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"Identification of Systemic Acquired Resistance–Related Volatile Organic Compounds and their Role in Plant Immunity"

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II. SUMMARY

Systemic acquired resistance (SAR) is an inducible immune response that depends on ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1), which is essential for SAR signalling. In contrast to SAR, local resistance remains intact in *Arabidopsis* (*Arabidopsis thaliana*) *eds1-2* mutant plants in response to *Pseudomonas syringae* delivering the effector protein AvrRpm1. I utilized the SAR-specific phenotype of the *eds1-2* mutant to identify volatile organic compounds (VOCs) related to SAR. To this end, SAR was induced by the *P. syringae* effector *AvrRpm1* that was expressed from a dexamethasone-inducible transgene (*pDEX:AvrRpm1-HA*). The volatile emissions of Col-0 *pDEX:AvrRpm1-HA* (Col-0 dex) and *eds1-2 pDEX:AvrRpm1-HA* (*eds1-2* dex) plants were collected and analysed by gas chromatography coupled to mass spectrometry (GC-MS). The monoterpenoid VOCs camphene, α-pinene, and ß-pinene were emitted at higher levels from Col-0 dex compared to *eds1-2* dex mutant plants; this associates them with SAR. Incubation of *Arabidopsis* wild type (wt) plants with a purchased volatile blend of α-pinene, ß-pinene, camphene, and limonene induced resistance against the virulent *P. syringae* pathovar tomato (*Pst*) strain DC3000. α-Pinene and ß-pinene were the crucial components of the volatile blend, while camphene and limonene contaminants likely had a contributing effect to resistance. This induced resistance was accompanied by elevated transcript accumulation of the salicylic acid (SA) marker gene *PATHOGENESIS-RELATED1* (*PR1*). Also, the SA pathway components *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*), *SA INDUCTION–DEFICIENT2* (*SID2*), and *NONEXPRESSOR OF PR GENES1* (*NPR1*) were essential for the resistance phenotype induced by the volatile blend. Furthermore, *GERANYL(GERANYL)DIPHOSPHATE SYNTHASE12* (*GGPPS12*, At4g38460) is essential for monoterpene biosynthesis, and *ggpps12* mutant plants showed a defect in SAR. However, resistance to *Pst* DC3000 was induced in *ggpps12* mutant plants in response to the volatile blend camphene/α-pinene/β-pinene/limonene. Together, the data provide a first link between monoterpene biosynthesis and SAR, suggesting that the monoterpenoid VOCs camphene, α- and β-pinene, and limonene function additively and in parallel with SA in plant immunity and in particular SAR.

Systemisch erworbene Resistenz (SAR) ist eine induzierbare Immunantwort der Pflanze, bei der das Protein ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) essentiell für den Transport von SAR-Signalen ist. Auf einen Angriff des Pflanzen-Pathogenes *Pseudomonas syringae*, welches das Effektor Protein AvrRpm1 in die Pflanze einschleust, können *eds12* mutierte Pflanzen keine SAR ausbilden, die lokale Resistenzantwort der Pflanze bleibt hingegen funktionsfähig. Dieser SAR spezifische Phänotyp der *eds1-2* Mutante wurde zur Identifizierung flüchtiger organischer Komponenten (VOCs) im Kontext von SAR herangezogen. Es wurden volatile Emissionen von *Arabidopsis thaliana* Col-0 *pDEX:AvrRpm1-HA* (Col-0 dex) und *eds1-2 pDEX:AvrRpm1-HA* (*eds1-2* dex) Pflanzen gesammelt und mittels Gaschromatographie mit Massenspektrometrie-Kopplung (GC-MS) analysiert. Col–0 dex Pflanzen emittierten im Vergleich zu *eds1-2* dex Pflanzen größere Mengen der monoterpenen VOCs Camphen, α-Pinen und ß-Pinen, was diese mit SAR assoziiert. Durch Inkubation von *Arabidopsis* Wild Typ Pflanzen mit einem gekauften Gemisch bestehend aus α-Pinen, ß-Pinen, Camphen und Limonen konnte eine Abwehrreaktion der Pflanzen gegenüber dem virulenten Pathogen *P. syringae* pathovar tomato (*Pst*) Stamm DC3000 induziert werden. α-Pinen und ß-Pinen stellten sich als die essentiellen Komponenten des Gasgemisches heraus, wobei die in geringeren Mengen enthaltenen Komponenten Camphen und Limonen auch zur Resistenzinduktion beitrugen. Die Resistenzinduktion war von einer Transkript-Akkumulierung des Salizylsäure (SA) Marker Gens *PATHOGENESIS-RELATED1* (*PR1*) begleitet. Wichtige Komponenten des Salizylsäure Signalweges wie *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*), *SA INDUCTION–DEFICIENT2* (*SID2*) und *NONEXPRESSOR OF PR GENES1* (*NPR1*) waren essentiell für die Resistenzinduktion.

Darüber hinaus ist das Protein GERANYL(GERANYL)DIPHOSPHATE SYNTHASE12 (GGPPS12, At4g38460) essentiell für die Biosynthese von Monoterpenen und *ggpps12* mutierte Pflanzen zeigten einen Defekt in SAR gegenüber *Pst* DC3000. Durch die Begasung mit einem Gemisch aus Camphen/α-Pinen/β-Pinen/Limonen konnte jedoch eine lokale Resistenzantwort in *ggpps12* mutierten Pflanzen induziert werden. Die Ergebnisse dieser Studie liefern eine erste Verbindung zwischen Monoterpen Biosynthese und SAR und weisen auf eine additive Funktion der monoterpenen VOCs Camphen, α- und β-Pinen und Limonen parallel zu SA in pflanzlicher Immunität und SAR im speziellen hin.

III. LIST OF FIGURES AND TABLES

1. INTRODUCTION

1.1 Basal resistance

Since plants are sessile organisms and cannot escape their enemies, they have developed a sophisticated multi-layered immune system with constitutive and inducible defence strategies (Jones et al. 2006, Spoel and Dong 2012, Dangl et al. 2013). Non-host resistance (NHR) displays the most robust and durable form of plant resistance and confers immunity to the majority of microbes that are not compatible (non-adapted) with the host under attack (Cheng et al. 2012). Alternatively, if an adapted pathogen penetrates the constitutive barriers of the plant's surface and cell wall, it encounters the extracellular space where, for example, pattern recognition receptors (PRRs) can recognize the pathogen via its conserved microbial structures (elicitors, pathogen associated molecular patterns, PAMPs) (Zipfel 2008, Macho and Zipfel 2014, 2015). PRRs are located in the plasma membranes of plant cells and contain extracellular ligand recognition domains and often intracellular kinase domains. Activation of these PRRs induces a battery of host responses including, stomatal closure, ethylene production, mitogen-activated protein kinase (MAPK) activation and changes in host gene expression, known collectively as PAMP-triggered immunity (PTI) (Chisholm et al. 2006, Jones and Dangl 2006, Bittel and Robatzek 2007, Boller and He 2009, Schwessinger and Ronald 2012, Macho and Zipfel 2014, 2015). The best-known example of PRR recognition is that of flg22, the elicitor-active epitope of bacterial flagellin, by the *Arabidopsis* receptor kinase FLS2 (Felix et al. 1999, Zipfel et al. 2004).

Normally PTI is sufficient to stop virulent pathogens from colonizing tissues and prevent further colonization (Boller and Felix 2009, Zipfel et al. 2014). But driven by natural selection, some host-adapted pathogens have evolved effectors (pathogen virulence factors) (Göhre and Robatzek 2008, Cunnac et al. 2009, Marrtin and Kamoun 2012, Giraldo and Valent 2013), which they secrete into the cytoplasm to suppress PTI, which results in effector-triggered susceptibility (ETS) (Jones and Dangl 2006, Boller and He 2009, Cui et al. 2009, 2013). To counteract ETS, plants express resistance genes (R genes) that can recognize specific pathogen effectors and induce effector-triggered immunity (ETI) also known as *R* gene-mediated resistance (Mackey et al. 2002, 2003, Chisholm et al. 2006, Jones and Dangl 2006, Bent and Mackey 2007, Narusaka et al. 2009). The current model for plant disease resistance suggests an interplay between plant and pathogen, leading to continued escalation of defence and counter-defence driven by natural selection (**Figure 1**) (Jones and Dangl 2006, Nishimura and Dangl 2010).

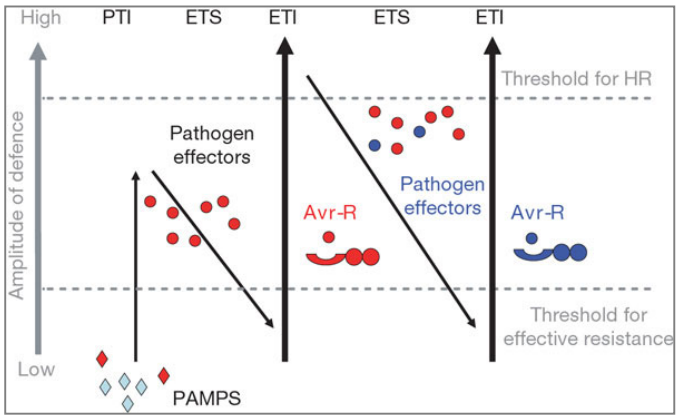

Figure 1: The „zigzag model" of plant defence. The model illustrates the quantitative output of the plant immune system and reflects defence and counter-defence of plants versus host-pathogens for immunity and susceptibility on both sides. (PAMP: Pathogen-associated molecular pattern, PTI: PAMP-triggered immunity, ETS: Effector-triggered susceptibility, ETI: Effector-triggered immunity, HR: Hypersensitive response, Avr: Effector, -R: Resistance protein). Illustration adapted from (Jones and Dangl 2006).

ETI displays an accelerated and amplified PTI response that usually comes along with a hypersensitive response (HR), a form of programmed cell death, at the infection site to keep the pathogen isolated from the remaining healthy tissue (Dangl et al. 1996, Morel and Dangl 1997, Jones and Dangl 2006, Mur et al 2008).

A variety of defence responses induced by PTI and ETI in the local leaves are production of reactive oxygen species (ROS), nitric oxide (NO) generation, secondary metabolite synthesis leading to accumulation of salicylic acid (SA), cell wall strengthening, and increased expression of pathogenesis-related (PR) genes (Dodds et al. 2010, Silipo et al. 2010, Macho et al. 2014). Both PTI and ETI are effective against biotrophic and hemibiotrophic pathogens that feed on living host tissue but not against necrotrophs that require dead host tissue for nutritional purpose and kill the host (Glazebrook 2005, Jones and Dangl 2006, Zipfel 2008, Dempsey et al. 2011).

1.2 Salicylic acid in basal resistance

Diverse plant hormones act in the plant immune signalling network during PTI and ETI. Recognition of PAMPs or pathogen effectors during PTI and ETI leads to SA accumulation (Grant and Lamb 2006), inducing NONEXPRESSOR OF *PR* GENES1 (NPR1)-mediated expression of antimicrobial *PR* genes that contribute to enhanced resistance (Dong 2004, Spoel and Dong 2012). NPR1 is a master regulator of SA-mediated defence signalling, which can reside both in the nucleus and the cytosol, where its nuclear localization is required for *PR1* transcription (Kinkema et al. 2000). In the cytosol, NPR1 is retained as oligomer connected by disulphide bridges. Long time a model was suggested in which SA accumulation during pathogen attack provokes changes in the cells redox status, driving the monomerization of the inactive cytosolic NPR1 oligomer and its translocation into the nucleus (Kinkema et al. 2000, Mou et al. 2003, Tada et al. 2008).

Even if SA was attributed a key regulatory function in plant immunity (Malamy et al. 1990, Metraux et al. 1990), a bona fide SA receptor has remained elusive so far (Shah and Zeier 2013). Recently, NPR1 was identified as being a direct SA receptor with SA binding via an

NPR1-linked copper. SA binding to NPR1 causes a conformational change that favours NPR1 oligomer disassembly thereby promoting its nuclear localization. Thus, direct SA binding to NPR1 rather then reducing conditions (Mou et al. 2003) induce NPR1 monomerization and nuclear translocation (Wu et al. 2012, Shah and Zeier 2013). In the nucleus NPR1 acts as a co-activator for TGACG MOTIF BINDING (TGA) transcription factors (TFs) to induce transcription of defence-related genes like *PR1* (Durrant and Dong 2004, Vlot et al. 2009). The key regulatory function of NPR1 comprises its downstream function in mediating the SA signal (Cao et al. 1994), on the other hand NPR1 is part of a negative feedback loop regulating SA signalling by suppression of SA biosynthesis (Shah 2003, Lu 2009).

In *Arabidopsis* SA can be synthesized via two different pathways, the PHENYLALANINE AMMONIUM LYASE (PAL) and the ISOCHORISMATE SYNTHASE (ICS) pathway (Garcion et al. 2006, Wildermuth et al. 2006, Vlot et al. 2009, An and Mou 2011, Dempsey et al. 2011). The majority of SA synthesized upon pathogen infection (approximately 90%) proceeds via the ICS pathway involving ICS and ISOCHORISMATE PYRUVATE LYASE (IPL) (Nawrath and Metraux 1999, Catinot et al. 2008, Okrent et al. 2009). *Arabidopsis* mutants lacking functional ICS (*ics1*, also known as *salicylic acid induction–deficient2*, sid2) are severely compromised in SA biosynthesis and pathogen resistance (Wildermuth et al. 2001, Garcion et al. 2008). To limit the adverse effects of free SA after pathogen attack, SA is converted into the biologically inactive forms methyl salicylate (MeSA), SA glucoside (SAG) and in smaller amounts to SA glucose ester (SGE) (**Figure 2**) (Vlot et al. 2009).

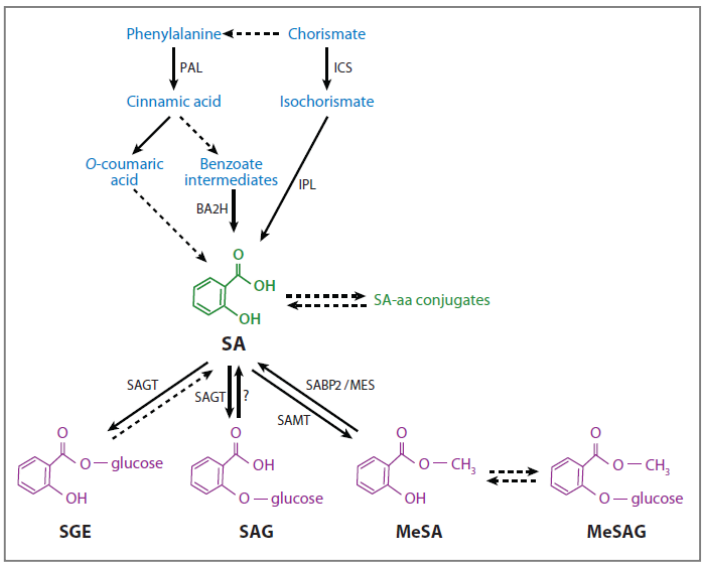

Figure 2: Salicylic acid biosynthesis and metabolism. SA can be synthesized via two different pathways starting from the common precursor chorismate (PAL: PHENYLALANINE AMMONIUM LYASE, ICS: ISOCHORISMATE SYNTHASE, IPL: ISOCHORISMATE PYRUVATE LYASE, BA2H: BENZOIC ACID-2-HYDROXYLASE, SA: Salicylic acid, SAGT: SA-GLUCOSYLTRANSFERASE, SABP2: SA-BINDING PROTEIN2, MES: MeSA ESTERASE, SAMT: SA METHYLTRANSFERASE, SGE: Salicyloyl glucose ester, SAG: SA O-ß-glucoside, MeSA: Methyl salicylate, MeSAG: MeSA O-ß-glucoside. Illustration adapted from (Vlot et al. 2009).

Modifications like glycosylation or methylation modulate the chemical properties of SA, its mobilization and activities (Wildermuth 2006). SAG is actively transported into the vacuole, as deactivation mechanism of bioactive free SA, where it functions as inactive storage form that can be converted back to SA (Dean and Mills 2004, Dean et al. 2005, Vlot et al. 2009).

1.3 Pipecolic acid in basal resistance

Alongside with SA, pipecolic acid (Pip) accumulates to high amounts in *Arabidopsis* leaves infected with avirulent *Pseudomonas syringae* patovar *tomato* (*Pst)*. Pip is a non-protein amino acid with widespread occurrence throughout flowering plants (angiosperms) (Morrison 1953, Zacharius et al. 1954). It was reported to accumulate in leaves infected with bacteria, fungi, or virus in different plants species, including rice, potato, tobacco, soybean, and *Arabidopsis* (Palfi and Dezsi 1968, Navarova et al. 2012, Vogel-Adghough et al. 2013). Biosynthesis of Pip derives from lysine by a two-step mechanism in which AGD2-LIKE DEFENCE RESPONSE PROTEIN1 (ALD1) catalyses the first aminotransferase step (Gupta and Spenser 1969, Song et al. 2004a, Navarova et al. 2012, Shah and Zeier 2013). SA and Pip provide additive contributions to basal resistance in *Arabidopsis*, reflected in elevated *ICS1* and *ALD1* transcript levels after *Pst* infection (Bernsdorff et al. 2016). Exogenous SA alone was able to induce *PR1* expression and disease resistance in the absence of Pip (*ald1*), whereas Pip-induced resistance was dependent on intact SA biosynthesis (*sid2*). But SA-induced resistance was markedly fortified in Pip-pretreated plants, indicating a synergism between SA and Pip in basal disease resistance (Bernsdorff et al. 2016).

A closer look into the synergistic interplay of Pip and SA provides *ALD1*, *FLAVIN-DEPENDENT MONOOXYGENASE1* (*FMO1)*, *PHYTOALEXIN-DEFICIENT4* (*PAD4*) and *NPR1* as important genes involved in SA and Pip defence pathways. ALD1 acts upstream of Pip and is responsible for Pip biosynthesis. FMO1 and PAD4 act downstream of Pip, but upstream of SA and are dispensable for SA signalling. The key regulator NPR1, which functions downstream of both Pip and SA is required for intact basal disease resistance (Bernsdorff et al. 2016).

Altogether Pip and SA act both synergistically and independently from each other in plant basal resistance with a redundant mode of action for Pip signalling and a higher relative contribution of SA to basal resistance than that of Pip (Bernsdorff et al. 2016).

1.4 Phytohormones - crosstalk between salicylic acid, jasmonic acid and ethylene

The phytohormones, SA, jasmonic acid (JA) and ethylene (ET) are the main signalling molecules in the regulation of plant defence against biotic stresses (Grant and Lamb 2006, Bari and Jones 2009, Pieterse et al. 2009, Vlot et al. 2009, Katagiri and Tsuda 2010, Verhage et al. 2010). Resistance conferred by biotrophic pathogens like *Pst* is mainly associated with the SA signalling sector whereas JA and ET are generally important for immunity to necrotrophic pathogens or insects (Bostock 2005, Glazebrook 2005, Verhage et al. 2010). Thus, two different signal transduction pathways in response to pathogen attack exist, the SA-dependent and the JA/ET-dependent pathway (Leon-Reyes et al. 2010). Crosstalk between the hormone signalling pathways equips the plant with a

powerful regulatory mechanism to regulate disease resistance and fine tune the immune response to the type of invader (Spoel and Dong 2008, Pieterse et al. 2009, Tsuda et al. 2009, Robert-Seilaniantz et al. 2011).

The SA- and JA-signalling pathways mainly act mutually inhibitory (Feys and Parker 2000, Kunkel and Brooks 2002, Glazebrook 2005, Bostock 2005, Spoel and Dong 2008, Grant and Jones 2009, Pieterse et al. 2012). For example, during *Pst* infection *Arabidopsis* plants activate the SA-signalling pathway whereas the JA-signalling pathway is suppressed, which makes these plants more susceptible to necrotrophic pathogens (Spoel et al. 2007). Conversely, *Pst* promotes its own virulence by suppressing the SA signalling pathway in plants via production of coronatine a molecular mimic of JA (Brooks et al. 2005). On the molecular level, the JA-responsive marker genes (*PLANT DEFENSIN1.2 (PDF1.2)* and *VEGETATIVE STORAGE PROTEIN2 (VSP2*)) were down regulated by exogenous SA (Van Wees et al. 2000, Koornneef et al. 2008).

ET is an important modulator of the crosstalk between SA- and JA-signalling pathways and is required for different processes in both pathways (Ndamukong et al. 2007, Leon-Reyes et al. 2009, Zander et al. 2010). For example, ET has a synergistic effect on SA-induced *PR1* expression (Lawton et al. 1994, De Vos et al. 2006, Pieterse et al. 2009), and regulation of the JA-responsive *PDF1.2* requires both JA and ET activation (Penninckx et al. 1998).

Although a few genes involved in the crosstalk between SA- and JA-signalling pathways are identified, the further molecular mechanisms underlying the crosstalk remain elusive (Zhang et al. 2012). In the nucleus, ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) with PAD4 controls the antagonism between SA and JA/ET-mediated defence responses (Gupta et al. 2000, Brodersen et al. 2006) and TGA1 is also known to be important for SA-JA cross talk (Ndamukong et al. 2007, Leon-Reyes et al. 2009, Zander et al. 2010). Nuclear NPR1 is not essential but cytoplasmic NPR1 seems to be involved in SA-JA crosstalk (Spoel et al. 2003, Leon-Reyes et al. 2009).

1.5 Systemic acquired resistance

Subsequent to the defence responses in the infected leaves, PTI and ETI can also trigger SA accumulation and *PR* gene expression in the upper uninfected leaves, leading to a resistance response called systemic acquired resistance (SAR). SAR is a long-lasting induced disease resistance that occurs in the distal tissues of locally infected plants and provides protection against a broad spectrum of harmful microbes, e.g. bacteria, fungi, or viruses (**Figure 3**) (Durrant and Dong 2004, Mishina and Zeier 2007, Vlot et al. 2008, 2009, Shah 2009, Dempsey et al. 2011, Dempsey and Klessig 2012, Fu and Dong 2013). The activation of SAR requires communication by the primary infected tissue with the uninfected healthy tissue. Simultaneously to the defence response in the primary infected leaves, long distance signals move from the infected site via the vasculature, most likely

the phloem (Guedes et al. 1980, Gaupels and Vlot 2013) to induce defence responses and to protect the systemic healthy tissue from subsequent infections (**Figure 3**) (Durrant and Dong 2004, Vlot et al. 2008, Shah and Zeier 2013).

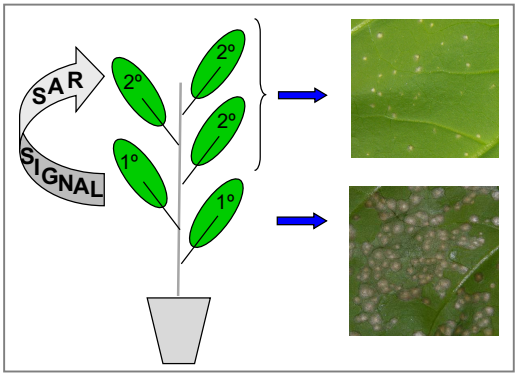

Figure 3: Systemic acquired resistance. In *Arabidopsis*, infection of the first two fully developed leaves (local leaves, 1°) with *Pst* induces SAR signalling. SAR signals move from the infected local leaves to the upper healthy uninfected leaves (systemic leaves, 2°) to induce SAR. Due to these defence responses infection of the systemic leaves of pre-infected plants results in less lesions compared to lesions in the local leaves.

SAR is associated with a transcriptional reprogramming response and priming of defence responses whereas both responses have overlapping regulatory principles (Shah and Zeier 2013). The transcriptional SAR response is reflected by changes in the gene expression in the systemic leaves of locally infected plants leading to increased readiness for following pathogen defence (Bernsdorff et al. 2016). Genes involved at different stages of defence signalling, such as elicitor perception, signal transduction, and transcriptional gene activation, were unregulated during SAR, and this was coupled with decreased photosynthesis as well as general metabolic- and growth-related processes (Bernsdorff et al. 2016).

The phenomenon of priming has evolved to save energy under enemy-free conditions and only involves costs when defence is activated during pathogen attack. In primed tissue the defence response is not directly activated but memorized and can be expressed in an accelerated manner during further pathogen attack (Pieterse et al. 2012). Primed tissue can switch on defence faster and stronger in case of a subsequent pathogen attack (Jung et al. 2009, Conrath 2011, Navarova et al. 2012). The SAR-mediated state of defence priming becomes apparent upon a second challenge of previously uninfected distal leaves. SAR associated defence priming conditions plants for timely and effective defence gene activation and SA biosynthesis (Bernsdorff et al. 2016).

Via epigenetics, the protective effect of SAR can also be transferred into the next generation and confer a fitness advantage for the progeny under conditions of high disease pressure (Traw et al. 2007, Jaskiewicz et al. 2011, Luna et al. 2012). It is evolutionarily advantageous for plants to give information about potential dangers in the circumjacent environment to the offspring and equip them with an already adapted immune system (Pieterse et al. 2012). Progeny of SAR-induced parents can switch on defence faster and stronger in case of a subsequent pathogen attack (Jung et al. 2009, Conrath 2011, Navarova et al. 2012). Transgenerational resistance is realized by epigenetic changes in genetic material, such as DNA methylation and histone modification (chromatin remodelling), and small interfering RNAs can heritably and reversibly modify the

expression of genes without changing the DNA sequence (Luna et al. 2012, Pieterse et al. 2012, Rasmann et al. 2012, Slaughter et al. 2012, Iwasaki and Paszkowski 2014).

1.6 Systemic acquired resistance signals

Even though SA is the key hormone in plant basal resistance and SAR, SA itself does not serve as the phloem-mobile signal for SAR as shown by grafting studies in chimeric tobacco and *Arabidopsis* plants (Gaffney et al. 1993, Vernooij et al. 1994, Pallas et al. 1996, Mauch-Mani and Metraux 1998).

Methyl salicylate (MeSA), the methylated derivative of SA, as first molecule was identified as a potential long-distance SAR signal in tobacco, potato and *Arabidopsis* (Park et al. 2007, 2009, Vlot et al. 2008, Manosalva et al. 2010, Liu et al. 2011a, 2011b). In *Pst* infected *Arabidopsis* leaves a part of the accumulating SA is converted into MeSA by BENZOIC ACID/SA CARBOXYLMETHYLTRANSFERASE1 (BSMT1) activity (Shah and Zeier 2013, Dempsey and Klessig 2012). MeSA travels from the infected leaves via the phloem to the distal tissue, where it is converted back to SA by MeSA ESTERASE (MES) activity to support SA biosynthesis and SAR (**Figure 4**) (Vernooij et al. 1994, Forouhar et al. 2005, Kumar and Klessig 2008, Dempsey and Klessig 2012).

Although MeSA is crucial for SAR in tobacco and potato (Park et al. 2007, Manosalva et al 2010), there are controversial opinions about the requirement of MeSA for SAR in *Arabidopsis* (Attaran et al. 2009, Park et al. 2009, Vlot et al. 2008, Liu et al. 2010, 2011a Chaturvedi et al. 2012, Dempsey and Klessig 2012). *Arabidopsis* plants exhibiting altered MeSA synthesis showed both SAR competence (*bsmt1-2*, *bsmt1-3*) and compromised SAR (*bsmt1-1*, chemical inhibition of MES activity) (Attaran et al. 2009, Park et al. 2009, Liu et al. 2010, Dempsey and Klessig 2012). Today we know that the necessity for MeSA to trigger SAR is dependent on the extent of light exposure after the first infection. Whereas AM-inoculated MeSA-metabolism-defective *Arabidopsis* still displayed SAR, MeSA and its metabolizing enzymes are required for SAR in PM-inoculated plants and for maximal SAR in AM-inoculated ones (Liu et al. 2011a, 2011b).

In the last five years, many more metabolites putatively involved in long-distance signalling in SAR have been identified in *Arabidopsis* (Shah 2009, Dempsey and Klessig 2012, Shah and Zeier 2013, Wendehenne et al. 2014). Besides MeSA, these include the dicarboxylic acid azelaic acid (AzA) (Jung et al. 2009), the abietane diterpenoid dehydroabietinal (DA) (Chaturvedi et al. 2012), a glycerol-3-phosphate (G3P)–dependent factor (Chanda et al. 2011, Mandal et al. 2012, Yu et al. 2013) and the amino acid-derivative Pip (Navarova et al. 2012). In addition to these chemicals, the SAR associated role of JA remains debatable, because some but not all JA-deficient mutants show an altered SAR (Cui et al. 2005, Attaran et al. 2009, Wendehenne et al. 2014). Some of these putative SAR signals interact with each other to induce SAR (**Figure 4**) (Dempsey and Klessig 2012).

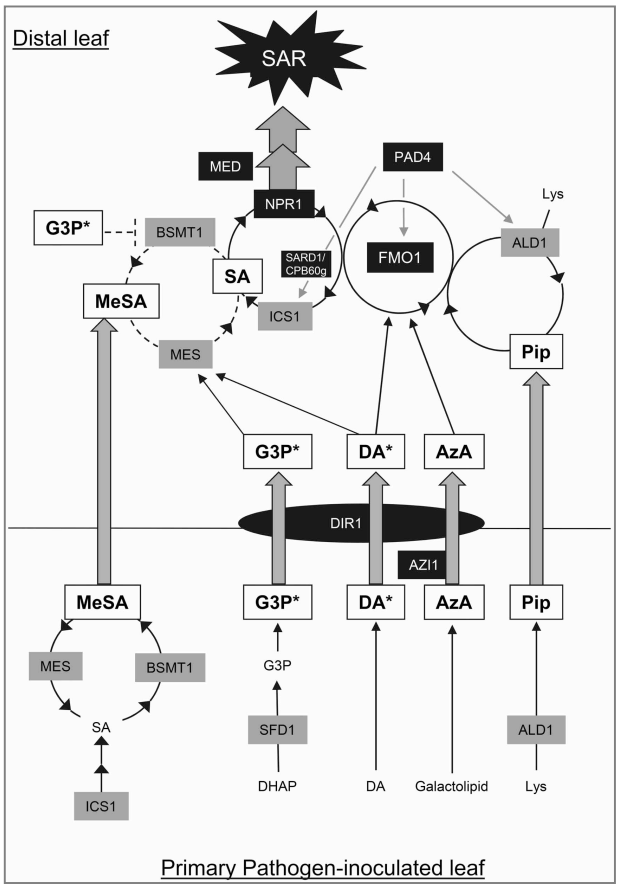

Figure 4: Putative long-distance SAR signals. SAR signals produced in the primary pathogen-infected leaves move to the systemic uninfected tissue to establish SAR. (ALD1: AGD2-LIKE DEFENCE RESPONSE PROTEIN1, AzA: Azelaic acid, AZI1: AZELAIC ACID INDUCED1, BSMT1: BENZOIC ACID/SALICYLIC ACID CARBOXYL METHYL TRANSFERASE1, CPB60g: CAM-BINDING PROTEIN 60-LIKE G, DA: Dehydroabietinal, DA*: Activated DA, DHAP: Dihydroxyacetone phosphate, FMO1: FLAVIN-DEPENDENT MONOOXYGENASE1, G3P: Glycerol-3-phosphate, G3P*: Activated G3P, ICS1: ISOCHORISMATE SYNTHASE1, MES: MeSA ESTERASE, MeSA: Methyl salicylate, Pip: Pipecolic acid, SA: Salicylic acid, SARD1: SAR DEFICIENT1, SFD1: SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY1 (SFD1/GLY1)). Illustration adapted from (Shah and Zeier 2013).

SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY1 (SFD1, also termed GLY1) catalysed the synthesis of G3P from dihydroxyacetone phosphate (Nandi et al. 2004, Chanda et al. 2008, 2011, Dempsey and Klessig 2012) and SFD1`s reductase activity was crucial for SAR (Nandi et al. 2004, Chanda et al. 2008, Mandal et al. 2011, Lorenc-Kukul et al. 2012). The G3P biosynthetic mutants *gly1*/*gli1* (Col-0 background), which display wt levels of systemic SA and AzA accumulation showed a SAR defect that could be restored by exogenous G3P. Since exogenous G3P alone was insufficient to trigger SA biosynthesis or SAR in wt *Arabidopsis*, G3P appears to be a necessary but not sufficient mobile signal for SAR (Zheng and Dong 2013). Since Pex from wt plants infected with avirulent *Pst* (*AvrPex*) was able to restore SAR in *sfd1* (Nössen background), but *AvrPex* from *sfd1* failed to induce SAR in wt, these data suggest that the SAR defect of *sfd1* is due to a defect in generation and/or translocation of a long-distance signal (Chaturvedi et al. 2012). Since G3P was inefficient to restore the SAR defect in *sfd1* and C^{14}-labeled G3P could not be recovered in the systemic leaves, G3P itself is not part of the translocated SAR signal. However, it is possible that a G3P-derived factor together with other factors present in the *AvrPex* is required for the accumulation and/or long-distance transport of a SAR signal (Shah 2009, Dempsey and Klessig 2012, Lorenc-Kukula et al. 2012, Shah and Zeier 2013, Zheng and Dong 2013). The identity of the SFD1/GLY1-derived SAR signal remains unclear (Chanda et al. 2011). In the systemic leaf the G3P-derived factor is suggested to function in the regulation of SA biosynthesis, since local treatment of wt plants with *AvrPex* enriched

with G3P suppressed systemic expression of *BSMT1*, and induced *MES9*, whose products converts MeSA to SA (**Figure 4**) (Chanda et al. 2011).

Dehydroabietinal (DA) is an abietane type diterpenoid that was identified as a SAR-inducing factor in *AvrPex* from *Arabidopsis* (Chaturvedi et al. 2012). As one of the most potent SAR inducers DA was active in *Arabidopsis*, tobacco and tomato in picomolar concentrations. Locally applied DA was rapidly translocated to the systemic tissue where it induced SA biosynthesis and SAR. In DA–treated as well as systemic leaves SA and *PR1* transcript levels were enhanced and SA biosynthesis enzymes like ICS1 and MES9. DA induced systemic resistance (SR) under requirement of the SA pathway components SID2 and NPR1, thus functioning upstream of SA accumulation and signalling. Although FMO1 was not essential for SA accumulation in DA-treated leaves, it is required for systemic SA accumulation and SAR. Surprisingly, comparable levels of DA were present in Pex and *AvrPex* from *Arabidopsis*. However, DA in *AvrPex* was enriched in a high-molecular-weight (HMW) fraction, biologically active for SAR induction, whereas the majority of DA in Pex was present in the biologically inactive low-molecular-weight (LMW) fraction. The mobilization of DA from the biologically inactive LMW pool into a biologically active signalling form present in the HMW pool is required for SAR (**Figure 4**). Other proteins that are associated with DA in the HMW pool and contribute to SAR remain unclear. If DA is essential for biologically induced SAR still has to be answered (Chaturvedi et al. 2012, Dempsey and Klessig 2012, Shah and Zeier 2013, Zheng and Dong 2013).

Azelaic acid (AzA) is a nine-carbon dicarboxylic acid found to be enriched in *AvrPex* from SAR induced wt *Arabidopsis* (Jung et al. 2009). A potential mechanism for its synthesis is by oxidation of 9-oxononanoic acid synthesized from fatty acids (FAs) (Shah and Zeier 2013, Wittek et al. 2014). Locally applied labelled AzA was recovered in Pex and distal leaves indicating that it can be systemically translocated. In contrast to MeSA and DA, AzA-treated plants did not show enhanced levels of SA and PR, but the defence response upon pathogen challenge was fortified and hastened. Thus, AzA is suggested to promote disease resistance through priming of SA signalling, rather than directly activating defence responses. Like the DA signal, AzA systemically requires FMO1 to induce SAR. AZELAIC ACID INDUCED1 (AZI1) encodes a putative lipid transfer protein that was accumulated at elevated levels in AzA-treated plants. The SAR associated priming of SA accumulation/signalling was attenuated in *azi1* and exogenously applied AzA as well as *AvrPex* failed to induce SAR in *azi1*. Thus, AZI1 was required for AzA as well as biologically induced SAR and it appeared to be involved in the production/translocation of a mobile SAR signal. In contrast to AZI1, the systemic translocation of AzA was not essential for establishment of SAR per se, but when translocated it is suggested to strengthen the SAR response (**Figure 4**) (Jung et al. 2009, Navarova et al. 2012, Zoeller et al. 2012, Shah and Zeier 2013).

DEFECTIVE IN INDUCED RESISTANCE1 (DIR1) encodes a putative lipid transfer

protein, which can bind long-chain FAs and displays proline-rich regions potentially involved in protein-protein interactions (Maldonado et al. 2002, Lascombe et al. 2008, Dempsey and Klessig 2012). The SAR-defective *dir1* mutant does not exhibit systemic *PR* gene expression when infected with *Pst* although ETI and PTI as well as local and systemic SA accumulation are comparable to that of wt *Arabidopsis* (Maldonado et al. 2002). Thus, DIR1`s function in defence seems to be specific to SAR (Maldonado et al. 2002, Chanda et al. 2011, Champigny et al. 2011, Yu et al. 2013). *AvrPex* from wt were able to restore the SAR defect in *dir1*, whereas *AvrPex* of *dir1* could not induce SAR in wt plants. This suggests DIR1 to be required for generation, accumulation and/or translocation of a SAR signal (Maldonado et al. 2012, Chaturvedi et al 2012, Shah and Zeier 2013, Dempsey and Klessig 2012). The SAR inducing activity of *AvrPex* in general is sensitive to proteinase K and trypsin treatment (Chanda et al. 2011, Chaturvedi et al. 2012) indicating that proteins are involved in SAR signalling (Shah and Zeier 2013). Its expression in the phloem companion cells and its proline-rich regions suggested DIR1 as a good candidate to chaperone such a long-distance SAR signal to the systemic tissue (**Figure 4**) (Chaturvedi et al 2012, Dempsey and Klessig 2012, Maldonado et al. 2012, Shah and Zeier 2013).

As mentioned above, some of these putative SAR signals interact with each other to induce SAR (Dempsey and Klessig 2012). The finding that *AvrPex* from *sfd1* and *dir1* were ineffective in SAR induction when applied individually, but effective inducers when co-applied as a mixture (Chaturvedi et al 2008), suggest that both a DIR1-depending activity and the G3P-dependent factor are required for SAR. The G3P-derived signal and DIR1 seem to be mutually interdependent for each other's systemic translocation and function together in long-distance signalling (**Figure 4**) (Chaturvedi et al. 2008, Chanda et al. 2011, Dempsey and Klessig 2012, Shah and Zeier 2013).

DIR1 was also required for AzA-induced resistance to *Pst* (Jung et al. 2009), and for SAR induction by local DA application (Chaturvedi et al. 2012). DA-treated *dir1* mutant plants accumulated lower levels of SA than *Pst* infected *dir1* or wt plants, thus DA-induced SA accumulation also may be partly dependent on DIR1. AzA, G3P, and DA all require DIR1 for their functions (Dempsey and Klessig 2012). If DIR1 binds directly to DA, AzA, and the G3P-derived signal, or whether DIR1 affects them through an indirect interaction has to be investigated (**Figure 4**) (Zheng and Dong 2013, Dempsey and Klessig 2012).

Additionally, to the interactions between DIR1 and other mobile signals, a synergistic effect of AzA and DA in the induction of SAR is suggested. Lower doses of DA were capable to induce SAR in wt but not in *azi1* and when DA and AzA were applied together their efficiency in SAR induction was increased (Zheng and Dong 2013).

Pipecolic acid (Pip) is the most recently identified potential long-distance SAR signal. Pip was highly enriched in *AvrPex* and distal leaves indicating for a specific transport of Pip out of inoculated leaves into the phloem to the distal tissue. As a water-soluble amino acid Pip has ideal physicochemical properties to travel via the phloem (Navarova et al. 2012). In

the systemic tissue Pip turned out to play a more critical and essential role in the establishment of SAR (see 1.7 Pipecolic acid in systemic acquired resistance) (**Figure 4**). The long-distance signals generated and released from the pathogen-infected tissue are supposed to be perceived in the distal leaves, although receptors of the individual mobile signals are yet not identified (Shah and Zeier 2013). In the systemic uninfected tissue the mobile SAR signals contribute to the regulation of the SA pathway via *ICS1* expression and SA accumulation (Shah and Zeier 2013). Part of systemic SA is synthesized from MeSA, which is hydrolysed to SA via MES activity, supported by the G3P signal, that also supresses BSMT1 activity, and DA (Shah and Zeier 2013). However, also de novo synthesis of SA in the pathogen free leaves via ICS1 is required for SAR (Wildermuth et al. 2001, Mishina and Zeier 2007, Chaturvedi et al. 2008, 2012, Attaran et al. 2009, Jung et al. 2009). Under requirement of FMO1, DA supports SA synthesis by up-regulation of *ICS1* expression and AzA contributes via priming to SA accumulation and signalling (Shah and Zeier 2013). Comparable to the mechanisms in the local leaf (see 1.1 Basal Resistance) systemic accumulation of SA induces the translocation NPR1 into the nucleus where it leads to SA downstream defence responses, including *PR1* expression (**Figure 4**) (Vlot et al. 2009, Dempsey and Klessig 2012, Shah and Zeier 2013). The arrival of Pip and early Pip accumulation in the systemic leaves drives further Pip production via up-regulation of *ALD1* and subsequent FMO1-mediated activation of SA biosynthesis. The Pip/FMO1 signalling module is proposed to act as the initial trigger of an SAR signal amplification mechanism that is essential for SAR and includes Pip and SA feedback loops (**Figure 4**) (Navarova et al. 2012, Shah and Zeier 2013).

In addition to the well-known critical SAR components ICS1 and NPR1, LEGUME LECTIN-LIKE PROTEIN1 (LLP1), FMO1, ALD1, PAD4, and the two transcription factors SAR-DEFICIENT1 (SARD1) and CALMODULIN- BINDING PROTEIN 60G (CBP60g) were identified as being crucial for systemic SAR induction (**Figure 4**) (Breitenbach et al. 2014, Song et al. 2004a, 2004b, Mishina and Zeier 2006, Zhang et al. 2010, Jing et al. 2011, Dempsey and Klessig 2012, Shah and Zeier 2013). In contrast to the SAR defect of *ald1* mutant plants, which was restored by Pip application, the SAR defect of *fmo1* mutant plants could not be restored with exogenous Pip, indicating that FMO1 systemically acts downstream of Pip, in between Pip and SA signalling (Bernsdorff et al. 2016).

Recently, a comparative proteomic analysis revealed APOPLASTIC, EDS1-DEPENDENT (AED) proteins that regulate different aspects of SAR. One of these is LLP1, one of 226 lectin genes encoding carbohydrate-binding proteins in the *Arabidopsis* genome (Peumans and Van Damme 1995, Sharon and Lis 2004, Armijo et al. 2013). In *llp1* mutant plants, SAR was compromised, whereas local resistance associated with EDS1 and SA as well as responses to exogenous SA appeared largely unaffected. This indicated that LLP1 promotes systemic rather than local immunity, possibly in parallel with SA (Breitenbach et al. 2014). Thereby LLP1 likely cooperates with additional components, including SA, since constitutive

over accumulation of LLP1 was not sufficient to enhanced systemic resistance to *Pst* (Armijo et al. 2013, Breitenbach et al. 2014). LLP1 is localized to the plasma membrane facing the apoplast (Armijo et al. 2013) and might regulate SAR by sensing changes in the glycan composition of the cell wall (Minic et al. 2004) or by recognizing components of the cuticle, which contributes to SAR signal perception (Xia et al. 2009, 2010, Breitenbach et al. 2014).
The transcription factors SARD1 and CBP60g are SAR relevant genes in *Arabidopsis*. Both were crucial for de novo SA biosynthesis and bind to the ICS promoter. *SARD1* and *CBP60g* were locally and systemically up–regulated upon *Pst* infection and *sard1 cbpg60g* double mutant plants were SAR defective (**Figure 4**) (Zhang et al. 2010).
The lipase-like protein PAD4 is a positive regulator of pathogen-induced SA and Pip biosynthesis (Zhou et al. 1998, Jirage et al. 1999, Navarova et al. 2012). PAD4 seems to exert its central defence regulatory role via transcriptional control of Pip- and SA-pathway genes, including *ALD1*, *FMO1*, *ICS1, SARD1*, and *CBP60g* (**Figure 4**) (Song et al. 2004a, 2004b, Bartsch et al. 2006, Navarova et al. 2012, Bernsdorff et al. 2016).

1.7 Pipecolic acid in systemic acquired resistance

Since 1980, SA was claimed to be the pivotal defence hormone in SAR (Vlot et al. 2009), but nowadays SA has to share its outstanding position in SAR with Pip. Beyond its role in basal resistance and function as long-distance SAR signal, Pip was recently identified as second important component in SAR (Navarova et al. 2012, Bernsdorff et al. 2016). SA shares the characteristic of systemic accumulation in plants upon local pathogen infection with Pip, which is the only amino acid substantially, increased in the distal leaves. Following pathogen-infection, Pip-deficient *ald1* mutant plants did not just completely lack Pip accumulation they also fail to accumulate SA in the distal leaf tissue and were fully compromised in SAR, indicating a critical role of Pip for SAR (Song et al. 2004a, Jing et al. 2011, Navarova et al. 2012).
Compared to PTI and ETI, local forms of induced resistance where pathogen effectors provide a direct, strong and long-standing trigger, the elicitor strength of the indirect endogenous long-distance SAR signal is probably much weaker. Thus, it is suggested that amplification of the stimulus in the systemic tissue is important for SAR establishment (Mishina and Zeier 2006, Shah and Zeier 2013). Since systemic Pip levels in SAR-induced *Arabidopsis* started to rise before marked elevations of systemic SA were detectable, the early increase of Pip at the onset of SAR was suggested to function as an initial trigger for such a signal amplification mechanism (Navarova et al. 2012). On the one hand Pip is critical for systemic SA accumulation and SAR, on the other hand Pip accumulation seems to rely on SA biosynthesis, strengthening the existence of a positive feedback amplification mechanism in SAR that integrates both Pip and SA signalling (Navarova et al. 2012, Shah and Zeier 2013).
The whole SAR response including transcriptional reprogramming and defence priming was

absent in *ald1*, whereas *sid2* mutant plants showed a slightly moderate SAR. It seems that a part of the SAR response, including Pip biosynthesis as indicated by enhanced levels of *ALD1* and *FMO1,* can be active in an SA independent manner (Bernsdorff et al. 2016). However, SA accumulation upon pathogen encounter is required to realize a full SAR response. Altogether the systemic accumulation of Pip is suggested to be the starting point of an SAR signal amplification mechanism that is indispensable for the establishment of SAR and integrates both Pip and SA signalling (Bernsdorff et al. 2016).

1.8 Reactive oxygen species in systemic acquired resistance

Depending on the concentration, reactive oxygen species (ROS) can either damage the plant by tissue necrosis (Girotti et al. 2001, Ochsenbein et al. 2006) or function as useful membrane-permeable signals during defence responses (Apel and Hirt 2004, Mittler et al. 2002, 2004, Torres et al. 2005, Ochsenbein et al 2006, Strauss et al. 2010, Torres 2010, Suzuki et al. 2011, Marino et al. 2012, Baxter et al. 2014). During pathogen attack hydrogen peroxide (H_2O_2) is mainly produced by plasma membrane-localized NADPH oxidases, named RESPIRATORY BURST OXIDASE HOMOLOGS (RBOHs) (Torres and Dangl 2005). Apoplastic production of H_2O_2 is one of the fastest physiological responses following pathogen recognition in plants (Torres and Dangl 2005, Jones and Dangl 2006, Torres 2010, Macho and Zipfel 2014) providing a direct antimicrobial effect to kill the pathogen or induce cell wall strengthening to inhibit pathogen invasion (Romero-Puertas et al. 2004, Torres 2010, O'Brien et al. 2012).

Upon pathogen attack ROS accumulation is characterized by two distinct peaks (biphasic ROS production) (Torres and Dangl 2005, Miller et al. 2009, Nishimura and Dangl 2010, Mittler et al. 2011, Dubiella et al. 2013, Baxter et al. 2014). The initial H_2O_2 increase activates EDS1-mediated SA accumulation. SA together with ROS, generated during the second phase of ROS accumulation, potentiate cell death and defence gene expression. Thereby, SA and H_2O_2 function in a self-amplifying loop enhancing each other´s synthesis and HR (Vlot et al. 2009, Overmyer et al. 2003). ROS triggers a cascade of cell-to-cell communication events that propagate throughout the plant tissue to regulate downstream pathways (Miller et al. 2009, Suzuki et al. 2011) and cell-to-cell spreading HR in adjacent cells (Romero-Puertas et al. 2004, Tada et al. 2004, Delledonne 2005). At the site of infection elevated SA, (nitric oxide) NO, and H_2O_2 levels collectively contribute to induce HR (Wendehenne et al. 2004, Grün et al. 2006, Mur et al. 2006, 2008, Caplan et al. 2008, Strauss et al. 2010, Torres 2010, Ochsenbein et al. 2016). In cells surrounding the site of infection, however, an antagonism between ROS and SA controls cell death. RBOHs can sense the enhanced levels of H_2O_2 (ROS signals) emanating from cells undergoing HR and stop H_2O_2 production to antagonize the SA-mediated but unwanted cell death (Durrant and Dong 2004, Torres et al. 2005, Strauss et al. 2010). ROS function as signal transducers that balance the cell fate between life and death (Torres 2010, Coll et al. 2011, O'Brien et

al. 2012). Supporting the SA dependent pro death signals at the site of infection while antagonizing them in cells around the infection site, explains how SA can activate proper defence in cells beyond the site of infection without causing cell death (Torres et al. 2005). Besides HR and basal resistance ROS are also associated with systemic signalling (Alvarez et al. 1998, Miller et al. 2009, Mittler et al. 2011) a crucial mechanism for SAR (Dempsey and Klessig 2012, Spoel and Dong 2012, Shah and Zeier 2013). Although it is unlikely that ROS themselves travel systemically because they are highly reactive and would be scavenged along the route (Mittler 2002), it was reported that the so-called „ROS wave" that displays a cell-to-cell relay mechanism (Miller et al. 2009, Suzuki et al. 2011) activated in cells along the systemic path to the distal tissue functions as rapid, long-distance, cell-to-cell systemic signal and general priming signal in plants (Mittler et al. 2011). With this mechanism a contribution of ROS signalling beyond the barriers of basal resistance as long-distance signal contributing to SAR signalling mechanism must be taken into consideration (Mittler et al. 2011).

1.9 Nitric oxide in systemic acquired resistance

NO is a highly toxic gas, which is emitted from plants during pathogen attack (Wendehenne et al. 2004, Delledonne 2005). Beside its direct antimicrobial effect to prevent further pathogen colonization its rapid reactivity and diffusibility across biological membranes makes NO an ideal signal transducer (Durner et al. 1998, Beligni and Lamattina 2001, Wendehenne et al. 2004). In *Arabidopsis,* the NITRIC OXIDE-ASSOCIATED PROTEIN1 (NOA1) and NITRATE REDUCTASES (NIA) are suggested to be essential for NO generation during SAR (Mandal et al. 2012, Wang et al. 2013). NO can have cytotoxic as well as beneficial protecting effects to the plant (Beligni and Lamattina 2001, Romero-Puertas et al. 2004, Delledonne 2005). The major intracellular antioxidant and regulator for NO reactivity is glutathione (GSH) (Rusterucci et al. 2007), which is a bioactive, mobile reservoir of NO (Stamler et al. 1992, Wendehenne et al. 2004, Lindermayr et al. 2005). Besides NO, S-nitrosoglutathione (GSNO) also represents a relevant signalling molecule during plant defence (Stamler et al. 1992, Lindermayr et al. 2005, Rusterucci et al. 2007). NO and ROS as signalling species act synergistically during the whole plant defence response. During HR, ROS is the key mediator channelling NO into the cell death pathway with NO having a crucial role in the regulation of the response (Zhang et al. 2003b, Wendehenne et al. 2004, Delledonne 2005). NO exert both agonistic and antagonistic interactions with ROS, for example by nitrosylating the RBOHD enzyme (Yun et al. 2011). Thus, NO function as bifunctional modulators either stimulating or inhibiting cell death by cross-communication with ROS levels (Beligni et al. 2002, Huang et al. 2002, Wendehenne et al. 2004, 2014). The combined and coordinated action of the ROS/NO signalling system is a crucial mechanism for the efficient activation of HR (Levine et al. 1994, Delledonne 1998, 2001, 2005, Romero-Puertas et al. 2004, Wendehenne et al. 2004, 2014).

NO is also involved in the induction of defence-related genes and to mediate a network that is involved in the establishment of SAR (Delledonne 1998, 2005, Durner 1998, Romero-Puertas et al. 2004, Rusterucci et al. 2007, Wendehenne et al. 2004, 2014). NPR1 is the key regulator of SA–mediated signalling during SAR (Klessig et al. 2000, Zhou et al. 2000, Durrant and Dong 2004) and NO/GSNO regulate this pathway via S-nitrosylation (Tada et al. 2008, Lindermayr et al. 2010). NO promotes the translocation of NPR1 into the nucleus and GSNO supports the interaction of NPR1 with the corresponding TGAs to increase DNA binding activity (Delledonne 2005, Tada et al. 2008, Lindermayr et al. 2010). Thus, NO/GSNO functions as redox regulator of the NPR1/TGA1 system in local as well as distal uninfected tissue (Delledonne 2005, Lindermayr et al. 2010).
GSNO is also suggested to play a role in systemic signalling as phloem long-distance signal in SAR (Durner and Klessig 1999). Recent findings of GSNO REDUCTASE (GSNOR) in the phloem companion cells of *Arabidopsis* and so far unidentified de novo NO synthesis in the systemic leaves reinforce this hypothesis (Espunya et al. 2012). Its localization in the vascular system makes GSNOR a promising candidate to regulate GSNO or SAR signal transport (Rusterucci et al. 2007).

1.10 The secrets of systemic acquired resistance

Recently, a new SAR model was proposed, which gives insight into the interrelationship between NO/ROS- and SA-signalling during SAR and the SAR-signalling compounds AzA, G3P, DIR1, and AZI1 (**Figure 5**) (Wang et al. 2014, Wendehenne et al. 2014, Gao et al. 2015, El-Shetehy et al. 2015). In *Arabidopsis*, *Pst* infection leads to the accumulation of ROS, NO and SA. Like SA, NO and ROS (H_2O_2) served as potent inducers of SAR and genetic mutations in NO/ROS accumulation (*rbohf*, *rbohd*, *gsnor1*, *nitric oxide-overproducing1 (nox1*)) abrogated SAR (Wang et al. 2014). 18:1 FAs, which can serve as precursor for AzA and G3P biosynthesis, also regulated NO levels via NOA1 (Mandal et al. 2012). This suggested not only a link between NO/ROS- and SA-signalling but also between NO and FA-AzA-G3P mediated SAR (**Figure 5**) (Wang et al. 2014).
NO is known to function upstream of SA (Durner et al. 1998) and crucial genetic evidence reinforces the importance of NO in SAR. Exogenous application of NO donors conferred SAR, while NO scavengers abolished SAR (Vernooij et al 1994, Song and Goodman 2001, Grün et al. 2006, Vlot et al. 2009, Wang et al. 2014) and mutants defective in NO accumulation during *Pst* infection (n*oa1 nia2*) were compromised in SAR (Mandal et al. 2012). Since *AvrPex* of wt could restore SAR in *noa1 nia2*, but *AvrPex* from *noa1 nia2* were unable to confer SAR in wt plants, NO seems to be important for generation of SAR signals (**Figure 5**) (Wang et al. 2014).

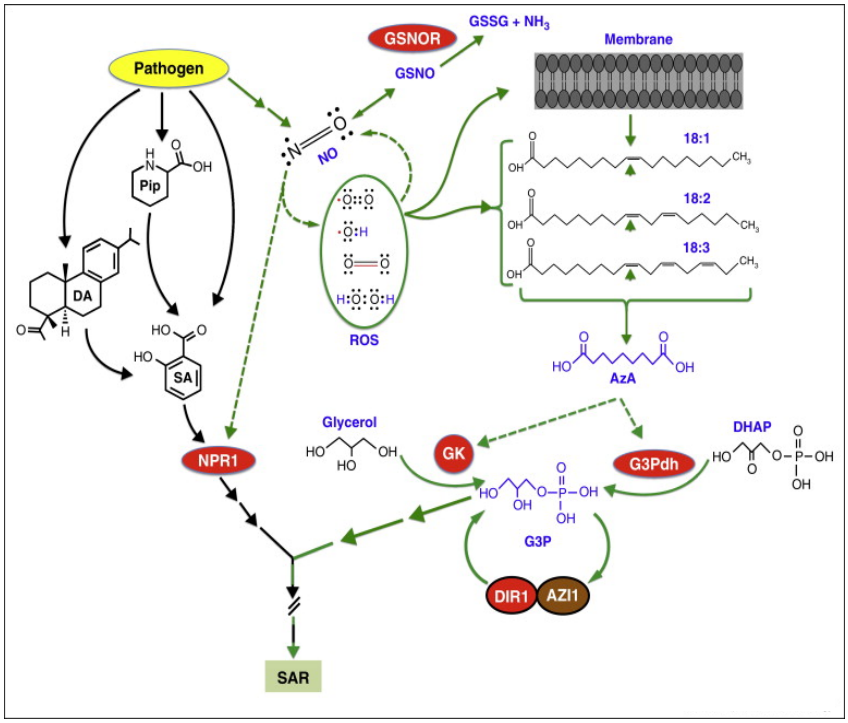

Figure 5: Simplified model illustrating chemical signalling during SAR. Interrelationship between NO/ROS-signalling and SA-signalling during SAR and the integration of the SAR-signalling compounds AzA, G3P, DIR1 and AZI1. (SA: Salicylic acid, NO: Nitric oxide, ROS: Reactive oxygen species, FAs: Fatty acids, AzA: Azelaic acid, G3P: Glycerol-3-phosphate, GK: Glycerol kinase, G3Pdh: G3P dehydrogenase, DIR1: DEFECTIVE IN INDUCED RESISTANCE1, AZI1: AZELAIC ACID INSENSITIVE1, GSNO: S-nitrosoglutathione, GSSG: Glutathione disulfide, GSNOR: S-NITROSOGLUTATHIONE REDUCTASE, NPR1: NONEXPRESSOR OF *PR* GENES1, SAR: Systemic acquired resistance). Illustration adapted from (Wendehenne et al. 2014).

The fact that SA was not able to restore the SAR defect in *noa1 nia2* and NO donors were unable to restore SAR in *sid2* (Wang et al. 2014) negates a linear relation between SA and NO in SAR (Vlot et al. 2009). On the contrary, NO and SA were suggested to confer SAR via two distinct independent pathway branches that merge in the induction of SAR. Mutant plants defective in SA or NO biosynthesis (*sid2*, *noa1 nia2*) accumulated normal levels of NO and SA, but co-application of an NO donor and SA induced a stronger SAR effect (**Figure 5**) (Wang et al. 2014).

NO was intricately connected to ROS (Scheler et al. 2013) and functions downstream of ROS in SAR (Wang et al. 2014). Pathogen infected *rboh* but also *noa1 nia2* mutant plants showed reduced levels of ROS (H_2O_2) and exogenous ROS conferred SAR in *noa1 nia2*. Additionally, the lack of NO accumulation in *rboh* mutants after pathogen attack and induction of *NOA1* by exogenous H_2O_2 proposed a regulatory feedback loop between NO and ROS (**Figure 5**) (Wang et al. 2014).

ROS comprised of various species including superoxide radicals, singlet oxygen, hydroxyl radical, and hydrogen peroxide function additively to catalyse oxidation and release of free C_{18} FAs from membrane lipids (Mao et al. 1995, Bruchey and Gonzalez-Lima 2008, Wang et al. 2013, 2014). Hydrolysis of C_{18} FAs generated AzA, which triggered the accumulation of G3P by up-regulation of glycerol kinase (GK) and G3P dehydrogenase (G3Pdh) activity. *Rboh* mutants showed reduced levels of AzA and G3P, and exogenous G3P could restore the SAR defect (Zoeller et al. 2012, Yu et al. 2013, Wang et al. 2014) indicating a linear connection of NO/ROS-AzA-G3P (**Figure 5**) (Wang et al. 2014, Wendehenne et al. 2014, El-Shetehy et al. 2015).

DIR1 and AZI1 also function downstream in this linear NO/ROS-AzA-G3P pathway. Exogenous NO did not induce SAR in *gli1*, *dir1*, and *azi1* but exogenous G3P was able to confer SAR in *noa1 nia2* and *rbohF*. Furthermore, G3P, DIR1, and AZI1 operate in a feedback regulatory loop being interdependent on each other for stability. G3P defective mutants (*gly1*/*gli1*) could not accumulate DIR1/AZI1 proteins but *dir1* and *azi1* mutant plants were also impaired in G3P accumulation (**Figure 5**) (Wang et al. 2014).

Altogether, the proposed model suggests NO to act upstream of ROS in a positive feedback regulatory loop. The importance of ROS in mediating FAs release during SAR was emphasized and NO/ROS were shown to function upstream of AzA/G3P and DIR1/AZI1. DA, Pip, AzA, and G3P all require SA to induce SAR, but only DA and Pip induce SA biosynthesis in the absence of pathogen infection (Chaturvedi et al. 2012, Navarova et al. 2012). Exogenous SA cannot restore SAR in NO, ROS or G3P defective mutants and conversely NO/ROS cannot confer SAR on mutants defective in SA synthesis or signalling. These findings suggest a branched pathway model, in which the ROS-AzA-G3P pathway complementary and non-redundantly acts together with the SA-signalling branch in SAR. Branched pathways are preferred in most metabolic networks, because they confer advantageous plasticity. The parallel networking of the NO/ROS and SA pathway provides opportunity for co-regulation, tighter regulation, or redundancies to ensure optimal induction of SAR. S-nitrosylation of NPR1, the key redox-regulator of SAR, is an example for such a cross talk (Wang et al. 2014, Wendehenne et al. 2014, Gao et al. 2015, El-Shetehy et al. 2015). NO also regulates ROS levels by nitrosylation of the RBOHD enzyme (Yun et al. 2011), what might serve as a checkpoint to prevent excessive ROS, which have a repressive effect on SAR (Wang et al. 2014). EDS1 is a further component that is involved in the two distinct pathways, regulating both SA and AzA levels (**Figure 5**) (Gao et al. 2015).

1.11 EDS1 – a key player in systemic acquired resistance signalling

ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) is a lipase-like protein, which is a central regulator of SA signalling (Falk et al. 1999, Wiermer et al. 2005, Strauss et al. 2010). EDS1 contains a non-catalytic N-terminal lipase-like domain with a classical α/ß-hydrolase fold connected to an α-helical bundle C-terminal domain, and both domains are critical for the function of EDS1 in immune signalling (Wagner et al. 2013).

EDS1 is essential for promoting SA-mediated basal resistance (PTI) to host-adapted biotrophic pathogens as well as ETI mediated by R proteins belonging to the TIR-NBS-LRR class (Aarts et al. 1998, Schurink et al. 1998, Falk et al. 1999, Feys et al. 2001, 2005, Wiermer et al. 2005, Ochsenbein et al. 2006, Truman et al. 2007, Birker et al. 2009, Vlot et al. 2009, Garcia et al. 2010, Heidrich et al. 2011, Rietz et al. 2011).

In *Arabidopsis* two major classes of R proteins exist (Meyers et al. 1999), the Toll/Interleukin-1 receptor-nucleotide binding site-leucine rich repeat (TIR-NBS-LRR) class (Aarts et al. 1998, Falk et al. 1999, Wiermer et al. 2005) and the N-terminal coiled-coil (CC)-NBS-LRR class (**Figure 6**) (Martin et al. 2003, Meyers et al. 2003, Nimchuk et al. 2003).

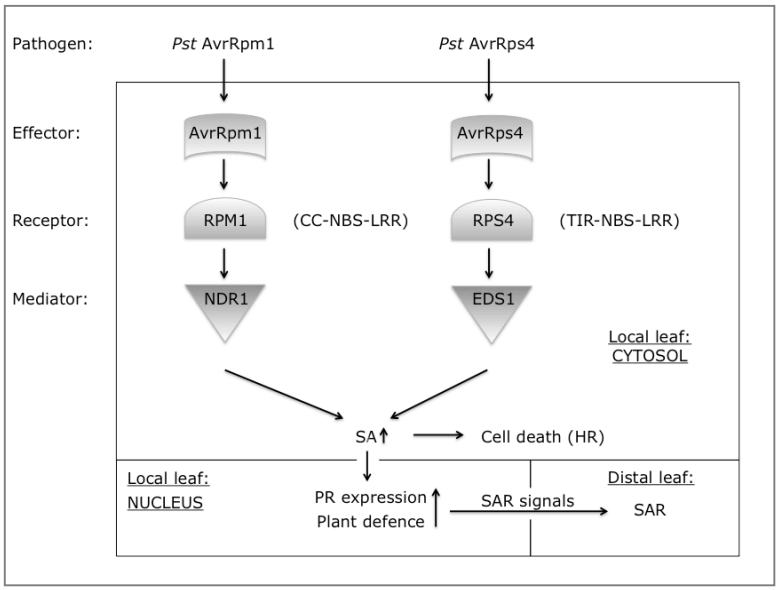

Figure 6: R gene-mediated resistance. Pathogens like *Pst AvrRpm1* or *Pst AvrRps4* secrete specific effectors into the cytosol of the host cell, which can be recognized by host receptors (R proteins). Different NBS-LRR receptors require different mediators to transfer the downstream defence signal. Most CC-NBS-LRR receptors function via NDR1 and TIR-NBS-LRR receptors via EDS1. (CC: Coiled-coil domain, EDS1: ENHANCED DISEASE SUSCEPTIBILITY1, NBS: Nucleotide-binding site, LRR: Leucine-rich repeat, NDR1: NONRACE SPECIFIC DISEASE RESISTANCE1, TIR: TOLL/INTERLEUKIN-1 RECEPTOR).

TIR-NBS-LRR (TNL) and CC-NBS-LRR (CNL) mediated defence pathways can be separated based on the requirement of EDS1. Whereas TNL receptors converge genetically on EDS1 to trigger resistance, most characterized CNL proteins instead require NONRACE SPECIFIC DISEASE RESISTANCE1 (NDR1) for defence activation (**Figure 6**) (Century et al. 1997, Aarts et al. 1998, Jones and Dangl 2006, Cheng et al. 2009, Vlot et al. 2009, Garcia et al. 2010). During PTI and TNL-mediated ETI, EDS1 interacts with its sequence-related interacting partners PAD4 and SENESCENCE ASSOSIATED GENE101 (SAG101) (Aarts et al. 1998, Falk et al. 1999, Wiermer et al. 2005, Rietz et al. 2011, Wagner et al. 2013). The fact that SA could rescue the enhanced disease susceptibility phenotype of *eds1* and *pad4* mutant plants indicates that EDS1 and PAD4 function upstream of SA (Feys et al. 2001, Rusterucci et al. 2001). Additionally, SA enhanced the expression of *EDS1* and *PAD4*, arguing that they are positively feedback regulated by SA (Zhou et al. 1998, Falk et al. 1999, Feys et al. 2001, Wiermer et al. 2005). The positive feedback loop, which likely potentiates SA action, is regulated by different EDS1 complexes including cytosolic EDS1 homodimers, nucleo-cytoplasmic EDS1-PAD4 heterodimers, and nuclear interactions between EDS1, PAD4, and the partly redundant SAG101 protein (Feys et al. 2001, 2005, Vlot et al. 2009, Rietz et al. 2011, Wagner et al. 2013).

During TNL-mediated ETI, EDS1 molecularly connects receptor recognition to downstream defence pathways (Garcia et al. 2010, Heidrich et al. 2011, Bhattacharjee et al. 2011). For example, EDS1 is intimately involved in the recognition process of the TNL receptor

RESISTANCE TO PSEUDOMONAS SYRINGAE4 (RPS4) with the *Pst* effector AvrRps4 (Gassmann et al. 1999, Bhattacharjee et al. 2011, Heidrich et al. 2011). Within the RPS4-EDS1 complex, EDS1 serves as the effector bait and is required for interception of the pathogen effector and following receptor activation. In contrast to the important role of EDS1 in TNL-mediated immunity, EDS1 is not required during CNL-induced ETI (Century et al. 1997, Aarts et al. 1998, Jones and Dangl 2006, Cheng et al. 2009, Vlot et al. 2009, Garcia et al. 2010) against pathogens like *Pst AvrRpm1* delivering the effector protein AvrRpm1 (Bent et al. 1994, Grant et al. 1995, Warren et al. 1998).

Subsequent to the defence responses in the infected leaves, PTI and ETI can induce a SAR response in the upper uninfected leaves (Durrant and Dong 2004, Mishina and Zeier 2007, Vlot et al. 2008, Fu and Dong 2013). Accordingly, functional EDS1-PAD4 signalling in the primary infected leaf is also required for systemic signalling beyond the infection site (Rusterucci et al. 2001, Truman et al. 2007). Nuclear EDS1-PAD4 induced transcriptional reprogramming enables the spread of resistance to systemic tissues (Wang et al. 2006, Garcia and Parker 2009, Garcia et al. 2010). Thus, besides its role in local resistance during PTI and TNL-triggered ETI, EDS1 is essential for systemic signalling and SAR (Vlot et al. 2008). During SAR, EDS1 is required for both SAR signal generation in the primary infected leaves and SAR signal perception in systemic uninfected tissues (Breitenbach et al. 2014).

In *Arabidopsis*, pathogen infection with *Pst AvrRpm1* leads to ETI mediated by the CNL protein RESISTANCE TO PSEUDOMONAS SYRINGAE pathovar MACULICOLA1 (RPM1) (Dangl et al. 1992). Although local resistance of the ETI response conferred by RPM1 genetically does not require EDS1 (Aarts et al. 1998), both *eds1* and *pad4* mutant plants show a SAR defective in response to local activation of RPM1 (Jing et al. 2011, Rietz et al. 2011, Truman et al. 2007, Rietz et al. 2011).

The fact that *eds1* mutant plants show a SAR-defect in response to *Pst AvrRpm1* while local resistance remains intact makes *eds1* a perfect tool to study systemic rather than local defence responses (Breitenbach et al. 2014, Vlot et al. 2009). In this work, I utilize the SAR-specific phenotype of the *eds1* mutant in response to *Pst AvrRpm1* to identify new SAR regulatory metabolites.

1.12 The DEX-system: A tool to study systemic acquired resistance-related metabolites

In contrast to Breitenbach et al. (2014) who identified new SAR-related proteins in the apoplast of wt compared to *eds1* mutant plants and Wittek et al. (2014) who focused on SAR-related soluble metabolites, this work focuses on the volatile emissions of *Arabidopsis* plants in response to AvrRpm1. For the analysis of plant-derived volatile emissions, a system is advantageous which can clearly distinguish between plant-derived volatile organic compounds (VOCs) and VOCs emerging from other sources. Transgenic plants carrying the dexamethasone (DEX)-inducible transgene *pDEX:AvrRpm1-HA* that encodes

C-terminally hemagglutinin (HA)-tagged AvrRpm1 provide such a pathogen free system (**Figure 7**) (Mackey et al. 2002, Breitenbach et al. 2014, Wittek et al. 2014).

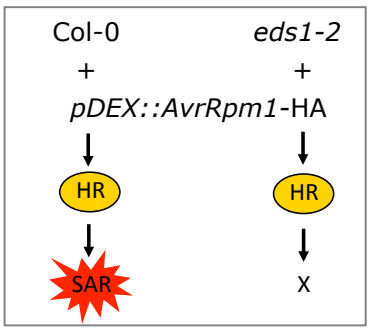

Figure 7: The DEX-system. SAR is induced by the bacterial effector AvrRpm1 expressed from a dexamethasone (DEX)-inducible transgene. Col0 and *eds1-2* mutant *Arabidopsis* plants are capable of mounting an HR, whereas only the Col-0 is capable of mounting a SAR response. Illustration adapted from (Wittek et al. 2014).

Application of 30µM DEX to the first two true leaves of *pDEX:AvrRpm1-HA* plants results in the appearance of HR lesions on the local treated as well as systemic untreated leaves showing that DEX treatment (30µM) leads to a systemic response in the plant presumably caused by DEX traveling from the treated tissues to the untreated systemic tissue. In contrast, application of 1µM DEX remains local and can be used to induce SAR (Breitenbach et al. 2014). In such analyses, a primary treatment with 1µM DEX induced SAR in Col-0 *pDEX:AvrRpm1-HA* but not in *eds1-2 pDEX:AvrRpm1-HA* indicating that *AvrRpm1-HA* expression triggered EDS1-dependent systemic immunity (**Figure 7**) (Breitenbach et al. 2014). *pDEX:AvrRpm1-HA* plants are a perfect tool to study plant derived VOC emissions in relation to SAR. It allows synchronization of the plants defence induction as much as possible, limits the presence of pathogen-derived proteins in the plants and eliminates pathogen-derived VOC emission. The conditional overexpression of *AvrRpm1* from the (DEX)-inducible transgene induces an EDS1-dependent systemic defence response (HR and ETI), with only Col-0 *pDEX:AvrRpm1-HA* plants showing SAR (**Figure 7**).

1.13 Volatile organic compounds in plant defence

The sophisticated defence strategies of plants to counteract attackers are not restricted to the inside of the plants organism. Secondary metabolite synthesis also includes production of VOCs, which are released into the plants surrounding as part of defence mechanisms against herbivores, bacteria, fungi, or viruses (Eigenbrode et al. 2002, McLeod et al. 2005, Mayer et al. 2008, Mauck et al. 2010, Dicke and Baldwin 2010, Hare 2011, Iason et al. 2012). Like introduced above, defence responses (ROS, NO, SA, HR, defence gene expression) are mediated and regulated by the SA- and JA/ET-signalling pathways. These phytohormone signalling pathway are also involved in the mechanisms underlying VOC production (Piel et al. 1997, Engelberth et al. 2001, Leitner et al. 2008), but the impact of the phytohormonal signalling pathway crosstalk on VOC emissions remains largely unexplored (Ponzio et al. 2013).

VOCs are lipophilic liquids with low molecular weight and high vapour pressure at ambient temperature. Due to their physical properties VOCs can be directly released through the

membranes of the epidermal tissue. VOCs can be emitted from flowering parts specialized in releasing volatiles, from vegetative parts (leaves and stems) through the stomata and from roots (Baldwin 2010, Pichersky et al. 2006, Dudareva et al. 2013, Matarese et al. 2014).

The emission of VOCs is either constitutive or induced by abiotic or biotic factors. Constitutive VOC emission is always present regardless of the experience of any stress, and the emission is largely controlled by genetic and environmental conditions, such as light or temperature (Niederbacher et al. 2015). Methanol used as marker for plant growth is one of the most commonly emitted constitutive VOCs, released from cell wall pectins when leaf shape changes (Baldwin 2010, Ghirardo et al. 2012, Niederbacher et al. 2015). (Stress-) induced VOCs are compounds that are only produced when needed. De novo synthesis is advantageous for the plant in terms of costs (carbon, energy usage) and elsewise do not reduce plant fitness (Holopainen 2004). VOC emission can be induced by abiotic stresses like temperature, water availability, salt stress, or oxidative stress (Niederbacher et al. 2015) as well as biotic factors. Among the biotic factors herbivore-induced VOC emission is the most well studied phenomenon, but also microbes and pathogens can induce VOC emission in plants (Baldwin 2010, Dudareva et al. 2013, Niederbacher et al. 2015). Inducible VOCs include alkanes, alkenes, carboxylic acids and alcohols, but the dominating compounds are terpenes and the so-called green leaf volatiles (GLVs) (**Figure 8**) (Holopainen 2004, Penuelas and Llusia 2004, Baldwin 2010).

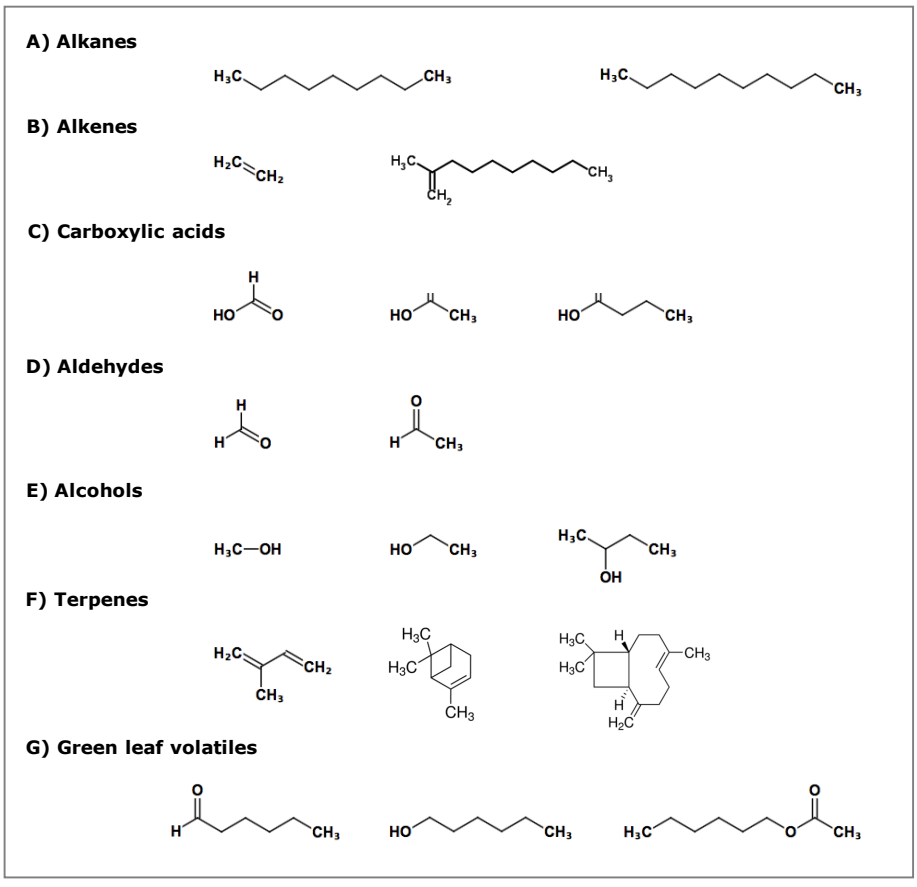

Figure 8: Typical VOCs emitted from plants. A) Alkanes: Nonane, decane. B) Alkenes: Ethylene, 2-methyl-n-1-tridecene. C) Carboxylic acids: Formic acid, acetic acid, butyric acid. D) Aldehydes: Formal-dehyde, acetaldehyde. E) Alcohols: Methanol, ethanol, 2-butanol. F) Terpenes: Isoprene, α-pinene, ß-caryophyllene. G) Green leaf volatiles: Hexanal, hexanol, hexyl acetate.

Plants can store VOCs in the vacuoles where they are released when needed, but also plants without storage structures can emit VOCs from their foliage (Baldwin 2010, Niederbacher et al. 2015). Constitutive VOCs normally released from healthy intact plants often become inducible VOCs emitted above the constitutive level after foliar damage (Holopainen 2004).

There is an ongoing debate about the function of VOCs. The hypothesis that VOCs as by-products of plant processes are just emitted as unavoidable result of their volatility with no apparent function is too simple-minded (Holopainen 2004, Niinemets et al. 2004, Penuelas and Llusia 2004, Rosenstiel et al. 2004). Although VOC emission is primarily determined by the principal physicochemical characteristics of compounds such as their solubility, volatility, and diffusivity (Niinemets et al. 2004), a new explanation for the emission of VOCs with apparently no function is the 'safety valve' hypothesis. VOCs act as 'safety valve' to remove an excess of energy or carbon that cannot be processed by the plant, as usually occurs under stressful conditions (Rosenstiel et al. 2004). However, and this is uncontroversial, natural selection has worked to take advantage of the volatility of VOCs (Niinemets et al. 2004, Penuelas and Llusia 2004, Rosenstiel et al. 2004). Many VOCs have well-known functions in plant defence, protection and communication (Ponzio et al. 2013, Holopainen 2004, Dudareva et al. 2013, Baldwin 2010, Loreto and Schnitzler 2010). VOCs can provide direct defence against biotic stress, by acting as toxins and feeding deterrents, directly repelling herbivores (Kessler and Baldwin 2001, Ponzio et al. 2013, Boulogne et al. 2012) or as pathogen-induced VOCs with antimicrobial activities to inhibit further pathogen colonization (Brown et al. 1995, Holopainen 2004, Neri et al. 2007, Boulogne et al. 2012, Huang et al. 2012). Microbes have adapted and as counter strategy detoxify and use the plants VOCs as carbon or nutrient sources for themselves, thus diminishing the role of VOCs as direct defence mechanism against biotic factors. However, VOCs are also involved in the protection of plants against abiotic stresses, such as high light, temperature or oxidative stress (Holopainen 2004, Dudareva et al. 2013, Baldwin 2010, Loreto and Schnitzler 2010).

Besides their role in direct defence and protection, VOCs are involved in different forms of (defence)-signalling and communication termed as intra-plant communication (within plant signalling), inter-plant communication (plant-plant signalling) or plant-animal communication. After microbial attack VOCs from attacked organs transmit information within the plant (within-plant signalling), affecting transcript abundance or directly activating defence responses in the distal unaffected tissue to elicit defence more rapidly when attackers arrive (Farmer et al. 2001, Heil and Ton 2008, Karban et al. 2006, Frost et al. 2007, Heil and Silva Bueno 2007). Since herbivores and pathogens can move independently of the vascular system, VOCs display a suitable instrument to reach parts of the plant that are spatially, but not anatomically, located close to the attacked organ

(Heil and Karban 2010). Although many studies suggest the induction of resistance via VOCs that move outside the plant as long-distance signals (Kiefer and Slusarenko 2003, Karban et al. 2006, Farmer et al. 2001, Heil and Silva Bueno 2007) studies that explicitly demonstrate the involvement of VOCs in the within-plant regulation of SAR are lacking (Heil and Ton 2008). Long-distance signalling via the vascular system is a well-known mechanism in SAR (Durrant and Dong 2004, Vlot et al. 2008, Shah and Zeier 2013) and MeSA functions as long distance SAR signal. It is likely that the SAR signal MeSA as a VOC does not only function via the vasculature but also via airborne transport as an additional mechanism to mediate SAR (Heil and Ton 2008).

Induced VOCs do not just distribute the information within the own organism but also to neighbouring plants termed plant-plant signalling. The neighbouring non-infested "receiver" plant gains a fitness benefit by monitoring these VOCs and is primed for resistance induction to future pathogen encounters (Baldwin and Schultz 1983, Shulaev et al. 1997, Karban et al. 2000, Yi et al. 2009, Heil and Karban 2010, Heil and Adame-Alvarez 2010).

Long-distance signalling, a well-known mechanism in SAR (Durrant and Dong 2004, Vlot et al. 2008, Shah and Zeier 2013) is not only caused by molecules that are transported in the vascular system. Also, VOCs that move outside the plant can function as long-distance signals and mediate systemic resistance within and between plants (Heil and Ton 2008, Yi et al. 2009). The already above mentioned constitutive GLV methanol is also well-known to mediate a priming effect within the plant and to neighbouring plants, when released in higher amounts from the cell wall after leaf damage through herbivores or pathogens (Komarova et al. 2014).

VOCs do not just play an important role in communication between plants, they also allow plants to communicate with other community members, such as herbivores, pathogens and natural enemies of herbivores, recapped as plant-animal communication (Kessler and Baldwin 2001, Dicke et al. 2003, Arimura et al. 2005, Unsicker et al. 2009, Dudareva et al. 2013, Ponzio et al. 2013). With the release of volatile scents from flowers or fruits, plants attract pollinators and seed dispersers to ensure reproduction (Pichersky and Gershenzon 2002, Pichersky et al. 2006, Dudareva et al. 2013). The guiding of insect predators or parasitoids by the release of VOCs is the most prominent indirect defence strategy and is well known as the plant`s 'cry for help' (Dicke and Baldwin 2010, Baldwin 2010).

To date, more than 1700 VOCs have been identified from 90 different plant families belonging to both angio- and gymnosperms (Dudareva et al. 2013). Due to their biosynthetic origin, VOCs are classified in terpenoids, phenylpropanoids/benzenoids, FA and amino acid derivatives (Dudareva et al. 2004, 2013, Matarese et al. 2014). In the following, the largest and most diverse class of VOCs will be introduced (reviewed by Tholl and Lee 2011, Dudareva et al. 2013): These are the terpenoids with well over 30.000

compounds providing much of the structural diversity in plant volatile blends (Baldwin 2010, Finefield et al. 2012).

Terpenoid VOCs are synthesized via two independent pathways, the mainly cytosolic mevalonic acid (MVA) pathway and the plastidial methylerythritol phosphate (MEP) pathway (**Figure 9**). The MVA pathway is giving rise to sesquiterpenes (C_{15}), irregular terpenes (triterpenes, (C_{30}) and geranyllinalool (C_{20}). The MEP pathway gives rise to hemiterpenes (C_5), monoterpenes (C_{10}), diterpenes (C_{20}), and volatile carotenoid derivatives (C_{40}). The MVA pathway involves six enzymatic reactions and is initiated by a stepwise condensation of three molecules of acetyl-CoA to hydroxymethylglutaryl-CoA (HMG-CoA), which undergoes reduction to MVA followed by two subsequent phosphorylations and a decarboxylation step with formation of isopentenyl pyrophosphate (IPP) as the final product. The MEP pathway consists of a total of seven enzymatic steps beginning with the condensation of D-glyceraldehyde 3-phosphate (GAP) and pyruvate to produce 1-deoxy-D-xylulose 5-phosphate (DXP), which is then subjected to isomerization with formation of MEP, followed by five consecutive steps leading to the end products IPP and dimethylallyl pyrophosphate (DMAPP) (at a ratio of 5:1). IPP and DMAPP are the universal precursors of all terpenes. Both pathways rely on IPP isomerase, which reversibly converts IPP to DMAPP and controls the equilibrium between them (**Figure 9**).

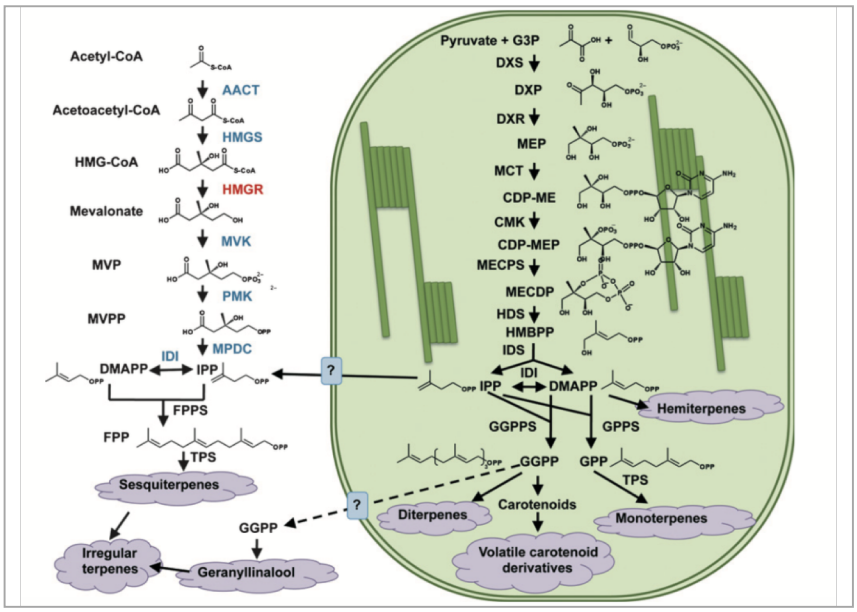

Figure 9: Terpenoid biosynthesis. Terpenoids are synthesized via the cytosolic mevalonic acid (MVA) pathway and the plastidial methylerythritol phosphate (MEP) pathway. GPPS is required for GPP synthesis. GERANYL (GERANYL) DI-PHOSPHATE SYNTHASE 12 (GGPPS12) as small subunit of GPPS is essential for monoterpenoid biosynthesis. Solid arrows represent established biosynthetic steps, whereas broken arrows point to hypothetical reactions (AACT: Acetyl-CoA acetyltransferase, CDP-ME: 4-Diphosphocytidyl-2-C-methyl-D-erythritol, CDP-MEP: CDPME 2-phosphate, CMK: 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase, DMAPP: Dimethylallyl pyrophosphate, DXP: 1-Deoxy-D-xylulose 5-Phosphate, DXS: DXP synthase, DXR: 1-Deoxy-D-xylulose 5-phosphate reductoisomerase, FPP: Farnesyl pyrophosphate, FPPS: FPP synthase, G3P: Glyceraldehyde 3-phosphate, GGPP: Geranylgeranyl diphosphate, GGPPS: GGPP synthase, GPP: Geranyl diphosphate, GPPS: GPP synthase, HDS: 4-Hydroxy-3-methylbut-2-en-1-yl diphosphate synthase, HMBPP: (E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate, HMG-CoA: Hydroxymethylglutaryl-CoA, HMGR; HMG-CoA reductase, HMGS: HMG-CoA synthase, IDI: Isopentenyl pyrophosphate isomerase, IDS: Isopentenyl diphosphate synthase, IPP: Isopentenyl pyrophosphate, MCT: 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, MECPD: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate, MECPS: ECPDsynthase, MVK: Mevalonate kinase, MPDC:

Mevalonate diphosphate decarboxylase, MVP: Mevalonate 5-phosphate, MVPP: Mevalonate 5-Pyrophosphate, PMK: Phosphomevalonate kinase, TPS: Terpene synthase). Illustration adapted from (Dudareva et al. 2013).

In the second stage of terpene biosynthesis, the building blocks geranyl diphosphate (GPP), farnesyl pyrophosphate (FPP) and geranylgeranyl diphosphate (GGPP) required for the formation of higher terpenoids are produced by isoprenyl diphosphate synthases. In the cytosol, condensation of two molecules IPP with one molecule of DMAPP gives rise to FPP, the precursor of volatile sesquiterpenes. In plastids, one molecule DMAPP with one or three IPP molecules forms GPP and GGPP, the precursors of mono- and diterpenes. GPP synthases (GPPSs) produce GPP, which is the precursor of most monoterpenes (**Figure 9**).
Terpene synthases (TPS) are the main cause of the tremendous diversity of volatile terpenoids in plants. They can act pluripotently and synthesize multiple products from a single prenyl diphosphate substrate. A single TPS can produce as many as twenty products or as few as one. In *Arabidopsis* 32 TPS genes with species-specific divergence and tissue- and cell-type specific expression profiles are known. The TPS gene family is divided into seven subfamilies (TPS-a, TPS-g), where TPS-a consists mostly of sesquiterpene and diterpene synthases and TPS-b and TPS-g clades contain mostly monoterpene synthases (Tholl and Lee 2011, Dudareva et al. 2013).

1.14 Aims of this study

The aim of this thesis was to identify new SAR regulatory metabolites. *Eds1-2* mutant plants show a SAR-defect in response to *Pst AvrRpm1* while local resistance remains intact. This makes *eds1-2* a perfect tool to study systemic rather than local defence responses (Breitenbach et al. 2014, Vlot et al. 2009). In contrast to Breitenbach et al. (2014) who identified new SAR-related proteins in the apoplast of wt compared to *eds1* mutant plants and Wittek et al. (2014) who focused on SAR-related soluble metabolites, this work focuses on the volatile emissions of *Arabidopsis* plants in response to AvrRpm1 and in relation to SAR. In collaboration with Prof. Dr. Schnitzler and Dr. Ghirardo (EUS/HMGU) volatile emissions of Col-0 *pDEX:AvrRpm1-HA* (hereafter referred to as Col-0 dex) and *eds1-2 pDEX:AvrRpm1-HA* (hereafter referred to as *eds1-2* dex) plants (Mackey et al. 2002, Breitenbach et al. 2014) were collected with a dynamic system and analysed by gas chromatography coupled to mass spectrometry (GC-MS).
The second aim of the study was to investigate a possible biological relevance of any newly identified EDS1-dependent VOCs in plant defence to the pathogen *Pst* DC3000. To this end, the potential of the VOCs to induce plant defence was investigated in an incubation set-up in comparison with the well-known resistance-inducing compound MeSA.
Third, the mechanism of VOC-induced resistance was to be elucidated by investigating *PR1* transcript levels and by using different SA-pathway mutants in the incubation set-up from goal two.

Finally, since three of the identified EDS1-dependent VOCs were monoterpenoid VOCs and displayed a potential biological relevance in terms of plant defence in the incubation set-up, the role of monoterpenoid biosynthesis in plant defence and SAR in particular was investigated. To this end, t-DNA insertion lines for *GERANYL(GERANYL)DIPHOSPHATE SYNTHASE12* (*AtGGPPS12*), which is suggested to play a key role in the regulation of monoterpene biosynthesis (Tholl et al. 2004, Wang and Dixon 2009), were investigated.

2. MATERIAL AND METHODS

2.1 MATERIAL

2.1.1 Plant material and growth conditions

All experiments were performed in *A. thaliana* ecotype Columbia-0 (Col-0). Mutants *eds1-2*, *npr1-1*, and *sid2-1* as well as transgenic plants expressing haemagglutinin (HA)-tagged AvrRpm1 from a dexamethasone (DEX)-inducible transgene (*pDEX:AvrRpm1-HA*) in Col-0 and *eds1-2* backgrounds were previously described (Cao et al. 1997, Wildermuth et al. 2001, Mackey et al. 2002, Bartsch et al. 2006, Breitenbach et al. 2014).
The Transfer-DNA (t-DNA) insertion lines SALK_208952C (*ggpps12-1*) and SALK_210207C (*ggpps12-2*) were obtained from the Nottingham Arabidopsis Stock Centre (Scholl et al. 2000). Seeds of selected homozygous plants were used for experiments.
Plants were grown on normal potting soil mixed with silica sand at a ratio 5:1 in plant growth chambers in 10h light, 14h dark cycles at 70% relative humidity, 22°C during the day at a light intensity of 100µE m^{-2} s^{-1}, and 18°C during the night. Seeds were sown on watered soil covered with wrapping film and stored for two days at 4°C to synchronize germination before being transferred into the plant growth chamber.
Seeds were stored in paper bags, permeable to air under dry and dark conditions.

2.1.2 Bacterial strains and culture conditions

For different experiments the bacterial strains *Pseudomonas syringae* pathovar *tomato* (*Pst* DC3000, virulent strain) and *Pst* carrying the bacterial effector AvrRpm1 (*Pst AvrRpm1*, avirulent strain) were used.
Pst strains (*Pst AvrRpm1*, *Pst* DC3000) were maintained on NYGA medium (**Table 9**) containing the selective antibiotics rifampicin and kanamycin (50µg ml^{-1} each, **Table 10**). Bacteria were grown at 28°C (MMM-Friocell 111, Munich, Germany).

2.1.3 Enzymes used for cDNA-synthesis

The enzyme SuperScript™II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) was used to produce cDNA from isolated *Arabidopsis* plant RNA.

2.1.4 Primers

The primers used in this study are outlined in **Table 1** and were used at a concentration of 10µM for qPCR.

Table 1: Primers used for qPCR analysis. Bp: base pairs, F: forward, R: reverse.

Name/Application	Composition/Concentration	Size [bp]	Annealing Temp. [°C]
GGPPS12_208952F	ACA TTT TGA AAG CGA CGT GTC	21	57.0
GGPPS12_208952R	AGC TCT TCT TCT TGT CCT CGG	21	61.0
GGPPS12_210207F	AAT GGA ATG AAT CAG GTG CAC	21	57.0
GGPPS12_210207R	TAT TGG ATA AGA TGG CCA ACG	21	57.0
LBb1(pBIN-pROK2)	GCG TGG ACC GCT TGC TGC AAC T	22	60.0
PR1_F	CTA CGC AGA ACA ACT AAG AGG CAA	25	60.0
PR1_R	C TTG GCA CAT CCG AGT CTC ACT G	22	60.0

2.1.5 Chemicals

All chemicals were used in a high purity grade and purchased either from Sigma-Aldrich GmbH (Taufkirchen, Germany), Carl Roth GmbH (Karlsruhe, Germany), Merck GmbH (Darmstadt, Germany), or from other sources as indicated in the tables. Applications of the chemicals are indicated in the title of the tables.

Table 2: Chemicals used for promotor activation in *DEX:AvrRPM1-HA* plants.

Name/Application	Composition/Concentration	Source
Promotor activation in *DEX:AvrRPM1-HA* plants	30 µM Dexamethasone 0.01% Tween-20 Ethanol	Sigma, Taufkirchen, Germany Bio Rad, Munich, Germany Sigma, Taufkirchen, Germany

The 30µM DEX solution was prepared freshly before the experiment in 100% ethanol and was filter-sterilized (pore size 0.22µm, Millipore, Billerica, MA, United States).

Table 3: Chemicals and VOC standard solutions used for GC-MS analysis.

Name/Application	Composition/Concentration	Source
BTEX	250µl in 50ml hexane	Resteck, Bad Homburg v. d. Höhe, Germany
Ethanol	Pure, undenaturated, in bidest. H_20 50:50	Merck, Darmstadt, Germany
Helium		Linde, Pullach, Germany
Hexane	Rotipuran, purity grade 99%	Roth, Karlsruhe, Germany
Methanol	Rotisolv, purity grade 99.9%	Roth, Karlsruhe, Germany
Nitrogen		Linde, Pullach, Germany

Nitrogen (liquid)		Helmholtz Center Munich
C_7-C_{30} saturated alkanes	Purity grade analytical standard	Sigma, Taufkirchen, Germany
(+)-2-Carene	7µl in 50ml hexane or methanol, Purity grade 97%	Sigma, Taufkirchen, Germany
ß-Caryophyllene	Purity grade 80%	Roth, Karlsruhe, Germany
Eucalyptol	Purity grade 99.57%	Roth, Karlsruhe, Germany
Trans-ß-farnesene	Purity grade 90%	Sigma, Taufkirchen, Germany
(+)-Limonene	Purity grade 99%	Roth, Karlsruhe, Germany
Linalool	Purity grade 97.79 %	Roth, Karlsruhe, Germany
Myrcene	Purity grade 90%	Roth, Karlsruhe, Germany
Trans-nerolidol	Purity grade 85 %	Fluka, Buchs, Schweiz
α-Pinene	Purity grade 97.17%	Roth, Karlsruhe, Germany

Table 4: Chemicals and VOC standard solutions used for the incubation set-up.

Name/Application	**Composition/Concentration**	**Source**
Camphene	Purity grade 95%	Sigma, Taufkirchen, Germany
n-Hexane	Rotisolv, Purity grade 99%	Roth, Karlsruhe, Germany
Isopropyl palmitate	Purity grade 90%	Sigma, Taufkirchen, Germany
(+)-Limonene oxide, mixture of cis and trans	Purity grade 97%	Sigma, Taufkirchen, Germany
(-)-Limonene oxide, mixture of cis and trans	Purity grade 99%	Sigma, Taufkirchen, Germany
Methyl salicylate	Purity grade 99%	Sigma, Taufkirchen, Germany
(±)-α-Pinene	Purity grade 99%	Sigma, Taufkirchen, Germany
(+)-β-Pinene	Purity grade 98.5%	Sigma, Taufkirchen, Germany
(-)-β-Pinene	Purity grade 99%	Sigma, Taufkirchen, Germany

Salicylic acid	Purity grade 99%	Roth, Karlsruhe, Germany

All the VOC solutions were prepared freshly and immediately before application in the desiccators. The particular concentrations of VOC solutions were achieved by dilution in 100% n-hexane.

A salicylic acid solution was used for testing the direct antimicrobial activity of the VOCs against *Pst* DC3000. Salicylic acid was solved in 100% MeOH and diluted in ddest. water to the particular concentration (500µM). The applied solution contained 0.05% MeOH.

Table 5: Chemicals used for DNA extraction

Name/Application	Composition/Concentration	Source
Chloroform	100%	Merck, Darmstadt, Germany
Lysis buffer CTAB	100mM TRIS	Roth, Karlsruhe, Germany
(per liter H_2O, pH 8.0)	20mM EDTA	Roth, Karlsruhe, Germany
+1% β-mercaptoethanol	1.4M NaCl	Merck, Darmstadt, Germany
	2%Hexadecyltrimethyl-ammoniumbromide (99%)	Roth, Karlsruhe, Germany
β-Mercaptoethanol	100%	Merck, Darmstadt, Germany
Phytopure	100%	GE Healthcare, Buckingham-shire, UK
Water	Licrosolv	Merck, Darmstadt Germany

Table 6: Chemicals used for RNA extraction.

Name/Application	Composition/Concentration	Source
TriReagent, per 100ml	3.05g Ammoniumrhodanide	Roth, Karlsruhe, Germany
(adjust pH 5.0 before	(Ammoniumthiocyanate)	
adding phenol)	9.44g Guanidinthiocyanat	Merck, Darmstadt, Germany
	5ml Glycerol	Roth, Karlsruhe, Germany
	3M Na-Acetate pH 5.2	Merck, Darmstadt, Germany
Phenol	100%	Roth, Karlsruhe, Germany
Chloroform	100%	Merck, Darmstadt, Germany
2-Propanol	100%	Roth, Karlsruhe, Germany
Ethanol	100%	Merck, Darmstadt, Germany

Table 7: Chemicals used for agarose gel electrophoresis.

Name/Application	Composition/Concentration	Source
Ethidiumbromid	100%	Roth, Karlsruhe, Germany
Agarose		Biozym, Oldendorf, Germany

1x TAE buffer (Tris-Acetate-EDTA)	40mM Tris acetate 1mM EDTA	Roth, Karlsruhe, Germany Roth, Karlsruhe, Germany
6x loading dye		Fermentas, St Leon-Rot, Germany

2.1.6 Media, buffers, and solutions

Applications of the media, buffers, and solutions are indicated in the titles of the tables.

Table 8: Solutions used in SAR experiments and spray infection experiments.

Name/Application	Composition/Concentration	Source
MOCK solution (pH 7.0)	10mM $MgCl_2$	Merck, Darmstadt, Germany
Bacteria isolation solution	10mM $MgCl_2$	Merck, Darmstadt, Germany
	0.01% Silwet	Lehle Seeds, Texas, USA

Table 9: Media for bacteria cultivation.

Name	Composition	Source
NYGA, per litre, pH 7.0	5g Bacto-proteose Peptone	Roth, Karlsruhe, Germany
	3g yeast extract	Roth, Karlsruhe, Germany
	20ml Glycerol	Roth, Karlsruhe, Germany

For NYGA agar plates 18g of Agar-Agar (Merck, Darmstadt, Germany) was added to the media after pH adjustment. Media were autoclaved for 20 min at 120°C. After autoclaving, the media were cooled down to ca. 55°C for addition of the selective antibiotics rifampicin and kanamycin.

2.1.7 Antibiotics

Table 10 shows the different antibiotics used in selective media with their final working concentrations.

Table 10: Antibiotics.

Name	Working concentration [µg/ml]	Source
Kanamycin	50	Roth, Karslruhe, Germany
Rifampicin	50	Duchefa Biochemie, Germany

Kanamycin was dissolved in ddest. water and rifampicin was dissolved in 100% dimethylsulfoxide (DMSO). Stock solutions were filter sterilized (0.22µm sterile filters, Millipore, Billerica, MA, United States) and stored as aliquots at -20°C.

2.2 METHODS

2.2.1 Induction of systemic resistance by *AvrRpm1* expression in dexamethasone-treated plants

Systemic resistance was induced in four-and-a-half-week-old *Arabidopsis* plants carrying a p*DEX:AvrRpm1-HA* transgene (Col-0 dex, *eds1-2* dex) by spray-application of 30µM DEX. To ensure that all leaves of the plants were evenly covered, 0.01% Tween-20 was added to the DEX-solution to reduce surface tension and allow better surface contact.

2.2.2 Measurement of volatile organic compounds

Plant material

The VOC emissions of four-and-a-half-week-old *Arabidopsis* plants carrying a *pDEX:AvrRpm1-HA* transgene (Col-0 *pDEX:AvrRpm1-HA*, *eds1-2 pDEX:AvrRpm1-HA*) were analysed. Plants were sprayed with 30µM DEX (0.01% Tween-20) to induce the *pDEX:AvrRpm1-HA* transgene. Half an hour post DEX-treatment the plants were transferred into gas-tight conic cuvettes (∅ 28cm/13cm, 5.5L, IKEA, Eching, Germany) made of glass material and containing a Teflon sheet (PTFE Teflon baking foil, Hightechflon GbR, Konstanz, Germany) sealed with Teflon paste on the bottom. Each cuvette was set with twelve plants. The plants were let acclimatizing to the new environmental conditions for half an hour.

Experimental set-up and volatile organic compound analysis

The collection of volatiles was performed in eight cuvettes that were run in parallel. Each cuvette was continuously flushed with 0.2 L min^{-1} of VOC-free synthetic air (79% N_2, 21% O_2) mixed with pure CO_2 to a final CO_2 concentration of 400µmol mol^{-1}. The light was provided for 10 hours (8am-6pm) and the intensity at leaf surface was 120-150µmol m^{-2} sec^{-1}. A part of the air exiting the cuvettes was diverted for the VOC sampling using Teflon t-pieces. A total of 18 litres of air were collected with a flow rate of 0.1 L min^{-1} for 180 min into glass cartridges filled with polydimethylsiloxane-foam (Gerstel, Mülheim an der Ruhr, Germany) and 50mg carbopack B (Sigma-Aldrich) adsorbents. Airflows were controlled using needle valves for the inlet and all the flows were measured before and at the end of each measurement using a calibrated mass flow meter (ADM 3000, Agilent Technologies, Palo Alto, CA, USA). VOC were collected during two different sampling periods. Sampling period one (SP1) was 1-4 hours post DEX-treatment, the second sampling period (SP2) was 4-7 hours post DEX-treatment. Background measurements of "pots without plant material" were performed twice, at the beginning and at the end of the experiments. The procedure followed exactly this used for sampling VOCs from plants, except that plants were removed out of the soil before enclosing the pots (Pöppelmann, TEKU, T06D, Lohne,

Germany) into the cuvettes. Half of the pots used for background measurements contained Col-0 *pDEX:AvrRpm1-HA* plants, the other half *eds1-2 pDEX:AvrRpm1-HA* plants. Two times eight "pots without plant material" were analysed with a total amount of 16 replicates for background samples. Cartridges containing the collected volatiles were kept at 4°C for approximately two weeks prior to chemical analysis. In additions, cartridges not used for the collection were stored in the same way as the samples and each day one was analysed as control of the storage procedures.

The analysis of the collected VOC samples was performed with a thermo-desorption unit (TDU, Gerstel GmbH) coupled to a gas chromatography coupled to mass spectrometry (GC-MS) (GC type: 7890A; MS type: 5975C Agilent Technologies, Palo Alto, CA, USA). The TDU-GC-MS followed established procedures (Ghirardo et al. 2012, Kreuzwieser et al. 2014, Ghirardo et al. 2016, Weikl et al. 2016). The samples were desorbed from 37 to 270°C at a rate of 280°C min^{-1} and holding for 2 min. The compounds were refocused on Tenax (cryocooling technique) at -50°C, then re-desorbed to 250°C at a rate of 12°C s^{-1} and by holding for 2 min. Separation of VOCs was achieved using a 5% phenyl 95% dimethyl arylene siloxane capillary column (60m × 250µm × 0.25µm DB-5MS + 10m DG, Agilent Technologies) with a constant flow rate of He of 1 ml min^{-1} and a temperature program of 40°C for 0 min, followed by ramping at 10°C min^{-1} to 130°C and holding for 5 min, followed by ramping at 80°C min^{-1} to 175°C and holding for 0 min, by ramping at 2°C min^{-1} to 200°C and holding for 0 min, by ramping at 4°C min^{-1} to 220°C and holding for 0 min, by ramping at 100°C min^{-1} to 300°C and holding for 6 min. All peaks of GC-MS chromatograms were taken into account. Chemical identification was achieved by comparing the mass spectra obtained from samples and commercially available authentic standards (Sigma-Aldrich, Taufkirchen, Germany). When standards were not available, sample spectra were compared to those available from the 2011 National Institute of Standards and Technology Mass Spectral Library (NIST, Wiley library v.275, USA) and by comparing the non-isothermal Kovats retention indices (RI). The calculation of RI followed the generally accepted standard procedure (Gonzalez and Nardillo 1999), based on chromatography retention times of a saturated alkane mixture (C_7-C_{40}; Sigma-Aldrich, Taufkirchen, Germany) and other alkanes (<C_7) occurring in the chromatogram background. Sensitivity changes during sample analysis were taken into account using a fix amount of δ-2-carene as an internal standard. Emission rates were calculated on a leaf area base (pmol m^{-2} s^{-1}).

Statistical analysis of GC-MS data

Collection of VOCs was performed seven times (for cuvettes per genotype) with independent plant material to obtain 28 biological replicates for each genotype and sampling period. All plants inside the same cuvette were pooled together for further biochemical analysis and considered as one single biological replicate.

For background correction, all peak areas of Col-0 dex and *eds1-2* dex samples were substracted by the average peak area of the corresponding peaks from background samples by using EXCEL. After background correction, peak areas were converted into emission rates of VOCs (pmol m^{-2} s^{-1}) by including the total leaf areas of the twelve plants of each cuvette (see 2.2.3 Measurement of leaf areas).

The background-corrected emission rates of VOCs emitted by *Arabidopsis* were analysed by a multivariate data analysis (MDA) approach using principal component analysis (PCA) and partial least square regression (PLSR) statistical methods. Both analyses were performed using the software package SIMCA-P version 13.0.0.0 (Umetrics, Umeå, Sweden). For the analysis, VOC emission rates from each individual biological replicate were used as X variable. Before PCA and PLSR analyses, data were pre-processed by log transformation [X = log(X+1)], mean centred, and scaled to unit variance. Cross-validation was used to validate the number of significant PCA and PLSR components (Eriksson et al. 2006) using a 99% confidence level on parameters and seven cross-validation groups. PCA was performed to describe the different blends of VOC emitted from plants, in an objective and unsupervised manner. PLSR was then performed using the regression type OPLS of SIMCA-P. PLSR was validated using analysis of variance testing of cross-validated predictive residuals (CV-ANOVA, Eriksson et al. 2008). The overall analysis aimed to identify which compound and at which degree the VOC was positively or negatively correlated with genotype (Col-0 dex/*eds1-2* dex) or sampling period SP1, SP2). A volatile compound was classified discriminant when it had both importance in the projection (VIP>1) and the uncertainty bar computed by jack-knife method (Efron and Gong, 1983) was smaller than its respective VIP value. Additionally, the discriminant VOCs resulted from MDA were repetitively verificated by student`s t-test (p<0.05, p<0.01).

2.2.3 Measurement of leaf areas

Calculation of VOC emissions was based on leaf areas (pmol m^{-2} s^{-1}). Therefore, the total leaf areas of the twelve plants of each cuvette were calculated by using the image-editing program GIMP. Prior to the DEX-treatment and VOC measurement pictures of the plants were taken with a fixed installed camera system. All pictures were taken under the same light conditions, camera settings and distance between plants and camera. To get a better contrast a black paper was placed between leaves and soil. The leaf areas were analysed with GIMP in number of pixels by using the contrast between leaves and the black sub face. The number of pixels was then converted into the unit square meters by using a picture (with same settings) of different reference areas. The calculated leaf areas were projection areas, since overlapping of leaves could not completely be eliminated. There was no significant difference between the leaf areas of Col-0 dex (68.63cm^2 ± 17.74 StDEV) and *eds1-2* dex (69.48cm^2 ± 20.38 StDEV) plants.

2.2.4 Volatile organic compound incubation set-up

The biological relevance of the possibly SAR-related VOCs was investigated in an incubation set-up. *Arabidopsis* plants were four-and-a-half-weeks old and grown on soil in stainless steel pots (Rotilabo-Messbecher high-grad steel, ∅ 30mm 30ml, Roth, Karlsruhe, Germany) to avoid VOC emissions from the pot material or trapping of the applied VOCs by pot plastic material. Eight wt *Arabidopsis* plants were placed in gas-tight glass desiccators (Rotilabo-Glas-Exsikkatoren, Typ200, ∅ 269mm/190mm, 5.5L, Roth, Karlsruhe, Germany) together with a filter paper. The desiccators were filled with fresh air from the inflow of the growth chamber where the plants were grown and closed gas-tightly. Since the tested VOCs were not soluble in water, hexane was used as diluting agent and negative control. Different concentrations of the VOCs (including 200µl hexane), the positive control MeSA (2µM, including 200µl hexane) or the negative control hexane (200µl) were applied with an HPLC-syringe through the gas tap onto the filter paper in the desiccators. The plants were incubated over three days in the gas-tight glass desiccators in the same growth chambers where the plants were cultivated. The supplemented air in the desiccators was replaced every day to supply fresh air and avoid high air humidity. Subsequently, the respective treatment with the VOC, MeSA, or hexane was applied again, in total three times on three consecutive days. After three days of pre-incubation the plants were removed from the desiccators and two fully expanded leaves were challenged with *Pst* DC3000 (according to the second challenge infection in SAR, see 2.2.6 Assessment of systemic acquired resistance in *Arabidopsis*). After four days, the bacterial growth was analysed (see 2.2.6 Assessment of systemic acquired resistance in *Arabidopsis*) as indicator for induced resistance.

2.2.5 Volatile organic compound application in the incubation set-up

All applied VOC solutions were diluted in hexane to the desired concentrations. According to the applied concentrations (**Table 11**, **column 5**), a stock solution with a volume of about 2ml was prepared in a gas-tight HPLC vial (**Table 11**, **column 1**). First, the hexane volume for the stock solution (**Table 11**, **column 1**) was injected into a 2ml HPLC-vial and gas-tightly sealed. Subsequently, the appropriate volume (depending on the concentration to be applied to the plants, **Table 11**, **column 1**) of VOC standard solution was injected with an HPLC-syringe onto the bottom of the hexane fraction.

For the VOC solution, which was applied in the desiccator, first 200µl of hexane were injected into a 300µl HPLC vial insert and gas-tightly sealed. Subsequent, the desired volume of stock solution (**Table 11**, **column 3**) was injected with an HPLC-syringe onto the bottom of the hexane fraction. With this approach, the amount of headspace was kept as small as possible and the VOC fraction was covered by hexane to avoid evaporation and ensure the application of precise VOC concentrations. Immediately after injection of the

VOC fraction into the HPLC vial, the whole volume of the VOC solution, containing hexane (200µl) and the VOC fraction, was injected with a HPLC syringe through the gas tap of the desiccator onto the filter paper.

Table 11: Applied VOC concentrations in the incubation set-up.

Compound	2ml Stock solution (hexane volume +VOC volume)	Volume of stock solution applied in 200yl hexane	Amount of substance In desiccator	VOC concentration in desiccator [5.5l]
Isopropyl palmitate	1903µl + 110µl	0.5µl	41.24nmol	7.50nM
	1903µl + 110µl	1.0µl	82.49nmol	15.00nM
	1903µl + 110µl	5.0µl	412.45nmol	74.99nM
	1903µl + 110µl	7.5µl	618.67nmol	112.48nM
	1903µl + 110µl	10µl	824.89nmol	149.98nM
	1903µl + 110µl	15µl	1237.34nmol	224.97nM
	1903µl + 110µl	20µl	1649.79nmol	299.96nM
Limonene	1848µl + 25µl	5µl	412.10nmol	74.93nM
	1848µl + 25µl	10µl	824.20nmol	149.85nM
	1848µl + 25µl	20µl	1648.84nmol	299.71nM
MeSA	1865µl + 40µl	10µl	1.620µmol	280.55nM
	1620µl + 170µl	10µl	8.10µmol	1402.75nM
	1667µl + 350µl	10µl	16.20µmol	2805.50nM
Pinene	1883µl +25µl	1.0µl	82.55nmol	15.00nM
	1883µl +25µl	5.0µl	412.77nmol	75.05nM
	1883µl +25µl	7.5µl	619.16nmol	112.57nM
	1883µl +25µl	10µl	825.54nmol	150.10nM
	1883µl +25µl	15µl	1238.31nmol	225.15nM
Camphene	1000µl + 10mg	1.24µl	82.4700nmol	14.99nM
	1000µl + 10mg	11.24µl	824.710nmol	149.95nM
	1000µl + 10mg	22.47µl	1649.420nmol	299.89nM
	1000µl + 1.0mg	17.98µl	13195.33nmol	2399.15nM

2.2.6 Assessment of systemic acquired resistance in *Arabidopsis*

SAR experiments were performed with bacteria that were grown over night (28°C) on NYGA plates containing selective antibiotics (**Table 10**). Bacteria were re-suspended in 10mM $MgCl_2$, pH 7.0 and diluted to a concentration of 10^8 colony-forming units (cfu ml^{-1}) by measuring the optical density at a wavelength of 600nm (Ultrospec 3100 pro, GE Healthcare, Munich, Germany). The required concentration was calculated using an OD_{600} of 0.2 equals 10^8 cfu of bacteria. The first two fully developed (local) leaves of four-and-a-half-week-old *Arabidopsis* plants were inoculated with $1x10^6$ cfu ml^{-1} of *Pst AvrRpm1* or with 10mM $MgCl_2$ (Mock) as control. Inoculation was performed from the abaxial side of the leaves by syringe-infiltration (without needle). After primary treatment with Mock, treated leaves looked green and healthy. Leaves treated with *Pst AvrRpm1* displayed yellow-spotted lesions. Three days after the primary treatment, the next two "upper" fully expanded (systemic) leaves were infiltrated with $1x10^5$ cfu ml^{-1} of *Pst* DC3000. Four days after challenge infection, the pathogen growth in the systemic leaves was analysed. Bacteria were extracted from three 6mm leaf discs per sample (in triplicate per genotype and first treatment). The discs were shaken at 600rpm in 500µl of 10mM $MgCl_2$ with 0.01% Silwet for 1h at 25°C. A tenfold serial dilution was performed with the bacterial suspension and 20µl of each dilution were spotted onto NYGA plates containing antibiotics (**Table 10**) and incubated for two days at 28°C. Colonies were counted in spots containing between 10 and 100 colonies. Subsequently, the bacterial titre in the leaf was calculated as cfu cm^{-2}.

2.2.7 DNA extraction

DNA was isolated from frozen and ground plant material by adding 500µl chloroform and 500µl lysis buffer (CTAB+1% β-mercaptoethanol) to 50-100mg of plant material. The mixture was shaken at 1400rpm at 8°C for 15 min. Subsequently, the samples were centrifuged at 14000rpm at 4°C for 10 min. The supernatant (approx. 600µl) was added to a new reaction tube, which was supplemented with 100µl phytopure (GE Healthcare, Buckinghamshire, UK) and 500µl chloroform. Samples were shaken at 1400rpm at 8°C for 15 min and centrifuged at 14000rpm at 4°C for 10 min. The supernatant (approx. 500µl) was added to new reaction tube, which was supplemented with 250µl isopropanol and incubated on ice for 10 min to precipitate the DNA. Subsequently, the samples were centrifuged at 14000rpm at 4°C for 10 min. Supernatant was decanted and 1 ml of 70% ethanol/0.1M sodium acetate was added to the pellet. Additionally, the samples were incubated for 5 min at room temperature. The pellet was washed twice, once with 80% ethanol and once with 100% ethanol, and subsequently dried. In order to resolve the pellet, 30µl of purified water was added. The samples were then shaken at 800rpm at 8°C for 20 min.

2.2.8 RNA extraction and cDNA-synthesis

Frozen plant material was ground under liquid nitrogen and RNA was isolated by the phenol-extraction method (Logemann et al. 1987). Quality and concentration of the (DNA and) RNA samples were determined by measuring the absorption at 260nm and 280nm using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). Absorption of 1.0 at 260nm equals a concentration of 40µg/ml of RNA. The A260/A280 ratio was used to assess the purity of total RNA and to detect the presence of protein, phenolics or other contaminants that absorb at or near 280nm. A ratio of approximately 1.8 to 2.0 is generally accepted for pure RNA. The A260/A230 ratio is a second purity measure, which should commonly be in the range of 2.0-2.2. An appreciably lower ratio may indicate the presence of contaminants absorbing at 230nm. RNA integrity was analysed by using 1% agarose gel electrophoresis.
The cDNA was generated using oligo (dT) (20-mer) and SuperscriptII reverse transcriptase following the manufacturer's instructions (Invitrogen, California, USA).

2.2.9 Polymerase chain reaction and quantitative real-time-PCR analysis

Polymerase chain reaction (PCR) is a method that allows the exponential amplification of defined DNA sequences within a double stranded DNA (dsDNA) molecule in vitro. Amplification of genes-of-interest was performed by a standard PCR protocol consisting of three repeating sections, including denaturing of the dsDNA, annealing of the primers to the single-stranded DNA, and elongation of the new DNA strand. dsDNA was denatured at 94°C. The annealing temperature of the primers depended on length and base pair composition of the primers used for amplification (**Table 1**). Elongation was performed at 72°C using the DNA polymerase Mango Taq™ (Bioline, Luckenwalde, Germany). PCR was executed in a MJ Research PTC-200 Peltier Thermal Cycler.
Quantitative real-time-PCR (qPCR) is a form of PCR, where quantification is accomplished by fluorescence measurements which are recorded during a PCR cycle. A cyanin-dye, in this case SYBR Green I, which is a major component in SensiMix™ SYBR Low-ROX Kit, (No. QT625-05, Bioline GmbB, Luckenwalde, Germany), binds to dsDNA and the resulting DNA-dye-complex emits green light (λmax = 520nm). The fluorescence increases directly proportionally to the amount of PCR products, which enables the target quantification. cDNA was used as a template, in order to quantify the expression of genes-of-interest with the primers in **Table 1**. To conduct the qPCR, the 7500 Real Time PCR System from Applied Biosystems (Darmstadt, Germany) and SensiMixTM SYBR Low-Rox Kit were used according to the manufacturer's instructions.

2.2.10 Agarose gel electrophoresis

Samples were supplemented with 6x loading dye (**Table 7**) to a final dye concentration of 1x. For the detection of the DNA, 0.05µg/ml ethidium bromide was added to the gel, which intercalates between double stranded DNA and fluoresces when irradiated with ultraviolet light of wavelengths between 254nm and 366nm. After gels were run at a voltage between 70 to 100V, depending on the size of the gel and the size of the DNA fragment, nucleic acids were visualized with UV light (302nm). Subsequently, the gels were photographed and documented using the BIO-Print M1 gel documentation system from Vilber Lourmat (Eberhardzell, Germany).

2.2.11 Identification of homozygous knock out plant lines

The t-DNA knock out (KO) lines of *A. thaliana* (SALK_208952C, SALK_210207C), obtained from the Nottingham Arabidopsis Stock Centre were tested to identify homozygous KO mutants. To screen the plants for chromosomal t-DNA insertions, an established PCR-based method was applied (**Figure 10**) (http://signal.salk.edu/tdnaprimers.2.html).

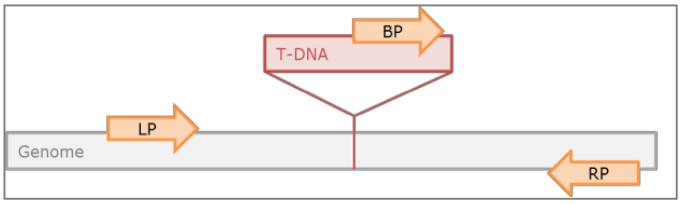

Figure 10: Identification of homozygous knockout mutants. Two PCR reactions were performed. The Left genomic (LP) and right genomic (RP) primers were used to identify the wild-type genomic fragment, whereas the (t-DNA border primer) BP and RP primer identified the t-DNA insert. With LP and RP the PCR product size of wt plants with no insertion is about 900-1100bp. PCR product size of homozygous lines, where the insertion is on both chromosomes, is 410+N bp - from BP (t-DNA border primer) to RP. Whereas heterozygous lines have the insertion of one of the pair chromosomes and the resulting PCR product will show both sizes (from LP to RP and BP to RP). N: Difference of the actual insertion site and the flanking sequence position, usually 0-300 bases. Illustration adapted from (http://signal.salk.edu/tdnaprimers.2.html).

Primers used for screening the *ggpps12-1* mutant were GGPPS12_208952F (LP), GGPPS12_208952R (RP) and LBb1(pBIN-pROK2) (BP), whereas GGPPS12_210207F (LP), GGPPS12_210207R (RP) and LBb1(pBIN-pROK2) (BP) were used to screen *ggpps12-2* (**Table 1**). Two PCR reactions were performed on DNA that was isolated from at least 10 plants of every line. As illustrated in **Figure 10**, the LP and RP primers were used to identify the wild-type genomic fragment, whereas the BP and RP primers identified the t-DNA insert. The PCR products were separated via electrophoresis on a 1% agarose gel and the occurring bands were evaluated. If there was only a PCR product with primer pair LP and RP, the plant was considered as wild-type and if both primer pairs yielded PCR products, the plant was identified as heterozygous. Plants that only showed a PCR product with the primer pair BP and RP were homozygous KO mutant plants and used for further analysis.

3. RESULTS

3.1 Induction of systemic resistance by *AvrRpm1* expression in dexamethasone-treated plants

For the analysis of plant-derived VOC emissions it is advantageous to have a system that can clearly distinguish between plant-derived VOCs and VOCs emerging from other sources. Transgenic plants carrying the dexamethasone (DEX)-inducible transgene *pDEX:AvrRpm1-HA* that encodes C-terminally hemagglutinin (HA)-tagged AvrRpm1 provide a pathogen free system (Mackey et al. 2002) that allows synchronized induction of the plants defence responses without having bacterial derived VOC components in the emissions.

Spray-application of 30µM DEX induces a systemic defence response in the plant that is EDS1-dependent. Col-0 *pDEX:AvrRpm1-HA* and *eds1-2 pDEX:AvrRpm1-HA* plants develop HR and ETI but only Col-0 *pDEX:AvrRpm1-HA* plants show SAR (Breitenbach et al. 2014). Due to the SAR-deficient phenotype of *eds1-2 pDEX:AvrRpm1-HA* plants in response to DEX-induced *AvrRpm1* expression, the DEX-system is a useful tool to study plant derived VOC emissions in relation to SAR (Breitenbach et al. 2014, Vlot et al. 2009).

3.2 Collection of VOCs possibly related to systemic acquired resistance

Col-0 *pDEX:AvrRpm1-HA* (hereafter referred to as Col-0 dex) and *eds1-2 pDEX:AvrRpm1-HA* (hereafter referred to as *eds1-2* dex) plants (Mackey et al. 2002, Breitenbach et al. 2014) were used to identify VOCs potentially related to SAR. In collaboration with Prof. Dr. Schnitzler and Dr. Ghirardo (EUS/HMGU) volatile emissions of *AvrRpm1*-expressing wt (Col-0 dex) and *eds1-2* mutant plants (*eds1-2* dex) were collected with a dynamic system and analysed by gas chromatography coupled to mass spectrometry (GC-MS) (**Figure 11**, **Figure 12**).

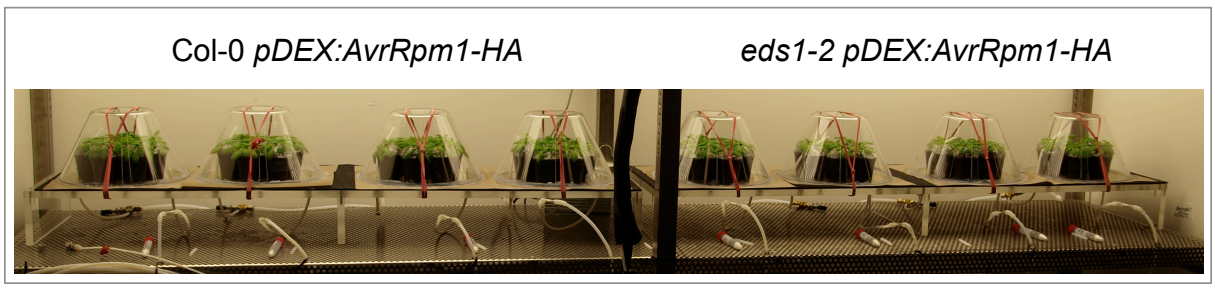

Figure 11: Experimental set-up. Collection of volatile emissions of *AvrRpm1*-expressing Col-0 dex and *eds1-2* dex mutant plants. Four cuvettes with DEX-induced Col-0 dex plants were run in parallel with four cuvettes containing DEX-induced *eds1-2* dex plants.

Four-and-a-half-week-old *Arabidopsis* plants (Col-0 dex, *eds1-2* dex) were sprayed with 30µM of DEX (0.01% Tween-20). Half an hour post DEX-treatment the plants were transferred into gas-tight glass cuvettes to collect the volatile emissions. Each cuvette was

set with twelve plants and the plants were let acclimatizing to the new environmental conditions for half an hour before starting the collection of the VOCs.
Background measurements of "pots without plant material" were performed twice, at the beginning and at the end of the experiment. The procedure followed exactly this used for sampling VOCs from plants, except that plants were removed out of the soil before enclosing the pots into the cuvettes.

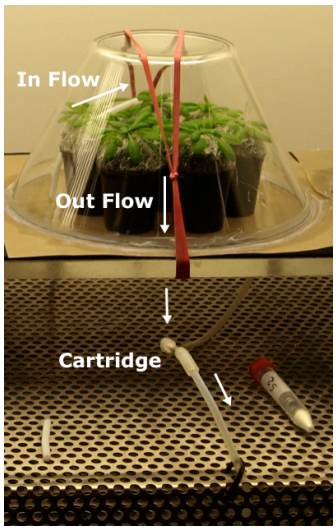

Figure 12: Dynamic system of VOC collection. Cuvettes were flushed with 0.2 L min^{-1} of VOC-free synthetic air (79% N_2, 21% O_2, CO_2 400µmol mol^{-1}). 18 litres of volatile extracts were sampled in glass cartridges with a flow rate of 0.1 L min^{-1}.

The collection of VOC emissions was run in eight cuvettes in parallel and each cuvette was continuously flushed with 0.2 L min^{-1} of VOC-free synthetic air (79% N_2, 21% O_2) mixed with pure CO_2 to a final CO_2 concentration of 400µmol mol^{-1}. The light was provided for 10 hours (8am-6pm) and the intensity at leaf surface was 120-150µmol m^{-2} sec^{-1}. A part of the air exiting the cuvettes was diverted for the VOC sampling. A total of 18 litres of air were collected with a flow rate of 0.1 L min^{-1} for 180 min into glass cartridges filled with polydimethylsiloxane-foam and 50mg carbopack B. Volatile emissions were collected in two different sampling periods. The first sampling period was 1-4 hours post DEX-treatment (SP1) the second sampling period was 4-7 hours post DEX-treatment (SP2).
Collection of VOCs from plants was done seven times with independent plant material and four cuvettes in parallel per genotype. After elimination of samples due to technical problems (breakage of cartridge, GC-MS break down) or data outliers, a total number of at least 18 biological replicates for each genotype was achieved (SP1: Col-0 dex 21 replicates, *eds1-2* dex 19 replicates; SP2: Col-0 dex 18 replicates, *eds1-2* dex 21 replicates). Cartridges containing the collected VOC extracts were kept at 4°C until analysis by GC-MS.

3.3 Volatile organic compounds possibly related to systemic acquired resistance

The collected VOC emissions from DEX-induced *AvrRpm1*-expressing wt and *eds1-2* dex mutant plants were analysed by GC-MS (see 2.2.2 Measurement of volatile organic compounds). GC-MS data analysis was performed as a non-targeted approach. Therefore, all peaks in the GC-MS chromatograms of Col-0 dex and *eds1-2* dex samples, which displayed peak areas above background levels, were taken into account.
With background correction VOCs deriving from other sources like soil, pot, or cartridge material, were eliminated. Therefore, the average peak area of each peak of background samples was determined. Then all peak areas of Col-0 dex and *eds1-2* dex samples were

substracted by the determined average peak area of the corresponding peaks from background samples to obtain background-corrected peak areas.

Of the 220 detected peaks present in GC-MS chromatograms of Col-0 dex and *eds1-2* dex samples 39 peaks were remaining after background correction. These 39 peaks can be attributed to volatile emissions of plants, rather than emission from other sources. The remaining 181 peaks were either derived from soil and therein microbes or algae, pot or cartridge material or any other sources present in samples with plants and as well as in background samples.

After background correction, all peak areas were converted into emission rates of VOCs (pmol m^{-2} s^{-1}). Therefore, the total leaf areas of the twelve plants of each cuvette were determined. Leaf areas were measured on basis of pictures taken two hours before the VOC measurements with the image-editing program GIMP. The emission rates of VOCs were calculated based on background-corrected peak areas and measured leaf areas.

Finally, the 39 VOCs detected in Col-0 dex and *eds1-2* dex extracts were chemically identified. Chemical identification of VOCs was achieved by comparing the mass spectra obtained from samples and commercially available authentic standards or by comparing sample spectra and retention indices with those available from the NIST library (National Institute of Standards and Technology Mass Spectral Library).

In extracts of volatile emissions of Col-0 dex and *eds1-2* dex plants 39 different VOCs could be detected and chemically identified (**Table 12**).

The background-corrected emission rates of VOCs emitted by Col-0 dex and *eds1-2* dex plants were analysed by a multivariate data analysis (MDA) approach using principal component analysis (PCA) and partial least square regression (PLSR) statistical methods. Both analyses were performed using the software package SIMCA-P version 13.0.0.0 (Umetrics, Umea, Sweden).

The overall analysis aimed to identify which VOC and at which degree the VOC was positively or negatively correlated with genotype (Col-0 dex/*eds1-2* dex) or sampling period (SP1/SP2). A VOC was classified discriminant when it had both importance in the projection (VIP>1) and the uncertainty bar computed by jack-knife method (Efron and Gong, 1983) was smaller than its respective VIP value (SE<VIP). Additionally, the discriminant VOCs resulted from MDA were repetitively verificated by student`s t-test (p<0.05, p<0.01) (**Table 12**).

Table 12: VOCs possibly related to SAR. The identity of compounds indicated with * was confirmed by comparison with pure standards. VOCs that were significantly correlated with plant genotype were identified by multivariate data analysis (VIP>1, SE<VIP, indicated in bold). Subsequently, significantly different emission rates from Col0 compared to *eds1-2* plants were determined by Student`s *t*-test. If P is not given, the value was >0.05. (RT: Retention time, RI: Retention index (Kovat's), VIP: Score of Variable of Importance for the Projection, SE: Standard error of the jack-knifing method, SP: Sampling period).

Number	Compound	RT (min)	RI (min)	VIP (SP1)	SE (SP1)	P (*t*-test) (SP1)	VIP (SP2)	SE (SP2)	P (*t*-test) (SP2)	Chemical class
01	ß-Pinene*	17.06	982	**2.97**	0.42	**<0.01**	**2.54**	1.49	**<0.01**	Terpene
02	α-Pinene*	15.09	936	**2.42**	0.68	**<0.01**	**1.63**	1.09	**<0.01**	Terpene
03	Camphene*	15.858	954	**2.23**	1.04	**<0.01**	**1.35**	1.28	**<0.01**	Terpene
04	Isopropyl palmitate*	47.32	2040	**2.16**	0.78	**<0.01**	**1.25**	0.54		Fatty ester
05	Decanal	27.25	1207	**1.43**	0.64		0.89	1.56		Fatty aldehyde
06	Sabinene*	16.74	975	**1.34**	0.95	**<0.01**	**1.89**	1.55	**<0.01**	Terpene
07	Alkane_01	46.14	1921	**1.19**	0.61		0.07	0.76		Alkane
08	Alkane_02	40.91	1580	1.00	1.43		0.20	1.19		Alkane
09	Alkane_17	37.54	1464	0.99	1.20		0.80	1.26		Alkane
10	Unknown sesquiterpene	35.6	1411	0.91	1.37		0.05	0.36		Terpene
11	Octanal	18.06	1006	0.86	0.64		**1.24**	1.20		Fatty aldehyde
12	Benzene, ethenyl	13.92	908	0.82	1.49		1.68	2.12		Aromatic
13	1-octanol,2-butyl-	31.13	1299	0.79	0.92		0.16	0.75		Alcohol
14	Unknown sesquiterpene	36.01	1422	0.74	1.32		0.59	0.69		Terpene
15	Alkane_04	39.85	1539	0.71	0.58		0.67	1.16		Alkane
16	Alkane_05	46.13	1920	0.68	1.02		0.16	0.73		Alkane
17	Alkane_10	20.32	1054	0.62	1.24		**1.39**	1.11		Alkane
18	Unknown sesquiterpene	35.87	1418	0.58	1.35		0.12	0.89		Terpene
19	Unknown	33.37	1355	0.54	1.04		0.28	0.60		
20	Alkane_06	39.75	1535	0.53	0.67		0.67	1.17		Alkane
21	Alkane_07	44.01	1752	0.47	0.93		0.37	1.15		Alkane

22	Alkane_08	43.32	1704	0.46	0.66		0.58	1.15		Alkane
23	Cis-pinen-3-ol	15.95	956	0.43	0.40		0.12	0.78		Terpenoid derivative
24	Alkane_09	40.68	1571	0.43	0.89		0.48	1.44		Alkane
25	Alkane_16	20.52	1059	0.41	0.79		1.33	1.61		Alkane
26	Alkane_18	35.17	1399	0.40	1.01		0.70	1.71		Alkane
27	Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	34.25	1376	0.38	0.86		0.52	1.39		Carboxylic acid ester derivative
28	Nonanal	22.69	1106	0.36	1.27		0.52	1.17		Fatty aldehyde
29	Alkane_11	41.49	1604	0.35	0.91		**1.39**	1.37		Alkane
30	Benzene, 1,3-bis(1,1-dimethyl)-	29.03	1250	0.28	0.48		0.71	1.27		Aromatic
31	Alkane_12	41.44	1601	0.26	0.91		0.26	1.25		Alkane
32	Decane (3,6-dimethyl-)	22.58	1103	0.19	0.68		1.53	1.58		Alkane
33	Phenol, 2,4-bis(1,1-dimethylethyl)-	39.41	1521	0.18	0.74		0.98	1.44		Aromatic
34	Alkane_13	43.33	1705	0.15	0.53		0.22	1.07		Alkane
35	Alkane_03	20.27	1053	0.13	0.59		0.72	1.92		Alkane
36	Decane (4-methyl-)	18.27	1010	0.08	0.74		**1.77**	0.92		Alkane
37	Alkane_14	43.32	1704	0.06	0.46		0.01	0.59		Alkane
38	Oxime-, methoxy-phenyl	13.7	903	0.04	0.91		0.18	0.88		Aromatic
39	Alkane_15	39.85	1539	0.01	0.46		0.83	1.40		Alkane

MDA revealed a significant separation of Col-0 dex and *eds1-2* dex samples for both sampling periods (SP1, SP2) (**Figure 13 A**). MDA of samples collected during SP1 revealed ß-pinene, α-pinene, camphene, isopropyl palmitate, decanal, sabinene, and alkane_01 as discriminant (SE<VIP>1) for the separation of Col-0 dex and *eds-1-2* dex samples from SP1 (**Figure 13 A, Table 12**). MDA of samples collected during SP2 revealed the VOCs ß-pinene, sabinene, decane 4-methyl), α-pinene, alkane_10, alkane_11, camphene, isopropyl palmitate, and octanal to be discriminant (SE<VIP>1) for the separation of Col-0 dex and *eds-1-2* dex samples from SP2 (**Figure 13 A, Table 12**).

Altogether, from SP1 and SP2 eleven VOCs (alkane_01, alkane_10, alkane_11, camphene, decanal, decane (4-methyl), isopropyl palmitate, octanal, α-pinene, ß-pinene, sabinene) were identified by MDA as being discriminatory for the separation of Col-0 dex and *eds-1-2* dex samples (**Figure 13 A, Table 12**). For these eleven VOCs revealed by MDA an additional t-test was performed. The five VOCs, which were indicated as being discriminant in both MDAs (SP1, SP2) (camphene, isopropyl palmitate, α-pinene, ß-pinene, sabinene) (**Figure 13 B, red dots**) also showed significant p-values in the t-test. In contrast, VOCs revealed as discriminant just from one MDA (alkane_01, decanal) did not show significant p-values in the t-test (**Table 1**). The discriminant VOCs camphene, isopropyl palmitate, α-pinene, ß-pinene, and sabinene were significantly negatively correlated with *eds1-2* dex (**Figure 13 C, red bars**), indicating that they were emitted in higher levels from Col-0 dex plants.

Altogether, MDA and repetitive verification by t-test revealed five VOCs to be significantly differentially accumulated between Col-0 dex and *eds1-2* dex extracts. Therefore, these five VOCs camphene, isopropyl palmitate, α-pinene, ß-pinene, and sabinene are possibly related to SAR (**Figure 13, Table 12**).

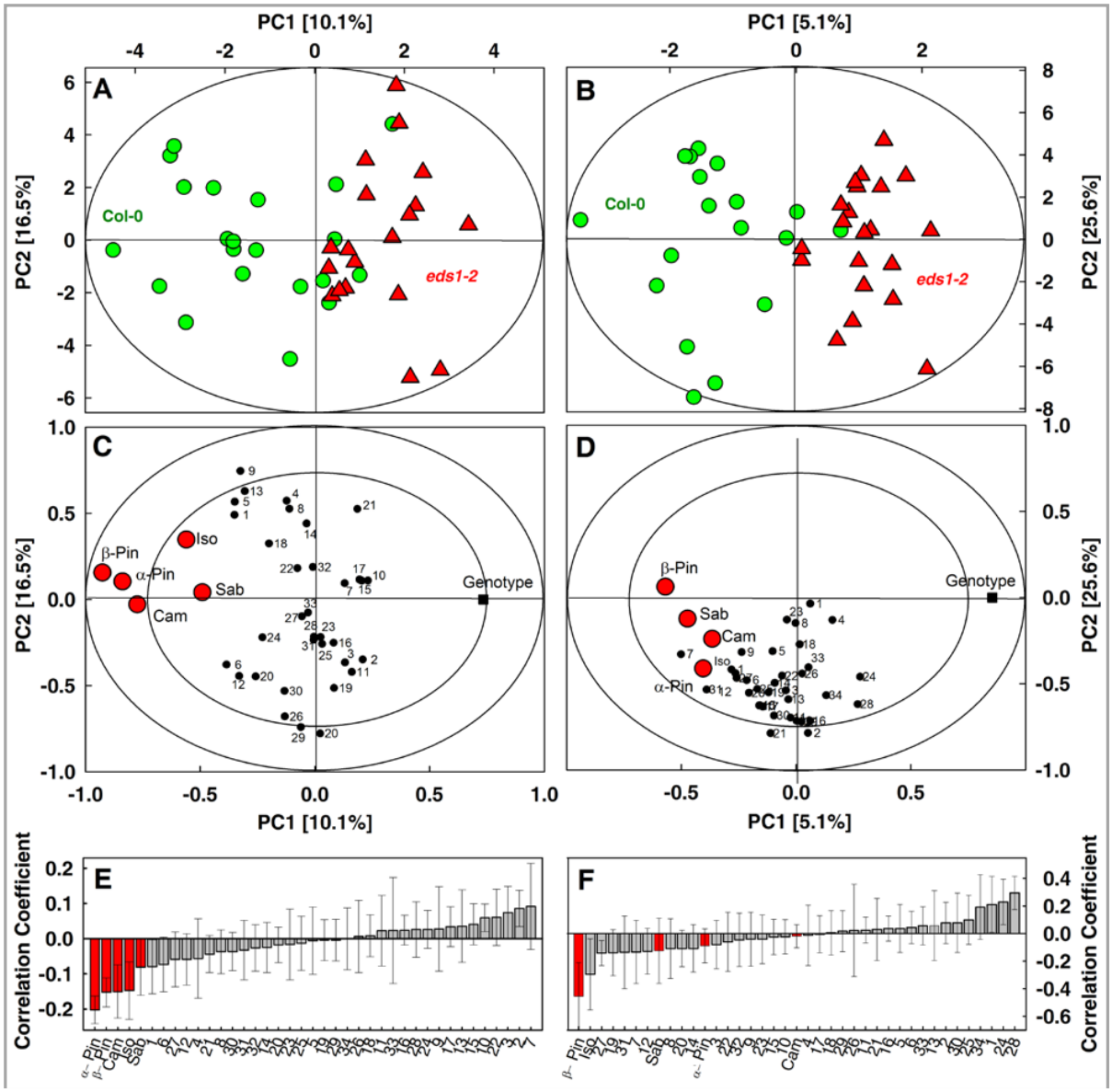

Figure 13: Principal component analysis of VOC emissions of wt and *eds1-2* mutant plants. A) Score plot: Significant separation of Col-0 dex and *eds1-2* dex samples from SP1 (left) and SP2 (right). **B) Loading plot:** VOCs emitted from Col-0 dex and *eds1-2* dex plants (black dots), discriminant VOCs for the separation of Col-0 dex and *eds1-2* dex samples (red dots) in the score plot (SP1 left, SP2 right). **C) Coefficient Plot:** Correlation of the VOCs with genotypes. VOCs significantly negatively correlated with *eds1-2* dex (red bars) (SP1 left, SP2 right). (α-Pin: α-Pinene, ß-Pin: ß-Pinene, Cam: Camphene, Iso: Isopropyl palmitate, Sab: Sabinene. Numbers in B-F refer to tentatively identified VOCs (see Table 12).

Isopropyl palmitate, and the four monoterpenoid VOCs camphene, α-pinene, ß-pinene, and sabinene were emitted in significantly higher amounts from Col-0 dex plants compared to *eds1-2* dex mutant plants and are thus possibly related to SAR (**Figure 14**). The emission rates of camphene, α-pinene, ß-pinene, and sabinene were lower in the second sampling period (SP2) compared to SP1. These four compounds were differentially emitted between Col-0 dex and *eds1-2* dex plants during both sampling periods (SP1, SP2). In contrast, the emission level of isopropyl palmitate in Col-0 dex was constant during both sampling periods, but the emission was only significantly different between Col-0 and *eds1-2* dex in

SP1 (**Figure 14**). In summary, the five VOCs isopropyl palmitate, camphene, α-pinene, ß-pinene, and sabinene were identified as possibly SAR-related and were used as candidate VOCs in further experiments.

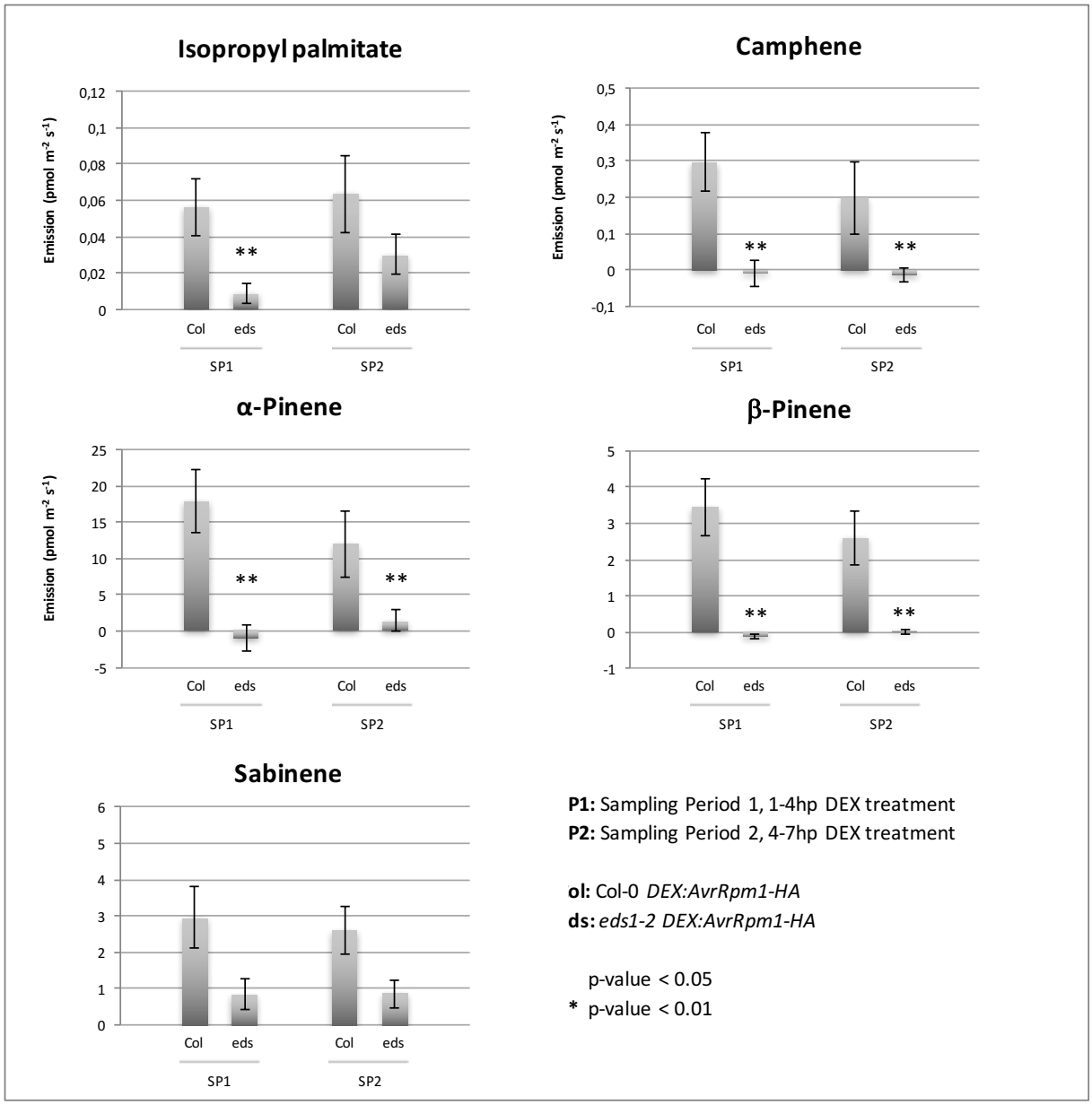

Figure 14: Volatile emission of VOCs potentially related to SAR. Isopropyl palmitate, camphene, α-pinene, ß-pinene, and sabinene were emitted in significantly higher amounts from Col-0 dex compared to *eds1-2* dex plants. Plotted values are the average ±SE of at least 18 replicates each. Asterisks indicate statistically significant differences between Col-0 dex and *eds1-2* dex (* p<0.05, ** p<0.01, student´s t-test).

3.4 Biological relevance of the newly identified volatile organic compounds

Here, camphene, isopropyl palmitate, α-pinene, and ß-pinene were investigated further to ascertain a possible biological relevance of these EDS1-dependent VOCs in plant defence to pathogens. To this end, the potential of the VOCs to induce plant defence was

investigated in an incubation set-up (2.2.4 Volatile organic compound incubation set-up) in comparison with the well-known resistance-inducing compound MeSA.

In the following experiments, *Arabidopsis* wt plants were incubated over three days with different concentrations of either camphene, isopropyl palmitate, or pinene (mixture of α-pinene:ß-pinene 1:1). Eight four-and-a-half-week-old wt *Arabidopsis* plants were placed in gas-tight glass desiccators that were subsequently filled with fresh air from the inflow of the growth chamber. The different VOCs, the positive control MeSA, or the negative control hexane (200µl) was applied with an HPLC-syringe through the gas tap onto a filter paper in the desiccators. The plants were incubated over three days in the dessiccators, whereby the supplemented air in the desiccators was replaced every day to supply fresh air. After changing the air, the treatment with either the VOC, MeSA or hexane was applied again, in total three times at three consecutive days. After pre-incubation, the systemic leaves were syringe-infiltrated with *Pst* DC3000 ($1x10^5$ cfu ml^{-1}) and four days after challenge infection the pathogen growth in the systemic leaves was analysed.

Because the VOCs were almost water insoluble hexane was used as diluting agent and negative control. Plants pre-incubated with hexane (200µl)-supplemented air showed enhanced levels of bacterial growth compared to plants pre-incubated with un-supplemented air (**Suppl. Figure 30**). To normalize the effect of hexane on resistance, all of the applied VOC and MeSA solutions were added by 200µl of hexane to avoid any side effect of the diluting agent.

The well-studied VOC MeSA, known to induce and mediate pathogen resistance in tobacco and *Arabidopsis* plants was used as positive control (Shulaev et al. 1997, Koo et al. 2007, Park et al. 2007, Vlot et al. 2008, 2009) and to establish the incubation set-up. Different concentrations of MeSA (2805.45nM, 1402.73nM, 280.55nM) and two different incubation periods (3 days, 6 days) were tested. The VOC concentrations stated in the figures indicate the VOC concentrations present in the air in desiccators (and not the concentration of the applied VOC solution, for applied amounts of VOC substances see **Table 11**). Wt plants pre-incubated over three days with 280.55nM of MeSA showed the lowest levels of *Pst* growth compared to hexane pre-incubated wt plants (**Suppl. Figure 30**). Due to this, 280.55nM of MeSA were used as positive reference VOC and an incubation time of three days was used for all pre-incubation experiments. Wt plants pre-incubated over three days with 280.55nM of MeSA showed significantly reduced levels of bacterial growth compared to wt plants that were pre-incubated with the negative control hexane. MeSA applied in a concentration of 280.55nM was able to induced resistance in wt *Arabidopsi*s plants against *Pst* DC3000 (**Figure 15**).

First, the VOC isopropyl palmitate was tested in the incubation set-up in concentrations from 74.99nM to 229.96nM. Wt plants pre-incubated with isopropyl palmitate did not show reduced bacterial growth compared to plants that had been pre-incubated with hexane. Isopropyl palmitate was not able to induce resistance against *Pst* DC3000 at any of the

concentrations tested. Hence, I could not identify a potential biological relevance for isopropyl palmitate in defence against *Pst* DC3000 in this range of experiments (**Figure 15**).

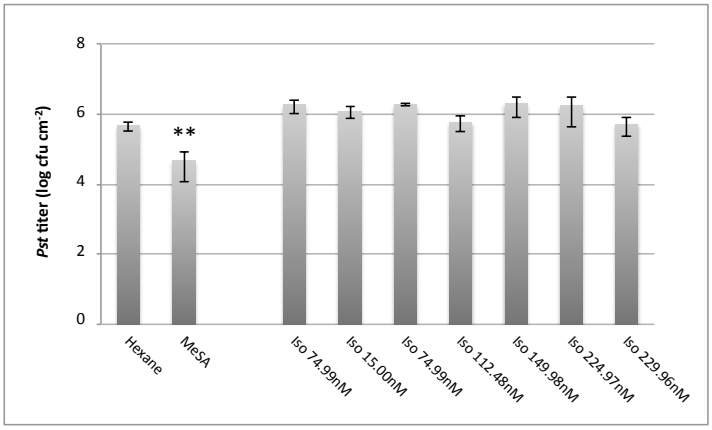

Figure 15: Isopropyl palmitate did not induce resistance. *Arabidopsis* wt plants were pre-incubated over three days with different concentrations of isopropyl palmitate and challenged with *Pst* DC3000. Resulting *Pst* titres are shown at 4 dpi. Positive control methyl salicylate (MeSA, 280.55nM), negative control Hexane (200µl), Iso (Isopropyl palmitate). Plotted values are the average ±SD of three replicates each. Asterisks indicate statistically significant differences from hexane control (* $p<0.05$, ** $p<0.01$, student´s t-test). This experiment was repeated two times with similar results [1].

Arabidopsis plants emitted both structural isomers of pinene, α-pinene and ß-pinene (**Figure 14**) and the production of both isomers in *Arabidopsis* was also reported (Tholl and Lee 2011, Chen et al. 2003, 2004). Since different isomers can display distinct functions, desired physiologic effects (Riva da Silvas et al. 2012, Finefield et al. 2012) and a synergistic function of both isomers could also be possible, first of all α-pinene and ß-pinene were tested in a mixture of both isomers (ratio 1:1).

The mixture of α-pinene and ß-pinene was tested in a concentration range of 15.00nM-225.15nM. Wt plants pre-incubated with pinene in a concentration of 112.57nM showed significantly reduced bacterial growth compared to hexane pre-incubated ones (**Figure 16**). Incubation with pinene reduced the bacterial growth to the same level as the positive control MeSA did. All the other tested concentrations of pinene (15.00nM, 75.05nM, 150.10M, 225.15nM) were not able to reduce bacterial growth and plants pre-incubated with these concentrations showed bacterial levels comparable to hexane pre-incubated plants. The mixture of both pinene isomers when applied in a concentration of 112.57nM was able to induce resistance against *Pst* DC3000 (**Figure 16**). Hence, I could identify a possible biological relevance for pinenes in defence against *Pst* DC3000.

[1] This experiment was repeated two times with similar results. The two repetitions could not be merged in one figure due to large variation in absolute values between experiments. This equally applies to all experiments, which were repeated several times with similar results.

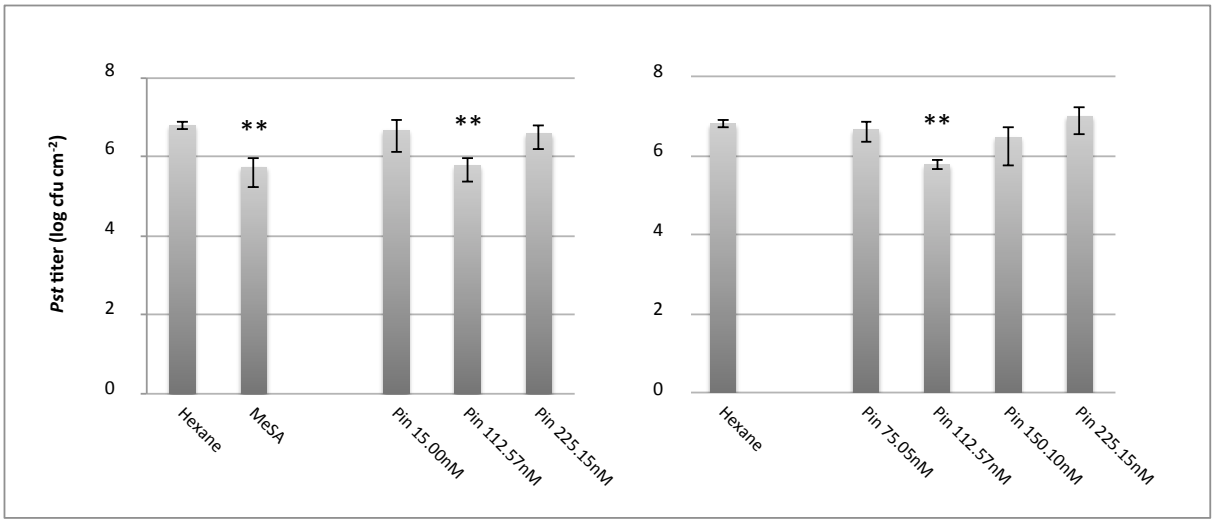

Figure 16: Resistance induction by pinene application. *Arabidopsis* wt plants were pre-incubated over three days with different concentrations of pinenes and challenged with *Pst* DC3000. Resulting *Pst* titres are shown at 4 dpi. Positive control methyl salicylate (MeSA, 280.55nM), negative control Hexane (200µl), Pin (α:ß-Pinene 1:1). Plotted values are the average ±SD of three replicates each. Asterisks indicate statistically significant differences from hexane control (* $p<0.05$, ** $p<0.01$, student´s t-test). This experiment was repeated two times with similar results.

The resistance inducing potential of the VOC camphene was investigated in a concentration range from 14.99nM to 599.79nM in the incubation set-up. Pre-incubation with camphene in a concentration of 14.99nM reduced bacterial growth of *Pst* DC3000 to the same level as 112.57nM of pinene did. Thus, camphene showed the same potential to induce resistance against *Pst* DC3000 like pinene but when applied in a lower concentration. In contrast, higher concentrations of camphene (149.95nM, 299.89nM, 599.79nM) were not able to induce resistance against *Pst* DC3000 (**Figure 17**). Thus, I could also identify a potential biological relevance for camphene in defence against *Pst* DC3000.

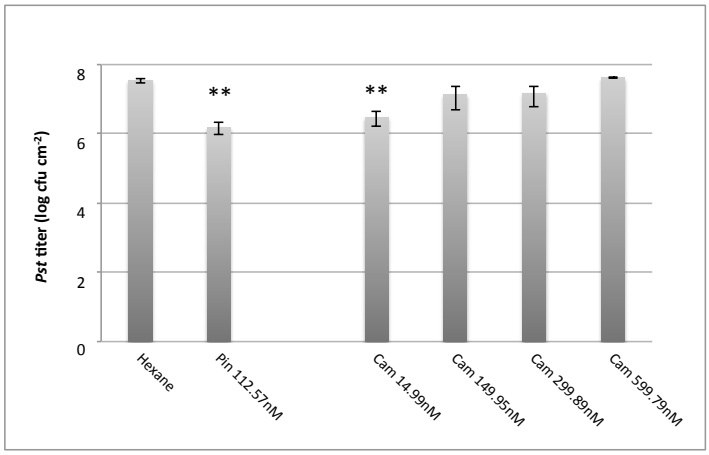

Figure 17: Resistance induction by camphene application. *Arabidopsis* wt plants were pre-incubated over three days with different concentrations of camphene and challenged with *Pst* DC3000. Resulting *Pst* titres are shown at 4 dpi. Negative control Hexane (200 µl), Cam (Camphene), Pin (α:ß-pinene 1:1). Plotted values are the average ±SD of three replicates each. Asterisks indicate statistically significant differences from hexane control (* $p<0.05$, ** $p<0.01$, student´s t-test). This experiment was repeated two times with similar results.

The four VOCs camphene, isopropyl palmitate, α-pinene, and ß-pinene were investigated for a potential biological relevance in resistance induction against *Pst* DC3000 in an incubation set-up, in which the VOCs were applied in their volatile state. Isopropyl

palmitate in concentrations between 74.99nM to 229.96nM could not be shown to have a resistance inducing potential against *Pst* DC3000. In contrast, for camphene and pinene a possible biological relevance could be detected. Camphene in a concentration of 14.99nM and pinene as a mixture of α-pinene and ß-pinene (ratio 1:1) in a concentration of 112.57nM were able to induce resistance in *Arabidopsis* wt plants against *Pst* DC3000. The effect of induced resistance on the phenotypical level is a first evidence for a potential biological relevance of the monoterpenoid VOCs pinene and camphene in plant defence and gives a first hint for a possible link between monoterpenoid biosynthesis and plant defence.

3.5 Constitutional and enantiomeric isomers of pinene

The pinene mixture, which was able to induce resistance in *Arabidopsis* wt plants against *Pst* DC3000 was a mixture of the two constitutional isomers α- and ß-pinene at a ratio of 1:1. Constitutional isomers as well as different enantiomers of an isomer can display differences in activity or even display different functions in biological systems (Riva da Silvas et al. 2012, Finefield et al. 2012). *Arabidopsis* produced both isomers α- and ß-pinene (**Figure 14**) (Tholl and Lee 2011, Chen et al. 2003, 2004).

In plants both enantiomers of α-pinene, (+)-α-pinene and (-)-α-pinene) exist and can co-occur with either enantiomer predominating. Contrary to this, β-pinene is almost always isolated as the optically pure (-)-isoform (Riva da Silvas et al. 2012, Finefield et al. 2012). Due to this the applied mixture of α- and ß-pinene (ratio of 1:1) was composed of both enantiomers of α-pinene ((±)-α-pinene) and just the (-)-isoform of β-pinene ((-)-β-pinene).

In the following, it was investigated if both isomers of pinene are necessary for the induction of the resistance phenotype or if one isomer alone may exhibit the biological activity. Therefore, *Arabidopsis* wt plants were incubated with just one of the two components/isomers of the active mixture, with either (±)-α-pinene or (-)-β-pinene (**Figure 18**).

During the three days of incubation, air in the desiccators was replaced every day followed by a new application of the VOCs. After the three days of pre-incubation with three times VOC application the systemic leaves of the plants were infected with *Pst* DC3000 ($1x10^5$ cfu ml^{-1}) and four days after challenge infection the pathogen growth in the systemic leaves was analysed (**Figure 18**).

Wt *Arabidopsis* plants that were pre-incubated with just one of the two components of the mixture ((±)-α-pinene or (-)-β-pinene) did not show reduced bacterial growth compared to hexane incubated plants. Thus, neither pre-incubation with (±)-α-pinene (112.57nM) nor pre-incubation with (-)-β-pinene (112.57nM) were able to induce resistance in *Arabidopsis* wt plants against *Pst* DC3000. Both components of the active mixture ((±)-α-pinene and (-)-β-pinene)) were necessary for the observed resistance phenotype

(**Figure 18**). These data indicate that both isomers of pinene are required to induce resistance against *Pst* DC3000 and that one isomer alone cannot induce the phenotype.
In regards to the enantiomeric function of pinene, (-)-β-pinene alone was not able to induce resistance and the fact that already the mixture of (±)-α-pinene did not induce resistance, indicates that (+)-α-pinene or (-)-α-pinene alone are also not able to induce resistance against *Pst* DC3000 (**Figure 18**).
Even if β-pinene is mostly isolated as (-)-β-pinene, the production of the (+)-isoform of β-pinene is also possible (Riva da Silvas et al. 2012, Finefield et al. 2012). Thus, also the isomer mixture with (+)-β-pinene instead of (-)-β-pinene was tested to see if there is a difference between the two enantiomers. The isomer mixture containing (+)-β-pinene instead of (-)-β-pinene was also able to induce resistance against *Pst* DC3000 and the resistance effect was even slightly stronger (**Figure 18**). This indicates that there was no difference in the function of the two β-pinene enantiomers ((+)-β-pinene, (-)-β-pinene) in terms of resistance induction against *Pst* DC3000.

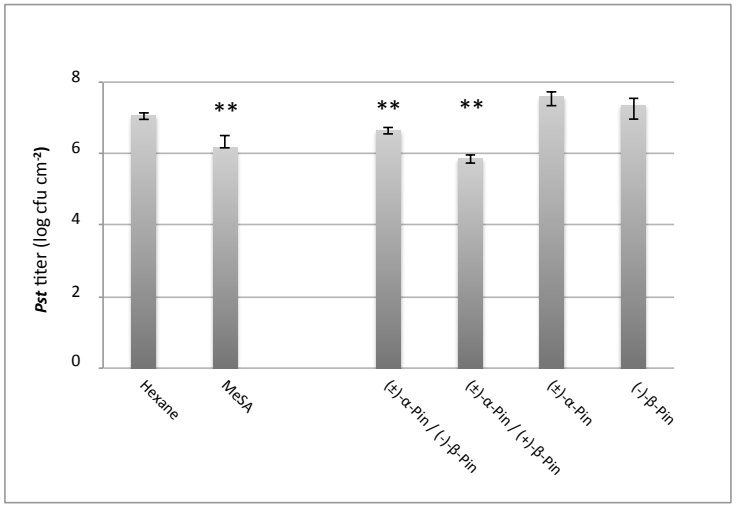

Figure 18: Resistance induction by isomers of pinene. *Arabidopsis* wt plants were pre-incubated over three days with a mixture of (±)-α-pinene and (-)-β-pinene, (±)-α-pinene and (+)-β-pinene, (±)-α-pinene, or (-)-β-pinene. Mixtures of isomers contained equal ratios of both isomers. All VOCs were applied in a concentration of 112.57nM. After pre-incubation plants were challenged with *Pst* DC3000. Resulting *Pst* titres are shown at 4 dpi. Positive control methyl salicylate (MeSA, 280.55nM), negative control Hexane (200 µl), Pin (Pinene). Plotted values are the average ±SD of three replicates each. Asterisks indicate statistically significant differences from hexane control (* $p<0.05$, ** $p<0.01$, student´s t-test). This experiment was repeated two times with similar results.

Altogether, wt plants pre-incubated with just one of the two pinene isomers ((±)-α-pinene, (-)-β-pinene) did not show reduced levels of bacterial growth. In contrast, plants pre-incubated with a mixture containing (±)-α-pinene and either the (+) or the (-) enantiomer of β-pinene both displayed reduced bacterial growth. These data show that both isomers of pinene are required to induce resistance against *Pst* DC3000. One isomer alone cannot induce resistance against *Pst* DC3000. The presence of either the (+) or the (-) enantiomeric form of β-pinene made no difference in terms of resistance induction, suggesting that the two β-pinene enantiomers do not display different or diverse functions for induction of resistance against *Pst* DC3000.
Since (+)-β-pinene is naturally rare, the pinene mixture containing both enantiomers of α-pinene and just the (-)-β-pinene ((±)-α-pinene:(-)-β-pinene, ratio 1:1), which was able to

induced resistance against *Pst* DC3000 in a concentration of 112.57nM was used as test solution for the following experiments.

3.6 Chemical composition of volatile organic compound standards

The quality and purity of the VOC standard solutions that were used for the experiments was analysed by GC-MS (**Figure 19**).

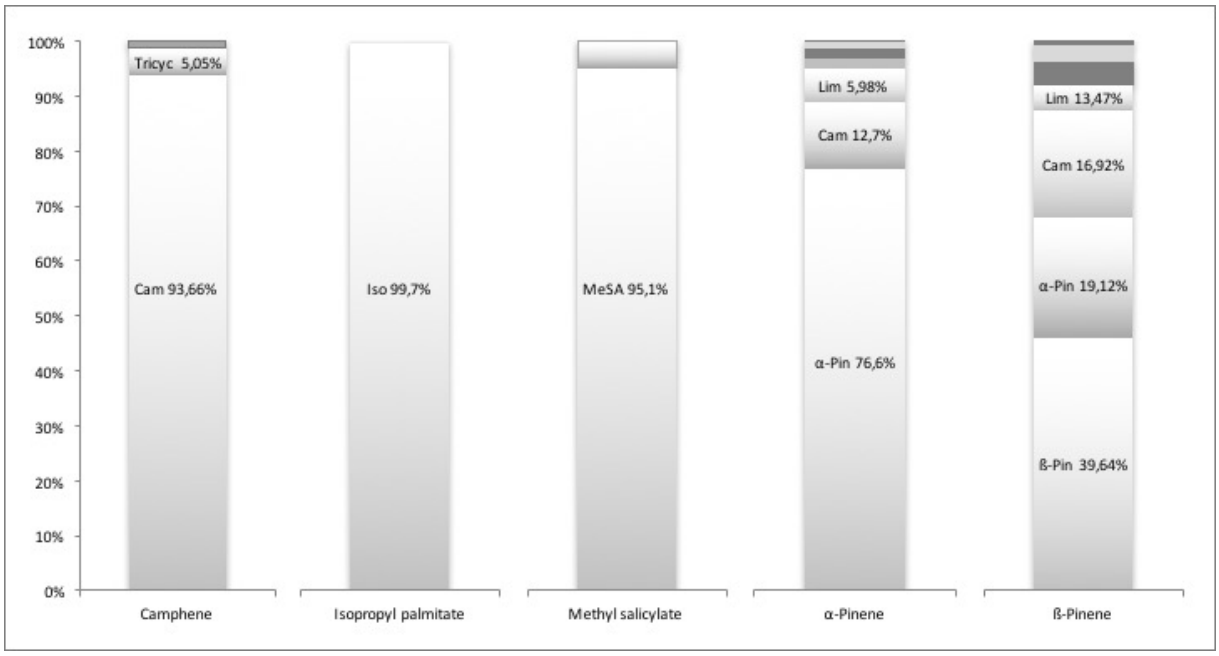

Figure 19: Chemical composition and purity of VOC standards. Standard (STD) compositions were analysed by GC-MS. Camphene STD composed of camphene 93.66%, tricyclene 5.05%, other VOCs 1.29%. Isopropyl palmitate STD composed of isopropyl palmitate 99.70%, other VOCs 0.30%. Methyl salicylate STD composed of methyl salicylate 95.10%, other VOCs 4.90%. α-Pinene STD composed of α-pinene 76.60%, camphene 12.7%, limonene 5.98%, γ-terpinene 2.06%, α-terpinolene 1.68%, α-fenchene 0.84%, ß-pinene 0.35%, other VOCs 0.22%. ß-Pinene STD composed of ß-pinene 39.64%, α-pinene 19.12%, camphene 16.92%, limonene 13.47%, α-terpinolene 3.34%, α-terpinene 2.76%, phellandrene 0.65%, other VOCs 4.09%. (Cam: Camphene, Iso: Isopropyl palmitate, Lim: Limonene, MeSA: Methyl salicylate, α-Pin: α-Pinene, ß-Pin: ß-Pinene, Tricyc: Tricyclene).

The camphene standard had a purity of 93.66% and contained only 5.05% tricyclene besides camphene. The isopropyl palmitate standard displayed the highest quality with 99.70% of isopropyl palmitate and also the MeSA standard had a high quality with 95.10% of MeSA and other components of below 5.0%.

In comparison to the camphene, isopropyl palmitate, and MeSA standard, the pinene standard solutions unfortunately had much lower quality in terms of purity. The α-pinene standard was composed of 76.60% α-pinene, 12.7% camphene, and 5.98% limonene. All other components in the α-pinene standard solution had percentage portions below 5% (2.06% γ-terpinene, 1.68% α-terpinolene, 0.84% α-fenchene, 0.35% ß-pinene, 0.22% other VOCs). The ß-pinene standard was consisting of 39.64% ß-pinene, 19.12% α-pinene,

16.92% camphene, and 13.47% limonene. Components below 5% percentage portions were 3.34% α-terpinolene, 2.76% α-terpinene, 0.65% phellandrene, and 4.09% of other VOCs. Altogether, GC-MS analysis of the VOC standard solutions revealed high purity for the camphene, isopropyl palmitate, and MeSA standards whereas the two pinene standards had much lower quality and contained other components with portions higher then 5%. The additional components in the pinene standards were camphene and limonene, two further closely related monoterpenoid VOCs. Due to the low purity of the α- and ß-pinene standard I cannot conclude that the observed resistance phenotype induced by the mixture of pinene (α:ß-pinene 1:1) was just due to pinene. It is likely that the other monoterpenoid VOCs camphene and limonene had a contributing effect to resistance against *Pst* DC3000 (**Figure 17, Suppl. Figure 31**). Limonene may contribute to the induced resistance against *Pst* DC3000 but limonene was just able to induce resistance to *Pst* DC3000 when applied at a relatively high concentration of 299.71nM and the induced resistance response was much weaker compared to the response induced by the pinene mixture (**Suppl. Figure 31**). Camphene was able to induce resistance against *Pst* DC3000 when applied alone in a concentration of 14.99nM comparable to its amount in the pinene mixture (16.44nM) but equal amounts of camphene present in the individually applied VOC solutions (±)-α-pinene (14.30nM) and (-)-β-pinene (18.05nM) were not sufficient to induce resistance (**Figure 18**). Thus, α-pinene and ß-pinene in the volatile blend seem to be crucial components for the induction of resistance against *Pst* DC3000.

In terms of individually tested pinene isomers (**Figure 18**) the GC-MS analysis of the standards revealed that just the applied α-pinene was a really isomer-pure solution. In contrast, the applied ß-pinene solution still contained lower amounts of α-pinene. Nevertheless, the provided data show that both components of the applied pinene mixture (ß-pinene STD and α-pinene STD 1:1) were required for the resistance inducing activity of the applied mixture what shows that both isomers of pinene are required to induce resistance against *Pst* DC3000.

GC-MS analysis of the VOC standards showed that a volatile blend consisting of the monoterpenoid VOCs α-pinene (47.86%), ß-pinene (19.82%), camphene (14.81%), and limonene (9.72%) was able to induce resistance against *Pst* DC3000 (**Figure 16**). This resistance effect induced by the volatile blend evidences a possible biological relevance of the monoterpenes α-pinene, ß-pinene, camphene, and limonene in plant defence and suggests a possible role of monoterpenoid VOCs in plant defence in general. Even if the applied pinene mixture (α:ß-pinene 1:1) was more a volatile blend of pinene, camphene, and limonene I stick to the term "mixture of pinenes" in the following text.

3.7 Antimicrobial effect of monoterpenoid volatile organic compounds

VOCs can display direct antimicrobial activity against bacterial pathogens (Brown et al. 1995, Holopainen 2004, Neri et al. 2007, Boulogne et al. 2012, Huang et al. 2012). In

terms of the monoterpenoid VOC pinene for example there is an on-going debate about its antimicrobial activity. Some studies attribute an antimicrobial activity of pinene while others reported no antimicrobial activity of pinenes (Sokovic and Griensven 2006, Rivas da Silva et al. 2012).

In the incubation set-up I do not expect a direct contact between the VOCs applied in the desiccators and the challenging pathogen *Pst* DC3000 which is applied subsequent to the incubation. Nevertheless, I reassured that the applied monoterpenoid VOCs camphene, isopropyl palmitate, α-pinene, and ß-pinene do not have a direct effect on the growth of *Pst* DC3000. For this purpose, 100µl of bacteria solution in 10mM $MgCl_2$ containing $1x10^5$ cfu ml^{-1} of *Pst* DC3000 were plated on a NYGA plate (containing antibiotics). Subsequent, 100µl of VOC solution containing either camphene, isopropyl palmitate, MeSA, or pinene (α:ß-pinene 1:1) solved in 200µl hexane in a concentration of 500µM were applied with an HPLC-syringe. 200µl of hexane were applied as negative control and SA, which is known to have direct antimicrobial activity (Gershon and Parmegiani 1962, Schaechter 2009, Carella et al. 2014), was applied as positive control (500µM). The plates were enclosed gas-tightly with parafilm and incubated for two days at 28°C. The bacterial growth of *Pst* DC3000 on plates with applied VOCs were compared *Pst* DC3000 on were only the negative control hexane was applied (**Figure 20**).

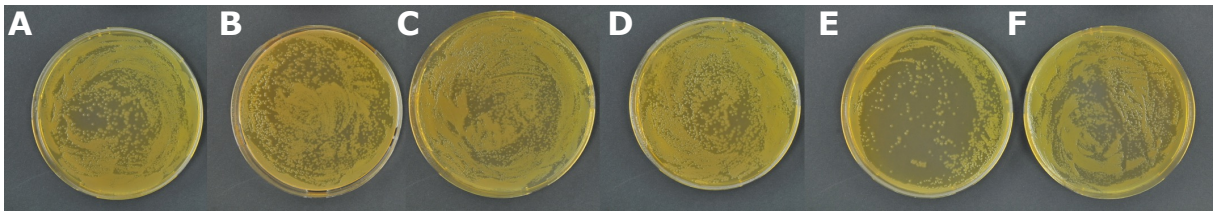

Figure 20: Effect of VOCs on *Pst* DC3000. Monitoring growth of *Pst* DC3000 grown in direct contact with VOC emission compared to *Pst* DC3000 grown in contact with the negative control hexane. VOC solutions were solved in hexane and applied in concentrations of 500µM. A) Campene B) Isopropyl palmitate. C) Pinene (α:ß-pinene 1:1). D) MeSA. E) SA. F) Hexane. Positive control methyl salicylate (MeSA 500µM), negative control hexane (200µl).

Any differences in the growth of *Pst* DC3000 grown in direct contact with either camphene, isopropyl palmitate, or pinene (α:ß-pinene 1:1) compared to *Pst* DC3000 grown in plates with the negative control hexane were not observed. In contrast to *Pst* DC3000 grown in direct contact with the VOCs, *Pst* DC3000 which was grown on plates were the positive control SA was applied displayed inhibited growth. In the way the VOCs, camphene, isopropyl palmitate, and pinene were applied, they had no direct antimicrobial effect on the growth of *Pst* DC3000. Therefore, I can be sure that the observed resistance phenotypes induced by camphene or pinene (**Figure 16, Figure 17**) were due to an effect of the VOCs on the plant during incubation and not due to an effect of the VOCs on the challenging bacterium *Pst* DC3000.

3.8 *PR1* response to pinene and methyl salicylate

To get a deeper insight in the molecular mechanism behind the pinene induced resistance the transcript accumulation of the resistance marker gene *PR1* (Thomma et al. 1988) was analysed.

Therefore, wt *Arabidopsis* plants were pre-incubated over three days with either the mixture of pinenes (α:β-Pin 1:1) in a concentration of 112.57nM, the positive control MeSA (280.55nM), or the negative control hexane (200µl). During incubation the VOC treatments were applied every day after replacing the air in the desiccators. After the three days of incubation with three repetitive VOC applications, at day four of the experiment the systemic leaves of the pre-incubated plants were harvested to monitor *PR1* transcript accumulation (T4). Non-harvested, pre-incubated plants were inoculated with *Pst* DC3000 ($1x10^5$ cfu ml^{-1}) and the infected systemic leaves were harvested 24h later at day five of the preceding experiment, to analyse *PR1* transcript levels after the challenge infection (T5). To see that the experiment worked, four days after challenge infection the pathogen growth in the systemic leaves of the pre-incubated plants was analysed. *PR1* expression was detected in pinene, MeSA, and hexane pre-incubated plants right after the pre-incubation before challenge infection (T4) and 24h after the challenge infection (T5) (**Figure 21**).

In general, two different options of molecular mechanisms for the observed resistance phenotype exist, induced resistance or priming. Induced resistance is a directly induced transcriptional defence response, which can be seen as enhanced *PR1* transcript level directly after the pre-incubation at day four of the experiment. In contrast, airborne priming is not a directly activated defence response. During priming the plant is sensitized and can react with a faster and stronger defence response to a subsequent challenge. A resistance phenotype based on priming gets obvious by enhanced *PR1* expression levels after the challenge infection (T5).

After pre-incubation (T4) *PR1* transcript levels were enhanced in MeSA and pinene pre-incubated plants compared to hexane pre-incubated ones. Incubation with MeSA induced ten-fold higher *PR1* expression compared to incubation with hexane. Pre-incubation with pinene also induced *PR1* transcript accumulation, but to a lower degree then MeSA did. This data show that pre-incubation with either pinene or MeSA directly activated a transcriptional defence response leading to induced resistance against *Pst* DC3000 (T4, **Figure 21**). After the challenge infection *PR1* transcript levels were elevated in hexane pre-incubated plants (T5) compared to hexane pre-incubated plants before the challenge infection with *Pst* (T4). This increase in the *PR1* transcript levels was due to infection with *Pst* DC3000, which induced *PR1* transcript accumulation compared to levels before the infection (**Figure 21**).

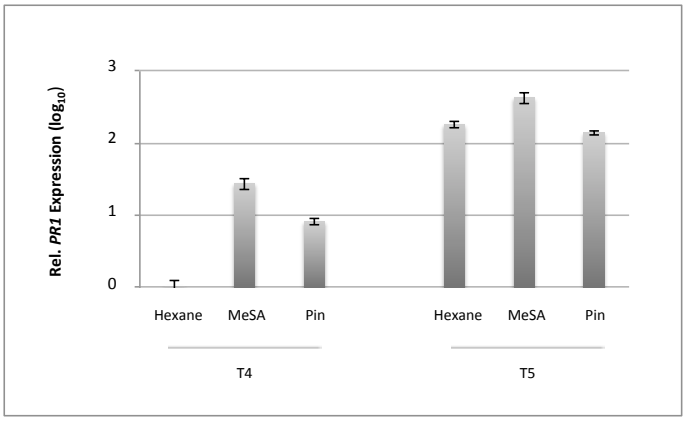

Figure 21: *PR1* transcript accumulation in response to MeSA and pinene incubation. A) *PR1* transcript accumulation in wt plants pre-incubated with Hexane, MeSA or pinenes after pre-incubation (T4) and 24h after bacterial challenge (T5). *PR1* transcript accumulation was analysed by qRT–PCR and normalized to that of the reference gene TUBULIN. The normalized expression is shown relative to that in hexane incubated plants. Positive control methyl salicylate (MeSA, 280.55nM, negative control Hexane (200µl), Pin (α:ß-pinene 1:1, 112.57nM). This experiment was repeated five times with similar results.

After the challenge infection *PR1* transcript levels were also significantly enhanced in MeSA pre-incubated plants compared to levels in hexane pre-incubated ones (T5). This implicates that the observed resistance phenotype in MeSA pre-incubated plants (**Figure 15, Figure 16, Figure 17**) was due to a directly induced transcriptional response and due to priming during the pre-incubation. In contrast, *PR1* transcript levels in pinene-incubated plants after the challenge infection were not enhanced and at the same level like in hexane incubated ones. Thus, the resistance phenotype in pinene pre-incubated plants was a result of directly induced resistance only and not due to a priming effect infection (**Figure 21**).

The analysis of *PR1* transcript levels shows that pre-incubation with pinene induced the classical resistance marker gene *PR1* like MeSA did. The underlying molecular mechanism of the phenotypically observed resistance phenotype in MeSA pre-incubated plants resulted of a directly induced transcriptional defence response together with an effect of priming. In contrast, our data show clearly that the induced resistance in pinene pre-incubated plants (**Figure 16**) is due to a direct transcriptional defence response and not a result of priming (**Figure 21**).

3.9 Incubation of salicylic acid-pathway mutants with pinene

PR1 is the classical resistance marker gene for SA (Thomma et al. 1998). Since pre-incubation with pinene induced *PR1* expression, I investigated the mechanism of pinene-induced resistance in different SA-pathway mutants.

Therefore, *Arabidopsis* wt, *sid2-1*, *eds1-2*, and *npr1-1* mutant plants were incubated over three days with either the mixture of pinenes (α:ß-pinene 1:1, 112.57nM), the positive control MeSA (280.55nM), or the negative control hexane (200µl). VOC treatments were applied three times on three consecutive days. Subsequent, the systemic leaves of the pre-incubated plants were infected with *Pst* DC3000 ($1x10^5$ cfu ml^{-1}) and harvested four days later to analyse the bacterial growth. The bacteria were extracted from leaf discs and a dilution series was grown on NYGA plates to count bacteria colonies (**Figure 22**).

Pinene pre-incubated wt plants showed reduced bacterial growth compared to hexane pre-incubated plants. The bacterial growth was reduced to the same level as in plants pre-incubated with the positive control MeSA. Thus, the mixture of pinenes (α:ß-pinene 1:1, 112.57nM) induced resistance against *Pst* DC3000 in wt *Arabidopsis* plants like MeSA did (**Figure 22**).

S*id2-1* mutant plants that were pre-incubated with MeSA or pinene showed no reduced bacterial growth compared to hexane-incubated plants. Thus, neither MeSA nor pinene pre-incubation was able to induce resistance against *Pst* DC3000 in *sid2-1* mutant plants. *Sid2-1* mutant plants have a defect in pathogen-induced SA biosynthesis and accumulation due to defective isochorismate synthase (Wildermuth et al. 2001, Strawn et al. 2007, Vlot et al. 2009). Even if MeSA acts downstream of SID2, the applied amount of MeSA during the incubation was not sufficient to restore the defect in SA accumulation of *sid2-1* mutant plants. The fact that pinene-incubation failed to induce resistance in *sid2-1* suggests pinene to function upstream of SID2. Pinene requires functional SA biosynthesis and accumulation by SID2 to induce resistance against *Pst* DC3000 (**Figure 22**).

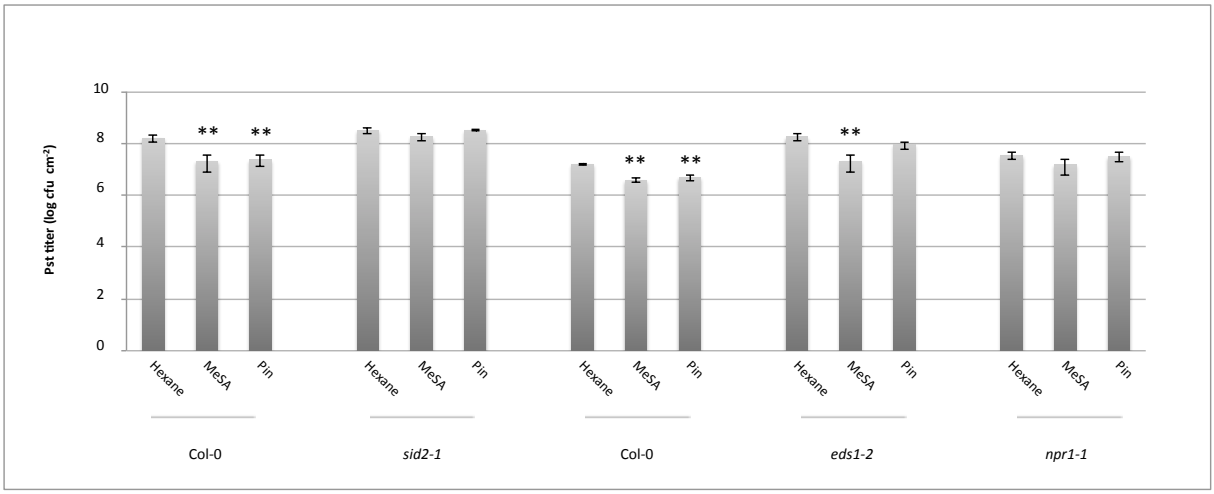

Figure 22: Incubation of different SA-pathway mutants with pinene. *Arabidopsis* wt (Col-0), *sid2-1, eds1-2*, and *npr1-1* mutant plants were pre-incubated over three days with either hexane, or methyl salicylate, or pinene and subsequently challenged with *Pst* DC3000. Resulting *Pst* titres are shown at 4 dpi. Positive control methyl salicylate (MeSA, 280.55nM), negative control Hexane (200µl), Pin (α:ß-pinene 1:1, 112.57nM). Plotted values are the average ±SD of three replicates each. Asterisks indicate statistically significant differences from hexane control (* $p<0.05$, ** $p<0.01$, student´s t-test). This experiment was repeated two times with similar results.

Eds1-2 mutant plants pre-incubated with hexane showed enhanced levels of bacterial growth compared to hexane pre-incubated wt plants, indicating that *eds1-2* mutant plants are more susceptible to *Pst* DC3000 then wt plants. *Eds1-2* mutant plants pre-incubated with the positive control MeSA showed reduced bacterial growth compared to hexane pre-incubated ones. MeSA acts downstream of EDS1 and exogenously applied MeSA was sufficient to restore the defect in SA biosynthesis in *eds1-2* mutant plants. In contrast, pinene incubated *eds1-2* mutant plants did not show reduced bacterial growth compared

to hexane-incubated plants. Since incubation with pinene did not induce resistance in *sid2-1* mutant plants, pinene may function upstream of EDS1 and require functional SA biosynthesis and EDS1 signalling to induce resistance against *Pst* DC3000.

Npr1-1 mutant plants pre-incubated with either MeSA or pinene did not show reduced levels of bacterial growth compared to hexane pre-incubated ones. Both MeSA and pinene were not able to induce resistance against *Pst* DC3000 in *npr1-1* mutant plants. NPR1 is a key regulator of SA-mediated defence gene induction (Mou et al. 2003, Dong 2004, Pieterse and Van Loon 2004, Spoel et al. 2009) and functions downstream of SA signalling and accumulation (Cao et al. 1994, Durrant and Dong 2004, Vlot et al. 2009, Spoel and Dong 2012). MeSA was not able to induce resistance in *npr1-1* mutant plants since it functions upstream of NPR1. The fact that pre-incubation with pinene could also not induce resistance in *npr1-1* mutant plants, suggests pinene to function upstream of NPR1 and intact NPR1 signalling is required for pinene-induced resistance against *Pst* DC3000.

Altogether, the data show that *EDS1*, *SID2*, and *NPR1* are required for pinene-induced resistance and suggests pinene to function upstream of all the three SA-pathway components. Pinene requires intact SA biosynthesis and accumulation via EDS1 and SID2 and the SA key regulator NPR1 to induce resistance against *Pst* DC3000. Since *EDS1*, *SID2*, and *NPR1* are important components of the SA-signalling pathway (Durrant and Dong 2004) this suggests that pinene functions via the SA-pathway to induce resistance against *Pst* DC3000.

3.10 Monoterpenoid biosynthesis and *GERANYL(GERANYL)DIPHOSPHATE SYNTHASE12*

Of the four VOCs camphene, isopropyl palmitate, α-pinene, and ß-pinene, which were tested in the incubation set-up only the three monoterpenoid VOCs camphene, α-pinene and ß-pinene displayed a potential biological relevance in terms of plant defence. Pre-incubation with either camphene or the mixture of pinenes induced resistance in *Arabidopsis* wt plants against *Pst* DC3000 (**Figure 16, Figure 17**). These resistance phenotypes provide evidence for a possible role of camphene, α- and ß-pinene in plant defence and a possible role of monoterpenoid VOCs in plant defence in general.

In the following, I wanted to investigate the role of the monoterpenoid biosynthesis in plant defence and SAR in particular. Camphene and pinene like all other monoterpenes are synthesized in the plastids via the MEP pathway. GERANYL DIPHOSPHATE SYNTHASE (GPPS, EC 5.2.1.1) displays a key enzyme in the MEP pathway responsible for the biosynthesis of geranyl diphosphate (GPP) the main precursor for all monoterpenes. GPPS catalyses the formation of GPP from the main building blocks IPP and DMAPP (Poulter and Rilling 1981, Ogura and Koyama 1997, Bouvier et al. 2000, Schmidt and Gershenzon 2008, Schmidt et al. 2010, Tholl et al. 2011, Dudareva et al. 2013). GPPS belongs to the class of short-chain isoprenyl diphosphate synthases, which catalyse the condensations of IPP and

DMAPP to GPP, FPP, and GGPP the precursors of all terpenes. By contrast, medium- and long-chain IDSs are responsible for the formation of products with more than twenty carbon atoms like ubiquinone, plastoquinone, or dolichol (Schmidt et al. 2010). The very latest state of data suggests GPPS to be a heterodimeric type GPPS comprising of a large (GPPS.LSU) and a small subunit (GPPS.SSU). In contrast to the long-lasting assumption that GPPS is responsible for the formation of GPP only, it was now shown that GPPS also displays multi-product medium/long chain IDS activity (Tholl et al. 2004, Wang and Dixon 2009, Van Schie et al. 2007, Hsieh et al. 2011) what suggests a role of GPPS in precursor synthesis not only for monoterpenes but also for other di-, tri-, tetra- and/or polyterpenes (Van Schie et al. 2007, Hsieh et al. 2011).

The recently identified *GERANYL(GERANYL)DIPHOSPHATE SYNTHASE12* (*GGPPS12*, At4g38460, also known as *GERANYLGERANYL REDUCTASE* (*GGR*)) encodes a type II small subunit (GPPS.SSU) of the heterodimeric GPPS in *Arabidopsis* which interacts with the large subunit of GPPS (GPPS.LSU) (Wang and Dixon 2009) and functions as a ''modifier'' to change the chain length of the product of GPPS from GGPP (C_{20}) to GPP (C_{10}) supporting the production of monoterpenes (Tholl et al. 2004, Wang and Dixon 2009). Since evidence is mounting that GPPS does not function for GPP formation and monoterpene biosynthesis only it denies *GPPS* (GPPS.LSU, At2g34630) as a candidate to investigate the impact of monoterpenes on plant defence. More importantly, the dwarfed phenotype of RNAi lines and the embryo lethality of transfer DNA (t-DNA) insertion lines suggests that *GPPS* is linked to other more basic developmental processes additionally to terpene biosynthesis (Arabidopsis tair webpage www.arabidopsis.org).

Here, the newly identified *GGPPS12* (GPPS.SSU) was used to investigate the impact of monoterpenes in plant defence. The expression of GPPS.SSU mRNA and GPPS.SSU protein was correlated with monoterpene biosynthesis and emission in *Antirrhinum majus* (Tholl et al. 2004) and the expression of the GPPS.SSU in hop was also directly correlated with the production of the monoterpene myrcene (Wang and Dixon 2009). This suggests *GGPPS12* (GPPS.SSU) to play a key role in regulating the formation of GPP and, thus, monoterpene biosynthesis. Due to this t-DNA insertion lines for *AtGGPPS12* are assumed to be compromised for biosynthesis of monoterpenes only whereas the formation of other higher terpenes should be unaffected.

3.11 *GERANYL(GERANYL)DIPHOSPHATE SYNTHASE12* is crucial for systemic acquired resistance

The monoterpenoid VOCs camphene, α-pinene, and ß-pinene have a biological relevance possible for local resistance in *Arabidopsis* wt plants. Pre-incubation with either camphene or pinene induced resistance in wt plants against *Pst* DC3000. These resistance phenotypes provide evidence for a possible role of monoterpenoid VOCs and monoterpene biosynthesis in plant defence in common. Due to the identification of these monoterpenoid VOCs via the

SAR-deficient phenotype of the *eds1-2* mutant, I was interested if monoterpenoid VOCs and monoterpene biosynthesis may also play a role in SAR.

For investigation of this possible link between monoterpene biosynthesis and SAR I performed SAR experiments (2.2.6 Assessment of systemic acquired resistance) with t-DNA insertion lines of the above-introduced *GGPPS12* (At4g38460). *GGPPS12* t-DNA insertion lines (*ggpps12-1*, *ggpps12-2*) were characterized by protein immunoblot for homozygosity and the *GGPPS12* gene expression level was analysed by qRT–PCR. *Ggpps12-1* mutant plants had a 98,9% reduced *GGPPS12* gene expression level compared to that of wt plants and can be designated as knockout (KO) plants. *Ggpps12-2* mutant plants with a 57,8% reduced *GGPPS12* gene expression level were identified as knock down plants (**Figure 23 B**).

The *GGPPS12* KO mutant *ggpps12-1* and the knock down mutant *ggpps12-2* were used for SAR experiments (**Figure 23 A**). The first two fully developed leaves (local leaves) of four-and-a-half-week-old wt *Arabidopsis* and *ggpps12* mutant plants were syringe-inoculated with $1x10^6$ cfu ml^{-1} of *Pst AvrRpm1* or with 10mM $MgCl_2$ (Mock) as control. After primary treatment with Mock, treated leaves looked green and healthy, whereas leaves treated with *Pst AvrRpm1* displayed yellow-spotted lesions as indicators for *Pst AvrRpm1* infection. Three days after the primary treatment, the next two "upper" fully expended leaves, called systemic leaves, were infiltrated with $1x10^5$ cfu ml^{-1} of *Pst* DC3000. Four days after the challenge infection with *Pst* DC3000, the pathogen growth in the systemic leaves was analysed. Bacteria were extracted from leaf discs and a serial dilution of the bacterial suspension was grown on NYGA plates to count bacteria colonies and determine the bacterial titres.

Arabidopsis wt plants that were pre-infected with *Pst AvrRpm1* showed reduced levels of *Pst* growth in the systemic challenged tissue compared to Mock pre-treated plants. The reduced bacterial growth in *Pst AvrRpm1* pre-treated plants is due to an induced and enhanced defence response in the systemic leaves resulting from the first infection with *Pst AvrRpm1*. The difference between the reduced bacterial titres in pre-infected plants compared to Mock pre-treated ones is defined as SAR effect (Durrant and Dong 2004, Mishina and Zeier 2007, Vlot et al. 2008, 2009, Dempsey and Klessig 2012, Shah 2009, Dempsey et al. 2009, Fu and Dong 2013). *Arabidopsis* wt plants displayed a significant SAR effect (**Figure 23 A**).

*Ggpps*12 mutant plants (*ggpps12-1*, *ggpps12-2*) pre-treated with Mock showed bacterial titres at the same level like Mock-inoculated wt plants. This indicates that *ggpps12* mutant plants do not have a general defect in basal resistance to *Pst* DC3000. *Ggpps12* mutant plants are as susceptible as wt plants to the infection with *Pst* DC3000. In contrast to wt, *ggpps12* mutant plants pre-infected with *Pst AvrRpm1* did not display reduced levels of *Pst* growth in the systemic, challenged tissue compared to Mock pre-treated plants. Thus, *ggpps12* mutant plants did not show a SAR effect against *Pst* DC3000 (**Figure 23 A**).

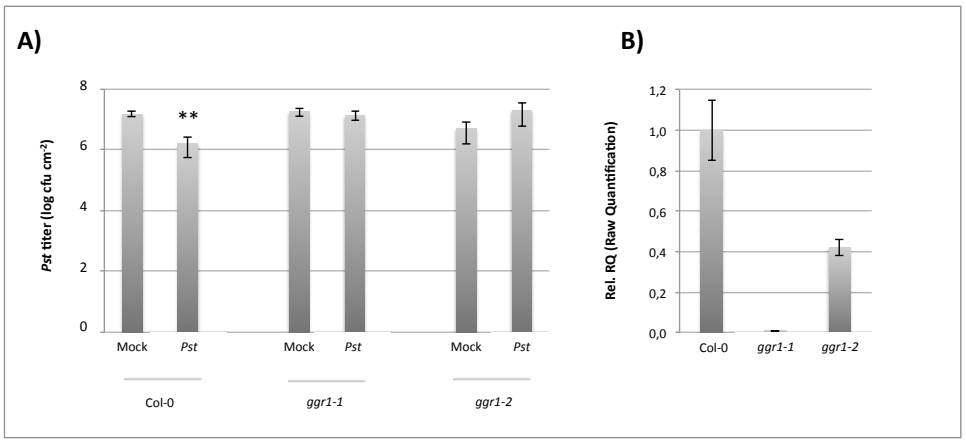

Figure 23: Characterization of *ggpps12* mutant plants. A) SAR defect in *ggpps12* mutant plants. *Arabidopsis* wt (Col-0) and *ggpps12* mutant (*ggpps12-1*, *ggpps12-2*) plants were locally treated with 10mM $MgCl_2$ (Mock) or *Pst AvrRpm1 (Pst).* Three days later, systemic leaves were challenge infected with *Pst* DC3000. Resulting *Pst* titres are shown at 4 dpi. Plotted values are the average ±SD of three replicates each. Asterisks indicate statistically significant differences from Mock control (* $p<0.05$, ** $p<0.01$, student´s t-test). This experiment was repeated five times with similar results. **B) *GGPPS12* gene expression level in *ggpps12* mutant plants.** *GGPPS12* gene expression was analysed by qRT–PCR and normalized to that of the reference gene TUBULIN. Rel. RQ (Relative raw quantification) means that the normalized expression is shown relative to that in wt plants.

Wt *Arabidopsis* plants displayed a significant SAR effect whereas *ggpps12* mutant plants (*ggpps12-1*, *ggpps12-2*) were defective for SAR (**Figure 23 A**). These data show a further newly identified SAR-deficient mutant. The SAR defect in *ggpps12* mutant plants indicates that *GGPPS12* is crucial for SAR. Since *GGPPS12* functions in monoterpene biosynthesis the SAR defect in *ggpps12* mutant plants also implicates a link between monoterpene biosynthesis and SAR.

Additionally to the SAR experiment, the *ggpps12* mutant plants were also tested in the incubation set-up (**Figure 24**). *Arabidopsis* wt and *ggpps12* mutant plants (*ggpps12-1*, *ggpps12-2*) were incubated over three days with either the mixture of pinenes (α:ß-pinene 1:1, 112.57nM), the positive control MeSA (280.55nM), or the negative control hexane (200 µl). VOC treatment was applied three times on three consecutive days. Subsequently, the pre-incubated plants were challenge with *Pst* DC3000 ($1x10^5$ cfu ml^{-1}) and four days later the infected leaves were harvested to analyse the bacterial growth. Wt plants incubated with either MeSA or pinene showed reduced levels of bacterial growth compared to hexane pre-incubated ones, indicating that both induced resistance in wt against *Pst* DC3000.

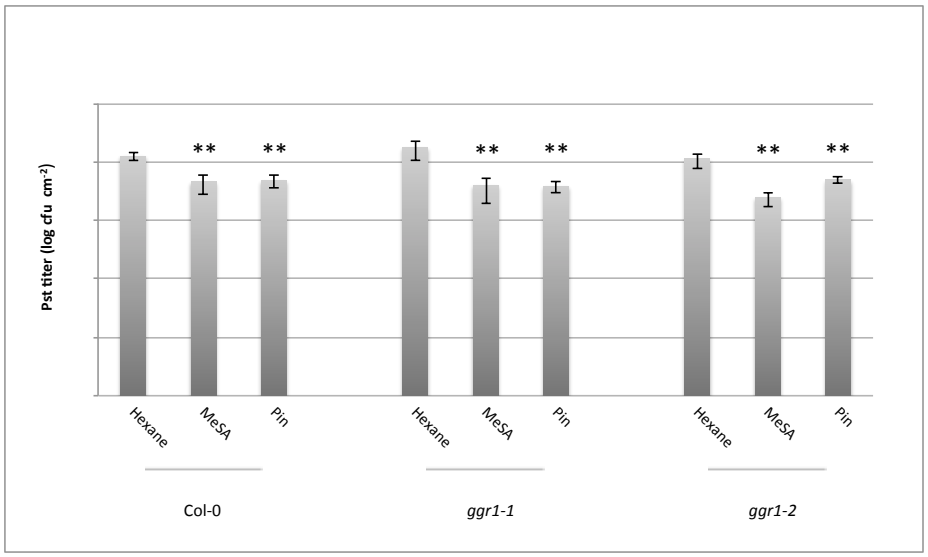

Figure 24: Incubation of *ggpps12* mutant plants with pinene. *Ara-bidopsis* wt (Col-0) and *ggpps12* mutant (*ggpps12-2*, *ggpps12-2*) plants were pre-incubated over three days with either hexane, or methyl salicylate, or pinene and sub-sequently challenged with *Pst* DC3000. Re-sulting *Pst* titres are shown at 4 dpi. Positive control methyl salicylate (MeSA, 280.55nM), negative control Hexane (200µl), Pin (α:ß-pinene 1:1, 112.57nM). Plotted values are the average ±SD of three replicates each. Asterisks indicate statistically significant differences from hexane control (* $p<0.05$, ** $p<0.01$, student´s t-test). This experiment was repeated two times with similar results.

Ggpps12 mutant plants (*ggpps12-1*, *ggpps12-2*) pre-incubated with hexane showed bacterial titres at the same level like hexane-incubated wt plants. This again shows that *ggpps12* mutant plants do not have a general defect in local resistance to *Pst* DC3000 and that *ggpps12* mutant plants are as susceptible as wt plants to *Pst* DC3000 (**Figure 23 A, Figure 24**).

Ggpps12 mutant plants (*ggpps12-1*, *ggpps12-2*) pre-incubated with either pinene or the positive control MeSA showed reduced bacterial titres compared to hexane pre-incubated ones. Although *ggpps12* mutant plants have a defect in SAR, pre-incubation with pinene was able to induce resistance. The exogenously applied pinene during pre-incubation can restore the defect in the *ggpps12* mutant, which indicates that pinene could be the crucial compound lacking during SAR establishment. Also, MeSA was able to induce resistance in *ggpps12*. This indicates that MeSA and the SA-mediated pathway function downstream of GGPPS12. GGPPS12 and possibly GPPS activity and monoterpene biosynthesis do not display a necessary prerequisite for SA/MeSA induced resistance against *Pst* DC3000.

Both pinene and MeSA are able to induce resistance against *Pst* DC3000 in *ggpps12* mutant plants. The fact that the positive control MeSA induced resistance in *ggpps12* mutant plants indicates that GGPPS12 is not a necessary prerequisite for MeSA induced resistance against *Pst* DC3000 and suggests MeSA to function downstream of GGPPS12. The fact that pinene is able to induce resistance in *ggpps12* mutant plants implicates that the exogenously applied pinene is able to restore the defect of *ggpps12* mutant plants and induce resistance downstream of GGPPS12.

4. DISCUSSION

4.1 Volatile organic compounds possibly related to systemic acquires resistance

In the volatile emissions of *AvrRpm1*-expressing *Arabidopsis* wt and *eds1-2* mutant plants 39 different VOCs were detected by GC-MS (**Table 12**). Five VOCs were differentially emitted from Col-0 dex as compared to *eds1-2* dex mutant plants with higher levels emitted from wt plants (**Figure 13, Figure 14**). Due to the SAR deficient phenotype of the *eds1-2* mutant these VOCs are possibly related to SAR and include isopropyl palmitate and the monoterpenoid VOCs camphene, α-pinene, ß-pinene, and sabinene.

Isopropyl palmitate (hexadecanoic acid, isopropyl ester) is the ester of palmitic acid (hexadecanoic acid), which displays one of the most common FAs in plants (**Figure 25**) (Farmer et al. 1998).

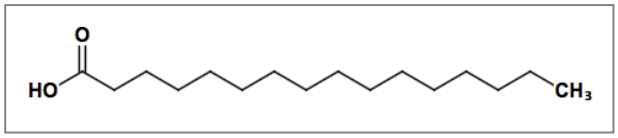

Figure 25: Chemical structure of isopropyl palmitate.

FAs play an important role in plant defence and are involved in the cross talk between various phytohormones including SA and JA (Weber 2002). FAs participate in defence to modulate basal, effector-triggered, and systemic immunity in plants against bacterial pathogens. C_{16} FAs are important for basal resistance to bacterial pathogens, C_{18} FAs affect the induction of multiple *R* genes, and both C_{16} and C_{18} FAs are important for the induction of SAR (Kachroo and Kachroo 2009). JA and its volatile methyl ester (MeJA) are by far the best-studied FA-derived signals in plants (Weber 2002). The importance of FAs for SAR is further supported by the FA-derived SAR signal AzA (Jung et al. 2009). Altogether the important role of FAs in plant defence responses asserts the higher levels of isopropyl palmitate in the emissions of *AvrRpm1*-expressing wt plants compared to *eds1-2* mutant plants and suggests isopropyl palmitate as a promising candidate for further investigations in terms of resistance induction.

Camphene, α-pinene, **ß-pinene**, and **sabinene** belong to the by far largest and most diverse class of VOCs, the terpenoid VOCs, and in particular to the monoterpenoid VOCs, the C_{10} representatives of the terpene family (**Figure 16**) (Tholl and Lee 2011, Dudareva et al. 2013, Schmidt and Gershenzon 2007).

Monoterpenoid VOCs are synthesized in the plastids via the MEP pathway (**Figure 9**). GPPS catalyses the formation of GPP the universal precursor of all monoterpenes (Poulter and Rilling 1981, Ogura and Koyama 1997, Bouvier et al. 2000, Schmidt and Gershenzon 2008, Schmidt et al. 2010, Tholl et al. 2011, Dudareva et al. 2013). In some species monoterpenes and the corresponding GPPS enzyme appear to be restricted to specific monoterpene storage and emission organs (Gershenzon et al. 1992, Dudareva et al. 1996, McConkey et al. 2000, Turner et al. 2000, Vuorinen et al. 2004, Wang et al. 2008, Niederbacher et al. 2015).

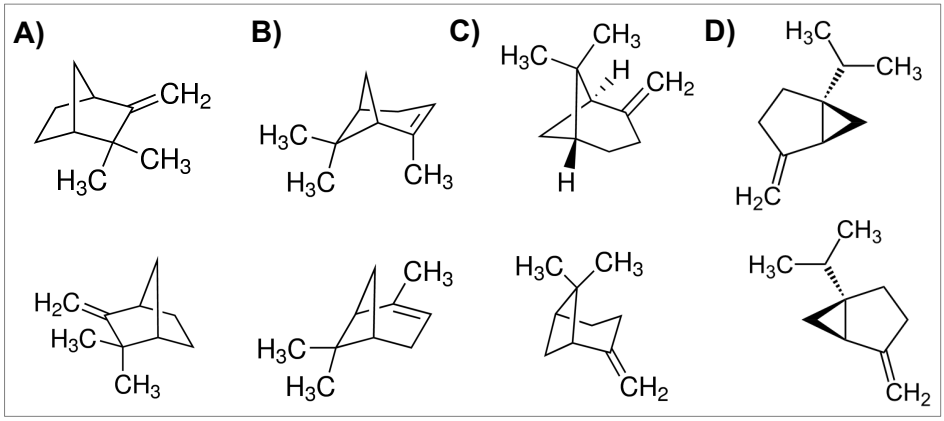

Figure 26: Chemical structures of the identified monoterpenes. A) Camphene B) α-Pinene C) ß-Pinene D) Sabinene. Upper structures show the (+) enantiomers, lower ones the (-) enantiomers.

In *Arabidopsis* monoterpenes are released without significant storage and not solely from specialized secretory structures (Bouvier et al. 2000, Tholl and Lee 2011). Monoterpenoid VOCs can be emitted from the leaves as well as from roots (Tholl and Lee 2011), where they are suggested to interact with rhizosphere organisms (Chen et al. 2004). Terpene synthases (TPS) are responsible for the final biosynthetic step in the synthesis of all terpenes. They catalyse the conversion of the terpene pathway intermediates, GPP, FPP and GGPP into different terpenes. TPSs targeted to plastids produce monoterpenes or diterpenes from GPP and GGPP, while TPSs located in the cytosol convert FPP to sesquiterpenes (Wise and Croteau 1999, Tholl and Lee 2011). In *Arabidopsis* only six TPS are suggested to be monoterpene synthases. Three of these monoterpene synthase genes are expressed in flowers and leaves and two are expressed exclusively in flowers (Aubourg et al. 2002). Fourteen TPSs have so far unknown functions and thus could also function in monoterpene synthesis. Of all thirty-two *Arabidopsis* TPSs, a considerable number of fourteen *TPS* genes are primarily or exclusively expressed in the roots. In contrast, only eight *TPS* genes are reported with expression in stem or leaves (Tholl and Lee 2011). Since it is very difficult to separate above- and belowground emission in plants with a fragile stem like *Arabidopsis*, I cannot provide information if the detected VOCs in this study were emitted from aerial parts (leaves, stem) or from the roots of *AvrRpm1*-expressing *Arabidopsis* plants.

A biogenic VOC emission can also appear from the soil and therein microorganisms (Penuelas et al. 2014). To ensure that the detected VOCs originated from the plant and not from the soil, an adequate number of background levels from "pots containing soil, but without plant material" were analysed.

Besides the emission of monoterpenes from flowers to attract pollinators (Knudsen et al. 1993, Chen et al. 2003, Pichersky and Gershenzon 2002, Tholl et al. 2004, Aharoni et al. 2005, Pichersky and Dudareva 2006, Schmidt and Gershenzon 2007) *Arabidopsis* leaves release only trace amounts of terpenes under physiologically normal growth conditions (Chen et al. 2003, Tholl and Lee 2011). Enriched (mono) terpene emission is induced by fungal or pathogen elicitors, application of JA, insect feeding and fungal or pathogen attack (Tholl and Lee 2011, Niederbacher et al. 2015). So far I cannot state if the difference in

the emission of the possibly SAR-related VOCs between Col-0 dex and *eds1-2* dex mutant plants was an induced phenotype due to induction by the effector *AvrRpm1* or if *eds1-2* mutant plants emitted constitutively lower levels of isopropyl palmitate, camphene, α-pinene, ß-pinene, and sabinene. An induced phenotype could indicate an important role of EDS1 in the emission of isopropyl palmitate, camphene, α-pinene, ß-pinene, and camphene during plant defence and SAR in particular. EDS1 signalling pathways are known to be essential for many defence responses to *Pst* DC3000 and maybe they are also crucial for the emission of certain VOCs during plant defence. However, a constitutive phenotype, which is due to a (constitutive rather than defence-induced) line effect of the *eds1-2 pDEX:AvrRpm1-HA* mutant, would also link the emission of isopropyl palmitate, camphene, α-pinene, ß-pinene, and sabinene to EDS1, which is essential for SAR signalling. Further experiments are planned to gain insight into the basal VOC emissions of Col-0 dex and *eds1-2* dex mutant plants.

Monoterpenes are crucial for numerous biological functions in plants (Bouvier et al. 2000, Attaran et al. 2008, Tholl and Lee 2011), including direct defence and protection against abiotic as well as biotic factors such as oxidative stress (Loreto et al. 2004, Loreto and Schnitzler 2010) and high temperatures (Loreto et al. 1996, 1998, Singsaas 1997). During biotic stress monoterpenoid VOCs can contribute in the very early stage of direct defence. Terpenoid VOCs can physically stabilize hydrophobic interactions in membranes and possibly contribute to inhibition of pathogen invasion (Vickers et al. 2009). Released from vegetative tissues monoterpenes can repel herbivores (Gershenzon and Croteau 1990) and serve as phytoalexins against fungi (Hammer et al. 2003) and pathogens (Loreto et al. 2000, Attaran et al. 2008, Hasegawa et al. 2010). Pinene for example is highly repellent to insects (Jump et al. 2010) and displays antimicrobial activity (Rivas da Silva et al. 2012). Since monoterpenes together with hemiterpenes, many sesquiterpenes, and a few diterpenes have high vapour pressure at ambient temperature and volatilize easily (Chen et al. 2003, Tholl et al. 2004, Tholl and Lee 2011) they function as airborne signals in chemical communication with other organisms. They indirectly protect plants by attracting natural enemies of herbivores such as parasitoids or predators (Kessler and Baldwin, 2001, Fäldt et al. 2003, Chen et al. 2004, Tholl and Lee 2011).

There are quite some reports about the emission of different monoterpenes from *Arabidopsis* (Bohlmann et al. 2000, Fäldt et al. 2003, Chen et al. 2003, Attaran et al. 2008, Huang et al. 2010, Snoeren et al. 2010, Tholl and Lee 2011, Van Poecke et al. 2001) and the emission of pinene and camphene from trees is also well documented (Loreto et al. 1996, Schmidt et al. 2010, Niederbacher et al. 2010). However, reports about the emission of pinene and camphene from *Arabidopsis* are rare (Chen et al. 2003), likely due to the fact that analysing VOCs in *Arabidopsis* is challenging because of low emission rates just slightly above background levels. Given that at least trace levels of monoterpenes are detectable in nearly all plant species investigated to date and that the very costly terpenoid

emission is sustained even when carbon budget becomes negative under stress conditions, the hypothesis is supported that monoterpenes may have important functions that are yet un-investigated (Schmidt et al. 2010, Attaran et al. 2008). The well-known functions of monoterpenoid VOCs in direct defence and defence signalling might explain the higher emission levels of these VOCs in the SAR competent Col-0 dex line in contrast to SAR-deficient *eds1-2* dex. Due to the various functions of monoterpenoid VOCs in plant defence the identified VOCs α-pinene, ß-pinene, and camphene were the most promising candidates for further investigations in terms of resistance induction.

4.2 Biological relevance of the newly identified volatile organic compounds

The four identified VOCs isopropyl palmitate, α-pinene, ß-pinene, and camphene were investigated for a potential biological relevance in plant defence. Therefore, A*rabidopsis* wt plants were pre-incubated over three days in gas-tight desiccators containing air supplemented with different concentrations of the VOCs. After VOC-incubation plants were challenged with *Pst* DC3000 and the bacterial growth was analysed as indicator for induced resistance.

The well-studied VOC MeSA known to induce and mediate pathogen resistance in tobacco and *Arabidopsis* plants was used as positive control (Shulaev et al. 1997, Koo et al. 2007, Park et al. 2007, Vlot et al. 2008, 2009). Since the VOCs that were tested in the incubation set-up are almost water insoluble, hexane was used as diluting agent and negative control. Plants pre-incubated with hexane-supplemented air showed enhanced bacterial growth compared to plants pre-incubated with un-supplemented air (**Suppl. Figure 30**). To normalize the effect of hexane on resistance, all the applied VOC and MeSA solutions were added by 200µl of hexane to avoid any side effect of the diluting agent. Even if the resistance phenotype observed in VOC treated plants (**Figure 16, Figure 17**) was partially influenced by hexane, the hexane proportions were higher in the applied VOCs solutions (200µl hexane plus the VOC fraction solved in hexane) compared to the negative control hexane (200µl hexane without VOC fraction). Thus, the effect of hexane should always be higher in VOC treated plants compared to control treated ones and, if at all, rather diminish and not increase the observed (induced resistance) phenotype. Therefore, the observed resistance phenotypes induced by camphene and the mixture of pinene were most likely due to the VOC treatment itself and not a side effect of the diluting agent hexane.

The incubation set-up was established with MeSA. Comparably to Shulaev et al. (1997) the supplemented air in the desiccators was replaced every day to supply fresh air and avoid high air humidity. In tobacco plants, incubation (6 days) with 250µg MeSA per litre air achieved best effects in terms of smallest lesion diameter of tobacco mosaic virus (TMV), highest SA content, and strongest *PR* induction. In contrast, A*rabidopsis* plants already showed induced resistance when incubated (6 days) with a five-fold lower concentration of MeSA (280.55nM ≅ 44.82µg/l) and displayed susceptibility to *Pst* when incubated like

tobacco plants (6 days, 1402.75nM ≅ 224.10µg/l). The lowest levels of bacterial growth were achieved with a shorter incubation period of just three days instead of six days (280.55nM), indicating that the MeSA induced resistance response in *Arabidopsis* works at lower concentrations compared to tobacco or that the resistance response in *Arabidopsis* is not as permissive in terms of concentrations compared to the response in tobacco (**Suppl. Figure 29**). MeSA in a concentration of 280.55nM, which induced resistance against *Pst* DC3000 with the lowest bacterial titres, was used as positive control for the incubation set-up. All incubation experiments were performed with an incubation period of three days with three times VOC application on three consecutive days.

The concentration of the positive control MeSA (280.55nM) was used as starting point to test the four identified VOCs in the incubation set-up. Depending on the observed bacterial titres the concentration range was adapted. Enhanced bacterial growth was an indicator for too high concentrations, bacterial levels comparable to or below levels in hexane pre-incubated plants were indicators to test lower concentrations.

Of the four tested VOCs, pre-incubation with isopropyl palmitate did not induce resistance against *Pst* DC3000 (**Figure 15**). Even if many reasons were in favour of isopropyl palmitate to be a promising VOC to induce resistance, pre-incubation with isopropyl palmitate alone in the tested concentration range (74.99nM to 229.96nM) was not sufficient to induce resistance against *Pst* DC3000. This result does not imply that isopropyl palmitate does not play a role in plant defence. VOCs often just function in a mix with other VOCs whereby the ratio of the single components is a crucial factor. Moreover, VOCs can function in a very dose-dependent manner and the tested concentrations could just have been not in the right range. In conclusion, although a role for isopropyl palmitate in defence against *Pst* DC3000 cannot be excluded, the current data set does not support biological relevance of this compound in defence.

In contrast to isopropyl palmitate, the other tested VOCs α-pinene, ß-pinene, and camphene showed a possible biological relevance for plant defence (**Figure 16, Figure 17**). The active concentrations of pinene (112.57nM), camphene (14.99nM) and MeSA (280.5nM) were in the same range. A comparison between pinene and camphene levels emitted from *Arabidopsis* and the active concentrations in the incubation set-up is difficult, since VOC emissions were collected in a dynamic system and the incubation set-up was performed as static system. Twelve wt *Arabidopsis* plants produced 3.17nmol of pinene (α- and ß-pinene) and 0.044nmol of camphene over three hours of sampling time (SP1). The active concentration of 112.57nM pinene mixture had a content of 419.98nmol pinene (α- and ß-pinene) and 90.40nmol camphene, which were applied in 5.5 litres of air over twelve hours (three times on three consecutive days). Eight wt plants were pre-incubated at the same time per desiccator, but 112.57nM of the pinene mixture were also sufficient to pre-incubate more than eight plants at the same time. VOCs often function in a volatile blend together with other VOCs whereby the ratio of the single components is a crucial factor. In

our experiment single VOCs were applied, which might explain why the concentrations needed to induce resistance against *Pst* DC3000 were higher than the amounts of VOCs that were emitted from the plants.

Although the α- and ß-pinene standards were declared with a purity grade of 98%, GC-MS analysis revealed also the closely related monoterpenoid VOCs camphene and limonene (percentage proportion >5%) in the standard solutions. Thus, the mixture of pinenes (α:ß-pinene 1:1) as it was used for the incubation experiments was more a volatile blend consisting of 47.86% α-pinene, 19.97% ß-pinene, 14.60% camphene, and 9.38% limonene (other VOC components had a percentage proportion <5%) (**Figure 19**). Due to the low purity of the α-pinene and ß-pinene standard it cannot be excluded that the observed resistance phenotype induced by the mixture of pinenes (α:ß-pinene 1:1) was not just due to pinene. It is likely that the other monoterpenoid VOCs camphene and limonene had a contributing effect to resistance against *Pst* DC3000, also because camphene and limonene each induced resistance when applied alone (**Figure 17, Suppl. Figure 31**). The assumption that the proportion of limonene present in the applied pinene mixture could be solely responsible for the induced phenotype is unlikely since higher concentrations of limonene (299.71nM) compared to camphene (14.99nM) and pinene (112.57nM) were necessary to induce resistance, whereas the proportion of limonene (10.55nM) present in the mixture was even lower than that of camphene and pinene. Furthermore, limonene (299.71nM) induced a much weaker resistance response (**Suppl. Figure 31**) compared to the response induced by camphene or the pinene mixture **(Figure 16, Figure 17**). Similarly, also the camphene proportion present in the mixture of pinenes (α:-β-pinene 1:1) probably did not exclusively induce the observed resistance phenotype. All of the three individually applied VOC solutions (±)-α-pinene, (-)-β-pinene, and the pinene mixture (α-:β-pinene 1:1) contained camphene in nearly the same amount (16.44nM, 14.30nM, 18.05nM). If the induced phenotype in plants pre-incubated with the pinene mixture would just be due to the proportion of camphene I would expect a resistance phenotype also in plants pre-treated with (±)-α-pinene or (-)-β-pinene. Since this was not the case (**Figure 18**) pinene itself most likely significantly contributed to the resistance inducing activity and seems to be a crucial component in the applied volatile blend (pinene mixture) for resistance induction against *Pst* DC3000. Altogether, our data show that a volatile blend consisting of the monoterpenoid VOCs α-pinene (47.86%), ß-pinene (19.97%), camphene (14.60%), and limonene (9.38%) was able to induce resistance against *Pst* DC3000 (**Figure 16, Figure 17, Suppl. Figure 31**). This indicates a possible biological relevance of the monoterpenoid VOCs α-pinene, ß-pinene, camphene, and limonene in plant defence and suggests a possible role of monoterpenoid VOCs and monoterpene biosynthesis in plant defence in general.

The pinene mixture was just active in a concentration of 112.57nM and camphene in a concentration of 14.99nM. Lower and higher concentrations of both VOCs solutions were

not capable of resistance induction. In two experiments higher concentrations (150.10nM, 225.15nM) of pinene were necessary to induce resistance (data not shown). This divergence from the normally active concentration of 112.57nM observed in all other experiments was due to degradation of the α- and ß-pinene content in the standards over time (as measured by GC-MS, data not shown), which again supports the importance of the pinene component in the volatile blend. These data suggest that the induced resistance by the monoterpenoid VOCs pinene and camphene is a dose-dependent mechanism. A reason why the observed resistance response is so sensitive in terms of concentration could be that pinene and camphene normally act in a volatile blend together with other resistance-inducing VOC components, which might contribute to a more robust effect.

Additionally, to the concentration, the ratio between the monoterpenoid VOC components in the volatile blend seemed to play a crucial role for the resistance inducing activity. (-)-ß-Pinene, which consisted of the same components as the mixture of pinenes (α:ß-pinene 1:1), but with differing relative ratios between the components, was not able to induce resistance (**Figure 18**). The (-)-ß-pinene standard contained 18.13% α-pinene, 37.59% ß-pinene, 16.04% camphene, and 12.7% limonene. The applied mixture (α:ß-pinene 1:1) contained 47.86% α-pinene, 19.97% ß-pinene, 14.60% camphene, and 9.38% limonene. Already the different ratio between the monoterpenoid VOC components was sufficient to destruct the resistance inducing potential of the applied ((-)-ß-pinene) solution. This indicates that the observed resistance phenotype induced by the pinene mixture (α:ß-pinene 1:1) is a dose-dependent resistance response in which the relative ratio of the single components is crucial.

The fact that both pinenes and camphene contributed to induce resistance to *Pst* DC3000 is not surprising, since these compounds share the same biosynthesis pathway (MEP pathway) in the plastids with GPPS being responsible for the production of their common precursor GPP. So far a TPS being responsible for the biosynthesis of camphene in *Arabidopsis* has not been identified (Tholl and Lee 2011). However, in other plant species pinene (camphene) synthases (EC 4.2.3.117, EC 4.2.3.119, EC 4.2.3.20) produced equal amounts of pinene and camphene (**Figure 26**) (Croteau et al. 1987, 1988, Bohlmann et al. 1999, Huber et al. 2005, Hyatt et al. 2005), which suggests that camphene is synthesized by the same TPSs that are responsible for the biosynthesis of pinenes. This might also hold true for *Arabidopsis*, and this hypothesis is strengthened by the pluripotent action of TPSs in *Arabidopsis* as well as by the similarity in the product profiles between pinene synthases from other plant species and the corresponding *Arabidopsis* enzymes (Chen et al. 2003, 2004, Tholl and Lee 2011).

The induced resistance phenotype against *Pst* DC3000 is a first evidence for a potential biological relevance of camphene, pinene, and limonene in plant defence and gives a first hint for a possible link between monoterpenoid VOCs and plant defence in general. Both incubation with the mixture of pinene and incubation with camphene reduced the bacterial

growth to the same level as the incubation with MeSA did, suggesting that monoterpenoid VOCs, including α-pinene, ß-pinene, and camphene, could have the same potential in plant defence as MeSA does. It would be interesting to investigate if camphene, α-pinene, and ß-pinene are able to induce systemic resistance in *Arabidopsis*, similarly to what was found for MeSA in tobacco and *Arabidopsis* (Shulaev et al 1997, Park et al. 2007, Vlot et al. 2009). However, an incubation set-up where just the lower part of the plant is pre-incubated and the upper part is gas-tightly separated and stays untreated, is difficult due to the fragile stem of *Arabidopsis*. Furthermore, the use of thin-layered plastic material like it is the case for the sealed plastic film chambers used by Park et al. (2007) is not suitable for monoterpenoid VOCs because the plastic material can easily trap these compounds.

4.3 Isomers and enantiomers of pinene and camphene

Pinene is found in nature as two active structural (constitutional) isomers; α- and β-pinene, of which both have enantiomers known as (+)-α-pinene, (-)-α-pinene, (+)-β-pinene, and (-)-β-pinene (**Figure 26**). The optical purity of camphene differs from country to country. While pine trees in North America contain racemic camphene with equal amounts of both camphene enantiomers, such trees in Europe may contain enantiopure camphene. When present enantiopure, (-)-camphene mostly dominates the (+) enantiomeric form (Atta-ur-Rahman 2016). Identification of (+)- and (-)-α-pinene synthases from *Salvia officinalis* (sage) determined that pinene as well as camphene (to a lesser degree) enantiomers can arise independently via stereochemically distinct routes in plants. Therefore, GPP stereoselectively binds in the active site as either a right-handed or left-handed helical conformer. For example, (-)-α-pinene is more common in European pines and (+)-α-pinene in North America (Riva da Silvas et al. 2012). On the other hand, many of the chiral monoterpenes are produced in both enantiomeric forms, often by the same plant species (Croteau et al. 1987, 1988).

Due to the fact that enzymes can be chiral and specialized for one or the other enantiomer, one enantiomer can be less active, inactive, or sometimes even produce the adverse effects as compared to the other. Thus, enantiomers can display distinct functions and have different physiologic effects (Riva da Silvas et al. 2012, Finefield et al. 2012). A well-known example of an enantiomeric monoterpene exhibiting distinct biological properties is carvon. (+)-carvone smells like spearmint and (-)-carvone like caraway (Russel et al. 1971). In plants both enantiomers of α-pinene exist and can co-occur with either enantiomer predominating. Contrary to this, β-pinene is almost always isolated as the optically pure (-)-isoform. The isolation of the (+)-β-pinene isomer is known, albeit the production of this metabolite is rare (Finefield et al. 2012). α-Pinene seems to be the more widely encountered isomer since its occurrence is reported in fifteen different plant species, whereas β-pinene is reported just in four (Finefield et al. 2012). Our data match these

findings, wt *Arabidopsis* plants in our hands emitted three times higher levels of α-pinene than β-pinene (**Figure 14**).

In *Arabidopsis* three pinene synthesizing TPS enzymes are known (corresponding genes: *TPS23 (At3g25830), TPS24 (At3g25810), TPS27 (At3g25830*)). The 1,8-cineole synthases *TPS23* and *TPS27* mainly produce 1,8-cineole and pinene in lower amounts (Tholl and Lee 2011, Chen et al. 2003, 2004). When expressed in *Escherichia coli* (*E. coli*) all three TPSs catalysed the formation of (-)-α-pinene and (-)-β-pinene, but not the formation of the (+) enantiomeric forms of both pinene isomers (Chen et al. 2003, 2004).

Lack of enantiomer identification and consideration of constitutional isomers may also be an explanation for the controversial results concerning the antimicrobial activity of pinenes. Some authors have attributed the antimicrobial activity of some essential oils to pinenes, whereas others reported no antimicrobial activity of pinenes. Constitutional isomers and enantiomers of pinenes may exhibit differences in toxicity and biological activity (Riva da Silvas et al. 2012).

Due to this information from plants in general and *Arabidopsis* in particular a mixture of a racemic (±)-α-pinene standard containing both enantiomers and an enantiomer pure (-)-β-pinene standard were used. Incubation with this mixture of (±)-α-pinene and (-)-β-pinene (α:ß-Pinene 1:1) induced resistance in *Arabidopsis* wt plants against *Pst* DC3000 (**Figure 16**). Since *Arabidopsis* emitted both isomers of pinene (**Figure 14**). I was interested if both isomers of pinene are necessary for the induction of the resistance phenotype or if one isomer alone may exhibit the biological activity. Plants pre-incubated with just the α-pinene isomer ((±)-α-pinene STD: 76.60% α-pinene, 12.7% camphene, 5.98% limonene) or the ß-pinene isomer ((-)ß-pinene STD: 39.64% ß-pinene, 19.12% α-pinene, 16.92% camphene, 13.47% limonene) did not show enhanced resistance (**Figure 18**), indicating that both isomers present in the applied pinene mixture were required to induce resistance. The fact that incubation with ß-pinene, which also contained a small amount of α-pinene (19.12%) was not sufficient to induce resistance suggests that not only the presence of both pinene isomers is important but also their (relative) ratio.

I also investigated the naturally occurring (+)-β-pinene enantiomer (Riva da Silvas et al. 2012, Finefield et al. 2012) for resistance induction against *Pst* DC3000. Both pinene mixtures, either containing the pinene (+)- or the (-)-β-pinene enantiomer, were able to induce resistance against *Pst* DC3000 (**Figure 18**). This indicates no difference in the function of the two β-pinene enantiomers when combined with α-pinene in terms of resistance induction against *Pst* DC3000.

In summary, both pinene isomers ((±)-α-pinene, (-)-β-pinene) as well as their relative ratio to each other were crucial for the resistance inducing activity of the pinene mixture, whereas the presence of the (+) or (-) enantiomeric form of β-pinene made no difference.

4.4 Pinene and camphene induce systemic resistance

For a deeper insight into the molecular mechanism of pinene- and camphene-induced resistance, the transcript levels of the classical resistance marker gene *PR1* were analysed (Van Loon and Kammen 1970, Henning et al. 1993, Lebel et al. 1998, Van Loo and Strien 1999, Sticher et al. 2007, Durrant and Dong 2004).

There are two well-known molecular mechanisms that can underlie the phenotypically observed resistance, termed "induced systemic resistance (ISR)" and "airborne priming" (Heil and Baldwin 2002, Frost et al. 2008, Goellner and Conrath 2008, Yi et al. 2009, Quintana-Rodriguez et al. 2015). Even though induced resistance by VOCs mostly leads to a resistance response comprising the whole plant (which is therefore termed "induced systemic resistance" (ISR)) we avoid this term to prevent confusion in the context of SAR and a related form of microbe-induced 'induced systemic resistance (ISR)'. Instead of "induced systemic resistance (ISR)" we use the term "induced resistance (IR)" for IR that is associated with VOCs. Although IR and priming have overlapping regulatory principles (Shah and Zeier 2013) their changes in the *PR1* gene expression level differ in terms of time course. IR displays a directly activated resistance response were transcriptional reprogramming occurs during VOCs incubation and leads to increased readiness for a following pathogen infection (Arimura et al. 2000, Farmer 2001, Engelberth et al. 2004, Ton et al. 2007, Yi et al. 2009). Thus, IR is associated with enhanced *PR1* transcript levels in the plant directly after pre-incubation. In contrast, priming is not a directly activated defence response. In primed plants, the defence arsenal is sensitized rather than fully induced and can be expressed in an accelerated manner during further pathogen attack (Frost et al. 2008, Goellner and Conrath 2008, Pieterse et al. 2012, Jung et al. 2009, Conrath 2011, Navarova et al. 2012, Van Hulten et al. 2006, Bruce et al. 2007, Ton et al. 2007). In the case of priming the transcriptional response is not directly activated during the pre-incubation but becomes obvious upon a subsequent challenge infection.

Our data showed significantly enhanced *PR1* expression in pinene and MeSA pre-incubated plants right after the incubation arguing for IR as the underlying molecular mechanism of the induced resistance response. After challenge infection *PR1* expression levels were enhanced further in MeSA pre-incubated plants but not in pinene pre-incubated ones (**Figure 21**). Pre-incubation with pinene induced the classical resistance marker gene *PR1* similarly to pre-incubation with MeSA. The phenotypically observed resistance phenotype in MeSA pre-incubated plants (**Figure 21**) seemed to result from a directly induced transcriptional defence response (IR) together with priming. In contrast, the induced resistance in pinene pre-incubated plants (**Figure 16**) seemed to be only due to directly induced resistance and not a result of priming (**Figure 21**).

From plant-plant signalling a phenomenon termed "associational resistance" is known, where the receiving plant adsorbs and deposits the VOCs in the cuticle, which mediate a passive resistance response to subsequent attacks (Quintana-Rodriguez et al. 2015). In

our set-up it is very unlikely that adsorbed levels of pinene and camphene outlast in the leaves when the plants are released from the gas-tight desiccators with VOC enriched air and kept under fresh air conditions during subsequent infection. Eventually absorbed levels of pinene and camphene should be evaporated quickly and not be present during the following four days of bacterial growth. Thus, I expect no direct contact or interaction of the applied VOCs pinene and camphene with the challenging bacterium *Pst* DC3000 in our set-up. To definitely exclude a direct antimicrobial effect of the applied VOCs on the challenging bacterium *Pst* DC3000 was also grown under direct contact with the VOCs and not any effect of growth inhibition by the mixture of pinene or camphene could have been observed (**Figure 20**).

Together the data indicate, that the observed resistance phenotype was due to a directly activated defence response of the plant that was associated with *PR1* induction after incubation with pinene or camphene and not due to a direct antimicrobial effect of the applied VOCs pinene or camphene on the challenging bacterium.

MeSA is the VOC with the most well investigated functions in plant defence (Shulaev et al. 1997, Park et al. 2007, Vlot et al. 2008, 2009, Manosalva et al. 2010). In *Arabidopsis* herbivore attack leads to the emission of MeSA that functions in the attraction of natural enemies of the feeding insects (Van Poecke et al. 2001). Tobacco plants infected with TMV emitted large amounts of MeSA from both infected and non-infected leaves. The data further suggested that MeSA as long-distance signal can move from the site of infection to the uninfected healthy tissue (Park et al. 2007). Also, tobacco and *Arabidopsis* plants could absorb the MeSA signal from neighbouring MeSA-emitting plants, convert it to SA, and thereby induce an SA-mediated defence response (Shulaev et al. 1997, Koo et al. 2007). Thus, MeSA functions as endogenous SAR signal via the vasculature (Shulaev et al. 1997, Park et al. 2007) and as exogenous long-distance signal in plant-plant signalling (Shulaev et al. 1997, Koo et al. 2007). In potato, MeSA was also shown to be important as phloem long-distance signal (Manosalva et al. 2001). In *Arabidopsis,* exogenously applied MeSA was also able to directly induce local resistance against *Pst* DC3000 and to mediate pathogen resistance via the vasculature as SAR signal (Park et al. 2007). Even if it is likely that MeSA as exogenous long-distance signal may contribute to the induction of SAR the involvement of other VOCs in the within-plant regulation of SAR is so far not shown (Heil and Ton 2008).

Besides MeSA a few VOCs were reported to have resistance inducing potential in different plant species. There are quite a few examples of VOCs emissions induced by pathogen attack that are suggested to contribute to the plants defence response due to their direct toxic activity. During HR, *Phaseolus vulgaris* leaves inoculated with *Pst phaseolicola* emitted several lipid-derived VOCs, including cis-3-hexenol and trans-2-hexenal, which had antibacterial activity (Croft and Slusarenko 1993). Peanut plants (*Arachis hypogaea*) infected with *Sclerotium rotium rolfsii* emitted (Z)-3 hexenyl acetate, the monoterpenoid

alcohol linalool, and MeSA, which displayed fungistatic activity against *S. rotium rolfsii* in solid-media cultures (Cardoza et al. 2002).

Besides the direct toxic effect of VOC emission, some studies also report examples of VOC-induced resistance or airborne priming. *Arabidopsis* exposed to the volatile leaf aldehydes trans-2-hexenal, cis-3-hexenal, or cis-3-hexenol for example supported induced enhanced disease resistance against the fungal pathogen *Botrytis cinerea*. Genes known to be induced by mechanical wounding or JA-application were induced directly after incubation, arguing for a directly VOC-induced resistance response. The SA-responsive gene *PR2* was not induced and airborne priming was not investigated in this study (Kishimoto et al. 2005). Similarly, lima bean (*Phaseolus lunatus*) plants showed airborne disease resistance when exposed to neighbouring plants infected with *Pst* DC3000. In this case, *PR2* expression was elevated in exposed compared to non-exposed individuals when subsequently challenged with *Pst* DC3000 indicating that plant-plant communication led to VOC-mediated airborne priming. The plant-derived VOC nonanal was identified as signalling compound responsible for the priming effect (Yi et al. 2009).

The most recent example of airborne priming comes from VOCs emitted from a resistant common bean cultivar (*Phaseolus vulgaris*). The VOCs limonene, linalool, nonanal, MeSA, MeJA, cis-hexenyl acetate, ß-pinene, farnesene, and α-terpineol were detected in the headspace of plants from the resistant cultivar. Susceptible plants that were exposed to the headspace of resistance-expressing plants became phenotypically as resistant as the resistant cultivar against the fungal pathogen *Colletotrichum lindemuthianum*. Several resistance marker genes (*PR1*, *PR2* and *PR4*) were primed in VOC-exposed susceptible plants. In vitro, the VOCs limonene, linalool, nonanal, MeSA and MeJA at natural concentrations directly inhibited fungal germination similar to VOCs in the headspace of resistance-expressing plants. The emitted VOCs were suggested to contribute to direct resistance in the emitter itself and resistance phenotypes of neighbouring individuals due to airborne priming (Quintana-Rodriguez et al. 2015).

A monoterpenoid VOC that induced resistance in *Arabidopsis* is ocimene. Exogenous application of ocimene directly induced resistance against *Botrytis cinerea*. It was not investigated if a mechanism of airborne priming contributed to the resistance. Incubation with ocimene induced molecular marker genes of ET/JA signalling and marker genes for mechanical wounding. SA marker genes like *PR1* and *PR2* were not induced by ocimene in *Arabidopsis* (Kishimoto et al. 2006), although exogenous ocimene treatment induced SA-associated defence genes, such as *PR* or *PAL*, in lima beans (Arimura et al. 2000).

Recently, a role of a terpenoid VOC in defence responses of the model plant *Arabidopsis* was investigated for the first time under physiological conditions. Inoculation with virulent or avirulent *Pst* strains induced emission of MeSA and the C16-homoterpene (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). Induced TMTT production proceeded via up-regulation of the TPS gene *TPS4* and *tps4* mutant plants were devoid in TMTT production.

Unfortunately, lack of TMTT synthesis in *tps4* mutants did not affect local resistance or SAR indicating that *Pst* induced TMTT formation is not a decisive event for disease resistance towards the bacterial pathogen *Pst* in *Arabidopsis* (Attaran et al. 2009).
Quite a few reports of VOCs with the potential to induce resistance or priming in plants against different microbes exist. In *Arabidopsis,* the only reports about terpenoid compounds comprise the monoterpene ocimene, which induced resistance against the fungal pathogen *Botrytis cinerea* independently of the SA marker genes (*PR1*, *PR2*) and the homoterpene TMTT, which turned out to be dispensable for resistance against *Pst*.

To my knowledge this study provides the first evidence for an induction of resistance against the bacterial pathogen *Pst* DC3000 by the monoterpenoid VOCs α-pinene, ß-pinene, camphene, and limonene in *Arabidopsis*.

4.5 *GERANYL(GERANYL)DIPHOSPHATE SYNTHASE12*: A candidate gene to investigate the role of monoterpenes in plant defence

Camphene and pinene like all other monoterpenes are synthesized in the plastids via the MEP pathway. GERANYL DIPHOSPHATE SYNTHASE (GPPS, EC 5.2.1.1) displays a key enzyme in the MEP pathway responsible for the formation of geranyl diphosphate (GPP) the main precursor for all monoterpenes from the main building blocks IPP and DMAPP (Poulter and Rilling 1981, Ogura and Koyama 1997, Bouvier et al. 2000, Schmidt and Gershenzon 2008, Schmidt et al. 2010, Tholl et al. 2011, Dudareva et al. 2013). GPPS belongs to the class of short-chain isoprenyl diphosphate synthases (IDSs), which catalyse the formation of the terpenoid precursors GPP, FPP, and GGPP (**Figure 8**). In contrast, medium- and long-chain IDSs are responsible for the formation of products with more than twenty carbon atoms like ubiquinone, plastoquinone, or dolichol (Schmidt et al. 2010).
In contrast to Bouvier et al. (2000), the very latest state of data suggests GPPS to be a heterodimeric type GPPS comprising of a large (GPPS.LSU) and a small subunit (GPPS.SSU). In contrast to the long-lasting assumption that GPPS is responsible for the formation of GPP only, it was now shown that GPPS also displays multi-product medium/long chain IDS activity (Tholl et al. 2004, Wang and Dixon 2009, Van Schie et al. 2007, Hsieh et al. 2011) suggesting a role of GPPS in precursor synthesis not only for monoterpenes but also for other di-, tri-, tetra- and/or polyterpenes (Schmidt and Gershenzon 2007, Van Schie et al. 2007, Hsieh et al. 2011). Furthermore, the dwarfed phenotype of RNAi lines and the embryo lethality of t-DNA KO lines implicate that GPPS in other more basic developmental processes additionally to terpene biosynthesis (Arabidopsis tair webpage www.arabidopsis.org). Altogether, this denies *GPPS* (EC 5.2.1.1, *At2g34630*) as a candidate to investigate the impact of monoterpenes on plant defence.
Recently, *GERANYL(GERANYL)DIPHOSPHATE SYNTHASE12* (*GGPPS12*, At4g38460) was identified, encoding a GGPP synthase-related protein, which contains two conserved CxxxC

motifs found to be essential for the interaction with the GPPS.LSU in hop (*Humulus lupulus*) and *Arabidopsis* (Wang and Dixon 2009). GGPPS12 encodes a type II small subunit (GPPS.SSU) of the heteromeric GPPS in *Arabidopsis,* which is localized to the chloroplast, expressed in petals and sepals and is involved in monoterpene biosynthesis (Arabidopsis tair webpage www.arabidopsis.org, Wang and Dixon 2009). The GRR subunit functions as a "modifier" to change the chain length of the product of GPPS from GGPP (C_{20}) to GPP (C_{10}) supporting the production of monoterpenes (Burke et al. 2002b, Wang and Dixon 2009, Tholl and Lee 2011). GGPPS12 is able to modify GGPP synthase11 *in vitro* by increasing its GPPS activity (Wang and Dixon 2009, Tholl and Lee 2011). In *Antirrhinum majus* flowers the expression of GPPS.SSU mRNA and GPPS.SSU protein was correlated with monoterpene biosynthesis and emission (Tholl et al. 2004) and the expression of the GPPS.SSU in hop was also directly correlated with the production of the monoterpene myrcene (Wang and Dixon 2009). These data suggest that *GGPPS12* (GPPS.SSU) might play a key role in regulating the formation of GPP and, thus, monoterpene biosynthesis (Tholl et al. 2004, Wang and Dixon 2009). T-DNA insertion lines for *AtGGPPS12* are assumed to be compromised for the biosynthesis of monoterpenes only whereas the formation of other higher terpenes should be unaffected. This makes the newly identified *GGPPS12* (*At4g38460*) a candidate gene to investigate the impact of monoterpenes in plant defence.

4.6 *GERANYL(GERANYL)DIPHOSPHATE SYNTHASE12* is crucial for systemic acquired resistance

Ggpps12 mutant plants were used to investigate the impact of monoterpene biosynthesis in SAR. In contrast to *Arabidopsis* wt plants, which supported SAR, two independent *ggpps12* t-DNA insertion mutants did not (**Figure 23 A**). The SAR defect in *ggpps12* mutant plants indicates that GGPPS12 is crucial for the establishment of SAR. The SAR defect in *ggpps12* mutant plants and the predicted function of GGPPS12 in monoterpene biosynthesis (Tholl et al. 2004, Wang and Dixon 2009) links monoterpene biosynthesis to SAR. It leads to the continuative assumption that monoterpene biosynthesis, monoterpenes themselves, or any degradation products of monoterpenes play a crucial role in SAR.

4.7 The mechanism of pinene induced resistance

The incubation of different SA-pathway mutants (*eds1-2*, *sid2-1*, *npr1-1*) with pinene provided a deeper insight into the molecular mechanism of pinene induced resistance. EDS1 is a nucleo-cytoplasmic protein with lipase homology and operates in the regulation of SA synthesis (Feys et al. 2001, Glazebrook et al. 1997, Jirage et al. 1999, Nawrath et al. 2002, Bartsch et al. 2006). Although EDS1 itself is not directly involved in SA

biosynthesis it is required for induced defence signalling leading to intact SA accumulation and defence gene induction. The *eds1-2* mutant is defective in SA biosynthesis induced by *Pst* DC3000 and in SAR signal generation and/or transmission leading to SAR (Breitenbach et al. 2014, Jirage et al. 1999, Zhou et al. 1998, Feys et al. 2001, Wiermer et al. 2005, Truman et al. 2007, Attaran et al. 2009). Incubation of *eds1-2* mutant plants with MeSA induced resistance to *Pst* DC3000 due to the fact that MeSA functions downstream of EDS1 signalling and replaces the lack of SA in *eds1-2* mutant plants. In contrast to MeSA, pinene was not able to induce resistance in *eds1-2*, suggesting that pinene functions upstream of EDS1 in resistance induction against *Pst* DC3000.

At the same time, eds*1-2* dex plants emitted lower levels of pinenes compared to Col-0 dex plants (**Figure 14**), suggesting a function of pinenes downstream of EDS1 in plant defence. The fact that pinenes seem to function upstream but also apparently downstream of EDS1 could implicate a positive feedback regulatory role of EDS1 in pinene-induced resistance against *Pst* DC3000.

SID2 encodes a putative chloroplast-localized isochorismate synthase (ICS), which converts chorismate to isochorismate, the main precursor of pathogen-induced SA (Wildermuth et al. 2001). Therefore, *sid2-1* mutant plants display a defect in pathogen-induced SA biosynthesis and accumulation (Wildermuth et al. 2001, Strawn et al. 2007, Vlot et al. 2009). In contrast to *eds1-2*, the *sid2-1* mutant did not support MeSA-induced resistance against *Pst* DC3000. In fact, MeSA acts downstream of SID2 and could function as a trigger to induce SA accumulation and NPR1 signalling. However, the ICS defect in the SA feedback loop needed for SA accumulation might have been too severe and could not be restored by the amount of MeSA, which was absorbed in the leaves during incubation. In fact, the data suggest that the MeSA-derived signal requires amplification in the form of SID2-dependent SA accumulation. Pre-incubation of *sid2-1* mutant plants with pinene also failed to induce resistance, demonstrating that pinene induced resistance relies on intact SA biosynthesis and accumulation via ICS/SID2.

NPR1 is a key regulator of SA-mediated defence gene induction and systemic resistance (Pieterse and Van Loon 2004, Spoel et al. 2009). On SA treatment and cognate redox imbalance, NPR1 translocates from the cytosol to the nucleus where it acts as a modulator of *PR* gene expression leading to defence (Dong 2004, Mou et al. 2003). Since NPR1 functions downstream of SA signalling/accumulation (Cao et al. 1994, Durrant and Dong 2004, Vlot et al. 2009, Spoel and Dong 2012), pre-incubation with MeSA could not induce resistance in *npr1-1* mutant plants. Pre-incubation with pinene showed the same pattern, suggesting that pinene, similarly to SA/MeSA, functions upstream of NPR1 and that intact NPR1 signalling is required for pinene induced resistance against *Pst* DC3000. Together, the data suggest that the monoterpenes camphene, pinene, and limonene require intact SA-mediated signalling in the establishment of SAR.

Ggpps12 mutant plants that were pre-incubated with hexane displayed bacterial titres

comparable to wt plants, indicating that *ggpps12* mutant plants do not have a defect in local resistance to *Pst* DC3000. Although *ggpps12* mutant plants have a defect in SAR, pre-incubation with pinene was able to induce resistance. This suggests that the exogenously applied pinene (and the additional monoterpenoid VOCs camphene and limonene present in the solution) during incubation was sufficient to restore the defect in the *ggpps12* mutant, supporting the notion that monoterpenoid VOCs could play a crucial role in SAR. Also, MeSA was able to induce resistance to *Pst* DC3000 in *ggpps12* mutant plants. This indicates that MeSA and the SA-mediated pathway function downstream of GGPPS12 and that GGPPS12 and possibly GPPS activity and monoterpene biosynthesis are not necessary for SA/MeSA induced resistance.

The observed phenotypes in *ggpps12* and SA-pathway mutants pre-incubated with pinene and MeSA provided insight into the mechanism of pinene-induced resistance against *Pst* DC3000. It can be stated that EDS1, SID2, and NPR1 are required for pinene induced resistance against *Pst* DC3000. The fact that all these components of SA-dependent defence responses (Durrant and Dong, 2004) were also required for pinene induced resistance suggests that pinene-induced resistance is mechanistically linked to SA-mediated defence responses. Pinene seemed to use the SA pathway for resistance induction against *Pst* DC3000, requiring intact SA synthesis, accumulation and perception. Together, the data suggest the following working model for the resistance against *Pst* DC3000 induced by the monoterpenoid VOC blend containing α-pinene, ß-pinene camphene, and limonene (**Figure 27**).

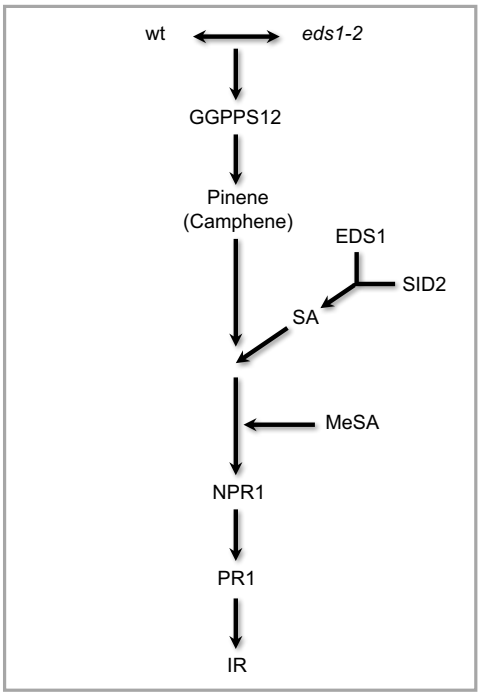

Figure 27: Monoterpenoid VOCs induce resistance against *Pst* DC3000. The working model provides insight into the molecular mechanism of monoterpenoid VOC induced resistance in relation to different SA-pathway components and SA-mediated plant defence (*EDS1*: *ENHANCED DISEASE SUSCEPTIBILITY1*, *GGPPS12*: *GERANYL(GERANYL)DIPHOSPHATE SYNTHASE12*, IR: Induced resistance, MeSA: Methyl salicylate, *NPR1*: *NONEXPRESSOR OF PR GENES1*, *PR1*: *PATHOGENESIS-RELATED1*, SA: Salicylic acid, *SID2*: SA *INDUCTION–DEFICIENT2*).

In *Arabidopsis* infection with *Pst AvrRpm1* induces *GGPPS12*, which is required for the biosynthesis of the monoterpenoid VOCs pinene and camphene. *AvrRpm1*-expressing wt plants emitted higher levels of these monoterpenoid VOCs compared to *eds1-2* mutant

plants. A volatile blend of α-pinene, ß-pinene, camphene, and limonene was able to induce resistance against *Pst* DC3000 via the induction of *PR1*. The SA pathway acts downstream of GGPPS12 and monoterpenoid VOCs and can induce resistance independently of GGPPS12. Resistance induction by the monoterpenoid VOC blend requires functional SA biosynthesis and accumulation via *EDS1* and *SID2*. Pinenes also function downstream of EDS1, suggesting a positive EDS1-pinene regulatory feedback loop.

NPR1 displays an important key regulator for monoterpenoid VOC-induced as well as SA-induced resistance. I suggest monoterpenoid VOCs like camphene, pinene, and limonene to function additionally to and upstream of SA in the induction of *PR1* and resistance against *Pst* DC3000 (**Figure 27**).

It could be shown that the monoterpenoid VOCs camphene, α-pinene, ß-pinene, and limonene play a role in plant defence, which gives a first hint for a possible link between monoterpene biosynthesis and plant defence in general. The two SA pathway components SID2 and EDS5 are chloroplast-localized proteins demonstrating the existence of an SA biosynthesis pathway localized in the chloroplast and revealing the importance of this organelle in mediating/controlling critical aspects of the plants defence response (Kachroo et al. 2003, Shah 2003). The co-localization of SA biosynthesis and monoterpene biosynthesis in the chloroplast together with our findings for a direct role of monoterpenes in plant defence supports this long-standing hypothesis.

Gil et al. (2005) provide further support for our suggested link between monoterpene biosynthesis and plant defence. The partial loss-of-function *hds-3* mutant showed strikingly enhanced resistance to *Pst* DC3000, suggesting a link between the MEP pathway and plant defence responses against biotrophic pathogens. In the MEP pathway HDS (4-hydroxy-3-methylbut-2-enyl diphosphate synthase) catalyses the penultimate step of the biosynthesis of IPP (Kollas et al. 2002), which serve as substrates for the precursors of all terpenoids. HDS is suggested to be a negative regulator of the SA-dependent defence pathway since *HDS* is down regulated following infection with *Pst* DC3000 and *hds* mutants displayed enhanced *PR1* transcript accumulation and enhanced resistance to *Pst* DC3000. Gil et al. (2005) could show by pharmacological complementation of the enhanced-resistance phenotype of *hds* mutant plants with fosmidomycin, an inhibitor of the MEP pathway upstream of HDS, that the observed resistance phenotype was not due to lack of downstream compounds. However, they could not exclude that the *hds* phenotype was an indirect effect due to stress caused by over-accumulation of an upstream substrate (Gil et al. 2005). During *Pst* DC3000 infection HDS seems to shift the plants focus to SA-mediated defence responses by down-regulating terpenoid biosynthesis. Preliminary data (not shown) showed reduced monoterpene levels (pinene, camphene) in extracts of *AvrRpm1*-expressing compared to non-induced Col-0 dex plants. And like fosmidomycin-treated *hds* mutant plants also *ggpps12* mutant plants displayed intact local resistance against *Pst* DC3000 (**Figure 23 A, Figure 24**), supporting the assumption that (mono-) terpene

biosynthesis seems to be not crucial during local resistance against *Pst* DC3000. In contrast to these findings, exogenously applied monoterpenoid VOCs induced resistance against *Pst* DC3000 (**Figure 16, Figure 17**) and monoterpene biosynthesis seems to be crucial for SAR (**Figure 23 A**). Although it is not common, it could be that HDS functions as negative regulator during local defence but positive regulator for SAR.

This study provides supporting evidence for the suggested link between the MEP pathway and plant defence. Additionally, the data provide a more detailed link between monoterpene biosynthesis and SAR in particular. Due to the fact that the *ggpps12* mutant displays normal basal resistance to *Pst* DC3000 comparable to wt plants indirect effects due to a possible over-accumulation of upstream substrates can be excluded. Gil et al. (2005) suggest a negative regulatory interaction link between the MEP pathway and SA-mediated plant defence responses in which a chloroplastic signal/factor derives from one of the early intermediary steps of the MEP pathway. This study suggests an (additional) interaction much more downstream of HDS, in which the end products of the monoterpene biosynthesis have an additional and supporting activity in the SA-mediated induction of *PR1* in systemic resistance.

4.8 Possible functions of monoterpenoid volatile organic compounds in plant defence

Finally, the question arises, why *Arabidopsis* plants emit monoterpenoid VOCs during pathogen attack. I want to attribute the question by suggesting different possible functions of monoterpenoid VOCs in plant defence and SAR.

First, monoterpenoid VOCs can function in the very early stage of direct plant defence to inhibit pathogen invasion (Vickers et al. 2009). Terpenoid VOCs can physically stabilize hydrophobic interactions in membranes leading to cell wall strengthening (Vickers et al. 2009) and monoterpenoids released from vegetative tissues can serve as phytoalexins against pathogens (Loreto et al. 2000, Attaran et al. 2008, Hasegawa et al. 2010). Monoterpenoid VOCs could function in early plant defence and contribute to inhibition of pathogen invasion (Vickers et al. 2009).

Second, within plant signalling has the aim to transmit the information of pathogen attack from attacked organs to the uninfected healthy tissue within the plant. Such signalling might affect transcript abundance or directly activate defence responses in the distal unaffected tissue to elicit defence more rapidly when attackers arrive (Heil and Ton 2008, Karban et al. 2006, Frost et al. 2007, Heil and Silva Bueno 2007). As long-distance signals camphene and pinene could either function as endogenous VOCs via the vasculature and/or as exogenously emitted VOCs that move outside the plant (Durrant and Dong 2004, Karban et al. 2006, Frost et al. 2007, Heil and Silva Bueno 2007, Heil and Ton 2008, Vlot et al. 2008, Yi et al. 2009, Shah and Zeier 2013). During SAR, just a part of the locally produced SA is converted into MeSA, which travels as long distance SAR signal via the phloem. The

majority of MeSA is released into the atmosphere (Attaran et al. 2009), maybe to function in within plant signalling outside the organism (Heil and Ton 2008). A similar function could be attributed to monoterpenes in SAR.

A clear hint for a role of terpenoids in SAR comes from dehydroabietinal (DA), an abietane type diterpenoid. DA was identified as a SAR signal in *Arabidopsis* (Chaturvedi et al. 2008) acting synergistically with glycerol-3-phosphate (G3P) and azelaic acid (AzA) to induce SAR (Dempsey and Klessig 2012). DA is a highly potent SAR inducer active in *Arabidopsis*, tobacco and tomato already in picomolar concentrations (Chaturvedi et al. 2012). DA and monoterpenes have certain elements in common: they share the same biosynthesis via the MEP pathway in the plastids and their carbon skeletons are composed of the same C_5 building blocks IPP and DMAPP, thus displaying structural similarities. The enzymes (GPPS, GGPPS) responsible for the synthesis of the monoterpene and diterpene precursors (GPP, GGPP) both belong to the family of short-chain prenyltransferases (Tholl et al. 2004). GPPS is suggested to have evolved from GGPPS based on the fact that diterpene biosynthesis evolutionarily predates monoterpene biosynthesis (Wang and Dixon 2009). The synthesizing enzymes are congeners that are equally compartmentalized and distributed in plastids and chloroplasts (Bouvier et al. 2010). Recently, GPPS was suggested to function not only in synthesis of the monoterpene precursor but also in the synthesis of GGPP the diterpene precursor. Thus, mono- and diterpenes have a common enzymatic origin in GPPS. Additionally, DA and the newly identified SAR-related monoterpene pinene show some parallels in the induction pathway of resistance. DA induces systemic resistance under requirement of *SID2*, *NPR1* and *FMO1*, indicating that DA functions upstream of SA accumulation and signalling (Shah 2009, Chaturvedi et al. 2012, Dempsey and Klessig 2012, Shah and Zeier 2013, Zheng and Dong 2013). Here, pinene was shown to require the SA-pathway components *SID2* and *NPR1* to induce resistance.

Unfortunately, it cannot be stated if pinene is able to induce SAR via the vasculature like DA does. Due to the high volatility of pinene it is not possible to decide if an observed SAR effect in the systemic leaves of locally pinene pre-treated plants would be due to signalling via the vasculature or due to pinene emission from the local treated leaves. Because of the fragile stem of *Arabidopsis*, it is not possible to separate upper untreated leaves gas-tightly from lower treated leaves to investigate a SAR inducing activity via the vasculature. Even if it cannot be stated that the monoterpenoid VOC pinene can induce SAR via the vasculature like the diterpenoid DA can, *GGPPS12*, which is essential for pinene biosynthesis is a necessary prerequisite for SAR. By contrast, it remains to be elucidated if DA is essential for biologically induced SAR (Shah 2009, Chaturvedi et al. 2012, Dempsey and Klessig 2012, Shah and Zeier 2013, Zheng and Dong 2013). I do not expect a connection between the SAR defect in *ggpps12* mutant plants and DA levels. GGPPS12 is just essential for the biosynthesis of monotepenes. Thus, DA levels should be not altered in *ggpps12*.

Additionally, to DA a further diterpene was reported to function as endogenous defence signal in resistance. In pathogen-infected tobacco plants increased levels of a diterpene alcohol, designated as WAF-1 (11 *E*, 13 *E*)-labda-11,13-diene-8_,15-diol) was detected during HR. Exogenously applied WAF-1 activated several SA-induced defence components like mitogen-activated protein kinase (MAPKs), enhanced *PR*-gene expression, and resulted in enhanced resistance to TMV infection, suggesting WAF-1 to function as an endogenous signal to mediate systemic resistance (Seo et al. 2003). Because (i) the diterpenoid DA functions as SAR signal and displays such a potent SAR inducer, (ii) a volatile blend of monoterpenoid VOCs induces resistance against *Pst* DC3000, and (iii) an important enzyme of monoterpene biosynthesis (GRR) is crucial for intact SAR, a more common role of mono- and diterpene(s) (biosynthesis) in SA-mediated plant defence signalling and especially in SAR should probably be considered. Monoterpenes could function in SAR already with the inhibition of pathogen invasion or in within plant signalling as endogenous or exogenous long-distance signals, or likely as a combination of these mechanisms.

Besides intra-plant signalling, pinenes and camphene could have been emitted to function in inter-plant or plant-plant signalling. VOCs can not only distribute the information within the own organism but also to neighbouring plants, which are "warned" and primed for resistance induction (Baldwin and Schultz 1983, Shulaev et al. 1997, Karban et al. 2000, Yi et al. 2009, Heil and Karban 2010, Heil and Adame-Alvarez 2010). This study provides a first hint for a possible function of monoterpenoid VOCs in plant-plant signalling, to warn neighbouring plants during pathogen attack. The preliminary data show, that *Pst AvrRpm1* infected wt plants were able to warn neighbouring wt plants whereas *eds1-2* and *ggpps12* mutant plants were not. Airborne signalling by VOCs emitted from the *Pst AvrRpm1* infected wt plants must have been responsible for the enhanced resistance in the naive receiver plant. The fact that *eds1-2* and *ggpps12-1* mutant plants were not able to emit VOCs, which induced resistance in the wt receiver plants supports the hypothesis that monoterpenoid VOCs mediate resistance in *Arabidopsis* against *Pst* DC3000 (Preliminary data, **Suppl. Figure 32**), possibly via plant-plant signalling.

Finally, another hypothesis about a possible function of monoterpenes in resistance is that monoterpenes such as α-pinene, ß-pinene, camphene, and limonene might play a role in plant defence and SAR by influencing NO/ROS signalling. The combined and coordinated action of ROS and NO signalling is crucial for the efficient activation and regulation of HR (Delledonne 2005), for the induction of defence associated genes (Lindermayr et al. 2010, Rusterucci et al. 2007) and to mediate a network that is involved in the establishment of SAR (Wendehenne et al. 2014) possibly including systemic signalling (Alvarez et al. 1998, Durner and Klessig 1999, Espunya et al. 2012, Rusterucci et al. 2007, Miller et al. 2009, Mittler et al. 2011). Cellular levels of ROS and NO are regulated by an efficient antioxidant defence system (Foyer and Noctor 2005), which likely includes unsaturated volatile terpenes as part of it (Vickers et al. 2009). ROS levels are reported to be regulated via the

chloroplasts (Foyer and Noctor 2005, Holuigue et al. 2007, Karpinski et al. 2003, Mur et al. 2008) where monoterpene biosynthesis is located and chloroplast-generated H_2O_2 production is regulated by SA (Mateo et al. 2004, 2006). The direct antioxidant behaviour of unsaturated volatile terpenes in scavenging ROS provides a proper tool to modulate ROS levels, and as consequence NO levels via the NO/ROS feedback loop (Vickers et al. 2009). Direct interaction of unsaturated volatile terpenes with ROS leads to production of reactive electrophile species, which are known to induce further antioxidants and other defence responses. Taken together, unsaturated volatile terpenes are suggested to alter signalling pathways by modulating to what extent and how rapidly ROS and NO signalling molecules are generated within the cell (Vickers et al. 2009, Vanzo et al. 2016), thus likely modulating the velocity and extent of the physiological response (Ahlfors et al. 2009, Wang et al. 2013). For DA it is suggested that an altered level of DA could negatively influence the NO-ROS-AzA-G3P branch of SAR and have an impact on SAR establishment (Wang et al. 2014, Wendehenne et al. 2014). The *ggpps12* mutant is likely defective for the production of monoterpenes, which display an important group of unsaturated volatile terpenes. Due to this defect, *ggpps12* mutant plants lack an important tool for modulating ROS and NO defence signalling. Thus, the SAR defect observed in *ggpps12* mutant plants might be due to troubles/issues in proper signalling mediated by the NO/ROS system.

In conclusion, this work analysed the volatile emissions of *AvrRpm1*-expressing *Arabidopsis* Col-0 dex and *eds1-2* dex mutant plants. The SAR-specific phenotype of the *eds1-2* mutant was used to identify possibly SAR-related VOCs. The monoterpenoid VOCs camphene, α-pinene, and ß-pinene were emitted in higher levels from Col-0 dex compared to *eds1-2* dex mutant plants. For the first time, monoterpene emission was associated with SAR in *Arabidopsis*. Incubation with a monoterpenoid VOC blend of α-pinene, ß-pinene, camphene, and limonene induced SA-dependent resistance against *Pst* DC3000 in *Arabidopsis* wt plants suggesting a role of monoterpenoid VOCs in SA-associated plant defence. Furthermore, *GGPPS12* is essential for monoterpene biosynthesis (Tholl et al. 2004, Wang and Dixon 2009) and for SAR, which provided further support to the putative link between monoterpenoid biosynthesis and SAR.

5. OUTLOOK

Preliminary data in this thesis showed a first hint for a possible function of monoterpenoid VOCs in plant-plant signalling to warn neighbouring plants during pathogen attack (**Suppl. Figure 32**). These data show, that *Pst AvrRpm1* infected wt plants were able to warn neighbouring wt plants whereas *eds1-2* and *ggpps12* mutant plants were not. It would be worthwhile to repeat the experiment for confirmation of the preliminary result and to include the second *ggpps12* mutant line (*ggpps12-2)*. A real evidence that monoterpenoid VOCs are the airborne signals that mediate the resistance in the neighbouring plants could be achieved by trapping the emitted VOCs in the desiccators with a twister followed by GC-MS analysis. The collected VOC extracts in the twister should be analysed by GC-MS.

GGPPS12 is the small subunit (GPPS.SSU) of the heterodimeric GPPS in *Arabidopsis* (Arabidopsis tair webpagewww.arabidopsis.org), which interacts with the large subunit of GPPS (Wang and Dixon 2009) and is believed to function as a ‘‘modifier’’ and “accelerator” supporting the production of monoterpenes (Tholl et al. 2004, Wang and Dixon 2009). The expression of GPPS.SSU was correlated with monoterpene biosynthesis in *Antirrhinum majus* and hop suggesting *GGPPS12* to play a key role in regulating the formation of GPP and, thus, in monoterpene biosynthesis (Tholl et al. 2004) and hop (Wang and Dixon 2009). *Ggpps12* mutant plants were defective for SAR (**Figure 23 A**), suggesting a crucial role of GGPPS12 and monoterpene biosynthesis in SAR. It would be interesting if the SAR defect in *ggpps12* mutant plants could be linked in more detail to further components of the monoterpene biosynthesis pathway. Downstream of GGPPS12, terpene synthases (TPSs) are responsible for the final biosynthetic step in the synthesis of monoterpenes. Plastid located TPSs catalyse the formation of monoterpenes from the main precursor GPP (**Figure 9, Figure 28**). At the same time TPSs also catalyse the formation of diterpenes from the precursor GGPP and sesquiterpenes from FPP (**Figure 9**). Thereby, TPSs act pluripotently and synthesize multiple products from a single prenyl diphosphate substrate.

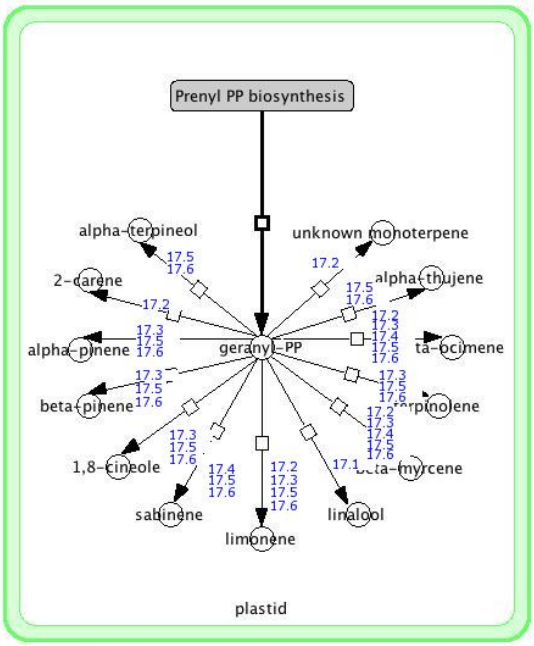

Figure 28: Terpene synthases catalyse the biosynthesis of monoterpenes. 17.1. (+)-3S-linalool synthase (*At1g61680*, TPS14), 17.2. ß-myrcene synthase (*At2g24210*, TPS10), 17.3. ß-myrcene synthase (At3g25810, TPS24), 17.4. Monoterpenoid synthase (At4g16730, TPS02), 17.5. 1,8-cineole synthase (*At3g25820,* TPS27), 17.6. 1,8-cineole synthase (*At3g25830*, TPS23). Illustration adapted from (Vranova et al. 2011).

A single TPS can produce as many as twenty products or as few as one. In *Arabidopsis* 32 *TPS* genes with species-specific divergence and tissue- and cell-type specific expression profiles are known. The *TPS* gene family is divided into seven subfamilies (TPS-a through TPS-g), where TPS-a consists mostly of sesquiterpene and diterpene synthases and TPS-b and TPS-g clades contain mostly monoterpene synthases (Tholl and Lee 2011, Dudareva et al. 2013). As follow-up work to this project, it would be interesting to investigate if the SAR defect of the *ggpps12* mutant can be attributed to one or more specific TPSs. This would provide more detailed information about the SAR critical component(s) of the monoterpene biosynthesis pathway. Furthermore, it could give insight into whether other monoterpenes beside the already investigated VOCs camphene, α-pinene, ß-pinene, and limonene may play a role in plant defence and if monoterpenoid VOCs in general are important for plant defence.

The data in this study do not allow a conclusion on which plant organs emitted camphene, α-pinene, and ß-pinene. Therefore, all *TPS* genes expressed in the roots, the leaves, and the stem of *Arabidopsis* plants (Tholl et al. 2011) should be taken into consideration to investigate a possible effect of TPS on SAR. SAR experiments with homozygous *tps* KO lines could link SAR to one or more particular *TPSs* being involved in the production of (mono-)terpenes which are important for SAR. Additionally, the experiments could provide new information about TPS with so far unknown functions. Also, the identification of a possible link between insect-induced TPS and SAR related-TPS could be shown with these experiments.

A further possible approach to investigate the role of monoterpenoid VOCs in SAR could involve incubation experiments with other plant species like for example tobacco. As mentioned previously, it would be interesting if the monoterpenoid VOCs α-pinene, ß-pinene, camphene, and limonene are able to induce not only local resistance but also systemic resistance against *Pst* DC3000 in *Arabidopsis* like MeSA does (Shulaev et al. 1997, Park et al. 2007, Vlot et al. 2009). With an incubation set-up where just the lower part of tobacco plants was pre-incubated with MeSA and the upper parts were gas-tightly separated, Park et al. (2007) could show that MeSA moves as SAR signal via the vasculature to induce systemic resistance against TMV. Because *Arabidopsis* and tobacco plants display many parallels in terms of SAR signalling (Shulaev et al. 1997, Park et al. 2007, Vlot et al. 2009), a local incubation of tobacco plants with the monoterpenoid VOCs α-pinene, ß-pinene, camphene, and limonene could clarify if monoterpenoids signal via the vasculature or via the air. Such experiments could provide further insight into the role of monoterpenoid VOCs in SAR and allow comparisons in terms of monoterpenoid-induced resistance in *Arabidopsis* and tobacco. Similarly, incubation of other plant species with the monoterpene blend might provide insight into a possible application of the VOCs for crop protection.

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V. SUPPLEMENT

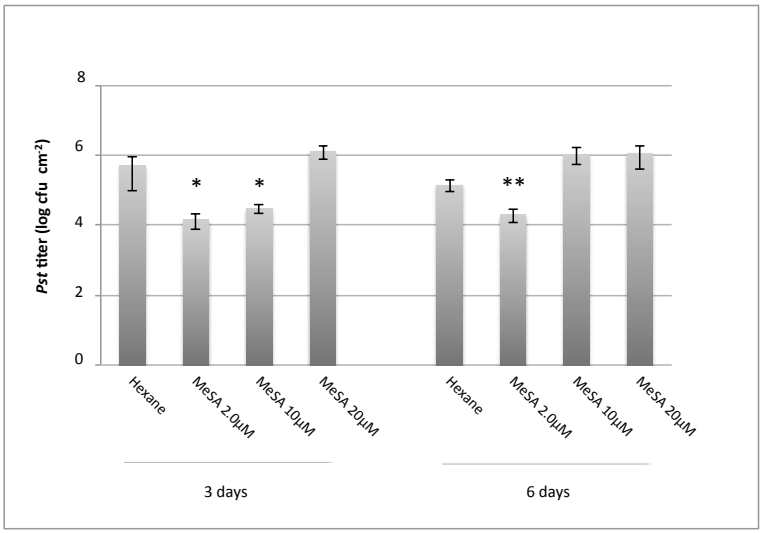

Figure 29: Establishment of incubation set-up with MeSA. *Arabidopsis* wt plants were pre-incubated over three or six days with different concentrations of MeSA and challenged with *Pst* DC3000. Resulting *Pst* titres are shown at 4 dpi. Negative control Hexane (200yl). Plotted values are the average ±SD of three replicates each. Asterisks indicate statistically significant differences from hexane control (* $p<0.05$, ** $p<0.01$, student´s t-test). This experiment was repeated two times with similar results.

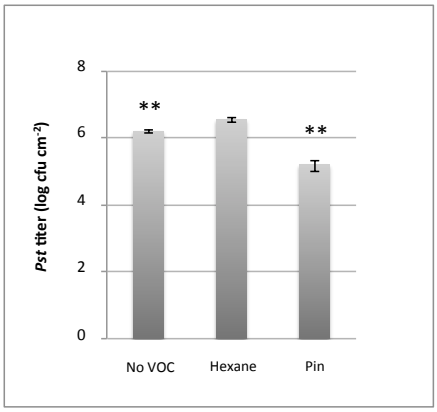

Figure 30: Negative control hexane in the incubation set-up. *Arabidopsis* wt plants were pre-incubated over three days with pinene or hexane supplemented air in comparison to un-supplemented air (No VOC). Subsequent, pre-incubated plants were challenged with *Pst* DC3000 and resulting *Pst* titres are shown at 4 dpi. Negative control Hexane (200yl), positive control Pin (α:ß-pinene 1:1, 112.57nM). Plotted values are the average ±SD of three replicates each. Asterisks indicate statistically significant differences from hexane control (* $p<0.05$, ** $p<0.01$, student´s t-test). This experiment was performed once.

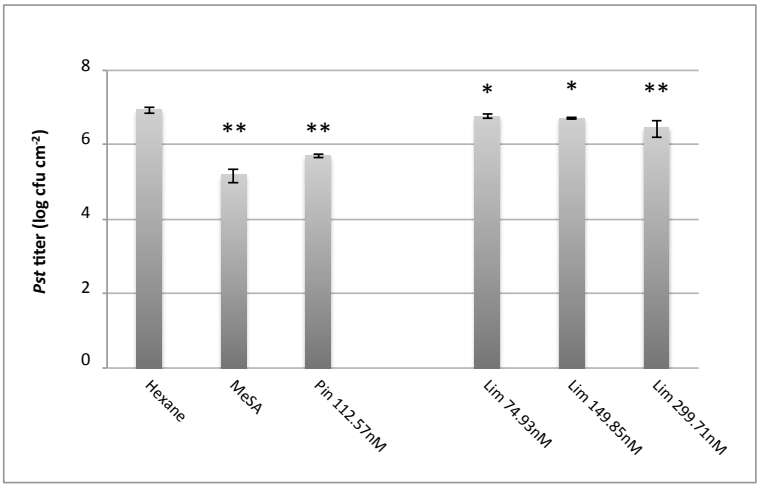

Figure 31: Resistance induction by limonene application. *Arabidopsis* wt plants were pre-incubated over three days with different concentrations of limonene and challenged with *Pst* DC3000. Resulting *Pst* titres are shown at 4 dpi. Positive control methyl salicylate (MeSA, 280.55nM), Pin (α:ß-Pinene 1:1), negative control Hexane (200yl), Lim (Limonene). Plotted values are the average ±SD of three replicates each. Asterisks indicate statistically significant differences from hexane control (* $p<0.05$, ** $p<0.01$, student´s t-test). This experiment was repeated two times with similar results. Experiment performed by Marion Wenig.

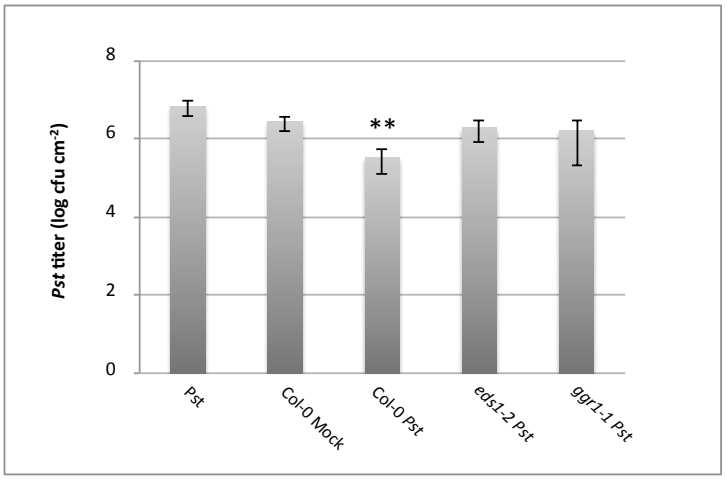

Figure 32: Plant-plant signalling in Col-0, *eds1-2* and *ggpps12-1* mutant plants. *Arabidopsis* wt plants were pre-incubated over three days with *Pst* (Nyga plate with *Pst*), Mock infected wt plants (Col-0 Mock), *Pst* infected wt plants (Col-0 *Pst*), *Pst* infected *eds1-2* plants, or *Pst* infected *ggpps12-1* plants (*ggpps12-1 Pst*). Subsequent, pre-incubated plants were challenged with *Pst* DC3000 and resulting *Pst* titres are shown at 4 dpi. Plotted values are the average ±SD of three replicates each. Asterisks indicate statistically significant differences from hexane control (* $p<0.05$, ** $p<0.01$, student´s t-test). This experiment was performed once and is considered as preliminary.

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"The metabolome of systemic acquired resistance in plants: Identification of metabolites that trigger immunity and their characterization in *Arabidopsis* and barley"

SFB924 Retreat Hersching, Germany 2013.

<u>Marlies Bichlmeier</u>, Andreà Ghirardo, Finni Wittek, Thomas Hoffmann, Wilfried Schwab, Jörg-Peter Schnitzler, A. Corina Vlot

„Metabolite profiling of *EDS1*-dependent systemic acquired resistance"

International Symposium SFB924, Freising, Germany 2013.

<u>Marlies Bichlmeier</u>, Andreà Ghirardo, Finni Wittek, Thomas Hoffmann, Wilfried Schwab, Jörg-Peter Schnitzler, A. Corina Vlot

„Metabolite profiling of *EDS1*-dependent systemic acquired resistance"

International Congress on Molecular Plant-Microbe Interactions, Rhodos, Griechenland 2014.

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„Metabolite profiling of *EDS1*-dependent systemic acquired resistance"

Joint International Workshop on PR Proteins and Induced Resistance against Pathogens and Insects – Prime time for induced resistance, Aachen, Germany 2015.

<u>Marlies Bichlmeier</u>, Andreà Ghirardo, Finni Wittek, Thomas Hoffmann, Wilfried Schwab, Jörg-Peter Schnitzler, A. Corina Vlot

„Metabolite profiling of *EDS1*-dependent systemic acquired resistance"

Botanikertagung, Freising, Germany 2015.