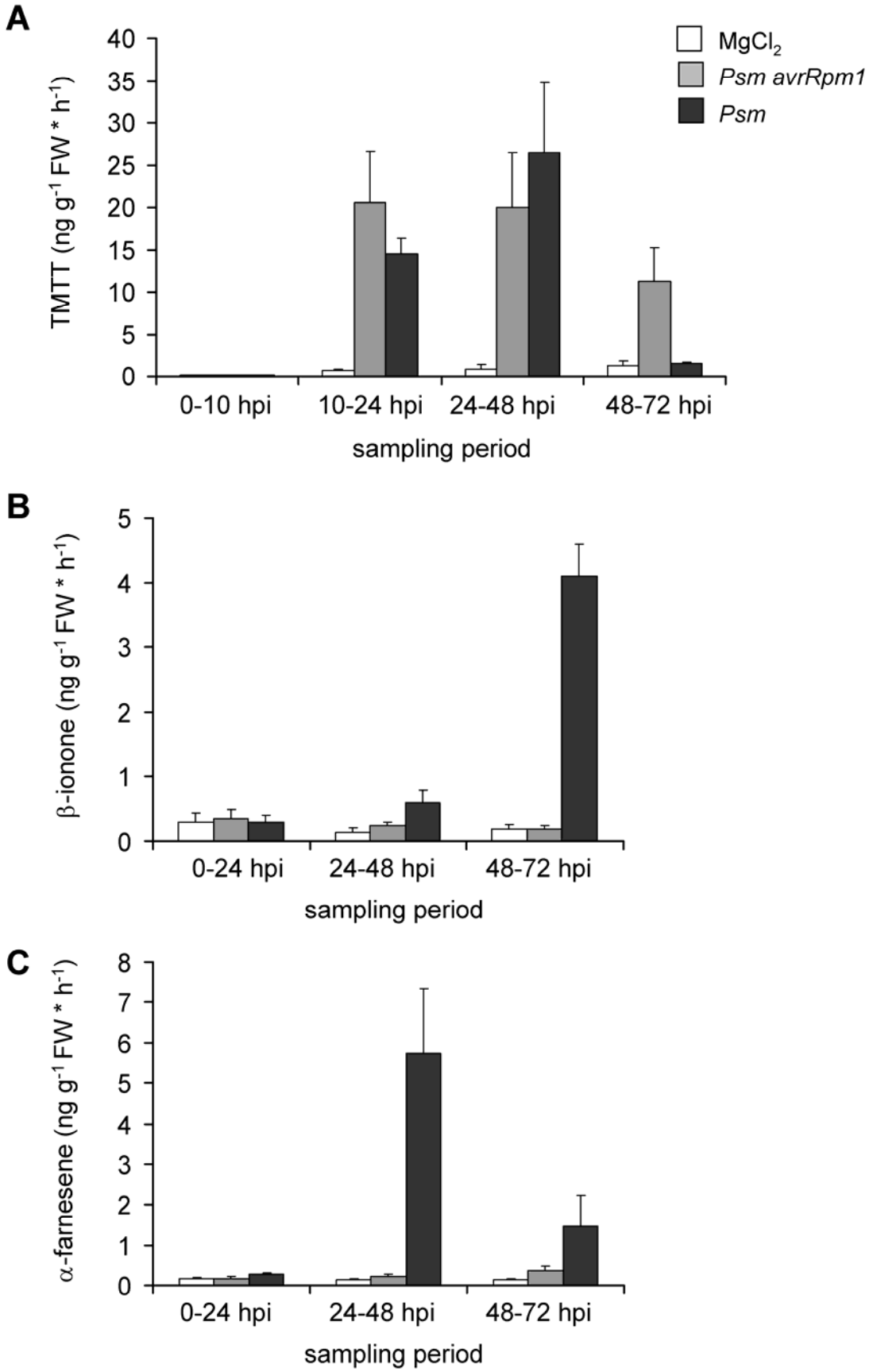

Fig. 2. Timecourse of terpenoid emission from *Arabidopsis* Col-0 plants inoculated with *Pseudomonas syringae* pv. *maculicola avrRpm1* (*Psm avrRpm1*) (incompatible interaction; light gray bars), *P. syringae* pv. *maculicola* (*Psm*) (compatible interaction; dark gray bars), or infiltrated with 10 mM $MgCl_2$ (control; white bars). Bars represent mean emission values (±standard deviation) from three independent plants. The time periods in which volatiles have been collected are indicated in hours postinoculation (hpi). Values are given in nanograms of volatile substance per gram of fresh weight (FW) per h. **A,** (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene emission, **B,** β-ionone emission, and **C,** α-farnesene emission.

tified (Fig. 1A). Leaf emission of either substance did not increase after treatments with both $MgCl_2$ and ES4326 avrRpm1 but was significantly elevated during later periods of the compatible Col-0–ES4326 interaction (Fig. 2B and C).

Emission of typical GLV such as (Z)-3-hexen-1-ol or hexanal was virtually absent in $MgCl_2$- and *P. syringae*-treated Col-0 plants, except for interactions with the compatible ES4326 strain during the sampling period between 24 and 48 hpi, when traces of (Z)-3-hexen-1-ol could be detected in the volatile blend. Similarly, volatile glucosinolate degradation products such as 4-methylthiobutyl-isothiocyanate or 5-methylthiopentane-nitril were not detected after $MgCl_2$ or *P. syringae* infiltrations. Above mentioned GLV and mustard oils, however, were emitted to significant amounts from artificially damaged Col-0 leaves (data not shown).

Regulation of *P. syringae*–triggered TMTT production.

Many plant responses to pathogens are either mediated by SA- or JA-induced signaling pathways (Halim et al. 2006; Reymond and Farmer 1998). Activation of terpenoid biosynthesis in response to herbivore attack has been reported in several plant species to depend on JA signaling (Ament et al. 2004; Arimura et al. 2008; Mercke et al. 2004). Using mutant lines that are either blocked in JA or SA biosynthesis or impaired in the respective signaling pathways, we tested whether microbial induction of TMTT synthesis in *Arabidopsis* would require JA- or SA-dependent signaling. In contrast to their corresponding wild-type background lines Col-0 and Ws, *P. syringae*-induced TMTT emission was virtually absent in *dde2* and *opr3* mutant plants, which are defective in the allene oxide synthase and 12-oxophytodienoic acid reductase JA biosyn-

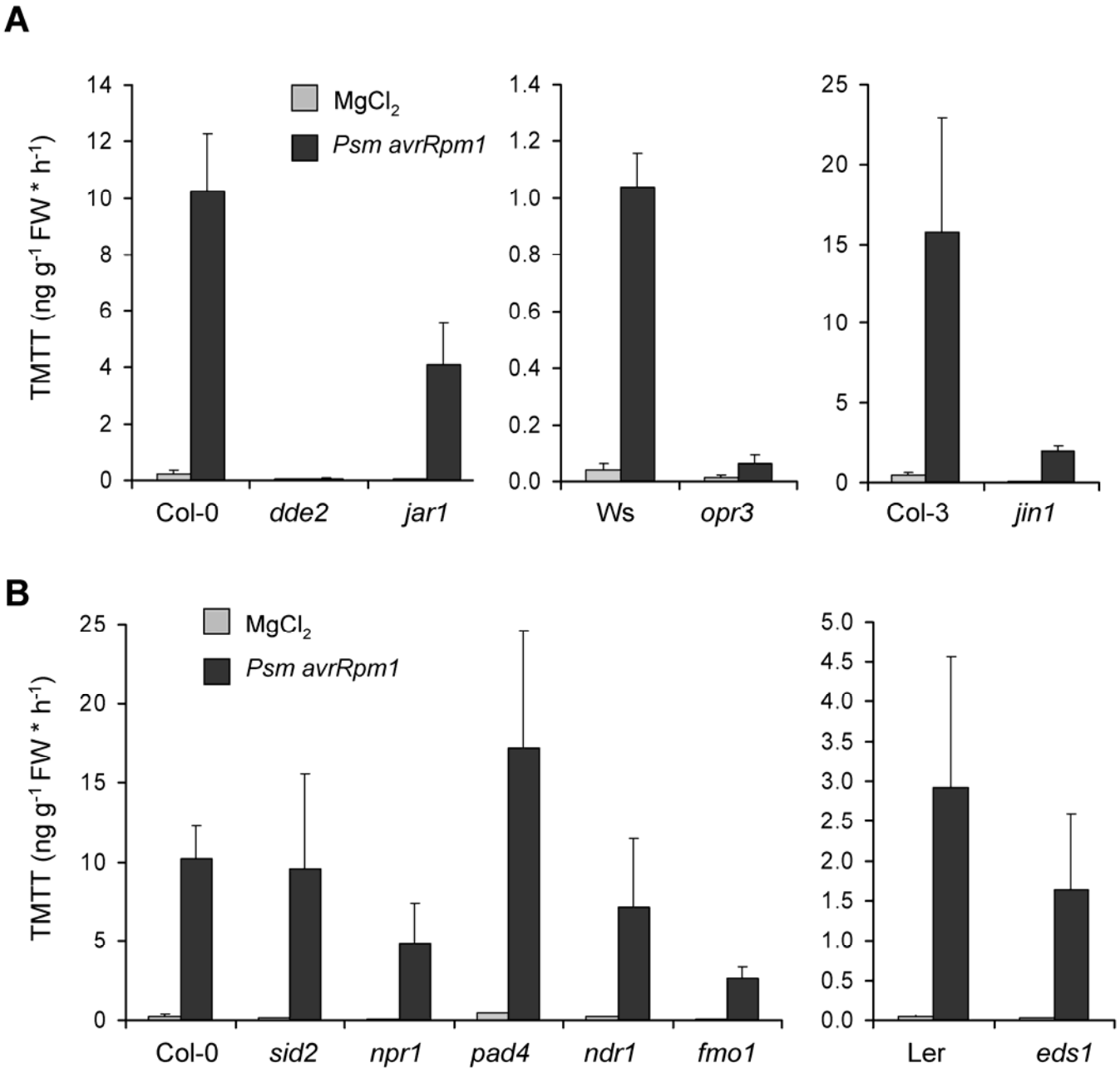

Fig. 3. *Pseudomonas syringae*–induced (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) emission in *Arabidopsis* defense mutants. TMTT emission from plants inoculated with *P. syringae* pv. *maculicola avrRpm1* (dark bars) or infiltrated with 10 mM $MgCl_2$ (control, light bars). Volatiles were collected from 0 to 24 h postinoculation. Bars represent mean emission values (±standard deviation) from three independent plants. **A**, Jasmonic-acid pathway mutants. **B,** Mutants directly or indirectly related to salicylic acid signaling.

thesis genes (Stintzi and Browse 2000; von Malek et al. 2002), respectively (Fig. 3A). Elicitation of TMTT production by avirulent *P. syringae* thus strongly depends on the plants capability to synthesize JA. Downstream components of JA signaling involve jasmonate–amino acid synthetase (JAR1) and the MYC transcription factor AtMYC2 (JIN1) (Lorenzo et al. 2004; Staswick et al. 2004). In both *jar1* and *jin1* mutant lines, *P. syringae*–induced TMTT production was significantly lower than in the respective wild-type plants Col-0 and Col-3, albeit its synthesis was not fully suppressed (Fig. 3A). This indicates that both JIN1 and JAR1 contribute to the JA-mediated control of pathogen-elicited TMTT synthesis.

In contrast to JA production, SA accumulation is not required for ES4326 avrRpm1 elicitation of TMTT synthesis in *Arabidopsis*, because the pathogen provoked wild-type-like TMTT emission in the SA biosynthesis mutant *sid2* (Fig. 3B) (Nawrath and Métraux 1999). Moreover the SA pathway–related defense components PHYTOALEXIN-DEFICIENT4 (PAD4) (Glazebrook et al. 1997), NON-RACE SPECIFIC DISEASE RESISTANCE1 (NDR1) (Century et al. 1995), and ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) (Parker et al. 1996) are not involved in *P. syringae*–induced TMTT synthesis, because respective mutant plants exhibited induced production of the homoterpene. This was also true for the SA-insensitive mutant *nonexpressor of PR-1* (*npr1*) (Cao et al. 1994) and the SAR-defective defense mutant *flavin-dependent monooxygenase1* (*fmo1*) (Mishina and Zeier 2006), although induced TMTT emission was lower in the latter two lines than in wild-type plants (Fig. 3B). Taken together, these data indicate that SA-related signaling pathways are not essential for pathogen-induced TMTT synthesis. Natural variation, on the other hand, has a stronger impact on the amount of produced TMTT, because its emission by the three *Arabidopsis* accessions that were used differed in relative levels of emitted TMTT (Col > Ler > Ws; Fig. 3).

Arabidopsis TPS4 is involved in induced TMTT synthesis.

In *Arabidopsis*, 32 functional TPS genes exist that are thought to mediate synthesis of terpenes out of prenyl diphosphate precursors (Aubourg et al. 2002). Microarray analyses indicate that four of these genes—TPS2, TPS3, TPS4 and TPS10—are upregulated in *Arabidopsis* leaves upon *P. syringae* pv. *tomato* inoculation (Fig. 4A). Thereof, expression of TPS4 (At1g61120) is induced most prominently by both avirulent and virulent *P. syringae* pv. *tomato* strains. Gel-blot analyses revealed that Col-0 leaves inoculated with the incompatible ES4326 avrRpm1 strain induce expression of TPS4 from at least 10 hpi onward and that expression of the gene in response to the compatible ES4326 strain was slower but reached a high value at 24 hpi (Fig. 4B). Taking these expression characteristics and our TMTT emission data as a basis for our rationale, we hypothesized that TPS4 might encode a TPS that is involved in TMTT biosynthesis. To directly test the presumed function of *TPS4* for TMTT production in planta, we intended to identify and characterize *Arabidopsis* T-DNA insertion lines with a *TPS4* knockout. The T-DNA Express *Arabidopsis* Gene Mapping Tool predicts several lines with putative disruptions of the *TPS4* coding region. When applying the polymerase chain reaction (PCR)-based protocol described by Alonso and associates (2003) to confirm the predicted insertions, we merely identified a single line, SALK_078187, that indeed harbors the T-DNA insert within the *TPS4* coding region (Fig. 4C). Plants homozygous for the insert, from here on designated as *tps4*, completely lack basal or pathogen-induced expression of the *TPS4* gene (Fig. 4D). When comparing volatile emissions from ES4326 avrRpm1-treated Col-0 and *tps4* plants, we obtained nearly identical profiles, except that the TMTT peak was totally absent in the *tps4* volatile blend (Fig. 5A through C). This confirmed our hypothesis that functional TPS4 is required for biosynthesis of TMTT in *Arabidopsis*. We additionally identified two *Arabidopsis* lines (*tps10-1*, SALK_108420 and *tps10-2*, SALK_041114) with inserts in the *TPS10* gene (At2g24210), which is also upregulated in response to *P. syringae*. Emission profiles and TMTT production of both *tps10* lines, however, were indistinguishable from those of Col-0, suggesting that TPS10 is not involved in the production of TMTT and other volatile terpenoids in *Arabidopsis* (Fig. 5C).

Is there a defensive role for TMTT against *P. syringae* attack?

The *tps4* mutant blocked in TMTT biosynthesis represented an excellent tool to study the functional relevance of induced terpenoid production in the *Arabidopsis–P. syringae* interaction. We reasoned that TMTT might function as a phytoalexin that directly contributes to restricting bacterial growth in inoculated leaf tissue. In this case, *tps4* mutants should exhibit decreased resistance towards *P. syringae* as compared with wild-type plants. However, when assessing bacterial growth in leaves inoculated with either ES4326 avrRpm1 or with ES4326, *tps4* did not allow the bacteria to multiply more vigorously in extracellular spaces than did Col-0. Instead, specific resistance to ES4326 avrRpm1 and basal resistance to ES4326 were similar in *tps4* and Col-0 plants, with a slight tendency to an even higher degree of resistance toward both strains for *tps4* (Fig. 6). These results clearly exclude a function for TMTT as a phytoalexin that is effective against the eliciting *P. syringae* pathogen.

Still, TMTT might play alternative roles in defense signaling, thus influencing or priming other defense reactions. Therefore, we tested whether differences existed in Col-0 and *tps4* with regard to the induction of typical defense responses at sites of ES4326 avrRpm1 inoculation. Accumulation of SA and JA as well as induced expression of the PR gene *PR-1*, however, were virtually identical in Col-0 and *tps4* mutants, demonstrating that TMTT production has no impact on these responses (Fig. 7A, B, and D). By contrast, we observed a reduced accumulation of the phytoalexin camalexin in *tps4* as compared with Col-0 (Fig. 7C). This difference could principally be based on positive cross-talk between TMTT formation and camalexin biosynthesis. Alternatively, it might be a simple consequence of reduced bacterial multiplication in *tps4* compared with Col-0 (Fig. 6A), resulting in an overall lower stimulatory activity towards camalexin production.

To clarify this point, we intended to examine plant responses toward a more constant abiotic stimulus possessing both camalexin- and TMTT-eliciting activity. Because heavy metal ions like Cu^{2+} are known to trigger camalexin biosynthesis in *Arabidopsis* (Pedras and Adio 2008), we treated leaves with 10 mM $CuSO_4$ and comparatively analyzed small metabolite content in and VOC emission from Col-0 and *tps4* plants. This leaf intoxication with $CuSO_4$ indeed evoked simultaneous camalexin production and TMTT formation in Col-0 (Fig. 8A and E) and, further, lead to β-ionone and α-farnesene emission. Remarkably, $CuSO_4$ treatment induced the biosynthesis of volatile terpenoids similar in both quality and quantity to those induced by *P. syringae* inoculation (Figs. 8A through C and 2). The absence of TMTT emission in $CuSO_4$-treated *tps4* mutant plants confirmed the requirement of functional TPS4 for TMTT biosynthesis (Fig. 8A). By contrast, emission of both β-ionone and α-farnesene from *tps4* plants was induced to at least wild-type levels, indicating that TPS4 is not involved in the biosynthesis of either of those terpenes (Fig. 8B and C). The similarities between the *P. syringae*–induced and the $CuSO_4$–triggered plant response were also evident when other

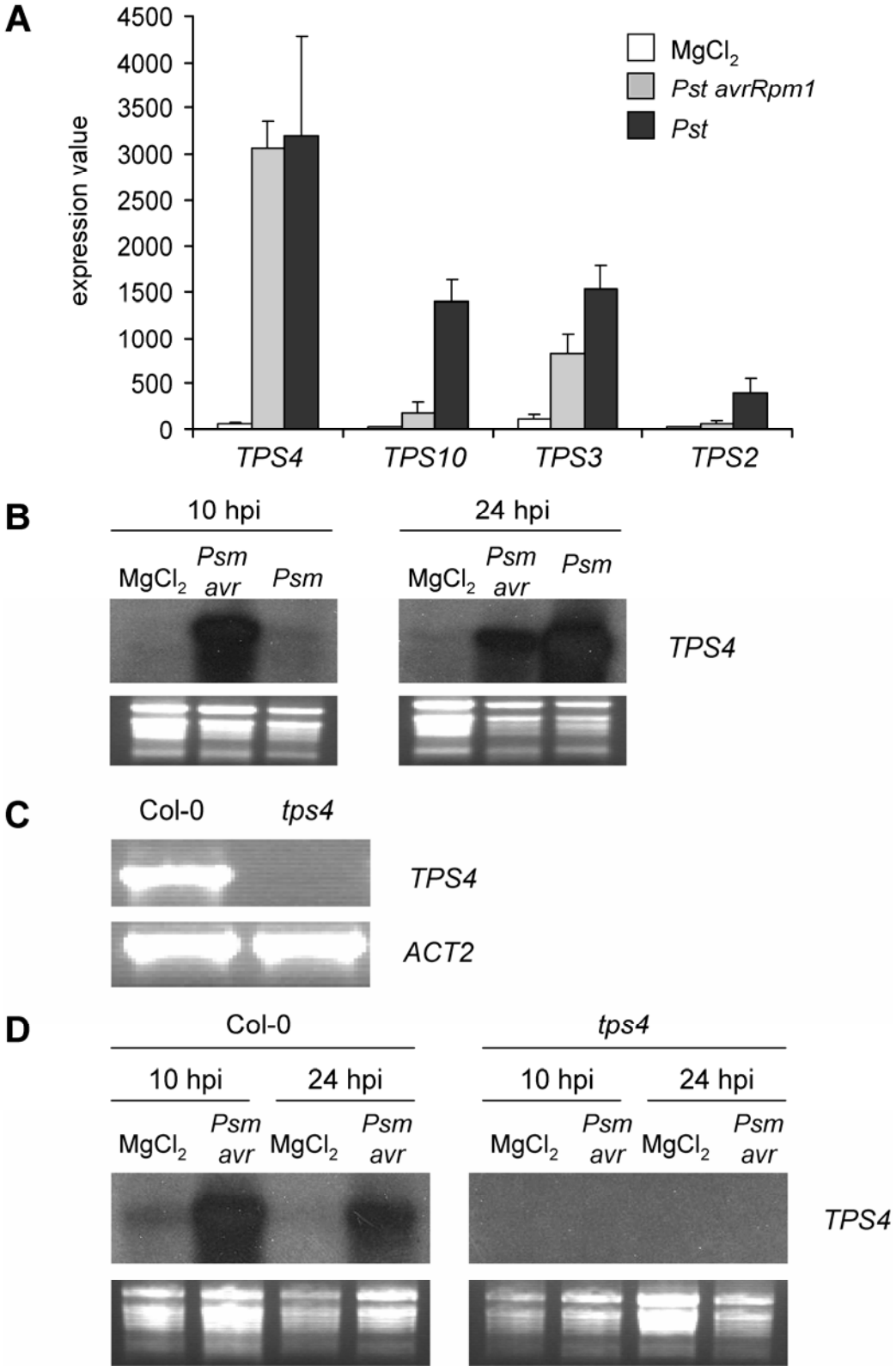

Fig. 4. Expression patterns of *TPS4* and other terpene synthase (TPS) genes in *Pseudomonas syringae*–inoculated *Arabidopsis* leaves. **A,** Expression levels (24 h postinoculation [hpi]) of TPS genes in Col-0 leaves challenged with *Pseudomonas syringae* pv. *tomato* (*Pst*) according to microarray analyses (The Arabidopsis Information Resource '*TAIR-ME00331*: Response to virulent, avirulent, type III secretion system deficient and nonhost bacteria'). Means (±standard deviation) of Affymetrix expression values originating from three independent replicates are given. The data are normalized according to the Affymetrix MAS 5.0 scaling protocol. **B,** Expression of *TPS4* in leaves inoculated with *P. syringae* pv. *maculicola avrRpm1* (*Psm avr*) or *P. syringae* pv. *maculicola* (*Psm*), as assessed by Northern blot analysis. Control samples were infiltrated with 10 mM $MgCl_2$. Leaf samples were taken at 10 and 24 hpi. **C,** Polymerase chain reaction analyses with genomic DNA from Col-0 or *tps4* mutant (T-DNA insertion line SALK_026163) plants as templates, indicating a T-DNA insert in the *TPS4* coding region of *tps4*. The following gene-specific primers were used for *TPS4* amplification: *TPS4-forward*, 5′-GCGTACGACAAG TATTTGCAG-3′ and *TPS4-reverse*, 5′-AAGTTCACGG-CCTAATGCTTC-3′. The actin gene *ACT2* was amplified as a positive control. **D,** Expression patterns of *TPS4* in Col-0 and *tps4* leaves infiltrated with 10 mM $MgCl_2$ or *P. syringae* pv. *maculicola avrRpm1*, as assessed by Northern blot analysis. Leaf samples are taken at 10 and 24 hpi.

low–molecular weight metabolites were analyzed. Like *P. syringae*, $CuSO_4$ evoked emission of MeSA as well as strong synthesis of camalexin, SA, and JA (Fig. 8D through G). Induced levels of all these defense-related metabolites were virtually identical in Col-0 and *tps4*, which excludes the above-mentioned possibility of cross-talk between camalexin and TMTT synthesis.

SAR involves the generation of one or more long-distance signals at sites of pathogen attack, signal translocation towards distant plant parts, and the initiation of defense responses in systemic tissue (Mishina et al. in press). To test whether TMTT production is required for the overall SAR process, we examined the capability of *tps4* mutant plants to mount defense responses and to enhance resistance at the systemic level. Therefore, three lower rosette leaves (1° leaves) of a given plant were either infiltrated with 10 mM $MgCl_2$ in a control treatment or inoculated with a suspension of ES4326 for biological induction of SAR. Three upper, previously untreated leaves (2° leaves) were collected 2 days later and were analyzed for SA content and PR gene expression or were subject to a subsequent ES4326 challenge. Systemic resistance was assessed by scoring bacterial growth in 2° leaves 3 days after the challenge infection. We found that SA contents of 2° leaves were considerably elevated in both Col-0 and *tps4* after ES4326 infection of 1° leaves and that expression levels of the SAR gene *PR-1* was increased in 2° leaves of both lines after the 1° ES4326-treatment (Fig. 9B and C). Although these systemic responses tended to be somewhat less pronounced in *tps4* than in Col-0, reduction of ES4326 growth in 2° leaves upon 1° leaf inoculation occurred to a similar degree in Col-0 and *tps4* mutant plants (Fig. 9A). This indicates that SAR establishment in *Arabidopsis* is essentially independent of TMTT production.

DISCUSSION

We have analyzed the induced production of VOC in *Arabidopsis* plants that were challenged with incompatible and compatible *P. syringae* strains (Figs. 1 and 2). Induced synthesis of

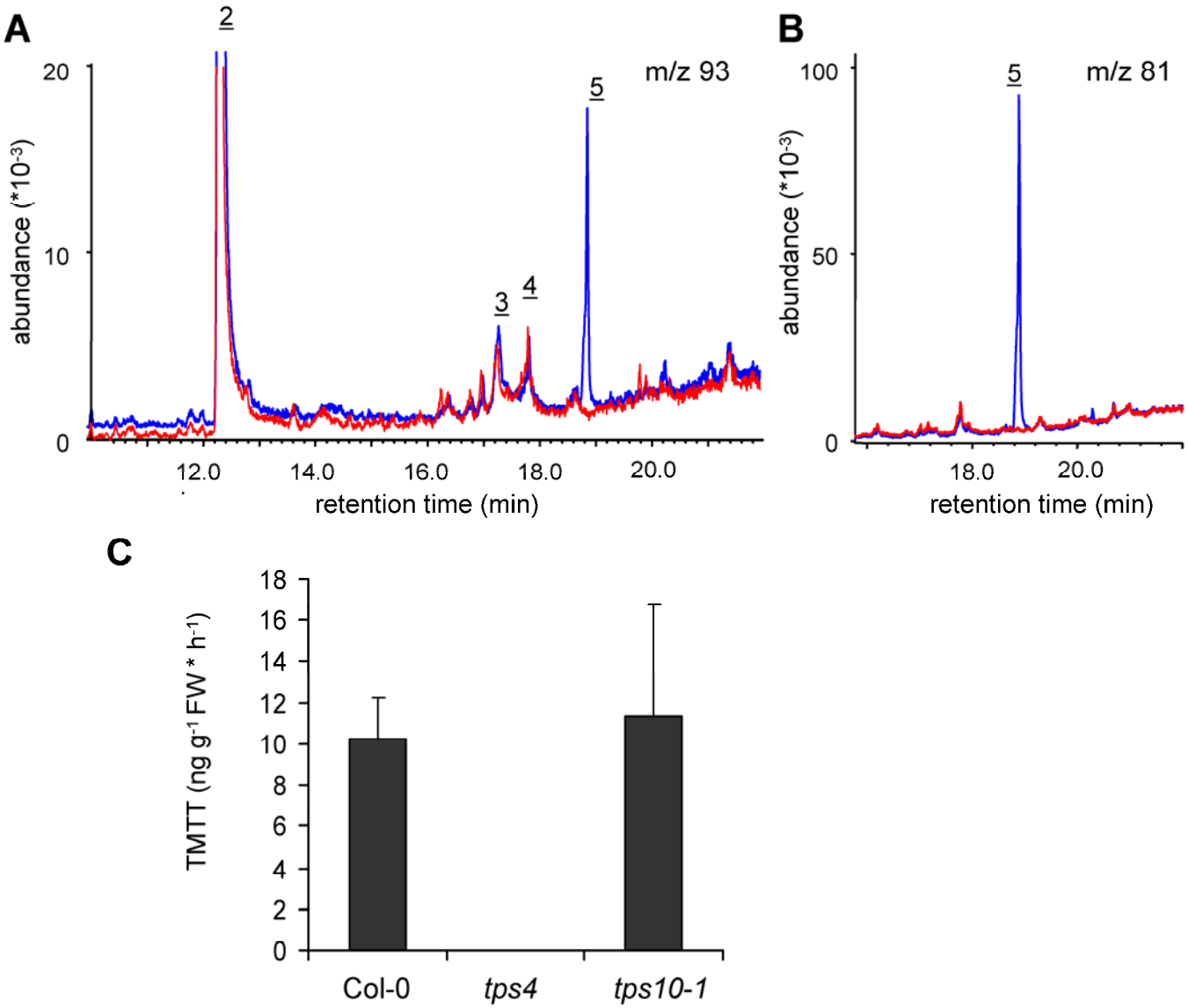

Fig. 5. *Pseudomonas syringae*–induced volatile and (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) emission from wild-type Col-0, *tps4* mutant, and *tps10* mutant plants. **A,** Ion chromatograms at m/z 93 of volatile samples from Col-0 plants (blue) and tps4 plants (red), simultaneously illustrating methyl salicylate (2), β-ionone (3), α-farnesene (4), and TMTT (5) emission. **B,** Ion chromatograms at m/z 81 of Col-0 (blue) and *tps4* (red) volatile samples more explicitly demonstrating the absence of TMTT in the *tps4* sample. **C,** Quantification of TMTT emitted from wild-type Col-0 plants, *tps4* mutant plants, and *tps10-1* mutant plants inoculated with *P. syringae* pv. *maculicola avrRpm1*. Volatiles were collected from 0 to 24 h postinoculation. Bars represent mean emission values (± standard deviation) from three independent plants. The *tps10-2* mutant exhibited similar levels of emission to *tps10-1* (data not shown).

two major volatile substances, the phenylpropanoid MeSA and the C_{16}-homoterpene TMTT, is part of the response repertoire of *Arabidopsis* against attack by avirulent ES4326 avrRpm1 and virulent ES4326. Later plant responses in compatible interactions of *Arabidopsis* with ES4326 comprise induced emission of the terpenoids β-ionone and α-farnesene. Induced VOC production in response to *P. syringae* has been previously observed for tobacco (Huang et al. 2003). The volatiles produced by this plant species include MeSA, indole, and a broad spectrum of terpenoids consisting of β-ocimene, linalool, α-farnesene, caryophyllene, and β-elemene as well as of two unidentified sesquiterpenes. Analogous to *Arabidopsis*, bacterial strains differing in their virulence properties elicit emission of distinct volatile blends from tobacco.

According to our results, *Arabidopsis* Col-0 leaves fail to provoke emission of typical green-leaf volatiles upon inoculation with avirulent ES4326 avrRpm1. Substantial amounts of the GLV (Z)-3-hexen-1-ol and (E)-2-hexenal, by contrast, are emitted from bean leaves after inoculation with incompatible *P. syringae* pv. *phaseolicola*. This and the fact that (Z)-3-hexen-1-ol bears bactericidal activity at low concentrations suggest that GLV contribute to pathogen resistance in bean (Croft et al. 1992). Our emission data, however, argue against a comparable role of GLV in *Arabidopsis* defense towards *P. syringae* attack. This might be particularly true for the examined Col-0 accession, which, compared with other ecotypes, exhibits reduced expression and activity of hydroperoxide lyase and has consequently only a weak ability to produce GLV (Duan et al. 2005). Nevertheless, in contrast to pathogen infection, severe mechanical damage of Col-0 leaves gave rise to the emission of clearly detectable amounts of the GLV (Z)-3-hexen-1-ol and hexanal in our experiments. Similarly, the lack of leaf emission of volatile mustard oils after *P. syringae* challenge indicates that the glucosinolate-myrosinase system, which is effective in *Arabidopsis* defense against insect herbivore attack (Barth and Jander 2006), is not an integral part of the defense arsenal operating against hemibiotrophic bacterial pathogens.

This study has focused particularly on the regulation of *P. syringae*–induced TMTT production in *Arabidopsis* and on its significance for defense and disease resistance against this microbial pathogen. Increased emission of TMTT as a plant response to biotic stress was first shown to occur in spider mite–infested lima bean and has since then been detected in many plant species attacked by herbivores (Ament et al. 2006; Hopke et al. 1994; Paré and Tumlinson 1997). Upon leaf feeding by *Pieris rapae* caterpillars and *Plutella xylostella* larvae, TMTT is also produced in *Arabidopsis* (Herde et al. 2008; van Poecke et al. 2001). The presence of TMTT in odors of lima bean positively influences the foraging behavior of natural enemies of spider mite herbivores feeding on this plant species, suggesting a signaling function for the homoterpene in indirect plant defense against herbivorous arthropods (De Boer et al. 2004). Moreover, TMTT and other terpenoids occurring in blends of herbivore-infested lima bean activate defense gene expression in naïve plants of the same species (Arimura et al. 2000).

Induction of TMTT synthesis after inoculation with ES4326 avrRpm1 is controlled through JA-mediated signaling pathways but is independent of plant SA production and SA-associated defense signaling (Fig. 3). The severely compromised synthesis of TMTT in JA biosynthesis mutants suggests that accumulation of JA is necessary for the production of the homoterpene in the incompatible *Arabidopsis*–*P. syringae* interaction. A low induction of TMTT in the *opr3* mutant, which is able to form the JA biosynthetic precursor OPDA but not JA, also indicates a certain signaling competency for OPDA for TMTT synthesis (Fig. 3A). OPDA and JA are both produced to substantial levels in leaves inoculated with avirulent *P. syringae* (Grun et al. 2007; Zeier et al. 2004), and their pathogen-induced accumulation is thus likely to trigger TMTT production. However, in response to infection with low or medium titers of the compatible ES4326 strain (e.g., optical density (OD) = 0.005 used in this study to trigger volatile emission), increases in leaf JA levels are much less pronounced than in response to avirulent ES4326 avrRpm1. In fact, JA levels do not rise significantly

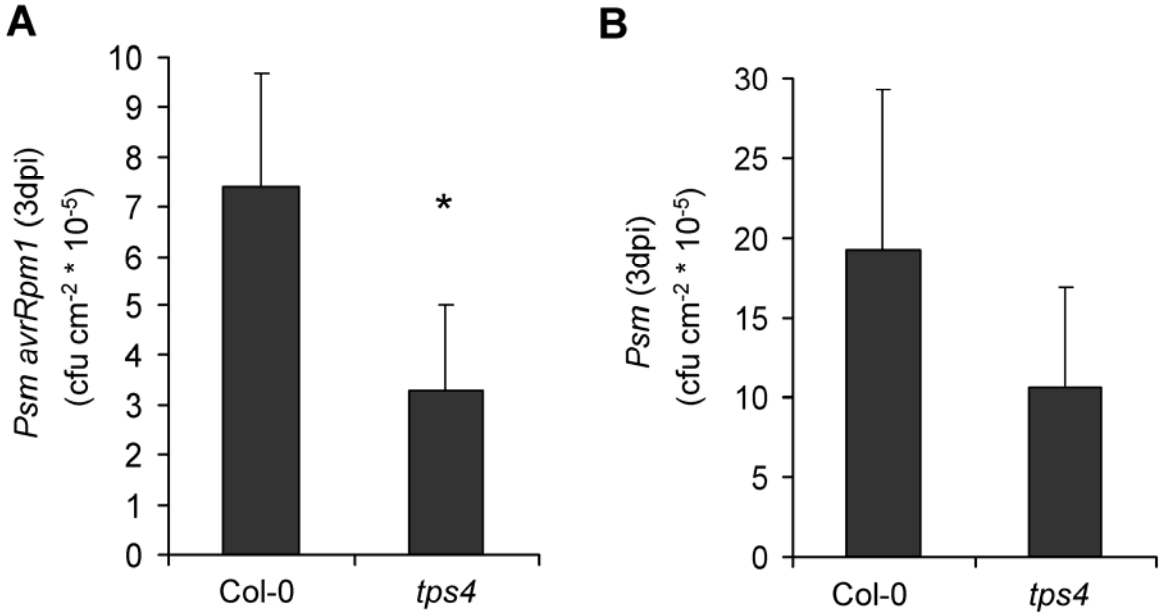

Fig. 6. Specific and basal disease resistance of wild-type Col-0 and *tps4* mutant plants. Bacterial growth quantification of **A,** *P. syringae* pv. *maculicola avrRpm1* (optical density [OD] = 0.005)- and **B,** *P. syringae* pv. *maculicola* (*Psm*) (OD = 0.001)–inoculated leaves of wild-type and *tps4* mutants 3 days after inoculation. Bars represent mean values (± standard deviation) of CFU per square centimeter from at least five parallel samples, each sample consisting of three leaf disks. Asterisk denotes *tps4* value with statistically significant differences to the wild-type value ($P < 0.05$; Student's *t*-test). To ensure the uniformity of infiltrations, initial bacterial numbers (1 h postinoculation [hpi]) were quantified. No significant differences in bacterial numbers were detected at 1 hpi for leaves of different lines (data not shown).

until day 2 after ES4326 inoculation (Mishina et al. in press). This almost excludes a signaling function of JA for TMTT production in the compatible interaction. Here, the phytotoxin and JA mimic coronatine, which is produced by several *P. syringae* pathovars, including ES4326 (Bender et al. 1996), might represent the predominant trigger for TMTT production. Signaling pathways that contribute to control of *P. syringae*–elicited TMTT synthesis downstream of JA or coronatine include both the jasmonate–amino acid synthetase JAR1 and the MYC transcription factor JIN1. The signaling events underlying microbial induction of TMTT synthesis in *Arabidopsis* are similar but not identical to those of herbivore-induced homoterpene synthesis in other species. For instance, induced TMTT production in tomato depends on functional JA biosynthesis. However, unlike wild-type, SA-deficient NahG tomato plants are blocked in TMTT synthesis upon spider mite herbivory, suggesting a requirement of SA signaling (Ament et al. 2006). The octadecanoid pathway also controls TMTT synthesis in

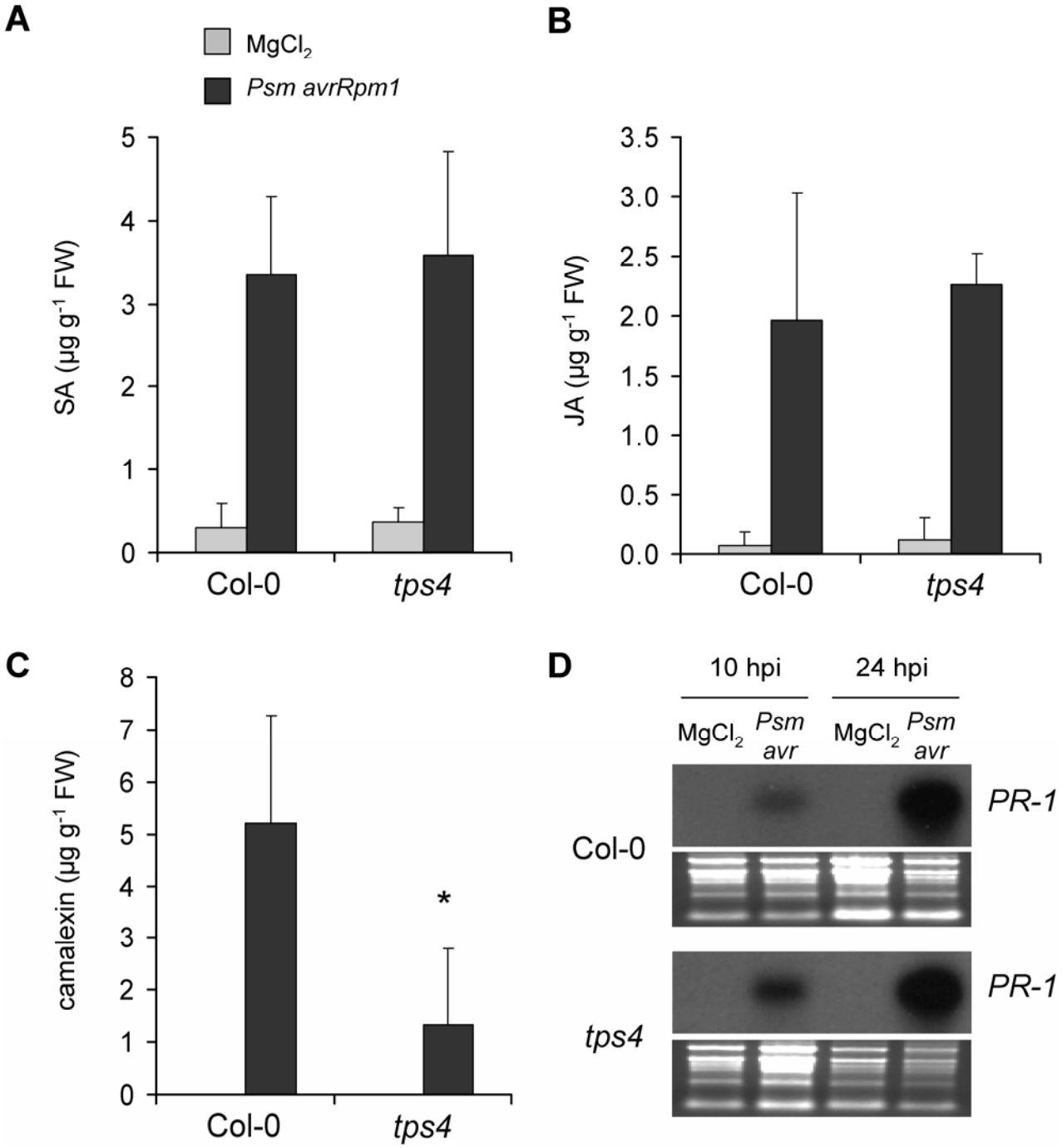

Fig. 7. Defense responses in leaves of wild-type Col-0 and *tps4* mutant plants at sites of *Pseudomonas syringae* pv. *maculicola avrRpm1* (optical density = 0.005) inoculation. Control samples were treated with 10 mM $MgCl_2$. **A,** Salicylic acid (SA) levels at 10 h postinoculation [hpi]. **B,** Jasmonic acid (JA) levels at 10 hpi. **C,** Camalexin accumulation at 10 hpi (camalexin was not detected in control leaves). In **A** through **C,** bars represent mean values (± standard deviation) of three independent samples, each sample consisting of six leaves from two different plants. The asterisk denotes *tps4* value with statistically significant difference to the respective wild-type value ($P < 0.05$; Student's *t*-test). Light bars indicate: $MgCl_2$-treatment and dark bars *P. syringae* pv. *maculicola avrRpm1* inoculation. **D,** Expression of the defense gene *PR-1*, assessed by Northern blot analysis. Control samples were infiltrated with 10 mM $MgCl_2$. Leaf samples were taken at 10 and 24 hpi.

lima bean. Here, exogenous treatment of leaves with the JA precursors linolenic acid and OPDA but not JA itself provoke TMTT emission (Koch et al. 1999). In *Medicago truncatula*, herbivore-induced emission of TMTT and other terpenoids is mediated by a concerted action of JA, ethylene, and calcium signaling (Arimura et al. 2008).

We have detected a remarkable similarity between metabolic changes occurring after *P. syringae* inoculation and $CuSO_4$

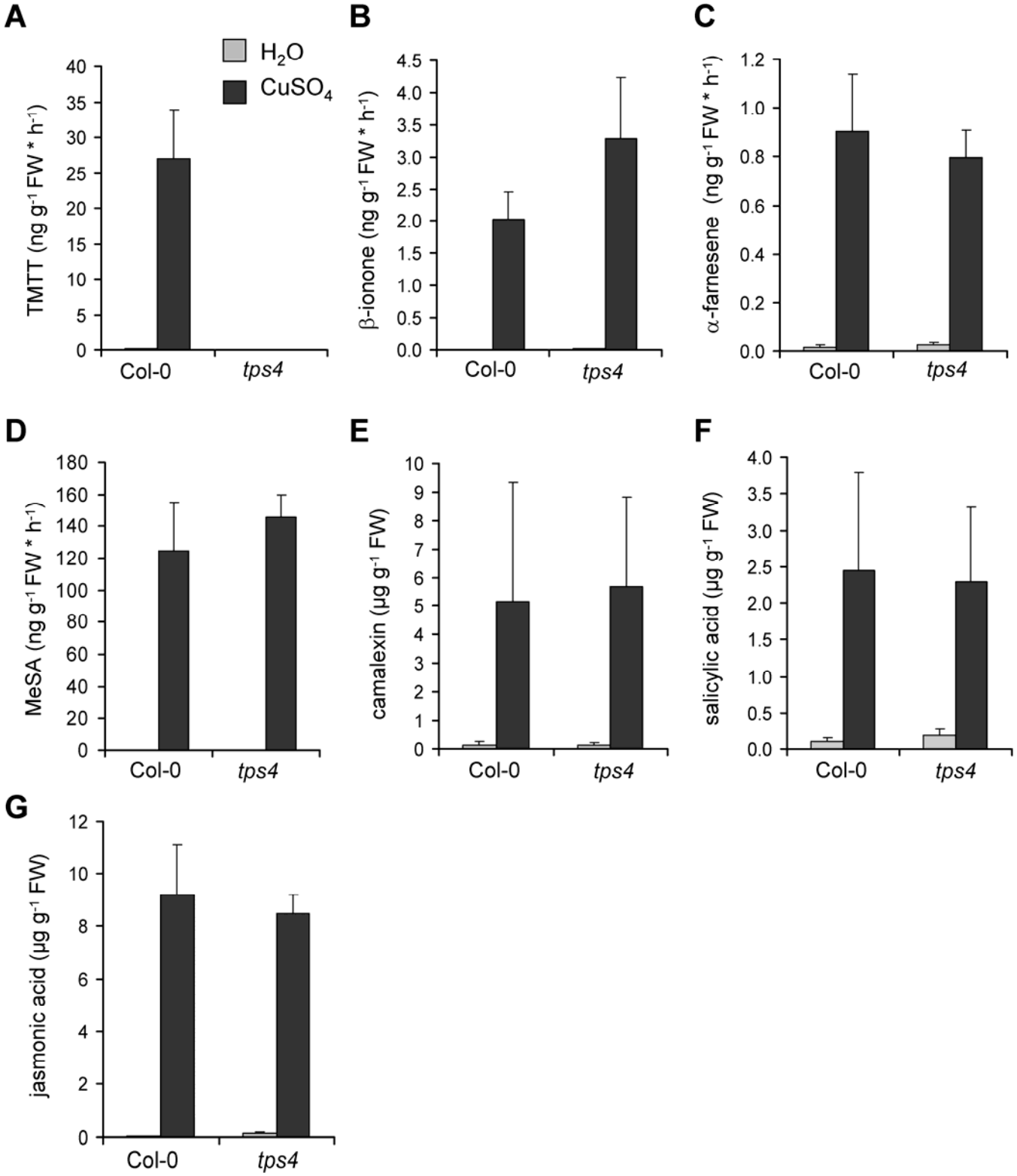

Fig. 8. Induction of leaf volatile organic compounds emission and defense metabolite production upon $CuSO_4$ stress in wild-type Col-0 and *tps4* mutant plants. **A** through **D,** Leaf volatiles were collected for 48 h after infiltration with 10 mM $CuSO_4$ or after infiltration with water as a control treatment. Values are given in nanograms of volatile substance per gram of fresh weight (FW) per h. **A,** E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) emission, **B,** β-ionone emission, **C,** α-farnesene emission, and **D,** methyl salicylate emission. **E** through **G,** Leaf metabolite levels were determined at 48 h posttreatment and are given in micrograms of substance per gram of FW. **E,** Camalexin levels, **F,** salicylic acid levels, and **G**, jasmonic acid levels. Bars represent mean values (±standard deviation) of at least five independent samples. Light bars indicate water infiltration and dark bars infiltration with 10 mM $CuSO_4$.

treatment, which involves induced synthesis of the volatiles TMTT, MeSA, β-ionone, and α-farnesene, as well as increased production of the nonvolatile defense metabolites camalexin, SA, and JA (Fig. 8). At the transcriptional level, such overlapping responses between pathogen-infected and heavy metal–treated plants have been previously reported. For instance, many cytochrome P450 genes, some of which are known to participate in secondary metabolite biosynthesis, are upregulated in *Arabidopsis* by both *Alternaria brassicicola* inoculation and copper stress (Narusaka et al. 2004). Similar to *P. syringae* infection, copper excess leads to increased production of ROS and oxidative stress (Drazkiewicz et al. 2004; Grun et al. 2007). Through activation of expression of genes involved in secondary metabolite production, ROS-induced signaling might thus account for the metabolic changes observed by both treatments. With regard to plant VOC production, it would be interesting to examine whether ROS indeed function as upstream signals in the biosynthesis of terpenoid volatiles and MeSA in future experiments.

Of the four *Arabidopsis* TPS genes upregulated after *P. syringae* infection, TPS4 is most prominently expressed in both incompatible and compatible interactions. Moreover, like other defense reactions in *Arabidopsis*, such as synthesis of SA, accumulation of camalexin, and expression of PR genes, *TPS4* expression is initiated earlier in response to avirulent than to virulent pathogens (Fig. 4). This difference is based on additional recognition events in incompatible interactions that are mediated by specific interaction of pathogen-derived avirulence proteins with plant resistance receptors (Nimchuk et al. 2003). The identified *tps4* knockout line completely fails to show both expression of *TPS4* and induction of TMTT emission. This demonstrates that expression of functional *TPS4* is required for TMTT biosynthesis (Figs. 4, 5, and 8). In planta conversion studies with ^{2}H-labeled precursors strongly suggest that TMTT is synthesized via the diterpene precursor (E,E)-geranyllinalool, which is produced from geranylgeranyl diphosphate (Boland and Gäbler 1989). As a member of the TPS family, TPS4 has been previously suggested to catalyze this

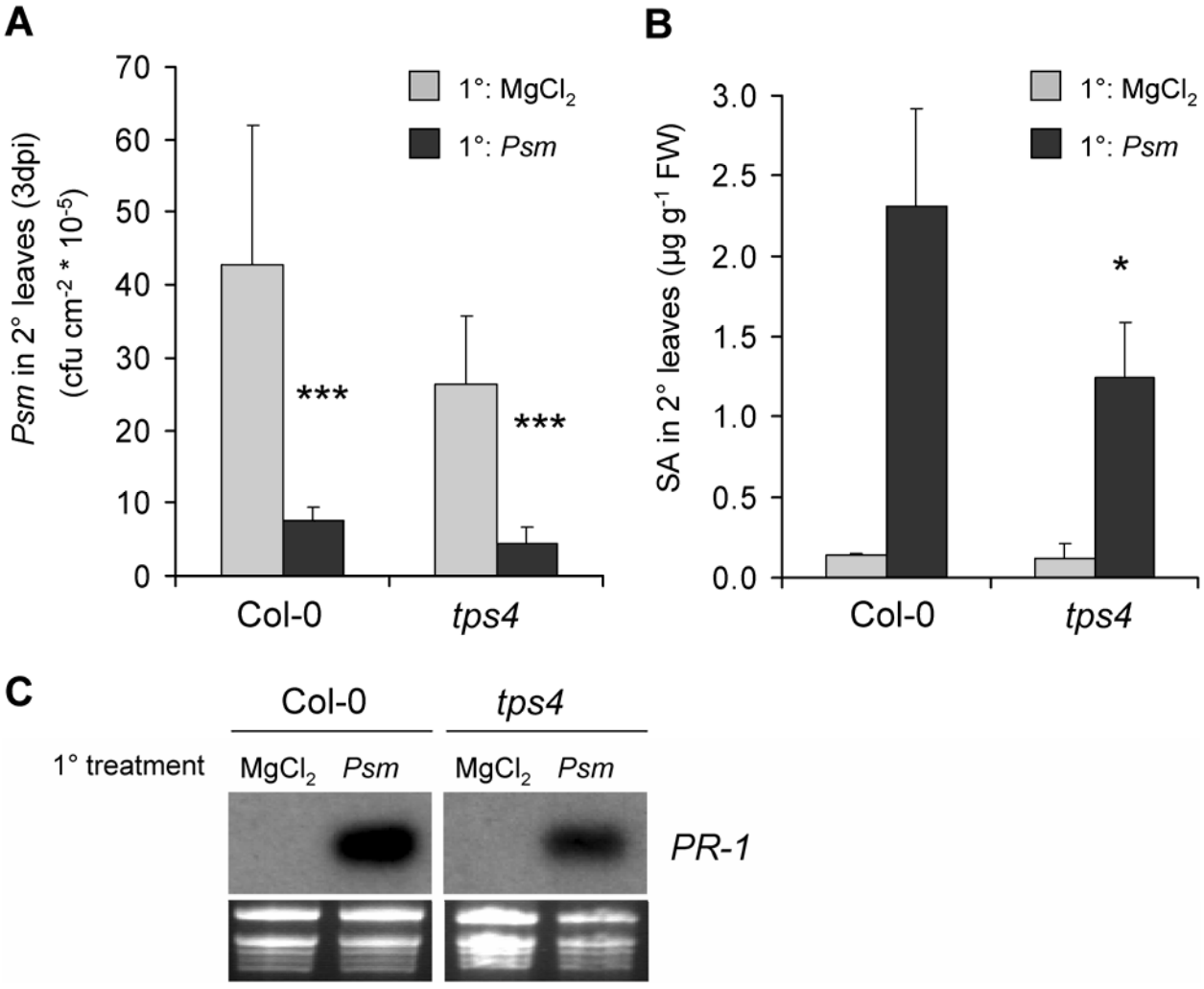

Fig. 9. Systemic defenses in wild-type Col-0 and *tps4* mutant plants. **A,** Bacterial growth quantification to directly assess systemic acquired resistance (SAR). Plants were pretreated with either 10 mM $MgCl_2$ or *Pseudomonas syringae* pv. *maculicola* (*Psm*) (optical density [OD] = 0.01) in three primary leaves (1° treatment), and 2 days later, three systemic (2°) leaves located directly above the primary leaves were inoculated with *P. syringae* pv. *maculicola* (OD = 0.001). Bacterial growth in systemic leaves was assessed 3 days after the 2° inoculation. Bars represent mean values (±standard deviation) of CFU per square centimeter from at least seven parallel samples, each consisting of three leaf disks. Asterisks denote statistically significant differences in growth between plants of a particular line pretreated with *P. syringae* pv. *maculicola* or $MgCl_2$ ($P < 0.001$; Student's *t*-test). **B** and **C,** Accumulation of salicylic acid (SA) and induction of *PR-1* expression in untreated 2° leaves. Treatments of 1° leaves were performed as described for A. The 2° leaves were harvested for analyses 2 days later. **B,** Systemic SA accumulation. Bars represent mean values (±standard deviation) of three independent samples, each sample consisting of six leaves from two different plants. The asterisk denotes statistically significant differences between SAR-induced *Col-0* and *tps4* plants ($P < 0.05$; Student's *t*-test). Light bars indicate 1° $MgCl_2$-treatment and dark bars 1° *P. syringae* pv. *maculicola* inoculation. **C**, Systemic expression of *PR-1*.

latter conversion (Aubourg et al. 2002). During the preparation of our manuscript, Herde and associates (2008) have confirmed TPS4 to act as a geranyllinalool synthase by biochemical characterization of the recombinant protein and analyses TMTT-deficient phenotypes of two independent T-DNA insertion lines, Salk_078187 (used in the current study) and Salk_039864. TPS4 thus catalyzes a first step in the formation of TMTT. The C_{20} carbon chain of geranyllinalool is supposed to be subsequently shortened by other enzymes, e.g., by cytochrome P450 monooxygenases (Dudareva et al. 2004), to yield the C_{16}-compound TMTT.

The wild type–like emission profiles from *TPS10* knockout mutants indicate that TPS10 is neither involved in TMTT production (Fig. 5C) nor in biosynthesis of β-ionone nor α-farnesene (data not shown). When functionally expressed in *Escherichia coli*, TPS10 converts geranyl diphosphate into the acyclic monoterpenes β-myrcene and (E)-β-ocimene (Bohlmann et al. 2000). Although *TPS10* is upregulated by *P. syringae* (Fig. 4A), we were unable to detect these monoterpenes in the volatile blend of ES4326 (±avrRpm1)–infected *Arabidopsis*.

The *tps4* mutant exhibits a growth phenotype indistinguishable from wild type, and except for TMTT production, volatile blends of *tps4* and Col-0 plants are identical. Comparative examination of resistance responses in Col-0 and *tps4* thus allowed us to functionally characterize the relevance of induced TMTT production in disease resistance against microbial pathogens. To our knowledge, this is the first study in which the role of in planta–produced terpenoids in pathogen defense has been directly assessed on a genetic basis.

If at all, untreated *Arabidopsis* plants only produce traces of TMTT, and a substantial emission only occurs after pathogen contact. This accumulation pattern is a characteristic feature of phytoalexins. Phytoalexins are defined as relatively lipophilic low–molecular weight compounds that are produced after contact with microbial pathogens and exhibit antimicrobial activity against fungi or bacteria (Kuć 1995). Because many terpenoids possess antimicrobial properties (Soković and Griensven 2006), we considered the possibility that TMTT might act as an *Arabidopsis* phytoalexin. Sesqui- and diterpenoid phytoalexins exist in several plant species, including tomato, sweet pepper, potato, cotton, and rice (Brooks and Watson 1991). Our bacterial growth data indicate, however, that *tps4* is at least as resistant to avirulent and virulent ES4326 as Col-0 (Fig. 6), which essentially excludes a function for TMTT as a phytoalexin that is effective against *P. syringae*. TMTT might either have no or insufficient bactericidal activity or the apoplast-colonizing bacteria do not come in contact with sufficient amounts of homoterpene vapors. On a fresh-weight base, *P. syringae*-induced TMTT emission from *Arabidopsis* plants is relatively low (10 to 20 ng per gram of fresh weight per h) compared with many other plants. It falls, for example, at least one order of magnitude below the levels of the main terpenoid emitted from herbivore-infested soybean plants (Rostás and Eggert 2008). Alternatively, ES4326 might have evolved strategies to tolerate a potential antimicrobial action of TMTT.

Exogenous application of the monoterpene allo-ocimene primes *Arabidopsis* defense reactions against the fungal pathogen *Botrytis cinerea* (Kishimoto et al. 2006). In a comparable way, TMTT produced upon *P. syringae* inoculation might positively or negatively influence other inducible defense responses. We have shown that *tps4* is not compromised in the production of SA and JA in inoculated leaves, and that *PR-1* expression in the mutant occurs in a wild type–like manner. This indicates that major inducible defense reactions towards *P. syringae* are not primed or otherwise influenced by TMTT in wild-type plants. This statement also holds true for camalexin production, although significantly lower levels of camalexin accumulated upon *P. syringae* challenge in *tps4* than in wild-type leaves. The latter finding can be ascribed to the reduced bacterial multiplication in *tps4*, resulting in attenuated elicitation of camalexin synthesis. $CuSO_4$ intoxication as a more robust abiotic stimulus, by contrast, entailed a wild type–like production of camalexin in *tps4*.

We can also exclude an essential function for TMTT during establishment of SAR, because bacterial-growth assays indicated that *tps4* is able to mount *P. syringae*-induced SAR as effectively as Col-0. The modest reduction of systemic SA and *PR-1* accumulation in *tps4* compared with Col-0 might be cautiously interpreted to mean that TMTT contributes to the realization of systemic defense responses (Fig. 9). However, a scenario in which TMTT emission from lower leaves would prime upper leaves for SAR responses is unrealistic because of the clear SAR response observed in *tps4*. The other major *Arabidopsis* volatile produced after *P. syringae* attack, MeSA, has been recently identified as a critical SAR long-distance signal in tobacco (Park et al. 2007). Whether methyl salicylate is a general SAR signal in *Arabidopsis* and other species has not yet been established.

In summary, we can state that although TMTT synthesis is markedly activated in *Arabidopsis* upon *P. syringae* inoculation, the significance of this response for defense and resistance against the bacterial pathogen is rather low. This is reminiscent of camalexin accumulation which is produced during the first 24 h of the *Arabidopsis–P. syringae* interaction at levels exceeding those of TMTT (approximately 0.25 to 0.5 μg per gram of fresh weight) by one to two orders of magnitude (Mishina and Zeier 2007b). Like TMTT, camalexin is dispensable for resistance against *P. syringae*, as it is for effective defense towards other pathogens with a biotrophic lifestyle (Zhou et al. 1999). Considering the fact that pathogens are recognized in a highly specific manner by plant resistance receptors (Nimchuk et al. 2003), it is surprising that, instead of specifically activating responses that efficiently help to restrict invasion of the particular intruder, plants rather invest nonspecifically in an array of defenses that includes a number of ineffective responses. In other words, a high recognition specificity is followed by a nonspecific, luxurious defense outcome. Our study supports a previously formulated hypothesis that plants can form VOC as byproducts of other processes and, due to their volatility, are emitted to the atmosphere with no apparent function (Holopainen 2004). Activation of JA signaling after microbial infection, herbivore feeding, and heavy-metal stress seems to entail TMTT production in a self-acting manner, regardless of whether or not it is biologically meaningful.

MATERIALS AND METHODS

Plant material and growth conditions.

Arabidopsis thaliana (L.) Heynh. plants were grown on an autoclaved mixture of soil (Klasmann, Beetpfanzensubstrat Typ R.H.P.16), vermiculite, and sand (10:0.5:0.5). Plants were cultivated in a controlled environmental chamber (J-66LQ4; Percival, Boone, IA, U.S.A.) with a 9-h day (photon flux density 70 μmol m^{-2} s^{-1}) and 15-h night cycle and a relative humidity of 70%. Growth temperatures were set to 21°C during the day period and to 18°C during the night. Naïve and unstressed 6-week-old plants showing a uniform appearance were used for experiments.

The *tps4* mutant line represents the Salk T-DNA insertion line SALK_078187, and *tps10-1* and *tps10-2* mutants originate from lines SALK_108420 and SALK_041114, respectively. All *Arabidopsis* insertion lines are in the Col-0 background. Homozygous insertion mutants were identified by PCR, using gene-specific (*TPS4*-forward: 5′-GCGTACGACAAGTATTTG

CAG-3′, *TPS4*-reverse: 5′-AAGTTCACGGCC-TAATGCTTC-3′, *TPS10-1*-forward: 5′-CATGGAAACTTGCATGT-GTTG-3′, *TPS10-1*-reverse: 5′-TTTGTTCATGCATATATACCAGCT C-3′, *TPS10-2*-forward: 5′-AATTCAA-CGACGACAAGGTTC-3′, *TPS10-2*-reverse: 5′-TTCAATATGGC-CACTCTCCTG-3′) and T-DNA-specific primers according to Alonso and associates (2003). The examined JA pathway mutants *dde2-2* (von Malek et al. 2002), *opr3* (Stintzi and Browse 2000), and *jin1* (Berger et al. 1996) have Col-0, Ws, and Col-3 backgrounds, respectively. All other defense mutants used in the present study are described in Mishina and Zeier (2007b).

Cultivation of bacteria.

ES4326 lacking or carrying the *avrRpm1* avirulence gene were grown in King's B medium containing the appropriate antibiotics at 28°C (Zeier et al. 2004). Overnight log phase cultures were washed three times with 10 mM $MgCl_2$ and were diluted to different final OD for leaf inoculations.

Collection of volatiles.

To assess *P. syringae*–induced plant volatile emission, bacterial suspensions at OD 0.01 were infiltrated from the abaxial side into seven full-grown rosette leaves per *Arabidopsis* plant, using a 1-ml syringe without a needle. Control treatments were performed by infiltrating a 10-mM $MgCl_2$ solution. For copper sulfate treatments, leaves were infiltrated with a solution of 10 mM $CuSO_4$.

Volatiles emitted by individual plants were collected in a push-pull apparatus essentially as described by Rostás and associates (2006). The system consisted of six independent circular glass chambers (13 cm in diameter, 12 cm in height) that allowed for simultaneous collection. Plants were placed in chambers about 30 min after leaf infiltrations, and trapping filters consisting of glass tubes packed with Super-Q absorbent (VCT-1/4X3-SPQ, Analytical Research Systems, Gainsville, FL, U.S.A.) were attached in a way so that the tip of each filter were a distance of 1 cm from each plant rosette. Charcoal-filtered and humidified air was pushed into each sampling chamber at a rate of 1.2 liters per min. The air flow containing plant volatiles was pulled through the trapping filter with a vacuum pump (ME2; Vacuubrand, Wertheim, Germany). After collecting volatiles for 10 to 24 h, trapping filters were removed, extracted, and analyzed as described below.

Chemical analysis of volatile extracts.

Trapping filters were eluted with 1 ml CH_2Cl_2 after each collection, and 200 ng of n-octane was added as internal standard. The mixture was concentrated to a volume of 25 µl under a gentle stream of nitrogen, strictly avoiding evaporation to dryness, and was analyzed by gas chromatography–mass spectrometry. Three-microliter aliquots of the sample mixture were separated on a gas chromatograph (6890N; Agilent Technologies, Waldbronn, Germany) that was equipped with a split-splitless injector and a fused silica capillary column (HP-1; 30 m × 0.25 mm ID, 0.25-µm film thickness) and were combined with a 5975 mass spectrometric detector (Agilent Technologies). Samples were injected in pulsed splitless mode, and helium was used as a carrier gas. The temperature of the oven was held at 50°C for 2 min and then was increased at 8°C per min to 300°C. Mass spectra were recorded at 70 eV. Substances were identified by comparison of mass spectra with those from the NIST 98 reference library. Compound identities were confirmed by comparison of mass spectra and retention times with those of standard substances. To allow sensitive quantification of volatiles, substance peaks originating from selected ion chromatograms were integrated (generally m/z 81 for TMTT and α-farnesene, m/z 177 for β-ionone, m/z 120 for MeSA). The resulting peak areas were related to the peak area of the n-octane standard (ion chromatogram m/z 114), whereby appropriate correction factors were considered for each substance.

Characterization of local and systemic resistance responses.

For the determination of local defense responses, bacterial suspensions at OD 0.005 (determination of gene expression, metabolite levels, ES4326 avrRpm1 growth assay) or OD 0.001 (ES4326 growth assays) were infiltrated into three full-grown leaves per plant. Bacterial growth was assessed 3 days after infiltration by homogenizing disks originated from infiltrated areas of three different leaves in 1 ml of 10 mM $MgCl_2$, plating appropriate dilutions on King's B medium, and counting colony numbers after incubating the plates at 28°C for 2 days.

For SAR experiments, plants were first infiltrated into three lower (1°) leaves with a suspension of ES4326 (OD = 0.01) or with 10 mM $MgCl_2$ as a control treatment. Two days after the primary treatment, upper (2°) leaves were either harvested for SA determination and gene expression analysis or were inoculated with virulent ES4326 (OD 0.001). Growth of ES4326 in 2° leaves was scored another 3 days later.

Determination of leaf SA, JA, and camalexin levels.

Determination of SA, JA, and camalexin levels in leaves was realized by vapor-phase extraction and subsequent gas chromatography-mass spectrometry analysis according to Mishina and Zeier (2006).

Analysis of gene expression.

Expression levels of *PR-1* (At2g14610) and *TPS4* (At1g61120) were determined by Northern blot analysis as described by Mishina and Zeier (2006).

ACKNOWLEDGMENTS

We thank T. Turlings and M. Held for providing TMTT standard, and S. Berger for donation of *Arabidopsis dde2*, *opr3*, and *jin1* mutant seeds. Financial support from the Deutsche Forschungsgemeinschaft (Graduiertenkolleg 1342) is gratefully acknowledged. E. Attaran is a Deutscher Akademischer Austauschdienst fellow.

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AUTHOR RECOMMENDED INTERNET RESOURCE

Salk Institute Genomic Analysis Laboratory T-DNA express *Arabidopsis* gene mapping tool:
signal.salk.edu/cgi-bin/tdnaexpress

PUBLICATION 2

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Methyl salicylate production and jasmonate signaling are not essential for systemic acquired resistance in Arabidopsis

The Plant Cell (2009) 21: 954-971

Thesis author's contribution:

Conception: 30 %
Experimental contribution: 80 %
Formulation of results: 30 %

This article is a *Plant Cell* Advance Online Publication. The date of its first appearance online is the official date of publication. The article has been edited and the authors have corrected proofs, but minor changes could be made before the final version is published. Posting this version online reduces the time to publication by several weeks.

Methyl Salicylate Production and Jasmonate Signaling Are Not Essential for Systemic Acquired Resistance in *Arabidopsis* [W]

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Systemic acquired resistance (SAR) develops in response to local microbial leaf inoculation and renders the whole plant more resistant to subsequent pathogen infection. Accumulation of salicylic acid (SA) in noninfected plant parts is required for SAR, and methyl salicylate (MeSA) and jasmonate (JA) are proposed to have critical roles during SAR long-distance signaling from inoculated to distant leaves. Here, we address the significance of MeSA and JA during SAR development in *Arabidopsis thaliana*. MeSA production increases in leaves inoculated with the SAR-inducing bacterial pathogen *Pseudomonas syringae*; however, most MeSA is emitted into the atmosphere, and only small amounts are retained. We show that in several *Arabidopsis* defense mutants, the abilities to produce MeSA and to establish SAR do not coincide. T-DNA insertion lines defective in expression of a pathogen-responsive SA methyltransferase gene are completely devoid of induced MeSA production but increase systemic SA levels and develop SAR upon local *P. syringae* inoculation. Therefore, MeSA is dispensable for SAR in *Arabidopsis*, and SA accumulation in distant leaves appears to occur by de novo synthesis via isochorismate synthase. We show that MeSA production induced by *P. syringae* depends on the JA pathway but that JA biosynthesis or downstream signaling is not required for SAR. In compatible interactions, MeSA production depends on the *P. syringae* virulence factor coronatine, suggesting that the phytopathogen uses coronatine-mediated volatilization of MeSA from leaves to attenuate the SA-based defense pathway.

INTRODUCTION

Systemic acquired resistance (SAR) is an enhanced state of broad-spectrum disease resistance that develops in the whole plant in response to a locally restricted leaf inoculation with microbial pathogens (Métraux et al., 2002; Durrant and Dong, 2004). Induction of SAR occurs at the site of pathogen inoculation where presumed mobile long-distance signals are generated. The latter are thought to be subsequently transferred to and perceived in distant, noninfected plant parts. Therein, they are supposed to initiate signaling and amplification processes that lead to an increase of systemic defense responses to boost whole-plant resistance (Mishina and Zeier, 2006).

Induction of SAR is not restricted to hypersensitive response (HR)-inducing or necrotizing pathogens but also takes place upon leaf contact with high inoculi of nonpathogenic microbes or after local treatment with bacterial pathogen-associated molecular patterns, such as flagellin or lipopolysaccharides (Mishina and Zeier, 2007). Irrespective of the eliciting stimulus, the molecular events set in motion in inoculated leaves to initiate SAR in distant leaves are only partially understood. The recent finding that ectopic expression of *Arabidopsis thaliana* mitogen-activated protein kinase kinase7 in local tissue induces pathogenesis-related (PR) gene expression and resistance to *Pseuodmonas syringae* in systemic tissue indicates that mitogen-activated protein kinase-based signaling cascades are involved in the initiation of SAR long-distance signaling (Zhang et al., 2007). However, the chemical nature of putative mobile SAR signals remains elusive (Vlot et al., 2008a).

Mutational analyses in *Arabidopsis* suggest that peptide and lipid derivatives participate in signal transduction from inoculated to distant leaves (Grant and Lamb, 2006; Chaturvedi et al., 2008). A peptide signal might be generated by the apoplastic aspartic protease CONSTITUTIVE DISEASE RESISTANCE1, which is required for the execution of both local and systemic resistance responses (Xia et al., 2004). Moreover, DEFECTIVE IN INDUCED RESISTANCE1 (DIR1) bears homology to lipid transfer proteins and is involved in local generation or subsequent translocation of a mobile systemic signal, possibly by acting as a chaperone for a lipid-related signal (Maldonado et al., 2002). A glycerolipid-derivative might be a DIR1-interacting partner because the dihydroxyacetone phosphate reductase SUPPRESSOR OF FATTY ACID DESATURASE ACTIVITY1 (Nandi et al., 2004) and the fatty acid desaturase FAD7, both components of plastid glycerolipid biosynthesis, are necessary for SAR establishment and, together with DIR1, are required for the accumulation of a SAR-inducing activity in *Arabidopsis* petiole exudates (Chaturvedi et al., 2008). Moreover, the plant defense hormone jasmonic acid (JA) or a JA pathway-related oxylipin was proposed as the signal mediating long-distance information transmission during SAR (Truman et al., 2007). JA-mediated signaling is well established to participate in induced plant resistance against both insect herbivory and attack by necrotrophic

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[W] Online version contains Web-only data.
www.plantcell.org/cgi/doi/10.1105/tpc.108.063164

pathogens, but its role in defense against biotrophic microbial pathogens is less well defined (Li et al., 2002; Glazebrook, 2005).

It has been known for more than a decade that salicylic acid (SA) acts as a major player during the establishment of SAR. SA accumulates both at inoculation sites and in distant leaves concomitant with the onset of SAR, and transgenic, SA hydroxylase (NahG) expressing plants not capable of SA accumulation are SAR deficient (Malamy et al., 1990; Métraux et al., 1990; Gaffney et al., 1993). The requirement for intact SA signaling during SAR is underlined by the failure of the *Arabidopsis* mutants *salicylic acid induction-deficient1* (*sid1*) and *sid2*, which are both defective in induced SA production, to enhance systemic resistance after pathogen infection. *SID1* and *SID2* code for a multidrug and toxic compound extrusion transporter protein and isochorismate synthase1 (ICS1), respectively (Nawrath and Métraux, 1999; Wildermuth et al., 2001; Nawrath et al., 2002). Grafting experiments using root stocks and scions from wild-type and NahG-expressing tobacco (*Nicotiana tabacum*) have indicated that SA itself is not a long-distance signal but that SA accumulation in distant leaves is critical for SAR (Vernooij et al., 1994).

SA can be biochemically modified to derivatives with altered physicochemical properties and bioactivity (Wildermuth, 2006). UDP-dependent SA-glucosyl-transferases transfer a glucose moiety to either the phenolic hydroxyl group or to the carboxyl group of SA, yielding the hydrophilic SA derivatives SA 2-*O*-β-D-glucose (SA glucoside [SAG]) or SA glucose ester (Lee and Raskin, 1999; Lim et al., 2002; Dean and Delaney, 2008). SAG, the most prominent glucosylated form of SA in many plant species, is produced from accumulating SA after pathogen infection (Malamy et al., 1992; Mishina et al., 2008). Furthermore, methylation of the free carboxyl group of SA yields the nonpolar and volatile SA methyl ester (methyl salicylate [MeSA]; Wildermuth, 2006). This reaction is catalyzed by SA methyl transferase (SAMT), which uses *S*-adenosine-L-methionine as methyl donor (Ross et al., 1999). In *Arabidopsis*, the *BSMT1* gene codes for a protein with both benzoic acid and SA methylating activities (Chen et al., 2003). *BSMT1* is highly expressed in flowers, and expression in leaves is upregulated by treatment with the antibiotic alamethicin, by methyl jasmonate application, and by herbivory. MeSA is a significant constituent of floral scents from various plant species and of volatile blends from herbivore-attacked vegetative plant parts, and it functions in pollinator attraction and defense against insects (Van Poecke et al., 2001; Effmert et al., 2005; Zhu and Park, 2005). Concomitant with SA biosynthesis, MeSA is produced in pathogen-infected tobacco and *Arabidopsis* leaves and emitted to significant amounts into the environment (Shulaev et al., 1997; Koo et al., 2007; Attaran et al., 2008).

Pathogen-elicited MeSA has been previously proposed as being an airborne signal involved in plant-to-plant communication (Shulaev et al., 1997). More recently, grafting experiments suggested that MeSA is a critical, phloem-mobile SAR long-distance signal in tobacco (Park et al., 2007). A model has been proposed in which the SA accumulating after tobacco mosaic virus (TMV) infection is converted to MeSA by SA methyl transferase (SAMT1) in inoculated tobacco leaves, and MeSA subsequently travels through the phloem to distant leaves. Here, by the methyl esterase activity of SA binding protein2 (Forouhar et al., 2005), MeSA is reconverted to active SA, which in turn triggers SAR in systemic tissue (Park et al., 2007). In addition to its movement through the phloem, MeSA has been suggested to act as a volatile intraplant signal that is capable of activating SAR in distant leaves of the same plant (Shulaev et al., 1997). Another recent study extended this putative signaling function of MeSA to SAR in *Arabidopsis* (Vlot et al., 2008b). In this species, 18 potentially functional methyl esterase genes exist, out of which five encode proteins with MeSA demethylase activity (Yang et al., 2008; Vlot et al., 2008b). Attempts to silence these five redundant methyl esterase genes by a combination of T-DNA knockout and RNA interference silencing strategies resulted in different transgenic lines with partial but not complete abrogation of SA methyl esterase expression. The failure of some of these lines to mount *P. syringae*–induced SAR was taken as supportive evidence for the notion that MeSA represents a universal mobile SAR signal in plants (Vlot et al., 2008a, 2008b).

In this study, we address the significance of MeSA during biologically induced SAR in *Arabidopsis*. We show that MeSA production strongly increases in leaves inoculated with SAR-inducing strains of *P. syringae* and that most of the generated MeSA is directly emitted into the atmosphere. Moreover, the SAR-deficient phenotype of several *Arabidopsis* defense mutants is not caused by a failure of MeSA production. Significantly, mutational defects in the *Arabidopsis* SA methyl transferase gene *BSMT1* completely abolish pathogen-induced MeSA production but do not affect SAR. Together, these data show that MeSA production is dispensable for SAR in *Arabidopsis* and that the systemic increase in SA, which is crucial for SAR, is not based on translocation of MeSA from inoculated to distant leaves. Instead, our findings support the hypothesis that the systemic rises in SA occur via de novo synthesis in distant leaves. Our data also show that MeSA biosynthesis is largely regulated via the JA pathway but exclude a role for JA signaling in SAR establishment. Since MeSA production in compatible interactions largely depends on the capability of *P. syringae* to produce the bacterial phytotoxin coronatine, a possible virulence mechanism of this phytopathogen includes volatilization of MeSA from leaves to negatively interfere with SA-associated defense responses.

RESULTS

The bacterial plant pathogen *P. syringae* pv *maculicola* ES4326 (*Psm*) is able to rapidly multiply in apoplastic spaces of *Arabidopsis* leaves, thereby causing yellowish disease symptoms (Dong et al., 1991). Leaf inoculation of accession Columbia-0 (Col-0), which carries the *Rpm1* resistance gene with *Psm* expressing the avirulence gene *AvrRpm1* (*Psm avrRpm1*), by contrast, elicits an HR associated with rapid cell death at inoculation sites (Bisgrove et al., 1994; Delledonne et al., 1998). Early defense responses associated with the HR do not fully abrogate but significantly restrict bacterial multiplication. Both virulent *Psm* and avirulent *Psm avrRpm1* trigger a robust SAR response in Col-0 plants (Mishina and Zeier, 2006; 2007).

Production and Fate of MeSA after Pathogen Attack

To assess the significance of MeSA during local and systemic resistance induction in *Arabidopsis* and its role in long-distance

transport, we first determined leaf MeSA production upon *P. syringae* inoculation. Because of the volatile nature of MeSA, leaf emission of volatile organic compounds (VOCs) was determined from intact plants (Attaran et al., 2008). Following leaf inoculation with the avirulent *Psm avrRpm1* strain, MeSA emission of Col-0 plants was not elevated before 6 h after inoculation (HAI) but strongly increased to ~15 ng g^{-1} leaf fresh weight (FW) h^{-1} between 6 and 10 HAI compared with $MgCl_2$-infiltrated control plants (Figure 1A). The release of MeSA further increased to 45 ng g^{-1} h^{-1} from between 10 and 24 HAI and then gradually decreased during the next 48 h of sampling. Comparatively, when plants were infected with virulent *Psm*, MeSA emission was delayed and not detectable before 10 HAI (Figure 1B). However, the quantity of emitted MeSA between 10 and 48 HAI was about one order of magnitude higher in the compatible than in the incompatible interaction, reaching values between 240 and 500 ng g^{-1} h^{-1}. This strong MeSA release markedly declined after 2 d after inoculation (DAI). Emission of MeSA in mock-infiltrated control plants was low throughout the entire sampling period (0.2 to 0.9 ng g^{-1} h^{-1}; Figures 1A and 1B). MeSA was the major *Arabidopsis* VOC induced after *P. syringae* infection. In addition, a significant amount of the volatile homoterpene (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) was emitted upon inoculation with both *Psm* and *Psm avrRpm1*, and lower increases in the amounts of the terpenes β-ionone and α-farnesene as well as of methyl benzoate were detected in the VOC blends during later stages of the compatible interaction (Attaran et al., 2008).

In addition to analyzing the MeSA vaporizing from leaves, we also determined its actual content in control and pathogen-inoculated leaf tissue through solvent extraction followed by gas chromatography–mass spectrometry (GC-MS) analysis (Figure 1C). While mock-treated leaves contained between 0.8 and 2.5 ng MeSA g^{-1}, the MeSA content was significantly higher in leaves inoculated with *Psm avrRpm1*, amounting to 17 and 24 ng g^{-1} at 10 and 24 HAI, respectively. Accordingly, the absolute value of MeSA retained in leaves after *Psm avrRpm1* inoculation equaled the amount emitted from leaves within ~30 min (Figure 1A).

An important requirement for SAR development is the accumulation of SA in distant, noninoculated leaves (Vernooij et al., 1994). Since systemic SA accumulation was proposed to be associated with phloem-based MeSA translocation from inoculated to distant leaves and subsequent MeSA to SA conversion (Park et al., 2007), we assessed MeSA emission and content systemically (i.e., in nontreated, distant leaves of pathogen-inoculated plants). A modest but statistically significant increase in emission of MeSA was observed in distant leaves after a remote *Psm* attack compared with a respective mock treatment (Figure 1D). However, emission rates from distant leaves were two to three orders of magnitude lower than the rates detected in pathogen-treated leaves and fell in the same range as those measured from $MgCl_2$-infiltrated control leaves (Figures 1A and 1B). Moreover, the leaf contents of MeSA in nontreated, distant leaves of remotely *Psm*-inoculated plants (Figure 1E) were similar to those of $MgCl_2$-infiltrated leaves (Figure 1C), and no significant differences in MeSA contents of systemic leaves existed between mock- and *Psm*-pretreated plants (Figure 1E).

In addition, we analyzed MeSA contents in petiole exudates collected from 6 to 48 HAI in mock- and pathogen-inoculated leaves. During this time period, a marked SAR response develops in Col-0 plants upon inoculation with the used inoculation density of *Psm* (OD 0.01), which is accompanied with systemic rises of 1 to 2 μg g^{-1} SA (Mishina and Zeier, 2007; Mishina et al., 2008). With 1.2 ng MeSA g^{-1} h^{-1}, *Psm*-inoculated leaves exhibited a threefold higher exudation of MeSA from petioles than control leaves (see Supplemental Figure 1A online). However, these values might underestimate the actual MeSA exudation, as a fraction of the volatile could have escaped into the atmosphere during the exudate collection period. Nevertheless, these values are in the same order of magnitude as the MeSA levels estimated in exudates from tobacco leaves (Park et al., 2007). We also detected and quantified free and glucosidic SA in the collected petiole exudates, and both SA forms were found in similar scales in the exudates as MeSA. Whereas exudation of SAG from petioles increased from 1.1 to 4.0 ng g^{-1} h^{-1} upon *Psm* inoculation (see Supplemental Figure 1B online), leaf pathogen treatment did not significantly alter the levels of exuded free SA. The latter was released to ~1 ng g^{-1} h^{-1} from both mock- and *Psm*-treated leaves (see Supplemental Figure 1C online).

In summary, these quantitative analyses show that MeSA production strongly increases in *P. syringae*–inoculated *Arabidopis* leaves. During the first 24 HAI, ~0.75 μg g^{-1} MeSA are produced in the incompatible interaction, whereas 3.5 μg g^{-1} are generated in the compatible interaction. However, most (97%) of the MeSA is directly emitted into the atmosphere, and only minor amounts are retained in leaves (Figure 1F). Lower amounts of MeSA and SAG but not of free SA also accumulate in petiole exudates after pathogen infection. The calculated sum of estimated MeSA and detected SAG exuded during a 48-h SAR induction period (~0.15 μg g^{-1}) falls well below the usually observed systemic rises in SA (1 to 2 μg g^{-1}; Mishina and Zeier, 2007; Mishina et al., 2008). Moreover, in leaves distant from pathogen attack, the content of MeSA is not elevated and its emission increases only marginally.

SA and MeSA Production in SAR-Deficient *Arabidopsis* Lines

SAR is fully compromised in the *Arabidopsis* SA biosynthesis mutant *ics1* (*sid2*), in the SA degrading NahG line, and in mutants of *NON-EXPRESSOR OF PR1* (*NPR1*), which encodes a regulatory protein acting downstream of SA (Cao et al., 1994; Delaney et al., 1995; Lawton et al., 1995; Nawrath and Métraux, 1999). Moreover, mutants defective in *NON RACE-SPECIFIC DISEASE RESISTANCE1* (*NDR1*), *FLAVIN-DEPENDENT MONOOXYGENASE1* (*FMO1*), and *PHYTOALEXIN-DEFICIENT4* (*PAD4*) are also SAR deficient (Shapiro and Zhang, 2001; Mishina and Zeier, 2006, 2007). A general hallmark of these SAR-defective lines is that, unlike SAR-competent Col-0 plants, they do not accumulate SA in distant leaves after a local inoculation with *P. syringae* (Figure 2A). However, except for the SA biosynthesis-defective *sid2* mutant and the SA nonaccumulating NahG line, these lines do produce SA in *Psm avrRpm1*-inoculated leaves to wild-type-like levels, or in the case of *npr1*, to levels even exceeding those of wild-type Col-0 (Figure 2B). These findings reflect the requirement of systemic but not local SA accumulation for SAR development, and they might be explained in two ways. The first

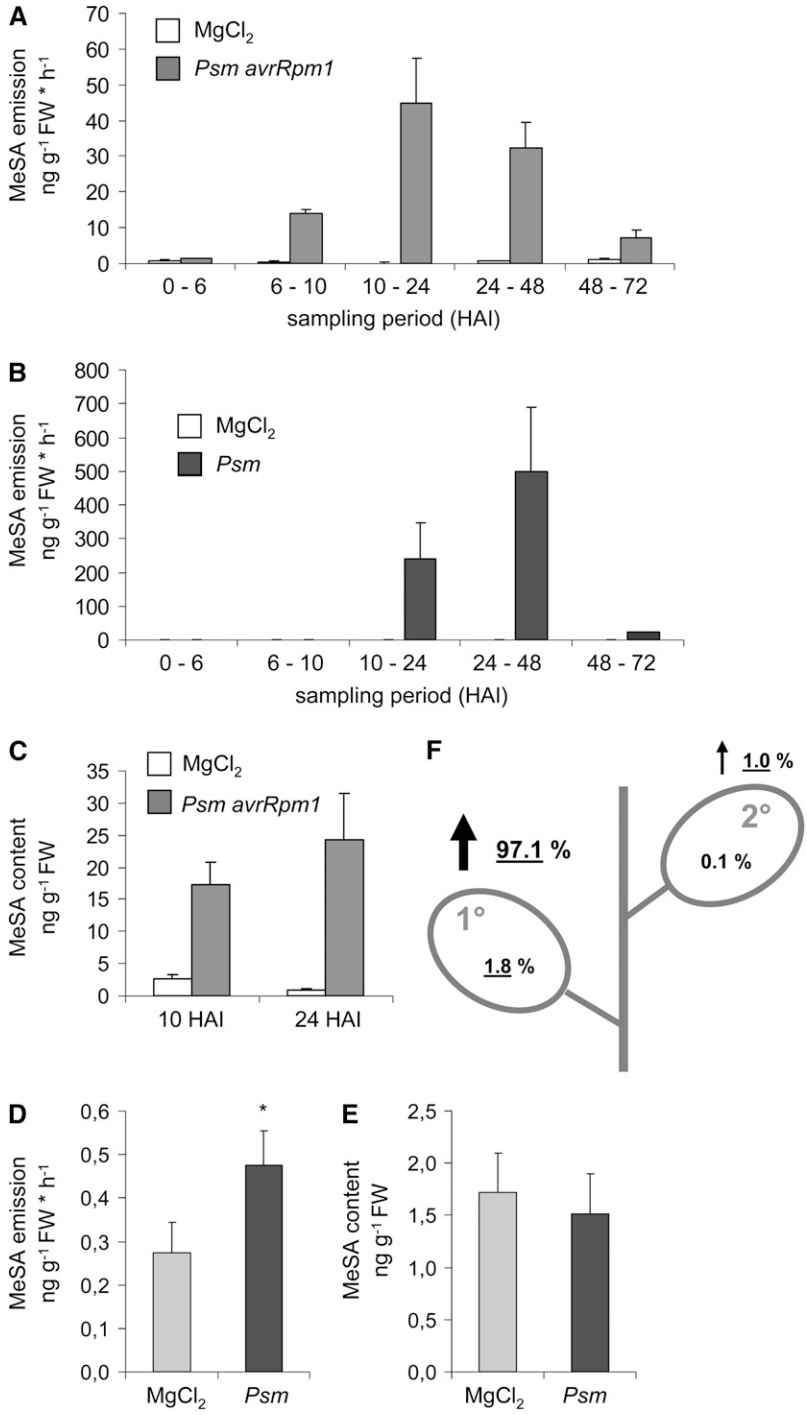

Figure 1. Leaf MeSA Production in *Arabidopsis* Col-0 Plants upon *P. syringae* Inoculation.

(A) and **(B)** Time course of MeSA emission after inoculation with HR-inducing *Psm avrRpm1* (gray bars) **(A)**, inoculation with compatible *Psm* (black bars) **(B)**, or infiltration with 10 mM $MgCl_2$ (white bars). Mean values of ng emitted substance g^{-1} leaf FW h^{-1} (±SD) from three independent plants are given. The time periods during which volatiles were collected are indicated. HAI, h after inoculation.

(C) Leaf MeSA contents in response to inoculation with *Psm avrRpm1* (gray bars) or infiltration with 10 mM $MgCl_2$ (white bars) at 10 and 24 HAI (means ± SD, $n = 3$).

(D) Emission of MeSA from nontreated, distant leaves of *Psm*-inoculated or $MgCl_2$-infiltrated Col-0 plants. Treated leaves were removed at the onset of SAR (at 2 DAI), and emission of the remainder of the plant was sampled from 2 to 3 DAI. Mean values of ng emitted MeSA g^{-1} leaf FW h^{-1} (±SD, $n = 5$) are given. Asterisk denotes statistically significant differences between *Psm* and $MgCl_2$ treatments ($P < 0.05$).

scenario is that the systemic rises in SA that normally occur during SAR in wild-type plants are generated by de novo synthesis in distant leaves. The second possibility is that the SA accumulating in inoculated leaves is transported to distant leaves in free or derivatized form in the wild type but that this translocation is blocked in the different SAR-defective mutants. If MeSA were the translocated SA derivative (Park et al., 2007), a failure of the SAR-deficient lines to produce MeSA would explain the lack of systemic SA accumulation in these mutants (Figure 2A). We therefore tested whether the SAR-defective lines under investigation were defective in MeSA production after *Psm avrRpm1* inoculation. However, except for *sid2* plants, which emitted low but still increased levels of MeSA after pathogen treatment and the NahG line in which MeSA emission was nearly abolished, all the other SAR-defective lines emitted considerable amounts of MeSA after *Psm avrRpm1* inoculation (Figure 2C). These data support the hypothesis that the majority of MeSA produced after pathogen inoculation is derived from SA synthesized by ICS1 and, more significantly for this study, indicate that the biosynthesis of MeSA is not impaired in several independent SAR-defective mutants.

Arabidopsis bsmt1 Mutants Do Not Elevate MeSA after Pathogen Inoculation but Are SAR Competent

Arabidopsis BSMT1 has been previously identified as a methyl transferase with in vitro activity for SA to MeSA conversion (Chen et al., 2003). Expression of the *BSMT1* gene in Col-0 leaves is virtually absent in mock-treated plants but is upregulated in response to *P. syringae* infection (Figure 3A). Whereas leaves inoculated with the incompatible *Psm avrRpm1* strain induce expression of *BSMT1* from 6 HAI onwards, expression of the gene in response to compatible *Psm* was slower but reached high values at 24 HAI. Thus, the temporal pattern and strength of leaf *BSMT1* expression during the incompatible and the compatible *P. syringae*–Col-0 interaction closely resemble the relative timing and magnitude of MeSA emission (Figures 1A and 1B). This suggests that BSMT1 is directly involved in *P. syringae*–induced MeSA production.

The T-DNA Express Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress) predicts several lines with putative T-DNA insertions in the *BSMT1* gene. We applied the PCR-based protocol described by Alonso et al. (2003) to confirm the predicted insertions and identified two lines, SALK_140496 and WiscDSLox430E05, which indeed harbor the T-DNA insert within the *BSMT1* gene (Figure 3B). Lines homozygous for the insert, from now on designated as *bsmt1-1* and *bsmt1-2*, do not exhibit any basal or pathogen-induced expression of *BSMT1* (Figure 3C). Analyses of VOC emission from mock- and *Psm avrRpm1*–treated Col-0 or *bsmt1* mutant plants revealed that MeSA was absent in blends of both *bsmt1-1* and *bsmt1-2* (Figures 4A and 4B). Moreover, the significant increase in leaf MeSA content that was detected in Col-0 upon *P. syringae* inoculation was not observed in *bsmt1* mutant plants. The latter showed marginal basal leaf contents of MeSA, which were lower than those of noninoculated Col-0 controls and close to the analytical detection limit of ~0.5 to 1 ng g^{-1} FW. These data demonstrate that BSMT1 is exclusively responsible for pathogen-induced MeSA production in Col-0 and suggest that a fraction of the already low basal MeSA levels might be produced independently from BSMT1. Compared with the wild type, neither *bsmt1-1* nor *bsmt1-2* plants had any obvious distinguishing morphological phenotype. Additionally, induced production of TMTT, the second most common volatile emitted from *P. syringae*–treated *Arabidopsis* leaves, was not affected in *bsmt1* mutants (Figure 4A; see Supplemental Figure 2 online).

Although our data collected so far argued against a role of MeSA as a critical mobile SAR signal in *Arabidopsis*, a direct genetic examination of this putative function was still missing. With the availability of *bsmt1* mutant plants lacking the ability to produce any pathogen-induced MeSA, the significance of MeSA during SAR could now be tested unequivocally. When plants of the different genotypes were inoculated with *Psm* in lower leaves to induce SAR, both *bsmt1-1* and *bsmt1-2* accumulated SA in upper, nontreated leaves, like the wild type, at day 2 after pathogen treatment (Figure 5A). Similarly, systemic expression of the SAR marker gene *PATHOGENESIS-RELATED1* (*PR-1*) was increased in all the lines under investigation upon *Psm* but not after a mock pretreatment (Figure 5B). To test the enhancement of systemic resistance directly, we challenge-inoculated upper leaves with *Psm* 2 d after the primary $MgCl_2$ or *Psm* treatment in lower leaves and assessed bacterial growth in upper leaves another 3 d later. When the primary, SAR-inducing *Psm* treatment in lower leaves was compared with the mock pretreatment, Col-0, *bsmt1-1*, and *bsmt1-2* plants exhibited a similar, statistically highly significant containment of bacterial multiplication during the challenge infection in upper leaves (Figure 5C). These findings show that *bsmt1* mutant plants are not affected in their abilities to enhance systemic SA levels, to systemically increase expression of the SAR gene *PR-1*, or to acquire resistance at the systemic plant level. Thus, MeSA is not required during SAR development and is not used as a long-distance signal ensuring systemic SA accumulation in *Arabidopsis*. As indicated by a strong upregulation of the SA biosynthesis gene *ICS1* in systemic tissue upon primary *Psm* infection in the three investigated lines, the systemic accumulation of SA might rather be accomplished by de novo synthesis of SA in distant leaves (Figure 5D).

The SAR process is often investigated by whole-plant treatment of resistance-enhancing chemical agents such as

Figure 1. (continued).

(E) MeSA content in nontreated, distant leaves of *Psm*-inoculated or $MgCl_2$-infiltrated Col-0 plants at 2 DAI (means ± SD, $n = 5$).

(F) Fate of MeSA after its production during SAR in a symbolized Col-0 plant. Percentages of total MeSA produced after a localized *P. syringae* inoculation are indicated. An underlined value indicates a significant increase after pathogen treatment. 1°, inoculated leaf; 2°, noninoculated, systemic leaf. Numbers given next to vertical arrows represent emission; numbers inside leaves represent leaf content.

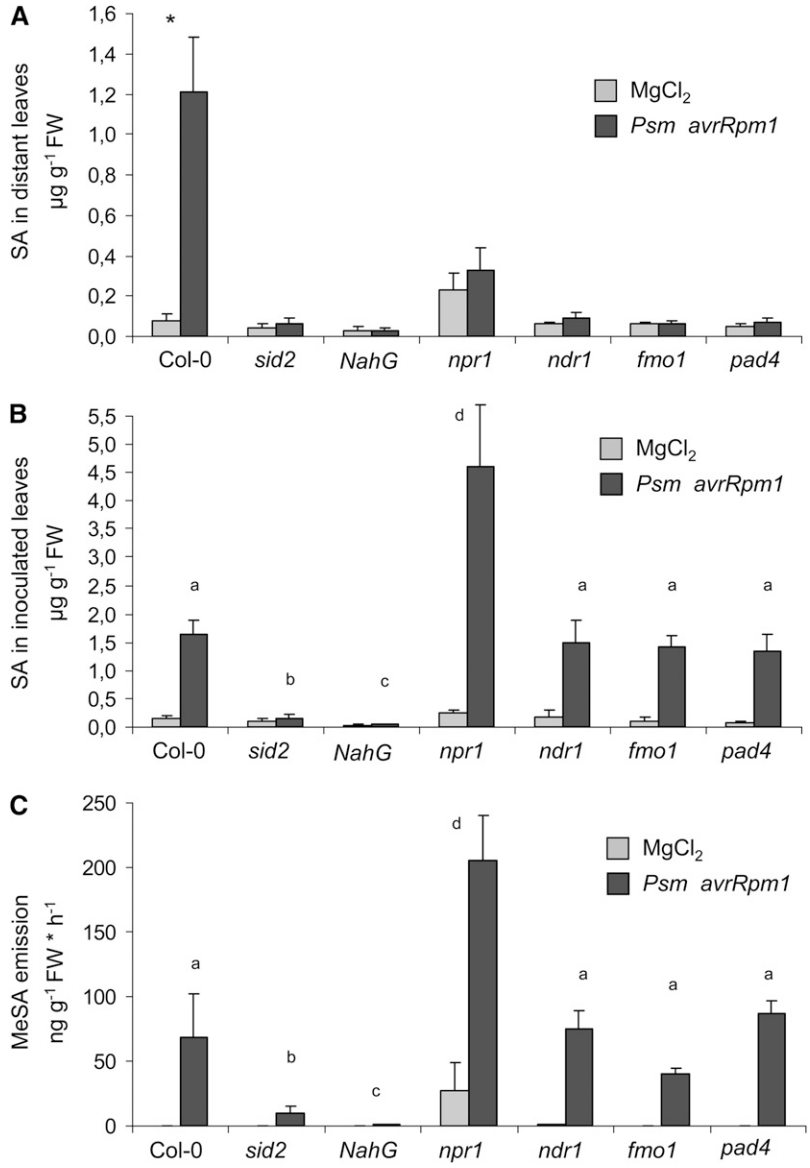

Figure 2. SA Accumulation and MeSA Production in *P. syringae*–Treated Wild-Type and SAR-Defective Mutant Plants.

(A) SA levels in nontreated, distant leaves of *Psm avrRpm1*–inoculated or $MgCl_2$-infiltrated plants at 2 DAI (means ± SD, $n = 4$). Asterisk denotes statistically significant differences between *Psm avrRpm1*- and $MgCl_2$-treated plants ($P < 0.01$).

(B) SA levels in *Psm avrRpm1*–inoculated leaves at 24 HAI (means ± SD, $n = 4$). Different characters symbolize statistically significant differences between *Psm avrRpm1*–treated plants from distinct lines ($P < 0.05$).

(C) MeSA emission from *Psm avrRpm1*- or mock-inoculated plants from 0 to 24 HAI (means ± SD, $n = 4$). Different characters symbolize statistically significant differences between *Psm avrRpm1*–treated plants from distinct lines ($P < 0.05$).

2,6-dichloroisonicotinic acid (INA), benzothiadiazole, or SA itself (Cao et al., 1994; Lawton et al., 1996), although such studies do not properly reflect the distinct spatial processes occurring after a localized induction of SAR with microbial pathogens. To test whether the chemical enhancement of resistance through SA analogs is dependent on functional *BSMT1*, we assayed leaf resistance against *Psm* of plants previously sprayed with a solution of 0.65 mM INA. Compared with water-sprayed control plants, a strong and highly significant enhancement of resistance by a factor of ~50 was detected in INA-treated Col-0, *bsmt1-1*, and *bsmt1-2* plants, indicating that INA-induced resistance is not affected by defects in *BSMT1* (Figure 6).

The *bsmt1* mutants also allowed us to test whether disease resistance at inoculation sites and associated local defense responses would be influenced by MeSA production. Local resistance against both the incompatible *Psm avrRpm1* strain and the compatible *Psm* strain were similar in wild-type and *bsmt1* mutant plants (Figures 7A and 7B). Moreover, local

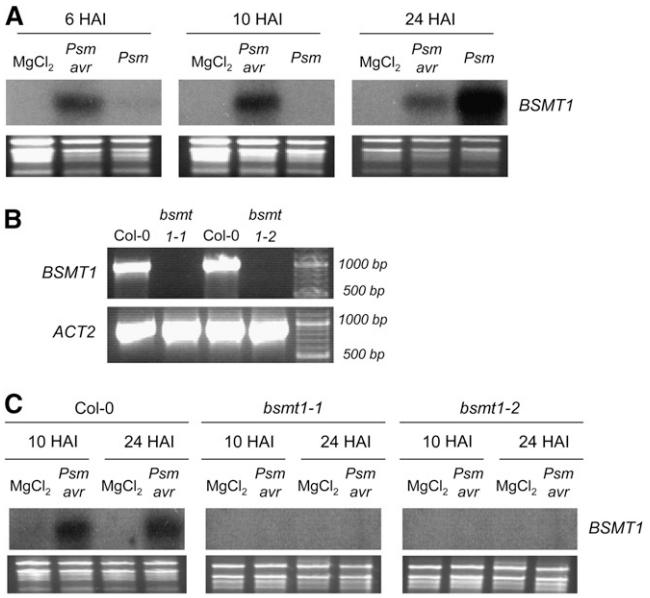

Figure 3. *P. syringae*–Induced Leaf Expression of the *BSMT1* Methyl Transferase Gene and Identification of Nonexpressing T-DNA Insertion Lines.

(A) Expression of *BSMT1* in Col-0 leaves inoculated with *Psm avrRpm1* (*Psm avr*) or *Psm*. Control samples were infiltrated with 10 mM $MgCl_2$. Leaf samples were taken at 6, 10, and 24 HAI for RNA gel blot analysis.
(B) PCR analyses using genomic DNA from Col-0, *bsmt1-1* (SALK_140496), and *bsmt1-2* (WiscDSLox430E05) mutant plants as templates and primers specific for the *BSMT1* gene sequence. The actin gene *ACT2* was amplified as a control.
(C) Expression patterns of *BSMT1* in Col-0 and *bsmt1* leaves infiltrated with 10 mM $MgCl_2$ or *Psm avrRpm1* (*Psm avr*) as assessed by gel blot analysis. Leaf samples were taken at 10 and 24 HAI.

accumulation of the defense signals SA and JA, and *PR-1* expression patterns at infection sites were not impaired in the *bsmt1* lines (Figures 7C to 7E). This indicates that, like SAR, induced resistance toward *P. syringae* at the site of pathogen inoculation is established independently of MeSA production.

JA Signaling Regulates MeSA Production but Not SAR

Induced biosynthesis of terpenoid volatiles in *Arabidopsis* and other plant species is dependent on JA signaling (Ament et al., 2006; Arimura et al., 2008; Attaran et al., 2008; Herde et al., 2008). By determining pathogen-induced MeSA emission from different *Arabidopsis* JA pathway mutants, we tested whether *P. syringae*–induced MeSA production would also require JA biosynthesis or associated downstream signaling events. The *Arabidopsis DDE2* and *OPR3* genes code for allene oxide synthase and 12-oxophytodienoic acid (OPDA) reductase, respectively (Stintzi and Browse, 2000; von Malek et al., 2002). The *dde2* mutant is therefore defective in the synthesis of both JA and its signaling competent precursor OPDA (Mueller et al., 2008), whereas *opr3* is compromised in JA but not in OPDA synthesis. Although *Psm avrRpm1* inoculation enhanced MeSA emission in *dde2* and *opr3*, the amounts of released MeSA were significantly lower in these mutants than the amounts emitted from the corresponding wild-type background lines Col-0 and Wassilewskija (Ws) after pathogen treatment (Figure 8A). The COI1 ubiquitin ligase is required for jasmonate-regulated defense responses (Xie et al., 1998), and *coi1* mutant plants displayed a strongly attenuated emission of MeSA after *Psm avrRpm1* inoculation (Figure 8A). Similarly, compared with the Col-3 wild type, induced MeSA production was markedly reduced in the *jin1* mutant carrying a defect in the transcription factor MYC2, which also acts downstream of JA (Lorenzo et al., 2004). By contrast, mutational defects in the *JAR1* gene, encoding jasmonate amino acid synthetase (Staswick and Tiryaki, 2004), only moderately affected *Psm avrRpm1*–induced MeSA production (Figure 8A). These data indicate that MeSA production induced by avirulent *P. syringae* partially requires JA biosynthesis and depends on COI1- and MYC2-mediated downstream signaling.

As part of the hypothesis that MeSA functions as a SAR signal (Park et al., 2007), JA was suggested to strengthen the MeSA component of SAR signaling (Vlot et al., 2008a, 2008b). Moreover, JA or related oxylipins were postulated to act as critical SAR long-distance signals in their own right (Truman et al., 2007), although the significance of JA for SAR long-distance signaling has recently been questioned (Chaturvedi et al., 2008). To clarify the importance of JA signaling during SAR, we examined whether biological induction of SAR occurs in *Arabidopsis* mutants defective in distinct steps of JA signaling. Compared with $MgCl_2$ pretreated control plants, *Psm* preinoculated plants of *opr3*, *jar1*, and *jin1* mutant lines were all able to significantly increase their resistance toward subsequent challenge infections in distant leaves (Figure 8B). Similarly, a statistically significant enhancement of resistance upon *Psm* pretreatment was observed for *dde2* and *coi1* mutant plants, which already exhibit a somewhat higher degree of basal resistance toward *P. syringae* than the Col-0 background line (Figure 8B; Kloek et al., 2001; Raake et al., 2006). These increases in whole-plant resistance upon localized *Psm* infection of the different JA-related mutants indicate that SAR can be established without a functional JA signaling pathway and thus rule out a function of JA or OPDA derivatives in SAR long-distance signaling. Together with our previous data (Figure 5), these findings also exclude a mechanism in which JA signaling strengthens SAR establishment through MeSA production.

Because most of the produced MeSA is emitted from leaves (Figure 1F), JA could negatively affect SA levels in plant pathogen interactions by promoting the conversion of SA to MeSA. However, considering this mechanism, the *bsmt1* mutants should exhibit higher SA levels after pathogen infection than wild-type plants and show increased *PR-1* gene expression, which is not the case (Figures 7C and 7E). To explain these unexpected results, we determined expression of *ICS1* after pathogen infection in *bsmt1* mutants and detected a slightly attenuated induction of the SA biosynthesis gene at 24 HAI compared with Col-0 (Figure 7F). Thus, although MeSA is not produced and emitted from *bsmt1* plants after pathogen infection, induced SA levels might remain at a wild-type-like level in the mutants because transcription of SA biosynthesis is alleviated to a certain extent.

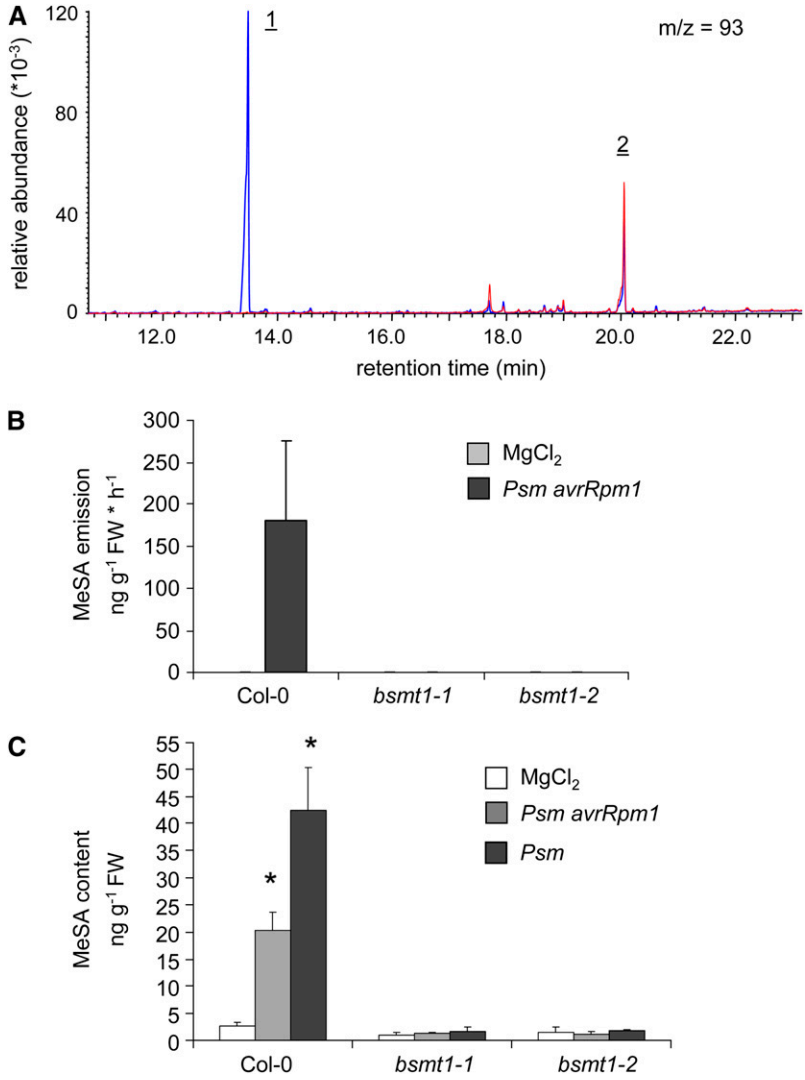

Figure 4. *bsmt1* Mutant Plants Are Completely Devoid of *P. syringae*–Induced MeSA Production.

(A) Ion chromatogram at *m/z* 93 of volatile samples from Col-0 plants (blue) and *bsmt1-1* plants (red), illustrating MeSA (1) and TMTT (2) emission.
(B) Quantification of MeSA emitted from wild-type Col-0 and *bsmt1* mutant plants inoculated with *Psm avrRpm1* or infiltrated with $MgCl_2$. Volatiles were collected from 0 to 24 HAI. Bars represent mean emission values (±SD, $n = 4$). MeSA emission was not detected in either *bsmt1* mutant line (detection limit ~0.05 ng g^{-1} FW h^{-1}).
(C) Leaf MeSA contents of Col-0 and *bsmt1* mutant plants in response to inoculation with *Psm avrRpm1* (gray bars), *Psm* (black bars), or infiltration with 10 mM $MgCl_2$ (white bars) at 24 HAI (means ± SD, $n = 3$). Asterisks denote statistically significant differences between *P. syringae*- and $MgCl_2$-treated plants of a particular line ($P < 0.003$).

Virulent *P. syringae* Mediate Leaf MeSA Release but Not SAR via Coronatine

Coronatine is a phytotoxin produced by several *P. syringae* pathovars, including *Psm* and *P. syringae* pv *tomato* DC3000 (*Pst*; Bender et al., 1999). It acts as a bacterial virulence factor that counteracts SA-dependent plant defense reactions by acting as a structural and functional mimic of bioactive jasmonates, most notably JA-Ile (Brooks et al., 2005; Thines et al., 2007; Katsir et al., 2008; Melotto et al., 2008). The availability of coronatine-deficient (cor$^-$) *Pst* mutants (Brooks et al., 2004) allowed us to test whether *P. syringae*–induced MeSA production would require the action of coronatine. Infection of Col-0 leaves with the coronatine-producing *Pst* wild-type strain evoked a strong emission of MeSA, which was similar in magnitude to the MeSA released after *Psm* infection (Figures 1B and 9A). By contrast, leaf MeSA emission from plants infected with the *Pst* cor$^-$ strain DB29 (Brooks et al., 2004) was only marginally elevated, falling by a factor of 60 below the amounts induced by wild-type *Pst* (Figure 9A). Because coronatine functions as a virulence factor to promote bacterial multiplication in planta (Brooks et al., 2005), we comparatively determined the growth of

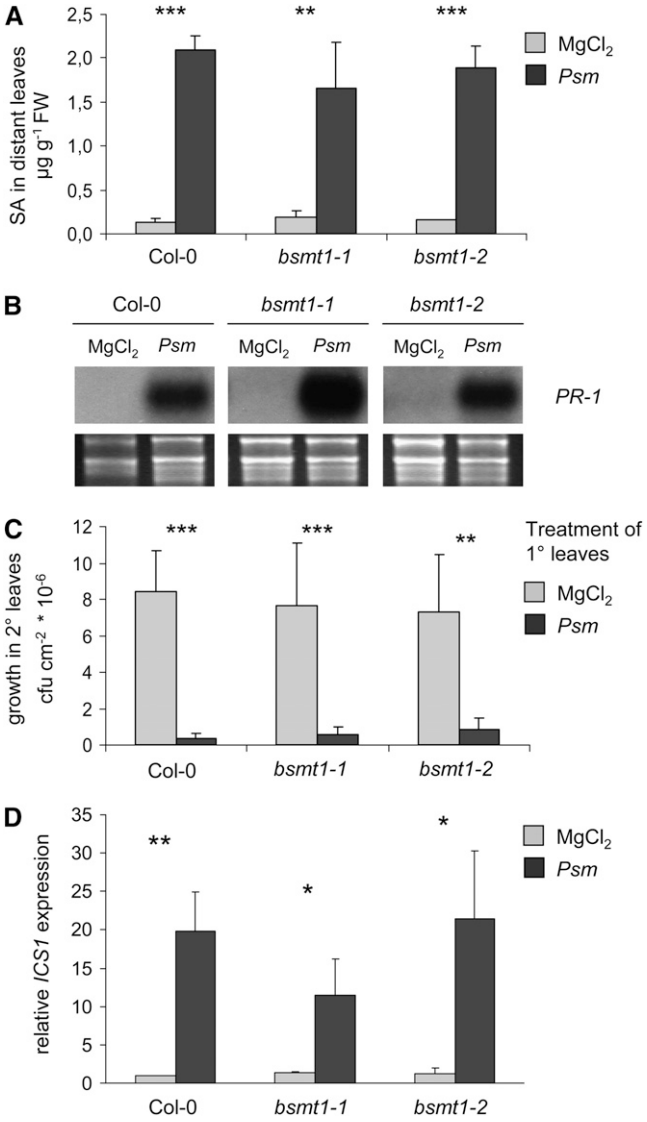

Figure 5. *P. syringae* Induces SAR in *bsmt1* Mutant Plants.

(A) Accumulation of SA in untreated, upper (2°) leaves after *Psm* inoculation, or $MgCl_2$ infiltration of lower (1°) leaves. Treatments of 1° leaves were performed as described in **(C)**. 2° leaves were harvested 2 d later for analyses. Bars represent mean values (±SD) of three independent samples, each sample consisting of six leaves from two different plants. Asterisks denote statistically significant differences in systemic SA levels between *Psm* and $MgCl_2$ pretreated plants of a particular line (***P < 0.001; **P < 0.01).

(B) Expression of the SAR marker gene *PR-1* in untreated, upper (2°) leaves after *Psm* inoculation or $MgCl_2$ infiltration of lower (1°) leaves, as assessed by gel blot analyses. 2° leaves were harvested 2 d after the 1° treatment for analyses.

(C) Bacterial growth quantification to directly assess enhancement of systemic resistance. Plants were pretreated with either 10 mM $MgCl_2$ or *Psm* (OD = 0.01) in three lower (1°) leaves. Two days later, three upper leaves (2°) were challenge infected with *Psm* (OD = 0.002). Bacterial growth in upper leaves was assessed 3 d after the 2° leaf inoculation. Bars represent mean values (±SD) of colony-forming units (cfu) per square centimeter from at least seven parallel samples each consisting of three leaf disks. Asterisks denote statistically significant differences of bacterial growth in 2° leaves between *Psm* and $MgCl_2$ pretreated plants of a particular line (***P < 0.001; **P < 0.01).

(D) Relative expression levels of *ICS1*, as assessed by quantitative real-time PCR analysis. *ICS1* expression values were normalized to those for the reference gene (At1g62930) and expressed relative to the wild-type $MgCl_2$ sample. For each expression value of one sample, three PCR replicates were performed and averaged. The depicted bars represent mean values (±SD) of three biologically independent samples. Asterisks denote statistically significant differences in systemic SA levels between *Psm* and $MgCl_2$ pretreated plants of a particular line (**P < 0.01; *P < 0.05).

wild-type *Pst* and of *Pst* cor⁻ at 24 HAI, the endpoint of MeSA sampling in the above experiment (Figure 9A). Leaf bacterial numbers were about twofold lower for *Pst* cor⁻ than for *Pst* (see Supplemental Figure 3 online). However, this relatively small growth difference is not likely to account for the large differences in leaf MeSA emission observed after treatments of plants with *Pst* and *Pst* cor⁻, respectively. Thus, MeSA release from *Pst*-infected leaves is mainly triggered by the action of the phytotoxin coronatine. Since MeSA is produced from SA by BSMT1 and predominantly lost into the atmosphere (Figures 1 and 4; Chen et al., 2003), coronatine-mediated MeSA volatilization has the potential to decrease SA levels at infection sites and thus to constitute a bacterial virulence mechanism that negatively influences SA-based plant defenses.

Finally, to test whether bacterial induction of SAR is affected by the ability of *Pst* to produce coronatine, we comparatively analyzed the systemic resistance of Col-0 plants after a remote infection with *Pst* and with *Pst* cor⁻. Since the primary infection with *Pst* cor⁻ triggered SAR to the same extent as infection with *Pst* (Figure 9B), SAR is established independently of coronatine in the *Arabidopsis–Pseudomonas* interaction. Because of the large discrepancies between MeSA production in *Pst*- and *Pst* cor⁻-infected plants, this result further corroborates our findings that MeSA formation is dispensable for SAR establishment in *Arabidopsis*.

DISCUSSION

The state of increased systemic disease resistance that develops during SAR requires elevated levels of SA and the mobilization of SA-dependent defenses in leaves distant from pathogen inoculation (Vernooij et al., 1994). The earliest candidate for a mobile long-distance signal traveling from inoculated to systemic tissue was SA itself. SA accumulates both at inoculation sites and in distant leaves concomitant with the onset of SAR, is found in phloem exudates of infected cucumber leaves, is distributed inside an *Arabidopsis* plant when applied externally to a single leaf, and its exogenous application increases whole-plant resistance in many species (Malamy et al., 1990; Métraux et al., 1990; Kiefer and Slusarenko, 2003). However, evidence from detailed physiological and grafting experiments has essentially excluded a function of SA as the phloem-mobile long-distance signal (Rasmussen et al., 1991; Vernooij et al., 1994).

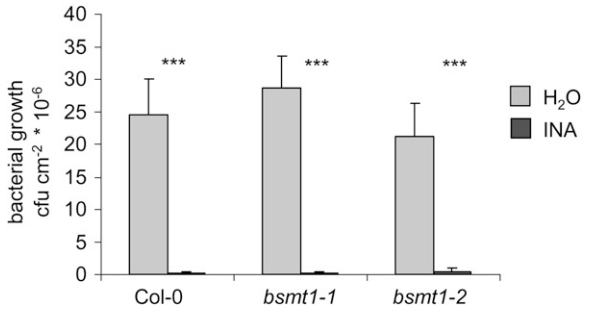

Figure 6. INA-Induced Resistance in Col-0 and *bsmt1* Mutant Plants.

Plants were sprayed with 0.65 mM INA or water, and three leaves per plant infected 2 d later with *Psm* (OD = 0.002). Bacterial growth was assessed 3 d after inoculation (***$P < 0.001$).

Still, instead of SA itself, modified forms, such as MeSA or SAG, are candidate molecules that might travel from inoculated to distant leaves. MeSA was recently proposed as being a critical, phloem-mobile SAR signal in tobacco. The respective model includes SA to MeSA conversion by SAMT in inoculated leaves, transport of MeSA to distant leaves, and subsequent reconversion to active SA by SA methyl esterase (Park et al., 2007). From SAR phenotypes of *Arabidopsis* lines in which different SA methyl esterase isoforms were concomitantly silenced, it was further concluded that MeSA functions as a conserved SAR signal in *Arabidopsis* and possibly other species (Vlot et al., 2008a, 2008b).

Our approach has tackled the problem from the side of MeSA production. BSMT1 belongs to a group of *Arabidopsis* methyl transferases and bears in vitro SA to MeSA converting activity (Chen et al., 2003). The *BSMT1* gene is strongly upregulated in response to *P. syringae* leaf inoculation (Figure 3A), and its expression kinetics closely correlates with the timing of MeSA production (Figures 1A and 1B). Two independent *Arabidopsis* lines, *bsmt1-1* and *bsmt1-2*, both with predicted T-DNA insertions in the *BSMT1* coding region, not only fail to express the gene but also lack any pathogen-induced elevation of MeSA production (Figures 3C and 4). This demonstrates that BSMT1 is the single methyl transferase that catalyzes induced production of MeSA in *Arabidopsis* leaves. If MeSA were critical for SAR in *Arabidopsis*, the *bsmt1* mutants would exhibit a SAR-compromised phenotype. Our findings that both *bsmt1-1* and *bsmt1-2* are able to mount a wild-type-like SAR response associated with conventional systemic SA elevation and PR gene expression shows that MeSA is dispensable for systemic SA accumulation and SAR in *Arabidopsis* (Figure 5). Thus, in this species, MeSA neither functions as a critical long-distance signal nor in any other SAR relevant process, including systemic SA accumulation. MeSA production is also not required for chemical induction of *Arabidopsis* resistance by the SA analog INA (Figure 6).

Our findings in *Arabidopsis* contradict the events described for TMV-induced SAR in tobacco (Park et al., 2007) and indicate the existence of species differences in the molecular nature of SAR long-distance signals. This is surprising because the SAR phenomenon has been observed in many plant species, and the associated responses, such as systemic SA accumulation, increased PR gene expression, or the timing of SAR induction, are well-conserved between species (Sticher et al., 1997). Nevertheless, we provide direct evidence that MeSA is not a conserved SAR signal in all species, and this is in sharp contrast with the previously proposed generalized model (Park et al., 2007; Vlot et al., 2008a, 2008b).

Mere physicochemical considerations and the experimentally determined in planta properties of MeSA also argue against a function of the molecule as an effective phloem-directed long-distance signal. Methylation of SA to MeSA does strongly increase membrane permeability and volatility, and this is reflected by our finding that the predominant part of the produced MeSA is lost into the atmosphere by emission, and only a small portion is retained in leaves or is detectable in petiole exudates (Figure 1; see Supplemental Figure 1 online). A directed and efficient mass flow of this volatile SA derivative through the phloem or other conductive parts of the stem therefore does not seem realistic. Moreover, the amount of MeSA accumulating after bacterial inoculation in leaf exudates during a 48-h SAR induction period is modest and falls well below the usually observed systemic elevation of SA levels observed during *P. syringae*–induced SAR in *Arabidopsis* (1 to 2 $\mu g\ g^{-1}$; Mishina and Zeier, 2007; Mishina et al., 2008). Finally, we did not observe increases in MeSA content and detected only a small elevation of MeSA emission in noninoculated leaves after pathogen treatment (Figures 1D to 1F), indicating that a flow of MeSA from inoculated to systemic leaves, if present at all, is only marginal. This is consistent with the minor and statistically barely significant elevations of systemic MeSA reported previously (Park et al., 2007; Vlot et al., 2008b).

The major part of MeSA produced in *P. syringae*–inoculated *Arabidopsis* leaves is released into the atmosphere. For the incompatible *Psm avrRpm1–Arabidopsis* interaction, emission rates of 50 ng g^{-1} h^{-1} are accompanied by leaf contents of 20 to 25 ng g^{-1}, meaning that the amounts retained in leaves equal the value emitted during ~30 min (Figure 1). Although MeSA production starts later in the compatible *Psm–Arabidopsis* interaction, the values emitted around 24 HAI are about one order of magnitude higher than in the incompatible one. In total, ~0.75 and 3.5 $\mu g\ g^{-1}$ MeSA are volatilized during the first 24 HAI from leaves inoculated with *Psm avrRpm1* and *Psm*, respectively (Figures 1A and 1B). Considering that in those interactions, SA and SAG accumulate in leaves at 24 HAI to ~1 to 1.5 $\mu g\ g^{-1}$ and 4 to 6 $\mu g\ g^{-1}$, respectively (Figure 4B; Mishina et al., 2008), a marked percentage of the totally produced SA is lost as volatilized MeSA. The MeSA amounts emitted from pathogen-treated tobacco plants are of the same order of magnitude as those emitted from *Arabidopsis*. Shulaev et al. (1997) detected emission rates from TMV-infected tobacco leaves of ~20 to 300 ng h^{-1} per plant.

We excluded MeSA as a phloem-mobile long-distance signal during SAR in *Arabidopsis*. However, considering the substantial levels of MeSA emitted from leaves, does MeSA act as an airborne SAR signal, as proposed previously (Shulaev et al., 1997)? The answer for *Arabidopsis* is clearly no, and this negative statement again relies on the wild-type-like SAR phenotype of the *bsmt1* mutant plants that fail to elevate production and emission of MeSA after inoculation (Figures 3 to 5). It is

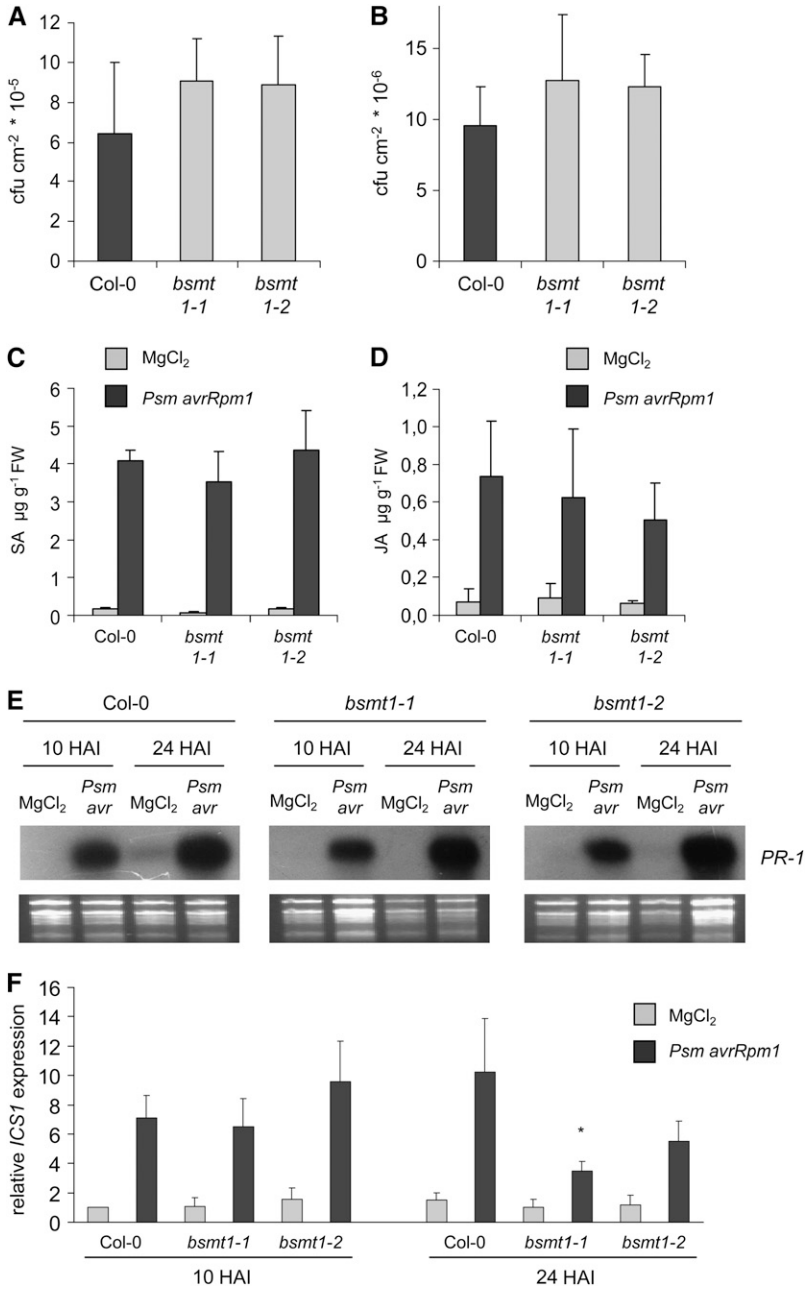

Figure 7. Local Defense Responses in *bsmt1* Plants Are Similar to Those in the Wild Type.

(A) and **(B)** Bacterial growth quantification of *Psm avrRpm1* (OD = 0.005) **(A)** and *Psm* (OD = 0.002) **(B)** in leaves of wild-type and *bsmt1* mutant plants 3 DAI. Bars represent means (±SD) of cfu per cm^2 from at least six parallel samples from different plants, each sample consisting of three leaf disks. No significant differences in bacterial numbers were detected at 3 DAI and 1 HAI (data not shown) for samples from different lines.

(C) and **(D)** Accumulation of the defense hormones SA **(C)** and JA **(D)** at sites of *Psm avrRpm1* inoculation (10 HAI). Control samples were infiltrated with 10 mM $MgCl_2$.

(E) RNA gel blot analysis of *PR-1* expression in Col-0 and *bsmt1* leaves infiltrated with 10 mM $MgCl_2$ or *Psm avrRpm1* (*Psm avr*). Leaf samples were taken at 10 and 24 HAI.

(F) Relative *ICS1* expression in Col-0 and *bsmt1* leaves infiltrated with 10 mM $MgCl_2$ or *Psm avrRpm1*, as assessed by quantitative real-time PCR analyses (see Figure 5D for details). Leaf samples were taken at 10 and 24 HAI. Asterisk indicates statistically significant differences between *Psm avrRpm1*–treated wild-type and mutant samples ($P < 0.05$).

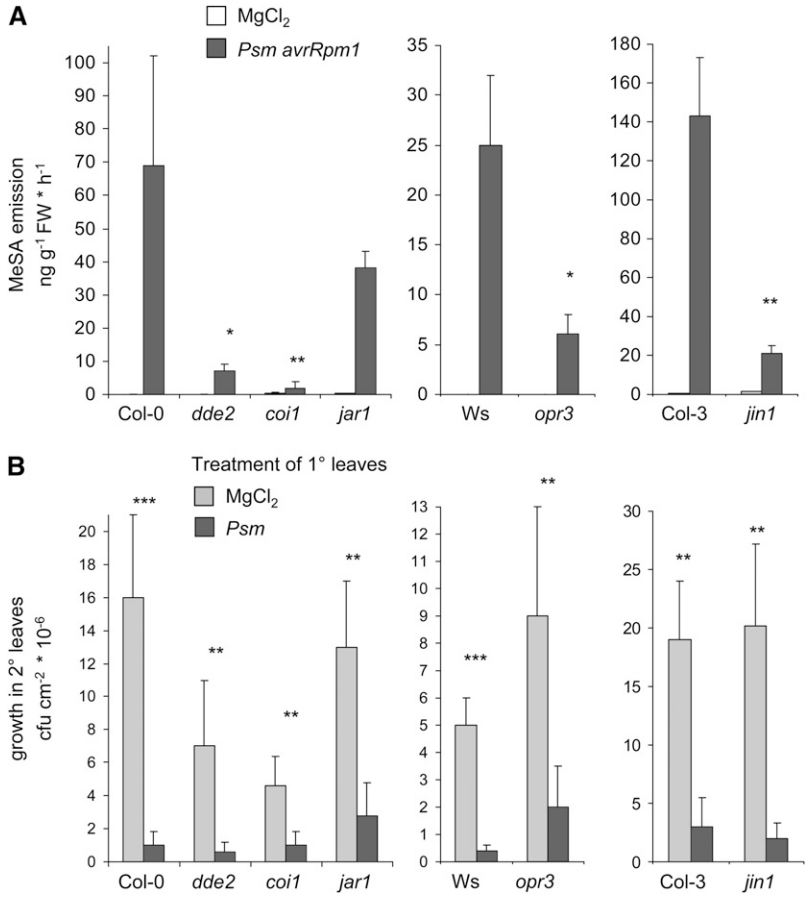

Figure 8. MeSA Production but Not SAR Is Regulated by JA Signaling.

(A) Leaf MeSA emission from *Psm avrRpm1*- or mock-inoculated JA pathway mutants and their corresponding wild-type lines (*dde2*, *coi1*, and *jar1* are in Col-0, *opr3* is in Ws, and *jin1* is in Col-3 background). Volatiles were sampled from 0 to 24 HAI, and mean values (±SD, $n = 4$) are given. Asterisks indicate whether statistically significant differences exist between *Psm avrRpm1*–treated JA mutant plants and the corresponding wild type (**P < 0.01; *P < 0.05). Note the different scales of the *y* axes.

(B) SAR assessment via bacterial growth quantification in challenge-infected upper (2°) leaves of pretreated (1°) JA pathway mutants and respective wild-type plants. For experimental details, see legend to Figure 5C. Bars represent means (±SD) of cfu per cm^2 from at least seven parallel samples. Asterisks denote statistically significant differences of bacterial growth in 2° leaves between *Psm* and $MgCl_2$ pretreated plants of a particular line (***P < 0.001; **P < 0.01). No statistically significant differences (P > 0.05) exist between *Psm*-treated wild-type and mutant samples with respect to a particular background, indicating a similar strength of SAR induction for the different lines. Note the different scales of the *y* axes.

noteworthy in this context that *bsmt1* mutants also develop SAR when wild-type plants, which are possible sources of MeSA, are absent from the experimental growth chamber. A second reasoning is that in our experimental setting for SAR assessments, mock-treated and pathogen-inoculated plants are routinely located in direct proximity, and several leaves of differently treated plants are often in close contact. Nevertheless, we observe statistically robust differences in acquired resistance between mock- and pathogen-treated plants (Figure 5), indicating that signaling processes within the plant but not airborne communication dominate during SAR. Further, SAR is suppressed in cucumber (*Cucumis sativus*) plants when petioles of inoculated leaves are girdled, suggesting an intraplant and more specifically a phloem-based signal transmission pathway (Guedes et al., 1980; van Bel and Gaupels, 2004).

This does not rule out that under certain artificially provoked and nonphysiological conditions, gaseous MeSA from external sources or from plants is able to heighten plant resistance, presumably by leaf uptake followed by conversion to bioactive SA (Shulaev et al., 1997; Koo et al., 2007; Park et al., 2007). The minimum concentration of externally applied gaseous MeSA at which tobacco plants start to significantly elevate resistance is ~10 $\mu g\ L^{-1}$ (Shulaev et al., 1997), and concentrations of up to 1 $mg\ L^{-1}$ have been used for this purpose in other experiments (Park et al., 2007). Considering the measured *Psm*-induced volatile emission in Col-0 plants during the first 48 h after inoculation (Figure 1B), and the 500-liter volume of the experimental compartment, and assuming a total of 50 *Psm*-treated plants from which three leaves (~0.1 g fresh weight) each have been inoculated, we calculate a concentration of 0.1 $\mu g\ L^{-1}$

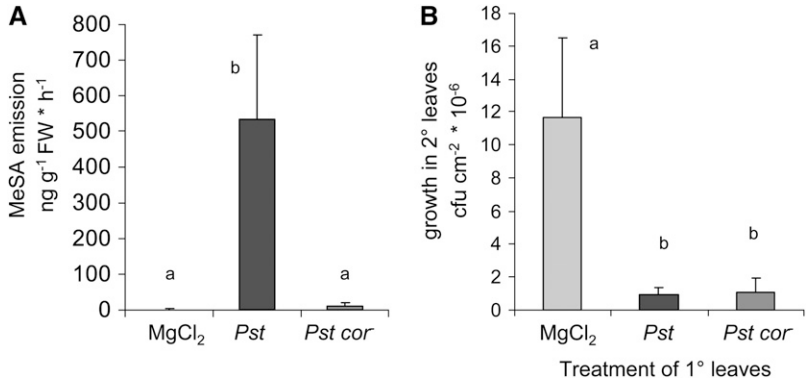

Figure 9. *P. syringae*–Induced MeSA Formation but Not SAR Is Dependent on Bacterial Production of the Phytotoxin Coronatine.

(A) MeSA emission from Col-0 leaves after inoculation with coronatine-producing *Pst*, coronatine-deficient *Pst cor*$^-$, and $MgCl_2$ infiltration. Volatiles were sampled from 0 to 24 HAI, and mean values of ng emitted substance g^{-1} leaf FW h^{-1} (±SD, $n = 7$) are given. Different letters symbolize statistically significant differences between treatments ($P < 0.002$).

(B) SAR induction by *Pst* and *Pst* cor$^-$ in Col-0 plants. 1° leaves were infiltrated with $MgCl_2$, *Pst*, or *Pst* cor$^-$ (OD 0.01 each), 2° leaves were challenge-infected 2 d later with *Psm* (OD 0.002), and quantities of *Psm* in 2° leaves were determined another 3 d later (see Figure 5C for details). Bars represent means (±SD) of cfu per cm^2 from at least six parallel samples. Different characters symbolize statistically significant differences between treatments ($P < 0.01$).

MeSA in our experimental chambers during a SAR experiment. Even with this relatively high plant density, the restricted volume, and the high inoculation frequency, the calculated value is about two orders of magnitude lower than the minimum concentration previously determined to be sufficient for resistance induction (Shulaev et al., 1997). By contrast, when MeSA produced by donor plants is pointedly directed into low volume vessels containing acceptor plants, plant resistance might be elevated in the acceptor plants. For instance, considerable amounts of MeSA that were emitted from 150 SA-treated *Arabidopsis* plants overexpressing the *BSMT1* rice (*Oryza sativa*) homolog were conducted into sealed 0.4-liter vessels containing Col-0 acceptor plants. This treatment increased expression of *PR-1* in the acceptor plants (Koo et al., 2007). However, this highly directed bulk flow of gaseous MeSA into a small-volume acceptor compartment is rather artificial and hardly reflects the physiological circumstances occurring during SAR.

As a relatively strong acid with a pK_a value of 3, nonderivatized SA predominantly exists as an anion in most subcellular compartments (an exception might be the fairly acidic vacuole), and its membrane permeability should therefore be low in the absence of a specific transport protein (Chatton et al., 1990). MeSA might thus represent a membrane-permeable, mobile form of SA able to travel over shorter cellular distances by diffusion. Our finding that MeSA but not SA levels increase in *Arabidopsis* leaf exudates after pathogen inoculation supports this view. Interestingly, SA glycosylation also enhances petiole exudation (see Supplemental Figure 1 online). However, overall exudation rates of SAG are too low to markedly contribute to the systemic rises of SA occurring during SAR via phloem-based long-distance transport. Moreover, the SAR-deficient *Arabidopsis* mutants *npr1*, *ndr1*, *fmo1*, and *pad4* are able to elevate local production of SA (Figure 2B), MeSA (Figure 2C), and SAG (see Supplemental Figure 4 online) but fail to increase SA levels in distant leaves (Figure 2A). The likewise SAR-deficient phytochrome photoreceptor double mutant *phyA phyB* exhibits a similar behavior (Griebel and Zeier, 2008). Because there is no obvious physiological reason why these different mutational defects should all block systemic translocation of locally accumulating SA derivatives, it seems reasonable to assume that neither SA itself nor a modified form of SA, such as MeSA or SAG, travels from inoculated to distant leaves during SAR. Together with the observation that the SA biosynthesis gene *ICS1* is strongly upregulated in distant leaves after local pathogen inoculation (Figure 5D), the above results support the hypothesis that the systemic rises in SA during SAR are achieved via de novo synthesis in distant leaves. This view is consistent with the outcome of SAR experiments using tobacco grafts with SA hydroxylase-expressing root stocks and wild-type scions (Vernooij et al., 1994).

A significant early production of JA occurs in *Arabidopsis* leaves following recognition of avirulent *P. syringae* (Mishina et al., 2008). According to the analyses of JA biosynthesis mutants (Figure 8A), this transient JA accumulation must be the main driving force for *Psm avrRpm1*–triggered MeSA production. By contrast, virulent strains, such as *Psm* or *Pst*, do not evoke significant rises in leaf JA levels during the first 2 d after infection when modest inoculum concentrations are applied (see below; Mishina and Zeier, 2007; Mishina et al., 2008). According to our results, the compatible bacteria rather use the phytotoxin and JA-Ile mimic coronatine to provoke leaf MeSA emission (Figure 9A). Further downstream of the JA pathway, both COI1 and MYC2-mediated signaling events are required for induced MeSA production (Figure 8A). The JA pathway-dependent regulation of MeSA formation is thus similar to the regulation of TMTT biosynthesis, the second significant *Arabidopsis* leaf volatile induced upon *P. syringae* attack (Attaran et al., 2008; Herde et al., 2008). Although production of the homoterpene TMTT is more tightly dependent on JA than synthesis of the phenylpropanoid MeSA, a common regulatory mechanism of these biochemically

unrelated, major *Arabidopsis* leaf volatiles is apparent. The regulation of MeSA synthesis through the JA pathway occurs at the transcriptional level because exogenous treatment with methyl jasmonate is sufficient to trigger *BSMT1* expression (Chen et al., 2003; Koo et al., 2007). Despite this coregulation, production of TMTT is not influenced by MeSA generation and vice versa (see Supplemental Figure 2 online; Attaran et al., 2008).

The significance of the JA pathway during SAR has recently been debated. On the one hand, a major role for JAs during SAR has been suggested, with JA or a related oxylipin derivative possibly initiating or directly mediating systemic long-distance signaling (Grant and Lamb, 2006; Truman et al., 2007). Experimental support for this proposition includes the finding that several JA pathway mutants show attenuated SAR in response to *Pst avrRpm1*, that foliar JA application enhances systemic resistance, and that JA levels increase in *Arabidopsis* leaf petiole exudates as well as in distant leaves after inoculation with high inoculum density (OD 0.2) of *Pst avrRpm1* (Truman et al., 2007). Other experiments, on the other hand, argue against a role for JA as a mobile SAR signal. Chaturvedi et al. (2008) have shown that a SAR-inducing activity collected from petiole exudates of *Pst avrRpm1*–inoculated leaves does not copurify with JA, and that neither JA nor MeJA reconstitute an inducer activity in SAR-inactive leaf exudates. Our presented results rule out a decisive role of the JA pathway during SAR because systemic resistance in the JA biosynthesis mutants *dde2* and *opr3*, as well as in the downstream signaling mutants *coi1*, *jar1*, and *jin1*, is significantly enhanced in response to a local *Psm* inoculation (Figure 8B). A SAR-positive phenotype for *coi1* mutants has also been reported by Cui et al. (2005). The correlation between SAR, JA petiole exudation, and systemic JA elevation reported by Truman et al. (2007) is questionable because it was not tested in this study whether the high inoculum (OD 0.2) used for analytical JA determinations indeed induces a SAR response. Instead, bacterial ODs that were several orders of magnitude lower than 0.2 were used by Truman et al. (2007) for SAR bioassays. Previous experiments with various bacterial inoculation densities conducted in our laboratory indicate that the magnitude of *P. syringae*–induced SAR is low for high inoculation densities (OD 0.2), although these ODs provoke, besides heavy tissue necrosis, strong JA elevation at inoculation sites. By contrast, modest inoculi (OD 0.005 to 0.02), which result in much lower or even no detectable rises of local JA, trigger a significantly stronger SAR response (Mishina and Zeier, 2007). In addition, we have never detected increased levels of JA or OPDA in distant tissue under these conditions (Mishina et al., 2008). Taken together, data from our and other laboratories (Cui et al., 2005; Chaturvedi et al., 2008) argue against a significant function of the JA pathway during SAR establishment and long-distance signaling. Moreover, the wild-type-like SAR-inducing capacity of *Pst* cor$^-$ mutants reveals that bacterial production of the JA-Ile-mimicking phytotoxin coronatine does not affect the SAR process, neither positively nor negatively (Figure 9B). SAR induction through *Pst* cor$^-$ is associated with a largely suppressed leaf MeSA production (Figure 9A), and this further corroborates the dispensability of MeSA during SAR in *Arabidopsis*.

In summary, our data exclude an essential function of both MeSA and JA signaling during systemic long-distance signaling and SAR in *Arabidopsis*. Other hitherto unidentified molecules are likely to travel from inoculated to distant tissue in this species to set in gear signal transduction and amplification mechanisms in distant leaves. The latter processes can then drive the systemic de novo biosynthesis of SA, which in turn is known to trigger expression of PR genes and SAR (Cao et al., 1994). A conceivable function of SA methylation in plant defense is to prevent SA levels from accumulating to toxic concentrations by vaporization of volatile MeSA into the atmosphere. JA may regulate this process because it promotes SA to MeSA conversion (Figure 8A). Analyses of *bsmt1* mutants cannot definitively prove this statement because MeSA depletion in these plants seems to negatively affect SA biosynthesis at the transcriptional level (Figure 7F). In addition to MeSA volatilization, SAG formation and subsequent vacuolar storage is an alternative way to handle an excess of SA (Lee et al., 1995; Dean et al., 2005). MeSA formation might also influence the interplay between SA and JA, which trigger distinct sets of defense responses and thereby often behave in a counteractive manner (Traw et al., 2003; Koornneef et al., 2008). JA-mediated MeSA production and subsequent release of the volatile might thus be one means by which negative crosstalk between SA and JA signaling is realized. Moreover, the strong induced production of MeSA by coronatine suggests a bacterial virulence mechanism through negative interference with the SA defense pathway: coronatine triggers SA to MeSA conversion, and the subsequent emission of volatile MeSA from the plant results in a lowering of the leaf SA pool. In support of this, coronatine-mediated attenuation of plant SA accumulation and downstream defenses have been reported previously (Brooks et al., 2005; Uppalapati et al., 2007). In this context, it is interesting to note that overexpression of the rice homolog of *BSMT1* in *Arabidopsis* resulted in constitutively enhanced MeSA emission and attenuated disease resistance due to SA depletion (Koo et al., 2007).

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana plants were grown on an autoclaved mixture of soil (Klasmann), vermiculite, and sand (10:0.5:0.5) in a controlled environmental chamber (J-66LQ4; Percival) with a 9-h day (photon flux density 70 μmol m^{-2} s^{-1})/15-h night cycle and a relative humidity of 70%. Growth temperatures during the day and night period were 21 and 18°C, respectively. Experiments were performed with 6-week-old naïve and unstressed plants exhibiting a uniform appearance. If not otherwise stated, *Arabidopsis* accession Col-0 was used for experiments.

The *bstm1-1* and *bstm1-2* mutant lines represent the T-DNA insertion lines SALK_140496 and WiscDSLox430E05, respectively, which are both in the Col background. Homozygous insertion mutants were identified by PCR, using gene-specific (*BSMT1-1*-forward, 5′-GCAAAAACTTCAAATATATTATGCATG-3′; *BSMT1-1*-reverse, 5′-GAAATCATTTTCCGGGAGATC-3′; *BSMT1-2*-forward, 5′-ATAAAACGGCATGTTGAATGC-3′; *BSMT1-2*-reverse, 5′- GGTCCAGTATCACATTATCACGG -3′) and T-DNA-specific primers as described by Alonso et al. (2003). The JA pathway mutants *opr3* (Stintzi and Browse, 2000) and *jin1* (Berger et al., 1996) are in the Ws and Col-3 backgrounds, respectively. All other *Arabidopsis* lines used in this study (*dde2-2* [von Malek et al., 2002], *coi1-35* [Staswick and Tiryaki, 2004], *jar1-1* [Staswick and Tiryaki, 2004], *sid2-1* [Nawrath and Métraux, 1999], *NahG* [Lawton et al., 1995], *npr1-2*

[NASC line N3801], *ndr1* [Century et al., 1995], *fmo1* [Mishina and Zeier, 2006], and *pad4-1* [Glazebrook et al., 1997]) have background Col-0.

Cultivation of Bacteria

Pseudomonas syringae pv *maculicola* strain ES4326 (*Psm*), *Psm* carrying the *avrRpm1* avirulence gene (*Psm avrRpm1*), *P. syringae* pv *tomato* DC3000 (*Pst*; strain KP105; Brooks et al., 2004), and *Pst* cor$^-$ (strain DB 29; Brooks et al., 2004) were grown in King's B medium containing the appropriate antibiotics at 28°C. Overnight log phase cultures were washed three times with 10 mM $MgCl_2$ and diluted to different final optical densities for leaf inoculations.

Assessment of SAR and Local Resistance Responses

For SAR experiments, plants were first infiltrated into three lower (1°) leaves with a suspension of *Psm* (OD = 0.01) or with 10 mM $MgCl_2$ as a control treatment. Two days after the primary treatment, upper (2°) leaves were either harvested for SA determination and gene expression analysis or inoculated with *Psm* (OD 0.002). Growth of *Psm* in 2° leaves was scored another 3 d later by homogenizing discs originating from infiltrated areas of three different leaves in 1 mL 10 mM $MgCl_2$, plating appropriate dilutions on King's B medium, and counting colony numbers after incubating the plates at 28°C for 2 d.

For the determination of local defense responses, bacterial suspensions of OD 0.005 (determination of gene expression, metabolite levels, and *Psm avrRpm1* growth assay) or OD 0.002 (*Psm* growth assays) were infiltrated into three full-grown leaves per plant. Bacterial growth was assessed 3 d after infiltration as described above.

INA-induced resistance was assessed by spraying whole plants with a solution of 0.65 mM INA or water as a control, leaf inoculation of *Psm* (OD 0.002) 2 d later, and determination of bacterial growth as described above.

Determination of VOC Emission Including MeSA

To assess *P. syringae*–induced plant VOC emission, including emission of MeSA, bacterial suspensions of OD 0.01 were infiltrated from the abaxial side into seven full-grown rosette leaves per *Arabidopsis* plant using a 1-mL syringe without a needle. Control treatments were performed by infiltrating a 10 mM $MgCl_2$ solution. To determine induced MeSA production in noninoculated systemic leaves, four lower leaves per plant were treated and removed at 2 DAI when SAR is just induced in the pathosystem (Mishina et al., 2008). The remainder plant was then sampled for VOC emission from day 2 to day 3 after inoculation.

Volatiles emitted by individual plants were collected in a push/pull apparatus as described by Attaran et al. (2008). Plants were placed in collection chambers ~30 min after leaf infiltrations and trapping filters consisting of glass tubes packed with Super-Q absorbent (VCT-1/4X3-SPQ; Analytical Research Systems) were attached. Charcoal-filtered and humidified air was pushed into each sampling chamber at a rate of 1.2 L min^{-1}. The air flow containing plant volatiles was pulled through the trapping filter with a vacuum pump (ME2; Vacuubrand), and volatiles were collected for 10 to 24 h.

After each collection, trapping filters were eluted with 1 mL CH_2Cl_2, and 200 ng of *n*-octane was added as internal standard. The mixture was concentrated to a volume of 25 μL under a gentle stream of nitrogen, strictly avoiding evaporation to dryness, and analyzed by GC-MS. Aliquots (3 μL) of the sample mixture were separated on a GC (6890N; Agilent Technologies) that was equipped with a split/splitless injector and a fused silica capillary column (HP-1; 30 m × 0.25 mm ID, 0.25 μm film thickness) and combined with a 5975 mass spectrometric detector (Agilent Technologies). Samples were injected in pulsed splitless mode, and helium was used as a carrier gas. The temperature of the oven was held at 50°C for 2 min and then increased at 8°C/min to 300°C. Mass spectra were recorded at 70 eV. Substances were identified by comparison of mass spectra with those of the National Institute of Standards and Technology (NIST 98) reference library. Compound identities were confirmed by comparison of mass spectra and retention times with those of standard substances. To allow sensitive quantification of VOCs, substance peaks originating from selected ion chromatograms were integrated (generally *m/z* 120 for MeSA and *m/z* 81 for TMTT). The resulting peak areas were related to the peak area of the *n*-octane standard (ion chromatogram *m/z* 114), whereby experimentally determined correction factors were considered for each substance.

Determination of Leaf MeSA Contents

Frozen leaf tissue (150 mg) was homogenized with 600 μL of extraction buffer (water:1-propanol:HCl = 1:2:0.005). After addition of 200 ng D_3-methylsalicylate (Sigma-Aldrich) as internal standard and 1 mL of methylene chloride, the mixture was shaken thoroughly and centrifuged at 14,000 rpm for phase separation. The lower, organic phase was removed, dried over Na_2SO_4, and subject to a vapor phase extraction procedure using a Super-Q collector trap. The final evaporation temperature was set to 200°C, and samples were eluted from the collector trap with 1 mL methylene chloride. Finally, the sample volume was reduced to 25 μL in a stream of nitrogen, and GC-MS analysis was performed as described above.

Determination of Leaf SA, SAG, and JA Levels

Leaf SA, SAG, and JA contents were determined by vapor-phase extraction and subsequent GC-MS analysis according to Mishina and Zeier (2006).

Collection of Leaf Petiole Exudates and Exudate Analyses

Petiole exudates were collected essentially as described previously (Maldonado et al., 2002; Chaturvedi et al., 2008). Plant leaves were either infiltrated with a suspension of *Psm* (OD 0.01) or with 10 mM $MgCl_2$ as a mock inoculation. Six hours after infiltration, leaves were cut at the base of their petioles and the cut surface sterilized by successive dipping for 10 s in 50% ethanol and in 0.0005% bleach. After rinsing petioles with sterile 1 mM EDTA, pH 8.0, they were submerged in fresh EDTA-solution for exudate collection. Twelve-well tissue culture plates were used for this purpose, whereas each well was filled with 2.5 mL of collection solution and equipped with 10 harvested leaves. Exudates were continuously collected in the period from 6 to 48 HAI.

For MeSA analyses, 10 mL of pooled exudate solution was extracted three times with 3 mL of CH_2Cl_2 after 200 ng D_3-MeSA was added as internal standard. The combined organic extracts were analyzed by vapor phase extraction and GC-MS as described above.

For SA determination, the aqueous phase remaining after solvent extraction was acidified with 0.1 M HCl to a final pH of 3, supplemented with internal standard (200 ng of D_6-SA; Sigma-Aldrich), and extracted three times with 3 mL of CH_2Cl_2/methanol (2:1, v/v). The combined organic phases were analyzed according to Mishina and Zeier (2006). For SAG analysis, the acidic aqueous phase remaining after solvent extraction was brought to pH 1.0 with HCl and heated for 30 min at 100°C, and the free SA liberated by hydrolysis was determined as described above.

Analysis of Gene Expression

Expression levels of *PR-1* and *BSMT1* were determined by RNA gel blot analysis as outlined by Mishina and Zeier (2006). *ICS1* expression was analyzed by quantitative real-time PCR, essentially as described by Schlaeppi et al. (2008). Total RNA was isolated from frozen leaves using peqGOLD RNAPure reagent (PeqLab). RNA samples were reverse

transcribed using an Omniscript Reverse Transcription kit (Qiagen) with 1 μg of total RNA. The resulting cDNA samples were diluted 10-fold with water, and quantitative real-time PCR was performed in triplicate using the SensiMixPlus SYBR kit (Quantace) in a Rotor-Gene 2000 apparatus (Corbett Research). In a 15-μL reaction volume, 5 μL of the cDNA sample was combined with 7.5 μL of 2 SYBR Green mix, 1.5 μL water, and 0.5 μL of each primer (both at 10 μM). The cycling included 95°C for 10 min, followed by 45 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s, and finally 72°C for 3 min. The following gene-specific primers were used: 5′-TTCTGGGCTCAAACACTAAA-AC-3′ (ICS1-forward) and 5′- GGC-GTCTTGAAATCTCCATC-3′ (ICS1-reverse). The At1g62930 gene, which is no-responsive to *P. syringae* inoculation (Czechowski et al., 2005), was used as a reference gene and amplified with the primers 5′-GAG-TTGCGGGTTTGTTGGAG-3′ (At1g62930-forward) and 5′-CAAGACAG-CATTTCCAGATAGCAT-3′ (At1g62930-reverse). The data were analyzed using the Rotor-Gene 6000 software, setting the threshold of the normalized fluorescence to 0.15, which corresponded to the exponential phase of the fluorescence signal. The resulting C_T and E values were used to calculate the relative mRNA abundance according to the $\Delta\Delta C_T$ method. The values were normalized to those for the reference gene and expressed relative to the $MgCl_2$-treated wild-type control sample.

Reproducibility of Experiments and Statistical Analyses

All pathogen experiments and the respective bacterial growth analyses, metabolite determinations, and gene expression analyses depicted in the figures were conducted three times with similar results or tendencies. Statistical analyses were performed using Student's *t* test for comparison of two data sets and using analysis of variance (Fisher's Least Significant Difference test) to analyze multiple data sets from comparable treatments.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At3g11480 (*BSMT1*), At2g14610 (*PR-1*), and At1g74710 (*ICS1*).

Supplemental Data

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

Supplemental Figure 1. Petiole Exudation of SA Derivatives from *P. syringae*- and Mock-Inoculated Col-0 Leaves.

Supplemental Figure 2. TMTT Emission from Wild-Type Col-0 and *bsmt1* Mutant Plants.

Supplemental Figure 3. Growth of *Pst* and *Pst* cor$^-$ in Col-0 Leaves.

Supplemental Figure 4. SAG Accumulation in *P. syringae*–Treated Wild-Type and SAR-Defective Mutant Plants.

ACKNOWLEDGMENTS

E.A. is a fellow of the German Academic Exchange Service (DAAD). Financial support from the German Research Foundation (DFG Graduiertenkolleg 1342) is gratefully acknowledged. We are indebted to Barbara Kunkel for kindly providing the *Pst* strains DB29 and KP105 and thank Jean-Pierre Métraux and Felix Mauch for critically reading the manuscript.

Received September 9, 2008; revised February 24, 2009; accepted March 17, 2009; published March 27, 2009.

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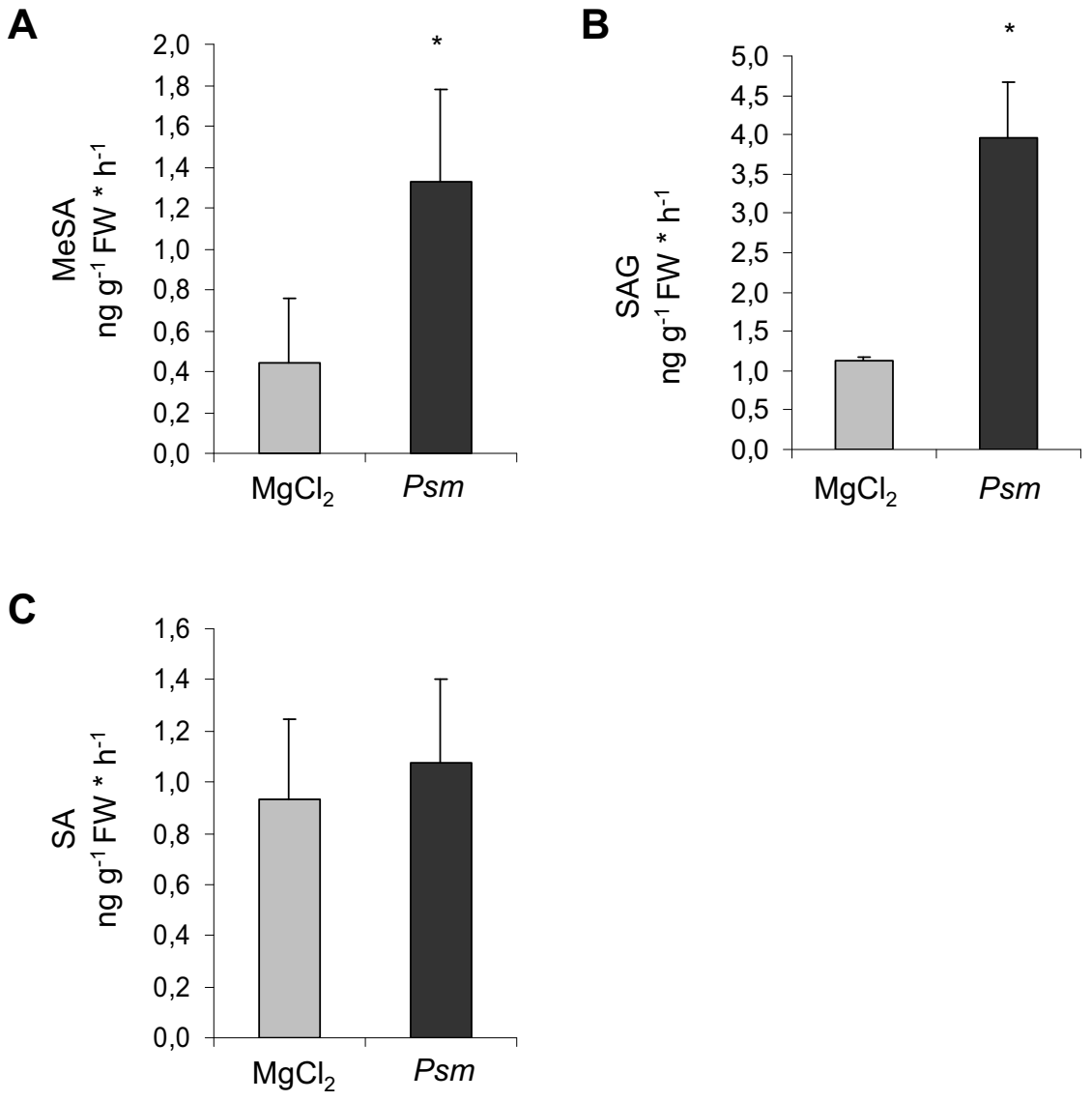
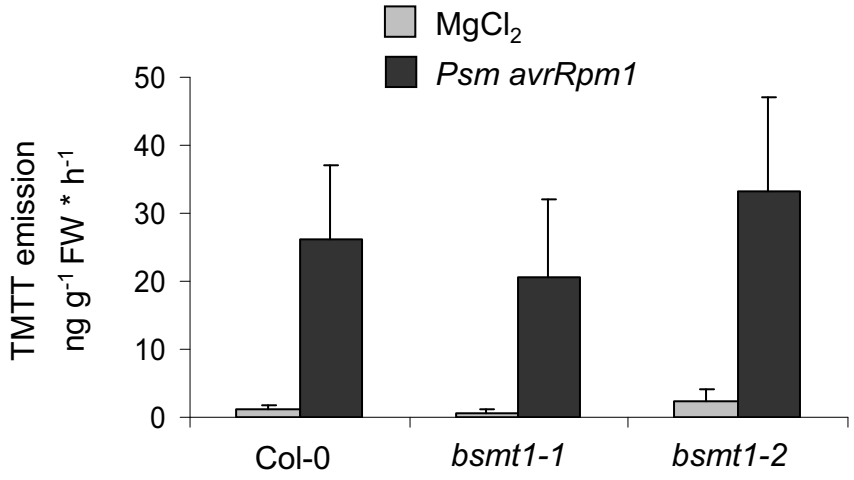
Supplemental Data. Attaran et al. (2009). Methyl salicylate production and jasmonate signaling are not essential for systemic acquired resistance in *Arabidopsis.*



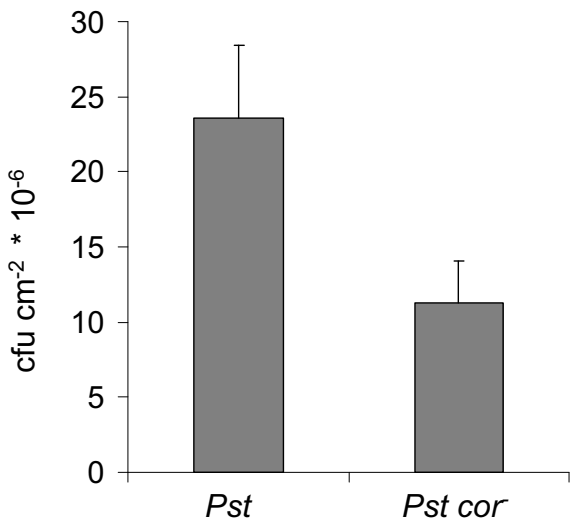
Supplemental Figure 1. Petiole exudation of SA derivatives from *P. syringae*- and mock-inoculated Col-0 leaves.

Exudates were collected between 6 and 48 hpi. Values (means ± SD, n = 5) represent ng exuded substance g^{-1} fresh weight h^{-1}. Asterisks denote statistically significant differences between *Psm*- and $MgCl_2$-treatments ($P < 0.02$).



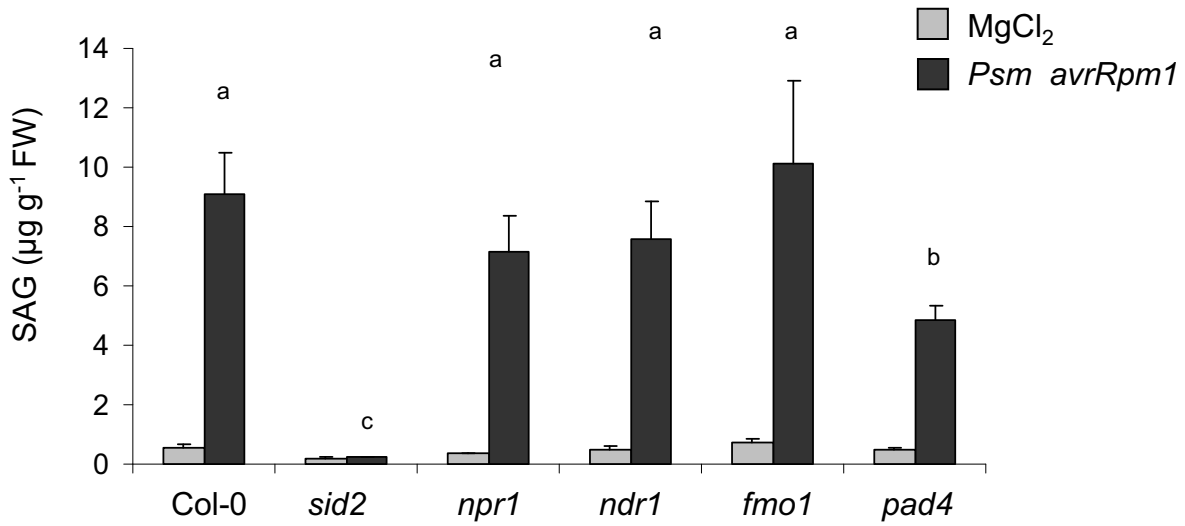
Supplemental Figure 2. TMTT emission from wild-type Col-0 and *bsmt1* mutant plants.

Plants were inoculated with *Psm avrRpm1* or infiltrated with $MgCl_2$. Volatiles were collected from 0 to 24 hpi. Bars represent mean emission values (± SD, n = 4).



Supplemental Figure 3. Growth of *Pst* and *Pst* cor⁻ in Col-0 leaves.

Bacterial numbers of *Pst* and *Pst* cor⁻ in leaves of Col-0 plants were determined one day after inoculation (OD = 0.01). Bars represent means (± SD) of cfu per cm^2 from six parallel samples from different plants, each sample consisting of three leaf disks.



Supplemental Figure 4. SAG accumulation in *P. syringae*-treated wild type and SAR-defective mutant plants.

SAG levels in $MgCl_2$-infiltrated and *Psm avrRpm1*-inoculated leaves at 24 hpi (means ± SD, n = 3). Different letters symbolize statistically significant differences between *Psm avrRpm1*-treated plants from distinct lines ($P < 0.05$).

4 DISCUSSION

4.1 Terpenoid production in the Arabidopsis-*Pseudomonas* interaction

Plants produce a large variety of volatile organic compounds that can function as airborn signals in chemical communication with other organisms. The largest group of plant volatiles comprises low-molecular-weight terpenoids, including monoterpenes, sesquiterpenes and homoterpenes, which serve to attract pollinators, fruit-dispersing animals, and enemies of herbivorours arthropods (Takbayshi and Dicke, 1996; Paré and Tumlinson, 1999; Gershenzon and Pichersky, 2002). Some terpenoids accumulate upon pathogen infection and function as part of the direct defence strategy of plants as phytoalexins. For instance, sesquiterpenes (e.g. capsidiol) are characteristic phytoalexins of the *Solanaceae* family (Chappell and Nable, 1987; Egea et al., 1996).

In recent years, *Arabidopsis thaliana* was shown to be an excellent model plant for investigating ecological interactions. The small white flowers of this species emit low amounts of monoterpenes and sesquiterpenes, with (E)-β-caryophyllene as the predominant compound (Chen et al., 2003; Tholl et al., 2005). Moreover, it has been reported that a blend of the sesquiterpene α-farnesene, the C_{16} homoterpene TMTT, and the benzenoid compound MeSA are released from rosette leaves of *A. thaliana* in response to insect herbivore attack (Van Poecke et al., 2001; Herde et al., 2008).

In the present work, it was shown that *Arabidopsis* plants produce and release two major VOCs, TMTT and MeSA, in response to inoculation with both incompatible and compatible *P.syringae* strains. Moreover, low level emission of β-ionone and α-farnesene occur, in particular during later stages of the compatible interaction.

To identify the terpene synthase responsible for the production of TMTT, the major terpenoid produced in leaves of the Arabidopsis ecotype Col-0 upon *P. syringae*-inoculation, publicly available microarray experiments have been evaluated. These data indicate that out of 32 Arabidopsis *TPS* genes, four are up-regulated (*TPS2, TPS3, TPS4* and *TPS10*) after *P. syringae* infection. Arabidopsis T-DNA knockout lines with insertions in two of these genes,

TPS4 and *TPS10,* could be identified. Among the induced *TPS* genes, *TPS4* showed the highest expression values in both the incompatible and the compatible interaction. The corresponding *tps4* insertion line completely lacked *TPS4* expression and emission of TMTT but not production of β-ionone and α-farnesene. This shows that the terpene synthase TPS4 is specifically involved in and essential for the biosynthesis of TMTT. By contrast, *tps10* mutant plants showed a wild type-like emission profile indicating that TPS10 is neither involved in production of TMTT, nor in biosynthesis of the two other detected terpenoids, β–ionone and α–farnesene.

TMTT is a diterpene-derived volatile produced by many plants in response to herbivory, which is produced by oxidative degradation of geranyllinalool (GL; Boland et al., 1998). Herde et al. (2008) have recently shown that TPS4 functions as a geranyllinalool synthase, catalyzing the conversion of geranylgeranyl diphosphate into geranyllinalool. Moreover, the involvement of CYP450 enzymes to form TMTT from GL is plausible (Herde et al., 2008). This suggests that GL is also the precursor of *P. syringae*-induced TMTT production. The molecular structures of the C16 tetradiene TMTT and the C20-alcohol GL suggest a higher volatility for TMTT than for GL. GL was not detected in the volatile assay in this work. However, as metabolite analysis of leaf extracts demonstrate (J. Zeier, personal communication), GL is indeed produced in Arabidopsis leaves upon *P. syringae* attack, suggesting that GL is also the precursor of TMTT in microbe-induced TMTT synthesis.

TMTT synthesis parallels the biosynthesis of 4, 8-dimethylnona-1,3,7-triene (DMNT), which is likely formed by oxidative degradation of the sesquiterpene (*E*)-nerolidol (Boland et al., 1998). Koch et al. (1999) showed that emission of TMTT in excised lima bean leaves can be induced by early intermediates of the JA biosynthetic pathway, linolenic acid and 12-oxo-phytodienoic acid. In addition, some reports indicate that the tomato mutant *def-1*, which is deficient in induced JA accumulation after wounding or herbivory (Li et al*.,* 2002), does not emit TMTT upon spider mite-infestation. However, emission of TMTT can be restored by pre-treating these plants with JA. These results indicate that in tomato, herbivore-induced biosynthesis of TMTT is regulated by JA (Ament et al., 2004 and 2006). Similarly, Herde et al. (2008) showed that *dde2* (von Malek et al., 2002), as well as *acx1/5* [unable to convert ODPA into JA)] (Schilmer et al., 2007) showed no emission of TMTT in

response to alamethicin. Therefore, JA or its derivatives are necessary for herbivore-induced TMTT production in Arabidopsis (Herde et al., 2008).

The regulation of induced terpenoid biosynthesis upon microbial pathogen attack has not been investigated so far. Therefore, we investigated this missing part about pathogen-induced VOC production. For this purpose, VOC profiles of different well-characterized Arabidopsis defence mutants, including JA- and SA-pathway mutants, have been analysed. Significantly, decrease in the emission of TMTT in JA biosynthetic *dde2* (a male-sterile mutant defective in ALLENE OXIDE SYNTHASE) declares that the presence of JA is essential for production of TMTT in the incompatible Arabidopsis-*P. syringae* interaction. Furthermore, since production of TMTT is strongly attenuated but not fully abrogated in the *opr3* mutant (capable to synthesize OPDA but not JA) upon avirulent *P.syringae* inoculation, OPDA might be capable to activate TMTT biosynthesis to a certain extend. Our observation also showed a reduced emission of TMTT in JA downstream signaling mutants *(jar1*, *jin1, coi1),* corroborating that the JA signaling pathway mediates the production of TMTT in *Arabidopsis.* By contrast, neither SA biosynthetic (*sid2*), SA-defense signaling (*pad4, npr1)*, nor other defence mutants tested *(ndr1* and *fmo1*) show alterations in the *P. syringae*-induced production of TMTT, demonstrating that the defence hormone SA and associated defence signaling pathways do not have a regulatory role in TMTT production. Our result is contradictory with the earlier report by Ament et al. (2006), which shows that unlike the wild-type, SA-deficient *NahG* tomato plant are not able to emit TMTT upon spider mite herbivory. However, they are consistent with data from Herde et al. (2008) which indicate that there is no alteration in production of TMTT when comparing the volatile profiles of SA-deficient/insensitive mutants (*sid2*, *nahG* and *npr1*) with the profile of wild-type plants.Taken together, these results suggest that induced emission of TMTT is dependent on JA production and signaling but not on the SA- or other defence pathways in the incompatible *Arabidopsis-P.syringae* interaction.

Moreover, induced production of TMTT has been observed in the compatible Arabidopsis-*Psm* interaction. JA is unlikely to be the triggering factor in this case, because JA levels are not elevated when leaves are inoculated with low OD's of the compatible *Psm* isolate (Mishina et al., 2008). However, COR, which is heavily produced by *Psm* and *Pst* as a virulence factor, might activate the JA pathway and trigger TMTT production. To prove this

hypothesis, JA-deficient and insensitive mutants have been inoculated with *Psm*. The obtained results show that JA biosynthesis mutant *dde2* indeed is able to produce TMTT upon virulent *Psm* infection [Supplemental data, Fig. 1A]. This is not the case upon avirulent *Psm avrRpm1* inoculation (Attaran et al., 2009). Moreover, the downstream signaling mutant *jar1* but not *coi1* does synthesize TMTT upon *Psm* infection [Suppl. Fig. 1A]. These data suggest that in the compatible Arabidopsis-*P.syringae* interaction, bacterial COR specifically activates the branch of the JA signaling pathway that depends on COI1. To directly test this hypothesis, emission profiles of a coronatine-deficient *Pst* strain (*Pst* DB29; Brooks et al., 2004) have been tested. However, since both the available *Pst cor*$^-$ and the corresponding wild-type *Pst* strain did not evoke TMTT production, this question could not finally be addressed (Suppl. Fig. 2). Obviously, some biochemical differences in the Arabidopsis-*Psm* and the Arabidopsis-*Pst* interaction exists with respect to TMTT synthesis.

To further investigate the regulation of TMTT synthesis, we intended to assess leaf TMTT emission after treatment with $CuSO_4$, which reflects a constant abiotic stess. Treatment of Arabidopsis leaves with 10mM $CuSO_4$ resulted in a massive emission of all detected VOCs (MeSA, TMTT, β-ionone, α-farnesene), as well as in increased production of the non-volatile defense metabolites camalexin, SA, and JA (Attaran et al., 2008; Fig. 8). Such overlapping responses between pathogen-infected and heavy metal-treated plants have been described before at the transcriptional level (Narusaka et al., 2004). Similar to pathogen infection, excess of copper causes ROS production and oxidative stress in plants. Thus, it seemed feasible that ROS constitute an upstream signal triggering the production of TMTT. Therefore, it was tested whether treatment of leaves with a combination of xanthine (X) and xanthin oxidase (XO), a substrate/enzyme mixture specifically generating superoxide (O_2^-), would trigger TMTT emission. Since X/XO treatment lead to a significant elevation of leaf TMTT emission [Suppl. Fig. 4B], it is likely that endogenously produced ROS upon biotic or abiotic stress is one trigger of TMTT biosynthesis.

It was previously shown by Zeier et al. (2004) and Griebel and Zeier (2008) that activation of several defense responses in Arabidopsis leaves upon *P. syringae*-inocularion is dependent on light. On the other hand, a light dependency of VOC emission in several plant species has been reported (Own et al., 2002). In order to investigate a possible light-regulation of *P.*

syringae-induced VOC production, emission of volatiles from Arabidopsis plants situated in different light conditions after *P. syringae* inoculation (constant darkness vs. 9h day/ 15h night cycle) was assessed. A compromised emission of MeSA and TMTT from dark-situated plants strongly suggests that induced production of TMTT in Arabidopsis is indeed light-dependent (Suppl. Fig. 3A). It would be interesting to examine the physiological principles underlying the light-dependency of VOC production in future experiments.

The identification of the *tps4* knockout line provided the opportunity to investigate the functional role of TMTT in the Arabidopsis-*P.syringae* interaction. Because several terpenoids show antimicrobial activity or have been implicated in defence signaling (Soković and Griensven, 2006; Kishimoto et al., 2006), it was expected that TMTT might act as an Arabidopsis phytoalexin or as a defence-activating compound. However, the bacterial growth of virulent and avirulent of *P.syringae* in leaves showed no marked differences between Col-0 and *tps4*, indicating that the contribution of TMTT on disease resistance of Arabidopsis against *P. syringae* is neglectible and that TMTT thus does not function as an effective phytoalexin in this interaction. Moreover, *tps4* is able to accumulate SA and JA in inoculated leaves like the wild type, which suggests normal defense activation in the absence of TMTT. However, accumulation of camalexin was somewhat lower in *tps4* upon *P.syringae* inoculation than in wild-type leaves. This lower accumulation of camalexin in *tps4* might be explained by a slightly reduced growth of the bacteria in *tps4,* which result in a lower initial stimulus and a concomitant decreas of camalexin biosynthesis. When leaves were treated with CuSO4 as a more constant stimulus, similar amounts of camalexin were observed in the wild-type and *tps4*. Toghether, these data indicate that the induced production of TMTT does not significantly alter the outcome of the Arabidopsis-*P. syringae* interaction. Therefore, TMTT does not act as an effective phytoalexin nor has a crucial function as a defense signal.

Airborn signaling from one plant part to the other or from one plant to a neighboring plant through volatiles leading to induction of resistance is a well-documented phenomenon (Baldwin and Schultz, 1983; Heil and Sila Bueno, 2007). Studies using mechanically damaged *Artemisia tridentata* revealed that airflow was necessary for systemic induction of resistance against herbivores, even among branches of the same individual (Karban et

al., 2006). Similarly, systemic induction of extra floral nectar secretion by leaves of wild lima bean in response to beetle feeding occurred only when air was moving freely between leaves (Heil and Sila Bueno, 2007), and volatiles from herbivore-damaged leaves of poplar (*Populus deltoides x nigra*) increased defense responses in adjacent leaves. However, similar studies that investigate the involvement of volatiles and airborn signalling in the regulation of SAR are lacking. Particularly, it has been speculated that possible signals contributing to the onset of SAR might be volatile compounds that freely move through the air (Heil and Ton, 2008). For instance, GLVs and other herbivore-induced VOCs can mediate systemic response of plants to local herbivore damage (Karban et al., 2006; Frost et al., 2007; Heil and Silva Bueno, 2007). The knockout line *tps4* lacking TMTT emission thus was a useful tool to test the involvement of volatile terpenoids in the establishment of the SAR. Although a slight reduction of systemic SA and *PR-1* accumulation in *tps4* compared with the Col-0 wild-type was observed upon *P. syringae* treatment, the bacterial growth assays showed that *tps4* is able to establish *P.syringae*-induced SAR to the same extend as Col-0. Thus, TMTT might have a minor role to amplify SAR related responses, but is not essential to establish a full SAR response under the experimental conditions used in this study.

Taken together, TMTT has no essential role in resistance induction but seems rather to be a by-product of JA signaling activated during the Arabidopsis-*P. syringae*-interaction. Whereas in the incompatible interaction, endogenous JA production activates the respective downstream signaling events, the bacterial virulence factor COR seems to induce JA signaling in the compatible interaction.

4.2 Regulation of *P. syringae*-induced MeSA production and its role in local resistance

The major VOC produced in Arabidopsis leaves upon *P.syringea*- inoculation is methyl salicylate. It was hypothesized that the herbivore-induced emission of a blend of volatiles including MeSA is part of an ecological mechanism to attract predatory insects preying on the herbivores which cause the initial damage to the plant (indirect defence; Van Poecke et al., 2001). MeSA also functions as an animal and insect toxin that exerts its deleterious effects internally after being ingested by the organism (Lindberg et al., 2000).

Moreover, MeSA has been shown to possess microbicidal properties (Demirci et al., 2000).

Chen et al. (2003) have identified an Arabidopsis gene (*BSMT1*) encoding a protein with both benzoic acid (BA) and SA carboxyl methyltransfrase activity. It was shown that recombinant BSMT1 is able to convert SA to MeSA and BA to methylbenzoate. The *BSMT1* gene is highly up-regulated in response to *P. syringae* leaf inoculation, and its expression kinetics of up-regulation is similar to MeSA production: whereas incompatible bacteria cause an early but less-pronounced up-regulation of *BSMT1*, compatible bacteria trigger a later but stronger up-regulation of the gene. These observations together with the findings of Chen et al. (2003) indicated that *BSMT1* might be involved in *P. syringae*-triggered MeSA production. We thus isolated two independent Arabidopsis lines, *atbsmt1-1* and *atbsmt1-2*, both with predicted T-DNA insertions in the *AtBSMT1* coding region. Both lines lack any basal or pathogen-triggered *BSMT1* expression. After determining leaf content and emission, it was also evident that these lines were totally impaired in pathogen-induced MeSA production. This demonstrates that BSMT1 is exclusively responsible for *P. syringae*-induced MeSA production *in planta*. Since we detected faint levels of basal MeSA in leaf extracts of wild-type and *bsmt1* mutant plants, other, pathogen-insensitive methyl transferases might be involved in the synthesis of these low basal MeSA levels.

To get a better understanding of the regulation of *P. syringae*-induced MeSA production, I assessed SA-related, JA-related, and other defense pathway mutants for their VOC profiles after pathogen attack. MeSA production was dramatically reduced in the SA-deficient *sid2* and NahG lines, corroborating that the substrate of BSMT1 in *planta* is SA (Chen et al., 2003; Effermt et al., 2005). However, a wild-type like MeSA production has been observed in other SA defense pathway mutants, indicating that induced MeSA production is independent of SA signaling. By contrast, MeSA production in the incompatible interaction is dependent on both JA biosynthesis and downstream signalling. Thus, the synthesis of the two major Arabidopsis volatiles produced after *P. syringae* attack, TMTT and MeSA, are regulated through similar mechanisms.

The availability of coronatine-deficient (*cor*$^-$) *Pst* mutants (Brook et al., 2004) gave me the opportunity to test whether the induction of MeSA in the

compatible interation would depend on COR. Whereas the wild-type *Pst* strain heavily triggered MeSA emission, the *Pst cor*$^{-}$ strain DB29 (Brook et al., 2004) is not able to stimulate a significantly enhanced leaf emission of MeSA. This demonstrates that MeSA emission from the wild-type *Pst* strain is predominantly induced by the phytotoxin coronatin. COR acts as a structural and functional analog of JA-Ile (Feys et al., 1994; Weiler et al., 1994; Bender et al., 1999; Feys et al., 1994; Lauchli and Boland, 2003). Thus, the emission of MeSA is dependent on SA biosynthesis and promoted by JA biosyntesis and signalling in the incompatible interaction, whereas in the compatible interaction, there is virtually no JA produced and MeSA is triggered by the bacterial virulence factor COR which activates JA downstream signaling. To verify that MeSA emission in the compatible interaction is COR dependent, JA-deficient/insensitive mutants have been inoculated with virulent *Psm*. The obtained results show that the JA biosynthesis mutant *dde2* is able to produce MeSA upon *Psm* infection [Suppl. Fig. 1B]. However, upon avirulent *Psm avrRpm1* inoculation, MeSA emission in JA biosynthesis mutants is attenuated (Attaran et al., 2009). Moreover, the downstream signaling mutant *jar1* but not *coi1* does synthesize MeSA upon *Psm* infection [Supplemental data, Fig. 3B]. These data suggest that in the compatible Arabidopsis-*P. syingae* interaction, bacterial COR specifically activates COI1-dependent JA signaling to mediate MeSA production.

When evaluating bacterial growth in leaves inoculated with either *Psm avrRpm1* or with *Psm*, we observed nearly similar resistance to both avirulent (*Psm avrRpm1)* and virulent (*Psm*) bacteria in *atbsmt1* and Col-0 plants. These outcomes rule out an essential function for MeSA as an antimicrobial or signaling compound in the Arabidopsis-*P.syringae* interaction. To test a possible role of MeSA to induce a typical defense response at sites of *Psm avrRpm1* inoculation, we conducted a series of experiments to asses the accumulation of SA and SAG at the site of infection. Metabolism and conjugation of SA are an important part of detoxification mechanism used by plants to protect them from toxic effect of excess of SA. Because *atbsmt1* plants are not able to emit MeSA, we expected to observe a higher accumulation of SA or SAG in *atbsmt1* mutants than in wild-type plants. However, we observed accumulation of wild-type-like amounts of SA, and even slightly lower SAG levels in mutant than in wild-type plants. Also the same *PR-1* gene expression pattern in the *bsmt1*

mutant like wild-type plant has been observed. These surprising results could be explained by determination of *ICS1* expression in mutants and Col-0 plants. Compared to Col-0, *ICS1* is expressed in *bmst1* after pathogen is attenuated, indicating that SA biosynthesis is somewhat lower in *bsmt1* than in Col-0 plants.

The conversion of SA to MeSA and its concomittant emission from the leaf could have a role in the detoxification of an excess of SA from the plant, and this process is regulated by endogenously produced JA in the incompatible interaction (Fig. 8). Moreover, JA-mediated MeSA production and loss from the plant could partly explain the often observed negative crosstalk between SA and JA. Furthermore, the strong production of MeSA in the compatible Arabidopsis-*P. syringae* interaction is mediated by the bacterial virulence factor COR. Thus, virulent *P. syringae* might deplete the plant SA pool via the action of COR to weaken the SA-defense pathway of the plant (Fig. 8).

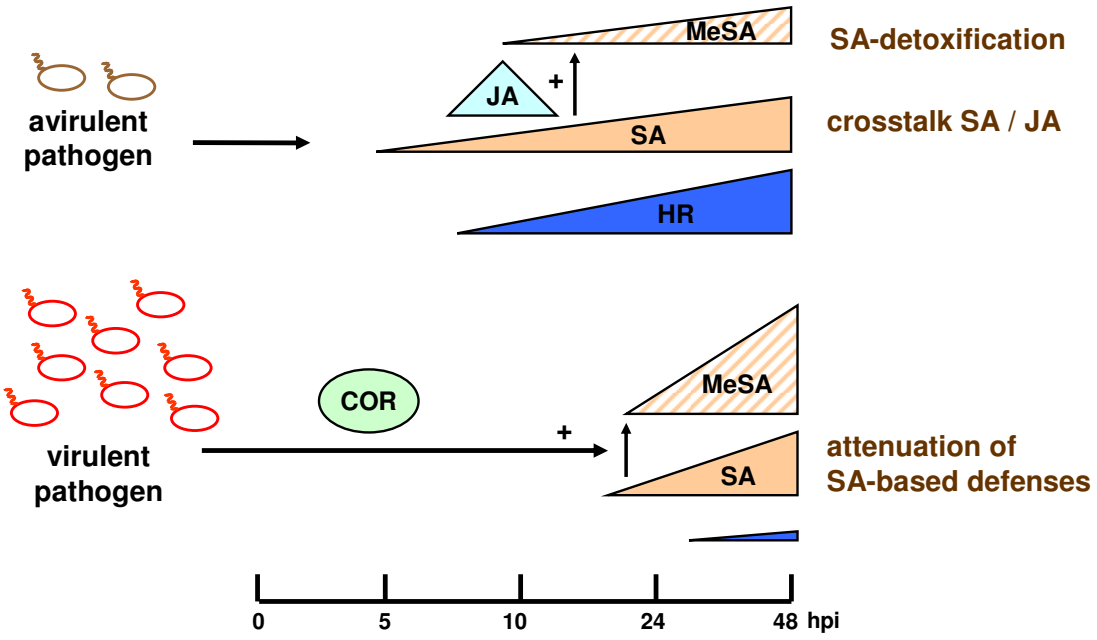


Fig. 8. Possible role of MeSA in the Arabidopsis-*Pseudomonas syringae* interaction (figure provided by J. Zeier).

4.3 Systemic acquired resistance in Arabidopsis against *P. syringae*

The idea that plants are capable to develop a form of acquired immunity to infection has existed since the discorvery of the animal immune system in

the later years of the nintheenth century (Lucas, 1999). Ever since the discovery of systemic resistance in plants, scientists were seeking the factors that function as long-distance signals to intitiate SAR. The conclusions from early work on plant immunity showed that the long-distance signal should stimulate a defensive response, be produced or released at the site of attack, be translocated from the attacked to the systemic tissue, and accumulate in the systemic tissue before resistance expression takes place (Heil and Ton, 2008). A previous publication provides the model that MeSA mediates long-lasting induction of resistance in tobacco (Park et al., 2007). In tobacco, two enzymes control the balance between SA and MeSA: SABP2, an esterase which converts biologically inactive MeSA into active SA, and SA methyltransferase1 (SAMT1), which catalyses the formation of MeSA from SA. *SABP2*-silenced tobacco is unable to express SAR. Therefore, according to this view, MeSA functions as a long-distance signal in tobacco (Shulaev et al., 1997; Park et al., 2007). Also, Vlot and colleagues (2008) analysed an 18-member methyl esterase gene family in Arabidopsis (At*MES*1-18) and concluded that MeSA is most likely a conserved SAR signal between plant species (Vlot et al., 2008a, 2008b).

In this project, we attempted to unravel the role of MeSA during SAR in Arabidopsis. To clarify whether MeSA is essential to establish SAR, we attempted to identify plants which cannot produce MeSA. As a first step, we showed that MeSA is a major *P. syringae*-triggered volatile emitted from Arabidopsis leaves, and that its production is mediated by *BSMT1*. Also, the expression kinetics of *BSMT1* closely correlates with the timing of MeSA production. BSMT1 belongs to a group of Arabidopsis methyltransferase and has the ability of converting SA to MeSA *in vitro* (Chen et al., 2003; Koo et al., 2007). Two independent predicted T-DNA insertions in the *BSMT1* coding region, Arabidopsis *bsmt1-1* and *bsmt1-2*, not only fail to express the *BSMT1* gene but are also unable to elevate MeSA production upon pathogen infection.

Therefore, the required tool to test the necessity of MeSA to establish SAR was available. In other words, if MeSA was important for SAR in Arabidopsis, the *bsmt1* mutant should display a SAR-compromised phenotype. However, the bacterial growth of secondary leaves of *bsmt1* plants upon secondary infection showed wild-type response in comparison to the control treatment. Also both lines (*bsmt1-1* and *bsmt1-2*) are able to elevate the usual SAR responses such as SA elevation and *PR* gene expression. Therefore; *bsmt1*

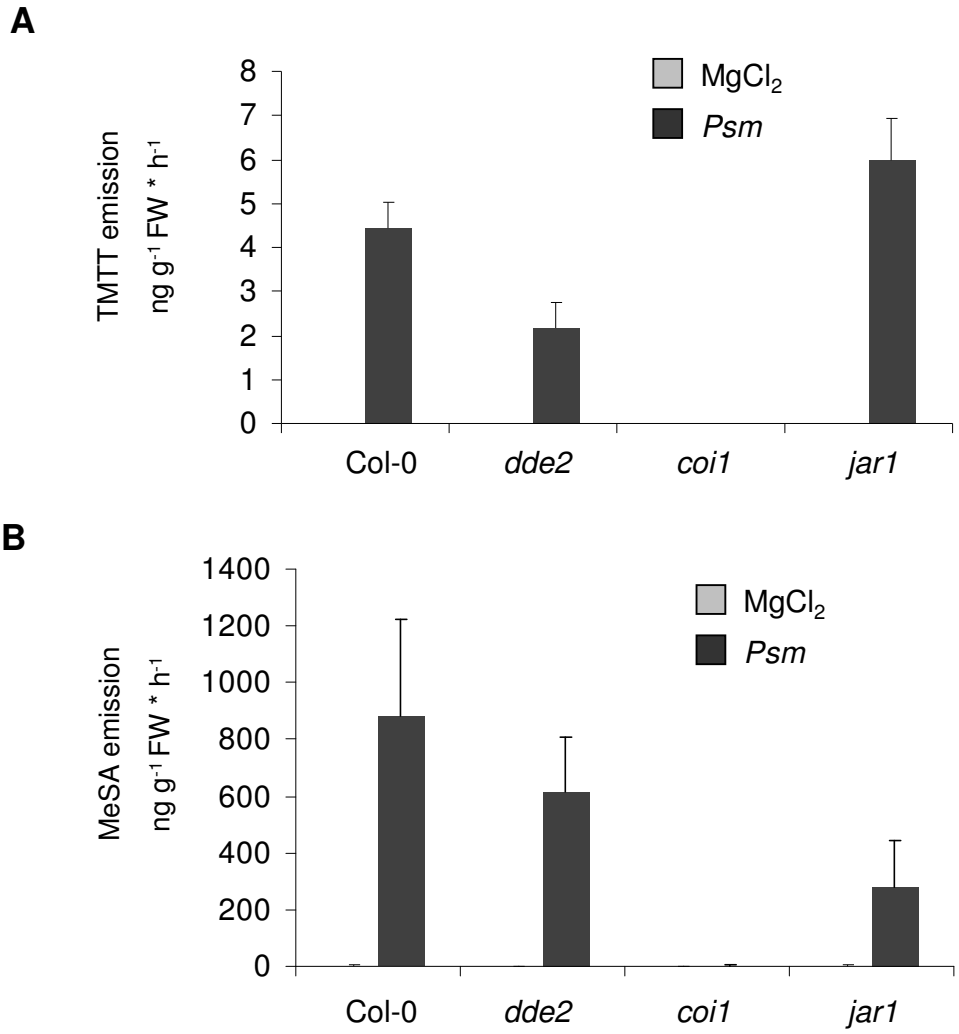
plants are capable to mount SAR in a wild-type like manner, unequivocally demonstrating that MeSA is dispensable for SAR in Arabidopsis. This data is opposing to the previous reports of Park et al. (2007), Vlot et al. (2008), and a very recent report from Liu et al. (2010). It thus seems that MeSA is not a conserved signal between all species. This is surprising because SAR has been reported in at least 20 plant species, and the mechanisms responsible for the induction and expression of SAR are thought to be conserved among different species (Métraux et al., 1997; Lucas, 1999).

This genetic evidence using Arabidopsis mutants is corroborated by our results obtained from infection experiments with the coronatine-nonproducing *Pst cor-* strain. On one hand, the wild-type-like SAR-inducing capacity of *Pst cor-* mutants reveals that bacterial production of COR does not affect the SAR process. On the other hand, SAR-induction through *Pst cor-* is associated with a suppressed leaf MeSA production. This further underlines the dispensability of MeSA during the SAR process in Arabidopsis

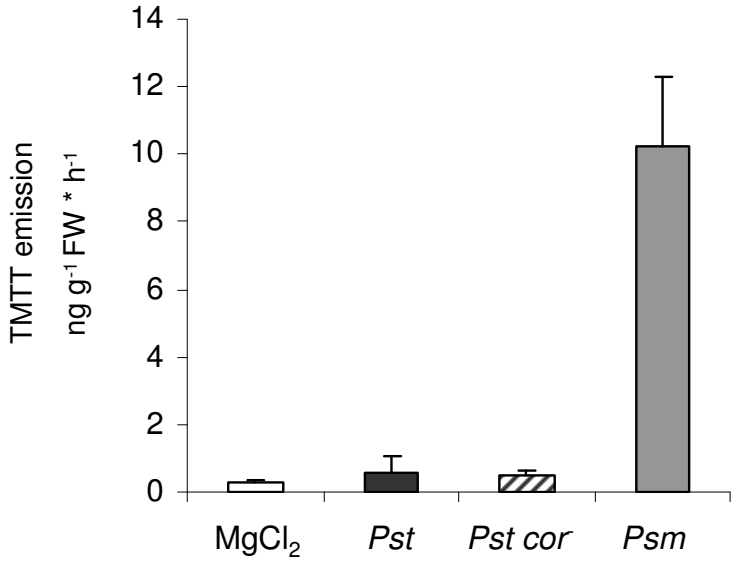
Our quantitative analyses indicate that the major part of MeSA (97-99%) is emitted from the inoculated leaves into the air, and only a minor part is contained in leaves or detectable in petiole exudates. Therefore, due to its high volatility, it is not possible that MeSA travels through the phloem to other plant parts in a controlled and directed manner. For example, we showed that in the incompatible *Psm avrRpm1–Arabidopsis* interaction, the amount of retained MeSA in leaves is equal to the value emitted during only 30 minutes. Our quantitative data also showed that the amount of accumulated MeSA after bacterial inoculation in leaf exudates during a 48h SAR induction period is much lower than the usually observed systemic elevation of SA levels during *P. syringae*–induced SAR in Arabidopsis (Mishina and Zeier, 2007; Mishina et al., 2008). Moreover, increase in MeSA content is not detectable and only a small elevation of MeSA emission in noninoculated leaves after pathogen treatment has been observed, indicating that the content of MeSA in systemic leaves is not elevated, and its systemic emission only slightly increases. This is consistent with the minor and statistically hardly significant elevations of systemic MeSA reported by Park et al., 2007. In summary, our genetic data and the physicochemical properties of MeSA strongly argue against the molecule being a phloem-mobile long distance signal and an essential role in the SAR process.

Recently, Truman et al. (2007) introduced JA as a mobile signal which might travel along the vascular system in Arabidopsis to mount SAR. They showed that systemic resistance was attenuated in the JA-signaling and biosynthesis mutants *jin1* and *opr3*, respectively. On the other hand, a report from Chaturvedi et al. (2008) argued against JA as mobile SAR signal. This work revealed that petiole exudates collected from Arabidopsis leaves which were inoculated with *Pst avrRpm1* does not copurify with JA, and none of the JA derivatives tested are able to activate SAR in SAR-inactive leaf exudates. Similarly, our data argue against JA as a mobile SAR signal, since the JA biosynthesis mutant *dde2* and *opr3* as well as JA signaling mutant *coi1*, *jar1*, *jin1* are able to mount a *Psm*-induced SAR response. Thus, the data from our as well as other laboratories (Cui et al., 2005; Chaturvedi et al., 2008) do not support a role of JA or its derivatives in SAR. In summary, it can be concluded that neither MeSA nor JA is required to induce SAR in Arabidopsis. Therefore, efforts to identify the signal(s) that initiate SAR in plants are still ongoing.

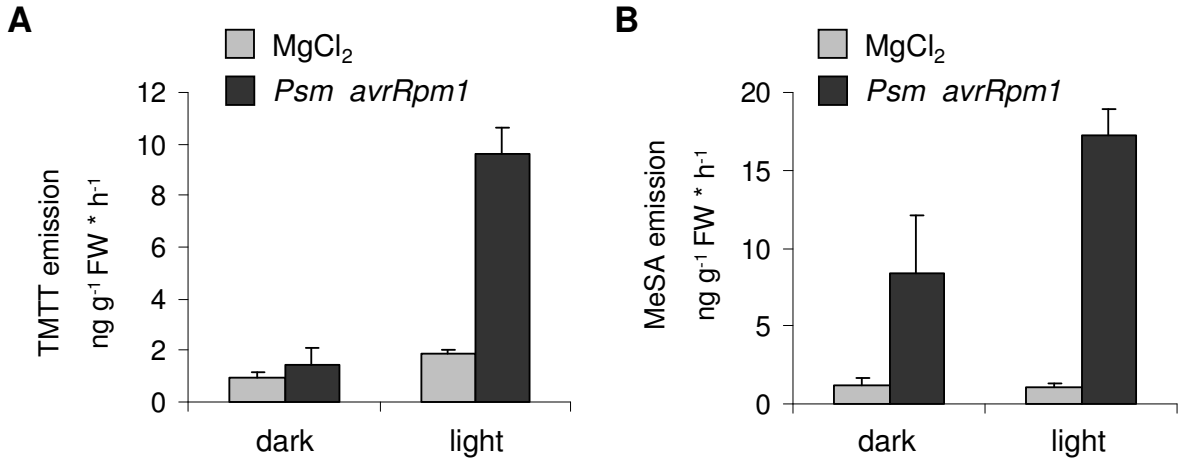
5 Supplemental Figures



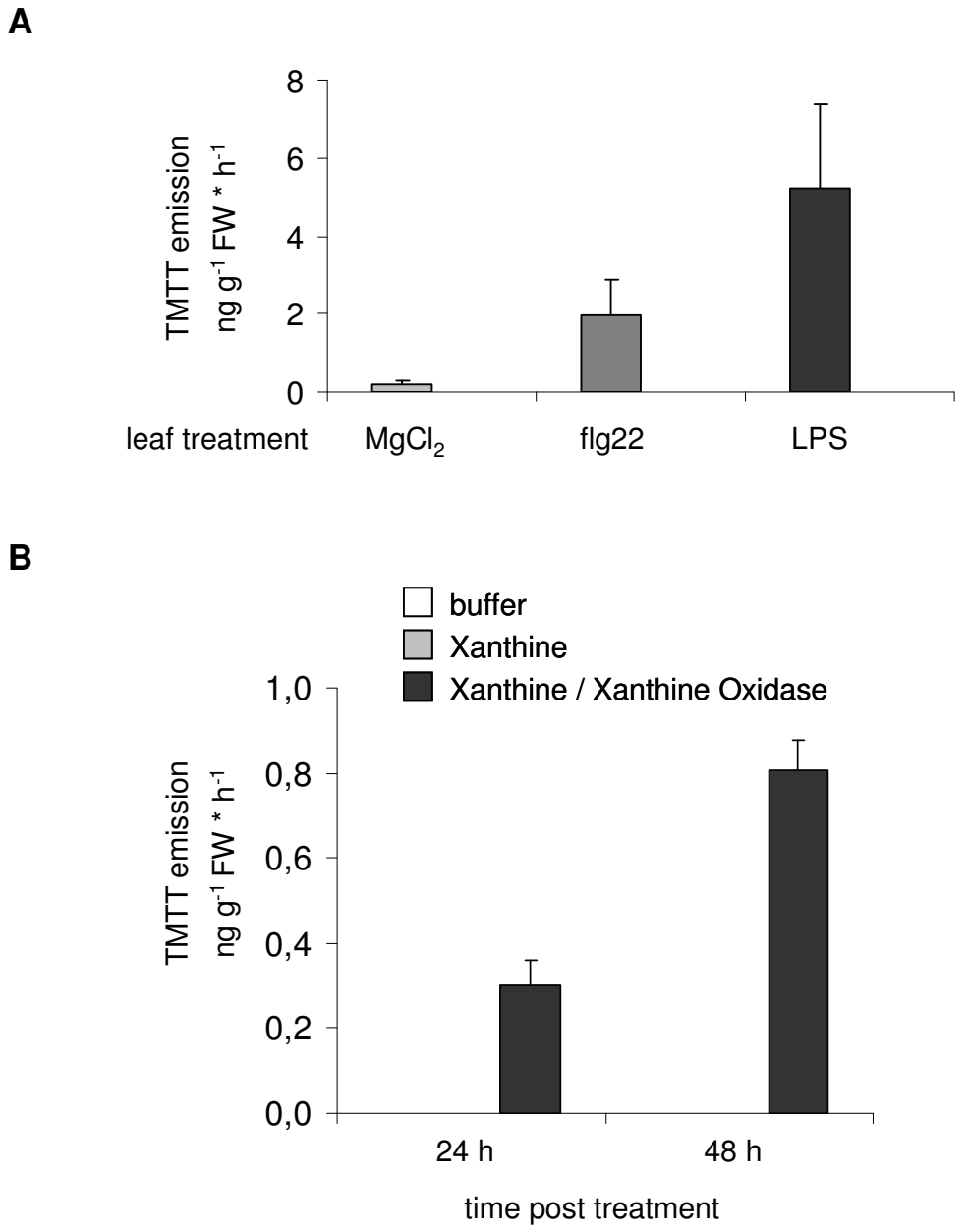
Supplemental Figure 1. *Pseudomonas syringae*-induced VOC (TMTT and MeSA) emission from wild-type Col-0 and jasmonic acid pathway mutants. Volatile emission was analysed from plants inoculated with *Psm* [dark bars] or infiltrated with 10mM $MgCl_2$ (control, light bars). Volatiles were collected from 0 to 24 h post inoculation. Bars represent mean emission values (± standard deviation) from three independent plants. **A**, TMTT emission. **B,** MeSA emission.



Supplemental Figure 2. TMTT emission from Col-0 leaves after inoculation with coronatine-producing *Pst*, coronatine-deficient *Pst cor*, and *Psm*, and after $MgCl_2$-infiltration. Volatiles were sampled from 0 to 24 hpi, and mean values of ng emitted substance g^{-1} leaf fresh weight h^{-1} (± SD, n = 3) are given.



Supplemental Figure 3. Emission of TMTT and MeSA from Col-0 leaves after inoculation with *P. syringae* pv. *maculicola avrRpm1* (dark bars) or infiltrated with 10mM $MgCl_2$ (control, light bars) in different light condition [short day conditions (9h light, 15h dark) vs. constant darkness]. Sampling time was from 0 to 24 hpi. **A**, TMTT emission. **B**, MeSA emission.



Supplemental Figure 4. TMTT production from wild-type Col-0 plants. Mean values of ng emitted substance g^{-1} leaf fresh weight h^{-1} (± SD, n = 3) are given **A**, Emission of TMTT from Col-0 leaves after infiltration with 10 mM $MgCl_2$ as control, 200 nM flg22, and 100 µg ml^{-1} LPS. Volatiles were sampled from 0 to 24 hpi. **B**, Emission of TMTT from Col-0-plants after leaf infiltration with control buffer (20 mM sodium phosphate buffer), 0.5 mM xanthine and 0.5 mM xanthine / 0.5 U ml^{-1} xanthine oxidase. The sampling period was between 0 and 24 or between 0 and 48 h post treatment. TMTT could not be detectd with buffer and xanthine treatments.

6 SUMMARY AND PERSPECTIVES

Plants are constantly attacked by pathogenic microbes. As a result, they have evolved a plethora of constitutive and inducible defense responses to defend against attempted pathogen infection. Although volatile organic compounds have been implicated in plant defense, direct evidence of their function in plant resistance is still lacking.

I have examined the role of VOCs in Arabidopsis defense against the hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv. *maculicola*. The obtained results show that the vegetative parts of Arabidopsis produces and emits the volatile phenylpropanoid MeSA and three kinds of terpenoids, (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), β-ionone and α-farnesene, upon avirulent and virulent *P. syringae* inoculation. Whereas the most abundant volatiles, MeSA and TMTT, are already produced at early stages of infection in the compatible and incompatible interaction, enhanced emission of β-ionone and α-farnesene can only be detected in later stages of the compatible interaction. It was revealed that pathogen-induced synthesis of TMTT in Arabidopsis requires the JA signaling pathway but occurs independently of SA defense signaling. Similarly, the production of MeSA is dependent on JA signaling but not on the SA defense signaling pathway. Furthermore, production of MeSA is dependent on the function of ISOCHORISMATE SYNTHASE1, which produces its precursor SA. Upon inoculation with avirulent *P. syringae*, endogenously produced JA activates the JA signalling pathway to mediate MeSA and TMTT synthesis. By contrast, in the compatible Arabidopsis-*Psm* interaction, production of MeSA predominantly depends on the *P. syringea* the virulence factor coronatine, which activates JA downstream signaling.

To learn more about the role of inducible VOCs in plant defense responses, I have identified an Arabidopsis T-DNA insertions line with a defect in the *TERPENE SYNTHASE4 (TPS4)* gene. Emission profiles from this mutant revealed that the induced production of TMTT but not of β-ionone, α-farnesene or MeSA are abolished, demonstrating that TPS4 specifically regulates the *P. syringae*-induced synthesis of TMTT in Arabidopsis. The lack of TMTT in *tps4* mutants, however, does not affect plant defense responses and resistance induction against *P. syringae*. This excludes a role

of the terpenoid as an effective phytoalexin in Arabidopsis leaves against the bacterial pathogen. Moreover, *tps4* mutant plants are still able to mount a SAR response, excluding a signaling function of TMTT during SAR.

An important aim of our studies was to address the defensive role of MeSA, the major VOC emitted from *P. syringae*-inoculated Arabidopsis leaves. MeSA has been recently proposed as a critical long distance signal in the development of SAR. I found that two independent T-DNA insertions lines with defects in expression of the pathogen-inducible SA methyl transferase gene *BSMT1* are completely devoid of pathogen-induced production of MeSA. However, *bsmt1* mutant plants are capable to increase the level of SA in systemic, non-infected leaves of Arabodopsis and develop SAR like wild-type plants upon local *P. syringae*-inoculation. Thus, MeSA does not function as a critical SAR signal in Arabidopsis. Further experiments showed that SA accumulation in distant leaves occurs due to *de novo* synthesis through isochorismate synthase. In addition, we also ruled out a critical defensive role of MeSA at inoculation sites, because *bsmt1* mutants are able to build up SA-dependent defense responses and local resistance in a wild-type-like manner. The conversion of SA to MeSA and subsequently emission of MeSA from the plant might help the plant to detoxify an excess of SA. This process is regulated by the JA pathway and might be one means to mediate negative crosstalk between JA and SA signaling. Moreover, the COR-triggered conversion of SA to MeSA and emission of the volatile methyl ester could be a way by which virulent *P. syringae* is able to attenuate the SA-defense pathway.

Perspectives

Release of VOCs in response to pathogens or herbiveres is energy-costly for a plant. However, this work demonstrates that the two main VOCs produced by Arabidopsis, the terpenoid TMTT and MeSA, do not have an obvious function in induced resistance against *P. syringae*. To better understand the defensive role of these VOCs, it might be necessary to investigate the resistance behaviour of *tps4* and *atbsmt1* mutant plants in response to inoculation with further microbes, such as the fungal and oomycete pathogens *Hyaloperonospora arabidopsidis*, *Botrytis cinerea,* or *Phytophthora infestans*. It might also be promising to examine the interaction

between *tps4* and *bsmt1* plants and insects to unreveal the role of TMTT and MeSA in herbivory and egg oviposition.

Another aspect to continue this research might be to further explore the regulation underlying pathogen-induced VOC production. For instance, similar to the establishment of plant SAR, the light-dependency of TMTT production might rely on a specific light signaling pathway. In addition, it would be interesting to further study the biosynthesis of TMTT in Arabidopsis and to look closer at the function of CYP450 enzymes in modification of the TMTT precursor geranyllinalool in this context. Finally, the determination of the subcellular localization of TPS4 and BSMT1 could contribute to a better understanding of the roles of TMTT and MeSA in the responses of Arabidopsis towards microbial pathogens.

ZUSAMMENFASSUNG

Pflanzen sind einer ständigen Bedrohung durch phytopathogene Mikroorganismen ausgesetzt und haben deshalb eine Vielzahl von konstitutiven und induzierbaren Abwehrstrategien entwickelt. Die Phytohormone Salicylsäure (SA), Jasmonsäure (JA) und Ethylen sind zum Beispiel entscheidende Regulatoren von induzierten Abwehrmechanismen. Eine Antwort der Pflanze auf mikrobielle Angriffe beinhaltet auch die Emission volatiler organischer Verbindungen (volatile organic compounds - VOCs). Antimikrobielle Wirkungen von VOCs wurden bisher jedoch nur in *in-vitro*-Assay beobachtet. Ein direkter Beweis für eine mögliche Rolle der VOCs in der Pflanzenabwehr wurde bisher nicht erbracht.

Die Rolle pflanzlicher VOCs und deren Bedeutung für die Pathogenabwehr im Modellsystems *Arabidopsis thaliana – Pseudomonas syringae* ist das zentrale Element dieser Arbeit.

Zunächst wurden Terpenoide, die die größte Gruppe der VOCs bilden, untersucht. Vegetative Teile von Arabidopsis emittieren nach Inokulation mit virulenten und avirulenten Stämmen von *P. syringae* pv. *maculicola* (*Psm*) vor allem drei Terpene: das Homoterpen (E,E)-4,8,12-Trimethyl-1,3,7,11-tridecatetraen (TMTT), β-Ionon und α-Farnesen, welches zur Gruppe der Sesquiterpene gehört. Als Hauptkomponente des pathogen-induzierten VOC-Profils wurde das Phenylpropansäurederivat Methylsalicylsäure (MeSA) identifiziert.

Um einen besseren Einblick in die Rolle der VOCs in der Pflanzenabwehr zu erhalten, wurden Arabidopsis T-DNA-Insertionslinien des Terpensynthase-gens *TPS4* isoliert. Die Emissionsmuster zeigten, dass die induzierbare Freisetzung von TMTT, aber nicht von β-Ionon, α-Farnesen oder MeSA reduziert war. Dies zeigt, dass TPS4 spezifisch die *Psm*-induzierte TMTT-Synthese in *A. thaliana* reguliert. Die verringerte Menge TMTT in den *tps4*-Mutanten hat jedoch keinen Einfluss auf die pflanzlichen Abwehrreaktionen und die Resistenzinduktion gegen *P. syringae*, was eine Rolle von TMTT als effektives Phytoalexin in *A. thaliana* gegen bakterielle Pathogene ausschließt. Ebenso hat TMTT keine Signalfunktion bei der Ausbildung der Systemisch erworbenen Resistenz (SAR), da *tps4*-Mutanten weiterhin in der Lage sind eine SAR-Antwort zu induzieren (Attaran et al. 2008).

Als weiteres Teilprojekt wurde die Regulation von pathogen-induzierten VOCs in *A. thaliana* untersucht. Viele induzierte Abwehrmechanismen beinhalten Signaltransduktionsnetzwerke an denen Salicyl- oder Jasmonsäure beteiligt sind. Mit *A. thaliana*-Mutanten, die in der SA- oder JA-Synthese oder den jeweiligen Signalwegen beeinträchtigt sind, konnte gezeigt werden, dass die pathogen-induzierte TMTT-Produktion in *A. thaliana* über den JA-Signalweg, aber unabhängig von Salicylsäure verläuft. Auch die MeSA-Produktion ist JA-abhängig. Für die Biosynthese von SA, genauso wie für deren Derivat MeSA, wird ISOCHORISMAT SYNTHASE1 benötigt, die den MeSA-Vorläufer SA bildet. Im Rahmen einer inkompatiblen Interaktion wird die Bildung von MeSA in Abhängigkeit von der JA-Biosynthese gesteuert. Im Gegensatz dazu ist in der kompatiblen Interaktion die MeSA-Produktion vom bakteriellen Virulenzfaktor Coronatin abhängig. Coronatin-defiziente Stämme von *P. syringae* sind nicht fähig, eine MeSA-Emission zu induzieren (Attaran et al., 2009).

Desweiteren wurde in der vorliegenden Arbeit die Rolle von MeSA in der Pflanzenabwehr untersucht. MeSA ist das VOC, welches von *P. syringae*-inokulierten *A. thaliana*-Blättern vorwiegend abgegeben wird. Kürzlich wurde für MeSA eine Signaleigenschaft als Langstreckensignal in der Etablierung der SAR postuliert (Park et al., 2007). Wir konnten zeigen, dass T-DNA Insertionslinien, bei denen keine Expression der pathogeninduzierten SA-Methyltransferase BSMT1 nachgewiesen werden konnte und die somit keine pathogen-induzierte MeSA-Produktion aufwiesen, auch in systemischen, nicht infizierten Blättern nach *P. syringae*-Inokulation einen erhöhten SA-Spiegel, eine verstärte Expression von Abwehrgenen und eine erhöhte Pathogenresistenz aufwiesen. Diese Mutantenlinien können also die SAR genauso und in demselben Maß wie Wildtyp-Pflanzen entwickeln. Damit konnte gezeigt werden, dass MeSA nicht als zentrales Signal für die Ausbildung der SAR in Arabidopsis wirken kann. Weitere Experimente machten deutlich, dass die SA-Akkumulation in distalen Blättern auf eine *de-novo*-Synthese durch die Isochorismat-Synthase zurückzuführen ist. Schließlich konnte auch eine wichtige Rolle von MeSA in der Pflanzenabwehr an den Infektionsstellen ausgeschlossen werden, da *bsmt1*-Mutanten SA-abhängige Abwehrreaktionen und lokale Resistenzantworten in gleicher Weise wie Wildtyp-Pflanzen zeigen (Attaran et al., 2009). Produktion und anschließende Emission von MeSA könnte daher in der Pflanze dazu beitragen, einen toxischen Überschuss an SA abzubauen. Reguliert wird

dieser Prozess durch den JA-Signalweg, der dadurch einen negativen Einfluss auf den SAHaushalt der Pflanze innehat. Die Auslösung der MeSA-Produktion von dem bakteriellen Virulenzfaktor COR in der kompatiblen Wechselwirkung könnte eine Strategie von *P. syringae* sein, die Effizienz der SA-basierenden Abwehr zu verzögern.

7 SUPPLEMENT

List of publications

1. **Attaran E**, Zeier TE, Griebel T, Zeier J (2009) Methyl salicylate production and jasmonate signaling are not essential for systemic acquired resistance in Arabidopsis. **Plant Cell** 21:954-971.
2. **Attaran E**, Rostás M, Zeier J (2008) *Pseudomonas syringae* elicits emission of the terpenoid (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene in Arabidopsis leaves via jasmonate signaling and expression of the terpene synthase TPS4. **Mol Plant-Microbe Interact** 21: 1482-1497.
3. Mishina TE, Griebel T, Geuecke M, **Attaran E**, and Zeier J (2008) New insights into the molecular events underlying systemic acquired resistance. Paper 81 in: M.Lorito, S.L. Woo, F.Scala, eds. Biology of Molecular Plant-Microbe Interactions CD. Vol 6. International Society for Molecular Plant-Microbe Interactions, St. Paul, MN.

Poster presentations

1. **Attaran E,** Zeier J (2009) Regulation of pathogen-inducible volatiles in Arabidopsis and their role in plant defence. 6th Tri-national Arabidopsis Meeting" Köln – Germany.
2. **Attaran E**, Zeier J (2007) Pathogen-inducible terpenoid volatiles in Arabidopsis and their role in plant defence. 8th Congress on "Molecular Plant-Microbe Interactions" - Sorrento – Italy.

7.1 *Curriculum Vitae*

Personal Information

Birthday / Place	21.09.1976 – Tehran, Iran
Nationality	Iranian

Education

2006- 2010: PhD in Molecular Plant Pathology Julius-Maximilians- Universität Würzburg, Germany.

Research focus: Pathogen-inducible volatiles in *Arabidopsis* and their role in plant defence

2000-2003: Master of Science in Plant Physiology Teacher Training University, Tehran-Iran; Research focus*:* Saponin and Anthocyanin concentrations in *Bellis Perennis* L. plants as affected by salinity and mineral deficiency.

1994-1998: Bachelor of Science in Botany .Shahid Bahonar University, Kerman-Iran

Training and workshops

29-30.09.2008: Self-confidence, Assertiveness and Communication Workshop, Würzburg-Germany.

08-17.01.2009: Training in qPCR to determine gene expression in plants, Fribourg – Switzerland.

Experiments / Employment

01.2000 -10.2005: Dr. Farhud Genetic Clinics as a scientific co-worker as well as laboratory manager, Tehran-Iran

10.2006- up to now: Dr. Jürger Zeier laboratory as a scientific co-worker (in the framework of a DAAD ferllowship) Würzburg-Germany.

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Erklärung

Hiermit erkläre ich, die vorliegende Dissertation selbst angefertigt und nur die angegebenen Quellen und Hilfsmittel verwendet zu haben.

Weiterhin erkläre ich, dass die vorliegende Dissertation weder in gleicher noch ähnlicher Form einem anderen Prüfungsverfahren vorgelegt wurde.

Hiermit bewerbe ich mich erstmals um den Doktorgrad der Naturwissenschaften der Julius-Maximilians-Universität Würzburg.

Würzburg, den 18. 1. 2010
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Elham Attaran