Induced intra- and interplant signaling mechanisms upon biotic interaction in *Ipomoea batatas* and *Arabidopsis thaliana*

Dissertation

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Segala yang terjadi dalam hidupku ini

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Perihnya cobaan hanya ujian kehidupan…

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1 Introduction

1.1 Plants amidst environmental challenges

Plants are widely defined as immobile organisms, which are exposed to a rapidly changing environment whilst constantly challenged by other surrounding organisms. The presence of detrimental external abiotic and biotic factors often results in stress for the plant (Gull et al., 2019). Abiotic stresses comprising extreme salt, heat, cold, humidity, and light conditions, are fluctuating environmental factors gaining importance over the years especially in the light of climate change and reduced crop yields (Bray et al., 2000;Ferguson, 2019). These agricultural losses can be amplified by simultaneously occurring abiotic and biotic stresses (Mittler, 2006;Dresselhaus and Hückelhoven, 2018). Biotic stress can be caused upon the interaction with pathogenic and/or herbivorous organisms; although beneficial symbionts and microbes can also serve as positive biotic regulators. Being subjected to such a perpetually occurring variety of potential threats and benefits, the plants' physiological adaptations are often depicted as a desperate attempt of survival caused by the inevitability to run away. However, these constant challenges over the course of millions of years led to a multilayered fortification and highly sophisticated response strategies (Maffei et al., 2012;Mithöfer and Boland, 2016). By implementing mechanical barriers (e.g. hair-like trichomes, epicuticular waxes) in combination with chemical defenses (e.g. toxic secondary metabolites, feeding deterrents) (Mithöfer and Boland, 2016), a solid toolbox was established enabling plants to grow, adapt, survive, and propagate irrespective of their immobility. In order to efficiently use these given tools, a reliable and tightly regulated perception and signaling network is required to activate responses appropriate to the given stimulus within a plant (intraspecific signaling) as well as its whole plant community (interspecific signaling). This dissertation aims to investigate intra- and interspecific signaling features involved in plant-herbivore and plant-microbe interactions.

1.2 Signal perception in plants upon damage

An efficient response to a given stimulus requires a specific recognition, decoding, and translation of the information to activate downstream elements resulting in appropriate reprogrammed cellular functions (Erb and Reymond, 2019). In the case of biotic stresses, the plant initially perceives pressure changes along the membrane caused by vibrations or mechanical impact (Alarcon and Malone, 1994;Huber and

Bauerle, 2016). These external stimuli are sufficient to trigger the generation of second messengers followed by a subsequent cascade of phosphorylation events and transcriptional changes (Zebelo and Maffei, 2015). Severe damage to the tissue can even result in ruptured cell structures, therefore releasing molecules which are usually constrained to specific cell compartments. Consequently, these endogenous regulators and/or their respective fragments are now exposed to the extracellular space serving as early indicators of injury, and activate immune and stress responses. These damage-associated molecular patterns (DAMPs) comprise different classes of molecules, including proteins and signaling peptides, saccharides, cell wall fragments, nucleotides in the form of extracellular adenosine triphosphate (eATP) and DNA (eDNA) (Hou et al., 2019), and volatile organic compounds (VOCs) (Quintana-Rodriguez et al., 2018;Meents and Mithöfer, 2020). In addition to purely mechanical damage, attacking herbivores and pathogens introduce herbivore- (HAMPs; e.g. volicitin, inceptin) (Mithöfer and Boland, 2008;Acevedo et al., 2015) and pathogen- (PAMPs; e.g. flagellin, peptidoglycans, lipopolysaccharides) (Schwessinger and Zipfel, 2008) associated molecular patterns into the plant, which are perceived by binding to pattern recognition receptors (PRRs) (Erb and Reymond, 2019). Being specific for the attacking organism and its mode of action, these elicitors are able to induce a complex network of signaling pathways transduced by either electrical or chemical signals (Zebelo and Maffei, 2015) leading to defense upregulation, growth promotion/inhibition, or enhanced/suppressed immunity.

The generation of electrical signals occurs within seconds to minutes after wounding or herbivory (Maffei et al., 2007;Zimmermann et al., 2009;Zimmermann et al., 2016), being able to travel up to a speed of 40 cm/s (Volkov, 2012) whilst serving as a systemic transmitter of information even over long distances. Electrical signals, e.g. depolarization of the plasma membrane potential occur simultaneously or precede chemicals signals such as Ca^{2+} fluxes in the framework of leaf-to-leaf signaling (Nguyen et al. (2018) Subsequently, each signaling pathway deploys a variety of chemical signals including Ca^{2+}, reactive oxygen (ROS) and nitrogen (RNS) species, phytohormones, volatiles, peptides, as well as protein phosphorylation events by mitogen-activated protein kinases (MAP-kinases) (Huber and Bauerle, 2016;Sözen et al., 2020). In the following sections, this thesis will focus on a selection of the aforementioned chemical signals and their involvement within biotic interactions.

1.2.1 Calcium

One of the earliest signaling events initiated by herbivory and microbe interaction (Maffei et al., 2004;Howe and Jander, 2008;Kiep et al., 2015;Yuan et al., 2017;Toyota et al., 2018) is the elevation of cytosolic free calcium $[Ca^{2+}]_{cyt}$. Calcium as an essential macronutrient serves in an accurately orchestrated manner structural, metabolic, and signaling purposes (Demidchik et al., 2018). Being a multifunctional key player within cell homeostasis, Ca^{2+} levels can range from 50-150 nM (Medvedev, 2005;Wilkins et al., 2016;Demidchik et al., 2018) in the cytoplasm up to 0.1-10 mM stored in specific organelles such as the endoplasmic reticulum, the vacuole or the apoplast (Stael et al., 2012;Demidchik et al., 2018). These massive compartment-dependent differences in Ca^{2+} concentrations can be targeted by the precise opening of ion channels localized at the plasma membrane or membranes of intracellular compartments. The main known Ca^{2+} channels in plants comprise cyclic nucleotide-gated channels (CNGCs) (Mäser et al., 2001), ligand-gated glutamate receptor-like channels (GLRs) (Davenport, 2002;Nguyen et al., 2018), and mechanosensitive, reduced hyperosmolality-induced $[Ca^{2+}]_i$ increase (OSCA) channel (Murthy et al., 2018). All of which are predominantly located at the plasma membrane with the exception of the vacuolar two-pore channel 1 (TPC1) at the tonoplast (Peiter et al., 2005;Peiter, 2011). The majority of the aforementioned channels were found to be involved in wounding- and herbivory-induced calcium signaling (Arimura and Maffei, 2010;Kiep et al., 2015;Vincent et al., 2017;Meena et al., 2019) leading to a systemic propagation of this initially local stimulus through the vascular-connected leaves, ultimately upregulating chemical defenses (Mousavi et al., 2013;Toyota et al., 2018).

The physiological responses following this channel-mediated calcium influxes, are triggered by the perception of the increased $[Ca^{2+}]_{cyt}$ by specific calcium sensing proteins, such as calmodulins (CaMs), calmodulin-like proteins (CMLs), calcineurin B-like proteins (CBLs), calcium-dependent protein kinases (CDPKs), and calcium/calmodulin-dependent protein kinases (CCaMKs) (Ku et al., 2018). The majority of the aforementioned Ca^{2+} sensors contain a specific helix-loop-helix motif, known as the “EF hand”, which binds single calcium molecules with high affinity (Tuteja and Mahajan, 2007). Additionally, several other proteins, e.g. phospholipase D (PLD) (Wang, 2001), calreticulin (Michalak et al., 1998), pistil-expressed Ca^{2+} binding protein (Furuyama and Dzelzkalns, 1999), and annexins (Clark and Roux, 1995) were found to bind Ca^{2+} independent of EF motifs (Tuteja and Mahajan, 2007). Especially the highly conserved multigene family of phospholipid-binding annexins gained increasing attention over the years (Tichá et al., 2020) due to their potential as unconventional Ca^{2+} channels (Laohavisit and Davies, 2011)

contributing to conductance at the plasma membrane. Up to now, eight annexins were found in *Arabidopsis thaliana* and their involvement in drought and salt stress responses was further unraveled (Konopka-Postupolska et al., 2009;Yadav et al., 2018). Apart from abiotic stress, insect feeding is a major elicitor able to induce rapid $[Ca^{2+}]_{cyt}$ elevations in plants, however to date evidence to what extent annexins might be involved in these calcium-mediated herbivore defenses is still lacking.

1.2.2 Plant peptides

Over the years, small peptides as key endogenous signaling factors gained increasing attention due to their bifacial role within plant immunity, plant microbe-, and plant herbivore interactions (Hu et al., 2018). Taking part in cell-to-cell communication, alteration of signaling pathways, and possessing antimicrobial properties, plant peptides serve as versatile molecules during insect attack or microbial infection, which can however also be reprogrammed and used to the intruder's benefit (Tavormina et al., 2015;Hu et al., 2018). Plant peptides are generally referred to as proteins with a length ranging from 2 to 100 amino acids originating from a longer precursor with various biological functions (Hu et al., 2018). Taking into account structural similarities and biosynthetic origins, plant peptides can be further subdivided into cysteine-rich peptides, post-translationally modified peptides, and others.

Cysteine-rich peptides such as plant defensins (PDFs), rapid alkalinization factor (RALF), and epidermal pattern factor (EPF), display the highest variability across species regarding their primary sequences and length while sharing a characteristic Cys-rich domain of 2-16 cystein residues (Tavormina et al., 2015). Although mainly known for their inherent antimicrobial activity, Cys-rich peptides were also found to be involved in plant growth, development, and overall immune response regulation.

In contrast to the aforementioned variability in length and the presence of cysteine residues, post-translationally modified peptides comprise a maximum of 20 amino acids and undergo glycosylation, hydroxylation, and Cys or Tyr sulfation (Matsubayashi, 2011) while possessing no or less than two Cys residues. Due to their small size and structural versatility, this class of peptides comprises multifunctional molecules involved in pathogen resistance, e.g. phytosulfokines (PSKs) and plant peptide containing sulfated tyrosine 1 (PSY1) (Mosher et al., 2013), as well as anti-herbivore protection *via* hydroxyproline-rich glycopeptide systemins (HypSys) (Pearce, 2011).

As the previous examples, the third and final group of plant peptides is also processed from large nonfunctional precursors, lacking post-translational modifications and more than 2 Cys residues with an

overall length of 8 to 36 aa. In 1991, Clarence A. “Bud” Ryan and his group isolated the first wounding-responsive peptide of 18 aa length from tomato leaves, which systemically travels through the plant and induces the accumulation of protease inhibitors (PIs) (Pearce et al., 1991). This groundbreaking discovery of the first mobile plant hormone named “systemin” paved the way for the identification of further systemins in other Solanaceae, e.g. black nightshade, bell pepper, and potato (Constabel et al., 1998). Systemin is processed from a 200 aa long precursor predominantly found in the cytosol of the vascular phloem, known as prosystemin (Fig. 1) (McGurl et al., 1992;Ryan and Pearce, 2003). Apart from its regulatory effect on PI’s, systemin was found to induce jasmonic acid (JA), the generation of anti-nutritive proteins, and herbivore-deterring volatiles (Orozco-Cardenas et al., 1993;Degenhardt et al., 2010); therefore triggering a potent local and systemic defense mechanism in the plant.

In 2006, a 23 aa long polypeptide called *At*Pep1 was isolated from *Arabidopsis thaliana*, serving as the first proteinaceous DAMP known in this model plant (Huffaker and Ryan, 2007). Together with six other homologues of this protein family, *At*Pep1 elicited the expression of defense-related genes (Fig. 1) and was shown to alkalinize suspension cell cultures (Huffaker and Ryan, 2007). Following studies highlighted the presence of plant elicitor peptides (Peps) in maize and various other species with the ability to regulate disease resistance (*Zm*Pep1) as well as the induction of anti-herbivore volatiles and JA in the case of *Zm*Pep3 (Huffaker et al., 2011;Huffaker et al., 2013). Although *At*Peps originate from a comparably shorter protein precursor (ProPep; 92 aa) (Huffaker and Ryan, 2007), Peps and systemin share multiple characteristics, e.g. the lack of a putative signal sequence, post-translational modifications, localization in the cytosol, and ultimately the wounding-inducibility, making both classes potent elicitors in plant defense (Narváez-Vásquez and Orozco-Cárdenas, 2008). Intriguingly, the presence of systemin was demonstrated for a variety of solanaceous species; however tobacco responded to wounding with the systemic induction of PI’s, which was independent from the so-far identified systemin (Pearce et al., 2001).

Following this observation, another peptide harboring the ability to induce systemic defense responses was isolated and characterized from tobacco leaves in 2001 (Pearce et al., 2001). Originating from a much larger precursor of 165 amino acids (*Nt*PreproHypSys) (Fig. 1), two forms of the subsequently processed *Nt*Hydroxyproline-rich glycopeptide systemin (*Nt*HypSys; 18 aa) were found to alkalinize suspension cell medium and induce the upregulation of protective trypsin proteinase inhibitors (Pearce, 2001). Studies by Del María et al. (2005) revealed that similar to tomato systemin, *Nt*PreproHypSys was stimulated during wounding and application of jasmonate, resulting in increased resistance against the

herbivore *Helicoverpa armigera* when overexpressed in *Nicotiana tabacum* (Ren and Lu, 2006). However, this enhanced insect defense was restricted to PreproHypSys overexpression in *N. tabacum* whereas *Nicotiana attenuata*-PreproHypSys-OE plants did not display any enhanced protection against herbivores, in this scenario the lepidopteran Manduca sexta (Berger and Baldwin, 2007).

Observations like these raised several questions regarding the herbivore- and plant specificity/compatibility as well as necessary post-translational modifications and peptidase processing within the plant. Apart from functional similarities displayed in their given names, the precursors and the following mature systemin and tobacco HypSys peptides share no sequence homologies. In contrast to systemin, which lacks a putative signal sequence (Narváez-Vásquez and Ryan, 2004), HypSys peptides possess an N-terminal signal sequence, indicating synthesis and transport through the secretory pathway followed by post-translational modifications, e.g. the attachment of hydroxyl groups or pentose residues (Ryan and Pearce, 2003). Regarding the localization of wound-inducible HypSys, studies by Narváez-Vásquez et al. (2005) revealed that the mRNA encoding the precursor of the tomato HypSys (*Le*HypSys 1-3) is synthesized in the phloem parenchyma cells with the functional *Le*HypSys being sequestered in the cell wall and not accumulated in the cytosol. In the ongoing decade, more PreproHypSys orthologs have been discovered in other solanaceous species such as potato (*Solanum tuberosum*) and a *Petunia* hybrid (Pearce and Ryan, 2003;Pearce et al., 2007). In the case of potato and tomato, PreproHypSys induced the synthesis of proteinase inhibitors, increasing their herbivore resistance, whereas HypSys in petunia activated *defensin*, a gene involved in pathogen defense (Thomma et al., 2002). Taken together, all of the aforementioned observations show a species-specific use of HypSys peptides against both herbivores and pathogens.

As the first non-solanaceous species, six highly active HypSys peptides deriving from a 291-aa precursor (*Ib*PreproHypSys) were discovered in sweet potato (*Ipomoea batatas,* Convolvulaceae) after wounding and methyl jasmonate (MeJA) treatment (Chen et al., 2008). All six synthetic peptides were shown to induce the alkalinization of suspension cell medium but only *Ib*HypSysIV purified from sweet potato leaves increased expression levels of *sporamin*, the major defensive trypsin inhibitor in sweet potato. Further studies by Li et al. (2016) unraveled the systemic effect of *Ib*PreproHypSys and the connected signal transduction pathway in overexpression and RNA interference (RNAi) lines in the insect-resistant sweet potato cultivar Tainong 57. After induction by wounding, jasmonate, and H_2O_2, *Ib*PreproHypSys was significantly upregulated whilst resulting in a local and systemic increased expression of defensive *ipomoelin* (IPO) (Li et al., 2016). This effect resulted from the presence of the active peptide *Ib*HypSys, which subsequently activated lignin biosynthesis, *IPO-*, and *IbPreproHypSys* expression. Taken together,

all of these upregulated processes lead to suppressed *Spodoptera litura* growth, highlighting the importance of *Ib*HypSys for the plants' fortification against attacking insects.

Upon the discovery of peptides as amplifiers in plant defense signaling, the question of suitable recognition mechanisms gained increasing attention. Cell surface receptors for *At*Pep1 (PEPR1) (Yamaguchi et al., 2006), *At*Pep2 (PEPR2) (Yamaguchi et al., 2010), and systemin (SYR1 & SYR2) (Wang et al., 2018) were found to be leucine-rich repeat receptor-like protein kinases (LRR-RLK) (Couto and Zipfel, 2016;Tang et al., 2017;Wang et al., 2018;Steinbrenner et al., 2020), able to mediate pathogen and herbivore resistance (Fig. 1). However, the isolation of HypSys receptors remained unsuccessful up to now and further investigations are required.

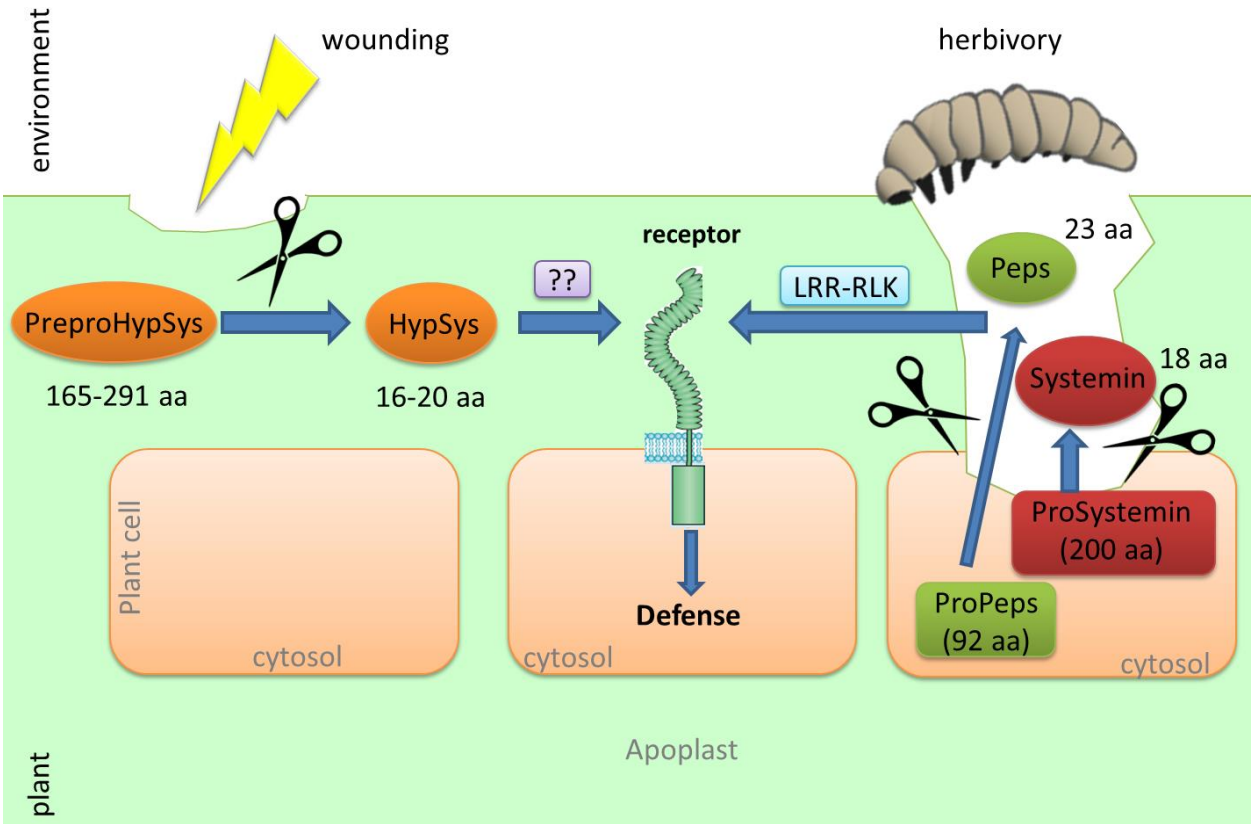

Fig. 1 Peptides triggering plant defense mechanisms. Tissue damage caused by wounding or herbivory activates the processing of hydroxyproline-rich systemin (HypSys) precursors (PreproHypSys) in the apoplast, yielding active HypSys. HypSys is then proposed to bind to cell-surface receptors triggering defense signaling cascades in the plant. Further, upon disruption of cells, cytosolic precursor proteins of systemin (ProSystemin→Systemin) and plant elicitor peptides (ProPeps→Peps) are cleaved into their active forms, which subsequently bind to their respective leucine-rich repeat receptor-like kinase (LRR-RLK). Scheme modified after Albert (2013).

1.2.3 Phytohormones

The role of phytohormones as plant signaling molecules involved in growth and development belongs to the best-studied phenomena in the history of botany. The concept that plant hormones serve as "organ-forming-substances" allowing communication between distant plant parts *via* the sap already evolved from 1758 and over the next centuries, carried further by pioneers such as Duhamel du Monceau, Julius von Sachs, as well as Charles and Francis Darwin (du Monceau, 1758;Sachs, 1882;Darwin, 1897). Since then, multiple plant hormones have been discovered and further investigated, comprising auxin, cytokinins, gibberellins, strigolactones, brassinosteroids, ethylene, abscisic acid, salicylic acid, and jasmonates (Bari and Jones, 2009;Brewer et al., 2013;Verma et al., 2016). Their functional versatility and the fact that the biosynthesis is not restricted to specialized organs or tissues, enables these phytohormones to tightly regulate physiological plant processes in response to external stimuli. This thesis will further highlight the involvement of often stress-related phytohormones such as jasmonates, auxin, abscisic acid (ABA), and salicylic acid (SA) during biotic interactions.

1.2.3.1 Jasmonates

Lipid-derived jasmonates are phytohormones regulating a broad range of biological processes in plants, including plant growth, development, sex-determination, tolerance to abiotic stresses (salt & drought), and the production of secondary metabolites (Ueda et al., 2020). In addition, jasmonic acid (JA; see Fig. 2) as the most prominent example, gained increasing attention as a mediator of plant defense in response to wounding, herbivory (Howe and Jander, 2008;Koo et al., 2009), as well as pathogen infection (Glazebrook, 2005).

The first steps of jasmonate biosynthesis (Fig. 2) occur inside the chloroplast starting from α-linolenic acid. Three enzymatic reactions catalyzed by lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC), convert α-linolenic acid into 12-oxophytodienoic acid (OPDA). Afterwards, OPDA is transported into the peroxisome where the second half of JA biosynthesis occurs. OPDA is then reduced by the OPDA reductase 3 (OPR3) into 8-(3-oxo-2-(pent-2-enyl)cyclopentenyl)octanoic acid (OPC-8:0) (Wasternack and Song, 2017) followed by three β-oxidation steps. During each step, two carbons are removed from the carboxyl side chain, yielding 6-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)hexanoic acid(OPC-6:0), followed by 4-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)butanoic acid (OPC-4:0), and ultimately JA. Subsequently JA is transported into the cytosol and catalyzed by the JA-amino acid

synthetase Jasmonate Resistant 1 (JAR1) into the bioactive form, jasmonoyl-isoleucine (JA-Ile) (Koo et al., 2009;Schaller and Stintzi, 2009;Wasternack and Song, 2017;Griffiths, 2020). Generation of JA-Ile can also occur *via* an OPR3-independent alternative pathway branching off from OPDA in the peroxisome. Chini et al. (2018) demonstrated that JA can be formed through the intermediates dinor-12-oxophytodienoic acid (dnOPDA), tetranor-12-oxophytodienoic acid (tnOPDA), and 4,5-didehydrojasmonic acid (4,5-ddh-JA) after three β-oxidations, followed by reduction of 4,5-ddh-JA to JA by OPR2.

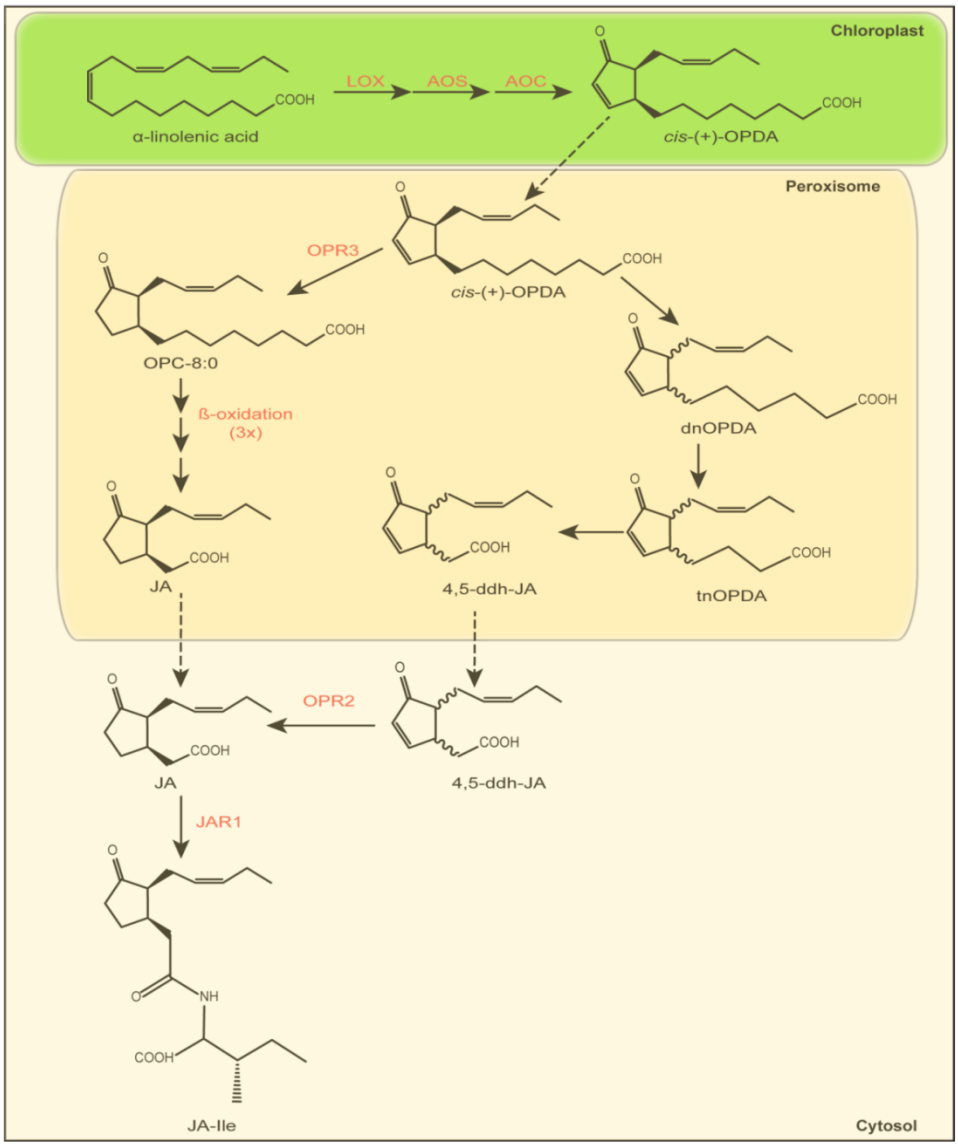

Fig. 2 Jasmonate biosynthesis. This simplified scheme shows the generation of jasmonoyl-isoleucine (JA-Ile) *via* two alternative jasmonic acid (JA) pathways. Enzymatic reactions and corresponding enzymes are indicated in red. Transport between cell compartments is depicted by black dotted arrows. Abbreviations: LOX, Lipoxygenase; AOS, Allene Oxide Synthase; AOC, Allene Oxide Cyclase; OPDA, 12-oxo-phytodienoic acid; dnOPDA, dinor-OPDA; tnOPDA, tetranor-OPDA; 4,5-ddh-JA, 4,5-didehydro-JA; OPR, OPDA Reductase; OPC-8, 8-(3-oxo-2-(pent-2-enyl)cyclopentenyl)octanoic acid; JA, jasmonic acid; JAR, Jasmonate Resistant; JA-Ile, jasmonoyl-isoleucine. Scheme modified after Chini et al. (2018), Griffiths (2020), Wasternack and Hause (2018), and Wasternack and Song (2017).

In the absence of stress, only low levels of JA-Ile are present in the cell (Fig. 3, I) (Hoffmann et al., 2011). In that scenario, MYC transcription factors involved in the transcription of JA-responsive genes, are repressed by the complex Topless (TPL)-Novel Interactor of JAZ adapter proteins (NINJA)-JA-ZIM-domain (JAZ). Upon certain stimuli, e.g. herbivory or wounding, JA-Ile accumulates and binds to the receptor Coronatine-Insensitive (COI1) (Fig. 3, II). COI1 forms together with Arabidopsis S-phase Kinase protein 1 (ASK1), Cullin1 (CUL1), and Ring-Box1 (RBX1) the SCF^{COI1}-E3 ligase complex. Binding of JA-Ile promotes the ubiquitination of the JAZ transcription repressors by the SCF^{COI1} complex, resulting in their degradation by the 26S proteasome (see Fig.3, III). Consecutively, MYC transcription factors are released and lead to the expression of JA-regulated defense- or stress-related genes (Fig. 3, IV).

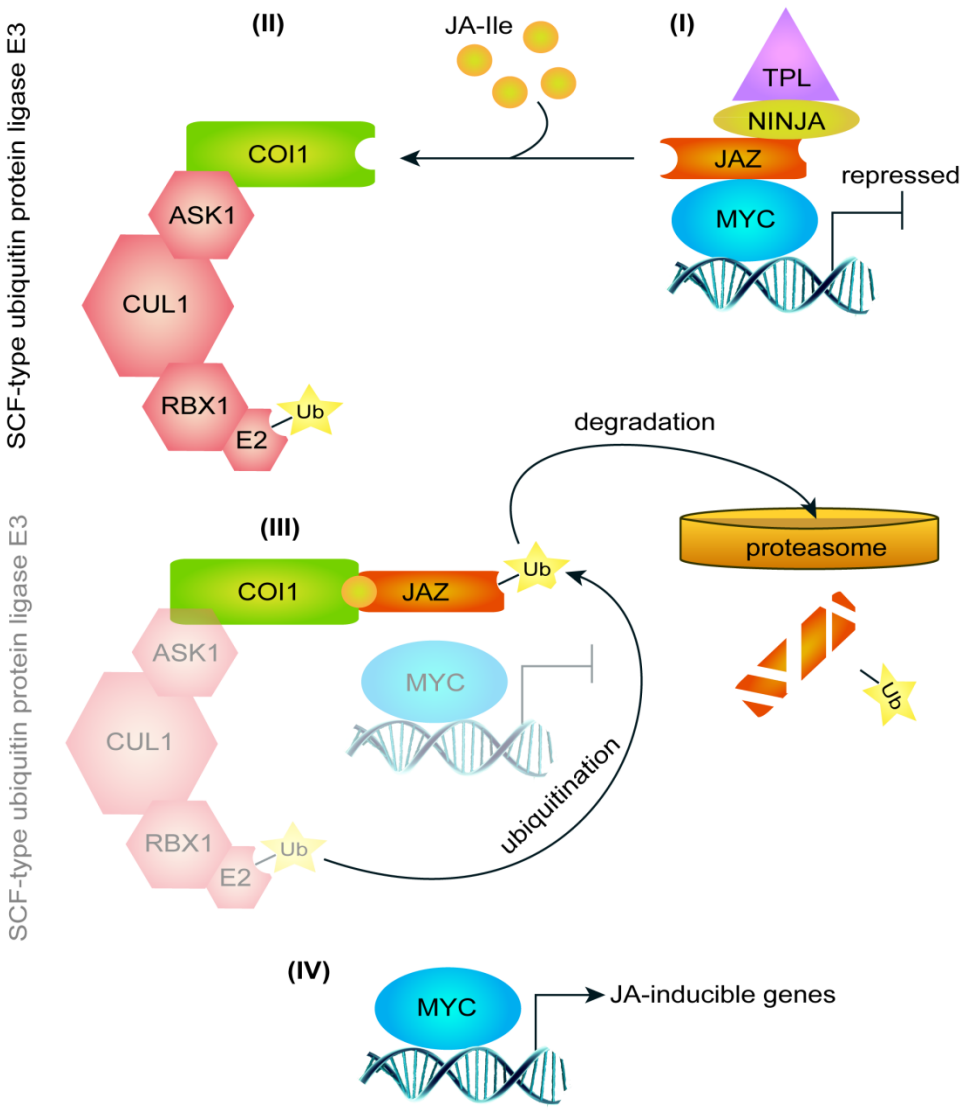

Fig. 3 JA-Ile perception and activation of JA-responsive genes. (I) During low concentrations of active jasmonates, downstream responses are repressed by JA-ZIM-domain (JAZ) proteins, which are recruiting the co-repressor Topless (TPL) through Novel Interactor of JAZ adapter proteins (NINJA). (II) When present at high levels, JA-Ile is perceived by the F-box protein Coronatine-Insensitive (COI1). COI1 forms together with the Arabidopsis S-phase Kinase protein 1 (ASK1), Cullin1 (CUL1), and Ring-Box1 (RBX1) the SCF^{COI1}-E3 ligase multiprotein complex. (III) Interaction with this multiprotein complex mediates ubiquitination (Ub) of JAZ repressors, resulting in their degradation by the 26S proteasome. (IV) Consecutively, the NINJA-TPL complex is released, leading to activation of JA-responsive genes *via* MYC transcription factors. Scheme adapted from Pérez-Alonso and Pollmann (2018).

1.2.3.2 Auxins

Auxin (greek “auxein” = “to grow”) is one of the most studied phytohormones, famously known for its involvement in almost every growth and developmental process throughout a plant’s life (Benjamins and Scheres, 2008). The predominantly occurring form of auxin, indole-3-acetic acid (IAA), regulates a plethora of growth responses, e.g. apical dominance, tropic responses to light and gravity, embryo polarity, and vascular differentiation (Woodward and Bartel, 2005;Brumos et al., 2018). All of the aforementioned processes rely on a tightly controlled auxin homeostasis to ensure proper concentration-dependent responses. Especially the storage of IAA conjugates plays an important regulatory role due to their inhibitory abilities (e.g. IAA-tryptophane) as well as the option to re-hydrolyze them back into free IAA (Ludwig-Müller, 2011).

In plants, IAA was found to be mainly – however not exclusively - synthesized from the aromatic amino acid tryptophan (Trp) *via* the indole-3-pyruvic acid (IPyA) pathway. Trp is first converted to IPyA by tryptophan aminotransferase (TAA) and subsequently metabolized by the flavin monooxygenase YUCCA (YUC) to form IAA (Fig. 4) (Brumos et al., 2014;Morffy and Strader, 2020). Further dissection of the IAA biosynthesis pathways revealed two additional Trp-dependent pathways. The involvement of the cytochrome P450 monooxygenase CYP79B which catalyzes Trp into indole-3-acetaldoxime (IAOx) (Zhao et al., 2002) unraveled the main components of the IAOx pathway, which was initially proposed to occur in crucifers only (Sugawara et al., 2009) (Fig. 4). The detection of the IAOx downstream product indole-3-acetonitrile (IAN) in maize revoked this hypothesis (Bak et al., 1998;Korasick et al., 2013). From IAN, nitrilases (NIT) catalyze the conversion into IAA. An alternative IAOx-derived intermediate is indole-3-acetamide (IAM) (Sugawara et al., 2009) which was proposed to be processed into IAA by AMIDASE1 (AMI1) (Pollmann et al., 2003) within the IAM pathway (Korasick et al., 2013;Brumos et al., 2014) (Fig. 4).

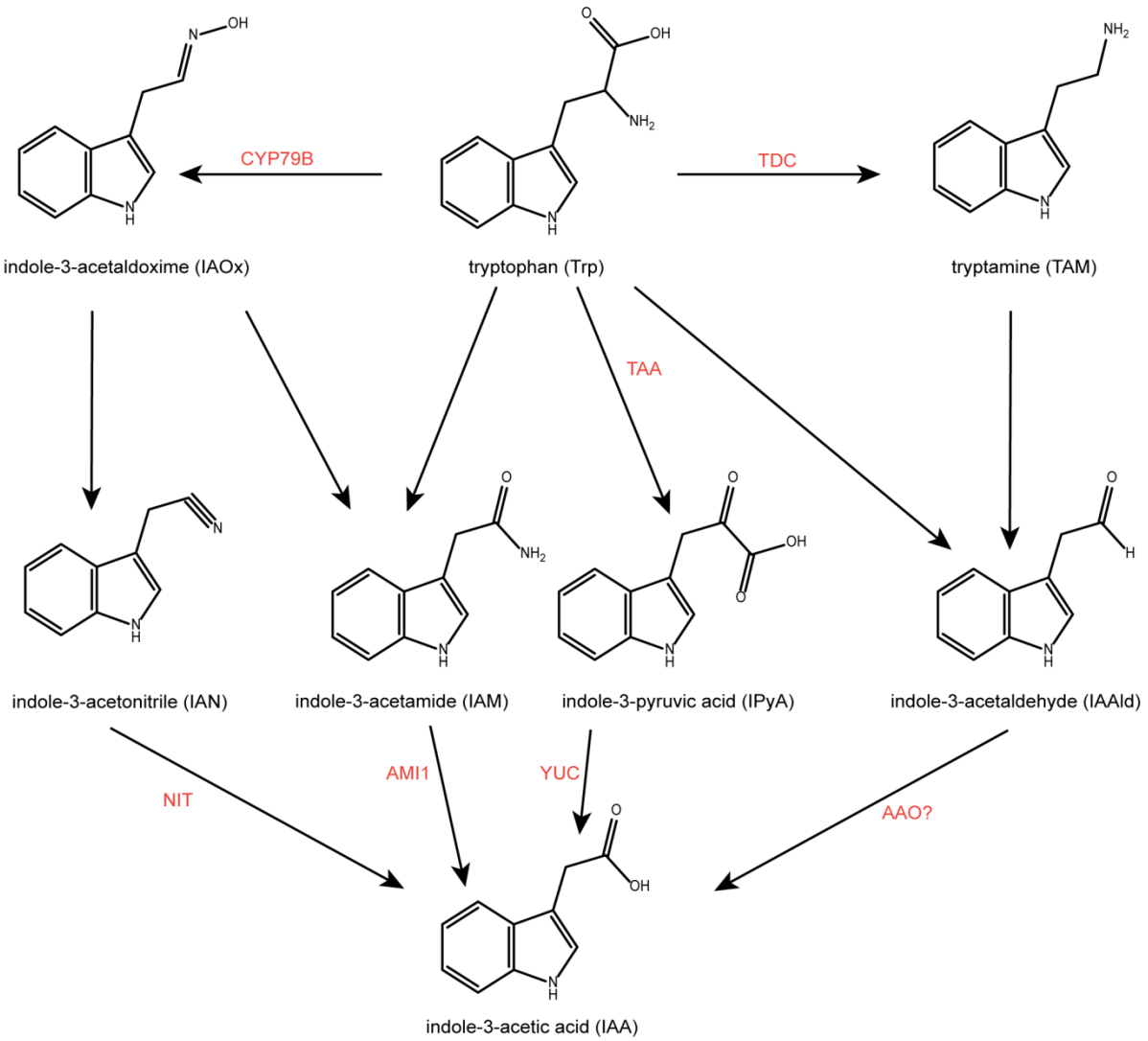

Fig. 4 Tryptophan-dependent IAA biosynthesis. Genetically confirmed biosynthetic steps in land plants are indicated by black arrows with their respective enzymes (red). Abbreviations: CYP, Cytochrome P;TDC, Trp Decarboxylase; TAA, Transferase of Arabidopsis; NIT, Nitrilase; AMI, Amidase; YUC, YUCCA; AAO, Arabidopsis Aldehyde Oxidase. Scheme modified after Morffy and Strader (2020).

The developmental programming of plant organogenesis is largely dependent on the biosynthesis, distribution, and modification of auxin - ultimately determining the signaling output and physiological responses (Ludwig-Müller, 2011, Brumos et al., 2018). Opposed to the classical notion that auxin is mainly produced in the shoot apical meristems, newly developed flower buds and leaves (Teale et al., 2006), more recent studies showed that auxin biosynthesis also locally occurs in the roots (Ljung et al., 2002;Stepanova et al., 2008). Subsequently, auxin is transported from the site of production *via* the phloem (Teale et al., 2006) or in a polar manner from cell to cell *via* auxin influx carriers (AUX1, LAXs), and auxin efflux transporters (PINs, MDR, PGPs) (Grones and Friml, 2015). These directed transport mechanisms lead to the generation of auxin gradients and distinct distribution patterns, forming so-called auxin maxima (Vanneste and Friml, 2009). The formation of these robust gradients dictates the coordination of organ development and plant growth (Zhao, 2010) by converting changes in auxin levels into a transcriptional activation (Chapman and Estelle, 2009).

During low IAA concentrations, auxin response factors (ARFs) are inhibited by auxin/indole-3-acetic acid proteins (AUX/IAA) and their co-repressor Topless (TPL) (Fig. 5, I). In the presence of increased IAA levels, auxin binds to the nuclear auxin receptor Transport Inhibitor Response1 and Auxin signaling F-Box (TIR1/AFB) (Tan et al., 2007) and the AUX/IAA/TPL repressor complex (Fig. 5, II). The auxin-bound proteins are then brought to the SCF^{TIR1}-E3 ubiquitin protein ligase complex (named after their subunits Skp1, Cullin, and F-box protein) (Smalle and Vierstra, 2004;Leyser, 2018) where AUX/IAA is ubiquitinated and degraded by the 26S proteasome (Fig. 5, III) (dos Santos Maraschin et al., 2009). After AUX/IAA degradation, ARFs are released from their inhibition, followed by the activation of auxin-responding genes (Fig. 5, IV) (Leyser, 2018).

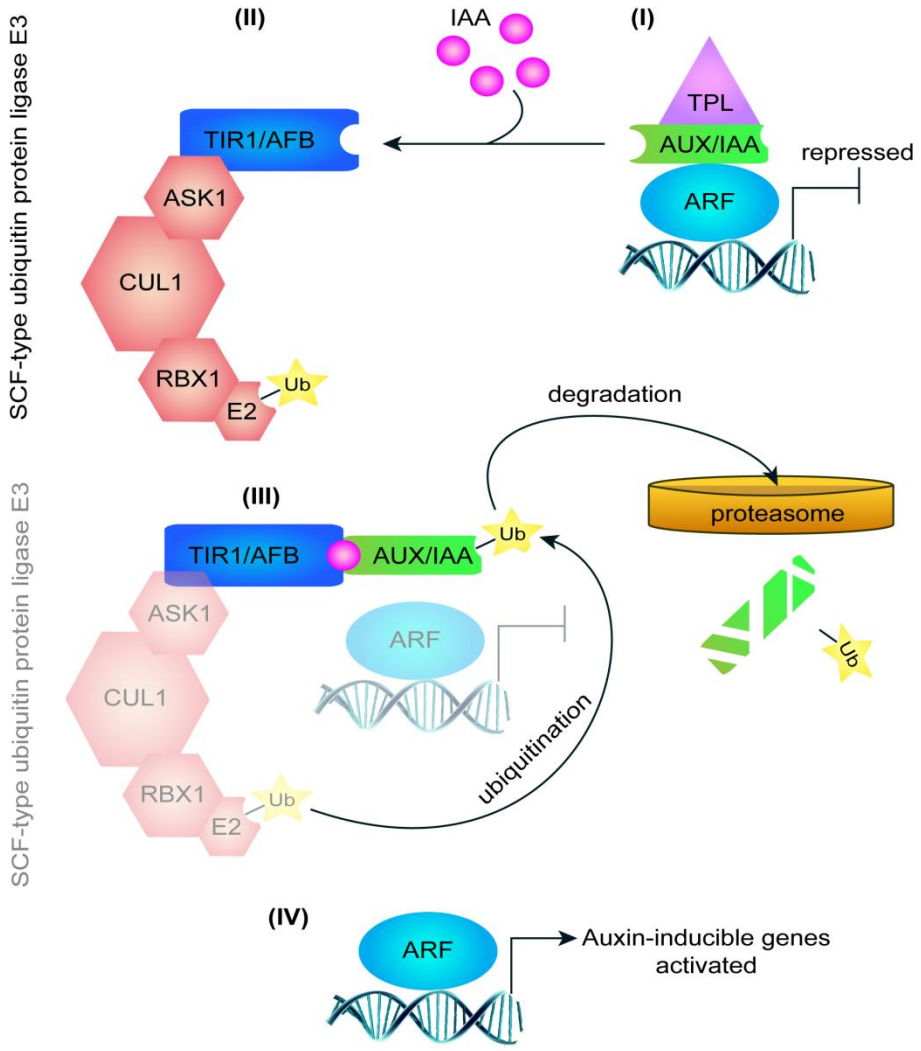

Fig. 5 IAA perception and activation of auxin-inducible genes. (I) In the absence of physiologically relevant IAA concentrations, Auxin Response Factors (ARFs) are repressed by the transcriptional repressor AUX/Indole-3-Acetic Acid (AUX/IAA) and its co-repressor Topless (TPL). (II) Upon increased IAA levels, free IAA binds to the cavity of the F-Box auxin receptor proteins Transport Inhibitor Response1 (TIR1) and Auxin signaling F-Box (AFB) together with AUX/IAA. (III) Similar to the previously described SCF^{COI1}-E3 ligase complex (S-phase Kinase protein 1 = ASK1, Cullin1 = CUL1, Ring-Box1 = RBX1), SCF^{TIR1}-E3 ubiquitinates AUX/IAA which is subsequently degraded by the 26S proteasome. (IV) Release from the AUX/IAA/TPL- repressor complex activates ARFs leading to the transcription of auxin-inducible genes. Scheme based on Pérez-Alonso and Pollmann (2018).

Although auxins were initially investigated as growth-related phytohormones, mounting evidence suggests a significant involvement during anti-herbivore defense and pathogen resistance (Kazan and Manners, 2009;Pérez-Alonso and Pollmann, 2018). Studies by Machado et al. (2016) demonstrated that IAA accumulates locally and systemically in herbivore-attacked *Nicotiana attenuata* plants, preceding local jasmonate bursts while activating biosynthetic YUCCA-like genes. Observations like these weaken the previously widespread notion that (JA-mediated) defense comes along with a trade-off visible in growth inhibition (Karasov et al., 2017). Additional evidence that jasmonate and auxin signaling and biosynthesis pathways are intimately connected was provided by Hentrich et al. (2013), showing that oxylipins can transcriptionally regulate auxin-related YUCCA genes. Reciprocally, Zhang et al. (2016b) demonstrated that members of the Arabidopsis IAA amidohydrolase family are able to metabolize JA-Ile. These findings combined with the striking similarities in the signal perception machineries (Hoffmann et al., 2011) underline the importance of a deeper understanding in hormone crosstalk between JA and IAA during biotic interactions.

1.2.3.3 Abscisic acid and salicylic acid

Apart from JA and IAA, additional phytohormones such as abscisic acid (ABA) and salicylic acid (SA) take part in the regulation of the plants' hormonal network - especially during stress or attack.

Abscisic acid (ABA, Fig. 6, A) is a 15 carbon- sesquiterpenoid which is synthesized from the 40 carbon-precursor β-carotene *via* the carotenoid pathway in the plastids (Nambara and Marion-Poll, 2005). It is a main regulator of abiotic stress responses, particularly drought stress, but also biotic stresses, e.g. pathogen attack. Both threats are met by ABA-mediated closure of the stomata to prevent infection and/or limit water loss (Chen et al., 2020). In response to herbivory or wounding, ABA was shown to act synergistically with JA by positively regulating MYC transcription factors (Abe et al., 2003;Anderson et al., 2004;Kazan and Manners, 2013). Depending on the given stimulus, studies by Anderson et al. (2004) proposed that upon pathogen infection the outcome is rather different as ABA negatively regulates JA. Therefore it has to be clearly distinguished which stress-specific signaling is triggered and how the corresponding phytohormone interactions shape the resulting gene expression changes in the plant.

Salicylic acid (SA; Fig. 6, B) is a phenolic compound, which is synthesized *via* the isochorismate- or the phenylpropanoid pathway (Wildermuth et al., 2001;Dempsey et al., 2011;Li et al., 2019). However, both pathways require chorismate originating from the shikimate pathway (Li et al., 2019). Regarding its functionality, salicylic acid is predominantly known as the antagonist of JA (Pieterse et al., 2012) and the

backbone of plant immune responses against biotrophic pathogens (Glazebrook, 2005;Li et al., 2019). Arabidopsis mutants impaired in SA biosynthesis and signaling displayed a higher susceptibility to specific pathogens (Thomma et al., 1998) which could be restored by the exogenous addition of SA (Gaffney et al., 1993;Wildermuth et al., 2001). Additionally, SA was shown to induce the upregulation of pathogenesis-related (PR) proteins, highlighting its crucial role within local- and systemic acquired resistance (SAR) (Vlot et al., 2009) through the activation of PAMP-triggered (PTI)- and effector-triggered (ETI)- immunity (Li et al., 2019). Depending on invading organism, SA signaling and the SA/JA antagonism can be specifically targeted by the attacker, ultimately rewiring the plants' hormonal machinery. Infection by virulent *Pseudomonas syringae* was shown to suppress SA-mediated immune responses using bacterial effector proteins. Injection of these effectors in combination with the bacterial toxin coronatine, a molecular mimic of JA-Ile, resulted in the suppression of SA-promoted host immunity (Nomura et al., 2005). In contrast to the aforementioned harmful interactions, beneficial microbes can temporarily suppress and reprogram PTI to the plants' benefit. The root-interacting beneficial fungus *Piriformospora indica* was shown to target the JA pathway while counterbalancing SA-defense mechanisms (Jacobs et al., 2011;Pieterse et al., 2012). This step then allowed rapid root-colonization before unfolding of the beneficial plant growth-promoting effect (Aslam et al., 2019). In addition to plant-microbe-interactions, SA also mediates defense mechanisms against herbivores. However, the mode of defense strongly depends on the mode of feeding, e.g. piercing-sucking by whiteflies and aphids (Moran and Thompson, 2001;Zarate et al., 2007) against chewing by lepidopteran caterpillars (Cipollini et al., 2004;Felton and Tumlinson, 2008). Infestation by phloem-feeding aphids triggered SA-related defense whereas wounding and the introduction of elicitors from *Spodoptera* larvae induced JA-mediated protection mechanisms (Vos et al., 2013;Huot et al., 2014).

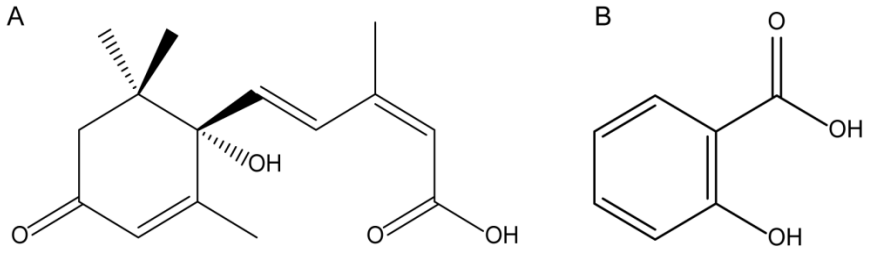

Fig. 6 Chemical structures of the phytohormones abscisic acid (A, ABA) and salicylic acid (B, SA).

Taken together, all of the phytohormones described contribute to an intricate network regulating plant responses throughout their whole life cycle, acting as highly specific chemical signals in dependence of

their combined concentrations. Apart from the aforementioned candidates, the gaseous phytohormone ethylene is also implicated in a plethora of growth, development, and defense-related mechanisms, playing a vital part within the plant's hormonal regulatory network (Kazan, 2015;Light et al., 2016). However, this thesis will focus on the non-volatile phytohormones highlighted in the sections above.

1.2.4 Volatile organic compounds (VOCs)

Volatile organic compounds (VOCs) emitted by plants provide an intriguing example of fast and efficient infochemicals involved in intra- and interspecific plant signaling. VOCs can originate from multiple different pathways with the majority being formed by fatty acid catabolism, isoprenoid/terpenoids, and the phenylpropanoid/benzenoid pathways (Dudareva et al., 2013). After undergoing multiple structural modifications (acetylation, methylation, hydroxylation), VOCs can occur in a tremendous chemical variety with green leaf volatiles (GLVs), terpenoids, phenylpropanoids, or benzenoids as the most well-studied examples (Dudareva et al., 2004;Ameye et al., 2018). Their structural diversity is intertwined with a plethora of functions which can trigger distinct responses in the emitter plant as well as on a community level (Karban and Maron, 2002;Das et al., 2013). *Via* the release of volatiles, plants are able to lure pollinators and seed dispersers towards their scented flowers (Pichersky and Gershenzon, 2002;Burkle and Runyon, 2019), induce distinct defenses in conspecific neighbors (Kost and Heil, 2006), and even guide predators towards infested tissues (Dicke and van Loon, 2000;Kessler and Baldwin, 2001;Van Poecke et al., 2001). This type of communication is not restricted to aboveground interactions, since roots are also able to emit (allelopathic) volatiles (Turlings et al., 2012;Zhang et al., 2012;Schulz-Bohm et al., 2018). The information encoded in the chemical bouquets is strongly dependent on the composition, concentration, and the context in which it is presented (Mumm and Hilker, 2005;Ninkovic et al., 2020). VOCs represent the plant's current physiological status driven by biotic and abiotic stress factors, e.g. the availability of resources, competitors, benefactors, or threats in general. Neighboring individuals can benefit from these constitutively emitted cues or induced signals to enhance their direct resistance against herbivores and pathogens, as well as to prepare for nutrient competition by recruiting beneficial microbes (Ninkovic et al., 2019). Apart from intra- and interspecific information transfer, volatiles harbor the potential to provide a fast and efficient shortcut to rapidly trigger signaling cascades in distant parts of the same plant (Karban et al., 2006;Heil and Bueno, 2007;Heil and Ton, 2008). The possibility to omit time-consuming vascular signaling steps *via* VOCs presents a well-structured mechanism which is implemented by various species (Meents and Mithöfer, 2020). However, the

receptors and downstream signal transduction cascade involved in defense-related VOC perception still remain elusive.

1.3 Aims of this study

Considering the broad toolbox of aforementioned signaling components, each plant as a living organism in a complex environment can tailor its responses in a multitude of different ways. Therefore, scientific research must thoroughly investigate as many combinations of interacting partners, stressors, physiological and chemical readouts as possible. Within this thesis, the complex interplay of several chemical signaling components described in the previous chapters will be addressed in the model organism *Arabidopsis thaliana* and the crop plant *Ipomoea batatas* (Fig. 7).

As one of the fastest signaling mechanisms, the rapid elevation of cytosolic calcium levels marks an excellent starting point for the investigation of stress-induced plant signaling mechanisms and the resulting responses. Over many years, a plethora of studies identified various calcium channels and sensors, including their function concerning the systemic signal propagation throughout connected leaves and tissues. Although the presence of annexins as calcium-binding proteins promoting calcium conductance has been demonstrated in plants, little is known regarding their function during biotic stress, especially herbivory. Wounding and/or insect attack pose as the most common threats triggering distinct local and systemic calcium signatures. Therefore, within this study standardized mechanical wounding (with and without elicitors) and feeding by the generalist *Spodoptera littoralis* (Fig. 7, right panel) were applied to address the following open questions:

1. Does ANNEXIN1 contribute to downstream local and systemic defense responses in *A. thaliana*?
2. Are $[Ca^{2+}]_{cyt}$ elevations, jasmonates, and defense-related gene expressions altered in *ANN1* overexpression- and *ann1* knock-out lines?
3. Are *ann1* knock-out mutants more susceptible to *S. littoralis* attack?

Apart from rapid - but solely within-plant - calcium signatures, wounded plant tissues are able to release distinct volatile organic compounds. These chemical bouquets can be emitted quickly within seconds after wounding and harness the ability to trigger responses on a community level. Volatiles gained increasing attention as eco-friendly resistance boosters within crop protection. Due to their potential as potent intra- and interspecific information transmitters, more and more crop species were investigated regarding their ability to release and perceive volatile blends. However, in many species the exact mechanism and overall potential is still unknown. Because volatiles are able to provide an interesting alternative to time-consuming vascular signaling, especially plants possessing vast and distant organs

structures (e.g. trees, vines), are of major interest to study volatile signaling. Thus, this thesis will discuss the potential of volatiles regarding the following aspects:

1. Can volatile organic compounds be classified as DAMPs and do they provide an applicable alternative to conventional pesticides?
2. Are there cultivar-dependent differences in wounding-induced volatiles within the crop vine sweet potato?
3. What chemical signaling components are regulating anti-herbivore defense in sweet potato (Fig. 7, left panel)?
4. Does trypsin inhibitor activation of sweet potato depend on the presence of jasmonates, peptides, and/or volatiles (Fig. 7, left panel)?
5. Are conspecific sweet potato plants able to communicate via VOCs?

Prior to all aforementioned aboveground interactions, each plant needs to anchor itself with a sophisticated network of roots into the soil, providing vital nutrients and stability. The necessity for efficient resource allocation to ensure optimal growth is mainly driven by a tight regulation of phytohormonal networks in the roots and throughout the whole seedling. As one of the main regulators in root growth and development, auxin is a central target of surrounding organisms (especially root-interacting fungi) for manipulation. These manipulations can be beneficial but also detrimental to the plant, depending on the interacting organism. Although it is widely acclaimed that microbes can utilize and reprogram the plants' auxin machinery, evidence regarding the early stages of infection and its effect on auxin distribution patterns in the root is still lacking for many fungal species. Therefore, this study will further investigate the following aspects:

1. How do beneficial (*Piriformospora indica* & *Mortierella hyalina*) and pathogenic (*Alternaria brassicicola* & *Verticillium dahliae*) fungi influence auxin-responsive genes in transgenic *Arabidopsis thaliana* plants (Fig. 7, right panel)?
2. How fast does infection occur upon exposure to spores or fungal plaques?
3. Does fungal colonization alter phytohormone distribution patterns in the plant?
4. Do fungi produce and accumulate phytohormones in the absence of plants?

This thesis aims to shed light on the complex interplay of these versatile yet specific signaling mechanisms during aboveground herbivore-, as well as belowground microbe interactions.

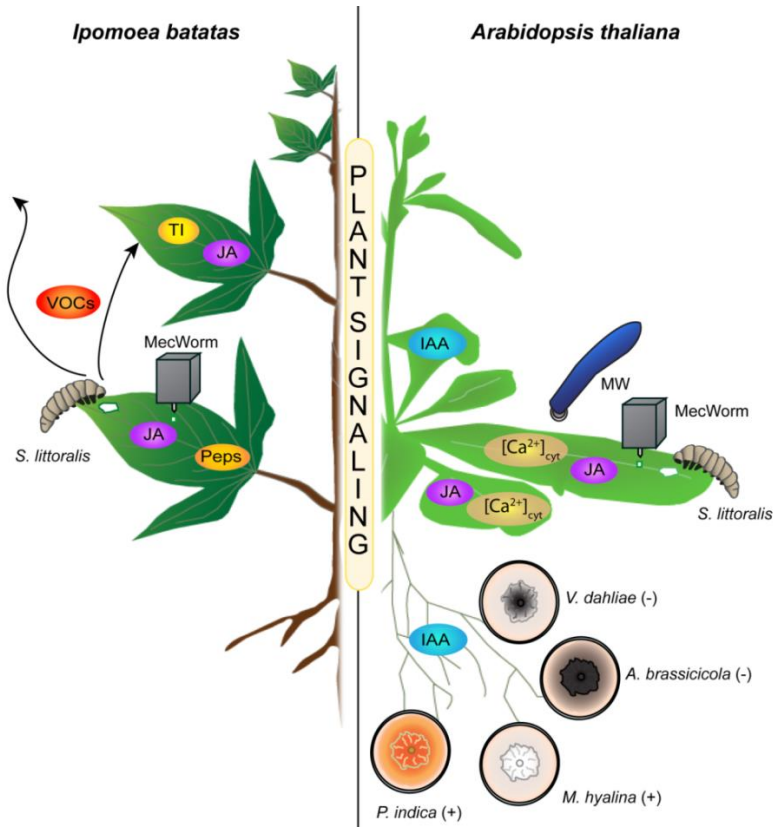

Fig. 7 Overview of investigated chemical signaling components in the two species *Ipomoea batatas* and *Arabidopsis thaliana* upon different types of stress. Left: One part of thesis will study the local and systemic involvement of jasmonates (JA), small signaling peptides (Peps), and volatile organic compounds (VOCs) during mechanical wounding inflicted by the robotic caterpillar MecWorm and herbivore attack by the generalist *Spodoptera littoralis.* As readout, sweet potato-specific signaling cascades resulting in the activation of the defensive trypsin inhibitor (TI) will be investigated. Right: Due to the availability of various transgenic reporter lines, the model plant *Arabidopsis thaliana* poses as an excellent organism allowing the visualization of specific chemical signaling components. Therefore this thesis will also focus on the local and systemic cytosolic calcium ($[Ca^{2+}]_{cyt}$) and jasmonate elevations in Arabidopsis lines expressing the bioluminescent Ca^{2+} reporter aequorin after mechanical wounding by a pattern wheel (MW), MecWorm, or feeding by *S. littoralis.* Investigation of biotic belowground interactions with beneficial (+) and pathogenic (-) root-interacting fungi combined with dual fluorescence reporter lines, will highlight the complex interplay of auxins (IAA) and jasmonates.

2. Manuscripts

2.1 Manuscript 1

2.1.1 Manuscript overview

Manuskript Nr. 1

Titel des Manuskriptes: ANNEXIN1 mediates calcium-dependent systemic defense in Arabidopsis plants upon herbivory and wounding

Autoren: Jaiana Malabarba, Anja K. Meents, Michael Reichelt, Sandra S. Scholz, Edgar Peiter, Julia Rachowka, Dorota Konopka-Postupolska, Katie A. Wilkins, Julia M. Davies, Ralf Oelmüller, Axel Mithöfer

Bibliographische Informationen: New Phytologist (2021), 231(1), 243-254; doi.org/10.1111/nph.17277

Der Kandidat / Die Kandidatin ist:

☐ Erstautor/-in, ☐ Ko-Erstautor/-in, ☐ Korresp. Autor/-in, X Koautor/-in.

Status: Publiziert

Anteile (in %) der Autoren / der Autorinnen an den vorgegebenen Kategorien der Publikation

Autor/-in	Konzeptionell	Datenanalyse	Experimentell	Verfassen des Manuskriptes	Bereitstellung von Material
Jaiana Malabarba	50 %	50 %	50 %	40 %	-
Anja K. Meents	-	30 %	40 %	20 %	-
Michael Reichelt	-	-	10 %	-	10 %
Sandra S. Scholz	-	5 %	-	-	-
Edgar Peiter	-	-	-	-	20 %
Julia Rachowka	-	-	-	-	5 %
Dorota Konopka-Postupolska	-	-	-	-	5 %
Katie A. Wilkins	-	-	-	-	5 %

Julia M. Davies	-	-	-	-	10 %
Ralf Oelmüller	-	5 %	-	-	5 %
Axel Mithöfer	50 %	10 %s	-	40 %	40 %
Summe:	100 %	100 %	100 %	100 %	100 %

New Phytologist

Research

ANNEXIN1 mediates calcium-dependent systemic defense in Arabidopsis plants upon herbivory and wounding

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Summary

- Cellular calcium (Ca) transients are endogenous signals involved in local and systemic signaling and defense activation upon environmental stress, including wounding and herbivory. Still, not all Ca^{2+} channels contributing to the signaling have been identified, nor are their modes of action fully known. Plant annexins are proteins capable of binding to anionic phospholipids and can exhibit Ca channel-like activity. Arabidopsis ANNEXIN1 (ANN1) is suggested to contribute to Ca transport.
- Here, we report that wounding and simulated-herbivory-induced cytosolic free Ca elevation was impaired in systemic leaves in *ann1* loss-of-function plants. We provide evidence for a role of ANN1 in local and systemic defense of plants attacked by herbivorous *Spodoptera littoralis* larvae.
- Bioassays identified ANN1 as a positive defense regulator. *Spodoptera littoralis* feeding on *ann1* gained significantly more weight than larvae feeding on wild-type, whereas those feeding on ANN1-overexpressing lines gained less weight. Herbivory and wounding both induced defense-related responses on treated leaves, such as jasmonate accumulation and defense gene expression. These responses remained local and were strongly reduced in systemic leaves in *ann1* plants.
- Our results indicate that ANN1 plays an important role in activation of systemic rather than local defense in plants attacked by herbivorous insects.

Introduction

Plants are challenged throughout their life by various abiotic and biotic stress factors. These changes in the environment require fast adaptation. Consequently, plants evolved a multi-layered metabolic barrier, composed of mechanical and chemical defenses (Maffei *et al.*, 2012; Mithöfer & Boland, 2012). An attack by herbivorous insects represents a major threat to the plant's survival. In particular, an attack by chewing insects is a combination of plant tissue wounding and application of insect-specific herbivore-associated molecular patterns, mainly present in their oral secretions (OSs; Mithöfer & Boland, 2008; Vadassery *et al.*, 2012b; Kiep *et al.*, 2015). The establishment of chemical defenses to such an insect herbivory is mediated by a network of signaling pathways (including calcium (Ca^{2+}) ions, protein phosphorylation, phytohormones, and reactive oxygen species (ROS) and reactive nitrogen (N) species) that finally initializes synthesis and accumulation of a plethora of defensive metabolites (Seybold *et al.*, 2014; Zebelo *et al.*, 2014). The elevation in cytosolic free Ca, $[Ca^{2+}]_{cyt}$, is one of the earliest signaling events initiated upon the plant's interaction with feeding insects (Maffei *et al.*, 2004; Kiep *et al.*, 2015; Toyota *et al.*, 2018). Jasmonates represent the most important class of wound-induced phytohormones to be activated, with the main components being jasmonic acid (JA) and its biologically active isoleucine conjugate (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile) (Wasternack, 2007; Mithöfer & Boland, 2008, 2012). The connection between jasmonates and $[Ca^{2+}]_{cyt}$ is likely mediated by Ca^{2+}-sensing proteins. In plants, canonical calmodulins, calmodulin-like proteins (CMLs), calcineurin B-like proteins, and Ca^{2+}-dependent protein kinases are good candidates (Swarbreck *et al.*, 2013; Yan

et al., 2018; Mohanta *et al.*, 2019; Tai *et al.*, 2019). In particular, a connection between $[Ca^{2+}]_{cyt}$ and jasmonate signaling has been shown for CML42 and CML37 (DeFalco *et al.*, 2010; Vadassery *et al.*, 2012a,b; Scholz *et al.*, 2014, 2016; Heyer *et al.*, 2018b).

However, upstream of jasmonates, Ca^{2+}-sensing proteins, and $[Ca^{2+}]_{cyt}$ changes, an influx of Ca^{2+} is necessary to cause $[Ca^{2+}]_{cyt}$ elevations. Here, the opening of ion channels localized at the plasma membrane or intracellular compartments is involved. In Arabidopsis, the ligand-gated channels glutamate receptor (GLR)-like channels and cyclic nucleotide-gated channels (CNGCs) plus the potentially stretch-activated Ca^{2+} channels reduced hyperosmolality-induced Ca^{2+} increase and MID1-complementing activity are the four main families of plasma membrane Ca^{2+}-permeable channels (Dodd *et al.*, 2010; Yuan *et al.*, 2014). In addition, the vacuolar two-pore channel 1 (TPC1) is localized at the tonoplast (Peiter *et al.*, 2005; Peiter, 2011).

Strikingly, Arabidopsis senses local herbivore attack and transmits this information to unwounded vascular-connected systemic leaves through a long-distance signaling system (Mousavi *et al.*, 2013; Kiep *et al.*, 2015). Systemic signaling has also been identified leading to activation of jasmonate accumulation and signaling in distal leaves (Mousavi *et al.*, 2013; Heyer *et al.*, 2018b). Recently, it has been shown that wound-induced electrical signals precede vascular Ca^{2+} fluxes as xylem contact cells and phloem sieve elements function together for leaf-to-leaf electrical signaling (Nguyen *et al.*, 2018). Probably, the systemic electrical signaling is mediated by GLR-type cation channels, because in *glr3.3 glr3.6* double mutants the wound-activated electrical signal propagation, as well as propagation of $[Ca^{2+}]_{cyt}$ signals between leaves, is attenuated (Toyota *et al.*, 2018). In addition, TPC1 was shown to be involved in systemic $[Ca^{2+}]_{cyt}$ elevations (Kiep *et al.*, 2015). This channel trio of GLR3.3, GLR3.6 and TPC1 also operates in local $[Ca^{2+}]_{cyt}$ elevations induced by aphid feeding (Vincent *et al.*, 2017). Very recently, it was demonstrated that a rapidly activated cyclic nucleotide-gated Ca^{2+} channel (CNGC19) also plays a partial role in wounding-induced Ca^{2+} influx (Meena *et al.*, 2019). Thus, it becomes increasingly evident that herbivory-induced $[Ca^{2+}]_{cyt}$ elevation involves multiple channels and pathways regulating local and long-distance $[Ca^{2+}]_{cyt}$ signals.

Nevertheless, some studies showed that conventional channels might not always be responsible for Ca^{2+} influx pathways. Thus, the involvement of other passive Ca^{2+} transport-mediating proteins, such as annexins, becomes an interesting possibility (Laohavisit & Davies, 2009, 2011; Davies, 2014; Ma *et al.*, 2019).

Annexins are found in eukaryotic organisms and form a diverse multigene superfamily of Ca^{2+}-dependent membrane-binding proteins that serve as targets for Ca^{2+} in most eukaryotic cells. In angiosperms, annexins are found in vegetative and generative organs (Laohavisit & Davies, 2011; Clark *et al.*, 2012). They are composed of motifs 60–70 amino acids long, repeated four times. The ability of annexins to conduct Ca^{2+} has become evident from *in vivo* and *in vitro* assays (Demidchik & Maathuis, 2007; Laohavisit *et al.*, 2009, 2012; Richards *et al.*, 2014; Ma *et al.*, 2019). Unlike conventional Ca^{2+} channels, which are routed from the Golgi complex to reside in a specific membrane, annexin proteins are able to occupy multiple cellular locations simultaneously. This characteristic makes annexins capable of a fast-recruitment response that can be driven by localized stimulation of membrane regions and might be independent of vesicular delivery – reviewed by Laohavisit & Davies (2009, 2011) and Clark *et al.* (2012).

Among the eight annexins described to date in *Arabidopsis thaliana*, ANNEXIN1 (ANN1) is the best-studied one. It was initially detected in the cytosol of cells, and later in the plasma membrane, endoplasmic reticulum, vacuole, mitochondria, chloroplast, and in the cell wall (Laohavisit & Davies, 2011). *ANN1* overexpression has a protective effect on plant survival under drought conditions, whereas lack of expression increases stress sensitivity (Konopka-Postupolska *et al.*, 2009). Studies on Arabidopsis roots have correlated its localization in the plasma membrane with the presence of a Ca^{2+} conductance, which is activated by voltage hyperpolarization and extracellular hydroxyl radicals and is involved in the elongation of root hair cells (Foreman *et al.*, 2003; Laohavisit *et al.*, 2012). The Arabidopsis *ann1* knockout mutant lacks this Ca^{2+}-channel-like activity in the plasma membranes of root epidermal cells and root hairs. Furthermore, *ann1* mutants also have shorter roots compared to wild-type (Columbia-0, Col-0) plants (Laohavisit *et al.*, 2012). More recent studies have shown that ANN1 is involved in root and seedling $[Ca^{2+}]_{cyt}$ elevation in response to hydrogen peroxide (Richards *et al.*, 2014; Zhao *et al.*, 2019).

As ANN1 is firmly implicated in $[Ca^{2+}]_{cyt}$ elevation and this occurs during insect feeding, our aim was to elucidate a putative role for this annexin in plant responses to herbivory-related cues. Therefore, we performed a set of assays to characterize *ann1* mutants. The effect of the lack of ANN1 was analyzed by observing larval growth of the crop-pest moth *Spodoptera littoralis* on two different *ann1* knockout and two ANN1-overexpressing lines. Further, the plant's response to mechanical injuries (i.e. mechanical wounding with and without the addition of larval OS) and after lesions caused by *S. littoralis* feeding on leaves was investigated. We found a role for ANN1 in Arabidopsis for both local and systemic defense responses against *S. littoralis* attack by mediating $[Ca^{2+}]_{cyt}$ elevations, jasmonate level, and defense-related gene expression. This study contributes to our understanding of the molecular identity of Ca^{2+} channels involved in the plant response to wounding and herbivory.

Materials and Methods

Plant growth and treatment

Arabidopsis thaliana Col-0 wild-type, *ANN1* knockout mutant lines *ann1-1* (SALK_015426 originally characterized by Lee *et al.* (2004) and *ann1-2* (WiscDsLox477–480P11), and *ANN1*-overexpressing lines *ANN1-OE10* and *ANN1-OE12* (Konopka-Postupolska *et al.*, 2009) were used. For $[Ca^{2+}]_{cyt}$ measurements, Col-0 and *ann1-1* were transformed by floral dip to express cytosolic (apo)aequorin driven by the 35S promoter. Floral dip employed *Agrobacterium tumefaciens* GV3101 pNEW 35S::AEQ

pGreen vector containing the 35S::AEQ insert cut from pMAQ (Knight *et al.*, 1991). All plants used in aequorin assays were at least three generations posttransformation.

Plants were kept in short-day conditions after stratification for 2 d at 4°C. Four to five-week-old plants grown in 10 cm round pots were used for all experiments. The growth chamber was adjusted to 50–60% relative humidity and 21°C with a 10 h : 14 h, light : dark photoperiod and a light intensity of 100 μmol m^{-2} s^{-1}. For experiments investigating the systemic response and translocation of metabolites, the leaves of each plant were counted according to their age (Dengler, 2006; Farmer *et al.*, 2013; Kiep *et al.*, 2015).

MecWorm (Mithöfer *et al.*, 2005) treatment was used for mechanical wounding of the plant with punches every 5 s (12 punches per minute) on treated leaf 8. To investigate the systemic response upon treatment of leaf 8, the local and systemic leaves 5, 8, 9 and 13 were analyzed. Untreated plants were used as control and had the same growth and handling conditions as the treated ones.

To study the mechanical-wounding-induced systemic response, wounds were generated on leaf 8 with a pattern wheel (six vertical movements on each side of the midrib), and 20 μl of water (MW + W: mechanical wounding + water) or of *S. littoralis* OS, diluted 1 : 1 in water (MW + OS) was applied to the wounds (Vadassery *et al.*, 2012a). Treated plants were kept in the growth chamber with a cover to prevent evaporation. Samples of leaf 8 and selected systemic leaves were harvested in liquid N_2 and kept at −80°C till further analysis.

Analysis of $[Ca^{2+}]_{cyt}$ elevations

For the analysis of $[Ca^{2+}]_{cyt}$ in whole plants, leaves of 4-wk-old Arabidopsis rosettes were numbered according to their phyllotactic sequence (Dengler, 2006). The day before the experiment, plants were sprayed with 10 μM coelenterazine in 0.01% (v/v) Tween 20 and incubated in the dark for 16 h for aequorin reconstitution. Aequorin imaging was performed according to Kiep *et al.* (2015) using a high-resolution photon-counting camera system (HRPCS218; Photek, St Leonards-on-Sea, UK) comprising an intensified CCD camera (ICCD218; Photek) and a camera controller (HRPCS4; Photek). The camera was mounted on a darkbox (DB-2; Photek). Signal acquisition and processing were performed with the IFS32 software (Photek). Photons were captured in photon-counting mode with a 200 ms frame rate, and cumulative images were integrated offline after the experiments as indicated in the figure legends. At the end of each treatment, the rosettes were flooded with 40 ml discharge solution (1 M calcium chloride, 10% (v/v) ethanol) to achieve a complete discharge of aequorin to enable calibration of the data obtained and determine the cytosolic Ca^{2+} concentration according to Knight *et al.* (1996). The identical regions of interest (ROIs) found in treatment and discharge images were identified, and the average signal intensity in the ROIs at a given time point, as well as the cumulative counts in the ROIs, were determined by using the IFS32 software. The wounding treatment consisted of mechanically wounding the midrib of leaf 8 with a pattern wheel and adding 20 μl of water (MW + W) or 20 μl of OS (MW + OS) across all holes of the mechanically wounded leaf.

Insect material and feeding assays

Larvae of the generalist herbivore *S. littoralis* were hatched and reared on artificial diet at 23–25°C with 10 h : 14 h, light : dark cycles (Bergomaz & Boppré, 1986). The OS was collected from *S. littoralis* larvae fed on *Arabidopsis* Col-0 plants and stored on ice. The OS was centrifuged at 10 000 ***g*** and 4°C to remove residual plant tissue pieces and then diluted with water (1 : 1) as previously described (Vadassery *et al.*, 2012a).

For short-term feeding assays, third instar *S. littoralis* larvae were used after being kept separately without food overnight. This treatment ensured an immediate start of feeding after placement on the plant. The locally fed leaves were collected in liquid N_2 after the indicated time points and kept at −80°C until further analysis.

For local larval feeding assays, overnight-starved third-instar larvae were placed on leaf 8 for direct feeding. Each plant received one larva. After feeding on *c.* 40% of the leaf, which took between 5 and 10 min, the larva was removed. After 90 min, the local and systemic leaves were harvested for phytohormone extraction and gene expression analysis.

For 1 wk feeding assays, 30 first-instar larvae were placed on each of 10 Col-0, *ann1* and *ANN1-OE* plants (three larvae per plant). To achieve similar starting conditions, all larvae determined for one plant genotype were pooled and weighed before the experiment. The minimal starting weight of 30 larvae was set to 60 mg. After 1 wk, the weight of all larvae found again was recorded separately. Owing to a limited number of first instar larvae available, the experiment was carried out several times and the weight data of each genotype were combined.

Gene expression

Total RNA was extracted from frozen material (*c.* 50–100 mg) using the Trizol method according to the manufacturer's protocol (Thermo Fisher, Darmstadt, Germany). Genomic DNA in total RNA samples was removed using the Turbo DNAse-free kit (Ambion, Thermo Fisher) according to the manufacturer's protocol. The integrity and amount of RNA were monitored by agarose gel electrophoresis and spectrophotometric quantification, respectively. Complementary DNAs were synthesized using the GeneAmp Core PCR RNA Kit (Applied Biosystems) according to the manufacturer's instructions. The pair of primers specific for *ANN1* (AT1G35720: forward 5′-ATGGCGACTCTTAA GGTTTCTGAT-3′ according to Clark *et al.* (2001) and reverse 5′-GCCTGATGACTTTCCTCTGTTCAG-3′) was used, producing a product size of 151 bp. For *VEGETATIVE STORAGE PROTEIN2* (*VSP2*; AT5G24770) we used forward 5′-ACGACT CCAAAACCGTGTGCAA-3′ and reverse 5′-CGGGTCGGT CTTCTCTGTTCCGT-3′ (Vadassery *et al.*, 2012b), and for *JASMONATE-ZIM-DOMAIN PROTEIN 10* (*JAZ10*; AT5G13220) we used forward 5′-TCGAGAAGCGCAAGGA GAGATTAGT-3′ and reverse 5′-AGCAACGACGAAGAAGG

CTTCAA-3′ (Scholz *et al.*, 2014). Quantitative reverse transcription PCR was performed on a CFX96 Real Time System (Bio-Rad). Brilliant II QPCR SYBR green Mix (Agilent, Böblingen, Germany) was used to monitor the synthesis of double-stranded DNA. Each biological sample was analyzed in technical triplicates. The cycle protocol consisted of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, and ended by a dissociation curve determined between 60°C and 95°C. The specificity of PCR amplifications was evaluated by the presence of a single peak in denaturation curves and by visualization of simple amplification products of expected size in ethidium bromide gel electrophoresis. The primer efficiencies were calculated using LinRegPCR (v.11.0, (Ruijter *et al.*, 2009). The mean relative expression of the gene was calculated according to Pfaffl (2001), using $\Delta\Delta C_t$ with the *ribosomal protein S18* gene (AT1G34030) as reference (Scholz *et al.*, 2014) (*RPS18*, forward 5′-GTCTCCAATGCCCTTGACAT-3′; reverse 5′-TCTTTCC TCTGCGACCAGTT-3′).

Extraction and quantification of phytohormones

A total of 250 mg of leaf material was used for phytohormone analyses. The extraction procedure and the determination of JA and JA-Ile were performed as previously described (Vadassery *et al.*, 2012a) with some modifications. An API5000 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) was used for detection (Heyer *et al.*, 2018a). Moreover, in this study, a different mixture of labeled jasmonates was used as internal standard. Instead of 15 ng of JA-[$^{13}C_6$]-conjugate used in the previous study, 12 ng of D_6-JA-Ile (HPC Standards GmbH, Cunnersdorf, Germany) was used. In addition, the 60 ng of 9,10-D_2-9,10-dihydrojasmonic acid was replaced by 60 ng of D_6-JA (HPC Standards GmbH) as previously reported by Scholz *et al.* (2017).

Statistics

To ensure reproducibility, all experiments were repeated with independent biological replicates. The exact number of replicates is indicated in the particular figure legends. For statistical analyses, one or two-way ANOVA followed by *post hoc* (Student–Newman–Keuls; Šidák; Tukey) tests or Student's *t*-tests were used as indicated in the figure legends. Different letters indicate significant differences between treatments or leaves. GraphPad Prism 6 and OriginPro 9.3 software were used for data analysis and graph composition.

Results

ANNEXIN1 is induced by mechanical wounding and herbivory

Initially, in order to learn whether ANN1 was involved in the plant's defense response against wounding and herbivory, we implemented different wounding-related treatments on Col-0 and *ann1-1* knockout plants and then analyzed *ANN1* expression levels. Different treatments were performed on leaf number 8: first, mechanical wounding (MW) with a pattern wheel and applying water (MW + W) or OS of *S. littoralis* (MW + OS) simulating herbivory; and second, direct feeding of *S. littoralis* larvae on one leaf. Col-0 plants showed a high accumulation of *ANN1* transcripts after all treatments, whereas in the *ann1-1* mutant no induction of *ANN1* was detected (Fig. 1). No significant difference between the different treatments was detected (Fig. 1a), suggesting a universal response of *ANN1* expression in response to mechanical wounding, with or without the presence of larval OS, and to herbivore attack.

Local and systemic calcium signaling is affected in *ann1* plants

Since, on the one hand, a $[Ca^{2+}]_{cyt}$ signal precedes jasmonate accumulation and subsequent defense-related responses upon wounding and herbivory (Fisahn *et al.*, 2004; Maffei *et al.*, 2007; Bricchi *et al.*, 2010; Toyota *et al.*, 2018; Meena *et al.*, 2019) and, on the other hand, annexins are components of $[Ca^{2+}]_{cyt}$ signal generation, we aimed to investigate how the elevation of $[Ca^{2+}]_{cyt}$ upon stress is influenced by the presence or absence of the ANN1 protein. Therefore, we used Col-0 and *ann1-1* plants, both containing the $[Ca^{2+}]_{cyt}$ reporter (apo)aequorin. The $[Ca^{2+}]_{cyt}$ elevation was analyzed in whole-plant rosettes according to Kiep *et al.* (2015). Leaf 8 was wounded with a pattern wheel before adding

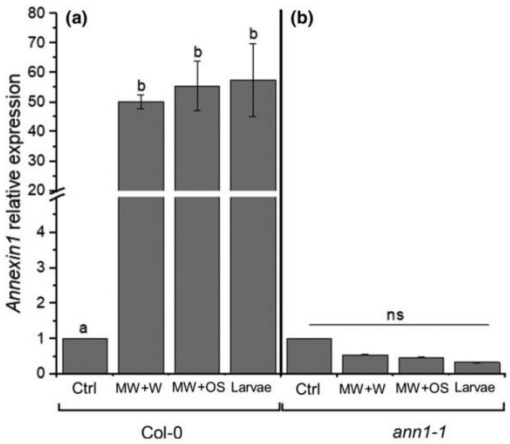

Fig. 1 Response of *ANNEXIN1* (*ANN1*) in *Arabidopsis thaliana* after treatment with different stresses. Levels of *ANN1* transcripts (± SE) were determined on (a) the wild-type (Col-0) and (b) the *ann1-1* mutant genotype after injuring leaf 8 with a pattern wheel, applying either water (MW + W) or oral secretion (MW + OS) to the wounds, or promoting *Spodoptera littoralis* larvae feeding (Larvae, third instar) feeding on leaf 8 until *c.* 40% of the leaf was eaten. Per genotype and treatment, nine replicates ($n = 9$) were done. All plants were incubated for 90 min before sampling leaves. Leaves of untreated plants were used as controls (Ctrl). Differences between treatments within the same genotype were analyzed using two-way ANOVA (Student–Newman–Keuls *post hoc* test); significant differences are indicated by different letters ($P < 0.05$); ns, not significant

20 µl water or OS (1 : 1 diluted) to the wounds. We observed an immediate and monophasic local elevation of $[Ca^{2+}]_{cyt}$, with a peak after *c.* 30–45 s, and a return to background levels after *c.* 4 min (Fig. 2; Supporting Information Fig. S1; Videos S1–S4). The local response was similar to those described in previous studies, but presenting a slightly faster $[Ca^{2+}]_{cyt}$ peak response (cf. 1 min: Verrillo *et al.*, 2014; Kiep *et al.*, 2015). In the case of MW + OS treatment, the Col-0 $[Ca^{2+}]_{cyt}$ elevation lasted longer than with the MW + W (water) treatment. Although slightly weaker than in the Col-0 plants, the local $[Ca^{2+}]_{cyt}$ response was clearly detectable in the wounded *ann1-1* leaf and moved into the petiole. Strikingly, neither water nor OS induced a systemic $[Ca^{2+}]_{cyt}$ response in the *ann1-1* genotype (Figs 2, S1). By contrast, upon MW + W treatment, a systemic $[Ca^{2+}]_{cyt}$ response (also monophasic) was observed in Col-0 plants (systemic leaf 5), which started after 4 min and reached a maximum at 4.5 min before decreasing to background levels (Fig. S1). Upon MW + OS treatment, the systemic response was more pronounced. Here, leaves 5, 6, 7, 10 and 11 responded in Col-0 (Fig. 2c). This response was highly significantly different when compared with *ann1*. It was possible to observe a $[Ca^{2+}]_{cyt}$ wave running from the treated leaf to the connected leaves in Col-0 (Videos S1, S3), whereas only the local response was detected in treated *ann1* plants (Videos S2, S4). Therefore, ANN1 seems to be an important player in the systemic $[Ca^{2+}]_{cyt}$ wave upon wounding and herbivory challenge.

ann1 plants are more susceptible to herbivore feeding, whereas *ANN1*-overexpressing plants are more resistant

To further evaluate the impact of ANN1 on the defense against chewing herbivores, we carried out feeding assays using first instar *S. littoralis* larvae on Col-0 wild-type plants and two different knockout lines (*ann1-1*, *ann1-2*) and two different *ANN1* overexpressor lines (*ANN1-OE12*, *ANN-OE10*). We performed two independent sets of experiments, each with Col-0 as wild-type control, one knockout, and one overexpressor line. Our results show that larvae feeding on *ann1* plants gained significantly more weight than wild-type-fed larvae (Fig. 3). This effect was also observed when we evaluated the larval growth on the APOAEQUORIN-containing *ann1* mutant (*ann1-1/AEQ*) (Fig. S2). The opposite happened when the larvae were feeding on the *ANN1-OE* plant lines, where they gained significantly less weight than those feeding on Col-0 plants (Fig. 3).

Spodoptera littoralis feeding-induced jasmonate accumulation is affected in *ANN1* mutant plants

Jasmonates are rapidly induced upon wounding and herbivory. To test if these were differentially induced in *ann1* or *ANN1-OE10* lines compared with the Col-0 wild-type, we analyzed jasmonate concentrations after herbivore feeding. In independent short-term (30, 90 min) feeding assays, the levels of both JA and JA-Ile increased significantly in the treated leaves of all genotypes at both time points (Fig. 4). Even fed leaves of *ann1-1* and *ann1-2* plants still accumulated both JA and JA-Ile; however, their levels were significantly lower than in Col-0 plants (Fig. 4). By contrast, in the overexpressing line *ANN1-OE10* the level of JA-Ile was significantly higher than in Col-0, although the JA level was not (Fig. 4c,d).

Systemic transcriptional responses to *S. littoralis* attack are impaired in *ann1-1* plants

In order to elucidate whether ANN1 is also involved in systemic defense, the effect of herbivory on local and systemic defense

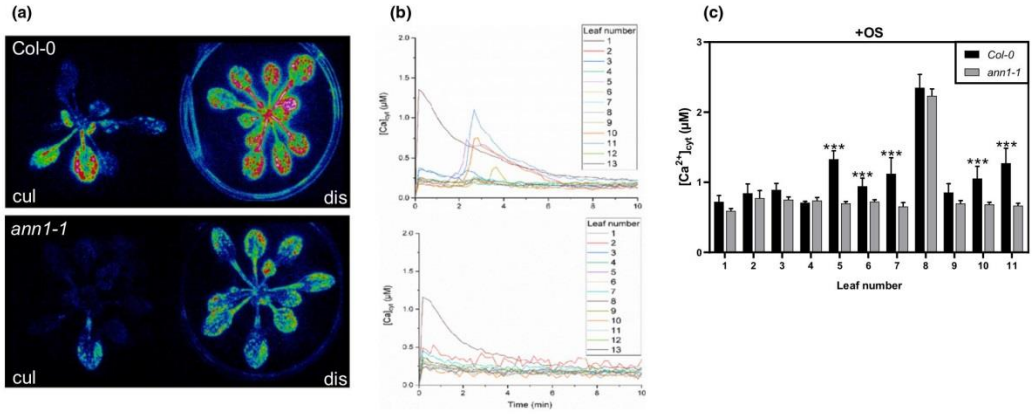

Fig. 2 Cytosolic free calcium ($[Ca^{2+}]_{cyt}$) response upon mechanical wounding (MW) and oral secretion (OS) in Col-0 and annexin1 mutant (*ann1-1*). (a) Cumulative images over 10 min of local and systemic $[Ca^{2+}]_{cyt}$ signals in 4-wk-old whole *Arabidopsis thaliana* Col-0 (top) and *ann1-1* (bottom) rosettes in response to MW + OS applied to leaf 8 (left) and after discharge (right). Discharge was used to determine L_{total} to calculate the $[Ca^{2+}]_{cyt}$ in individual leaves. (b) Kinetics of a representative $[Ca^{2+}]_{cyt}$ response in individual leaves in Col-0 and *ann1-1* after treatment. (c) $[Ca^{2+}]_{cyt}$ response (mean ± SE) in individual leaves in Col-0 and *ann1-1* after treatment. Per genotype and treatment, nine replicates ($n = 9$) were done. cul, cumulative; dis, discharge. Two-way ANOVA (Sidak *post hoc* test; ***, $P < 0.001$).

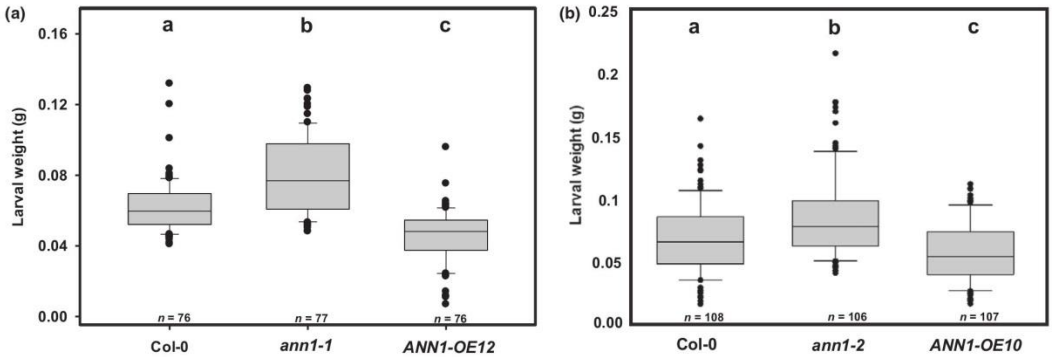

Fig. 3 Weight of *Spodoptera littoralis* larvae after feeding on *Arabidopsis thaliana* Col-0 plants, two annexin1 (*ann1*) mutants, and two *ANN1*-overexpression lines. Thirty first-instar larvae of *S. littoralis* were preweighed, and three larvae were placed on each plant. In each independent experiment, 10 plants per genotype were used. Larva weight was measured individually after 7 d of feeding. Each set of experiments was independently repeated: (a) $n = 3$; (b) $n = 4$. The combined total number of larvae n recovered after 1 wk is indicated. The box indicates the middle 50% of the data points; the black line within the box is the median. Whiskers are defined as 1.5-fold interquartile range; dots represent outliers. Statistical differences between the genotypes after feeding were analyzed using one-way ANOVA (Tukey's *post hoc* test) and indicated by different letters ($P < 0.001$).

responses was analyzed in parallel, focusing on the full knockout line *ann1-1* (Fig. 1b). We performed an experiment in which *S. littoralis* was allowed to feed on one defined local leaf (leaf 8) followed by leaf sampling after 90 min – leaf numbering according to Dengler (2006), Farmer *et al.* (2013), and Kiep *et al.* (2015). In addition to the treated leaf 8, unwounded systemic leaves 5 and 13 (vascularly connected to leaf 8) and leaf 9 (unconnected to leaf 8) were sampled and analyzed for the expression of two jasmonate-responsive genes, *VSP2* and *JAZ10*. Compared with the nontreated Col-0 plants, *VSP2* was induced 10-fold in the local leaf 8 and even more strongly in the directly vascularly connected leaf 13, whereas no change in expression was

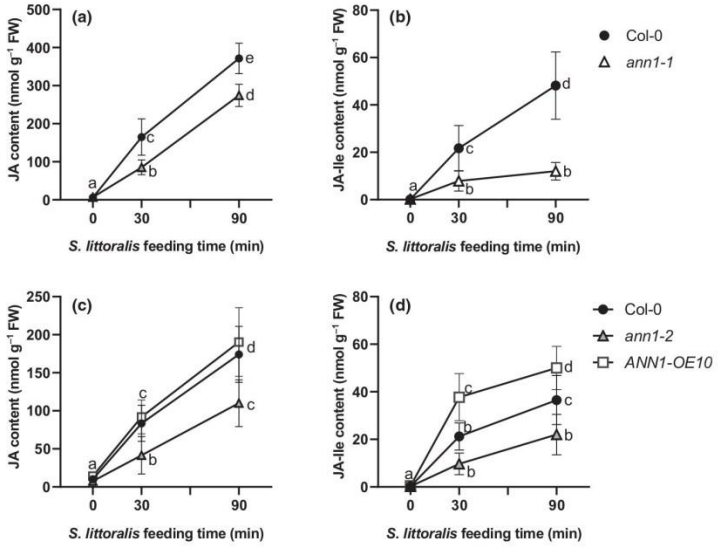

Fig. 4 Local accumulation of jasmonates in *Arabidopsis thaliana* leaves after short-time feeding assay with *Spodoptera littoralis*. (a, c) Jasmonic acid (JA) and (b, d) (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile) levels (mean ± SE) from *A. thaliana* leaves were analyzed after 30 and 90 min of feeding from a single third instar *S. littoralis* larva. Phytohormones were determined only from fed leaves. Five to eight replicates ($n = 5$–8) were done per time point. Leaves of untreated plants were used as controls. The differences of JA and JA-Ile levels between time points and genotypes were analyzed using two-way ANOVA (Sidak *post hoc* test); significant differences are indicated by different letters ($P < 0.005$).

detectable in the *ann1-1* mutant (Fig. 5a). Also, *JAZ10* was significantly induced in the treated leaf 8 in Col-0 as well as in all systemic leaves, again with the highest expression in leaf 13. In addition, in *ann1-1* plants, *JAZ10* showed a significant induction in systemic leaves 5 and 13 (Fig. 5b).

Herbivory-like feeding-induced systemic jasmonate accumulation is abolished in *ann1-1* plants

Considering that ANN1 is involved in jasmonate-related defense induction in systemic leaves, we wanted to study whether mechanical wounding alone employs ANN1 or if a chemical signal of the OS of the larvae is necessary. Thus, we tested the systemic jasmonate response after local leaf wounding with a pattern wheel followed by either water or OS application to the small wounds. The results show a clear local jasmonate response in Col-0 and in *ann1-1* plants upon wounding and water (Fig. 6). This local response was significantly higher when wounded sites were treated with OS. Interestingly, only OS treatment induced a systemic response in the vascularly connected leaves 5 and 13 in Col-0, represented by a strong increase of JA and JA-Ile. This was not the case in *ann1-1* plants, where the systemic effect was completely absent (Fig. 6). These results were supported by other measured jasmonates. The biosynthetic precursor *cis*-12-oxo-phytodienoic acid and the catabolite hydroxy-JA both showed very similar results to those found for JA and JA-Ile (Fig. S3).

Previous work has shown that pattern-wheel wounding does not fully represent the insect feeding-like wounding such as, for example, the use of MecWorm does (Mithöfer *et al.*, 2005). Therefore, MecWorm-mediated wounding was applied in an additional experiment. Based on the studies of Heyer *et al.* (2018b), we wounded leaf 8, including the midrib. As shown in Fig. 7, the continuous mechanical wounding of leaf 8 inflicted by MecWorm significantly elevated the local levels of JA-Ile, which we focused on as the bioactive form of the jasmonates, in both Col-0 and *ann1-1* plants. Strikingly, in contrast to Col-0, in *ann1-1* plants, local accumulation of jasmonates in leaf 8 was not accompanied by a comparable systemic increase of jasmonates. Moreover, a highly significant difference in JA-Ile accumulation in directly connected leaf 13 was observed when Col-0 and *ann1-1* plants were compared (Fig. 7).

Discussion

Various studies have demonstrated that a rapid, early and transient increase of $[Ca^{2+}]_{cyt}$ is involved and essential for the successful induction and regulation of jasmonate accumulation and further downstream plant defense strategies upon wounding and insect herbivory (Fisahn *et al.*, 2004; Maffei *et al.*, 2004, 2007; Arimura *et al.*, 2008, 2011; Scholz *et al.*, 2014; Toyota *et al.*, 2018; Yan *et al.*, 2018; Kumari *et al.*, 2019; Meena *et al.*, 2019). Such stress-induced $[Ca^{2+}]_{cyt}$ elevation occurs both locally and systemically (Kiep *et al.*, 2015). There are various channels that have been shown to be involved in wounding or herbivory-related Ca^{2+} influx into the cytosol such as the TPC1 (Kiep *et al.*, 2015; Vincent *et al.*, 2017), glutamate receptor-like channels (GLRs; Mousavi *et al.*, 2013; Toyota *et al.*, 2018), and cyclic nucleotide-gated channel 19 (CNGC19; Meena *et al.*, 2019). However,

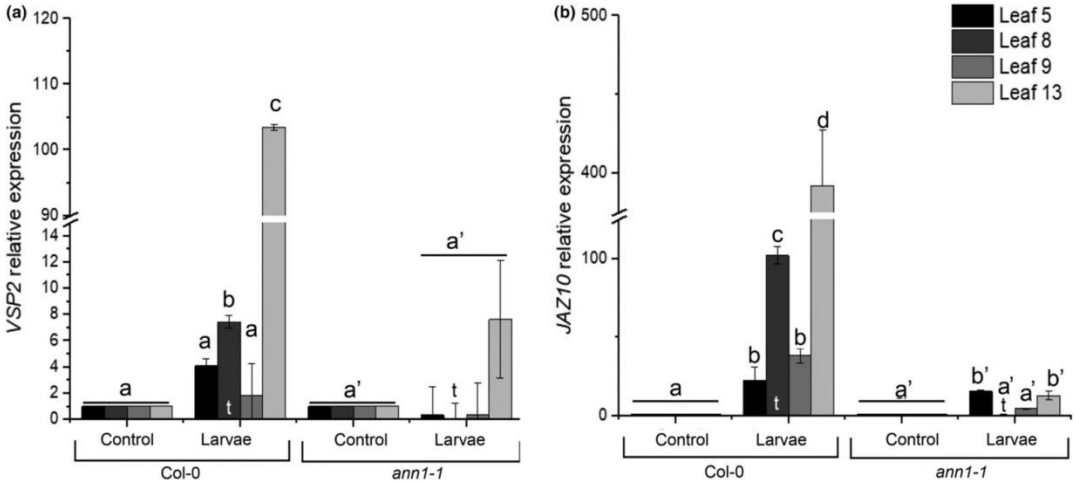

Fig. 5 Local and systemic transcriptional responses to *Spodoptera littoralis* feeding. (a) Levels of *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) and (b) *JASMONATE-ZIM-DOMAIN PROTEIN 10* (*JAZ10*) transcripts were determined in *Arabidopsis thaliana* Col-0 and annexin1 (*ann1-1*) genotypes in different leaves after local *S. littoralis* (Larvae) feeding for 90 min on leaf 8 (t). Treated leaf 8 and untreated leaves 5, 9 and 13 were analyzed (mean ± SE). Per genotype and treatment, nine replicates ($n = 9$) were done. Leaves of untreated plants were used as controls. *RPS18* was used as a reference gene to normalize the data. Statistical differences between treatments were analyzed using two-way ANOVA (Student–Newman–Keuls *post hoc* test); significant differences are indicated by different letters ($P < 0.05$).

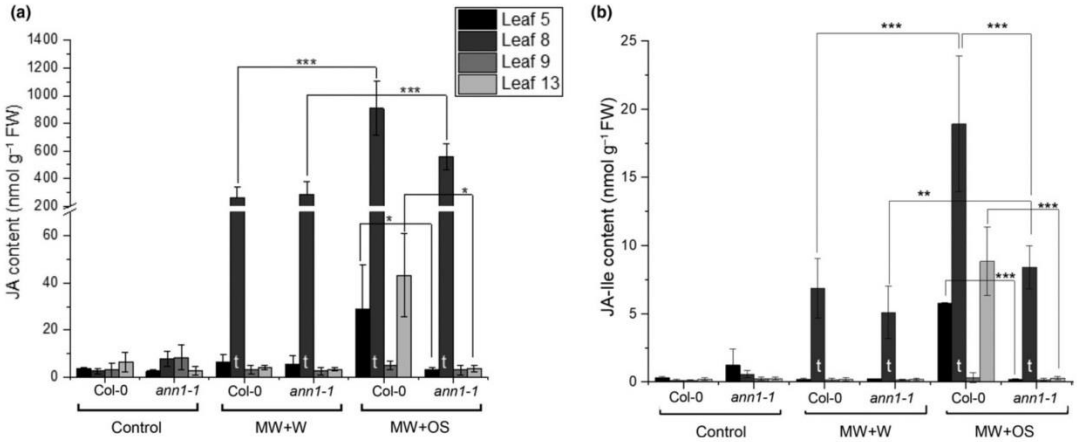

Fig. 6 Local and systemic accumulation of jasmonates after mechanical wounding (MW) and oral secretion (OS) treatment. Levels of (a) jasmonic acid (JA) and (b) (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile) were determined in *Arabidopsis thaliana* Col-0 and annexin1 (*ann1-1*) genotypes in different leaves 90 min after wounding of leaf 8 with a pattern wheel (MW) and applying water (MW + W) or oral secretion (MW + OS) on the wounds (mean ± SE). Per genotype and treatment, seven replicates ($n = 7$) were done. Untreated leaves 5, 9 and 13 and treated leaf 8 (t) were analyzed. Leaves of untreated plants were used as controls. Statistical differences found in leaves between treatments, or between genotypes were analyzed using two-way ANOVA (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$; Student–Newman–Keuls test). No indication, no significant difference.

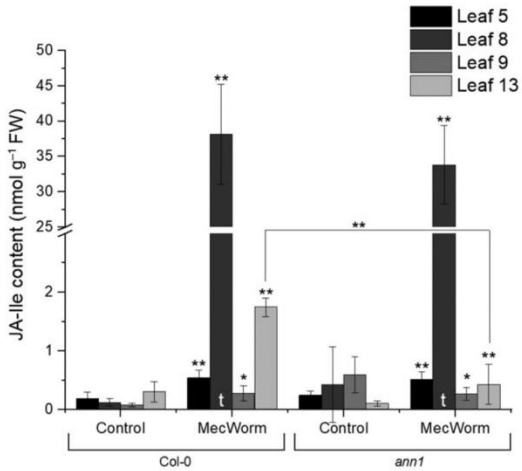

Fig. 7 Accumulation of (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile) in leaves of *Arabidopsis thaliana* after mechanical wounding with MecWorm. JA-Ile levels were analyzed in Col-0 and annexin1 (*ann1-1*) genotypes in different leaves after mechanical wounding for 90 min, including the midrib. In treated plants, leaf 8 (t) was subjected to mechanical damage; untreated leaves 5, 9 and 13 and treated leaf 8 were analyzed (mean ± SE). Per genotype and treatment, six to eight replicates ($n = 6$–8) were done. Leaves of untreated plants were used as controls. Statistical differences between leaves of control and treated plants on the same genotype and between Col-0 and *ann1-1* leaves upon MecWorm treatment were analyzed using an unpaired Student's *t*-test (*, $P < 0.05$; **, $P < 0.005$). No indication, no significant difference

Ca^{2+} influx may not be mediated only by conventional channels – additional, unconventional ones such as annexins might also contribute (Laohavisit & Davies, 2011; Laohavisit *et al.*, 2012; Davies, 2014; Ma *et al.*, 2019). Diverse studies in plants have gathered evidence of annexins' ability to influence Ca^{2+} transport. A growing body of data suggests that annexins play a role in plant response to nematode parasitism. Some cyst-secreted effectors are annexin-like and able to affect plant defense possibly by mimicking the endogenous annexin functions and impairing H_2O_2-induced $[Ca^{2+}]_{cyt}$ transients (Patel *et al.*, 2010; Zhao *et al.*, 2019). Moreover, it was shown that compared with wild-type, overexpression of *ANN1* and *ANN4* decreases susceptibility against *Meloidogyne incognita* nematode infection of roots while the *ann1* and *ann4* lines were more susceptible (Zhao *et al.*, 2019). Our study demonstrates a role for *ANN1* in systemic leaf defense responses against herbivore attack and mechanical wounding.

ANNEXIN1 is induced upon insect herbivory

Biotic interaction has been shown to influence annexin transcription in crops such as alfalfa (*Medicago sativa*), Indian mustard (*Brassica juncea*), tomato (*Solanum lycopersicum*), and wheat (*Triticum aestivum*) (Kovács *et al.*, 1998; Jami *et al.*, 2009; Lu *et al.*, 2012; Xu *et al.*, 2016). It was shown in multiple studies that Arabidopsis *ANN1* gene expression is influenced by diverse environmental signals (Konopka-Postupolska *et al.*, 2009; Clark *et al.*, 2010; Guelette *et al.*, 2012). Here, we demonstrate the expression response of Arabidopsis *ANN1* to herbivory. The assays were designed to understand the attack in a mechanistic and holistic way, which we achieved by dissecting the insect

attack into different modules of stress: mechanical wounding alone (MW + W), mechanical wounding plus OS (MW + OS), or the complex stress of larval feeding (Fig. 1). The high expression of *ANN1* upon the different but complementary stresses showed that *ANN1* transcription activation is triggered quickly (here, after 90 min) by insect-feeding-related damage, but also by wounding alone. The latter confirms earlier results showing *ANN1* induction 24 and 48 h after wounding (Konopka-Postupolska *et al.*, 2009).

ANNEXIN1 is involved in the defense against herbivores

As with other cellular components that are involved in $[Ca^{2+}]_{cyt}$ signaling (Vadassery *et al.*, 2012b; Scholz *et al.*, 2014; Meena *et al.*, 2019), we show here that ANN1 is also an important player in the regulation of insect-feeding-induced defense. Using two different knockout and two different overexpression lines, we showed that *S. littoralis* larvae feeding on *ann1* plants gained substantially more weight (in total, +27.6%), whereas those feeding on ANN1-OE plants were much smaller (−26.9%) than larvae feeding on Col-0 plants (Figs 3, S2). Thus, ANN1 is a positive regulator in herbivory-induced defense in Arabidopsis and contributes to resistance against *S. littoralis*, comparable to CNGC19 and GLRs.

To gain further insight into the putative role of ANN1 in systemic defense-related signaling, we performed a series of experiments in which a defined local leaf was treated and systemic leaves were analyzed (Mousavi *et al.*, 2013; Kiep *et al.*, 2015). Using (apo)aequorin-expressing *ann1-1* and wild-type plants, we demonstrated that ANN1 is indispensable for the systemic $[Ca^{2+}]_{cyt}$ response; this is comparable to what has been found previously for TPC1 and GLRs (Kiep *et al.*, 2015; Toyota *et al.*, 2018). By contrast, the local response was slightly but not significantly reduced in the *ann1-1* mutant (Figs 2, S1). However, compared with other studies (Nguyen *et al.*, 2018; Toyota *et al.*, 2018), the systemic $[Ca^{2+}]_{cyt}$ response was found to be weaker. This can be explained by different experimental conditions; first, in the other studies, the GCaMP3 fluorescent-protein-based $[Ca^{2+}]$ sensor was used, a highly sensitive calcium fluorescence reporter, whereas we used bioluminescent aequorin. Second, their mode of wounding was much harsher. Whereas Nguyen *et al.* (2018) destroyed half of the leaf tissue, we used a pattern wheel that caused only a few small holes in the tissue. This supports the view that the intensity of wounding correlates with the intensity of the response (Nguyen *et al.*, 2018).

As a readout for the defense response, the expression of jasmonate-responsive genes *VSP2* and *JAZ10* was examined. Upon larval feeding, a local increase was detected in Col-0 for both genes, as well as a strong increase in the vascularly connected leaf 13 that was even higher than the local response (Fig. 5). Such a strong systemic increase was not found for either *VSP2* or *JAZ10* in *ann1-1* plants, suggesting that the feeding-related signals necessary to induce the systemic gene activation do not reach the distal leaves. The fact that in this and other experiments leaf 13 and (to a lesser extent) leaf 5 respond more strongly can be explained by the direct (leaf 13) and indirect (leaf 5) vascular connections to leaf 8 (Dengler, 2006).

As $[Ca^{2+}]_{cyt}$ elevations initiate Ca^{2+} signaling (Kudla *et al.*, 2010; Mithöfer & Boland, 2012; Scholz *et al.*, 2014; Vadassery *et al.*, 2014) and precede downstream signals, such as phytohormone accumulation, we further investigated the concentrations of the jasmonates JA and JA-Ile at two time points (30 and 90 min). These jasmonates were found to be strongly induced locally in both Col-0 and *ann1* upon herbivore attack; but accumulation was significantly lower in *ann1* plants, whereas in the *ANN1-OE10* at least the JA-Ile level was significantly higher (Fig. 4). This is in accordance with the finding that $[Ca^{2+}]_{cyt}$ signals in *ann1* plants are affected in local leaves to a certain extent and suggests that a full induction of jasmonates in response to insect herbivores is not possible in those plants. Strikingly, the induction of *ANN1* upon herbivory or wounding (Fig. 1) is high if compared with downstream responses such as jasmonate or gene induction. Very likely, the reason behind this finding is that gene expression does not always reflect the corresponding protein expression. Lee *et al.* (2004) already noted that *ann1-1* transcript level does not necessarily correspond to the ANN1 protein. Besides using larvae, we further evaluated the phytohormonal responses in local and systemic leaves of plants treated with mechanical wounding. We chose this approach because such experiments can be better standardized than exposure to larvae that might feed or not, particularly when only a short time period is investigated. Pattern-wheel-mediated mechanical wounding, as well as wounding by MecWorm, supported the finding of local jasmonate induction in wild-type and mutant (Figs 6, 7). Strikingly, after MW + OS treatment, the response of JA-Ile in *ann1-1* plants was significantly reduced compared with wild-type (Fig. 6). In addition, an elevation of jasmonates was also observed in systemic leaves of OS-treated plants in wild-type but not in *ann1-1* mutant plants. This further indicates that ANN1 is involved in systemic $[Ca^{2+}]_{cyt}$-dependent jasmonate elevation. This notion was supported by results obtained by mechanical wounding alone, using MecWorm treatment. In *ann1-1* plants, leaf 13 showed significantly lower accumulation of JA-Ile than in Col-0 plants (Fig. 7), suggesting that ANN1 might not only be involved in systemic OS-specific signaling but also in systemic wound-induced jasmonate accumulation.

Conflating the data obtained, we propose that ANN1 is a positive factor of the $[Ca^{2+}]_{cyt}$-dependent systemic defense response against wounding and larval feeding. Thus, the absence of this unconventional Ca^{2+} channel causes an impaired systemic response. ANN1 can exist as an integral plasma membrane protein (Alexandersson *et al.*, 2004; Marmagne *et al.*, 2007). Nevertheless, as small amphipathic proteins, annexins are distributed throughout cells and can be transported within the plant via the phloem (Guelette *et al.*, 2012). It is possible that annexins may be recruited directly to membranes, independently of vesicle delivery, to operate in stimulus-specific signaling (Laohavisit & Davies, 2011; Laohavisit *et al.*, 2012; Davies, 2014; Espinoza *et al.*, 2017). Therefore, a plant that contains the conventional and unconventional Ca^{2+} channels should be able to recruit annexins to the tissues under stress when needed, whereas the *ann1* genotype can only launch reduced $[Ca^{2+}]_{cyt}$-mediated defense responses. It should be kept in mind that annexins might

also act via the regulation of channel activities, for example by selective channel delivery to or retraction from membranes, in a similar way as the KAT1 plasma membrane potassium ion (K^+) channel is cycled during abscisic acid induced stomatal closure (Sutter *et al.*, 2007). However, the observed effect of abolishing systemic $[Ca^{2+}]_{cyt}$-induced responses in *ann1* plants may be specific for certain stresses, such as herbivory, and is not necessarily involved in all types of biotic and abiotic stress responses. An example is a study showing that ANN1 was not necessary for systemic signaling and development of acquired resistance in uninfected leaves during challenge with avirulent bacteria *Pseudomonas syringae* pv tomato (Carella *et al.*, 2016).

The success of the plant's defense response against stresses does not depend on one single signaling component alone. Instead, it is a coordinated local and systemic communication between cells and distant organs. For that, various signals, such as ROS, hydraulic pressure, as well as electropotential waves, $[Ca^{2+}]_{cyt}$ and others, are employed in a tightly linked manner (Foreman *et al.*, 2003; Zimmermann *et al.*, 2009; Maischak *et al.*, 2010; Farmer *et al.*, 2013; Mousavi *et al.*, 2013; Davies, 2014; Seybold *et al.*, 2014; Ranjan *et al.*, 2015; Peiter, 2016; Alonso *et al.*, 2019; Gully *et al.*, 2019; Saijo & Loo 2019). In previous studies using epidermal root tissue, hydroxyl-radical-activated plasma membrane conductance of Ca^{2+} and K^+ were absent in the *ann1-1* mutant (Laohavisit *et al.*, 2012). Expression and protein levels of Arabidopsis *ANN1* also correlated with the occurrence of the radical-activated plasma membrane Ca^{2+} conductance in the root epidermis and at the apex of root hairs (Clark *et al.*, 2001; Dinneny *et al.*, 2008). These results strongly suggest that ANN1 is very likely a Ca^{2+}-permeable protein in Arabidopsis and might provide a molecular link between ROS and $[Ca^{2+}]_{cyt}$ in the systemic defense-related signaling in plants. Further studies will address this hypothesis.

In conclusion, we investigated the role of ANN1 in local and systemic plant defense against wounding and herbivorous insects in Arabidopsis. Plant tissue wounding and cell disruption caused by feeding insects strongly induced *ANN1* expression, demonstrating that it is part of the rapid defense response against invertebrate pests; neither jasmonates nor defense-related genes were upregulated systemically in *ann1* mutants. ANN1 mediates plant defense, affecting larval growth, and is crucial for the induction of signaling upon herbivory within the whole plant. ANN1 is an important part of systemic $[Ca^{2+}]_{cyt}$ signaling, thereby connecting $[Ca^{2+}]_{cyt}$ to subsequent downstream signals and defense responses against herbivores.

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Author contributions

JM and AM conceived the project and research plans; JM, AKM, and MR performed the experiments; JM, AKM, SSS, RO and AM designed the experiments and analyzed the data; EP provided and assisted with the equipment for Ca analyses; DK-P, JR, JMD and KAW generated and provided seed material; JM, AKM, SSS and AM wrote the article with contributions from all authors.

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Supporting Information

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

Fig. S1 $[Ca^{2+}]_{cyt}$ response upon mechanical wounding (MW) and water (H_2O) in Col-0 and annexin1 mutants.

Fig. S2 Weight of *Spodoptera littoralis* larvae fed on Arabidopsis Col-0 and *ann1-1* plants expressing *Aequorin*.

Fig. S3 Accumulation of related jasmonates after wounding and oral secretion (OS) treatment.

Video S1 Time-lapse of $[Ca^{2+}]_{cyt}$ response in *Arabidopsis thaliana* Col-0 rosette induced by mechanical wounding + oral secretion (OS) of the leaf lamina.

Video S2 Time-lapse of $[Ca^{2+}]_{cyt}$ response in *Arabidopsis thaliana ann1-1* rosette induced by mechanical wounding + oral secretion (OS) of the leaf lamina.

Video S3 Time-lapse of $[Ca^{2+}]_{cyt}$ response in *Arabidopsis thaliana* Col-0 rosette induced by mechanical wounding + water of the leaf lamina.

Video S4 Time-lapse of $[Ca^{2+}]_{cyt}$ response in *Arabidopsis thaliana ann1-1* rosette induced by mechanical wounding + water of the leaf lamina.

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2.1.2 Supplementary Material Manuscript 1

New Phytologist

New Phytologist Supporting Information

Article title: **ANNEXIN1 mediates calcium-dependent systemic defense in Arabidopsis plants upon herbivory and wounding**

Authors: **Jaiana Malabarba, Anja K. Meents, Michael Reichelt, Sandra S. Scholz, Edgar Peiter, Julia Rachowka, Dorota Konopka-Postupolska, Katie A. Wilkins, Julia M. Davies, Ralf Oelmüller, Axel Mithöfer**

Article acceptance date: 05 February 2021

The following Supporting Information is available for this article:

Figure S1. $[Ca^{2+}]_{cyt}$ response upon mechanical wounding (MW) and water (H_2O) in Col-0 and annexin1 mutants.

Figure S2. Weight of *Spodoptera littoralis* larvae fed on Arabidopsis Col-0 and *ann1-1* plants expressing *Aequorin.*

Figure S3. Accumulation of related jasmonates after wounding and oral secretion (OS) treatment.

Video S1. Time-lapse of $[Ca^{2+}]_{cyt}$ response in *Arabidopsis thaliana* Col-0 rosette induced by mechanical wounding + oral secretion (OS) of the leaf lamina.

Video S2. Time-lapse of $[Ca^{2+}]_{cyt}$ response in *Arabidopsis thaliana ann1-1* rosette induced by mechanical wounding + oral secretion (OS) of the leaf lamina.

Video S3. Time-lapse of $[Ca^{2+}]_{cyt}$ response in *Arabidopsis thaliana* Col-0 rosette induced by mechanical wounding + water of the leaf lamina.

Video S4. Time-lapse of $[Ca^{2+}]_{cyt}$ response in *Arabidopsis thaliana ann1-1* rosette induced by mechanical wounding + water of the leaf lamina.

Figure S1. $[Ca^{2+}]_{cyt}$ response upon mechanical wounding (MW) and water (H_2O) in Col-0 and annexin1 mutants. (a) Cumulative images over 10 min of local and systemic $[Ca^{2+}]_{cyt}$ signals in 4-week-old whole *A. thaliana* Col-0 (top) and *ann1-1* (bottom) rosettes in response to MW+water applied to leaf 8 (left) and after discharge (right). Discharge was used to determine L_{total} to calculate the $[Ca^{2+}]_{cyt}$ in individual leaves. (b) Kinetics of a representative $[Ca^{2+}]_{cyt}$ response in individual leaves in Col-0 and *ann1-1* after treatment. (c) $[Ca^{2+}]_{cyt}$ response in individual leaves in Col-0 and *ann1-1* after treatment. Per genotype and treatment 9 replicates (n=9) were done. cul= cumulative; dis= discharge. 2-way ANOVA (Sidak post-hoc test) (*** P <0.001).

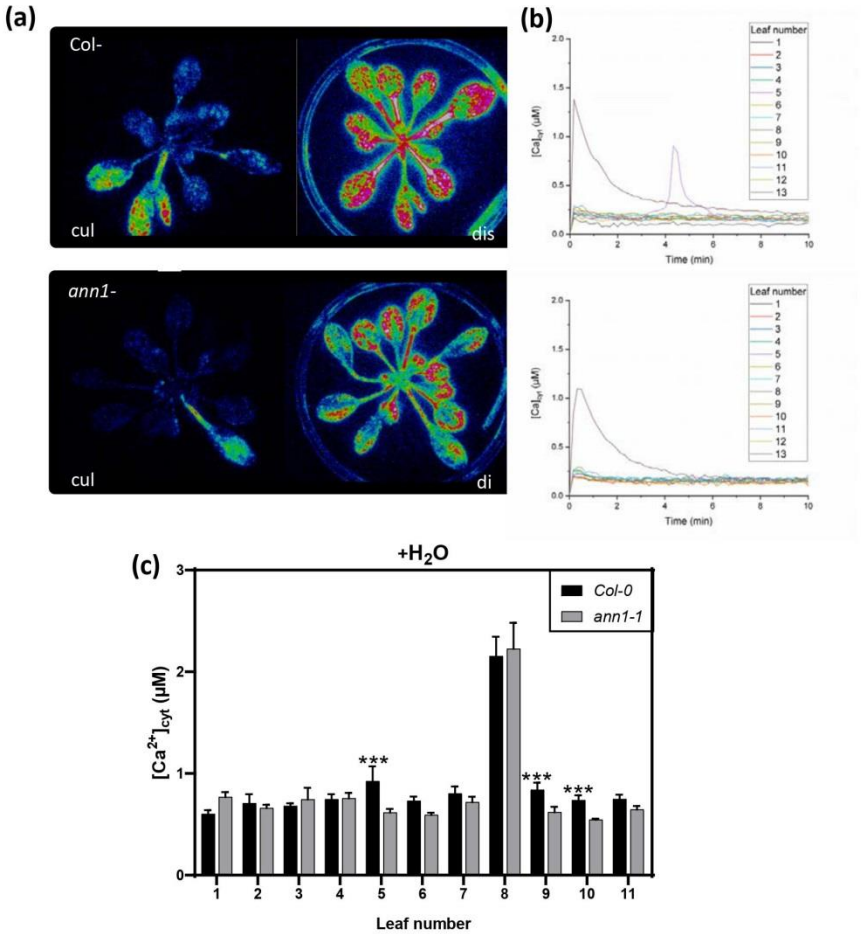


Figure S2. Weight of *Spodoptera littoralis* larvae fed on Arabidopsis Col-0 and *ann1-1* plants expressing *Aequorin*. 30 first instar larvae of *S. littoralis* were pre-weighed and 3 larvae were placed on each plant. In each independent experiment, 10 plants per genotype were used. The larval weight (mean ± SE) was measured after 7 days of feeding. Experiment was independently repeated 5 times (n=5). The combined total number of larvae (N) is indicated (a) Final larval weight after feeding on wild-type (white) and *ann1-1* (black) plants. (b) Macroscopic view of larvae after one week of feeding on the indicated genotypes. Statistical difference between the genotypes after feeding was analyzed by Student's *t*-test (*** $P \leq 0.001$).

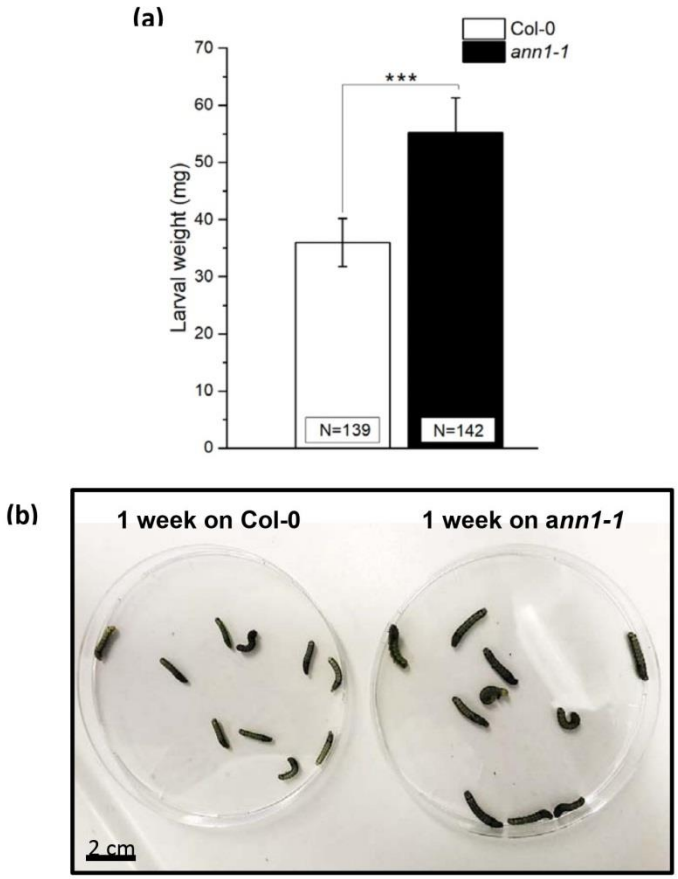
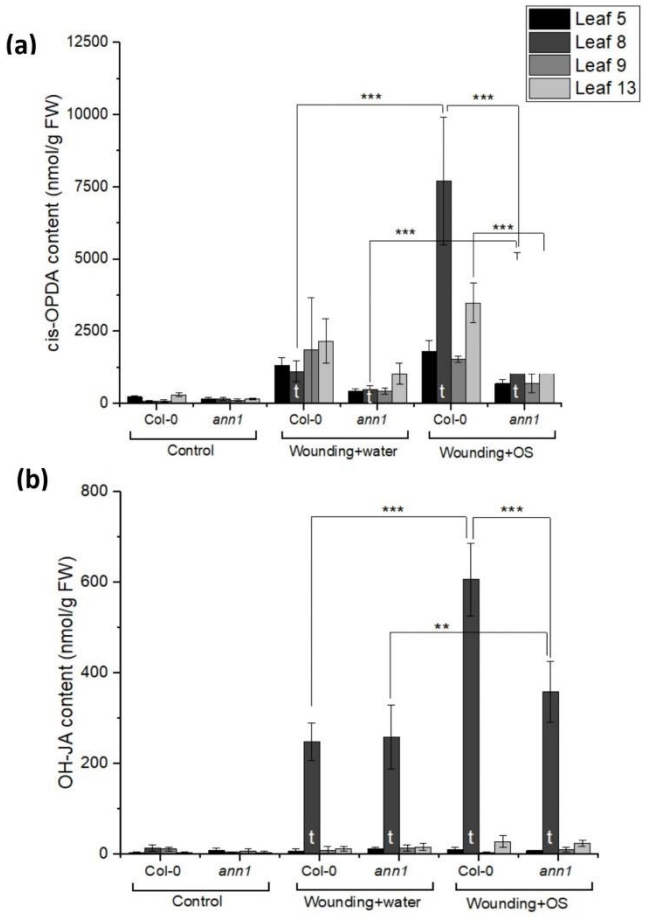


Figure S3. Accumulation of related jasmonates after wounding and oral secretion (OS) treatment. Levels of (a) *cis*-OPDA, (b) OH-JA were determined in different leaves 90 min after mechanical wounding (MW) of leaf 8 with a pattern wheel and applying water (MW+Water) or oral secretion (MW+OS) on the wounds (mean ± SE). Per genotype and treatment 7 plants (n=7) were used. Untreated leaves 5, 9, and 13 and treated leaf 8 were analyzed. Leaves of untreated plants were used as controls. Statistical differences between treatments were analyzed by 2-way ANOVA (post hoc SNK test; ** P <0.005; *** P <0.001).



Video/Movie S1

Video S1. Time-lapse of $[Ca^{2+}]_{cyt}$ response in *Arabidopsis thaliana* Col-0 rosette induced by mechanical wounding + oral secretion (OS) of the leaf lamina. Leaf number 8 of 4-week-old Col-0 Arabidopsis rosettes was mechanically wounded using a pattern wheel with six vertical motions including the midrip. Subsequent to wounding, 20 µl of freshly diluted OS from *S. littoralis* (1:1) were evenly spread across all holes and the aequorin luminescence was recorded for 10 minutes.

Video S2. Time-lapse of $[Ca^{2+}]_{cyt}$ response in *Arabidopsis thaliana ann1-1* rosette induced by mechanical wounding + oral secretion (OS) of the leaf lamina. Leaf number 8 of 4-week-old *ann1-1* Arabidopsis rosettes was mechanically wounded using a pattern wheel with six vertical motions including the midrib. Subsequent to wounding, 20 µl of freshly diluted OS from *S. littoralis* (1:1) were evenly spread across all holes and the aequorin luminescence was recorded for 10 minutes.

Video S3. Time-lapse of $[Ca^{2+}]_{cyt}$ response in *Arabidopsis thaliana* Col-0 rosette induced by mechanical wounding + water of the leaf lamina. Leaf number 8 of 4-week-old Col-0 Arabidopsis rosettes was mechanically wounded using a pattern wheel with six vertical motions including the midrib. Subsequent to wounding, 20 µl of water were evenly spread across all holes and the aequorin luminescence was recorded for 10 minutes.

Video S4. Time-lapse of $[Ca^{2+}]_{cyt}$ response in *Arabidopsis thaliana ann1-1* rosette induced by mechanical wounding + water of the leaf lamina. Leaf number 8 of 4-week-old *ann1-1* Arabidopsis rosettes was mechanically wounded using a pattern wheel with six vertical motions including the midrib. Subsequent to wounding, 20 µl of water were evenly spread across all holes and the aequorin luminescence was recorded for 10 minutes.

2.2 Manuscript 2

2.2.1 Manuscript overview

Manuskript Nr. 2

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REVIEW
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Plant–Plant Communication: Is There a Role for Volatile Damage-Associated Molecular Patterns?

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Damage-associated molecular patterns (DAMPs) are an ancient form of tissue-derived danger or alarm signals that initiate cellular signaling cascades, which often initiate defined defense responses. A DAMP can be any molecule that is usually not exposed to cells such as cell wall components, peptides, nucleic acid fragments, eATP and other compounds. DAMPs might be revealed upon tissue damage or during attack. Typically, DAMPs are derived from the injured organism. Almost all eukaryotes can generate and respond to DAMPs, including plants. Besides the molecules mentioned, certain volatile organic compounds (VOCs) can be considered as DAMPs. Due to their chemical nature, VOCs are supposed to act not only locally and systemically in the same plant but also between plants. Here, we focus on damage-induced volatiles (DIVs) that might be regarded as DAMPs; we will review their origin, chemical nature, physiochemical properties, biological relevance and putative function in plant–plant communications. Moreover, we discuss the possibility to use such airborne DAMPs as eco-friendly compounds to stimulate natural defenses in agriculture in order to avoid pesticides.

Keywords: DAMP, defense, plant–plant communication, signaling, volatiles, wounding

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INTRODUCTION

As other eukaryotic organisms, plants are able to perceive typical, endogenous cell molecules or fragments thereof, when these are released at increased concentrations into the extracellular space. This occurs during cellular stress or mechanical damage upon herbivore and pathogen attack. Subsequently, the endogenous compounds contribute to activate local and systemic defense-related responses or the plant innate immunity (Howe and Jander, 2008; Boller and Felix, 2009). The whole dynamic immunity response is induced by the recognition of specific insect-derived [herbivore-associated molecular patters (HAMPs) (Mithöfer and Boland, 2008)] or pathogen-derived [pathogen-associated molecular patters (PAMPs) (Ausubel, 2005)] signals, and signals from the injured plant cells. These latter signaling molecules function as danger signals, stress signals, (endogenous) elicitors, alarmins, or damage-associated molecular patterns (DAMPs). Although various synonyms exist for the aforementioned molecules, the term DAMP is to our knowledge the most prominent example and will be further referred to in this review. With the increasing acceptance of the "damaged-self recognition" concept (Heil, 2009) for plants, the number of DAMPs, their putative reception and signaling and the corresponding literature continuously increased. Thus, here we avoid providing another collection of DAMPs and refer to recent reviews

that give comprehensive overviews (Boller and Felix, 2009; Heil and Land, 2014; Gust et al., 2017; Quintana-Rodriguez et al., 2018; Hou et al., 2019; Ferrusquía-Jiménez et al., 2020). Nevertheless, some typical examples must be mentioned such as peptides, cell wall components, nucleic acid fragments, and extracellular ATP (eATP). However, a new putative class of DAMPs that would be unique for plants (Heil and Land, 2014) will be addressed in the following: volatile DAMPs.

In recent years, plant-derived volatile organic compounds (VOCs) gained much attention as cues in plant–plant communication. However, the concept of VOCs released by attacked plants transmitting information to warn neighboring individuals is far from posing as a novelty, being described almost 40 years ago in various caterpillar-infested tree species (Baldwin and Schultz, 1983; Rhoades, 1983). Criticism regarding the lack of true replication and artificial experimental conditions (Fowler and Lawton, 1985) resulted in the rejection of this popular phenomenon known as "talking trees." It took almost 20 years to revisit and revive the concept of plant–plant communicating via volatile cues by intensely searching for evidence of VOC-induced plant protection against herbivory (Heil and Karban, 2010; Karban et al., 2014). This review focuses specifically on wounding-/damage-induced plant volatiles that fulfill the criteria of DAMPs in *stricto sensu*. We highlight their chemical nature and their ability to induce defense responses in neighboring plants and critically examine their putative role in the field.

A SHORT SURVEY OF PLANT VOLATILES

A plethora of studies is available highlighting the versatility of VOCs and in particular of herbivory-induced plant volatiles (HIPVs). Apart from activating direct and indirect plant defenses against herbivores, HIPVs are also known to mediate a diverse array of interactions between plants and insects (Turlings et al., 1990; De Moraes et al., 1998, 2001; Hoballah and Turlings, 2001). In numerous plant species HIPVs are involved in repelling herbivores, attracting their predators of a higher trophic level as well as upregulating and priming defense responses (Kessler and Baldwin, 2002; Arimura et al., 2004; Engelberth et al., 2004; Kessler et al., 2006; Arimura and Pearse, 2017). Although plants release distinct volatile bouquets with differing compositions and concentrations depending on the given stimulus, e.g., herbivory, mechanical wounding, or touch (Mithöfer et al., 2005; Bricchi et al., 2010; Meents et al., 2019), many taxa share common constituents (McCormick et al., 2012). The most well-known representatives described within the past decades are terpenoids, phenylpropanoids as well as fatty acid and amino acid derivatives (**Figure 1**) (Dudareva et al., 2004, 2006).

Among the most ubiquitous VOCs emitted after mechanical damage, herbivory, or microbial infection are green leaf volatiles (GLVs, for a full review see, Ameye et al., 2018) named after their typical odor of freshly cut green leaves. GLVs are C_6 alcohols, aldehydes, and esters such as (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, and (*Z*)-3-hexen-1-yl acetate generated via oxidation of fatty acids such as linoleic and α-linolenic acid within the oxylipin pathway (for example, see, Matsui, 2006).

Considering the largest class of plant secondary metabolites, terpenes provide a wide array of volatile compounds which are subdivided depending on the number of C_5 units (Dudareva et al., 2004; McCormick et al., 2012). The main representatives of this family are hemiterpenes (C_5; e.g., isoprene), monoterpenes [C_{10}; e.g., linalool, *(E)*-β-ocimene], sesquiterpenes [C_{15}; *(E)*-β-caryophyllene, *(E,E)*-α-farnesene, α-humulene], and homoterpenes displaying irregular structures such as *(E)*-4,8-dimethyl-1,3,7-nonatriene (DMNT; C_{11}) and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT; C_{16}) (Boland et al., 1992; Maffei et al., 2011; McCormick et al., 2012). The formation of the abovementioned terpenes from the basic C_5 building blocks occurs via two compartmentalized pathways: the cytosol-localized mevalonate pathway (MVA) and the methylerythritol phosphate (MEP) pathway localized in the plastids (Dudareva et al., 2004). Both pathways are strictly enzymatically regulated by a large family of terpene synthases (Dudareva et al., 2013).

Another structurally diverse category of VOCs are the shikimate pathway-derived phenylpropanoids and benzenoids, originating from the amino acid phenylalanine. Sharing a single or multiple benzene rings, these two classes undergo miscellaneous modifications such as acetylation, hydroxylation or methylation, thereby creating a variety of side chains and resulting compounds (Dudareva et al., 2004, 2006). Being often specific to certain plant species and genera, methyl salicylate (MeSA), benzaldehyde, chavicol, eugenol, phenylethanol, and benzylalcohol are typical compounds of this category which can be found in numerous volatile bouquets (Dudareva et al., 2006; Arimura and Pearse, 2017). By performing radioactive labeling studies, several other amino acid-derived VOCs ranging from, e.g., isothiocyanates, sulfides, nitriles, oximes, and amines have been discovered over the years (Dudareva et al., 2006; McCormick et al., 2012). One of the key volatiles released after herbivore damage is indole, which is biosynthesized via anthranilate as an intermediate product in the tryptophan pathway (Paré and Tumlinson, 1996; Frey et al., 2000).

In the context of plant volatiles and their effects on atmospheric chemistry, short-chain oxygenated volatiles (oxVOCs) such as formic and acetic acids, formaldehyde, acetone, methanol, and ethanol, have gained increasing importance in research respective to climate change and contribution to formation of aerosol particles and ozone (Seco et al., 2007).

MECHANICAL DAMAGE-INDUCED VOCs

While studies investigating HIPVs became increasingly popular over time, volatiles solely induced by and emitted after mechanical damage [from now on *damage-induced volatiles* (DIVs)] without any contribution of other organisms, were predominantly shortly mentioned or being considered as not representative for natural processes. In more recent years, VOCs received increasing attention as a DAMP-related cue whilst

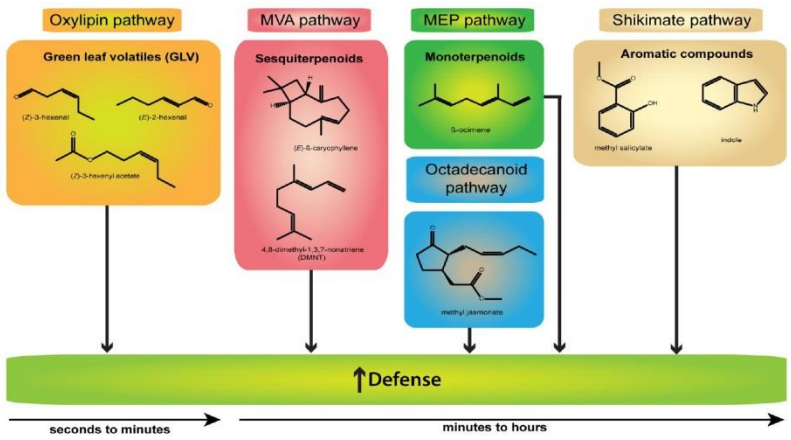

FIGURE 1 | Representative damage-induced volatiles (DIVs). Shown are structures and the biosynthetic origin (indicated by different colors) of the main DIVs involved in defense response induction (↑) and released after wounding or mechanical damage only. Compounds of the oxylipin pathway appear early within seconds to minutes, other compounds later within minutes to hours.

serving as reliable responses upon damage in various plant tissues. In **Figure 2** different key players involved in volatile induction and their relationship among each other are depicted. Particular studies by Quintana-Rodriguez et al. (2018) placed volatiles emitted upon wounding-induced tissue damage in the absence of elicitors in an entirely new context. They pointed out that such DIVs are synthesized upon cell disruption and possess the ability to trigger systemic responses and herbivore resistance, therefore functioning as a DAMP in plants (**Figure 3**) (Heil, 2009; Duran-Flores and Heil, 2016). However, it is also conceivable that DIVs are generated downstream of classical DAMPs such as oligosaccharines or peptides and therefore should be seen as second messengers rather that the initial signals. Most likely, DIVs are synthetized *de novo* after damage. However, here we must discriminate two situations. First, synthesis is initiated within seconds upon tissue damage by constitutively present enzymes as in case of GLVs. Second, synthesis is induced only upon damage perception within hours as for example in case of phenolic compounds and many terpenes. In any case the release of all these volatile compounds can be considered as early and late damage-induced responses, respectively; in contrast to classic DAMPs which are not synthesized upon damage. Only some stored terpenes are released immediately upon disruption of tissue containing pre-existing secretory structures.

Considering that damaging of a plant without the introduction of foreign molecular patterns (e.g., insects) completely omits evolutionary factors such as arms race (Heil, 2009), investigation of the underlying mechanisms will improve the understanding of "ancient" plant defense responses. Thus, we next would like to take a closer look which volatiles are actually released solely upon mechanical damage without associated herbivore feeding or other stress factors in order to identify DIVs, which might serve as potential ancient DAMPs.

One of the most well-known DIVs is the characteristic smell of freshly cut grass, mainly caused by the emission of GLVs. Karl et al. (2001) identified predominantly C_6 compounds

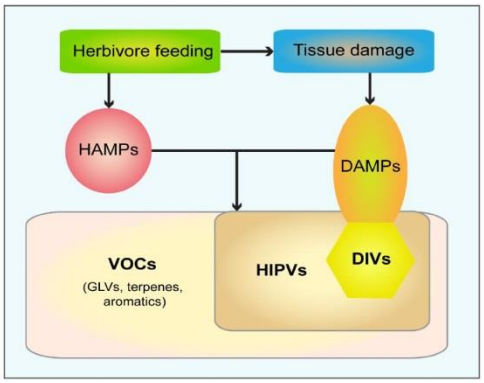

FIGURE 2 | Scheme of the relationship between groups of volatile compounds induced upon herbivore feeding and tissue damage. Herbivore feeding provides chemical signals, HAMPs (herbivory-associated molecular patters), and causes tissue damage, which in turn generates DAMPs (damage-associated molecular patters). The combination of HAMPs and DAMPs induce the emission of HIPVs (herbivory-induced plant volatiles), which all belong to the huge group of VOCs (volatile organic compounds) that includes GLV (green leaf volatiles), terpenes and aromatic compounds. DAMPs are also generated by tissue damage/wounding alone. DIVs (damage-induced volatiles) represent a sub-group of DAMPs, due to their volatile character; all DIVs belong to HIPVs. For simplicity damage-induced electrical signals are neglected.

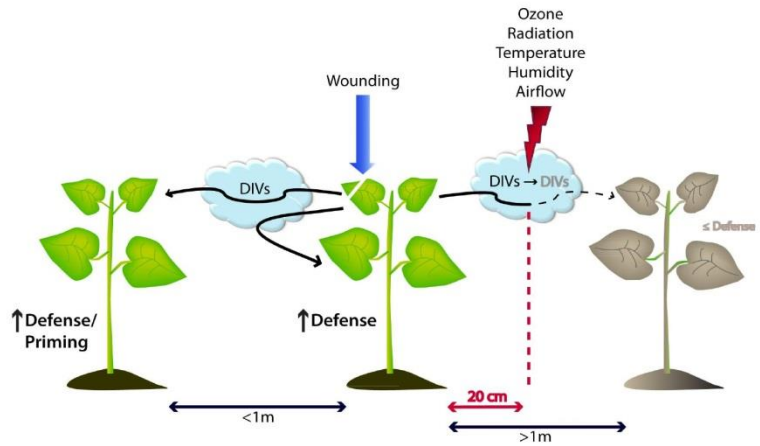

FIGURE 3 | Model of damage-induced volatile (DIV) emissions that trigger intra- and interspecific defense responses in plants. Upon wounding events without contribution from other organisms, plants release specific DIVs possessing the ability to upregulate molecular and chemical defense mechanisms within the same individual as well as in neighboring plants up to a distance of 1 m. The signal intensity and distance of DIVs is highly dependent on environmental factors such as tropospheric reagents (ozone), temperature, radiation as well as the direction of airflow. All of the aforementioned conditions can drastically reduce the effectiveness of DIV signaling by lowering it to a ∼20 cm radius.

including (*Z*)-3-hexenal, (*E*)-2-hexenal, hexenol, hexanal, and acetaldehyde to be emitted within minutes after lawn mowing and lasting for several hours in the field, therefore causing this distinct bouquet. The rapid emission reported in this field study confirmed previous findings in aspen (*Populus tremuloides*), beech (*Fagus sylvatica*), and clover (*Trifolium repens*), where cutting of leaves with scissors elicited a release of (*Z*)-3-hexenal within 1–2 s paving the way for the release of the aforementioned compounds plus hexenyl acetates (Fall et al., 1999). The sensitivity of such measurements was immensely improved by new measuring techniques, such as proton−transfer−reaction mass spectrometry (PTR-MS), enabling monitoring of the release of selected VOCs simultaneous and on-line in the laboratory or in the field.

In addition to the aforesaid rapidly emitted GLVs, mechanical wounding has been shown to generate a variety of DIVs in many different species ranging from common agricultural crops (tomato, *Solanum lycopersicum*; potato, *Solanum tuberosum*; lima bean, *Phaseolus lunatus*), model organisms (*Arabidopsis thaliana;* common liverwort, *Marchantia polymorpha*), herbs, shrubs, and grasses (sagebrush, *Artemisia tridentata*; common reed, *Phragmites australis*; *Plantago lanceolata*) to even trees (aspen, *Populus tremula*; beech, *Fagus sylvatica*; poplar, *Populus nigra*). Although the emission of such DIVs occurs in a species and/or cultivar dependent manner, similar constituents are found in the emitted bouquets.

Jackson and Campbell (1976) observed the release of the plant hormone ethylene after excision of petiole segments from tomato plants. In the following years the list of known DIVs became increasingly refined, adding β-caryophyllene, (*E*)-β-farnesene, germacrene D, and β-bisabolene discovered in potato and common broad bean (*Vicia faba*), to the mix (Agelopoulos et al., 1999). Headspace analyses in *A. thaliana* revealed, apart from GLVs, an increased emission of various terpenoids (β-ionone, β-cyclocitral), sulfides (dimethyl disulfide, dimethyl trisulfide), alcohols (3-pentanol, 1-penten-3-ol, 2-ethyl-1-hexanol), and ketones (3-pentanone, 1-penten-3-one) after rubbing of the leaf midrip with carborundum powder (Van Poecke et al., 2001). Using a more common wounding approach by punching holes into lima bean leaves, Arimura et al. (2000) paved the way for extensive VOC studies using this species by demonstrating the upregulated release of, e.g., DMNT, MeSA, α-pinene (in addition to previously mentioned compounds). From the 2000s onwards, more and more DIVs comprising methyl jasmonate (MeJA) in sagebrush (Preston et al., 2001), linalool and linalool oxide in damaged wheat (*Triticum aestivum*) (Piesik et al., 2006), acetaldehyde, methanol, isoprene, and additional C_6 compounds in common reed (Loreto et al., 2006), the essential oils pulgeone and menthone in the medicinal plant *Minthostachys mollis* (Banchio et al., 2005), as well as C_8 VOCs in the model liverwort species *Marchantia polymorpha* (Kihara et al., 2014), were identified and further investigated. In addition to DIVs found in agriculturally relevant species such as cotton (*Gossypium hirsutum*), Brussel sprouts (*Brassica oleracea*), and sweet potato (*Ipomoea batatas*) (Röse and Tumlinson, 2005; Connor et al., 2007; Meents et al., 2019), more recent studies included traditional medicinal plants and trees (Fontana et al., 2009; Martins et al., 2017; Kanagendran et al., 2018; Portillo-Estrada and Niinemets, 2018). Although the inclusion of a wider array of species highlights common DIV constituents, the potential as a functional DAMP yet remains to be verified for the majority of them. Quintana-Rodriguez and colleagues compiled

valuable information regarding VOCs triggering responses at multiple levels, identifying, e.g., GLVs, methanol, and MeJA as resistance-enhancing compounds (Duran-Flores and Heil, 2016; Quintana-Rodriguez et al., 2018). In recent studies, a combination of wounding and additional abiotic stresses (e.g., gasses, temperatures, dark treatments) revealed more volatile profiles in various species (Loreto et al., 2006; Brilli et al., 2011; Kanagendran et al., 2018). However, the focus of these investigations was mainly on the combined stress treatments, just mentioning DIVs for the sake of completeness of individual effects and not for its sole purpose. Apart from studies investigating the physiological and ecological role of DIVs, research unraveling the impact of wounding on plant volatile composition during food processing has also entered the global industry (Moretti et al., 2002; Farneti et al., 2013; Zeng et al., 2016).

WOUNDING VERSUS WOUNDING – PITFALLS IN STANDARDIZATION

A crucial aspect of all studies implementing artificial wounding is the standardization and reproducibility of such methods, especially regarding the comparability of obtained results. As discussed by Heil, mechanical damage was shown to be sufficient to induce responses in various species that are comparable to those observed after herbivore feeding – however not in all cases (Heil, 2009). The ambiguity of reports containing artificial wounding is mainly caused by the flexibility of the treatment itself. As recently highlighted by Waterman et al. (2019), the execution of mechanical damage can comprise cutting, scratching, piercing, grinding, or pinching of leaf areas differing in size whilst in- or excluding the midrip; therefore resulting in highly variable responses even within the same species (Mithöfer et al., 2005). Regarding its effect on VOC release, artificial wounding is known to produce elevated DIV levels (see above) although not as intense and diverse as during herbivory (Fontana et al., 2009; Holopainen and Gershenzon, 2010). These shortcomings were omitted by adding specific elicitors or oral secretion obtained from the respective herbivore. Furthermore, the construction of a robotic caterpillar ('MecWorm') revealed that continuous mechanical damage simulates herbivory more accurately than single wounding events, yielding DIV patterns comparable to actual herbivory (Mithöfer et al., 2005). Taken together, although the possibility of standardized wounding patterns to study DAMPs and DIVs in a comparable manner exists, the extent of reported artificial damage still varies tremendously.

WHICH DIVs CAN ELICIT DOWNSTREAM RESPONSES ON A MOLECULAR LEVEL?

While DAMPs activate defense-related signaling such as membrane depolarization, cytosolic Ca^{2+} concentration changes, generation of reactive oxygen species (ROS), MAPKinase activation, octadecanoid (jasmonate) and/or salicylic acid (SA) signaling, as well as downstream defense responses like the expression of digestion inhibitors and of defense-related genes (Duran-Flores and Heil, 2016; Li et al., 2020), our knowledge of VOC-induced defense-related responses is still fragmentary. In particular studies on early signaling events are missing. To answer the question whether any of the volatiles mentioned above could actually function as a DAMP, either systemically or between plants, it is crucial to consider whether they are (i) emitted after mechanical damage only and (ii) possess the ability to trigger detectable downstream responses on a molecular or physiological level. Although the exact mechanism of volatile perception still remains an enigma, evidence for perception of DIVs in trees, e.g., sugar maple (*Acer saccharum*) and poplar (*Populus x euroamericana*), was already found by Baldwin and Schultz (1983). This study demonstrated that airborne cues emitted from trees with artificially torn leaves triggered an enhanced accumulation of phenolic compounds and condensed tannins in nearby undamaged individuals. Over the following 20 years, an extensive amount of research was published, identifying specific DIVs and their ability to induce a plethora of responses in a broad spectrum of species ranging from trees, shrubs (sagebrush) to crops (cotton, tomato, potato), and model organisms (*A. thaliana*). The main observed responses to DIVs included accumulation of secondary metabolites, especially phenolic compounds and tannins (Baldwin and Schultz, 1983; Zeringue, 1987; Choi et al., 1994), upregulation of proteinase inhibitor gene expression and proteinase inhibitor biosynthesis (Farmer and Ryan, 1990; Reid, 1995), activation of defensive oxidative enzymes (Karban et al., 2000) by compounds such as MeJA or ethylene, which could even lead to an enhanced herbivore resistance (Karban et al., 2003).

A groundbreaking study by Arimura et al. (2000) continued to disentangle the impact of individual compounds in the upregulation of defense-related genes in lima bean. It was demonstrated that only VOCs emitted by *T. urticae*-infested leaves resulted in the upregulation of defense-related genes encoding pathogen-related (PR) proteins including PR-2 (β-1,3-glucanase), PR-3 (chitinase), as well as lipoxygenase (LOX), phenylalanine ammonia-lyase (PAL), and farnesyl pyrophosphate synthetase (FPS), whereas exposure to VOCs from artificially damaged plants only slightly triggered PR-2 gene upregulation. Although VOC emission profiles revealed the presence of (*Z*)-3-hexenol, α-pinene, (*E*)-β-ocimene, DMNT, α-copaene, junipene, β-caryophyllene, and MeSA after artificial wounding by punching holes into the detached leaves, the available concentration of the individual compounds was seemingly not sufficient to trigger defense mechanisms. Follow-up studies with whole plants revealed that GLVs such as (*Z*)-3-hexenol, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate were in fact able to induce the expression of defense genes in non-infested plants (Arimura et al., 2001; Farag et al., 2005). Findings by Bate and Rothstein (1998) corroborated the importance of C_6- GLVs (mainly (*E*)-2-hexenal) triggering plant defense response genes in *A. thaliana*. Additionally, the potential of DIVs such as DMNT or β-ocimene to activate transcript accumulations, if present in high enough amounts, was shown by their individual application resulting in upregulation of several

defense genes (Arimura et al., 2000). A similar observation was made by Meents et al. (2019) showing that VOCs released after mechanical wounding with tweezers or the application of DMNT only induced several defense genes and trypsin inhibitors in sweet potatoes in a cultivar- and concentration-specific manner. Both studies highlight the potential of single components as putative DAMPs; however the experimental setup, execution and magnitude of artificial wounding, air exchange, incubation time, and concentration of applied volatiles need to be critically taken into account.

More recent findings focused on the effect of mainly HIPVs in intra- and interspecific plant signaling, omitting artificial treatments and placing VOC signaling in a more ecological context. Matthias Erb and his team found the mainly herbivory-induced aromatic compound indole (**Figure 1**) to be a potent priming agent in maize (*Zea mays*) which increased the accumulation of defense-related phytohormones and volatiles in undamaged neighboring plants (Erb et al., 2015). Although the indole-mediated priming response was specific for maize only, exposure to synthetic indole triggered the emission of DMNT, α-pinene, and (*E*)-β-caryophyllene also in cotton and cowpea (*Vigna unguiculata*) (Erb et al., 2015). This highlights the potential of indole as a putative universal information transmitter among various species based on the fact that – although in small amounts only – it can be found in other species as well (Zeng et al., 2016; Li et al., 2019; Meents et al., 2019). Again, the mode of damage seems to play a crucial role for defense upregulation, based on studies showing the occurrence of small amounts of indole only after continuous mechanical wounding in certain species (Bricchi et al., 2010; Zeng et al., 2016; Meents et al., 2019) and not after single wounding events (Zhuang et al., 2012). These observations highlight that VOCs mainly declared as HIPVs are not necessarily limited to herbivory, but might also act as a damage-inducible priming agent and triggering DAMP mechanisms with sufficient indole released after wounding. Taken together all of these findings, there is a strong evidence for some DIVs regulating as volatile DAMPs various plant responses *via* different pathways.

How these DAMP signals act on and in neighboring plants and the receiving tissue is still not known. For sure, plants harbor the potential to perceive and transmit volatile signals. Some scientists highlighted the ability of DIVs to further induce VOC emissions in the receiver plant, e.g., via upregulating ethylene biosynthesis genes in lima bean (Arimura et al., 2002), local and systemic terpene production in tomato (Farag and Paré, 2002), or production of HIPVs-mimics in cotton, tobacco (*Nicotiana attenuata*), or maize as a response to MeJA or (*Z*)-3-hexen-1-ol (Halitschke et al., 2000; Rodriguez-Saona et al., 2001; Ruther and Kleier, 2005). Especially airborne MeJA connects different possible pathways, being taken up by the plant and consecutively converted into jasmonic acid and its active conjugates (Tamogami et al., 2008). Jasmonic acid and its conjugates are then able to regulate defense responses including VOC emission; sometimes in cooperation with peptide signaling as shown for prosystemin in tomato (Degenhardt et al., 2010). However, as shown for sweet potato, DIV-induced defense is not necessarily connected with the activation of the jasmonate pathway (Meents et al., 2019). These observations support the possibility of dual functions of certain volatile DAMPs such as DMNT, which could act with and without including known defensive pathways. Moreover, such DAMPs can either directly initiate defense as in the case of sweet potato (Meents et al., 2019) or being involved in priming (Erb et al., 2015).

It should be noticed that DIVs must also be seen in the original sense of tissue damage; i.e., this cue is not necessarily exclusively triggered in the event of an herbivore or pathogen attack but might be involved in activation in vital wounding repair mechanisms within the damaged individual, therefore serving as a shortcut. However, to our knowledge, volatile DAMPs-related to wound healing processes in plants have not been described yet.

SPECIFICITY, STABILITY, AND RANGE OF INFLUENCE

One recurring point of controversy has been the distance over which HIPV signals can be received by plants (Baldwin et al., 2002; Karban et al., 2003; Kessler et al., 2006). Recent work has shown that vascular constraints on systemic induction can be overcome with HIPVs (Karban et al., 2006; Frost et al., 2007; Heil and Bueno, 2007), as hypothesized by Farmer (2001) and Orians (2005). However, the potential of emitted VOCs to trigger a systemic response in the emitter or conspecific individuals is a complex interplay of various factors starting from released concentrations of active compound, cue specificity, stereochemistry-related configuration, field *vs* laboratory conditions, and the distance to the emitter (**Figure 3**) (Preston et al., 2004).

Among several well-studied volatiles, MeJA gained increasing attention from the 90s on after a study by Farmer and Ryan (1990) finding its emission significantly increased after excision of branches from sagebrush. Being conducted in enclosed bell jars only, Karban and colleagues transferred this knowledge to the field, performing further experiments demonstrating that wild tobacco plants growing near clipped sagebrush exhibit less herbivore damage than individuals without a wounded neighbor present (Karban et al., 2000), highlighting the defensive ability of DIVs. Upon further characterization of the emitted plume after mechanical damage in sagebrush, Preston et al. (2004) identified *cis*-MeJA as the main released epimer compared to the *trans* conformation. Subsequent experiments aiming to reproduce the emission of MeJA via application of lanolin paste or aqueous sprays revealed that neither *cis*- nor *trans*-MeJA elicited direct defenses in *N. attenuata* when applied in concentrations consistent with sagebrush emissions. This study exquisitely addressed that besides structural specificity, the application and the released amount of compounds is a crucial aspect making it tremendously difficult to treat plants in physiologically relevant quantities in order to reproduce observations made in the field.

Follow-up field studies on sagebrush conducted by Karban et al. (2006) found air contact and proximity of conspecific plants to be key to intra- and interplant communication. It was shown that adjacent conspecifics of clipped sagebrush were not

only influenced within a range of 15 cm but even up to 60 cm. Additionally, a downwind airflow toward the neighboring plant was necessary to establish volatile-mediated contact, ultimately triggering induced resistance among branches as well as within the wounded individual itself which was not observed by clipping and trapping released DIVs (Karban et al., 2000, 2006). In the case of neighboring tobacco plants, 5 days of exposure to clipped sagebrush increased the overall resistance for the whole season with up to 48% decreased herbivore damage (Karban, 2001; Karban et al., 2003). All of these findings underlined the possible longevity of volatile-based protective mechanisms even across different species, however suffering limitations based on airflow and spatial distribution of such cues. Considering the proximity of neighboring individuals, MeJA-based communication appears to be useful in sagebrush due to adjacent plants growing within a maximum distance of 60 cm apart (Karban et al., 2006) including the branches of the clipped individual itself.

Apart from warning neighboring (potentially eavesdropping) individuals, DIVs might also provide a fast and efficient mechanism of within-plant-signaling, reaching further locations of the wounded plant itself as has been demonstrated in lima bean, poplar, blueberry (*Vaccinium corymbosum*), and sagebrush (Karban et al., 2006; Frost et al., 2007; Heil and Bueno, 2007; Rodriguez-Saona et al., 2009; Heil and Adame-Álvarez, 2010). Depending on the growth form, Heil and Karban predicted that large and anatomically complex plants (especially lianas and vines) are more prone to use VOC-mediated protective mechanisms, omitting a time-consuming signaling cascade via the vascular system (Heil and Karban, 2010). Evidence for this hypothesis and the distance over which VOCs can travel was found in lima bean plants grown in the field. Heil and Adame-Álvarez (2010) demonstrated that cues from emitter plants triggered with JA or benzothiadiazole (BTH) increased secretion of extrafloral nectar as an output for resistance in independent receiver plants at a distance up to 50 cm. Interestingly, over 80% of the leaves located around a single leaf at this range still belonged to the same plant, therefore inducing resistance mainly in the same individual (**Figure 3**) (Heil and Adame-Álvarez, 2010). Additional findings were presented by Girón-Calva et al. (2012) highlighting the specificity of plant perception in lima bean, depending on the applied VOC and the dose and exposure time. Taken together, volatiles are representing a cue for within-plant-signaling as well as an alarm signal for surrounding plants of a possible threat, however in a limited range from 15 up to 60 cm, which was extended to 100 cm by work of Piesik et al. (2010) for some cereal crops and recently by Sukegawa et al. (2018) in a mint (*Mentha* × *piperita*) emitter – soybean (*Glycine max*) receiver system.

HOW ATMOSPHERIC EFFECTS CAN SHAPE VOLATILE DISTRIBUTION PATTERNS

In nature, plants are exposed to a vast number of environmental stimuli and stress factors, leading to drastic physio-chemical changes in the plant. These external factors are often omitted in studies that are performed in the laboratory. As addressed in a review by Holopainen and Gershenzon (2010), the co-occurrence of biotic and abiotic stresses such as high temperatures, nutrient availability in the soil, and increasing herbivore attacks, can significantly alter the volatile profiles in plants. These effects can be additive and result in an increased VOC emission, as observed in maize and lima bean (Gouinguené and Turlings, 2002; Vuorinen et al., 2004) under high temperature or ozone stress combined with herbivory, or prioritize a single response, e.g., anti-pathogen instead of anti-herbivore defense (Rostás et al., 2006). Strikingly, after degradation or condensation on leaf surfaces VOCs can play an entirely different biological role (Holopainen and Gershenzon, 2010).

As worked out recently, many different physico-chemical parameters can affect the occurrence and concentration of released VOCs in the close environment. Their particular vapor pressure, but also temperature, wind speed, relative humidity, and radiation are such factors (**Figure 3**) (Douma et al., 2019). In addition, an important key factor for volatile communication is the atmospheric lifetime of emitted VOCs which can range from 30 s up to several days (Atkinson and Arey, 2003). As stated again by Douma and colleagues, the chemical class of a certain compound is less important than its reactivity with atmospheric oxidants, biosynthesis rate, and volatility (Douma et al., 2019). Thus, the longevity of such a signal strongly depends on the presence of reactive radicals (OH, NO_3, O_3) and the number of C double bonds (Mofikoya et al., 2017). Especially ozone, known as the most important tropospheric air pollutant in rural areas (Ashmore, 2005), is highly reactive with a variety of VOCs (Pinto et al., 2007). As demonstrated by Blande et al. (2010) in laboratory studies, this can lead to a significantly decreased signaling distance and, hence, limited plant–plant communication. In numbers, the exposure of *T. urticae*-infested lima beans to 80 ppb ozone (representing concentrations of semi-urban areas) reduced VOC signaling distances from 70 cm (control) to 20 cm, mainly due to degradation of compounds such as (*E*)−β−ocimene, DMNT, and TMTT. Additionally, recent field studies revealed that priming of cabbage (*Brassica oleracea* var. *capitata*) after exposure to HIPVs of *Pieris brassicae*-infested neighbors was significantly disturbed (Girón-Calva et al., 2017) by elevated tropospheric ozone levels, therefore inhibiting a crucial VOC-mediated protective mechanism of plant communication. However, this adverse effect does not apply to all compounds and plant responses. Compounds such as MeSA or 2-butanone were not significantly affected and exposure to even higher ozone concentrations (160 ppb) stimulated extrafloral nectar production in lima bean, representing an increased defense mechanism (Blande et al., 2010). Apart from its influence in the plant itself, oviposition by *P. xylostella* was generally lower in plots under elevated ozone (Mofikoya et al., 2017), indicating that behavioral patterns by the herbivore are also altered in the process. The question whether this activation of defensive mechanisms might be used as a plant protection strategy or simply puts the plant under constant stress, still remains to be answered. All of these findings create a rather puzzling image regarding the benefit or drawback of air pollutants on plants and their VOCs; however representing

a major external factor that has to be considered when applying VOCs in the field.

VOLATILE DAMPs – ARE THEY USEFUL IN AGRICULTURE?

Over the last decades, numerous studies proposed the use of plant-based VOCs (DIVs as well as HIPVs) for crop protection as means for an environment-friendly pest management (**Table 1**). All having the same aim, various strategies have been suggested targeting different volatile-based mechanisms. Groundbreaking field studies by Pickett and colleagues (Cook et al., 2007; Hassanali et al., 2008; Pickett et al., 2014) introduced the push-pull-system by intercropping repellant and attractant plant species, luring pests toward attractive odors whilst protecting the important crop from damage.

Following up, various publications aimed to identify suitable crop species and cultivars based on their natural ability to release and induce VOC-mediated defenses in adjacent plants. Studies by Piesik et al. (2010) investigated the influence of mechanical damage and herbivory on the VOC emission in common cereals, e.g., wheat, barley (*Hordeum vulgare*), and oat (*Avena sativa*), revealing tremendous differences in quantities of especially GLVs emitted by injured plants. These species-specific differences in DIV quantity could even be observed in different cultivars of the same species in sweet potato (Meents et al., 2019). In both cases, herbivory resulted in the emission of higher amounts and more different VOCs compared to mechanical injury. However, low amounts of released DIVs after mechanical damage were already sufficient to induce the release of GLVs in uninjured crop plants within 1 m distance (**Figure 3**) (Piesik et al., 2010). The ability of DIVs to trigger an upregulated VOC release in adjacent plants might serve as an interesting starting point of signal amplification within an agricultural land plot. Supposing that artificial wounding of few individuals can trigger upregulation of VOCs in uninjured neighbors, which subsequently serve as relays amplifying the signal, it would be intriguing to test whether it could actually prime or induce resistance in larger areas of one plot. However, the feasibility of this concept strongly depends on the intensity and frequency of the given stimulus, stability and complexity of the signal, the ability of the receivers to perceive and respond to the given stimulus, the longevity of the response, and whether there is a trade-off between defense and yield. At this point, it might be worth to mention a very recent study demonstrating that among released VOCs – GLVs in particular – were the best candidates to indicate herbivore occurrence, suggesting their longer presence in the environment compared with other VOCs (Douma et al., 2019).

Independent of initial stimuli or wounding events, studies by Sukegawa et al. (2018) suggested mint species due to their constitutive emission of resistance-enhancing volatiles as suitable companion plants for soybean, *Brassica rapa,* and kidney bean (*Phaseolus vulgaris*). Cultivation or pre-incubation for up to 7 days in the greenhouse next to mint plants resulted in lowered herbivore damage and transcript accumulation of defense marker genes for up to 8 days. These promising findings confirmed previous studies in potato by Vucetic et al. (2014) highlighting the potential of constitutively emitted aromatic VOCs to elicit defense or priming in crop species. Another field study showed convincingly that repeated weeding-induced release of DIVs from goldenrod (*Solidago altissima*) plants reduced both leaf and seed damage in soybeans. It could be further shown that at least three different goldenrod-derived monoterpenes were involved in the induction of the respective soybean defense (Shiojiri et al., 2017). However as critically pointed out by Sukegawa et al. (2018), one has to consider whether the recipient crop species (such as soybean) is grown in large monocultures in the field, which might drastically attenuate the beneficial effect of mint as companion plants, making it more suitable for small scale house gardening and glasshouse cultivation.

Another interesting principle regarding volatile-based protection comprises the addition of a third trophic level. Various studies (Dicke et al., 1990, 2003; Turlings et al., 1990; Takabayashi and Dicke, 1996; Arimura et al., 2009; Baldwin, 2010) revealed that plants release distinct volatile blends upon herbivory in order to attract natural enemies of the attacking herbivore. Making direct use of this knowledge, researchers tested common HIPVs such as DMNT or (*Z*)-3-hexenyl acetate among many others, in field studies regarding their attractiveness toward parasitoids. In the process, MeSA as both a DIV and an HIPV, was revealed to be a promising candidate for commercial application due to its luring ability of predatory mites, bugs, and lacewings whilst repelling aphid plant pests (Dicke and Sabelis, 1987; Dicke et al., 1990; Drukker et al., 2000; Ozawa et al., 2000; James, 2003, 2005). Although being able to bait certain insect species in hop yards over a distance of 15 m away from the dispenser, studies using commercially available MeSA lures in strawberry (*Fragaria* × *ananassa*) fields did not result in decreased local pest abundance (Lee, 2010). This study just posing as an example, it nevertheless reveals the complexity of this strategy due to the predator's preferences and the potential lack of a rewarding system.

Combining aforementioned strategies, studies by von Mérey et al. (2011) constructed dispensers in maize fields releasing synthetic GLVs in order to induce and/or prime defense in neighboring plants while simultaneously monitoring predator and herbivore attractiveness. Although GLV-exposed maize plants emitted increased concentrations of sesquiterpenes, the hypothesis this would improve herbivore resistance could not be maintained but caused even higher numbers of herbivores, depending on the distance to the dispenser. Another crucial aspect is again the emitted concentration of each compound especially in complex mixtures, since repellent cues can be turned into attractants in the process or covering the desired function, especially when presented in the wrong context (D'Alessandro and Turlings, 2005; Mumm and Hilker, 2005; Snoeren et al., 2010). As addressed by Heil and Walters (2009) (VOC-mediated) induced systemic resistance seems to come with ecological costs. This effect is again highly species-specific and strongly dependent on the applied volatile, which was shown in a field study where lima bean and pepper (*Capsicum annuum*) were exposed to low doses of (*Z*)-3-hexenyl acetate for 7 days (Freundlich and Frost, 2019). Volatile treatment resulted in increased leaf

TABLE 1 | Overview of plant-derived DIVs and their application in the field.

Compound/molecule class	Classification	Emitter/source	Receiver plant	Applied VOC dosage	Distance emitter-receiver	Response	References (and ref. therein)
Methyl jasmonate (MeJA)	DIV	*Artemisia tridentata* (clipped)	*Nicotiana attenuata*	20–30 ng/g FW/h	15 cm	↑Polyphenol oxidase ↑Herbivore resistance	Karban et al., 2000
Methyl jasmonate (MeJA)	DIV	*Artemisia tridentata* (clipped)	*Artemisia tridentata*	n.a.	0–60 cm	↑Herbivore resistance	Karban et al., 2006
Methyl jasmonate (MeJA)	DIV	Dispenser (Chem-Tica sachet)	*Vitis labrusca* (var. Concord)	1 g; 7 mg/d released	0–30 m	↑Parasitoid abundance	James and Grasswitz, 2005
Methyl salicylate (MeSA)	DIV	MeSA dispenser (Predalure)	*Fragaria* × *ananassa*	2 g /lure	0–10 m	→Pest abundance	Lee, 2010
Methyl salicylate (MeSA)	DIV	Dispenser (Chem-Tica sachet)	*Vitis labrusca* (var. Concord)	5 g; 40 mg/d released	0–30 m	↑Parasitoid abundance	James and Grasswitz, 2005
Methyl salicylate (MeSA)	DIV	Dispenser (Chem-Tica sachet)	*Vitis labrusca* (var. Concord)	5 g; 60 mg/d released	0–30 m	↑Parasitoid abundance	James and Price, 2004
Methyl salicylate (MeSA)	DIV	Dispenser (Chem-Tica sachet)	*Humulus lupulus*	5 g; 60 mg/d released	0–30 m	↑Parasitoid abundance	James and Price, 2004
(*Z*)-3-Hexenyl acetate	DIV	Dispenser (Chem-Tica sachet)	*Vitis labrusca* (var. Concord)	1 g; 7 mg/d released	0–30 m	↑Parasitoid abundance	James and Grasswitz, 2005
(*Z*)-3-Hexenyl acetate	DIV	Lanolin paste	*Phaseolus lunatus*	30 ng/μl; 10 ng/h released	1 m	↑Height and biomass ↑Flower and fruit production ↓Herbivore damage ↓Cyanide production	Freundlich and Frost, 2019
(*Z*)-3-Hexenyl acetate	DIV	Lanolin paste	*Capsicum annuum* (var. Cayenne)	30 ng/μl; 10 ng/h released	1 m	↓Height and biomass ↓Flower and fruit production →Herbivore damage	Freundlich and Frost, 2019
(*E*)-β-Caryophyllene	DIV/ HIPV*	*Zea mays* ssp. *parviglumis*	n.a.	n.a.	1 m	↑Parasitoid abundance	Rasmann et al., 2005
n.a.	DIV/ HIPV°	*Mangifera indica* (var. Criollo)	n.a.	n.a.	20 cm	↑Parasitoid abundance	Carrasco et al., 2005
VOC mixture monoterpenes, GLVs, terpenes, N- and S-containing VOCs, DMNT, (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene	DIV/HIPV	*Brassica oleracea* (var. Capitata)	*Brassica oleracea* (var. Capitata)	n.a.	30 cm	↑VOC emission (priming)	Girón-Calva et al., 2017
VOC mixture (*E*)-2-hexenal, (*Z*)-3-hexen-1-yl acetate, (*E*)-β-ocimene	DIV	*Solidago altissima* (cut)	*Glycine max* (cv. Hyokei Kuro-3)	500 mg cut *S. altissima* pieces	0–15 m	↓Leaf damage ↓*Spodoptera litura* damage	Shiojiri et al., 2017
GLV mixture (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate	DIV	Dispenser	*Zea mays* (var. Tuxpeño Sequía)	0.2 ml	<0.1–1 m	↑Sesquiterpene emission ↑Herbivore damage ↑Herbivore abundance →Parasitism rate	von Mérey et al., 2011
(*E*)-β-farnesene	HIPV/cVOC (GMO)	*Triticum aestivum* (cv. Cadenza)	n.a.	Maximum 30.7 μg/plant/h released	0.5 m	→Grain yield →Aphid abundance →Parasitoid abundance	Bruce et al., 2015
VOC mixture 1,8-cineole, menthone, menthol	cVOC	*Mentha* × *piperita* (cv. Candy)	*Glycine max* (cv. Tanba–Kuro) *Brassica rapa* *Phaseolus vulgaris* (cv. Nagauzuramame)	n.a.	50–100 cm	↓Herbivore damage ↑Defense genes	Sukegawa et al., 2018
Push-pull-intercropping systems	DIV/cVOC	For a full review see					Pickett et al., 2014
Plant extracts	DAMP	For a full review see					Quintana-Rodriguez et al., 2018

DIV, damage-induced volatiles; HIPV, herbivore-induced volatiles; cVOC, constitutive volatiles; GLV, green leaf volatiles; GMO, genetically modified organism; n.a., not applicable. ↓ decrease; ↑ increase; → no change.
**VOC emission induced by herbivore feeding. °Beneficial effect observed after herbivory only.*

and flower formation, overall taller growth and decreased herbivory in lima bean plants, however coming at the cost of a reduced cyanide induction (trade-off). An entirely different output was observed in pepper, producing fewer flowers and fruits conjoined with reduced above- and belowground biomass and unaltered herbivore damage. These observations illustrate the effect of a single VOC on traits such as reproductive fitness and growth in a species-specific manner which very carefully needs to be considered while choosing a suitable VOC-plant pairing in agriculture. Having a large scale application of volatile treatments in agriculture in mind, in addition to the compounds' environmental compatibility and efficacy also their production costs must be considered, which may become a limiting factor.

CONCLUSION

Within the past decades, plant-based signaling compounds became increasingly popular as eco-friendly priming compounds or resistance boosters in the fields of biotechnology and agriculture. Unfortunately, up to now most of the proposed concepts have not yet proven to be successful enough to pose as viable alternatives for conventional crop protection strategies. This observation is mainly based on the variety of drawbacks addressed by Brilli et al. (2019) which still need to be further discussed and overcome in the future. However, new concepts exploring the potential of DAMPs as plant protective compounds found especially eDNA (Ferrusquía-Jiménez et al., 2020) to be a new candidate for application in the field. In addition to such treatments directly spraying compounds produced by wounded plant tissues on unwounded crops, we would like to focus onto damage-induced volatile compounds (DIVs). These DIVs are (i) specifically synthesized and emitted upon tissue disruption and (ii) can serve as intra- and interplant signals initiating immune responses as well. Due to their generation upon injuries or damage, these compounds can also be classified as DAMPs. Mainly GLVs but also DMNT and indole fulfill the criteria to be classified as volatile DAMPs in *stricto sensu*. Their airborne nature opens new possibilities for applications but also reveals new challenges. A general issue is the volatile-based communication itself, involving the plant as an emitter as well as a receiver. On the one hand, even in conspecific plants a high genetic identity does not guarantee a functioning communication between varieties as shown for sweet potato (Meents et al., 2019). On the other, VOC-emitting plants do not necessarily release "private messages" and may attract unwanted organisms as well as advantage eavesdropping adjacent plants competing for nutrients (Gershenzon, 2007). The intensity and longevity of the volatile "messages" itself is highly fluctuating as well since environmental conditions can strongly reduce the efficiency of the particular volatile compound not only on a physico-chemical level but simply by fast dilution due to strong winds. On a physiological scale, the cost-benefit ratio for the emitting plant and the effect on conspecific individuals need to be further investigated to prove an actual profit and not simply a trade-off. Taken together, up to this point DIVs pose as a promising approach for DAMP-based crop protection – however, mainly restricted to a controlled and space-limited area such as phytochambers and greenhouses.

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Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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2.3 Manuscript 3

2.3.1 Manuscript overview

Manuskript Nr. 3

Titel des Manuskriptes: Volatile DMNT systemically induces jasmonate-independent direct anti-herbivore defense in leaves of sweet potato (*Ipomoea batatas*) plants

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Autor/-in	**Konzeptionell**	**Datenanalyse**	**Experimentell**	**Verfassen des Manuskriptes**	**Bereitstellung von Material**
Anja K. Meents	-	40 %	45 %	45 %	-
Shi-Peng Chen	-	30 %	35 %	20 %	-
Michael Reichelt	-	5 %	15 %	-	10 %
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Volatile DMNT systemically induces jasmonate-independent direct anti-herbivore defense in leaves of sweet potato (*Ipomoea batatas*) plants

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Plants perceive and respond to volatile signals in their environment. Herbivore-infested plants release volatile organic compounds (VOCs) which can initiate systemic defense reactions within the plant and contribute to plant-plant communication. Here, for *Ipomoea batatas* (sweet potato) leaves we show that among various herbivory-induced plant volatiles, (*E*)-4,8–dimethyl–1,3,7-nonatriene (DMNT) had the highest abundance of all emitted compounds. This homoterpene was found being sufficient for a volatile-mediated systemic induction of defensive Sporamin protease inhibitor activity in neighboring sweet potato plants. The systemic induction is jasmonate independent and does not need any priming-related challenge. Induced emission and responsiveness to DMNT is restricted to a herbivory-resistant cultivar (Tainong 57), while a susceptible cultivar, Tainong 66, neither emitted amounts comparable to Tainong 57, nor showed reaction to DMNT. This is consistent with the finding that *Spodoptera* larvae feeding on DMNT-exposed cultivars gain significantly less weight on Tainong 57 compared to Tainong 66. Our results indicate a highly specific, single volatile-mediated plant-plant communication in sweet potato.

Plants are constantly subjected to different kinds of biotic stress. Especially herbivorous insects pose as a major threat based on their variability in ways of attacking the plant. An inevitable consequence of herbivory is the mechanical wounding of the infested tissue together with the introduction of signaling compounds from the feeding organisms that can be recognized by the plant to initiate the appropriate defense reactions[1]. Particularly chewing insects from the orders Lepidoptera and Coleoptera can cause severe tissue damage but simultaneously trigger distinct defense-related signaling pathways in the plant[2,3]. Generally, strategies in plant defense are classified as direct or indirect[4]. Direct defense strategies often rely on compounds that are toxic, repellent or anti-nutritive and contribute directly to the plants' defense[4]. Indirect defense describes the involvement of additional trophic levels apart from the host and the feeding insect. For instance, the production and emission of herbivore-induced plant volatiles (HIPV) such as terpenoids or fatty acid derivatives (green leaf volatiles), can attract predators and/or parasitoids of the herbivores and reduce infestation[5,6]. This generation and emission of HIPVs is regulated by phytohormones, in particular jasmonates[7]. In general, phytohormones play an essential role in regulatory processes during plant defense. Beyond the jasmonates, i.e. jasmonic acid (JA) and its active form jasmonoyl-isoleucine (JA-Ile), salicylic acid (SA), abscisic acid (ABA), and ethylene have been identified as signaling molecules that mediate and orchestrate the defense against pathogen and herbivore attacks[8,9].

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Important for the success of plant defense against herbivores is a coordinated local and systemic communication between cells of the infested tissue and distant organs. As recent studies demonstrated, for that purpose plants own fast, systemic, stress-related signaling mechanisms that include electrical signals, Ca^{2+} ions, and reactive oxygen species (ROS) traveling within the vascular system in order to trigger systemic responses in a plant under attack[10–13]. However, here only the leaves that are connected via the vascular system are integrated. In addition, it was shown that especially HIPVs can also be involved in signaling thereby inducing defense-related genes or priming systemic tissue within the plant as well as in a plant-plant communication where neighboring plants may receive information about an upcoming herbivore threat[14–20].

Sweet potato (*Ipomoea batatas* Lam.; Convolvulaceae) is one of the most important tuber crops worldwide with a rich phenotypic variability exhibited in many cultivars. Especially the development of insect-resistant cultivars attracted attention in agricultural sciences because sweet potato is subjected to a tremendous variety of pests. *I. batatas* has a high nutritional value, which is mainly due to an abundant storage protein, Sporamin, that is constitutively present in the tuberous roots. This protein also gained importance as a defensive protein against herbivores as it has insect–defense features due to its trypsin protease inhibitory (TPI) activity[21,22]. Previous studies showed that overexpression of *Sporamin* in tobacco or sweet potato led to severe growth retardation in larvae of *Spodoptera litura*, confirming the ability of this sweet potato trypsin inhibitor to confer insect resistance[21]. Sporamin protease inhibitor (SPI) induction in the leaves depends on jasmonates[23,24]. Recently, functional studies in sweet potato showed that the NAC-domain transcription factor, *Ib*NAC1, positively regulates *SPI* expression and thus contributes to the protection against wounding and herbivory[25].

Strikingly, upon inflicted mechanical damage on sweet potato leaves, *SPI* is locally but even stronger systemically induced in both leaves and stems[22,23]. This finding raised the question for the nature of the systemic signaling and the underlying mechanisms, in particular because *I. batatas* stems can reach a length of several meters; thus, a solely involvement of the vascular system seems unlikely and VOC mediated signaling is conceivable as discussed[19,26].

The overall goal of our study was to identify signals or compounds that are involved in and responsible for the systemic defense induction after mechanical wounding and herbivory in both an insect herbivory resistant (Tainong 57, TN57) and a susceptible (TN66) cultivar of *I. batatas*. Therefore, we analyzed wounding and herbivory-induced phytohormones including jasmonates, *SPI* expression, its inherent trypsin-inhibitor activity, and emission of volatile compounds in local and systemic leaves as defense-related readouts. In addition, we tested the impact of inducible defense in both cultivars on the performance of the insect herbivore *S. litura*. The knowledge gained from the present study provides better understanding of plant-plant communication in sweet potato. It highlights potential resistance traits that can be targeted in order to develop new approaches, which increase plant resistance against herbivore attacks.

Results and Discussion

Induction of defensive *sporamin* and jasmonate accumulation. Previous studies have shown that a complex wounding-inducible signaling cascade is activated by different modes of damage in sweet potato plants[22–25,27]. Strikingly, *SPI* transcripts were found to accumulate preferentially in non-wounded compared to wounded leaves[22]. We decided to first re-examine this finding and performed experiments using herbivorous insect larvae of the generalist lepidopteran species *Spodoptera littoralis* or the robotic caterpillar MecWorm (MecW)[28] mimicking only the mechanical wounding part during herbivory. After either treatment of the 3rd fully expanded sweet potato TN57 leaf for 0.5, 1 and 3 h, both the treated local leaf and the non-treated adjacent 4th entirely developed systemic leaf, which are not directly connected via the vascular system (Supplementary Fig. S1), were harvested for qRT-PCR to investigate SPI gene expression levels. While *S. littoralis* treatment induced only a small transient increase of *SPI* level in the local leaf, systemically, feeding resulted in a significant increase of *SPI* transcripts after 0.5 h (nearly 6-fold; Supplementary Fig. S2a). SPI gene expression after MecW treatment showed no increase in the local leaf whereas in the systemic leaves a clear trend of *SPI* upregulation (up to 8-fold after 1 h; Supplementary Fig. S2b) was observed. These findings confirm and further support the hypothesis that the wounding-inducible signaling cascade in sweet potato - which ultimately contributes to the plants' protection against the herbivore by the production of the trypsin inhibitor - is triggered mainly systemically.

In order to elucidate further whether the bioactive JA-Ile and other phytohormones play a role in local and systemic defense regulation, MecW-wounded and *S. littoralis*-fed TN57 leaves were analyzed for phytohormone levels. Compared to non-treated controls, the concentrations of both JA and JA-Ile showed a significant increase locally in the MecW-wounded leaves as well as in the *S. littoralis*-treated leaves at all time points (Fig. 1a–d). Strikingly, in contrast to the strong local response throughout both treatments, systemic leaves showed no significant differences compared to control plants (Fig. 1). So far, in sweet potato little was known about the production of other jasmonates apart from JA. Here, in addition to JA, JA-Ile was demonstrated to accumulate after both types of treatment: mechanical wounding and herbivory (Fig. 1c,d). Moreover, a pronounced accumulation of jasmonate metabolites was found; i.e. after the rapidly enhanced production of JA and JA-Ile in wounded and infested leaves, metabolites such as JA-OH, JA-Ile-OH and JA-Ile-COOH were detected as well. The accumulations of these metabolites occurred in a similar manner as found for JA and JA-Ile (Fig. 1e–j). This confirms the catabolism of JA and JA-Ile, which has been described by Wasternack and Hause[9].

Upon mechanical wounding, SA only showed a slight but significantly elevated level in the damaged leaf (MecW) after 3 h of treatment (Supplementary Fig. S3a). Analyses of the drought–stress related phytohormone abscisic acid (ABA) showed no obvious differences to control plants (Supplementary Fig. S3b). Also, SA levels were not significantly affected by herbivore feeding (Supplementary Fig. S3c) but herbivory treatment generated a significant amount of ABA in the damaged leaf compared to the untreated control after 3 h of treatment (Supplementary Fig. S3d). In conclusion, there was no induced production of stress–related phytohormones in local leaves that was nearly comparable to what was found for jasmonates.

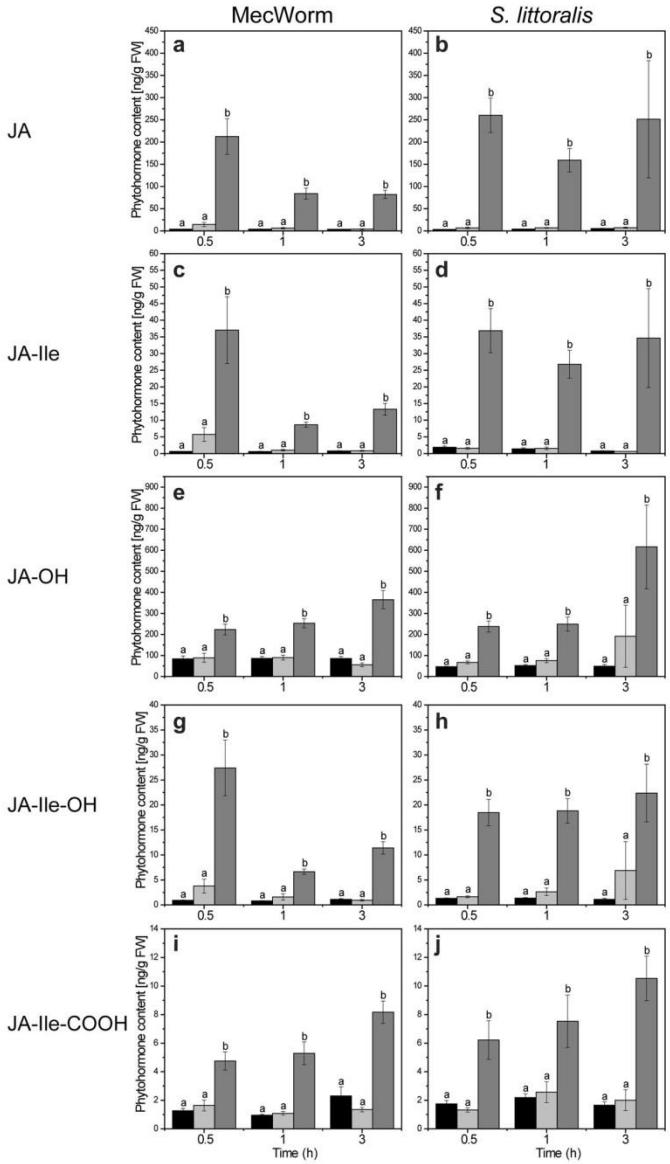

Figure 1. Local but no systemic increase of jasmonates after wounding in *Ipomoea batatas*. (**a–j**) Jasmonate levels after mechanical wounding by MecW (**a**,**c**,**e**,**g**,**i**; n = 10–11) and insect feeding by *S. littoralis* (**b**,**d**,**f**,**h**,**j**; n = 7–12) measured in *I. batatas* TN57 after 0.5 h, 1 h and 3 h. Phytohormone levels were measured in locally wounded leaves (dark gray bars) and the adjacent unwounded systemic leaf (light gray bars). Leaves from undamaged plants served as controls (black bars). Statistically significant differences between each treatment group after treatment were analyzed for each time point separately using one–way ANOVA. Different letters indicate significant differences among groups for $p < 0.05$, determined by Tukey's test. In (**a–j**), data are presented as mean ± SEM.

Since there was no detectable accumulation of jasmonates after herbivore or MecW treatment in unwounded sweet potato leaves but an induction of *SPI*, systemic activation of the defense-signaling cascade appears to be independent of increased JA levels on-site. These findings contradict previously proposed models by Rajendran *et al.*[24] and support the possibility of JA-independent pathways for *SPI* induction in unwounded leaf tissues.

Alternatively to a direct jasmonate-dependent upregulation of defense-related proteins, the activation by bioactive hydroxyproline-rich glycopeptides (HypSys peptides) was reported by Chen *et al.*[29]. These cell wall localized peptides are processed from wound- and jasmonate- inducible sweet potato precursor proteins with the ability to induce expression of *SPI* in *I. batatas* leaves. Thus, it is conceivable that the activation of *SPI* expression in unwounded sweet potato leaves is triggered without JA accumulation in the target leaves but by endogenous *Ib*HypSys peptides. However, an initial herbivory- or wounding-induced JA burst in the local, treated leaf seems necessary to start the whole cascade of signals and those peptides mainly act locally. Nevertheless, an example of JA-independent systemic defense responses has been observed in *Arabidopsis* plants in which the herbivore-induced accumulation of γ-amino-butyric acid (GABA) follows the same pattern[30]. Overall, these considerations lead to the conclusion that an accumulation of phytohormones in systemic tissues is not essential; at least for a systemic induction of defense-related genes like *SPI*.

Upregulated volatile emission in response to wounding and herbivory. Excluding the involvement of jasmonates in systemic defense upregulation in sweet potato TN57, other putative signaling mechanisms were investigated. Although the mechanism of volatile perception in plants remains so far unknown[4], VOCs can have a systemic effect on neighboring or within plants. These compounds may serve as a direct elicitor (e.g. methyl jasmonate), as a precursor for further transformation into a defensive compound[31] or by priming the adjacent plants in preparation for an imminent herbivore infestation[20,32]. In order to test whether VOCs released by wounded sweet potato plants can activate defense mechanisms in neighboring plants, a first proof-of-principle experiment was conducted. Two *I. batatas* plants were placed in a closed glass tank (34 L) without any physical contact; one plant was mechanically wounded using tweezers[24,25,27] with the second plant remaining non-wounded. After 24 h, in the non-wounded plants gene expression of *SPI* and the upstream transcription factor *IbNAC1* were analyzed. Compared to the control, both gene transcripts were found to be significantly induced (Supplementary Fig. S4).

For a deeper analysis, volatile compounds released by *I. batatas* after biotic/abiotic damage were collected and identified. Application of mechanical damage (MecW) and herbivore feeding (*S. littoralis*) on single sweet potato leaves led to an emission of at least 40 identified compounds (Supplementary Table S1) in TN57 with differences in terms of volatile bouquet quality as well as in quantities of individual compounds (Fig. 2a and Supplementary Table S1)[28,33]. Investigation of emitted volatiles in unwounded control plants of *I. batatas* TN57 and a second cultivar TN66 showed a basic composition comprising different classes of hydrocarbons including alkenes, aldehydes and mono-, sesqui-, and homoterpenes. Focusing on TN57, mechanical damage for 18 h revealed an emission of additional compounds such as (*Z*)-hex-3-enyl acetate, (*Z*)-jasmone or nerolidol. Apart from the previously detected compounds, feeding of *S. littoralis* resulted in a more complex volatile blend (Fig. 2a and Supplementary Table S1). Multiple sesquiterpenes like α-copaene, α-humulene, germacrene D as well as another monoterpene, (*E*)-β-ocimene, were added to the volatile bouquet. The homoterpenes (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) as well as (*E*)-β-caryophyllene and also indole and pentadecane increased upon *Spodoptera* treatment. Compared to all other compounds, a strikingly enhanced emission of DMNT was observed during treatments (Supplementary Table S2). Thus, the amount of emitted DMNT per cm^2 leaf area after both types of wounding was determined in TN57 (Fig. 2c). Control values showed that the experimental set-ups already triggered the emission of small amounts of DMNT due to the fixation of the leaf in a plexiglas cabinet (MecW) or enclosing the leaf into a feeding cage with additional bagging into plastic foil (*S. littoralis* feeding). The latter procedure – being more invasive compared to the MecW treatment - resulted in an even higher amount of emitted DMNT in the *S. littoralis* control samples. Nevertheless, feeding of *S. littoralis* resulted in a significantly higher emission of DMNT in comparison with MecW wounding and controls.Damaging the leaf with MecW for 18 h resulted in an 6-fold increased averaged total emission after 24 h of volatile collection compared to the control. DMNT emission during 24 h was even more pronounced in herbivore–infested leaves resulting in an average amount of 132 ng $\pm$ 31 DMNT cm^{-2} leaf area. Differences between emitted DMNT upon insect feeding and other treatments were shown to be significantly different (Fig. 2c). These results suggested that the strong increase of DMNT emission might play a role in the interaction of sweet potato against herbivores. Thus, in addition to TN57, we analyzed the volatile emission of the susceptible sweet potato TN66 using the same treatments (Fig. 2b; Supplementary Tables S1 and S2). In general, the VOC patterns in the non-treated control and after MecW treatment are only slightly different compared to the patterns found for TN57. Unwounded TN66 control plants emitted 25 detectable compounds with an even higher number of VOCs during MecW treatment (32 VOCs). The variety of volatiles emitted after mechanical wounding (30 VOCs) or control (22 VOCs) in TN57 was slightly lower. Upon *S. littoralis* feeding less compounds showed up in TN66 (33 identified VOCs) than in TN57 with 38 detectable emitted VOCs (Fig. 2; Supplementary Table S1). Regarding DMNT, after both types of treatment the emission of this particular homoterpene was also less pronounced in TN66 (Fig. 2d). The exact quantification of DMNT released from TN66 showed that neither after MecW nor *S. littoralis* treatment a significant increase was detectable. Again, DMNT emission upon larval feeding was significantly higher than upon sole mechanical wounding. The increased number of the individual compounds released after herbivore infestation can be explained by the combination of mechanical wounding with the contribution of HAMPs (herbivory-associated molecular patterns) provided by oral secretion[1]. In TN57, indole was induced only after herbivory while among all VOCs, DMNT showed the most pronounced upregulation after both MecW and herbivory, followed by caryophyllene. The very strong induction of DMNT upon both treatments was the main reason to choose this compound for further experiments. The most prominent compounds after feeding in TN66 were DMNT, TMTT, (*E*)-β-caryophyllene and pentadecane. Previous studies also showed the variability of

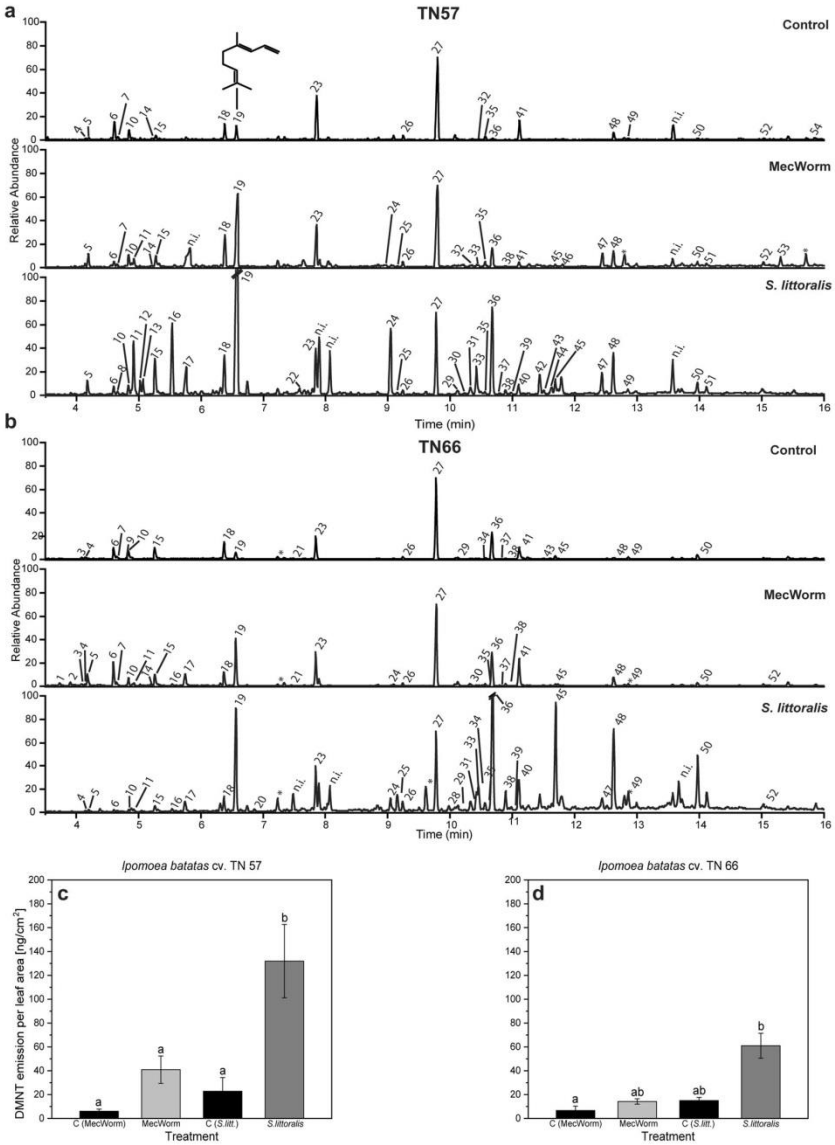

Figure 2. Volatile emission and upregulation of (*E*)-4,8–dimethyl–nonatriene (DMNT) in *Ipomoea batatas*. (**a**,**b**) Gas chromatograms of volatiles emitted by *I. batatas* TN57 (**a**) and TN66 (**b**): Controls without wounding; volatiles induced by mechanical damage (MecW) inflicted over 18 h; volatiles induced by feeding of *S. littoralis*. All volatiles were collected over 24 h and eluted with internal standard. Asterisks mark contamination by plasticizer or column residuals. Identified compounds are marked as follows: (1) α-Pinene; (2) 1-Butoxy-2-propanol; (3) 2-Ethylhexanal; (4) Benzaldehyde; (5) 5-Ethyl-(5 H)-furan-2-one; (6) 6-Methyl-5-hepten-2-one; (7) Mesitylene; (8) 1-Decene; (9) *n*-Decane; (10) *n*-Octanal; (11) *(Z)*-Hex-3-enyl acetate; (12) Hexyl acetate; (13) *(E)*-Hex-2-enyl acetate; (14) Limonene; (15) 2-Ethyl-hexanol; (16) *(E)*-β-Ocimene; (17) unidentified monoterpenoid (93, 136); (18) *n*-Nonanal; (19) 4,8-Dimethylnona-1,3,7-triene; (20) Phenyl acetonitrile; (21) Naphthalene; (22) *(Z)*-Hex-3-enyl butanoate; (23) *n*-Decanal; (24) Indole; (25) *n*-Tridecane; (26) *n*-Undecanal; (27) Internal standard (*n*-bromodecane); (28) *(E)*-2-Undecenal; (29) α-Copaene; (30) β-Cubebene; (31) 7-epi-Sesquithujene; (32) 1-Tetradecene; (33) *(Z)*-Jasmone; (34) *n*-Tetradecane; (35) Dodecanal; (36) *(E)*-β-Caryophyllene; (37) β-Copaene; (38) *(E)*-α-Bergamotene; (39) Sesquisabinene; (40) α-Humulene; (41) Geranyl

acetone; (42) Germacrene D; (43) β-Ionone; (44) Bicyclogermacrene; (45) *n*-Pentadecane; (46) Tridecanal; (47) Nerolidol; (48) (3E,7E)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene; (49) *n*-Hexadecane; (50) *n*-Heptadecane; (51) *n*-Pentadecanal; (52) *n*-Octadecane; (53) Isopropyl tetradecanoate; (54) *n*-Hexadecanol. Identification of compounds is shown in Supplementary Table S1. (**c**,**d**) DMNT emission after mechanical damage and herbivore feeding in *I. batatas* TN57 (**c**) and TN66. (**d**) VOCs were collected over 24 h with 18 h mechanical wounding by MecW (light gray bars; n = 5–8) or 24 h infestation with *S. littoralis* (dark gray bars; n = 7–12) and the respective control (black bars; n = 5–7). Bars represent the mean ± SEM of emitted DMNT in ng cm^{-2} leaf area. Statistically significant differences between each group were analyzed using a Kruskal-Wallis one-way ANOVA on ranks. Different letters indicate significant differences among groups for $p < 0.05$, determined by Dunn's test and adjusted p-values according to Benjamini & Hochberg. (**c**) TN57: $p < 0.001$. (**d**) TN66: $p = 0.004$.

emitted compounds by *I. batatas* in a cultivar-specific manner[34,35] and the resulting effect on the behavior of specialist herbivores[36]. The latter study focused mainly on root-emitted volatiles; we focus on emitted volatiles from leaves and their putative protective effect against feeding generalists like *Spodoptera* (Fig. 3). Because the emission of DMNT is a common phenomenon after insect feeding, though in a species- and herbivore- dependent manner[28,37,38], especially DMNT with its many biological functions[39,40] might be a regulator in wound-inducible signaling within and among sweet potato plants.

Airborne DMNT increases herbivore resistance in *I. batatas* TN57. To explore further, whether airborne DMNT alone is sufficient to induce defense responses and enhanced herbivore resistance in undamaged *I. batatas*, we synthesized DMNT and incubated this VOC (at comparable amounts that were emitted from treated leaves, i.e. app. 6 µg $leaf^{-1}$) together with sweet potato plants in a closed glass tank (34 L). Either DMNT or dichloromethane (DCM, serving as solvent for DMNT dilutions) as the respective control were applied on a cotton ball to avoid any physical contact with the plant and was incubated in adjusted concentrations and durations. The induction of defense-related SPI gene expression was fast; the incubation with 20 µg DMNT in a volume of 34 L (3.9 nM) showed already after 15 min an 11-fold upregulation of *SPI* (Supplementary Fig. S5a). This concentration of DMNT applied for 1 h resulted in an even higher *SPI* upregulation (21-fold) in sweet potato leaves while lower concentrations, i.e. 5 µg in the same volume, showed no detectable induction (Supplementary Fig. S5b), suggesting a concentration dependent reaction.

Based on these findings, further experiments were performed to elucidate whether DMNT-treated plants show increased resistance against herbivores. Therefore, sweet potato plants were exposed for 3 h to 3.9 nM DMNT and subsequently treated with *S. litura* for several days. By exposing *S. litura* larvae to volatile DMNT it was demonstrated beforehand that DMNT itself had no toxic effect on the larvae (Supplementary Fig. S6). A significantly reduced larval weight compared to the control treatment could already be observed after feeding for 7 d on TN57. This effect became even more pronounced after 10 d of feeding (Fig. 3a). In contrast to TN57, in TN66 no differences in the larval performance after feeding either on DMNT-exposed or control plants could be detected (Fig. 3b). After determining the *SPI* transcript accumulation, the trypsin inhibitory activity was analysed. Compared to the control treatment, DMNT-exposed TN57 plants displayed a significantly higher trypsin inhibitory activity, whereas in TN66 no induced activity was detected (Fig. 3c,d). Regarding the finding that herbivory and wounding induced jasmonates only locally, the ability of DMNT to induce JA or JA-Ile itself was investigated in the systemic leaves. As shown in Fig. 4 and consistent with the former results, no significant induction of jasmonates was measured.

Thus, we could demonstrate that the external application of synthetic DMNT to both sweet potato cultivars caused a significant induction of TPI activity only in TN57, which likely results in a reduced larval performance when feeding on those TN57 leaves.

In contrast, TN66 was more susceptible to insect feeding due to the lack of TPI-depending defense upregulation (Fig. 3). Based on these findings TN57 appears to be not only more effective in DMNT release but also more sensitive to DMNT compared with TN66, resulting in the induction of defense mechanisms.

Overall, DMNT was identified as a potent trigger for cultivar-specific systemic defense upregulation. Whether or not other VOCs such as caryophyllene or indole may have a similar function needs to be investigated in further studies.

Cultivar-specific volatile emission of sweet potato is known to play a crucial role in attraction of specialist attackers[35,36]. However, here not the plants' defense but their attractiveness towards the herbivore (*Cylas formicarius*) made them more susceptible or resistant. Herbivory-induced volatile release is also involved in the communication within and between plants. HIPV can be perceived and prime the receiving tissues and plants for a rapid and enhanced response upon subsequent wounding and insect attack[41]. It is well known that some green leaf volatiles (GLV) have priming activities in various plant species such as maize, Arabidopsis, tomato, and wheat[16,42–44]. Moreover, for maize it was recently shown that pretreatment with indole can increase the production of JA-Ile, ABA and certain volatiles after wounding, representing another example for priming[17]. For lima bean (*Phaseolus lunatus)* it has been shown that volatile blends emitted by *Tetranychus urticae*-infested lima bean plants as well as single compounds such as GLV but also DMNT were able to induce expression of some defense-related genes in neighboring plants[14,15]. Beyond priming or gene induction, the upregulation of a distinct direct defensive activity in neighboring plants simply by a volatile signal has never been shown for a crop plant. To our knowledge, the only example for a direct defense is β-ocimene emitted from *Myzus persicae*-infested *Brassica pekinensis* plants that was recently shown to induce glucosinolates in un-infested plants[45]. Strikingly, our study not only confirmed that in sweet potato upregulation of defense by VOCs alone is possible; it showed also that this was independent of jasmonate accumulation (Fig. 4). In addition, in TN57 for an increased TPI activity induction no further treatment was necessary, which does not represent a classical priming scenario.

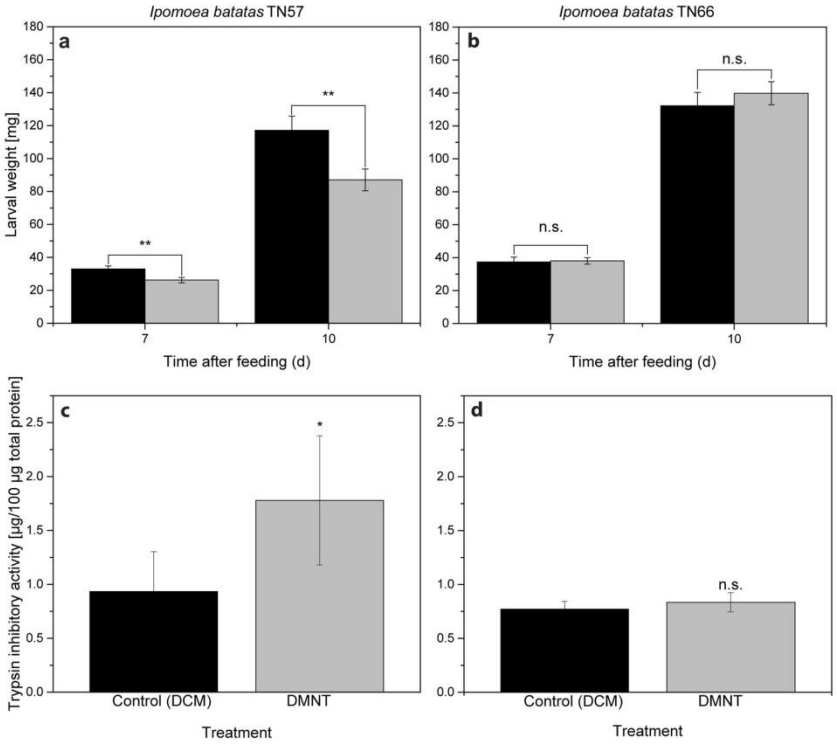

Figure 3. Airborne DMNT increases defense capabilities in *Ipomoea batatas* TN57. (**a**,**b**) Larval weight of *S. litura* after feeding for 7 d and 10 d on DCM (control) or DMNT- treated TN57 (**a**, n = 16) or TN66 (**b**, n = 25) plants. For DMNT treatment, plants were incubated with 3.9 nM for 3 h. (**c**,**d**) Trypsin inhibitory activity of TN57 (**c**, n = 5) and TN66 (**d**, n = 6) after incubation with 3.9 nM of DMNT for 3 h. Bars represent the mean ± SEM of larval weight or trypsin inhibitory activity for control (DCM, black bars) and DMNT treatment (gray bars). Significance levels are indicated by the asterisks (n.s. = non-significant; *$p < 0.05$; **$p < 0.01$). (**a**) TN57: p (CxDMNT) = 0.002; (**b**) TN66: p (CxDMNT) = 0.468. (**c**) TN57: p (CxDMNT) = 0.028; (**d**) TN66: p (CxDMNT) = 0.207. Asterisks indicate significant differences between control and DMNT treatment, based on a t-test (**c**,**d**) and ANOVA followed by a Tukey- adjusted comparison based on a linear model (**a**,**b**).

All of these findings show a cultivar-dependent specificity of volatile-mediated defense in sweet potato with DMNT as a signal compound able to trigger protective mechanisms for resistance against herbivores in non-attacked conspecific TN57 plants (Fig. 5). Next, it is necessary to study how DMNT and other VOCs function on the molecular level as has been shown for mint volatiles in soybean where histone acetylation in the promotor regions of defense genes caused enhanced RNA levels[46]. The ability to emit DMNT conjoined with the corresponding volatile perception ability emphasizes the relevance of this signaling compound for the defense in cultivar TN57. Morphological distant but adjacent parts of the same plant might also interact via volatiles, thereby omitting long-distance signaling along the vascular connections within the plant. Such a volatile shortcut may represent an efficient protection mechanism to enhance plant resistance against attackers. However, in particular systemic signaling within the plant may rely on a combination of both the vascular system and volatiles[26].

In any case, the demonstrated DMNT-SPI relation in sweet potato can serve as a promising model for plant-plant communication. Nevertheless, in the near future it is essential to gain more information on the systemic signaling processes in *I. batatas* during herbivory to further improve the resistance against insect pests. Identification and generation of cultivars with higher natural emission levels of DMNT might be useful to strengthen the overall resistance of this crop in the field.

Materials and Methods

Plant material and growth conditions. Sweet potato scions (*Ipomoea batatas* Lam.; cultivars Tainong 57 and Tainong 66) were grown in a substrate mix (200 L Klasmann TS1 mixed with 70 L Klasmann Tonsubstrat, Klasmann- Deilmann, Germany) in 10 cm diameter round pots under long day conditions (16 h light:8 h dark) at 28 °C (day) and 25 °C (night) and 70% relative humidity for 3 weeks. With light, illumination in the growth

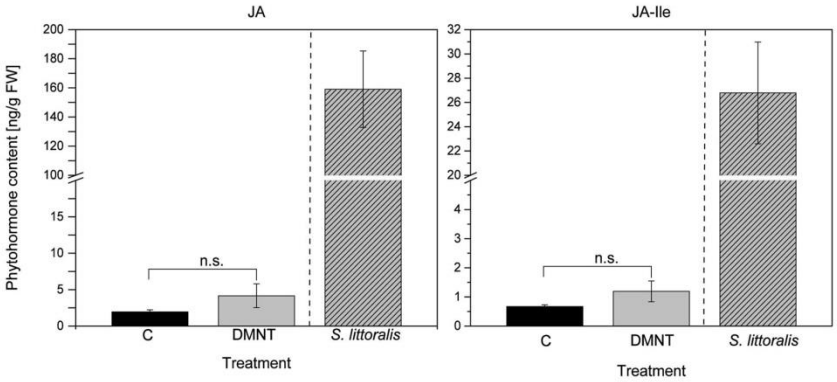

Figure 4. DMNT does not systemically induce jasmonate production. JA and JA-Ile levels of single *I. batatas* TN57 plants incubated with 1.41 µg of DMNT dissolved in dichloromethane in 2.4 L glass desiccators for 1 h (light gray bar, n = 10). Black bars indicate the control samples treated with dichloromethane (n = 7). *S. littoralis* feeding (light gray with stripes) was used as a positive control for the visualization of jasmonate induction. Bars represent the mean ± SEM of detected JA and JA-Ile. Significant differences were determined using a Shapiro–Wilk normality test and a subsequent Mann-Whitney rank sum test for the treatment and the respective control (n.s = non-significant); p JA (CxDMNT) = 0.130; p JA-Ile (CxDMNT) = 0.661.

chamber was kept at 100 µmol m^{-2} s^{-1}. All plants were fertilized once per week with 0.01% Ferty Basisdünger 3 (Planta Düngemittel, Germany). Plants used for studies at NTU (National Taiwan University, Taipei) were cultivated under the same growth conditions at a temperature of 25 °C (day) and 20 °C (night). Experiments conducted at MPICE in Jena were set up between 9 and 12 o'clock (am). In each experiment, 3-week-old plants with six to eight fully expanded leaves were used. Each 3rd fully expanded leaf was locally treated (MecWorm or *S. littoralis* feeding) and harvested together with the adjacent 4th leaf (systemic) using scissors.

Mechanical wounding with MecWorm. In order to mimic herbivore feeding without its elicitors in the oral secretion, MecWorm[28], known as the mechanical caterpillar was used to inflict continuous mechanical wounding to the 3rd fully developed leaf of each plant. Wounding sites of rectangular shapes were set with a punching speed of 12 punches per min lasting for 30, 60 and 180 min. For headspace volatile collection the continuous damage was extended to 18 h. Additionally, the treated leaf was enclosed in a Plexiglas cabinet of the MecWorm apparatus.

Insect rearing. *Spodoptera littoralis* (Boisd., Lepidoptera, Noctuidae) larvae were hatched from eggs (Bayer Cropscience, Germany) and reared on an artificial diet consisting of 500 g hackled beans, 9 g ascorbic acid, 9 g 4-ethylbenzoic acid, 9 g vitamin E Mazola oil mixture (7.1%), 4 ml formaldehyde, 1.2 l water, 1 g-sitosterol, 1 g leucine, 10 g AIN-76 vitamin mixture and 200 ml agar-water solution (7.5%). Insects were reared at 23–25 °C with a photoperiod of 14 h. Experiments conducted at the National Taiwan University applied 2nd-instar *Spodoptera litura* larvae for insect feeding assays. *S. litura* larvae were provided by Prof. Y. Wu (Department of Entomology, NTU). Twenty-four hours prior to herbivore application, 3rd to 4th–instar larvae of *S. littoralis* or 2nd–instar larvae of *S. litura* were separated into plastic cups for starving.

Herbivore infestation with *S. littoralis* larvae. For herbivore treatment with *S. littoralis*, the third fully expanded leaf of each plant was infested with a single 3rd to 4th–instar larva of *S. littoralis* and enclosed in a feeding cage for 0.5, 1 or 3 h. Modifications for volatile collection included that the treated leaf and the feeding herbivore with the surrounding feeding cage were enclosed in odorless PET foil (Bratschlauch, Toppits, Germany) for 24 h. All herbivore experiments were conducted in the growth chamber. Measuring of the treatment period started after the first observed feeding by the herbivore.

RNA extraction and quantitative real time (qRT)-PCR. Collected sweet potato leaves were ground to a fine powder in liquid nitrogen. RNA was extracted using TRIzol Reagent (Invitrogen, USA) by adding 1 ml reagent to 100 mg of ground leaf tissue. After thorough mixing, all samples were incubated at room temperature for 10 min. The following centrifugation steps were performed at 12,000 × g at 4 °C. After incubation, samples were centrifuged for 15 min and the supernatant was transferred into 200 µl chloroform. Subsequently samples were inverted for 1 min and centrifuged again for 15 min. Addition of 200 µl chloroform to the supernatant was repeated and samples were centrifuged for 15 min. The aqueous phase was added to approximately 600 µl isopropanol (1:1, sample: isopropanol) and precipitated overnight at 20 °C. For sedimentation, samples were centrifuged for 15 min and the supernatant was removed. RNA pellet was washed with 1 ml of cooled 80% ethanol, mixed and centrifuged for 15 min. The washing step was repeated once more and the resulting pellet was vacuum-dried

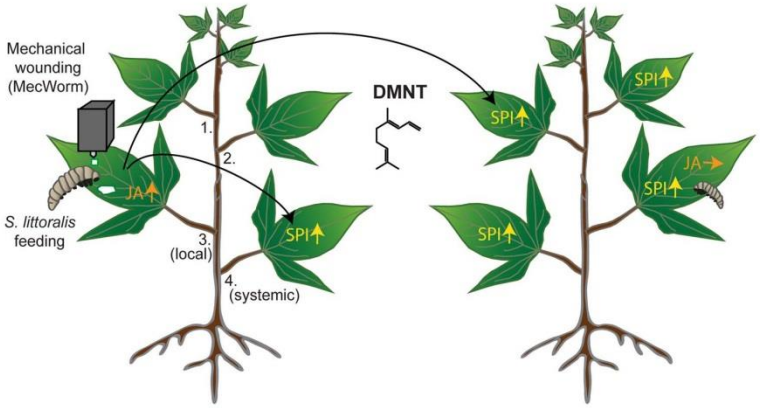

Figure 5. Model of DMNT emission that triggers SPI-dependent resistance enhancement in TN57 after mechanical wounding and *Spodoptera* herbivory. Upon mechanical wounding (MecWorm) or herbivore (*S. littoralis)* feeding, jasmonates (JA) are locally upregulated in the treated leaf. Sporamin protease inhibitor (SPI) is upregulated mainly systemically. In parallel, (*E*)-4,8–dimethyl–nonatriene (DMNT) is emitted to the environment and induces the generation of SPI in leaves of non-treated neighboring *I. batata*s plants without changes in JA levels. As a consequence, these plants show higher resistance against feeding *Spodoptera* larvae.

in an Eppendorf Concentrator plus (Eppendorf AG, Germany) for 10 min at 30 °C. Subsequently, the dried pellet was dissolved in 50 µl of preheated water for 15 min at 42 °C with mixing every 5 min. RNA concentration was measured using NanoDrop One microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). First strand cDNA was synthesized from 6 µg of total RNA using RevertAid First Strand cDNA synthesis Kit (Thermo Fisher Scientific, USA) with Oligo$(dT)_{18}$ Primer. Synthesis was conducted according to the manufacturers' instructions with slight modifications. After mixing 6 µg of template RNA with primer and water, additional incubation at 65 °C for 5 min with subsequent chilling on ice for 10 min was conducted before adding the reaction mix. The sample was incubated at 42 °C for 60 min. The reaction was stopped at 72 °C for 5 min. Subsequently cDNA was diluted by addition of 40 µl of water for a total cDNA concentration of 100 ng μl^{-1}.

Real-time qPCR analysis was performed using Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, USA) and gene-specific primers (Supplementary Table S3; Eurofins Genomics, Luxembourg). For normalization of gene expression levels, *IbACTIN-2* was used as housekeeping gene. The master mix was prepared according to the manufacturers' instructions using 400 nM of each gene-specific primer and 100 ng cDNA per well for a total reaction volume of 25 µl.

Gene amplification was achieved using Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA) comprising the following steps: 95 °C (3 min); 45 cycles of [95 °C (30 s), 60 °C (30 s), 72 °C (30 s)]; 95 °C (10 s), 65 °C (5 s) and 95 °C (50 s). The generated data was processed by using Bio-Rad CFX Manager (Bio-Rad Laboratories GmbH, USA). For analysis, the normalized fold expression was calculated according to the ΔΔCP method described by Pfaffl[47]. Expression levels were calculated according to the respective control treatment including the gene of interest and the housekeeping gene. For each experiment at least 6 (up to 11) biological replicates were used. Detected CT-values from technical replicates deviating more than 0.5 from each other were not used for calculation. The total RNA from DMNT-induced samples was obtained using the method of Chang, *et al.*[48]. Subsequent cDNA synthesis and qPCR was performed as previously described by Chen, *et al.*[25].

Phytohormone extraction and quantification. Collected sweet potato leaves were ground to a fine powder in liquid nitrogen and 200–250 mg of finely ground leaf material was weighed into an Eppendorf tube (Eppendorf AG, Germany). The extraction and detection was performed as previously described by Vadassery, *et al.*[49] with minor modifications. For phytohormone extraction, weighed powdery leaf material was mixed with 1.5 ml methanol containing 60 ng D_6–abscisic acid (Santa Cruz Biotechnology, USA), 60 ng of D_6–jasmonic acid (HPC Standards GmbH, Germany), 60 ng D_4–salicylic acid (Sigma-Aldrich, USA) and 12 ng of jasmonic acid-$^{13}C_6$-isoleucine conjugate as internal standard. After brief mixing, samples shook for 30 min at 4 °C using a Rotator Mixer RM-Multi 1 (STARLAB GmbH, Germany) using the program 100 rpm: 15 s, 75 °C: 16 s, 3 °C: 5 s. Samples were centrifuged afterwards at 13,000 × g at 4 °C for 20 min, the supernatant was collected and the remaining pellet resuspended in 500 µl methanol, shaken and centrifuged again as previously described. The combined supernatants were concentrated for 3 h using Eppendorf Concentrator plus (Eppendorf AG, Germany), re-suspended in 500 µl methanol and centrifuged at 16,000 × g at 4 °C for 10 min. Finally, 400 µl of supernatant were used for LC-MS/MS measurements.

Phytohormone analysis was performed using Agilent 1200 HPLC system (Agilent, USA) with subsequent API 5000 tandem mass spectrometer (Applied Biosystems, USA) with a Turbo spray ion source in negative ionization

mode. The elution profile was: 0–0.5 min, 10% B; 0.5–4.0 min, 10–90% B; 4.0–4.02 min, 90–100% B; 4.02–4.5 min, 100% B and 4.41–7.0 min, 10% B at a flow rate of 1.1 ml min^{-1}.

Multiple reaction monitoring (MRM) was applied to monitor analyte parent ion → product ion: *m/z* 209.1 → 59.0 collision energy (CE) −24 V, declustering potential (DP) −35 V) for jasmonic acid; *m/z* 215.1 → 56.0 (CE −24, DP −35 V) for D_6-jasmonic acid, *m/z* 322.2 → 130.0 (CE −30 V, DP −50 V) for jasmonic acid-isoleucine conjugate, *m/z* 328.2 → 136.1 (CE −30 V, DP −50 V) for jasmonic acid-$^{13}C_6$-isoleucine conjugate, *m/z* 136.9 → 93.0 (CE −22 V, DP −35 V) for salicylic acid, *m/z* 140.9 →97.0 (CE −22 V, DP −35 V) for D_4-salicylic acid, *m/z* 263.0 → 153.2 (CE −22 V, DP −35 V) for abscisic acid, *m/z* 269.0 → 159.2 (CE −22 V, DP −35 V) for D_6-abscisic acid and *m/z* 290.9 → 165.1 (CE −24 V, DP −45 V) for *cis*-(+)-12-oxophytodienoic acid (*cis*–OPDA).

Visualization of the plant vascular system. To determine the vascular connections of the sweet potato cultivar used in this study, the stem of 3-week-old *I. batatas* was cut at the plant base and split in two halves. The stem halves were submerged in tap water and commercial purple colored ink (Violett Pelikan 4001, Germany). After 3 h the ink was resorbed and translocated into the leaves of the plant as described in Zimmermann, *et al.*[50]. The staining of the vascular system was monitored with a digital camera.

Volatile collection and GC–MS analysis. Volatiles were collected over 24 h using the closed-loop stripping technique as previously described by Kunert, *et al.*[51]. Plants were either treated with MecWorm or *S. littoralis* as described above. Each single third and adjacent neighboring fourth leaf, still connected to the whole sweet potato plant, was enclosed in odorless PET foil bags (Toppits, Germany) to avoid contamination by soil volatiles. Each third fully developed leaf of the untreated plant was enclosed in odorless PET bags and used as control. Emission patterns of Plexiglas feeding cages used in herbivore bioassays showed no detectable influence on the induction of volatile release. For constant air circulation and continuous volatile collection each foil bag was connected to a circulation pump (Fürgut GmbH, Germany) attached to a charcoal trap with 1.5 mg absorption material (CLSA filter, 6 cm long, 0.5 cm diameter, Gränicher & Quartero, France). After collection, volatiles were eluted with 2 × 20 µl dichloromethane containing 50 µg ml^{-1} *n*-bromodecane as internal standard. Analysis was conducted using GC-MS (Finnigan TRACE GC 2000, Thermo Fisher Scientific, USA) equipped with a Zebron ZB-5 column (25 m × 0.25 mm × 0.25 µm, Phenomenex, USA) and the following temperature profile for separation of VOCs: Initial temperature was set at 45 °C for 2 min, heating up 10 °C min^{-1} to 200 °C followed by 30 °C min^{-1} to 280 °C. Helium was used as carrier gas with a flow rate of 1.5 ml min^{-1}. Split ratio was set at 1: 10 and 1 µl of the eluate was automatically injected. Injector temperature was set to 220 °C. The MS was run in EI mode (70 eV) with a scan range of 35 to 450 amu, a transfer line temperature of 280 °C, and an ion source temperature of 250 °C. Data acquisition was performed using Xcalibur 1.1 (Thermo Fisher Scientific).

Identification of VOCs. A mixture of n-alkanes C_8 – C_{20} in *n*-hexane (Sigma-Aldrich, USA) was measured before and after a sample sequence under the same conditions. Retention times of the *n*- alkanes were used to calculate the retention index (RI) for each peak in the GC-MS chromatogram according to the method of van Den Dool and Kratz[52].

Compounds were tentatively identified based on their mass spectra (MS) in combination with their individual RIs in comparison to Mass Spectral Library (NIST/EPA/NIH)[53], Adams[54] and Massfinder[55] MS and RI databases using Massfinder software in combination with Mass Spectral Library (NIST/EPA/NIH)[53] MS Search. Authentic reference compounds were used additionally for identification, if at hand. Retention indices deviating more than ±2 from the authentic references and ±5 compared to the database were regarded as mismatches and not considered. For relative quantification, identified peaks of the GC-MS total ion chromatogram (TIC) were integrated and the peak areas were divided by the peak area of the internal standard. According to Massfinder[55] instructions, peak areas below a minimum peak width of 2 with a sensitivity of 2 and an area threshold of 1000000 were regarded as below the quantification limit. For detailed information, see Supplementary Tables S1 and S2.

Absolute quantification of DMNT. The absolute amount of emitted DMNT was calculated using a standard curve. DMNT was synthesized as described in Maurer, *et al.*[56]. Different quantities of DMNT were dissolved in pure dichloromethane to generate solutions with the following concentrations of DMNT: 2.5; 5; 10; 20; 50; 100; 150; 200 and 250 µg ml^{-1}. All dilutions contained 50 µg ml^{-1} of *n*-bromodecane as internal standard for comparison with the previous volatile measurements. Emitted quantities of DMNT were calculated by division of DMNT peak areas through the respective peak areas of the internal standard. The output value was then inserted into the regression line formula of the measured DMNT standard curve and calculated according to the applied 40 µl elution volume per single leaf and its respective leaf area.

Induction of plants by volatiles and DMNT. To test whether VOCs of a wounded sweet potato plant can activate defense mechanisms in unwounded neighboring plants, two *I. batatas* plants were placed in a closed glass tank (34 L) without additional air flow and without any physical contact. One plant was then mechanically wounded using tweezers and placed next to an unwounded neighboring plant. Two unwounded plants were placed next to each other in the same type of glass container as control treatment. After 24 h, a single leaf of the non-wounded plants was harvested, used for RNA extraction and subsequent qRT-PCR. Gene expression of *SPI* and the upstream transcription factor *IbNAC1* were then analyzed.

In order to verify induction of defense mechanisms in sweet potato plants by DMNT only and whether there is a concentration- dependency, 5 and 20 µg of DMNT dissolved in 1 ml of pure dichloromethane were impregnated into a single piece of cotton wool to ensure accurate application. The cotton wool was placed without any physical contact centrally between sweet potato plants in a closed glass container (34 L; no additional air flow) for 1 h. As control equal volumes of pure dichloromethane were incubated on cotton wool and placed centrally between three plants.

Trypsin inhibitory activity assay in sweet potato. After a 3 h- exposure to 3.9 nM DMNT in DCM or pure DCM as control in an enclosed container, the third fully expanded leaf of each treated plant was harvested and used for protein extraction. The leaf material was grinded in liquid N to a fine powder and further homogenized in 2 ml extraction buffer (1x PBS pH 7.4; supplemented with 1 mM PMSF; Santa Cruz Biotechnology, USA). To determine the extracted amount of protein, Bradford assay was performed using Quick Start Bradford 1x Dye Reagent (Bio-Rad, Germany) according to the manufacturer's instructions. Subsequently, 100 µg of extracted protein were incubated with 2 µg trypsin (Sigma-Aldrich, USA) at 37 °C for 30 min. Afterwards 5 µl N-α-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (50 mg ml^{-1}, Sigma-Aldrich, USA) were added and incubated for 20 min with following absorbance measurements at 410 nm using Infinite M200 Microplate reader (Tecan, Switzerland). A regression curve was made using soybean trypsin inhibitor (0, 0.1, 0.2, 0.5, 1 and 2 µg; Sigma-Aldrich, USA) to normalize the quantity of trypsin inhibitor in the total amount of protein.

Feeding assay. To ensure an authentic volatile exposure, 3-week-old sweet potato plants were incubated with DMNT (20 µl of 1 mg ml^{-1} DMNT in DCM on a cotton ball) or 20 µl pure DCM (control) without any direct physical contact in a glass container (34 L) at 25 °C for 3 h. Afterwards, eight *S. litura* larvae (2nd instar) were placed on the pre-exposed sweet potato plants and allowed to feed for up to 10 d. In order to provide freshly DMNT- induced plants for feeding, the sweet potato plants were replaced after 3 d, 5 d, and 7 d. The larval weights were determined at 7 d and 10 d of feeding using an analytical balance.

DMNT toxicity assay. Second instar *S. litura* larvae reared on artificial diet were exposed to DMNT (20 µl of 1 mg ml^{-1} DMNT in DCM) or 20 µl pure DCM (control) without direct physical contact in a glass container (34 L) at 25 °C. The larval weights were determined after 10 d of feeding using an analytical balance.

Statistical analysis. Statistical significances of phytohormone contents were tested using Shapiro–Wilk normality test with a subsequent one–way ANOVA or Kruskal-Wallis one-way ANOVA on ranks in SigmaPlot (V 12.1.0). ANOVA was conducted for each single time point during all treatments. Tukey's test was selected for all pairwise multiple comparison procedures. Statistical significance between groups was given when $p < 0.05$. Statistically significant differences of quantified DMNT within each cultivar comparing all treatments were calculated using a Kruskal-Wallis one-way ANOVA on ranks in R (V 3.6.1; R Core Team) followed by Dunn's test with a Benjamini & Hochberg correction using the package "FSA v0.8.25" (R Core Team). Statistical significance between groups was given when $p < 0.05$. Relative quantification of emitted volatile organic compounds after mechanical wounding and herbivore feeding were analyzed using SigmaPlot (V 12.1.0). One-sample t-test (against 0) was used for cultivars that only showed a quantifiable amount in a single treatment group whereas samples with two comparable groups were analyzed using Mann-Whitney Rank Sum test. Cultivars emitting a detectable amount in more than two treatments were analyzed with a Kruskal-Wallis ANOVA on ranks. A linear model (lm) was used to examine the effect of DMNT on the larval weight of *S. littoralis*. In this model, the larval weight of *S. littoralis* was set as the dependent variable with treatment and time as the independent variables including the interaction of time and treatment. Significant interactions between the main effects in this model were analyzed using ANOVA. Tukey- adjusted pairwise comparisons of the DMNT treatment with the control based on the model were performed using the package "lsmeans" (R Core Team). These analyses were conducted using R (V 3.6.1; R Core Team). Levels of statistical significance are marked as the following: $p < 0.05$ (*); $p < 0.01$ (**) and $p < 0.001$ (***). Data generated using qRT-PCR was analyzed as described above (see "RNA extraction and quantitative real time (qRT)-PCR") according to Pfaffl[47] followed by a Shapiro-Wilk normality test and a one-sample t-test. Statistics for experiments comprising the determination of toxicity and trypsin inhibitory activity were done using Shapiro-Wilk normality test for data exploration followed by a t-test in SigmaPlot (V 12.1.0).

Data availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions
A.K.M., S.P.C., K. -W.Y. and A.M. designed the experiments. A.K.M., S.P.C., M.R. and H.H.L. performed the experiments. A.K.M., S.P.C., M.R., S.B. and A.M. analysed data. S.B. synthesized DMNT. A.K.M. and A.M. wrote the manuscript with contribution from all authors.

Competing interests
The authors declare no competing interests.

Additional information
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2.3.2 Supplementary Material Manuscript 3

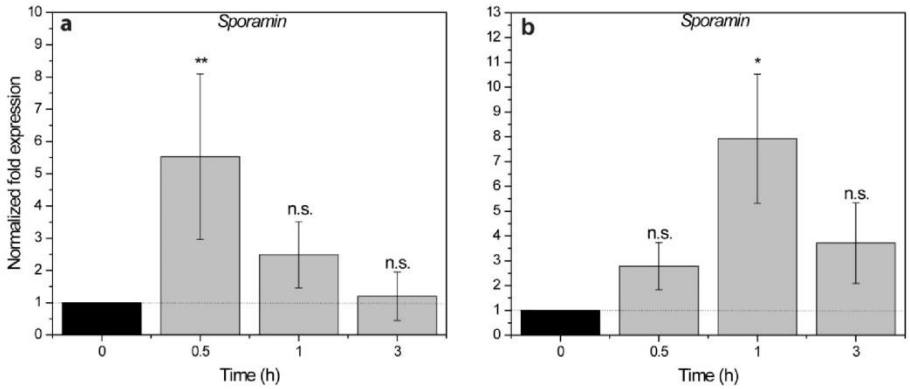
Supplementary Information

Volatile DMNT systemically induces jasmonate-independent direct anti-herbivore defense in leaves of sweet potato (*Ipomoea batatas*)

Anja K. Meents, Shi-Peng Chen, Michael Reichelt, Hsueh-Han Lu, Stefan Bartram, Kai-Wun Yeh, Axel Mithöfer

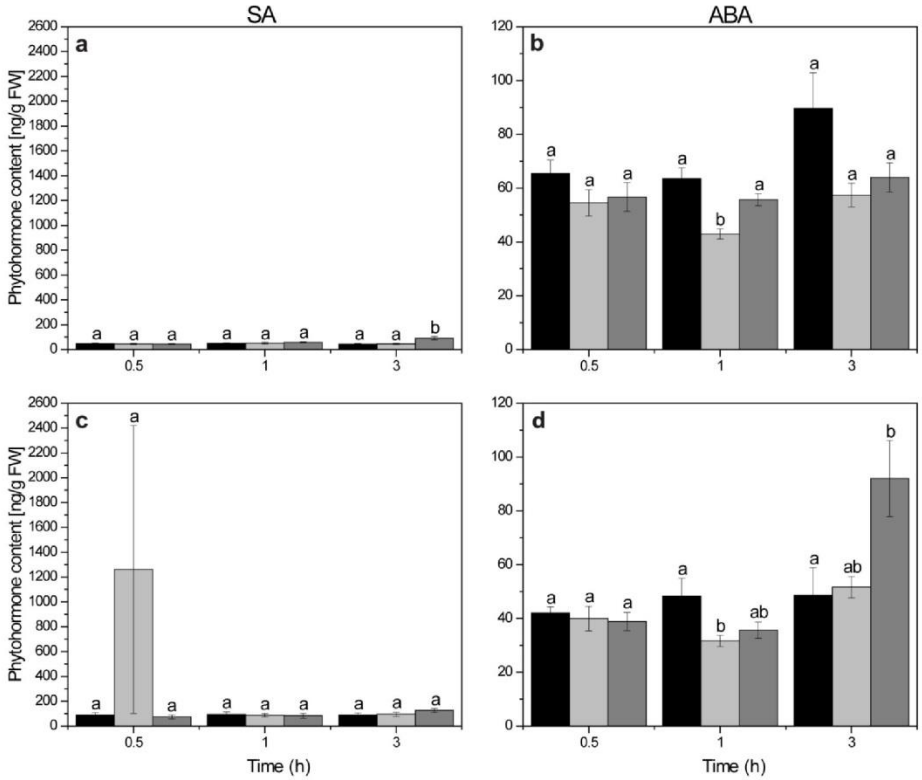
Supplementary Figure S1. No direct vascular connection between adjacent leaves 3 and 4 in 3-week-old *Ipomoea batatas*.

To demonstrate the vascular branching in *I. batatas*, the stems of 3-week-old sweet potato plants were cut at the plant base and split in two halves. The left stem half was placed in tap water with the right stem half submerged in purple ink and incubated for 3 h. (a) *I. batatas* before cutting (t0). (b) Stained *I. batatas* plant after 3 h incubation.



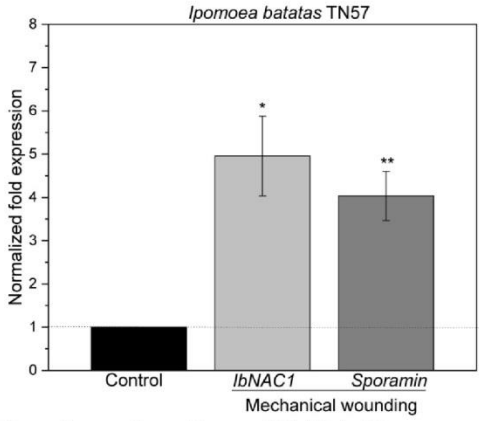
Supplementary Figure S2. Systemic induction of defensive *Sporamin* after *S. littoralis* feeding and MecWorm wounding in *Ipomoea batatas.*

Changes in the transcript level of *Sporamin* were determined in the intact systemic leaves (light gray bars) of *I. batatas* TN57 after 0.5, 1 or 3 h infestation with (a) a single larva of *S. littoralis* (0.5 h: n=10; 1 h: n=11; 3 h: n=6; p (controlx*S. littoralis* 0.5h) = 0.006) or wounding with (b) MecWorm (0.5 h: n=11; 1 h: n=11; 3 h: n=8; p (controlxMecW 1h) = 0.011) relative to the respective untreated control plants (black bar). Subsequent to a Shapiro-Wilk normality test, a one-sample t-test was selected to calculate statistical significant differences within each timepoint. Bars represent the mean ± standard error of the mean (SEM). Significance levels are indicated by the asterisks (*p<0.05;** p<0.01).



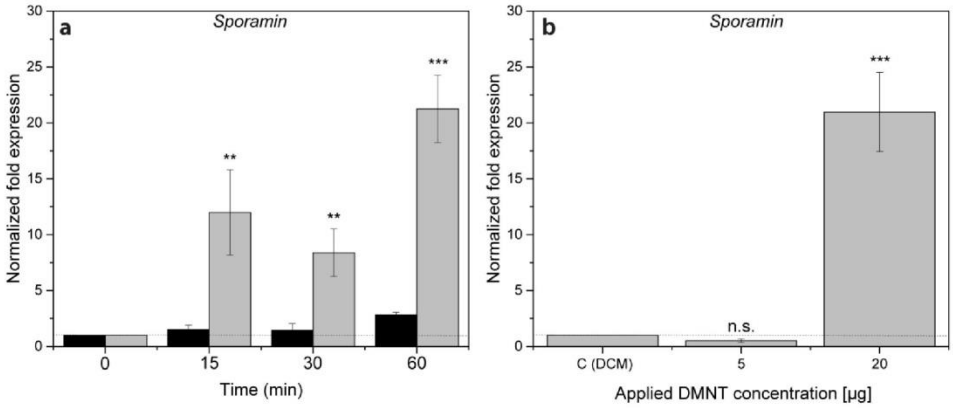
Supplementary Figure S3. Stable amount of salicylic acid and abscisic acid after wounding in *Ipomoea batatas* TN57.

(a-d) Salicylic acid (SA) and abscisic acid (ABA) levels after mechanical wounding by MecWorm (a, b; n = 10-11) and insect feeding by *S. littoralis* (c, d; n = 7-12) measured in *I. batatas* TN57 after 0.5 h, 1 h, and 3 h of treatment. Bars show the mean ± SEM of SA (a, c) and ABA (b, d) concentrations. Phytohormone levels were measured in locally wounded leaves (dark gray bars) and the adjacent unwounded systemic leaf (light gray bars). Leaves from undamaged plants were used as controls (black bars). Statistically significant differences between each treatment group after treatment were analyzed for each time point separately using one–way ANOVA or Kruskal-Wallis one-way ANOVA on ranks. Different letters indicate significant differences among groups for $p<0.05$, determined by Tukey's test.



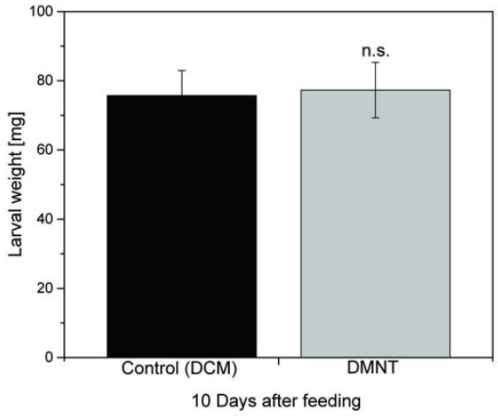
Supplementary Figure S4. Volatiles emitted by mechanically wounded plants induce *IbNAC1* and *Sporamin* in neighboring TN57 plants.

qRT-PCR of *IbNAC1* and *Sporamin* in unwounded TN57 leaves after incubation for 24 h with mechanically damaged neighboring plants. Bars represent the mean ± SEM of normalized fold *IbNAC1* (light gray bar) and *Sporamin* (dark gray bar) expression levels with undamaged plants (black bar) used as a respective control. Significance levels are indicated by the asterisks (* = $p<0.05$; ** = $p<0.01$) with n = 3. p (Cx*IbNAC1*) = 0.016; p (Cx*Sporamin*) = 0.005. Statistical analyses were performed for the treatment and the respective control with a Shapiro–Wilk normality test and subsequent one–sample t–test.



Supplementary Figure S5. Systemic induction of defense-related *Sporamin* by airborne DMNT occurs in a concentration- and time- dependent manner.

(a) Sweet potato plants (n = 3) incubated in an odorless glass container with 20 µg DMNT in a volume of 34 L (3.9 nM) dissolved in dichloromethane (DCM; light gray bars) for 15, 30 and 60 min. Plants incubated with DCM were used as control (black bars). (b) Sweet potato plants (n = 3) incubated for 1 h with either 5 µg or 20 µg of DMNT dissolved in pure DCM. Changes in the transcript level of Sporamin were determined in the 3rd fully expanded leaf of each plant. The gene fold expression change was calculated relative to DCM–treated control plants. Bars represent the mean ± SEM of normalized fold expression levels of *Sporamin*. Significance levels are indicated by the asterisks (n.s. = non-significant; ** $p<0.01$; *** $p<0.001$). (a) p (Cx*Sporamin* 15 min) = 0.009; p (Cx*Sporamin* 30 min) = 0.006; p (Cx*Sporamin* 60 min) < 0.001. (b) p (Cx*Sporamin* 5 µg) = 0.094; p (Cx*Sporamin* 20 µg) < 0.001 . Statistical analyses were performed for each treatment and the respective control with a Shapiro–Wilk normality test and subsequent one–sample t–test.



Supplementary Figure S6. DMNT is not toxic to *Spodoptera litura*.

Second instar *S. litura* larvae (n = 7) reared on artificial diet were exposed to DMNT (20 µl of 1 mg ml-1 DMNT in DCM) or 20 µl pure DCM (control) without direct physical contact in a glass container (34 L) at 25 °C. The larval weights were determined after 10 d of feeding. Bars represent the mean ± SEM of measured larval weight. Significance levels are indicated by n.s. = non-significant; p (CxDMNT) = 0.886. Statistical analyses were performed for the treatment and the respective control with a Shapiro–Wilk normality test and a subsequent t–test.

Supplementary Table S1. Identification of volatiles collected in the headspace of *I. batatas* TN57 and TN66 after mechanical wounding and feeding by *S. littoralis.*

See separate Excel file Supplementary Table S1

Supplementary Table S2. Quantification of volatiles (ng g^{-1} fresh weight) collected in the headspace of *I. batatas* TN57 and TN66 after mechanical wounding and feeding by *S. littoralis* relative to the internal standard.

See separate Excel file Supplementary Table S2

Supplementary Table S3. Primers used for real-time qPCR.

Primer name	Sequence
IbACTIN-2 F	GACTACCATGTTCCCCGGTA
IbACTIN-2 R	TTGTATGCCACGAGCATCTT
Sporamin F	TACTACATGTCTCCGCCATATGGG
Sporamin R	CTCAATCTTGAACTGGTTGCTATTGTC
IbNAC1 F	CGGCCGGGGATACAAATTTGTAAGCTT
IbNAC1 R	GAATCGGAATCCCGGCGGCATCTC

Supplementary Table S1. Identification of volatiles collected in the headspace of *I. batatas* TN57 and TN66 after mechanical wounding and feeding by *S. littoralis* .

No. (chromatogram)	RI (exp)	RI (DB)	Compound	Database	TN57	TN66
	901	900	*n*-Nonane	au	2	3
	903	901	*n*-Heptanal	ni	1,2	1,2
1	931	930	α-Pinene	au	-	2
2	942	945	1-Butoxy-2-propanol	ni	-	2
3	954	955	2-Ethylhexanal	ni	-	1,2
4	958	957	Benzaldehyde	au	1	1,2,3
5	961	966	5-Ethyl-(5H)-furan-2-one	ni	1,2,3	2,3
6	987	987	6-Methyl-5-hepten-2-one	au	1,2,3	1,2,3
7	991	994	Mesitylene	ad	1,2	1,2
8	991	989	1-Decene	ni	3	-
9	1000	1000	*n*-Decane	au	-	1
10	1003	1003	*n*-Octanal	ni	1,2,3	1,2,3
11	1008	1008	(*Z*)-Hex-3-enyl acetate	au	2,3	2,3
12	1015	1011	Hexyl acetate	ni	3	-
13	1018	1016	(*E*)-Hex-2-enyl acetate	ni	3	-
14	1027	1027	Limonene	au	1,2	2
15	1031	1030	2-Ethyl-hexanol	ni	1,2,3	1,2,3
16	1048	1049	(*E*)-β-Ocimene	ni	3	2,3
17	1100	-	unidentified monoterpenoid (93, 136)	-	3	2,3
18	1103	1103	*n*-Nonanal	au	1,2,3	1,2,3
19	1117	1118	4,8-Dimethylnona-1,3,7-triene	au	1,2,3	1,2,3
20	1137	1134	Phenyl acetonitrile	ad	-	3
21	1178	1178	Naphthalene	ad	-	1,2
22	1187	1187	(*Z*)-Hex-3-enyl butanoate	ni	3	-
23	1205	1205	*n*-Decanal	au	1,2,3	1,2,3
24	1293	1295	Indole	ni	2,3	2,3
25	1300	1300	*n*-Tridecane	au	2,3	3
26	1306	1307	*n*-Undecanal	ni	1,2,3	1,2,3
27	1349	1349	Internal standard (*n*-bromodecane)	au	1,2,3	1,2,3
28	1363	1365	(*E*)-2-Undecenal	ni	-	3
29	1374	1375	α-Copaene	au	3	1,3
30	1389	1389	β-Cubebene	ni	3	2
31	1390	1391	7-epi-Sesquithujene	ni	3	3
32	1392	1392	1-Tetradecene	au	1,2	-
33	1397	1394	(*Z*)-Jasmone	ni	2,3	3
34	1400	1400	*n*-Tetradecane	au	3	1,3
35	1408	1409	Dodecanal	ni	1,2,3	2,3
36	1417	1417	(*E*)-β-Caryophyllene	au	1,2,3	1,2,3
37	1428	1430	β-Copaene	ad	3	1,2
38	1435	1435	*(E)*-α-Bergamotene	ni	2,3	1,2,3
39	1443	1444	Sesquisabinene (1)	-	3	3
40	1452	1452	α-Humulene	au	3	3
41	1453	1453	Geranyl acetone	ni	1,2	1,2
42	1479	1479	Germacrene D	au	3	-
43	1484	1486	β-Ionone	ni	3	1
44	1494	1495	Bicyclogermacrene	ni	3	-
45	1500	1500	*n*-Pentadecane	au	2,3	1,2,3
46	1509	1509	Tridecanal	ad	2	-
47	1564	1564	Nerolidol	ni	2,3	3
48	1580	1580	(3*E*,7*E*)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene	au	1,2,3	1,2,3
49	1600	1600	*n*-Hexadecane	au	1,3	1,2,3
50	1700	1700	*n*-Heptadecane	au	1,2,3	1,2,3
51	1714	1715	*n*-Pentadecanal	ni	2,3	-
52	1800	1800	*n*-Octadecane	au	1,2	2,3
53	1827	1827	Isopropyl tetradecanoate	ni	2	-
54	1881	1880	*n*-Hexadecanol	ni	1	-

(1) tentatively Sesquisabinene A according to: Weissbecker B. et al.
J. Chem. Ecol., 2000, 26, 6, 1433-1445. [doi:10.1023/A:1005535708866]

Abrreviations:

au	Authentic ref	**Treatment:**	
Tentative identification with:		1	Control
ad	Adams	2	MecWorm
ni	NIST	3	*Spodoptera littoralis*

Supplementary Table S2. Quantification of volatiles (ng/g fresh weight) collected in the headspace of *I. batatas* TN57 and TN66 after mechanical wounding and feeding by *S. littoralis* relative to the internal standard.

				Control (MecWorm)		MecWorm		Control (Herbivore Feeding)		Herbivore feeding		Statistics	
No. (chromatogram)	RI (exp)	RI (DB)	Compound	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Test	p-value
	901	900	*n*-Nonane	bql	bql	bql	bql	bql	bql	bql	bql	-	-
	903	901	*n*-Heptanal	2537.19	1559.80	15075.70	11785.14	bql	bql	bql	bql	mw	0.328
4	958	957	Benzaldehyde	6391.53	2977.15	bql	bql	bql	bql	bql	bql	ost	0.069
5	961	966	5-Ethyl-(5H)-furan-2-one	bql	bql	bql	bql	bql	bql	2611.05	2611.05	ost	0.339
6	987	987	6-Methyl-5-hepten-2-one	4824.21	2885.85	3639.25	1958.43	3095.66	2185.28	4153.58	2617.20	aov	0.730
7	991	994	Mesitylene	4206.37	4206.37	10723.23	4978.29	bql	bql	bql	bql	mw	0.130
8	991	989	1-Decene	bql	bql	bql	bql	bql	bql	839.61	839.61	ost	0.339
10	1003	1003	*n*-Octanal	27817.07	16542.64	11680.06	8060.51	14266.23	7651.90	31546.55	11365.68	aov	0.068
11	1008	1008	*(Z)*-Hex-3-enyl acetate	bql	bql	10164.43	4053.53	6851.72	4076.40	16750.30	14578.58	aov	0.494
12	1015	1011	Hexyl acetate	bql	bql	bql	bql	bql	bql	3782.76	2552.03	ost	0.166
13	1018	1016	*(E)*-Hex-2-enyl acetate	bql	bql	bql	bql	bql	bql	2041.28	2041.28	ost	0.339
14	1027	1027	Limonene	163.39	163.39	581.20	417.54	bql	bql	bql	bql	mw	0.645
16	1048	1049	*(E)*-β-Ocimene	bql	bql	bql	bql	bql	bql	73669.73	37330.63	ost	0.200
18	1103	1103	*n*-Nonanal	4012.64	4012.64	14378.09	13978.76	2995.73	1455.77	21211.18	9505.34	aov	0.086
19	1117	1118	4,8-Dimethylnona-1,3,7-triene	47074.88	6276.82	209217.97	55472.86	140085.00	53851.82	649718.38	149030.15	aov	1(a),2(a),3(a),4(b) <0.001
22	1187	1187	*(Z)*-Hex-3-enyl butanoate	bql	bql	bql	bql	bql	bql	2731.41	2157.96	ost	0.232
23	1205	1205	*n*-Decanal	8026.09	7735.94	25973.04	25973.04	1329.21	866.71	10455.92	7573.00	aov	0.676
24	1293	1295	Indole	bql	bql	bql	bql	bql	bql	99031.16	31411.78	ost	0.313
25	1300	1300	*n*-Tridecane	bql	bql	1264.70	620.79	bql	bql	bql	bql	ost	0.081
26	1306	1307	*n*-Undecanal	185051.01	70444.21	76359.24	49988.87	788.68	788.68	31669.24	15322.81	aov	0.116
29	1374	1375	α-Copaene	bql	bql	bql	bql	bql	bql	32288.89	13229.11	ost	0.033
31	1390	1391	7-epi-Sesquithujene	bql	bql	bql	bql	bql	bql	12929.71	2864.87	ost	<0.001
33	1397	1394	*(Z)*-Jasmone	bql	bql	3527.83	1659.29	828.86	475.11	12250.13	7450.51	aov	0.308
35	1408	1409	Dodecanal	bql	bql	1156.66	757.82	bql	bql	bql	bql	ost	0.171
36	1417	1417	*(E)*-β-Caryophyllene	3658.82	946.14	38580.42	13401.63	4798.08	1783.28	179021.92	29825.04	aov	0.051
38	1435	1435	*(E)*-α-Bergamotene	bql	bql	27245.63	16607.11	1950.37	878.26	117163.86	33965.80	aov	1(a),2(a),3(b)=0.007
39	1443	1444	Sesquisabinene (1)	bql	bql	bql	bql	443.82	217.67	3171.03	676.94	mw	3(a),4(b)=0.008
40	1452	1452	α-Humulene	bql	bql	bql	bql	278.18	186.33	4555.59	2742.64	mw	3(a),4(b)=0.082
42	1479	1479	Germacrene D	bql	bql	bql	bql	562.02	267.35	5214.36	5214.36	mw	0.139
44	1494	1495	Bicyclogermacrene	bql	bql	bql	bql	bql	bql	2727.42	2492.05	ost	0.297
45	1500	1500	*n*-Pentadecane	bql	bql	2533.63	2015.18	535.30	317.52	77829.21	12255.34	aov	0.206
46	1509	1509	Tridecanal	bql	bql	1319.06	1319.06	bql	bql	bql	bql	ost	0.351
47	1564	1564	Nerolidol	1092.72	535.00	4223.56	2017.76	2120.90	1056.89	15858.65	5987.44	aov	1(a),2(a),3(a),4(b)=0.002
48	1580	1580	(3*E*,7*E*)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene	247.71	174.01	6920.59	3278.90	36966.77	19931.48	70276.16	12789.51	aov	1(a),2(a),3(a),4(b) <0.001
49	1600	1600	*n*-Hexadecane	bql	bql	bql	bql	10536.28	7247.98	26500.49	6996.54	mw	0.155
50	1700	1700	*n*-Heptadecane	301.21	150.99	2554.82	1275.17	1113.17	389.41	2338.22	1068.66	aov	0.370
51	1714	1715	*n*-Pentadecanal	bql	bql	bql	bql	3654.54	2271.95	21177.99	4171.06	mw	3(a),4(b)=0.002
52	1800	1800	*n*-Octadecane	1729.99	783.31	2297.50	784.25	bql	bql	bql	bql	mw	0.279
53	1827	1827	Isopropyl tetradecanoate	bql	bql	2935.40	1043.30	bql	bql	bql	bql	ost	0.026

(1) tentatively Sesquisabinene A according to: Weissbecker B. et al. J. Chem. Ecol., 2000, 26, 6, 1433-1445. [doi:10.1023/A:1005535708866]; **Abbreviations:** Bql (below quantification limit); **Treatment:** (1) Control; (2) Control (MecWorm); (3) Control (*Spodoptera littoralis*); (4) *Spodoptera littoralis*; **Statistical test used:** Ost (one sample t-test); Mw (Mann-White rank sum test); Aov (Kruskal-Wallis AOV).

Supplementary Table S2 (contin.). Quantification of volatiles (ng/g fresh weight) collected in the headspace of *I. batatas* TN57 and TN66 after mechanical wounding and feeding by *S. littoralis* relative to the internal standard.

				Control (MecWorm)		MecWorm		Control (Herbivore Feeding)		Herbivore feeding		Statistics	
No. (chromatogram)	RI (exp)	RI (DB)	Compound	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Test	p-value
	901	900	*n*-Nonane	bql	bql	bql	bql	2414.80	437.95	4287.19	987.07	mw	0.295
	903	901	*n*-Heptanal	bql	bql	3706.54	1811.86	bql	bql	bql	bql	ost	0.177
4	958	957	Benzaldehyde	bql	bql	265.41	187.91	bql	bql	bql	bql	ost	0.293
5	961	966	5-Ethyl-(5H)-furan-2-one	bql	bql	bql	bql	bql	bql	bql	bql	-	-
6	987	987	6-Methyl-5-hepten-2-one	1155.60	586.43	675.81	675.81	2578.00	1582.06	7194.52	2137.44	aov	0.093
7	991	994	Mesitylene	bql	bql	bql	bql	bql	bql	bql	bql	-	-
8	991	989	1-Decene	bql	bql	bql	bql	bql	bql	bql	bql	-	-
10	1003	1003	*n*-Octanal	bql	bql	2338.76	1531.17	bql	bql	bql	bql	ost	0.266
11	1008	1008	*(Z)*-Hex-3-enyl acetate	bql	bql	1427.07	1427.07	bql	bql	bql	bql	ost	0.423
12	1015	1011	Hexyl acetate	bql	bql	708.01	708.01	bql	bql	bql	bql	ost	0.423
13	1018	1016	*(E)*-Hex-2-enyl acetate	bql	bql	bql	bql	bql	bql	bql	bql	-	-
14	1027	1027	Limonene	bql	bql	690.15	491.10	bql	bql	bql	bql	ost	0.295
16	1048	1049	(*E*)-β-Ocimene	1124.33	1124.33	bql	bql	843.93	567.32	267.12	267.12	aov	0.643
18	1103	1103	*n*-Nonanal	bql	bql	bql	bql	157.79	157.79	7045.16	4985.16	mw	0.628
19	1117	1118	4,8-Dimethylnona-1,3,7-triene	15305.16	6522.85	47689.17	23659.61	25254.29	8591.66	33862.73	6877.90	aov	1(a),2(ab),3(ab),4(b); 0.004
22	1187	1187	*(Z)*-Hex-3-enyl butanoate	bql	bql	bql	bql	bql	bql	bql	bql	-	-
23	1205	1205	*n*-Decanal	1147.86	739.58	71.94	71.94	4434.44	2542.35	14306.06	7163.07	aov	0.181
24	1293	1295	Indole	bql	bql	1142.87	909.94	428.35	302.10	11143.32	3959.34	aov	0.122
25	1300	1300	*n*-Tridecane	bql	bql	bql	bql	1839.30	1114.87	19419.98	9167.51	mw	0.014
26	1306	1307	*n*-Undecanal	38351.04	38351.04	275.21	275.21	26380.11	26380.11	61785.89	61785.89	aov	0.942
29	1374	1375	α-Copaene	bql	bql	bql	bql	4277.08	1742.02	2123.49	902.73	mw	0.276
31	1390	1391	7-epi-Sesquithujene	bql	bql	bql	bql	bql	bql	bql	bql	-	-
33	1397	1394	*(Z)*-Jasmone	bql	bql	bql	bql	bql	bql	bql	bql	-	-
35	1408	1409	Dodecanal	bql	bql	287.98	287.98	9526.81	4252.70	21270.09	5441.72	aov	0.051
36	1417	1417	*(E)*-β-Caryophyllene	1420.42	681.81	1085.41	619.57	bql	bql	6502.82	5149.96	aov	0.570
38	1435	1435	*(E)*-α-Bergamotene	21037.56	11165.92	24085.06	14706.20	9414.99	2744.27	344803.18	145774.80	aov	0.073
39	1443	1444	Sesquisabinene (1)	bql	bql	bql	bql	bql	bql	bql	bql	-	-
40	1452	1452	α-Humulene	bql	bql	bql	bql	1830.82	1005.57	41532.08	12235.25	mw	0.001
42	1479	1479	Germacrene D	bql	bql	bql	bql	bql	bql	bql	bql	-	-
44	1494	1495	Bicyclogermacrene	bql	bql	bql	bql	bql	bql	bql	bql	-	-
45	1500	1500	*n*-Pentadecane	618.88	318.25	100.92	100.92	bql	bql	bql	bql	mw	0.196
46	1509	1509	Tridecanal	bql	bql	bql	bql	bql	bql	bql	bql	-	-
47	1564	1564	Nerolidol	2599.29	2599.29	29.73	29.73	1280.75	1110.58	1918.05	1276.94	aov	0.989
48	1580	1580	(3*E*,7*E*)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene	302.23	302.23	526.64	314.33	557.53	376.10	16112.35	12800.32	aov	0.826
49	1600	1600	*n*-Hexadecane	bql	bql	bql	bql	bql	bql	3420.72	3420.72	ost	0.356
50	1700	1700	*n*-Heptadecane	bql	bql	bql	bql	bql	bql	bql	bql	-	-
51	1714	1715	*n*-Pentadecanal	bql	bql	bql	bql	bql	bql	bql	bql		
52	1800	1800	*n*-Octadecane	bql	bql	bql	bql	bql	bql	bql	bql	-	-
53	1827	1827	Isopropyl tetradecanoate	bql	bql	bql	bql	bql	bql	bql	bql	-	-

(1) tentatively Sesquisabinene A according to: Weissbecker B. et al. J. Chem. Ecol., 2000, 26, 6, 1433-1445. [doi:10.1023/A:1005535708866]; **Abbreviations:** Bql (below quantification limit); **Treatment:** (1) Control; (2) Control (MecWorm); (3) Control (*Spodoptera littoralis*); (4) *Spodoptera littoralis*; **Statistical test used:** Ost (one sample t-test); Mw (Mann-White rank sum test); Aov (Kruskal-Wallis AOV).

2.4 Unpublished results

2.4.1 Sweet potato peptides *Ib*HypSys4 and *Ib*PepI differentially regulate anti-herbivore defense in sweet potato (*Ipomoea batatas* L.)

2.4.1.1 Material and Methods

Plant material and growth conditions

Sweet potato scions (*Ipomoea batatas* Lam.; cultivar Tainong 57) were grown as previously described in Meents et al. (2019) (Manuscript 3) for 3 weeks under long day conditions (16 h light : 8 h dark) at 28 °C (day) and 25 °C (night) in 70% relative humidity.

Peptides

Peptides (*Ib*HypSys4: REEKPOOOAOETDDPN; *Ib*PepI: LSSRPPRPGLGNSGDPQTNDTSS; *Sl*Pep6: ATDRRGRPPSRPKVGSGPPPQNN; scrambled: PEROEDDNEOPKORPC) were ordered from GenScript Biotech (Leiden, Netherlands) and dissolved prior to each experiment in double-distilled water to a final concentration of 25 µM.

Peptide spray treatments

To study the local effects of peptide solutions on DMNT emission and gene expression, whole plants with six to eight fully expanded leaves were evenly sprayed with peptide solution or double-distilled water (control) until all leaves were fully covered in liquid. After a 1 h incubation period, single plants were placed for 24 h in 2.4 L glass desiccators (VWR international) for headspace volatile collection. For RNA-Seq, qRT-PCR, and phytohormone analyses each 3rd fully expanded leaf was locally sprayed with peptide solution or ddH_2O (control) and harvested together with the adjacent 4th leaf (systemic) after 1 h of incubation.

VOC collection and quantification

Volatiles were collected over 24 h from peptide- or water-treated sweet potato plants enclosed in 2.4 L desiccators using the closed-loop stripping technique (Kunert et al., 2009). Throughout the headspace collection, each desiccator was connected to an air circulation pump (Fürgut GmbH, Germany) containing a charcoal trap with 1.5 mg absorption material (CLSA filter, 6 cm long, 0.5 cm diameter, Gränicher & Quartero, France). After collection, volatiles were eluted and measured as described in Meents et al. (2019) (Manuscript 3) with minor modifications. In this study, samples were eluted with 2

x 20 µl of dichloromethane containing 10 µg ml^{-1} *n*-bromodecane as internal standard used for further relative quantification.

RNA extraction and quantitative real time (qRT)-PCR

Harvested sweet potato leaves were processed and used for real-time PCR as described in Meents et al. (2019) (Manuscript 3) with the additional primer pair *IbLRR-RLK1* (5'-3'; F: TGGCCCATTTCCTGAGTCTTTAC; R: GACAGGCAAGGTTCCAACAAGATT; Eurofins Genomics, Luxembourg) on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA).

Phytohormone extraction and quantification

Local and systemic leaves collected after 1 h peptide treatment were extracted and measured according to Meents et al. (2019) (Manuscript 3) using Agilent 1200 HPLC system (Agilent, USA) with subsequent API 5000 tandem mass spectrometer (Applied Biosystems, USA) with a Turbo spray ion source in negative ionization mode.

RNA-Seq

RNA from single 3rd leaves treated for 1 h with *Ib*HypSys4, *Ib*PepI, and water (control) was extracted according to Meents et al. (2019) (Manuscript 3) using TRIzol Reagent (Invitrogen, USA). Four biological replicates per treatment were used for RNA-Seq experiments conducted by Novogene Europe (Cambridge, UK). RNA quality was monitored using NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). 1 µg of RNA per sample was used as template material for further sample preparations. Sequencing libraries were generated *via* NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's instructions. 20 M paired end reads of 150 bp per sample were generated, sequenced on an Illumina NovaSeq 6000 instrument (San Diego, USA), and mapped in reference to the genome of *Ipomoea trifida* (http://sweetpotato.plantbiology.msu.edu). Differential gene expression analysis was conducted using DESeq2 (v1.22.2) R package followed by a Benjamini and Hochberg FDR correction of the resulting p-values. Genes with an adjusted p-value of < 0.05 were considered as significantly differently expressed. Statistical enrichment analyses of differential gene expression in KEGG pathways was executed with KOBAS (v3.0).

Statistical analysis

Data generated using qRT-PCR was analyzed as described in Meents et al. (2019) (Manuscript 3) followed by a Shapiro-Wilk normality test with subsequent t-test or Mann-Whitney rank sum test based on the data distribution. Phytohormone levels were analyzed using a two-way ANOVA with initial Shapiro-Wilk-normality and equal variance test. For all analyses, phytohormone content was set as the dependent variable with treatment and leaf type as independent variables. For identification of significant differences between groups, pairwise multiple comparison procedure via the Holm-Sidak method was implemented with a significance level of $p < 0.05$. All statistical analyses were conducted in SigmaPlot (V 11.0).

2.4.1.2 Results and Discussion

***Ib*HypSys4 induces the emission of anti-herbivore DMNT in sweet potato**

In our previous study we demonstrated that the wound-inducible volatile (*E*)-4,8–dimethyl–1,3,7-nonatriene (DMNT) is sufficient to induce the defensive trypsin inhibitor sporamin in the sweet potato cultivar Tainong 57 in a jasmonate-independent manner (Manuscript 3). Apart from this novel volatile shortcut, the endogenous 18 aa hydroxyproline-rich glycopeptide *Ib*HypSys 4 was also shown to activate *sporamin* expression upon wounding and methyl jasmonate treatment (Chen et al., 2008) therefore serving as an additional systemic signaling component during anti-herbivore defense. In order to elucidate whether *Ib*HypSys4 contributes to DMNT-mediated protection, whole sweet potato plants were sprayed with the active peptide and volatiles were collected over 24 h to test for DMNT induction. Application of *Ib*HypSys4 resulted in a significantly increased DMNT emission compared to water controls (Fig. 1 a). In addition to previously discovered HypSys signaling peptides, plant elicitor peptides (Peps) were also found to induce defense-related genes and anti-herbivore volatiles in maize and *Arabidopsis thaliana* (Huffaker and Ryan, 2007;Huffaker et al., 2013;Huffaker, 2015). Based on amino acid sequences shared with known Peps in other species, the novel peptide candidate *Ib*PepI was identified in *Ipomoea batatas* in reference to the genome of *Ipomoea trifida* and tested for activity using ROS-based luminescence assays (Lu, Meents, et al., in prep). Although *Ib*PepI was confirmed to possess bioactive properties, 24 h incubation with *Ib*PepI did not lead to DMNT induction (Fig. 1 b), highlighting the specificity of HypSys and Pep-induced volatile responses in sweet potato. Scions incubated with the tomato-derived peptide *Sl*Pep6 (Fig. 1 c) or an inactive scrambled peptide (Fig. 1 d) also only displayed

basal DMNT levels comparable to the control treatment, therefore underlining the functionality of the peptide application method and the (species-)specificity of the elicitor.

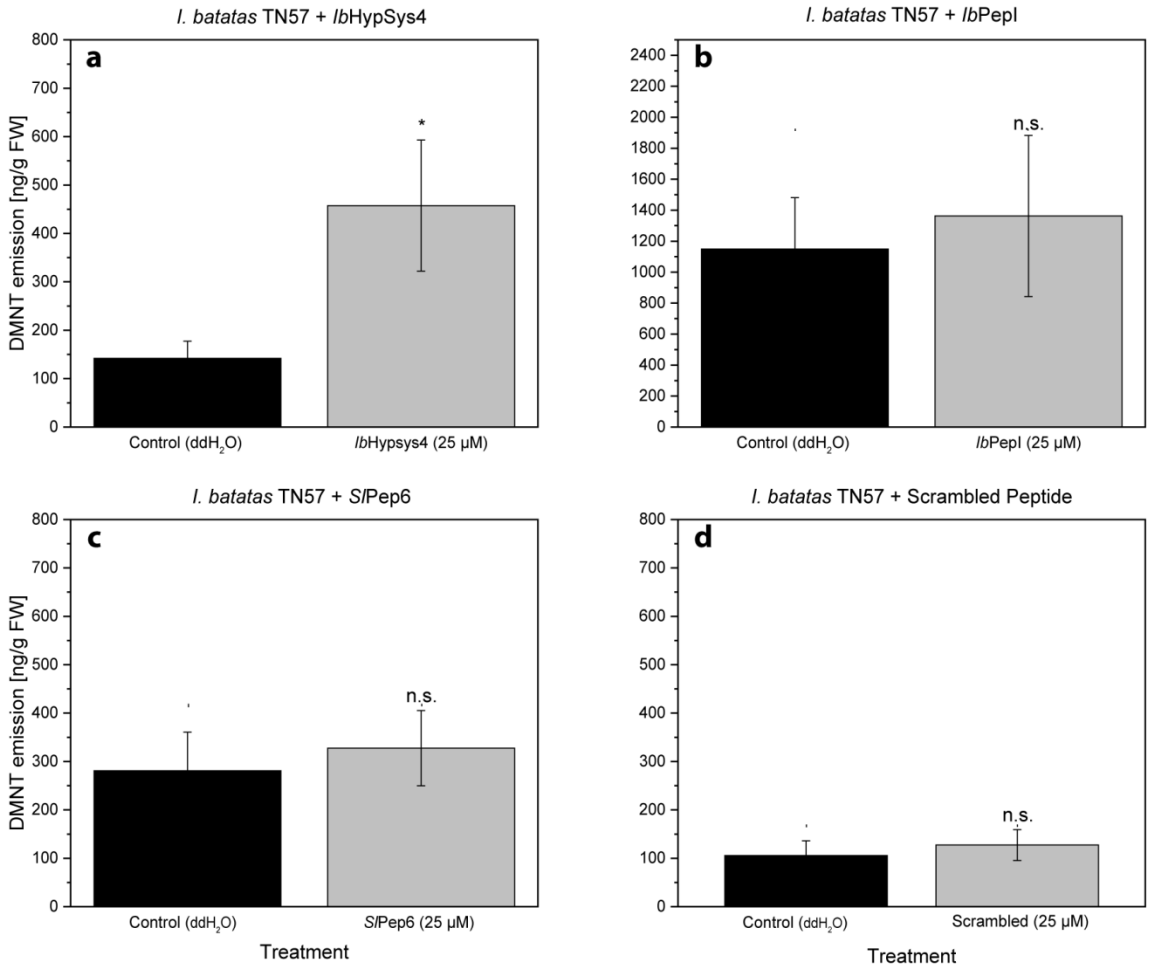


Fig. 1 Incubation with *Ib*HypSys4 induces emission of (*E*)-4,8–dimethyl–nonatriene (DMNT) in *Ipomoea batatas* TN57. DMNT emission of whole *I. batatas* plants sprayed with 25 µM of (a) *Ib*HypSys4 (n = 8-10), (b) *Ib*PepI (n = 10), (c) *Sl*Pep6 (n = 11), (d) scrambled peptide (n = 7) solution (gray bars) or water (control, black bars). Single plants were evenly sprayed with liquid and incubated for 1 h until no more droplets were visible. Volatiles were then collected over 24 h and eluted with internal standard. Bars represent the mean ± SEM of DMNT emission in ng/g fresh weight. Significance levels are indicated by the asterisks (n.s. = not significant; *$p < 0.05$). Asterisks indicate significant differences between control and peptide treatment, based on a Shapiro-Wilk normality test followed by a Mann-Whitney rank sum test.

Upregulated *sporamin* transcript levels in response to *Ib*HypSys4 spray

To further examine whether our peptide treatment is sufficient to reproduce previously described *sporamin* upregulation, sweet potato plants were sprayed and incubated with *Ib*HypSys4 solutions for 1

h. Subsequent qRT-PCR analysis revealed approx. 10-fold increased *sporamin* transcript levels upon contact with *Ib*HypSys4 (Fig. 2 a), confirming the ability of HypSys peptides to trigger *sporamin*-related signaling cascades.

Although increasing information is available about signaling mechanisms leading to the activation of defensive sporamin in sweet potato, it stills remains unclear which receptors are involved. As outlined in the introductory chapter 1.2.2, various peptide-binding cell surface receptors have been identified with the majority belonging to the family of leucine-rich repeat receptor kinases (LRR-RK) (Tang et al., 2017). Bioinformatic analyses conducted by Dr. Hwang (Academic Sinica, Taiwan) identified the novel *Ib*HypSys4 receptor candidate *Ib*LRR-RLK1 by comparison of amino acid sequences from known LRR-RK to sequences found in *Ipomoea trifida* (Lu, Meents, et al., in prep). Unfortunately, spraying of *Ib*HypSys4 did not upregulate transcript levels of the putative receptor candidate (Fig. 2 b) confirming ROS- and ethylene-based assays by H. Lu (data unpublished). Taken together, our results indicate that *Ib*HypSys4 is involved in sporamin-mediated defense, however not by binding to *Ib*LRR-RLK1.

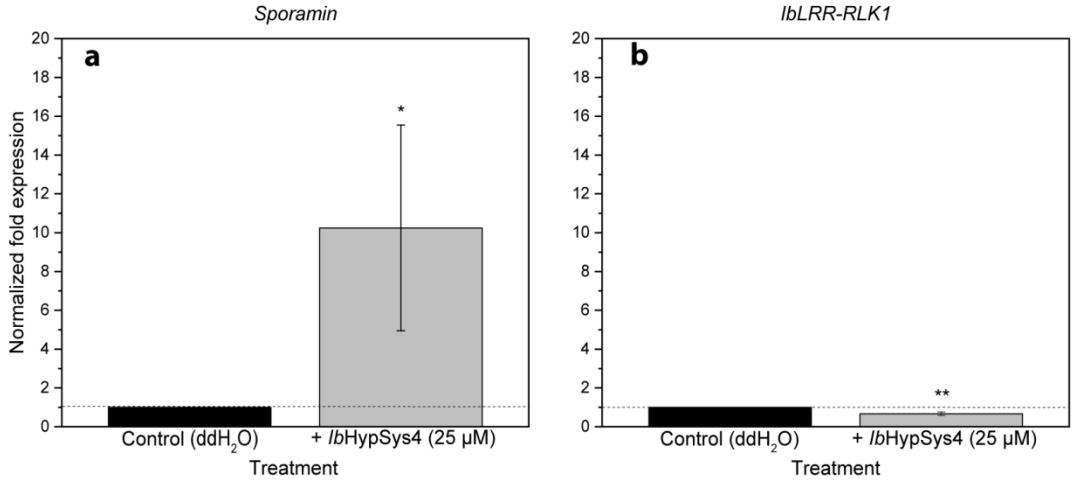


Fig. 2 *Ib*HypSys4 spray induces defensive *sporamin* but not the putative receptor candidate *IbLRR-RLK1.* qRT-PCR of trypsin-inhibitory *sporamin* (a) and the putative receptor candidate gene (b) *IbLRR-RLK1* after 1 h incubation with 25 µM of sprayed *Ib*HypSys4 (gray bars) or ddH_2O (black bars). Bars represent the mean ± SEM of normalized fold gene-expression levels with water-sprayed leaves used as a respective control. Significance levels are indicated by the asterisks (*$p < 0.05$; **$p < 0.01$; $n = 8$). Statistical analyses were performed for the peptide treatment and the respective control using a Shapiro-Wilk normality test followed by a Mann-Whitney rank sum (a) and a t-test (b).

*Ib*PepI treatment induces expression of upstream *Ib*NAC1 but not s*poramin*

Strikingly, ROS burst assays using chimeric receptor constructs revealed that the novel peptide candidate *Ib*PepI is able to bind and activate the putative receptor *Ib*LRR-RLK1 at concentrations of 0.01-0.1 nM (Lu, Meents, et al., in prep). Additional RT-PCR experiments by Lu, Meents, et al. (unpublished) confirmed that *IbLRR-RLK1* transcripts can be induced by *Ib*PepI treatment and wounding of *I. batatas* TN57. Based on these findings, our study aimed to investigate whether wound-inducible *Ib*PepI can elicit sporamin defense. Incubation experiments with 25 µM of sprayed *Ib*PepI for 1 h induced expression of upstream transcription factor *IbNAC1* (Fig. 3 a) however not of the downstream trypsin inhibitor sporamin (Fig. 3 b). The fact that *Ib*PepI activated universal transcription factors, e.g. *Ib*NAC1 (Meng et al., 2018) in the absence of defense-related sporamin or DMNT output, suggests a different role than the anticipated involvement in anti-herbivore defense.

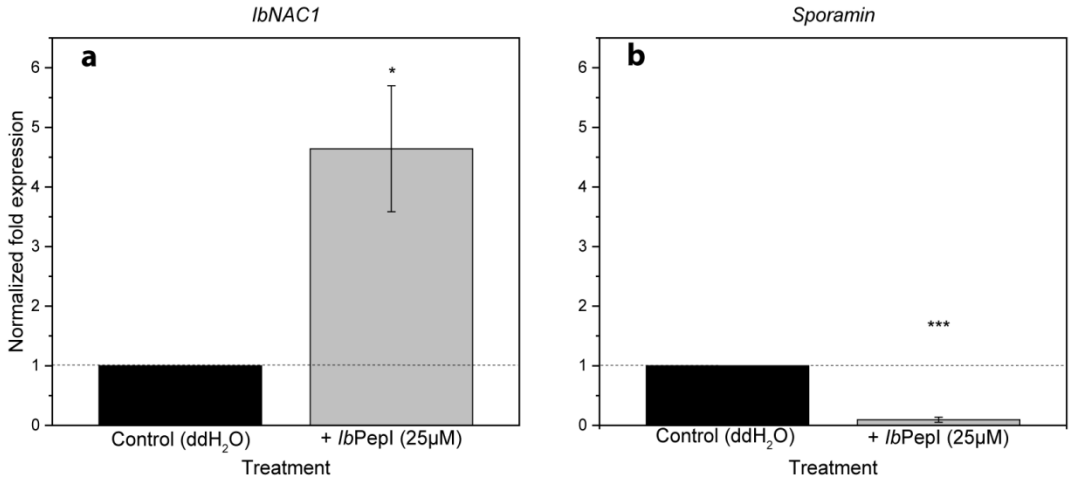


Fig. 3 *Ib*PepI spray induces *IbNAC1* but not downstream defensive *sporamin*. qRT-PCR of *IbNAC1* (a) and trypsin-inhibitory *sporamin* (b) after 1 h incubation with 25 µM of sprayed *Ib*PepI (gray bars) or ddH_2O (black bars). Bars represent the mean ± SEM of normalized fold gene-expression levels with water-sprayed leaves used as a respective control. Significance levels are indicated by the asterisks (*$p < 0.05$; ***$p < 0.001$; $n = 8$). Statistical analyses were performed for the peptide treatment and the respective control using a Shapiro-Wilk normality test followed by a t-test (a) and a Mann-Whitney rank sum test (b).

Local *Ib*HypSys4 and *Ib*PepI differentially affect phytohormones

Our previous studies have shown that mechanical wounding and herbivory induced local jasmonate accumulation without a systemic increase in JA levels (Manuscript 3). At that point, we mainly focused

on unraveling the DMNT-mediated volatile shortcut, rendering systemic JA responses unnecessary to activate sporamin defenses. However, our recent findings confirmed that jasmonate-inducible HypSys peptides such as *Ib*HypSys4 can induce *sporamin* expression and DMNT release (see Fig. 1 & 2), indicating its importance for signaling during herbivore defense. In order to further elucidate which role peptides play within the *Ipomoea* defense framework, local and systemic TN57 leaves were analyzed for phytohormone levels after peptide contact. In comparison to water-treated controls, no significant differences in local and systemic JA concentrations could be observed after *Ib*HypSys4 treatment (Fig. 4 a). Interestingly, *Ib*HypSys4-treated leaves showed a significantly increased amount of bioactive JA-Ile, however only locally (Fig. 4 d). For the JA metabolite JA-OH, differences could be observed between control and peptide-treated groups with a decreased concentration of systemic leaves sprayed with *Ib*HypSys4 (Fig. 4 g). A similar trend was detected for *cis*-OPDA, however not significant due to strong concentration fluctuations during measurements (Fig. 5 a). For the stress-related hormones SA and ABA (Fig. 5 d & g), no significant differences to control treatment could be observed except for a local decrease in SA concentrations upon contact with *Ib*HypSys4 (Fig. 5 d). Overall, it has to be noted that the measured phytohormone concentrations were relatively low compared to previously reported levels (Manuscript 3). This indicates that in order to determine significant changes and plant responses, longer incubation periods and increasing peptide concentrations might be necessary to highlight specific responses on a phytohormone level. As reported by Li et al. (2016), application of *Ib*HypSys4 to leaf-petiole cuttings increased expression levels of *Ib-13-LOX* and *IbAOS* after 1 h, providing first evidence for its participation in jasmonate biosynthesis. Within our experimental setup using intact plants, we could confirm a local accumulation of the active jasmonate, JA-Ile, after 1 h of *Ib*HypSys4 treatment. This indicates that *Ib*HypSys4 is able to induce jasmonate production – however on a very low level, which might be insufficient to trigger downstream defense signaling mechanisms requiring higher jasmonate bursts as e.g. after feeding or wounding.

Treatment with our newly identified *Ib*PepI did neither alter jasmonate nor SA levels although possible effects might have been masked by low concentrations and strong fluctuations (Fig. 4 b, e, h & Fig. 5 e). Exposure to *Ib*PepI resulted in significantly increased *cis*-OPDA concentrations in the systemic leaf compared to the control group. An opposing effect was observed for ABA with decreasing amounts mainly found in the local leaf (Fig. 5 h). Although no tremendous changes in phytohormone levels were overall visible, we noted a clear tendency that for hormones responding to *Ib*HypSys4, no response

would occur during exposure to *Ib*PepI and *vice versa* (Fig. 4 & 5). On that note, the treatment implemented in this study appears to be species-specific as spraying with tomato-derived *Sl*Pep6 did not affect any stress-or defense related hormones (Fig. 4 & 5 c, f, i).

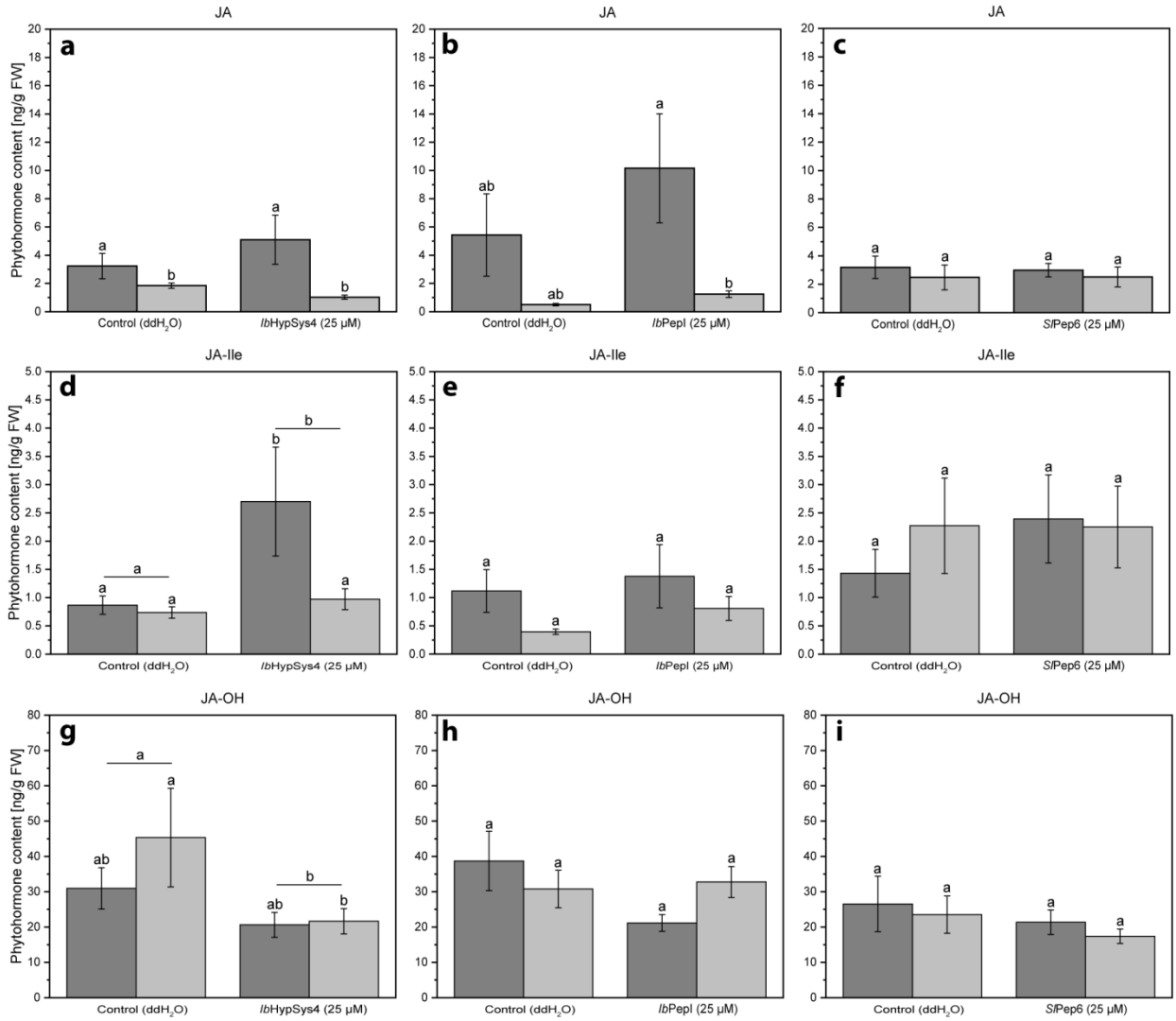


Fig. 4 Alternating jasmonate induction of *I. batatas* after treatment with *Ib*HypSys4 and *Ib*PepI. (a-i) Jasmonate levels after *Ib*HypSys 4 (a,d,g), *Ib*PepI (b,e,h), and *Sl*Pep6 (c,f,i) treatment measured in *I. batatas* TN57 after 1 h. Phytohormone contents were measured in the locally treated 3rd leaf (dark gray bars) and the adjacent untreated 4th systemic leaf (light gray bars). Leaves from ddH_2O-sprayed plants served as controls. Statistically significant differences between groups were analyzed using a two-way ANOVA with initial normality and equal variance tests. Different letters indicate significant differences among groups for $p<0.05$, determined by the Holm-Sidak method. For all analyses, phytohormone content was set as the dependent variable with treatment and leaf type as independent variables. Data are presented as mean ± SEM.

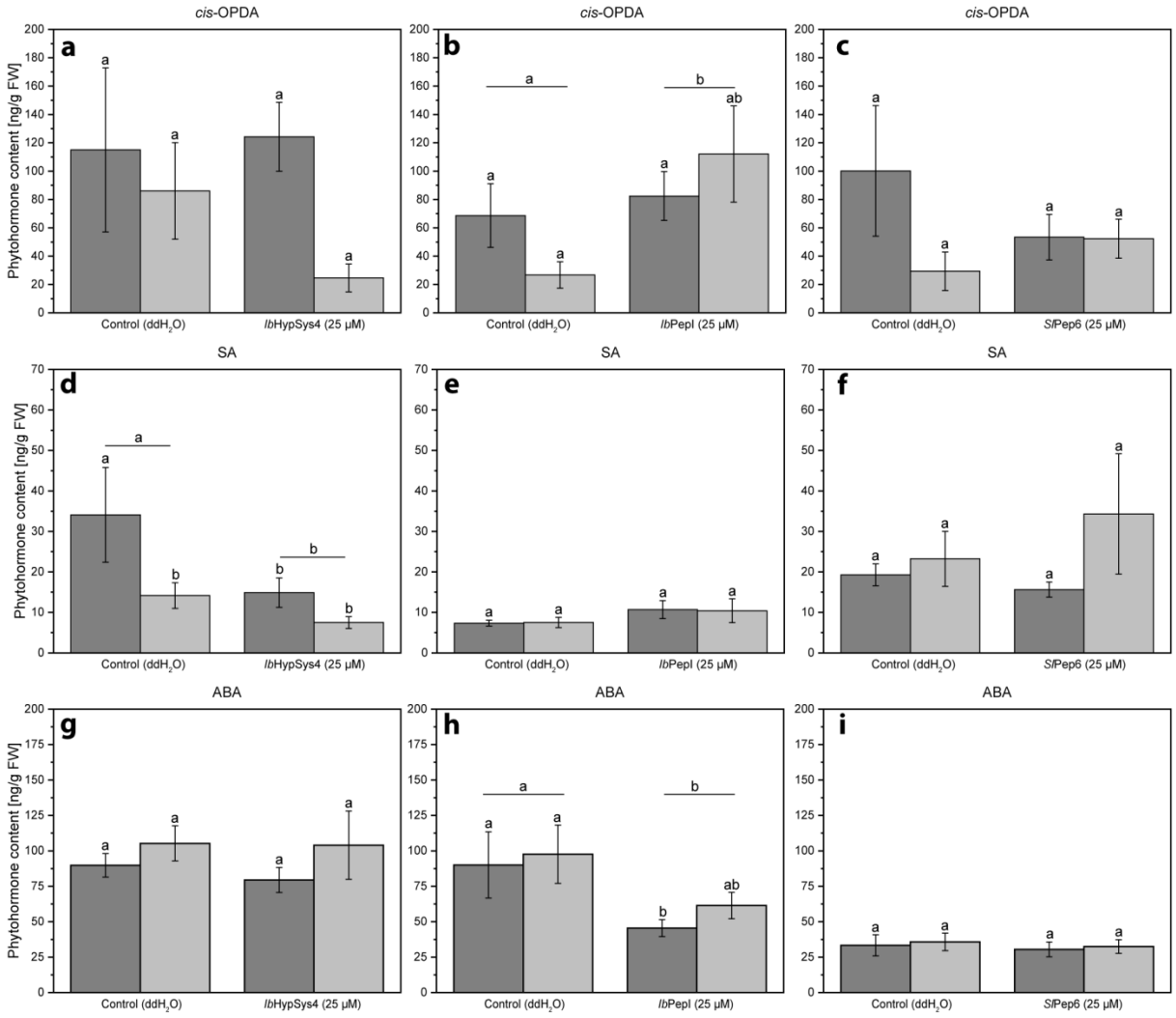


Fig.5 Opposing phytohormone accumulation patterns in *I. batatas* after treatment with *Ib*HypSys4 and *Ib*PepI. (a-i) Jasmonic acid precursor *cis*-OPDA, salicylic acid (SA), and abscisic acid (ABA) levels after *Ib*HypSys 4 (a,d,g), *Ib*PepI (b,e,h), and *Sl*Pep6 (c,f,i) treatment measured in *I. batatas* TN57 after 1 h. Phytohormone contents were measured in the locally treated 3rd leaf (dark gray bars) and the adjacent untreated 4th systemic leaf (light gray bars). Leaves from ddH_2O-sprayed plants served as controls. Statistically significant differences between groups were analyzed using a two-way ANOVA with initial normality and equal variance tests. Different letters indicate significant differences among groups for $p<0.05$, determined by the Holm-Sidak method. For all analyses, phytohormone content was set as the dependent variable with treatment and leaf type as independent variables. Data are presented as mean ± SEM.

RNA-Seq of *I. batatas* reveals high number of differentially expressed genes (DEGs) upon *Ib*PepI and *Ib*HypSys4 peptide treatment

In order to better understand the functionality and similarities between *Ib*PepI and *Ib*HypSys4, RNA-Seq experiments were conducted on single leaves treated with *Ib*HypSys4, *Ib*PepI, and water (control) for 1 h. Overall, 24482 differentially expressed genes (DEGs) were detected based on comparisons to the reference genome *Ipomoea trifida*. A total of 22702 DEGs was shared among all treatments including control samples; however *Ib*HypSys4 elicited expression of 241 specific DEGs whereas plants responded even stronger to *Ib*PepI incubation with 485 DEGs (Fig. 6). The amount of shared transcripts between both peptide treatments was found to be 326 genes (Fig. 6).

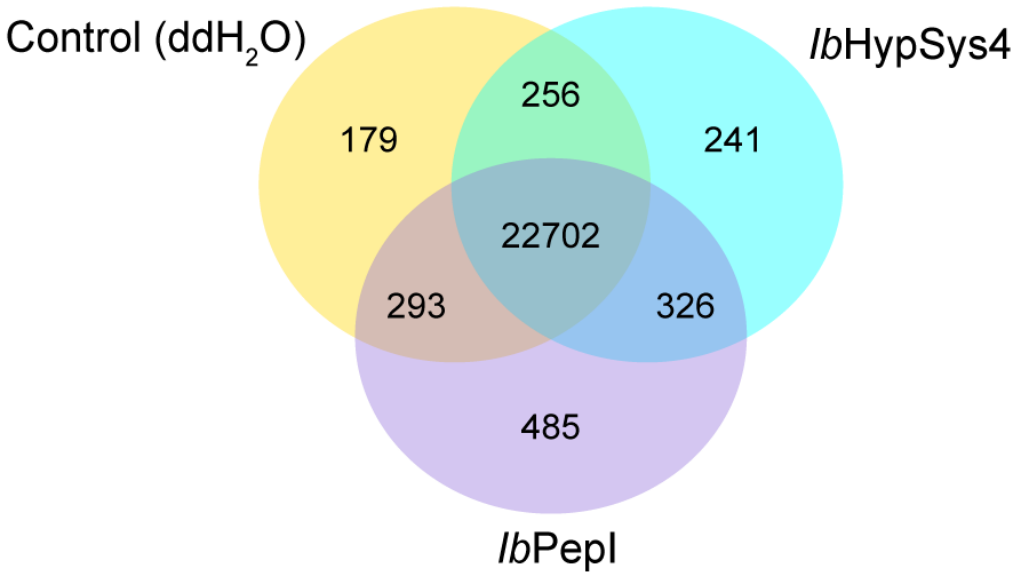


Fig. 6 Venn diagram of DEGs in leaves locally treated with 25 µM of *Ib*HypSys4, *Ib*PepI, or ddH_2O (control) for 1 h. Overlapping circle parts represent the shared differentially expressed genes (DEGs) between the three treatments.

Compared to water-treated control samples, spraying of *Ib*HypSys4 induced upregulation of 555 genes whereas 826 were significantly downregulated (Fig. 7 a). After *Ib*PepI incubation, an even stronger response was observed with 1749 DEGs up- and 2694 downregulated (Fig. 7 b). This suggests that *I. batatas* responds more strongly to *Ib*PepI, indicating that this peptide can trigger a wider range of mechanisms within the plant compared to *Ib*HypSys4. However, this hypothesis requires further

confirmation with KEGG pathway analyses and RT-qPCR of selected genes. Nevertheless, an overall comparison of both peptide treatments revealed that the so far highest detected amount of 2607 DEGs was shown to be upregulated whilst 2229 displayed downregulated patterns (Fig. 7 c). These findings highlight the proposed different functions of both investigated sweet potato-derived peptides, based on their ability to trigger a diverging variety of genes. However at this point, a deeper analysis has to be conducted to verify this assumption.

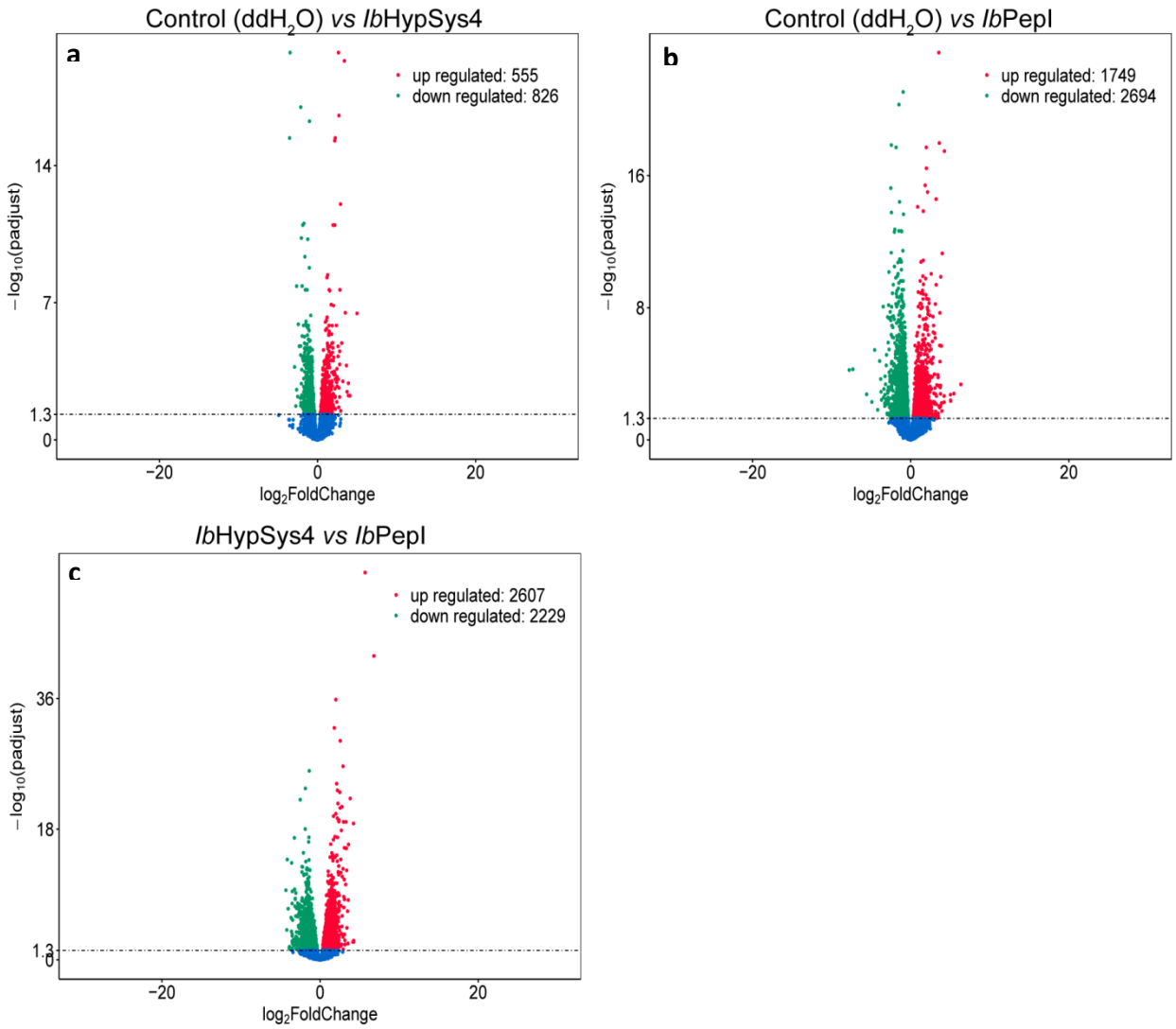


Fig. 7 Volcano plot of statistical significance (-$\log_{10}$ adj. p-value) against enrichment of DEGs ($\log_2$-fold change) after 1 h treatment of *I. batatas* leaves with 25 µM of *Ib*HypSys4, *Ib*PepI, or ddH_2O (control). The number of significantly upregulated genes is indicated in red with the downregulated ones highlighted in green. DEGs not meet significance thresholds are expressed in blue.

2.4.1.3 Outlook

The defensive role of the sweet potato peptide precursor *Ib*preproHypSys and the resulting *Ib*HypSys4 has been demonstrated in the past, shedding light on its importance within sporamin-induced herbivore resistance (Chen et al., 2008;Li et al., 2016). We could confirm that spraying of *Ib*HypSys4 elicited DMNT emission and *sporamin* transcript upregulation in sweet potato (Fig. 8, left panel). Based on these findings, this peptide serves as an intriguing new component within our proposed DMNT-mediated systemic signaling. As we could only find a local jasmonate accumulation upon wounding in sweet potato, HypSys signaling peptides provide an elegant solution to systemically activate sporamin as well as enhancing DMNT release. As shown by Chen et al. (2008), transcript levels for the *Ib*HypSys4 precursor *IbpreproHypSys* were induced upon exposure to methyl jasmonate vapors, showing once more the plant's perceptiveness to VOCs. As *vice versa* the active peptide is able to induce DMNT emission, it would be crucial to investigate whether DMNT can trigger *Ib*HypSys4 production, ultimately closing the feedback loop. Additionally, future analyses of the transcriptome data will provide a deeper insight into additional regulatory functions of *Ib*HypSys4. As the proposed receptor candidate *IbLRR-RLK1* did not respond to this specific peptide, further experiments are necessary to identify the actual binding partner for *Ib*HypSys4 (Fig. 8, left panel).

Strikingly, the receptor candidate *IbLRR-RLK1* was identified as the binding partner for the novel discovered peptide *Ib*PepI (Lu, Meents, et al., in prep) therefore presenting the first known peptide-receptor pairing in sweet potato. Regarding its mode of action, we found that *Ib*PepI did not affect DMNT emission and *sporamin* transcript levels, therefore ruling out an involvement in known trypsin-inhibitory herbivore defense (Fig. 8, right panel). Based on the divergent expression patterns observed within the RNA-Seq analysis, *I. batatas* appears to be responsive to *Ib*HypSys4 as well as *Ib*PepI, however upon activation of different gene patterns. The identification of pathways induced by *Ib*PepI as well as its biological relevance will be a key element addressed in future studies. Due to the structural similarities shared with e.g. the tomato peptide *Sl*Pep6, we predict that *Ib*PepI participates in broader immune responses or pathogen defense, comparable to the *At*Pep1/PEPR1 pairing (Yamaguchi et al., 2006;Huffaker and Ryan, 2007;Yamaguchi et al., 2010). As *Sl*Pep6 was also able to bind to the sweet potato receptor *IbLRR-RLK1* (Lu, Meents, et al., in prep), the receptor type is also able to recognize and bind peptides from other species – underlining its versatility and involvement in a wider range of

functions. However, further evidence needs to be gathered, which can ultimately be confirmed by RT-qPCR and bioassays.

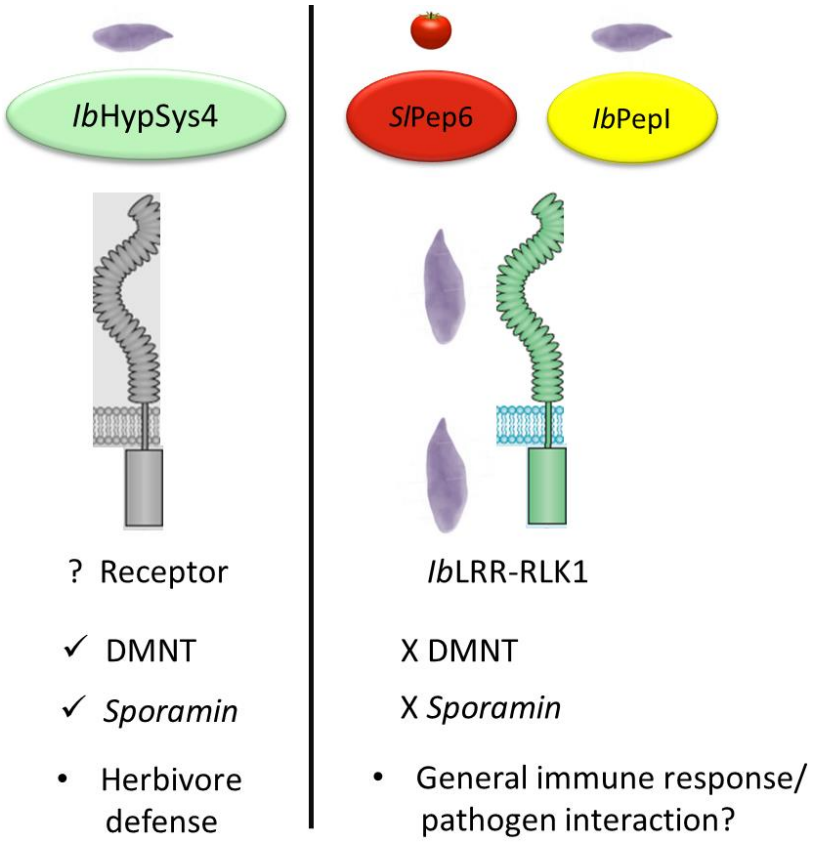


Fig. 8 Overview of the current status of peptide-receptor pairings and its signaling functions in *I. batatas* TN57. Left panel: Our studies revealed the involvement of the signaling peptide *Ib*HypSys4 within anti-herbivore defense by mediating DMNT emission and *sporamin* upregulation. A receptor binding this peptide yet remains to be discovered. Right panel: The novel sweet potato peptide *Ib*PepI does not take part in DMNT-*sporamin*-signaling. Investigation of putative receptor candidates revealed *Ib*LRR-RLK1 as a binding partner for *Ib*PepI and tomato-derived *Sl*Pep6, indicating a role in general immune responses and pathogen interaction.

2.5 Manuscript 4

2.5.1 Manuscript overview

Manuskript Nr. 4

Titel des Manuskriptes: Beneficial and pathogenic Arabidopsis root-interacting fungi differently affect auxin levels and responsive genes during early infection

Autoren: Anja K. Meents, Alexandra C. U. Furch, Marília Almeida-Trapp, Sedef Özyürek, Sandra S. Scholz, Alexander Kirbis, Teresa Lenser, Günter Theißen, Veit Grabe, Bill Hansson, Axel Mithöfer, Ralf Oelmüller

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Ralf Oelmüller	50 %	5 %	-	25 %	50 %
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ORIGINAL RESEARCH
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Beneficial and Pathogenic Arabidopsis Root-Interacting Fungi Differently Affect Auxin Levels and Responsive Genes During Early Infection

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Auxin (indole-3-acetic acid, IAA) is an important phytohormone involved in root growth and development. Root-interacting beneficial and pathogenic fungi utilize auxin and its target genes to manipulate the performance of their hosts for their own needs. In order to follow and visualize auxin effects in fungi-colonized Arabidopsis roots, we used the dual auxin reporter construct *DR5*::*EGFP-DR5v2*::*tdTomato* and fluorescence microscopy as well as LC-MS-based phytohormone analyses. We demonstrate that the beneficial endophytic fungi *Piriformospora indica* and *Mortierella hyalina* produce and accumulate IAA in their mycelia, in contrast to the phytopathogenic biotrophic fungus *Verticillium dahliae* and the necrotrophic fungus *Alternaria brassicicola*. Within 3 h after exposure of Arabidopsis roots to the pathogens, the signals of the auxin-responsive reporter genes disappeared. When exposed to *P. indica*, significantly higher auxin levels and stimulated expression of auxin-responsive reporter genes were detected both in lateral root primordia and the root elongation zone within 1 day. Elevated auxin levels were also present in the *M. hyalina*/Arabidopsis root interaction, but no downstream effects on auxin-responsive reporter genes were observed. However, the jasmonate level was strongly increased in the colonized roots. We propose that the lack of stimulated root growth upon infection with *M. hyalina* is not caused by the absence of auxin, but an inhibitory effect mediated by high jasmonate content.

Keywords: auxin, phytohormones, plant-fungus interaction, *Piriformospora indica*, *Mortierella hyalina*, *Alternaria brassicicola*, *Verticillium dahliae*, light sheet fluorescence microscopy

INTRODUCTION

Auxin plays a central role for root growth and participates in many aspects of root development, including cell elongation, differentiation (Rahman et al., 2007), lateral root, and root hair formation (Masucci and Schiefelbein, 1994, 1996; Pitts et al., 1998; Reed et al., 1998; Casimiro et al., 2001; Bhalerao et al., 2002), and gravitropic responses (Rashotte et al., 2000; Sukumar et al., 2009). Auxin action depends on its differential distribution within plant tissues, where it forms local maxima or gradients between cells. The different auxin levels arise from auxin metabolism (biosynthesis, degradation, and conjugation), long-distance transport and directional cell-to-cell translocation (Petrášek and Friml, 2009). Auxin interacts with other phytohormones and imbalances in the phytohormone levels have severe consequences. Well-studied examples are the auxin/cytokinin balance (De Rybel et al., 2014) and the control of lateral root growth by an interplay between auxin, abscisic acid, brassinosteroids, ethylene, and jasmonates (cf. Fukaki and Tasaka, 2009; Ishimaru et al., 2018). In addition, auxin produced by plant-associated microorganisms mediates phytostimulatory effects on plants. Pathogens can manipulate auxin biosynthesis, signaling, and transport pathways to promote host susceptibility. Auxin responses are also coupled to antagonistic and synergistic interactions with salicylic acid (SA)- and jasmonic acid (JA)-mediated defenses, respectively (Hoffmann et al., 2011; Naseem et al., 2015; Huang et al., 2017). Jasmonates participate in the regulation of primary root growth and reproductive development, thereby interacting with auxins. Crosstalk can occur at multiple levels, including hormone perception, since indole-acetic acid (IAA) and JA-isoleucine (JA-Ile) are perceived by SCF E3-ligases through the interaction of IAA- and JA-related regulators of gene expression and the modulation of each other's homeostasis (Hoffmann et al., 2011; Naseem et al., 2015). Auxin induces JA biosynthesis and JA controls the expression of some of the auxin biosynthetic genes (Dombrecht et al., 2007; Sun et al., 2009; Hentrich et al., 2013). Furthermore, high JA concentrations reduce the accumulation of the PIN-FORMED1 (PIN1) and PIN2 auxin transporters (Gutjahr et al., 2005; Hoffmann et al., 2011; Sun et al., 2011). IAA binds to the receptor AUXIN SIGNALING F-BOX PROTEIN TIR1 and inhibits a family of transcriptional repressors known as AUXIN/IAAs (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Salehin et al., 2015). In the presence of IAA, the SKP1-CULLIN1-F-BOX TIR1 ubiquitin ligase complex binds to AUXIN/IAAs and triggers their degradation (Calderon-Villalobos et al., 2010).

The vast majority of roots in the ecosystems is associated with beneficial fungi. They form mycorrhizal symbiosis or interact with endophytes. Plants benefit from these associations in many ways, such as better access to water and nutrients, promotion of growth and biomass production and resistance to biotic and abiotic stress. The fungi are supplied with reduced carbon from the host photosynthesis and live in a protected shelter. The beneficial symbiosis results in alterations of the host phytohormone levels. For example, beneficial microbes synthesize auxin or auxin precursors, interfere with the plant auxin biosynthesis and metabolism or manipulate auxin signaling and responses. In many cases, the microbes utilize the plant phytohormone machinery and reprogram it to their own needs (Xu et al., 2018, and references therein).

In this study, we used the previously described auxin-responsive reporter system *DR5::EGFP-DR5v2::tdTomato* (Ulmasov et al., 1997; Liao et al., 2015) to monitor how the root-interacting microbes *Piriformospora indica*, *Mortierella hyalina*, *Verticillium dahlia,* and *Alternaria brassicicola* manipulate the root auxin metabolism during early phases of the interaction with Arabidopsis roots. *P. indica*, a member of *Sebacinales*, grows inter- and intracellularly and forms pear shaped spores, which accumulate within the roots and on the root surface (Peškan-Berghöfer et al., 2004; Oelmüller et al., 2009; Camehl et al., 2011). The fungus promotes the growth of the host plants (Peškan-Berghöfer et al., 2004), induces early flowering (Pan et al., 2017) and confers resistance against abiotic and biotic stress (Narayan et al., 2017; Zhang et al., 2017; Vahabi et al., 2018). The endophyte produces indole derivatives but they are not required for growth promotion in barley roots (Hilbert et al., 2012). *P. indica* releases cellotriose that induces root-specific $[Ca^{2+}]_{cyt}$ elevation required for the activation of a mild defense response (Vadassery et al., 2008; Johnson et al., 2018a; Oelmüller, 2018). Root-specific $[Ca^{2+}]_{cyt}$ elevation is also induced by an exuded info-chemical from *M. hyalina* (Johnson et al., 2018b), a growth promoting fungus of *Mortierellales* (Shinmen et al., 1989) which results in defense gene activation. The fungus promotes growth of the aerial parts of Arabidopsis via a fungus-released volatile, while the growth behavior of colonized roots resembles that of un-colonized controls (Johnson et al., 2018b). *V. dahliae* is hemibiotroph with an initial biotrophic life phase in the root xylem, followed by a necrotrophic phase once the hyphae reach the aerial plant tissues. While infected roots show little or no disease symptom development during the biotrophic phase, the fungus blocks xylem transport and synthesizes a cocktail of toxins during the necrotrophic phase, which results in wilting and disease symptom development in the leaves of *Brassicaceae* species (Pemberton and Salmond, 2004; Gijzen and Nürnberger, 2006; Qutob et al., 2006; van der Does and Rep, 2007; Bolton et al., 2008; de Jonge and Thomma, 2009; van Esse et al., 2009; de Jonge et al., 2010, 2011; Oliva et al., 2010; Klosterman et al., 2011; Scholz et al., 2018). The necrotrophic fungus *Alternaria* causes black leaf spot disease in crucifers and was used in this study since it infects both leaves and roots. Root infection induces rapid $[Ca^{2+}]_{cyt}$ elevations which results in host defense gene activation, jasmonate accumulation, ROS production and innate immunity. A few days after root infection, the Arabidopsis seedlings are dead (Johnson et al., 2018b).

In this study, we addressed the hypothesis that interactions of Arabidopsis roots with pathogenic and beneficial fungi are accompanied by different activation of auxin-responsive genes during very early phases of contact. Our results suggest that the activation of auxin-responsive genes and auxin-induced developmental programs in the colonized roots are controlled by the phytohormone balance, rather than by the auxin concentration alone.

MATERIAL AND METHODS

Plant Material and Growth Conditions

Seedlings of *Arabidopsis thaliana* containing the dual auxin reporter construct *DR5::EGFP-DR5v2::tdTomato* were generally described in Ulmasov et al. (1997) and Liao et al. (2015). Here, plants containing T-DNA comprising construct *pGWB601-DR5::EGFP-DR5v2::tdTomato* were used, which had been generated as described (Kirbis, 2017). The seeds were surface-sterilized and placed on petri dishes containing full Murashige-Skoog nutrient medium (MS) (Murashige and Skoog, 1962) supplemented with 40 mM sucrose, 2.6 mM 2-(N-morpholino)ethanesulfonic acid and 1% Kobe Agar (all supplies from Carl Roth, Karlsruhe, Germany). After a 48 h stratification at 4°C, plates were incubated vertically for 10 days at 22°C under long day conditions (16 h light/ 8 h dark; 80 μmol m^{-2} s^{-1}).

Fungal Material and Growth Conditions

P. indica (syn. Serindipita indica) was grown on petri dishes with modified Kaefer's medium (KM) as previously described (Verma et al., 1998; Peškan-Berghöfer et al., 2004) and kept in the dark at room temperature for 3 weeks. *A. brassicicola*, *M. hyalina*, and *V. dahliae* were cultivated for 2 weeks at 22 ± 1°C on potato dextrose agar (PDA) medium as reported in Johnson et al. (2014). *A. brassicicola*, *M. hyalina*, and *V. dahliae* were obtained from the Jena Microbial Resource Center and *P. indica* was provided by Ajit Varma (Amity Institute of Microbial Technology, India).

Spore suspensions of the fungi were prepared according to Johnson et al. (2014) by rinsing the plates containing the fungi with sterile H_2O, gently scraping the spores and hyphae off the plate followed by filtration through a sterilized nylon membrane (75 μm pore size). The spore concentration was determined using a hemocytometer and adjusted with sterile H_2O containing 0.01 % Tween-20 to 2 × 10^6 spores/ml. As control the same solution was used without spores. Viability of the spores was routinely checked via germination tests.

Co-cultivation for Fluorescence Microscopy

In order to investigate the impact of different fungi on the distribution of auxin maxima, Arabidopsis seedlings containing the reporter construct *pGWB601-DR5::EGFP-DR5v2::tdTomato* were co-cultivated with fungal plaques of *P. indica, A. brassicicola*, *M. hyalina*, or *V. dahliae* as described previously by Johnson et al. (2011, 2014) with modifications. For co-cultivation with subsequent fluorescence microscopy, 12 day-old plants were placed on microscope slides on top of a thin layer of full MS medium containing 1% Kobe agar and a plaque (5 mm diameter) of medium (PDA or KM, for control), or fungus agar cultures were applied (see **Figure 1A**). The seedling and fungal plug were coated with sterile tap water, covered with a cover slip and placed in a petri dish until microscopy was performed. The plates were then sealed with Parafilm (American National Can Company, Greenwich, USA) to prevent drying out of the sample and kept at 22°C under long day conditions (16 h light/8 h dark; 80 μmol m^{-2} s^{-1}) for 1 to 3 days. The fluorescence of the reporter construct was analyzed by fluorescence microscopy (see below) after 24 h of incubation. To analyze the dose-dependent induction of fluorescence, the MS agar was supplemented with 0, 5, 10, or 20 μM of IAA (Merck, Darmstadt, Germany) or 0, 1, 5, or 10 μM of JA (Sigma-Aldrich, Taufkirchen, Germany).

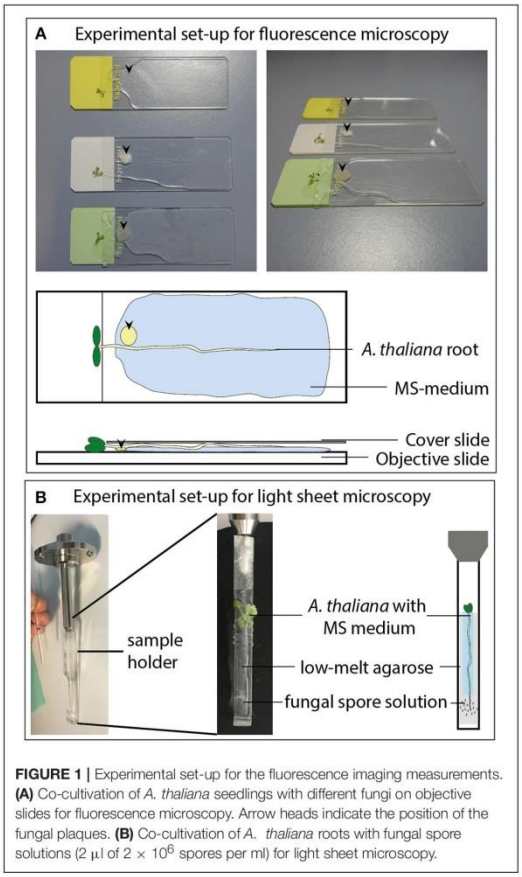

FIGURE 1 | Experimental set-up for the fluorescence imaging measurements. **(A)** Co-cultivation of *A. thaliana* seedlings with different fungi on objective slides for fluorescence microscopy. Arrow heads indicate the position of the fungal plaques. **(B)** Co-cultivation of *A. thaliana* roots with fungal spore solutions (2 μl of 2 × 10^6 spores per ml) for light sheet microscopy.

Fluorescence Microscopy

The entire Arabidopsis roots, elongation zones, root tips and primordia were imaged using an AXIO Imager.M2 (Zeiss Microscopy GmbH, Jena, Germany) equipped with a 10x objective (N-Achroplan 10x/0.3). The bright field and fluorescence images (EX 545/25 and EM 605/70) were recorded with a color camera (AXIOCAM 503 color Zeiss, Jena, Germany) by use of an EGFP (EM 525/50 nm) and DsRED filter (EM 605/70 nm). Digital images were processed with the ZEN software (Zeiss, Jena, Germany), treated with Adobe® PhotoShop to optimize brightness, contrast and coloring and to overlay the photomicrographs. The quantification of fluorescence was measured using the ZEN software by analyzing a region of interest at the root tip and/or the whole root.

Light Sheet Fluorescence Microscopy

Twelve day-old Arabidopsis seedlings (grown as described above) were mounted on a custom plastic holder (see **Figure 1B**). Afterwards the root tip was infected with 2 μl of a *P. indica, A. brassicicola, M. hyalina,* or *V. dahliae* spore suspension (2×10^6 spores/ml) or sterile water containing 0.01 % Tween-20 as a control. The whole plant was fixed using 2% low melting agarose (Carl Roth, Karlsruhe, Germany) and Parafilm to prevent unspecific movement (see **Figure 1B**). The measuring chamber was flooded with full Murashige-Skoog nutrient medium to ensure optimal nutrient supply during the whole measurement. Time series of the root response to the spore treatments were recorded on a LightSheet.Z1 (ZEISS, Oberkochen, Germany) equipped with a W Plan Apochromat 20x/1.0 UV-VIS (ZEISS, Oberkochen, Germany) and two lasers (laser lines: Argon 488 nm and Helium-Neon 561 nm). The red fluorescence was visualized using the 561 nm Helium-Neon laser at 20% transmission with a LBF 405/488/561 emission filter BP 575-615. For all recordings an exposure time of 350 ms was used at a zoom of 0.36 and a light sheet thickness of 6.68 μm. Image stacks series were acquired at 1,920·pixels, 20 μm z-thickness and 16 bit every 30 min for 24 h. Representative intensity kinetics were generated with the ZEN software (Zeiss, Jena, Germany).

Co-cultivation for Phytohormone Analyses

For phytohormone analyses, 15 Arabidopsis seedlings were positioned on a full MS square plate on a sterilized nylon membrane, stratified for 2 days at 4°C and vertically grown for 10 days at 22°C under long day conditions (16 h light/8 h dark; 80 μmol m^{-2} s^{-1}). In accordance with the previous plaque treatment, a 5 mm broad stripe of PDA medium with or without (control) fungal mycelium of *A. brassicicola, M. hyalina*, or *V. dahliae* was placed on the roots and incubated for 1 day. For co-cultivation with *P. indica,* a stripe of KM medium with or without fungus was used for treatment. The plates were co-cultured for 24 h before the entire root of each plate was collected, immediately frozen in liquid nitrogen and stored at −80°C before phytohormone extraction. Mycelium from each fungus grown alone was additionally collected from a whole plate, frozen and kept at −80°C.

Quantification of Phytohormones

Prior to phytohormone extraction, the collected fungal samples (40–200 mg fresh weight, $n = 3$) were freeze-dried in a Modulyo®D Freeze Dryer (Thermo Scientific, Waltham, USA) for 48 h. The freeze-dried fungal samples and the collected root tissue of co-cultivated seedlings (1 sample = 12–15 seedlings from the same plate; which corresponded to 30–150 mg fresh weight, $n = 8$) were weighed and homogenized using a Geno/Grinder® (Spex SamplePrep, Stanmore, UK) at 1,100 rounds per min for 1 min. As described in Almeida Trapp et al. (2014), 1 ml of methanol: water (7:3) containing 20 μg/ml of d4-SA and d5-IAA as well as 10 μg/ml d5-JA and d6-ABA was added to the powdered root and fungal material. After mixing, samples were shaken for 30 min and centrifuged at 16,000 *g* at 4°C for 5 min. Subsequently the supernatant was transferred into a new tube and concentrated for 4 h at 45°C in an Eppendorf Concentrator plus (Eppendorf AG, Hamburg, Germany). The concentrate was resuspended in 100 μl of 50% methanol with 0.05% formic acid, mixed and centrifuged at 16,000 *g* at 4°C for 10 min. Afterwards the supernatant was collected and measured on an Agilent 1100 HPLC system (Agilent Technologies, Böblingen, Germany) connected to a LTQ Orbitrap mass spectrometer (Thermo Scientific, Waltham, USA) (Almeida Trapp et al., 2014).

Statistics

Statistical analyzes of phytohormone data were performed in R studio (version 1.1.456), using one way ANOVA on log transformed data to ensure that the residues followed a normal distribution. Tukey's HSD test was used as *post-hoc* test to examine the differences between factor levels (treated plants vs. control) or multiple comparison for the amount of phytohormones present in fungi samples.

RESULTS

The Reporter Construct *DR5::EGFP-DR5v2::tdTomato* Responds to Exogenously Applied IAA in a Concentration-Dependent Manner in Arabidopsis Roots

To test the expression of the auxin reporters to exogenously applied IAA, the roots of 12 day-old Arabidopsis seedlings were incubated with increasing IAA concentrations. After 24 h, the EGFP and tdTomato fluorescence was monitored (for experimental set-up see: **Figures 1A**, **2A,B**). Without exogenously applied IAA, the fluorescence from both reporters was detectable in the quiescent center, columella, pericycle, and vascular system of the roots (**Figure 2A**, second image, EGFP; third image, tdTomato; fourth image, merged). Incubation with the lowest applied concentration of 5 μM IAA resulted in a significant increase in both fluorescence signals which were extended to the rhizodermal cell layers (**Figure 2B**). The intensity of the signals after the application of 5 μM IAA was twice as high as in the untreated controls and increased almost linear with increasing IAA concentrations (**Figures 2B,C**). Because the fluorescence signals were detectable throughout the entire root tissues (**Figure 2B**) exogenously applied IAA did not cause cell- or tissue-specific induction of the reporter genes. For these experiments and the pictures for the physiological experiments shown below, the fluorescence from both reporters was always measured; however, the overlay analysis never discovered meaningful differences (see **Figure 2B**). Since the fluorescence signal for the EGFP reporter was much lower than the one observed for the tdTomato reporter, the cell- and tissue-specific resolution was better for the latter reporter system. Therefore, for the presentation of the data, we show the results for the stronger fluorescent tdTomato reporter. Furthermore, the linear increase of the fluorescence signals with increasing exogenous applied IAA (**Figure 2B**) makes it likely that the fluorescence reports fungus-induced changes in the expression of auxin-responsive genes in the root tissues.

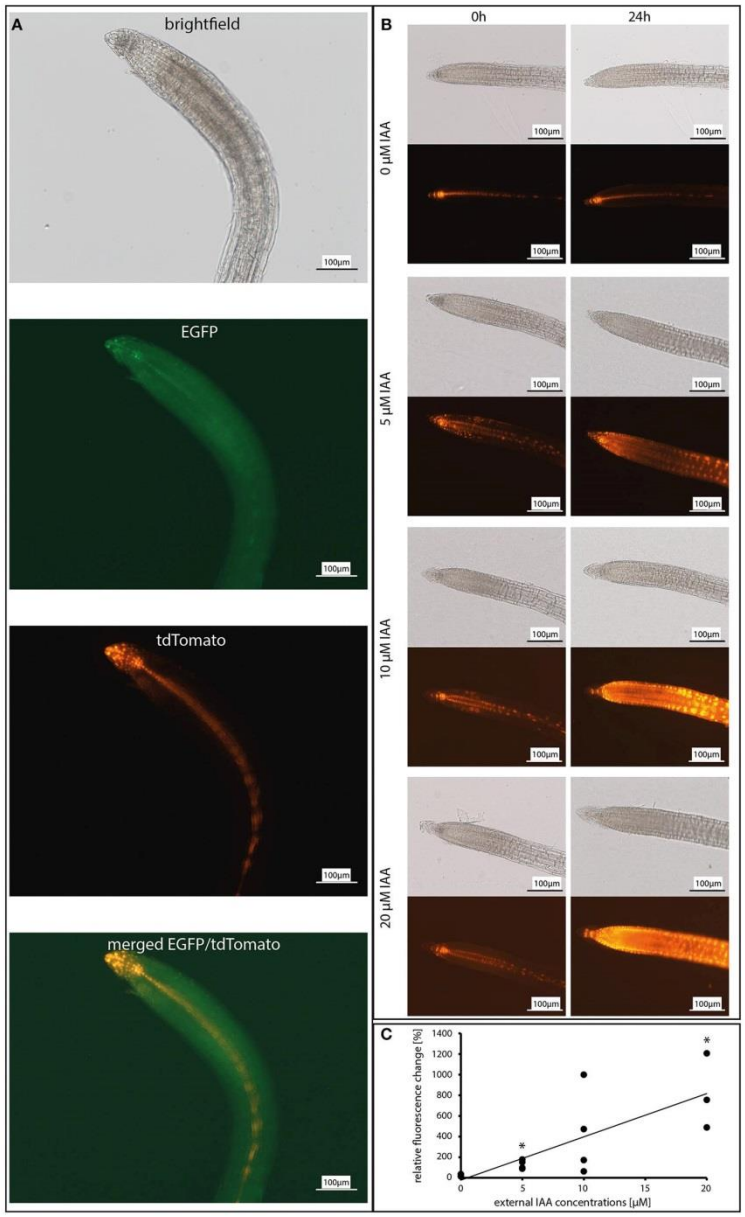

FIGURE 2 | Dose-response curve of the reporter construct *DR5::EGFP-DR5v2::tdTomato* in response to incubation with 0, 5, 10, and 20 µM IAA (indole-3-acetic acid). **(A)** Co-localization of the *EGFP* and *tdTomato* expression in 12 day-old *A. thaliana* roots. Shown are the brightfield image, GFP channel (for EGFP), DS red channel (for tdTomato), and a merged image of the GFP and the DS red channel. **(B)** Fluorescence images of the tdTomato signal after 0 and 24 h. **(C)** Graphic presentation of the relative fluorescence change in response to exogenous IAA concentrations ($n = 3$–4). Asterisks indicate significant differences to 0 µM IAA ($p < 0.05$).

Live Imaging of Root Infection Reveals Fungus-Specific Redistribution of Auxin Maxima in *A. thaliana*

The auxin response was monitored over 24 h after spore application to the roots by measuring the fluorescence emitted from the DR5v2::tdTomato reporter using light sheet fluorescence microscopy (**Figures 1B**, **3A,B**). Representative pictures from the movies (**Supplementary Videos S1–S5**) are shown in **Figure 3A**. **Figure 3B** shows quantified fluorescence signals of the entire roots and of the root tip separately. Without spores, the tdTomato-derived fluorescence displayed initially a stable signal in the quiescent center, columella, pericycle and vascular system (**Figure 3A**). After 10–12 h, the overall fluorescence in the entire root increased. In the root tip, we observed a continuous decrease of the fluorescence (see **Figure 3B**), while the fluorescence from cells located in the elongation zone and from rhizodermal cells increased. The measurements were stopped after 24 h because the reporter system showed the first bleaching symptoms at the root tip.

Application of spores from *P. indica* led to an almost linear increase of the fluorescence in the entire root which started after a lag phase of ∼1 h. Most of the fluorescence was emitted from cells of the basal meristem and the transition zone. Furthermore, the decrease of the fluorescence emitted from the root tip, which was observed for uncolonized roots, was stopped. This clearly indicates that signals from *P. indica* activate the auxin-responsive reporter gene in Arabidopsis roots.

A quite different scenario was observed for *M. hyalina*. The overall emission from the entire root remained almost constant over the measuring period (<20% decrease over the 24 h period), indicating that the fungus inhibits the stimulation which is observed for uncolonized and *P. indica*-colonized roots (more than 70% increase over the 24 h period). Furthermore, after initial stimulation of the fluorescence emission, the activity also declines at the root tip, similar to the decline observed for the uncolonized control. These results demonstrate that *M. hyalina* prevents the activation of the reporter gene.

The two pathogenic fungi *A. brassicicola* and *V. dahliae* showed almost identical effects on the expression of the reporter gene, although the first fungus is a necrotroph and the second a biotroph. A few hours after spore application the fluorescence in the roots and root tips completely disappeared. Thus, signals from the fungi very likely inhibit the activation of the reporter gene.

In summary, compared to uncolonized roots, *P. indica* stimulated and the pathogens inhibited the expression of the reporter gene. Besides an initial positive effect on the root tip, the reporter gene activity is not altered by *M. hyalina*.

Phytohormone Levels in Colonized *A. thaliana* Roots

The phytohormone levels in Arabidopsis roots are strongly influenced by the 24 h incubation period with the different fungi (**Figure 4**). Compared to the uncolonized control, the IAA level in *P. indica-*, *M. hyalina-*, and *A. brassicicola*-colonized roots increased, whereas *V. dahliae* colonization had no effect. Interestingly, co-cultivation with *M. hyalina* resulted in the highest stimulation of the IAA level (∼4-fold stimulation); *P. indica* and *A. brassicicola* showed a similar, but lower induction (∼3-fold stimulation). These results are not consistent with expression data of the auxin reporter gene (**Figures 2**, **3**).

However, quantification of the amounts of the defense-related phytohormones revealed that only *M. hyalina*, but not the other three fungi, stimulated the accumulation of the jasmonates JA and JA-Ile. In addition to *M. hyalina,* the JA precursor 12-oxophytodienoic acid (OPDA) showed a low, however significant increase after infection with *P. indica* and *V. dahliae*. The only other significant alteration induced by any of the fungi was a slight stimulation of the SA level in *P. indica*-infected roots. These results suggest the jasmonates play an important role during the early phase in the *M. hyalina*/Arabidopsis interaction, and that the sharp decline in the reporter gene expression in response to the two pathogens is probably not directly caused by the phytohormones but by factors influencing the cell fate in the symbiotic roots (cf. section Discussion).

Phytohormone Levels in the Fungal Mycelia

To investigate whether the observed differences in the regulation of the reporter gene in Arabidopsis roots are caused by differences in fungal auxin levels, the phytohormone concentrations were measured in the mycelia. Strikingly, the IAA contents in the four fungi varied tremendously. The highest level was found in *P. indica*, *M. hyalina* contained ∼5 times less auxin, and the levels in the two pathogens was quite low (**Figure 5**). We also measured the amounts of the stress related JA, SA and abscisic acid. None of the mycelia contained JA and JA-Ile (data not shown). The lowest SA level was found in *M. hyalina*. *P. indica* contained twice as much and the pathogens 6–7 times more SA than *M. hyalina*. No abscisic acid was found in the two beneficial fungi, while the pathogens contained comparable levels (**Figure 5**). Taken together, the auxin levels that were detectable in the mycelia were consistent with the regulation of the auxin-responsive reporter gene in Arabidopsis roots.

Jasmonate Impairs *DR5::EGFP-DR5v2::tdTomato* Fluorescence

Due to the elevated jasmonate contents found during the *M. hyalina*/Arabidopsis interaction (**Figure 4**), the question arose whether jasmonates can affect the expression of the auxin reporter gene. Therefore, roots of 12 day-old *DR5::EGFP-DR5v2::tdTomato* seedlings were incubated with increasing JA concentrations for 24 h and the fluorescence was monitored. Incubation with 1 and 5 μM JA resulted in a stable but decreased tdTomato fluorescence intensity compared to control samples (**Figures 6A,B**). Application of 10 μM JA showed the significantly highest detectable loss of fluorescence (∼20%) in the roots, supporting the hypothesis that increased JA levels during early *M. hyalina*/Arabidopsis interaction impede the activation of the IAA-responsive reporter construct.

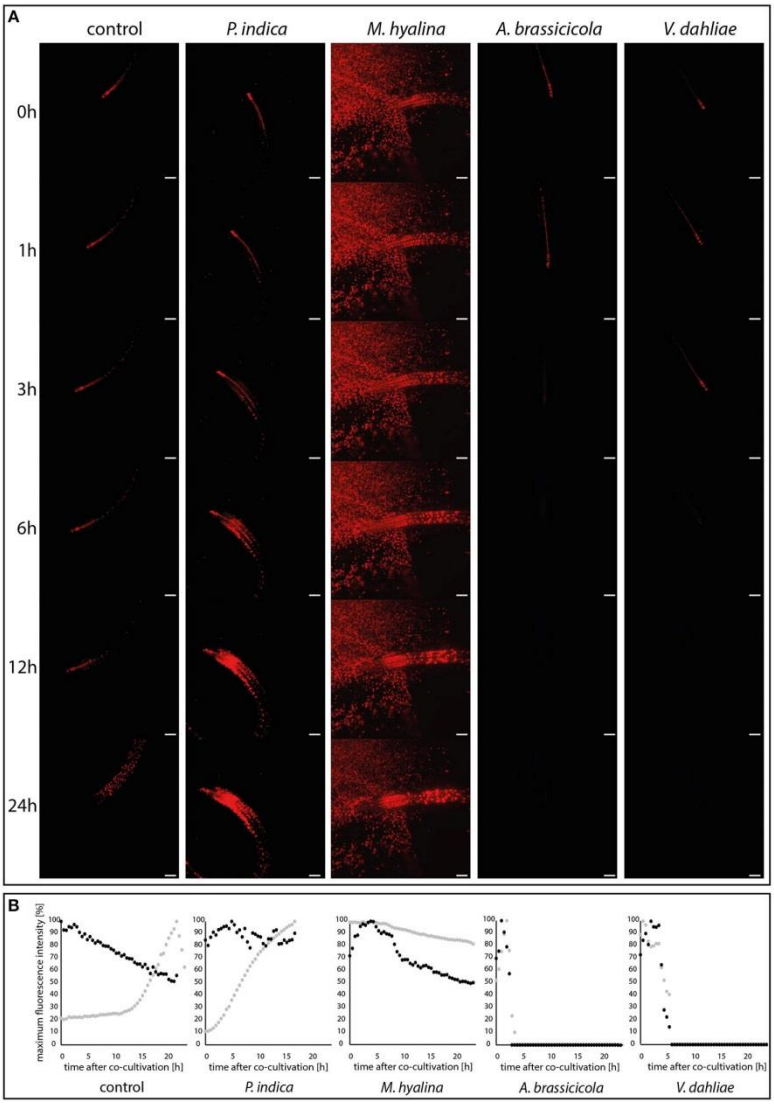

FIGURE 3 | Visualization of auxin maxima using the reporter construct *DR5::EGFP-DR5v2::tdTomato* during plant-fungus interaction. **(A)** Light sheet images of 24 h-recordings of control and co-cultivation with spore solutions of *P. indica*, *M. hyalina*, *A. brassicicola*, and *V. dahliae*. Scale bar = 200 μm. Background fluorescence in *M. hyalina* co-culture is due to autofluorescence of spores. **(B)** Quantification curves of fluorescence maxima of each co-culture in a time-dependent manner. Black dots, root tip; gray dots, whole root ($n = 3$).

P. indica Induces the Formation of Lateral Root Primordia

The former results suggest that only *P. indica* can initiate processes which may result in the promotion of root development within the 24 h of experimentation. In accordance with this idea, we found that the initiation of lateral root primordia was stimulated by *P. indica* but not by *M. hyalina* (**Figure 7**).

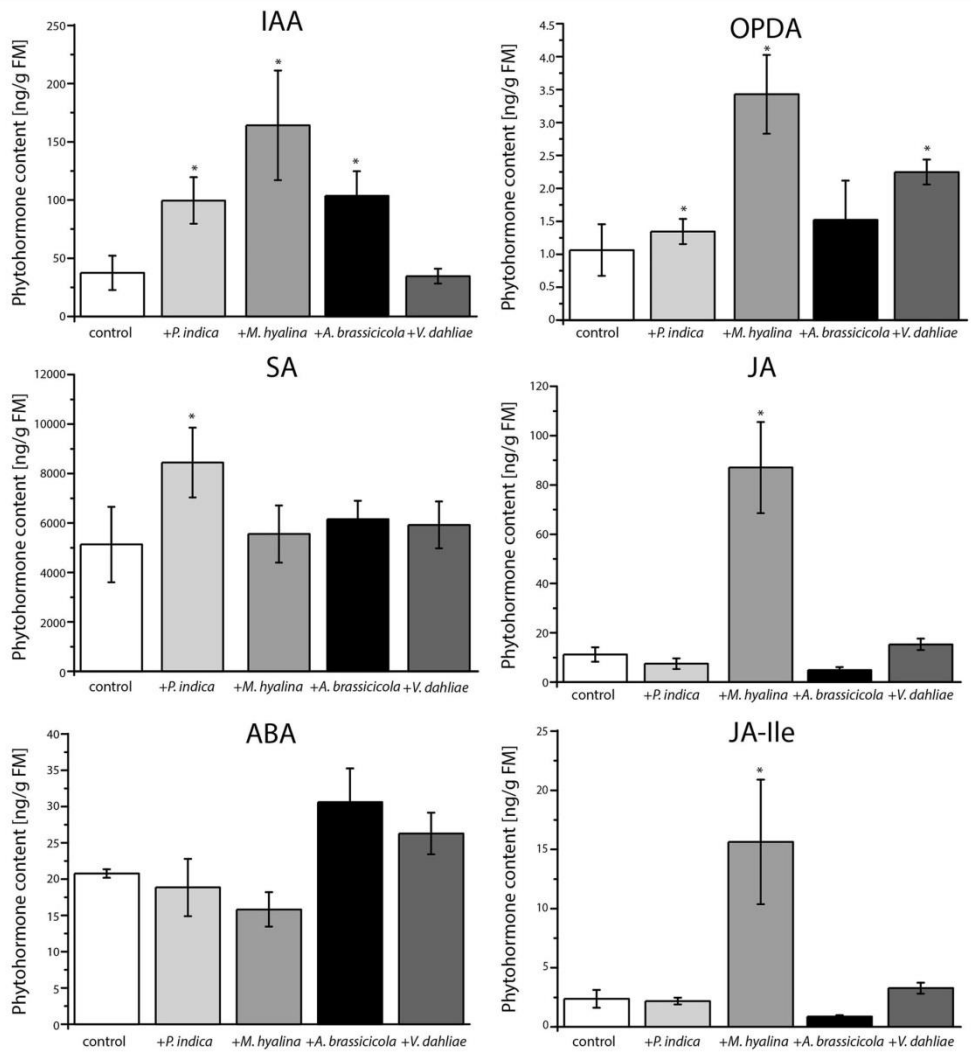

FIGURE 4 | Phytohormone measurements of *A. thaliana* roots after one day of co-cultivation with *P. indica*, *M. hyalina, A. brassicicola,* and *V. dahliae*. (IAA, indole-3-acetic acid; ABA, abscisic acid; SA, salicylic acid; JA, jasmonic acid; OPDA, 12-oxophytodienoic acid (*cis* and *trans*); JA-Ile, jasmonoyl-isoleucine conjugate). Asterisks mark statistically significant differences against control treatment ($n = 8$; $^*p < 0.05$) as determined by one–way ANOVA followed by Tukey's test.

DISCUSSION

Expression of Auxin Reporter Is Dose-Dependent in Arabidopsis Roots

Liao et al. (2015) demonstrated that the auxin reporter construct *DR5::EGFP-DR5v2::tdTomato* induced a dose- and time-dependent fluorescence development as well as IAA-induced gene expression when Arabidopsis plants are treated with 0.0001–1.0 μM IAA. The construct was used to monitor the auxin level in different tissues of transformed *A. thaliana, P. tremula × alba, H. vulgare,* and *N. benthamiana* plants (Hilbert et al., 2012; Chen et al., 2013; Liao et al., 2015; Kirbis, 2017). A previous study showed that the EGFP signal was relatively low compared to the tdTomato signal (Kirbis, 2017). We

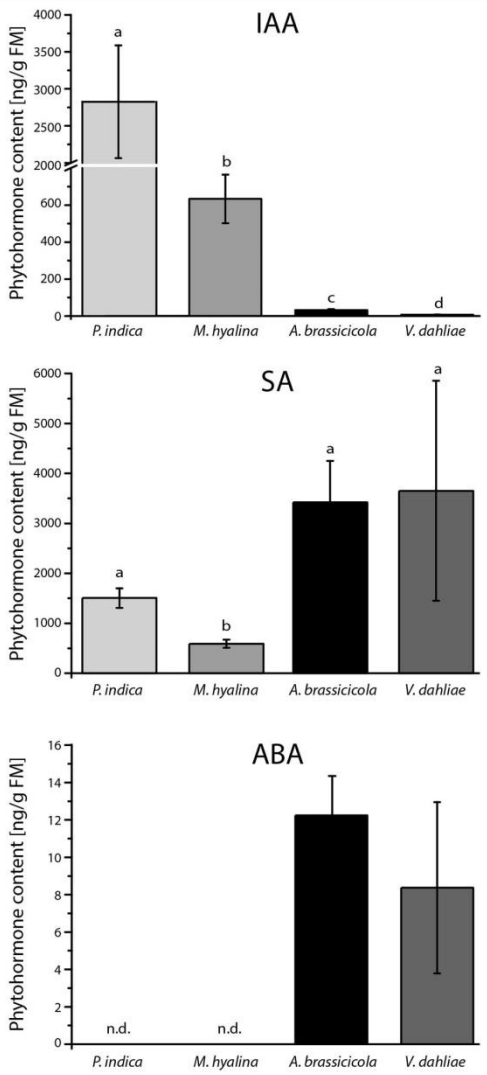

FIGURE 5 | Phytohormone content of mycelia from *P. indica* cultivated on Kaefer's medium for three weeks and *M. hyalina, A. brassicicola,* and *V. dahliae* grown for 2 weeks on potato dextrose agar medium (IAA, indole-3-acetic acid; ABA, abscisic acid; SA, salicylic acid). Statistically significant differences between all fungal species were analyzed using one–way ANOVA. Different letters indicate significant differences among groups for $p < 0.05$, determined by Tukey's test ($n = 3$).

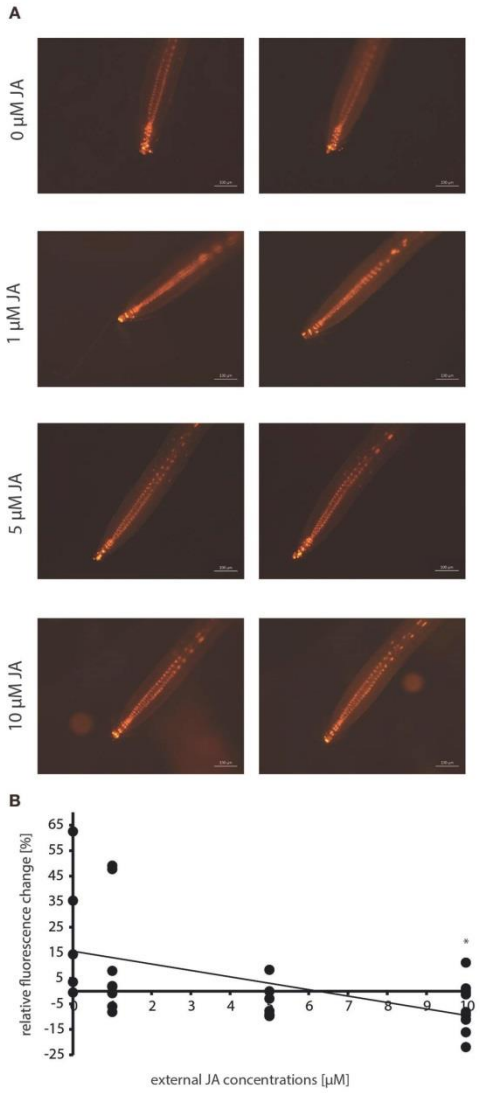

FIGURE 6 | Dose-response curve of the reporter construct *DR5::EGFP-DR5v2::tdTomato* in response to incubation with 0, 1, 5, and 10 µM JA (jasmonic acid) before and after 24 h. **(A)** Fluorescence images of the tdTomato signal after 0 and 24 h. **(B)** Graphic presentation of the relative fluorescence change in response to exogenous JA concentrations ($n = 6$–10). Asterisks indicate significant differences to 0 µM JA ($p < 0.05$).

observed the same for fungus-exposed and control Arabidopsis roots but did not observe differences in the expression for the two reporters (**Figure 2A**). Due to better resolution, we used the tdTomato fluorescence (**Figures 2A,B**) for our study and demonstrate that the expression is dose-dependent and shows expression in tissues and cells which are known to be

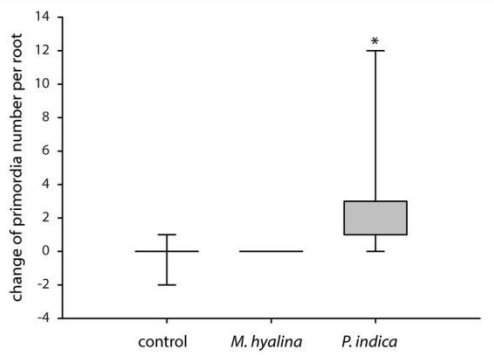

FIGURE 7 | Measurement of lateral root primordia induction in *A. thaliana* after 1 day of co-cultivation with *M. hyalina* and *P. indica*. Graphical illustration of the absolute change of primordia number. The asterisk mark statistical significant differences against control and *M. hyalina* treatment ($n = 5–7$; $^*p < 0.05$).

involved in root growth (**Figures 2B,C**). With an increase in the applied IAA concentration, the fluorescence appeared also in tissues which are located shootwards of the elongation zone in the roots. The expression pattern matches known polar auxin transport mechanisms where IAA is translocated to the root apex and toward the root–shoot junction (Bennett et al., 1998; Reed et al., 1998; Rashotte et al., 2000; Casimiro et al., 2001). Therefore, the construct was used to monitor rapid changes in the auxin distribution in Arabidopsis roots in response to different root-interacting fungi.

P. indica, but Not *M. hyalina* and the Pathogenic Fungi Activate the *DR5v2* Promoter in Arabidopsis Roots

The endophytic fungus *P. indica* promotes the growth of *A. thaliana* by enhancing both root and shoot biomass production (Peškan-Berghöfer et al., 2004; Camehl et al., 2011; Lee et al., 2011; Das et al., 2012). In Chinese cabbage it was demonstrated that colonization by *P. indica* also strongly enhances lateral root development (Lee et al., 2011; Dong et al., 2013). It is known that auxin plays a role in the beneficial interaction and that the fungus produces auxin and auxin precursors which interfere with hormones inside the plant (Sirrenberg et al., 2007; Vadassery et al., 2008; Hilbert et al., 2012; Xu et al., 2018). The auxin reporter was utilized to analyze the auxin kinetic in the early infection stages of *P. indica*-colonized roots as well as in roots colonized by *M. hyalina*, *A. brassicicola* and *V. dahliae* (**Figure 3**). Colonization with *P. indica* showed a strong, 10-fold increase in fluorescence in the entire root while the expression at the root tip stayed at a rather constant level (**Figure 3B**). Already 3 h after co-culture, the increase in the fluorescence (**Figure 3A**) indicates a higher amount of available IAA in the root tissue, which may be provided by the fungus or is produced by the plant. A similar (but only 3-fold) elevation of free IAA was observed in barley roots co-cultured with *P. indica* for 3 d (Hilbert et al., 2012). At later time points (5 and 14 days after infection) this effect was gone in barley which was also observed for Arabidopsis seedlings where the IAA level in colonized seedlings was not different from uncolonized controls 7, 10 and 14 days after infection (Vadassery et al., 2008; Hilbert et al., 2012). Our data suggests that *P. indica* induces a rapid increase in auxin levels during early recognition phases which might be crucial for reprogramming root development. Initiation of the formation of lateral root primordia can already be detected 24 h after co-cultivation with *P. indica*, but not with *M. hyalina* (**Figure 7**).

Phytohormones controlling the development of colonized roots can be of fungal or plant origin. Hilbert et al. (2012) showed that indole derivative production by *P. indica* is not required for growth promotion but for biotrophic colonization of barley roots. In other symbiotic interactions, promotion of root or plant growth was proposed to be caused by microbial phytohormones—in particular auxin (e.g., Contreras-Cornejo et al., 2009; Dudeja et al., 2012; Khan et al., 2014; Lee et al., 2015; Liao et al., 2017, and references therein). The examples demonstrate that fungal auxin or metabolites which can be converted to auxin in the plant, participate in growth regulatory effects in the host. Apparently, phytohormones that are active in the roots can be of either plant or microbial origin. Which one of the two sources might play a more important role in the symbiotic interaction and how these processes are related to circuits, which manipulate plant's phytohormone metabolism and function is probably symbiosis-specific.

M. hyalina did not induce such an auxin increase in the entire root during the first 24 h of co-culture, in fact, the level even decreased slightly about 20% (**Figure 3B**). Five h after co-cultivation, the fluorescence at the root tip increased by 30% and decreased again afterwards to half of the initial level. The initial increase in fluorescence during the recognition of the fungus by the plant could be caused by fungus-derived auxin which is later on stopped by other processes (cf. below). These results match the phenotype observed for *M. hyalina*-colonized Arabidopsis seedlings: we did not observe any significant increase in the root biomass while the aerial parts of the colonized seedlings became bigger (Johnson et al., 2018b).

In contrast, the fluorescence in the entire root decreased in response to the two pathogenic fungi *V. dahliae* and *A. brassicicola* after short periods of co-cultivation (**Figure 3A**). For *V. dahliae*, this was unexpected since it is well known that its interaction starts with a biotrophic phase associated with the stimulation of growth which is followed by a necrotrophic phase later on (Schnathorst, 1981; Reusche et al., 2014; Cho, 2015). In the *A. brassicicola* co-culture, the fluorescence was no longer detectable after 3 h while *V. dahliae*-infected plants lost the fluorescence ~6 h after co-cultivation (**Figure 3B**). This is accompanied by visible destruction of plant tissue and the root tip (data not shown). Both pathogens colonized the roots rapidly. For *V. dahliae*, the roots were covered with conidia already 6 h after infection (Zhao et al., 2014). Downregulation of the auxin response is

likely caused by toxic effects: *V. dahliae* produces different phytotoxins and other molecules that induce the hosts' cell death associated with the degradation of host tissue (Fradin and Thomma, 2006). *A. brassicicola* produces the AB-toxin which is released by the germinating spores on host tissue (Oka et al., 2005).

M. hyalina Induces Jasmonate and *P. indica* SA Accumulation in the Host Roots

Arabidopsis plants respond to colonization by different fungi with the accumulation of different phytohormones. Beneficial fungi like *P. indica* often induce SA, while necrotrophic fungi induce jasmonates (Schäfer et al., 2009; Sun et al., 2014; Johnson et al., 2018b; Scholz et al., 2018; Vahabi et al., 2018; Xu et al., 2018). To analyze whether the observed IAA-induced fluorescence changes during root colonization (**Figure 3**) are exclusively mediated by auxin changes or whether other phytohormones might also be involved, we measured the phytohormone profiles in the roots (**Figure 4**). Compared to uncolonized control roots, both beneficial fungi showed a significant accumulation of IAA within the first 24 h of co-culture. For later time points, quite different results for IAA accumulation were reported for *P. indica*-colonized roots (Vadassery et al., 2008; Lee et al., 2011; Hilbert et al., 2012; Hua et al., 2017). *M. elongata*-colonized maize roots and *M. alpina*-colonized *Crocus sativus* L. plants also showed significantly elevated IAA contents (Wani et al., 2017; Li et al., 2018). However, expression of auxin-responsive genes has never been investigated at the early time points studied here (**Figure 3**). Apparently, although *P. indica* and *M. hyalina* colonization results in the accumulation of auxin, the outcome is quite different. While *P. indica* induces fluorescence, *M. hyalina* does not. This suggests that auxin in the *M. hyalina*-colonized roots cannot (fully) activate the downstream IAA signaling cascade. A possible explanation could be that IAA signaling is antagonistically regulated by other hormones or elicitors.

Furthermore, *V. dahliae* did not induce IAA accumulation in Arabidopsis roots which matches the observed fluorescence. However, the IAA increase was similar for *A. brassicicola* and *P. indica*, although the pathogen did not induce the fluorescence from the auxin reporter (**Figure 3**). Similar observations were made in previous studies (Qi et al., 2012; Riet et al., 2016). This might be due to the rapidly induced cell death by the fast growing pathogen and its release of toxins. The importance of auxins for resistance against different pathogens has been repeatedly demonstrated: For instance, auxin biosynthesis defective mutants are more susceptible to *A. brassicicola* infection compared to wild-type plants (Bari and Jones, 2009; Kazan and Manners, 2009; Qi et al., 2012). Furthermore, *A. brassicicola* infection leads to the degradation of AUX/ IAA proteins indicating that the fungus activates the auxin signal transduction pathway (Qi et al., 2012). Since the hormone data in previous studies were measured for entire roots or even plants, it is difficult to compare them with locally occurring infections and generation of auxin maxima.

Recent studies have demonstrated an antagonistic crosstalk between IAA and SA, which in turn regulates plants' resistance to different fungi. It has been hypothesized that downregulation of auxin signaling is part of the SA-mediated disease-resistance mechanism (Navarro et al., 2006; Chen et al., 2007; Wang et al., 2007; Kazan and Manners, 2009). Our analysis of the SA content of colonized roots is consistent with this assumption, since roots with high IAA content did not show a strong accumulation of SA upon fungal infection at least with *A. brassicicola* and *M. hyalina* (**Figure 4**). However, *P. indica*-colonized roots showed a small increase in both—IAA and SA levels; the latter was also observed in our previous studies (Sun et al., 2014; Vahabi et al., 2018). This seemingly contradiction was addressed in a previous study showing that SA does not directly affect the IAA concentration but the auxin-dependent signaling (Wang et al., 2007). Colonization by *M. hyalina* did not result in elevated SA levels as already shown by Johnson et al. (2018b). The two pathogenic fungi did not induce SA. Similar results were obtained in previous studies where SA content was not elevated and SA mutants did not show a different susceptibility (Botanga et al., 2012; Sun et al., 2014; Scholz et al., 2018). Apparently, significant amounts of SA accumulate only during early phases of the *P. indica*/Arabidopsis interaction.

The class of jasmonates is mainly induced by wounding of plant tissue and plays a major role in defense against necrotrophic fungi (Thaler et al., 2004; Kachroo and Kachroo, 2009; Acosta and Farmera, 2010). Our study revealed that after 24 h of co-culture, the JA and JA-Ile contents were not significantly up-regulated in the roots colonized by the two pathogens (**Figure 4**). A previous study with *V. dahliae* showed the same result (Scholz et al., 2018), although a precursor of JA, OPDA, was stimulated by *V. dahliae*. In this study, we could not distinguish between the active *cis*- and inactive *trans*-OPDA, so a statement about the relevance of this JA precursor would not be appropriate. Interestingly, there was a clear difference in the induction of jasmonates between the two beneficial fungi. While *P. indica*-colonized roots contained the same levels of JA and JA-Ile as the uncolonized control roots, *M. hyalina* colonization resulted in a significant increase in the OPDA (3-fold), JA and JA-Ile levels (8-fold) (**Figure 4**). This appears to be restricted to the early recognition phase of the two partners, since we did not detect an increase of jasmonates in *M. hyalina*-colonized plants during later time points (Johnson et al., 2018b). A recent study in Arabidopsis indicated that JA interferes with auxin signaling independent of the JA-receptor complex COI1. The expression of auxin-inducible genes and lateral rooting is inhibited in Arabidopsis seedlings treated with JA (Ishimaru et al., 2018). These findings are supported by our experiment showing that the incubation with increasing concentrations of JA impairs the expression of the IAA-responsive reporter construct (**Figures 6A,B**) in spite of the presence of elevated IAA levels in *M. hyalina* colonized roots (**Figure 4**). Finally, although ABA is involved in plant defense (**Figure 4**), we did not find a significant accumulation of this hormone during the early phase of interaction for any of the four fungi with Arabidopsis roots.

The crosstalk of phytohormones during recognition and early phases of plant/fungus interaction is not well understood

and most studies focus on defense-related hormones. The presented results and measuring techniques open new research fields, which will shine more light on the first steps in symbiotic interactions.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

AKM, AF, SS, and RO designed the experiments. AKM, AF, SÖ, and MA-T performed the experiments. AKM, AF, and MA-T analyzed the data. VG and BH provided and assisted with the light sheet microscope. AK, TL, and GT generated and provided the transgenic seed material. AKM, AF, SS, AM, and RO wrote the manuscript with contributions from all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00380/full#supplementary-material

Supplementary Video S1 | Visualization of auxin maxima using the reporter construct *DR5::EGFP-DR5v2::tdTomato* during plant-fungus interaction. Light sheet videos of 17 h-recordings of control without addition of fungal spores.

Supplementary Video S2 | Visualization of auxin maxima using the reporter construct *DR5::EGFP-DR5v2::tdTomato* during plant-fungus interaction. Light sheet video of 17 h-recording of co-cultivation with *P. indica* spore solution.

Supplementary Video S3 | Visualization of auxin maxima using the reporter construct *DR5::EGFP-DR5v2::tdTomato* during plant-fungus interaction. Light sheet video of 17 h-recording of co-cultivation with *M. hyalina* spore solution. Background fluorescence in *M. hyalina* co-culture is due to autofluorescence of spores.

Supplementary Video S4 | Visualization of auxin maxima using the reporter construct *DR5::EGFP-DR5v2::tdTomato* during plant-fungus interaction. Light sheet video of 17 h-recording of co-cultivation with *A. brassicicola* spore solution.

Supplementary Video S5 | Visualization of auxin maxima using the reporter construct *DR5::EGFP-DR5v2::tdTomato* during plant-fungus interaction. Light sheet video of 17 h-recording of co-cultivation with *V. dahliae* spore solution.

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2.6 Manuscript 5

2.6.1 Manuscript overview

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Anja K. Meents	25 %	25 %	25 %	35 %	-
Sedef Özyürek	-	50 %	50 %	30 %	-
Ralf Oelmüller	50 %	-	-	-	100 %
Alexandra C. U. Furch	25 %	25 %	25 %	35 %	-
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RESEARCH PAPERS

Fungal-Induced Formation of Auxin Maxima in *Arabidopsis thaliana* Roots

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Abstract—Phytohormones are crucial molecules for plant development and the interaction with microbes. This study focused on the phytohormone auxin (indole-3-acetic acid, IAA) and its role in the interaction of *Arabidopsis thaliana* (L.) Heynh. roots with two beneficial (*Piriformospora indica* and *Mortierella hyalina*) and two pathogenic (*Alternaria brassicicola* and *Verticillium dahliae*) root-colonizing fungi. *Arabidopsis* plants expressing the dual reporter construct *DR5::EGFP-DR5v2::tdTomato* allow visualization of auxin maxima during early stages of the plant–fungus interaction. Fluorescence microscopy was used to monitor changes in auxin levels and distribution patterns. We hereby demonstrate that only the beneficial *P. indica* activates the IAA reporter system. *M. hyalina*- but not *P. indica*-colonized roots accumulate jasmonates which might prevent the activation of the IAA reporter system. Additionally, both the necrotrophic fungus *A. brassicicola* and the biotrophic fungus *V. dahliae* completely inhibit the fluorescence emission from the IAA reporter system within 3–6 h. The results indicate that the reporter system responds to IAA accumulation in symbiotic roots, but the activation process might be controlled by a crosstalk with other phytohormones, such as jasmonates.

Keywords: Arabidopsis thaliana, *Alternaria brassicicola*, *Mortierella hyalina*, *Piriformospora indica*, *Verticillium dahliae*, auxin, fluorescence microscopy, phytohormones, plant–fungus interaction

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INTRODUCTION

Phytohormones are a group of vital organic substances which affect numerous physiological processes within the life cycle of a plant. They play an essential role during growth, differentiation and many other developmental processes—even at low concentrations [1]. Phytohormones can be divided into 11 subclasses. Among them, 10 phytohormone subclasses are known to occur in higher plants. These important organic molecules are generally synthesized in specific tissues, such as apical meristems, young leaves or fruits. They can be subsequently transported to their target sites (e.g., leaves, roots, fruits) [1, 2].

One well-known class of phytohormones are auxins. Auxin itself is the first discovered plant phytohormone and was named IAA in the mid-1930s. The term "auxin" is derived from the Greek word *auxein*, which means "to increase" or "to grow" [2]. IAA is the most abundant auxin form occuring in plants [2]. Its biosynthesis is related to rapidly dividing and growing tissues, particularly in the shoot [2]. So far, the two proposed IAA biosynthesis pathways in plants are the tryptophan (Trp)-independent and (Trp)-dependent pathway [3]. In plant tissues, auxin can be synthesized in different locations such as the apical meristem, leaf primordia, young leaves and seeds [1]. The primary mode of action relies on its differential distribution within plant tissues. The main determinant of differential auxin distribution depends on its directional transport between cells [4]. Auxin transport mechanisms include passive and active processes that transport auxin over long and short distances [4]. Consequently, the distribution of auxins is controlled by the abundance, the location, and transport activity of plasma membrane-based auxin influx and efflux carriers [4].

Auxin plays a crucial role for root growth and development especially during cell elongation and differentiation, lateral root hair formation and gravitropic responses which are promoted in the presence of IAA [5].

The necessity to gain more information about the abundance and distribution of auxin in plants led to the discovery of transgenic reporter constructs for better visualization of IAA-dependent processes. Thus, several auxin reporter constructs were generated over the last decades [6]. The most prominent auxin reporter constructs are based on IAA-responsive pro-

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motor elements coupled to fluorescent proteins which are triggered within the auxin signalling pathway. Expression of genes that play a role in auxin signaling are often controlled by a *cis*-regulatory element found in their promoter regions, called auxin responsive elements (AuxREs) which are activated by members of the auxin response family (ARF) [2]. In general, auxin signaling pathways stimulate ARF proteins, which play a crucial role in regulating the expression of auxin responses [7]. However, auxin transcription factors can have both stimulatory and repressive effects on their targets. When the auxin level is low, these ARFs are bound by the transcriptional repressor proteins auxin/IAAs (Aux/IAAs) which again are linked to the transcriptional corepressor TOPLESS (TPL) [8]. Thus, this repressive protein complex impedes the transcriptional activation of auxin sensitive genes [2]. In the presence of auxin in the nucleus, auxin triggers signaling cascades by binding to TIR1/AFB in SKP1–CUL1–F-box (SCF) ubiquitin ligase complexes [9]. This binding raises the affinity between $SCF^{TIR1/AFB}$ ubiquitination ligase complex with their substrates, thereby catalysing the repressor proteins Aux/ IAAs, which serve as ARF inhibitors [10]. ARFs regulate expression of auxin-responsive genes when Aux/IAA repressor proteins are no longer present at the ARF transcription factors [2].

A widely known reporter used for monitoring auxin responses is the DR5 promoter, which was first discovered in soybean by Ulmasov et al. in 1997. It consists of 7–9 TGTCTC AuxRE repeats and can be coupled to various reporters like fluorescent proteins or luciferase thereby marking sites of transcriptional auxin responses [6]. This fusion of the DR5 promoter allows the auxin-responsive transcription of a reporter gene and with that a recognition of auxin at the cell level. Frequently an increased activation of the DR5 promotor is considered as the presence of so called "auxin maxima" in cells or tissues [6]. However, the AuxRE in the DR5 promoter has limited sensitivity for ARF transcription factors since it is not a high-affinity binding site. In 2015, a new reporter element named DR5v2 was generated [6] by replacing the nine original AuxREs in the DR5 promoter with a novel binding site showing higher affinity (TGTCGG). Therefore, in this work the analysis of auxin levels in *Arabidopsis* roots during fungal treatment was based on the detection of a reporter construct which contained the original DR5 promoter fused to the enhanced green fluorescent protein (EGFP) and the optimized DR5v2 promoter fused to the red fluorescent protein tandem dimer Tomato (tdTomato).

In natural habitats, the majority of roots is associated with microorganisms. These relations can be symbiotic between plant and fungi or bacteria [11, 12]. The interactions between plant roots and fungi can result in a mycorrhizal symbiosis or beneficial cooperation with endophytes. Such an association results in better access of the plants to water and nutrients from the soil, while the fungi receive reduced carbon from the host which originates from their photosynthetic activity. The symbiosis also results in better resistance of the host against biotic and abiotic stress [12]. As a result of this beneficial symbiosis, changes in the phytohormone levels in the colonized roots are often observed [12]. In many cases, the microbes use the plant phytohormones according to their needs, e.g., to obtain access to nutrients from their hosts or stimulate their growth for a shelter or even habitat [12].

The endophytic fungus *P. indica*, a member of the *Sebacinales* (family Sebacinaceae), was discovered in the Thar Desert of India, and colonizes the roots of monocots and dicots, mosses and trees [13]. The spores of this fungus proliferate within the roots, on the root surface, growing inter- and intracellularly thereby supporting the growth of the host plants [13]. It also affects the auxin production and signaling processes in the host [12]. Another endophytic fungus is *Mortierella hyalina*, a member of the *Mortierellales* (genus *Mortierella*). The genus *Mortierella* was first isolated from soil since the fungus lives as saprobes on decaying organic material [14]. The genus comprises 100–170 species with a worldwide distribution [15]. *M. hyalina* has been shown to colonize *Arabidopsis* roots and to increase growth of the host plant's aerial parts via a fungal volatile [16]. In contrast to *P. indica*, *M. hyalina* does not induce growth promotion in the plant roots itself [16].

Opposing to interactions with beneficial fungi, plants also developed various defense strategies against pathogens and pathogenic fungi, including the activation of plant hormone-dependent defense signaling pathways. Depending on their lifestyle, plant pathogens can be divided into necrotrophs and biotrophs. Necrotrophs obtain nutrients from dead or dying cells whereas biotrophs obtain them from living host tissue [17]. *Alternaria brassicicola* is a necrotrophic fungus, which initiates the black leaf spot disease in crucifers and can additionally infect both leaves and host roots [18]. The plant's response to a pathogen attack is controlled is controlled by genetic reprogramming. This includes a phytohormone-specific activation of defense genes, ROS production and other innate immune responses, which are often initiated by a rapid increase in $[Ca^{2+}]_{cyt}$ levels in response to root or leaf infections [19]. Infection with necrotrophs like *A. brassiciola* results in the activation of jasmonate-dependent defense pathways in their hosts followed by a high plant mortality rate [16]. In contrast to the solely pathogenic fungus *A. brassicicola, Verticillium* spp. is a soil-borne plant pathogen which causes vascular wilt disease after an initial biotrophic growth phase. The verticillium wilt disease, one of the most aggressive fungal diseases in the world [20] affects over 200 hosts, many of which are important crops. *V. dahliae* is classified as a hemibiotroph due to the fact that its pathogenicity initially starts with a biotrophic phase in the

root xylem. *Verticillium* infection overall stimulates the synthesis of the defense-related hormone jasmonic acid (JA), its precursor *cis*-12-oxo-phytodienoic acid (*cis*-OPDA) and the biologically active form jasmonic acid-isoleucine (JA-Ile) [20].

Combining these four fungi, which display highly different modes of interactions with their host plants, the purpose of this study was to visualize the auxin abundance and distribution in *Arabidopsis* roots treated with *P. indica*, *M. hyalina*, *V. dahliae* or *A. brassicicola* during the early stages of the interaction. In order to monitor the auxin distribution in the roots of infected *Arabidopsis*, the auxin sensitive reporter system containing the two auxin-responsive promoter elements DR5 and DR5v2, fused to two different fluorescent reporters, the enhanced green fluorescent protein (EGFP) and the red fluorescent protein tandem dimer Tomato (tdTomato), was used. Changes in the auxin levels, predicted from the expression of the reporter gene system, were ultimately correlated with the levels of other phytohormones which were determined by HPLC [5].

MATERIALS AND METHODS

Plant material and growth conditions. *Arabidopsis thaliana* (L.) Heynh. seedlings containing the dual auxin reporter construct *pGWB601-DR5::EGFP-DR5v2::tdTomato* were kindly provided by the Department of Genetics, Friedrich Schiller University Jena, Jena, Germany [5, 21]. The seeds were surface-sterilized and placed on full Murashige & Skoog medium (MS; Sigma Aldrich, Germany) supplemented with 40 mM sucrose, 2.6 mM 2-(N-morpholino)ethanesulfonic acid and 1% Kobe Agar (all supplies from Carl Roth, Germany). The pH of the MS medium was adjusted to 5.7 with 1 M KOH. For germination, the plates were kept at 4°C for 48 h and afterwards grown under long day conditions (16 h light/8 h dark; 80 μmol/(m^2 s)) for 9 to 14 days at 22°C.

Incubation with IAA. To test whether the auxin reporter construct *DR5:EGFP-DR5v2::tdTomato* responds in a dose-dependent manner to low concentrations of externally applied IAA, 12-day-old seedlings were mounted on microscope slides as described in Meents et al. [5] and incubated with 0, 0.1, 1 and 2.5 μM of IAA (Merck, Germany) for 24 h.

Fungal material for co-cultivation. For co-cultivation experiments, *Piriformospora indica* (syn. *Serindipita indica*; kindly provided by Ajit Varma, Amity Institute of Microbial Technology, Amity University Uttar Pradesh, India), *Mortierella hyalina* (FSU-509), *Alternaria brassicicola* (FSU-218), and *Verticillium dahliae* (FSU-343; provided from the Jena Microbial Resource Centre Jena, Germany) were used. *P. indica* was grown on Kaefer medium (KM) as previously described [22, 23] and kept at room temperature in the dark for 3–4 weeks. *M. hyalina*, *A. brassicicola* and *V. dahliae* were grown on Potato Dextrose Agar (PDA) [19] for 2 weeks at 20 ± 1°C in a temperature-controlled chamber (Ehret, Germany) in 75% relative humidity and 12/12 h light/dark cycles.

Co-cultivation of *Arabidopsis thaliana* with fungal plaques. For the co-cultivation experiments, Plant Nutrient Medium (PNM) was prepared as described by Johnson et al. [24]. For a uniform plate preparation, 20 mL PNM were poured into sterile round Petri dishes and stored at 4°C until further use. Nylon membranes (Sefar nitex 03-70/33: pore size 65–70 μm; mesh count 81 cm^{-1}; Sefar GmbH, Switzerland) were cut into discs with a diameter of 80–85 mm, subsequently soaked 3 times with sterile water and boiled at approximately 250–300°C for about 15–20 min. Afterwards, the membrane was washed again with sterile water for 3 times, dried and autoclaved. For experimental use, the nylon discs were placed on top of the PNM medium.

To ensure optimal growth conditions before and during the co-cultivation of *A. thaliana* with the aforementioned fungi, *Arabidopsis* seedlings were pre-grown on MS medium for 12 days. *P. indica* was cultivated on KM for 4 weeks as well as *M. hyalina, A. brassicicola* and *V. dahliae* on PDA for 2 weeks. For the co-cultivation, a plaque (5 mm diameter) with or without (control) fungal mycelia was placed on the center of the nylon membrane. Four 12-day-old *Arabidopsis* seedlings per Petri dish were then transferred onto the membrane as previously described in Johnson et al. [24]. All Petri dishes were sealed with Parafilm and incubated under long day conditions for up to 14 days (16 h light/8 h dark; 80 μmol/(m^2 s)) and used for fluorescence microscopy. While KM medium (without fungal mycelium) was used as a control for *P. indica* treatment, PDA medium (without mycelium) was used for the other species (*A. brassicicola*, *M. hyalina,* and *V. dahliae*). For each experiment at least 5 biological replicates were used per time point and treatment.

Auxin imaging with fluorescence microscopy. *Arabidopsis* root tips expressing the fluorescent proteins EGFP and tdTomato were imaged using an AXIO Imager.M2 (Zeiss Microscopy GmbH, Germany) equipped with a 10× objective (N-Achroplan 10×/0.3). The bright field and fluorescence images (EX 545/25 and EM 605/70) were taken using a color camera (AXIOCAM 503 color Zeiss) with an EGFP (EM 525/50 nm) and DsRED filter (EM 605/70 nm). The images were processed with ZEN software (Zeiss) and subsequently Adobe® PhotoShop to optimize brightness, contrast, colouring and to overlay the photomicrographs. The quantification of fluorescence was measured using the ZEN software by analyzing a region of interest at the root tip and/or the whole root

Statistical analysis. Statistics for co-cultivation experiments and IAA incubation were done using *t*-test or Mann-Whitney rank sum test in SigmaPlot (V 12.1.0). In order to detect significant differences

between the fungal treatment and the respective controls within one time point, the test was chosen according to the distribution of the values included in the dataset using Shapiro-Wilk normality test for data exploration. Levels of statistical significance are marked as $P < 0.05$ (*) with the standard error of the mean depicted in the figures. Statistical significances between the time points within the *P. indica* treatment were tested using Shapiro–Wilk normality test with a subsequent one–way ANOVA in SigmaPlot (V 12.1.0) and Tukey's test. Statistical significance between groups was given when $P < 0.05$ (*).

RESULTS

The Reporter Construct DR5::EGFP-DR5v2::tdTomato Shows a Specific Linear Response to Low Concentrations of Exogenously Applied IAA in Arabidopsis Roots

It was previously demonstrated that the dual reporter construct *DR5::EGFP-DR5v2::tdTomato* responds in a linear manner to the exogenous application of 0 to 20 μM IAA [5]. To test whether the linearity and sensitivity of the construct's response also applies during exposure to lower concentrations, the roots of 12-day-old *Arabidopsis* seedlings were incubated in IAA ranging from 0 to 2.5 μM. After 24 h the tdTomato fluorescence was monitored and quantified (Fig. 1). Control treatment without IAA added to the medium resulted in a detectable fluorescence in the columella, pericycle and the vascular system of the roots (Fig. 1a). Application of 0.1 μM IAA did not lead to a significant increase in the fluorescence intensity. However, incubation with a concentration of 1 μM IAA resulted in an almost 200% increased fluorescence intensity compared to the control with the signal extending throughout the root tissue (Figs. 1a, 1b). The observed concentration-dependent increased signal intensity showed a linear trend with an almost upregulation of both reporter constructs during treatment with 2.5 μM IAA (Fig. 2b), therefore demonstrating the suitability of the used reporter construct to monitor the expression of auxin-responsive genes in the root tissue even in the presence of low IAA concentrations.

Piriformospora indica Induces Auxin Maxima in the Roots of Arabidopsis thaliana

To investigate the effect of beneficial fungi on the activity of the auxin reporter system in *A. thaliana* roots, 12-day-old seedlings were co-cultivated with a *P. indica* (Fig. 2a) or KM plaque without fungal mycelium as control (Fig. 2b). The auxin response was monitored from 24 h up to 14 days of co-cultivation using fluorescence microscopy (Figs. 2a–2c). After one day of co-cultivation, the tdTomato-derived fluorescence signal in the control without the fungus was detectable in the quiescent center, pericycle, columella and vascular system of the root (Fig. 2b) while in roots treated with *P. indica*, the tdTomato fluorescence dramatically extended throughout the whole root tissue (Fig. 2a) with a 4-fold increased intensity (Fig. 2c). Interestingly, prolonged co-cultivation of 4 up to 14 days with *P. indica* led to a decreased fluorescence intensity of the root (Figs. 2a, 2c) displaying auxin maxima mainly around the colonization area in the cortex, columella and the entire apical and basal meristems (Fig. 2a). Although a slightly elevated fluorescence emission was still visible after 4 days of infection, no significant differences in the signal intensity could be detected compared to the control (Fig. 2c). This effect could be continuously observed for later time points with the fluorescence intensity returning to a basal level (Figs. 2a–2c). Within *P. indica* treatment, 1 day of infection was the only time point displaying a significantly increased signal compared to later time points. These results indicate that *P. indica* rapidly activates the auxin-responsive reporter in *Arabidopsis* roots within 24 h of colonization—however not in a continuous manner.

Beneficial Mortierella hyalina Does Not Upregulate the DR5v2 Promoter in Arabidopsis Roots

Consecutive experiments were performed using another beneficial root-colonising fungus, *M. hyalina*. The previously described setup was applied for all following experiments placing a PDA plug as a respective control treatment. After 1 day of co-cultivation, the tdTomato fluorescence with and without *M. hyalina* was visible in the quiescent center, pericycle, columella and vascular system (Figs. 3a, 3b) as described before. In contrast to the drastic upregulation of the reporter activity observed during 1 day of *P. indica* treatment (Figs. 2a, 2c), no meaningful differences between control and *M. hyalina* treatment could be detected (Figs. 3a–3c). This experiment shows that *M. hyalina* did not induce DR5v2 promoter activity in the root tips.

Pathogenic Alternaria brassicicola and V. dahliae Inhibit Reporter Gene Activation

A completely different sccenario was observed during the co-cultivation experiments with two pathogenic fungi, *A. brassicicola* and *V. dahliae.* The images obtained from both fungal treatments were almost identical. After 1 day of inoculation with *A. brassicicola* and *V. dahliae*, no tdTomato fluorescence could be detected (Figs. 4a, 4c and 5a, 5c) throughout all time points in contrast to a stable *DR5v2* signal in the control samples (Figs. 4b and 5b). This indicates that both pathogenic fungi—although *V. dahliae* is initially biotrophic—inhibit the fluorescence signal in *Arabidopsis* roots by severely attacking the root tissue and killing it within 24 h.

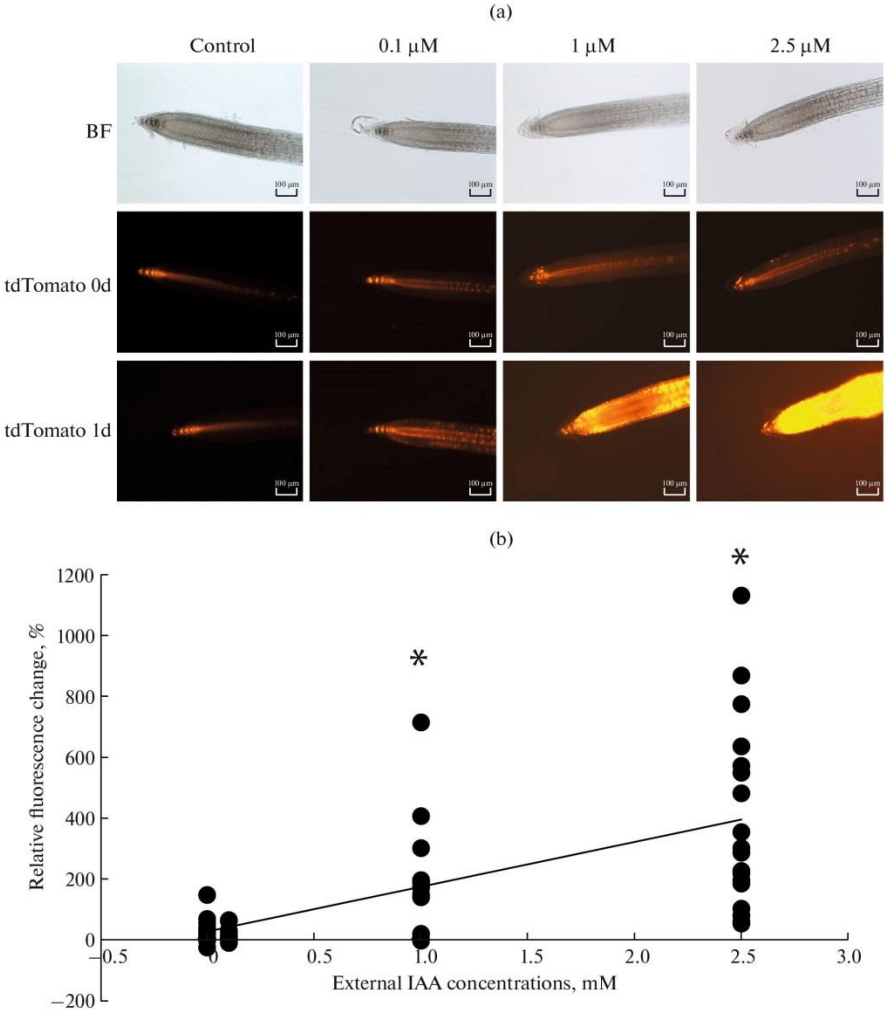

Fig. 1. Fluorescence images of *Arabidopsis* roots harboring the reporter construct *DR5::EGFP-DR5v2::tdTomato* in response to incubation with 0, 0.1, 1 and 2.5 μM IAA for 24 h. (a) *tdTomato* expression in root tips of 12 day-old *Arabidopsis thaliana*. Shown are the bightfield image (BF) and the tdTomato fluorescence directly after IAA application (0d) and after 24 h incubation (1d); (b) graphic presentation of the relative fluorescence change in response to exogenous IAA application ($n > 5$). Asterisks indicate significant differences to 0 μM IAA ($P < 0.05$).

DISCUSSION

Liao et al. [6] generated the *DR5::EGFP-DR5v2::tdTomato* auxin reporter construct and treated *Arabidopsis* tissues harboring this construct with 0.001–1.0 μM IAA to evaluate the auxin response. It was demonstrated that the construct responded with a dose-dependent fluorescence emission [6]. Thus, the *DR5::EGFP-DR5v2::tdTomato* construct enables visualization of auxin responses in *Arabidopsis* roots. Moreover, *DR5::EGFP-DR5v2::tdTomato* expression was tested in different tissues and plants in order to study fruit development in *A. thaliana*, *Lepidium campestre* and *Aethionnema arabicum* [21]. In this study, it was mentioned that the EGFP- derived signal

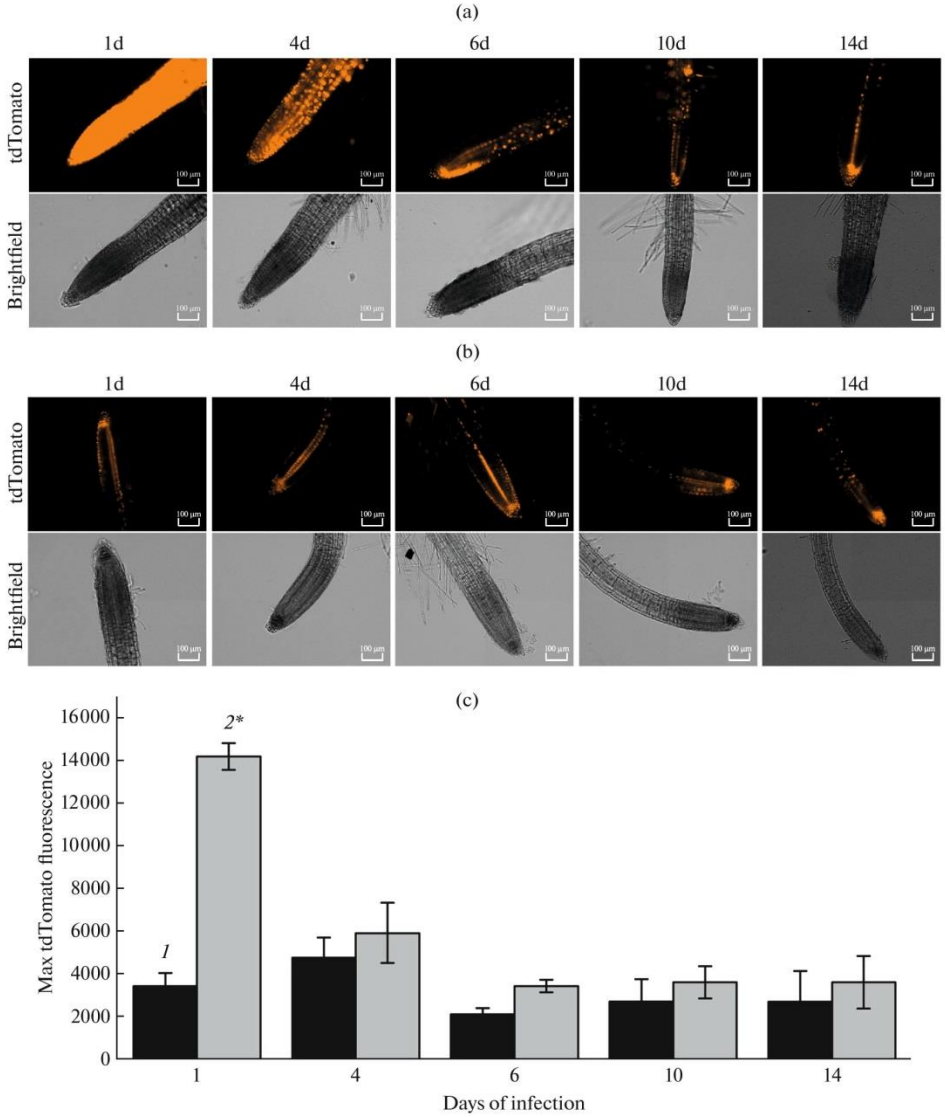

Fig. 2. Visualization of auxin maxima using the reporter construct *DR5::EGFP-DR5v2::tdTomato* during inoculation with the beneficial fungus *Piriformospora indica*. (a, b) Fluorescence images of the *Arabidopsis* root tip after 1 up to 14 days of co-cultivation. Shown are the brightfield image and the tdTomato fluorescence in presence of a *P. indica* plaque (a) and a plaque with KM medium only as the respective control (b). Scale bar = 100 μm; (c) graphic presentation of the maximum tdTomato fluorescence intensity of the mock treatment (plaque without mycelium, black bar) and in the presence of *P. indica* (with fungal mycelium, gray bar). Significance levels are indicated by the asterisks (*$P < 0.05$). Statistical analyses were performed for fungal treatment and the respective control with a Shapiro–Wilk normality test and subsequent two-sample *t*-test or Mann–Whitney rank sum test according to the data distribution. For statistical differences within the *P. indica* treatments, one-way ANOVA followed by Tukey's test ($n = 5$) was performed to determine significant differences. (*1*) Mock; (*2*) *P. indica*.

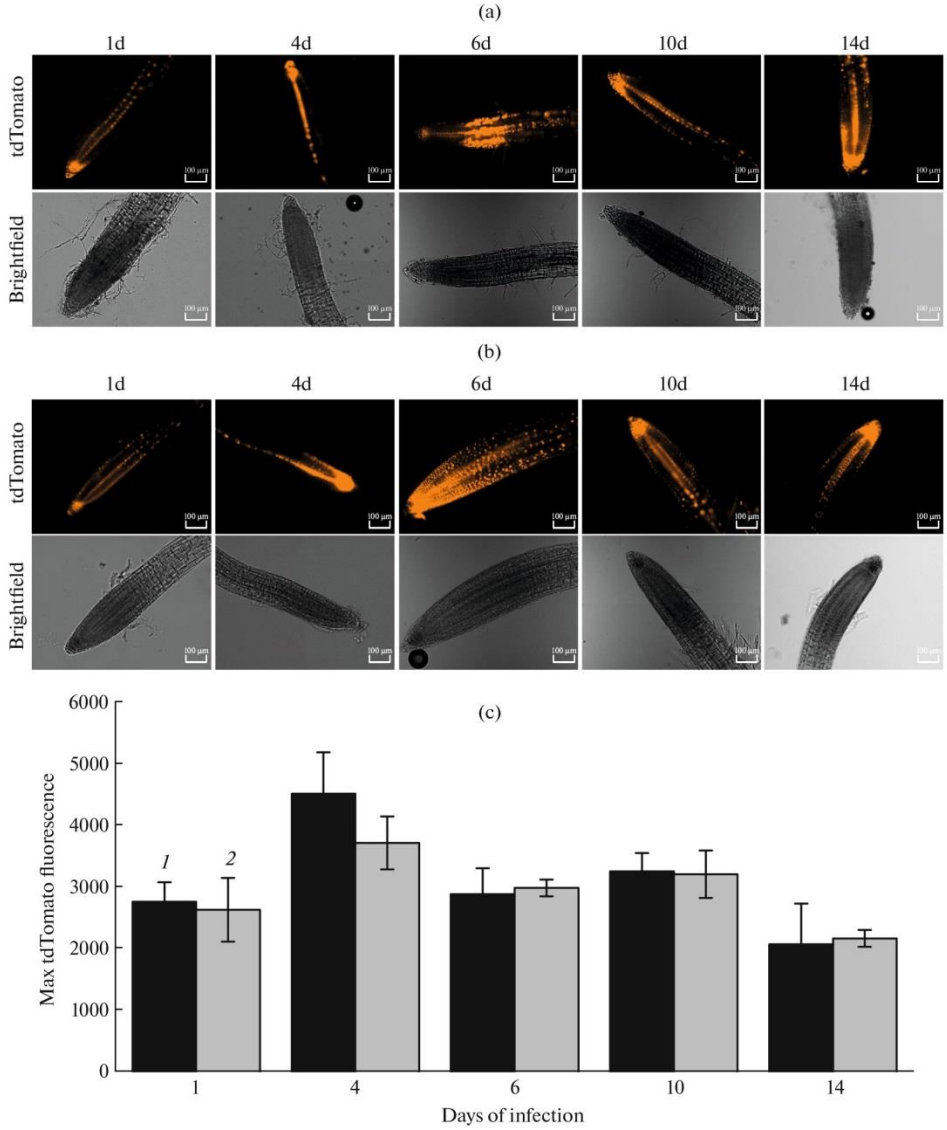

Fig. 3. Visualization of auxin maxima using the reporter construct *DR5::EGFP-DR5v2::tdTomato* during inoculation with the beneficial fungus *Mortierella hyalina*. (a, b) Fluorescence images of the *Arabidopsis* root tip after 1 up to 14 days of co-cultivation. Shown are the brightfield image and the tdTomato fluorescence in presence of a *M. hyalina* plaque (a) and a plaque with PDA medium only as the respective control (b). Scale bar = 100 μm; (c) graphic presentation of the maximum tdTomato fluorescence intensity of the mock treatment (plaque without mycelium, black bar) and in the presence of *M. hyalina* (with fungal mycelium, gray bar). No significant differences could be detected between control and fungal treatment ($P > 0.05$). Statistical analyses were performed for fungal treatment and the respective control with a Shapiro–Wilk normality test and subsequent two-sample *t*-test or Mann–Whitney rank sum test according to the data distribution ($n = 5$). (*1*) Mock; (*2*) *M. hyalina*.

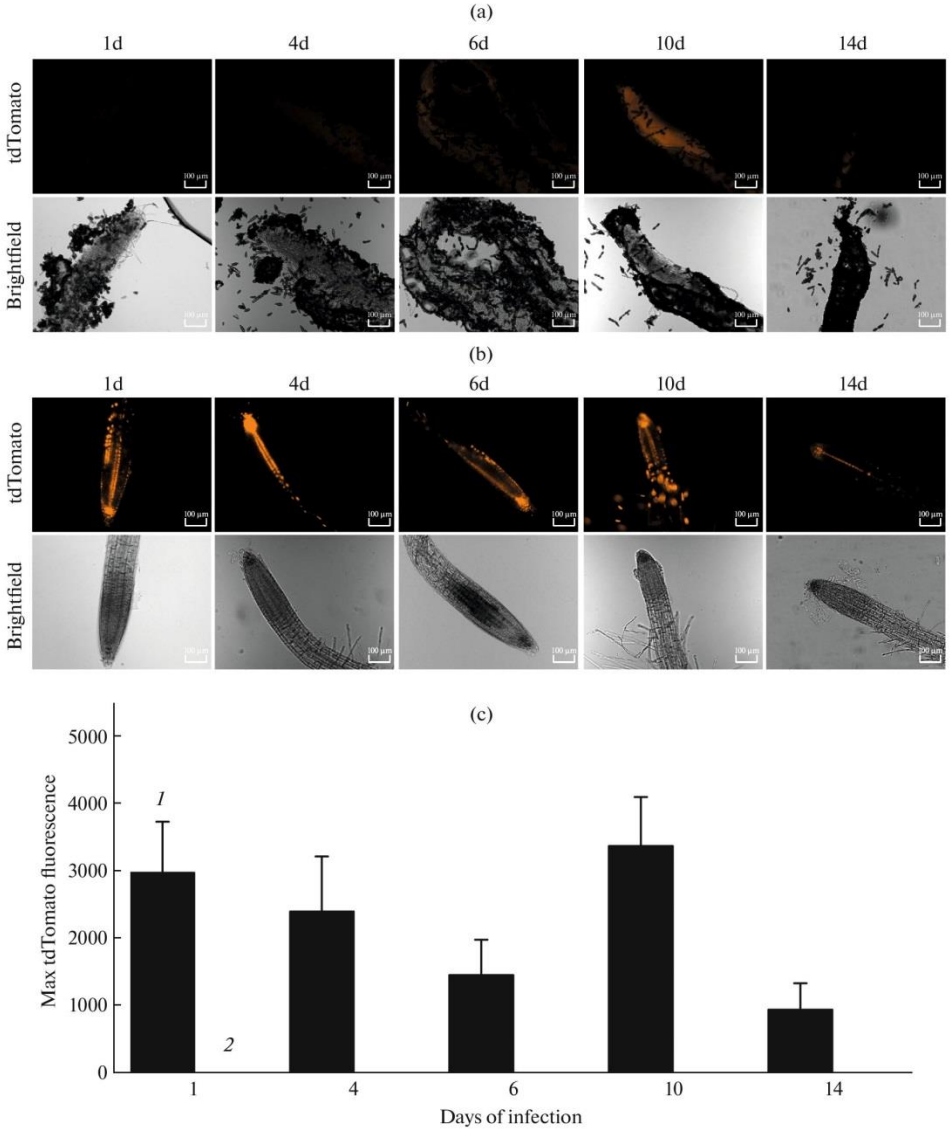

Fig. 4. Visualization of auxin maxima using the reporter construct *DR5::EGFP-DR5v2::tdTomato* during inoculation with the necrotrophic fungus *Alternaria brassicicola*. (a, b) Fluorescence images of the *Arabidopsis* root tip after 1 up to 14 days of co-cultivation. Shown are the brightfield image and the tdTomato fluorescence in presence of an *A. brassicicola* plaque (a) and a plaque with PDA medium only as the respective control (b). Scale bar = 100 μm; (c) graphic presentation of the maximum tdTomato fluorescence intensity of the mock treatment (plaque without mycelium, black bar) and in the presence of *A. brassicicola* (with fungal mycelium, gray bar). No fluorescence was detectable in the *A. brassicicola* treatment due to the rapid infection during the plant–fungus interaction ($n = 5$). (*1*) Mock; (*2*) *A. brassicicola*.

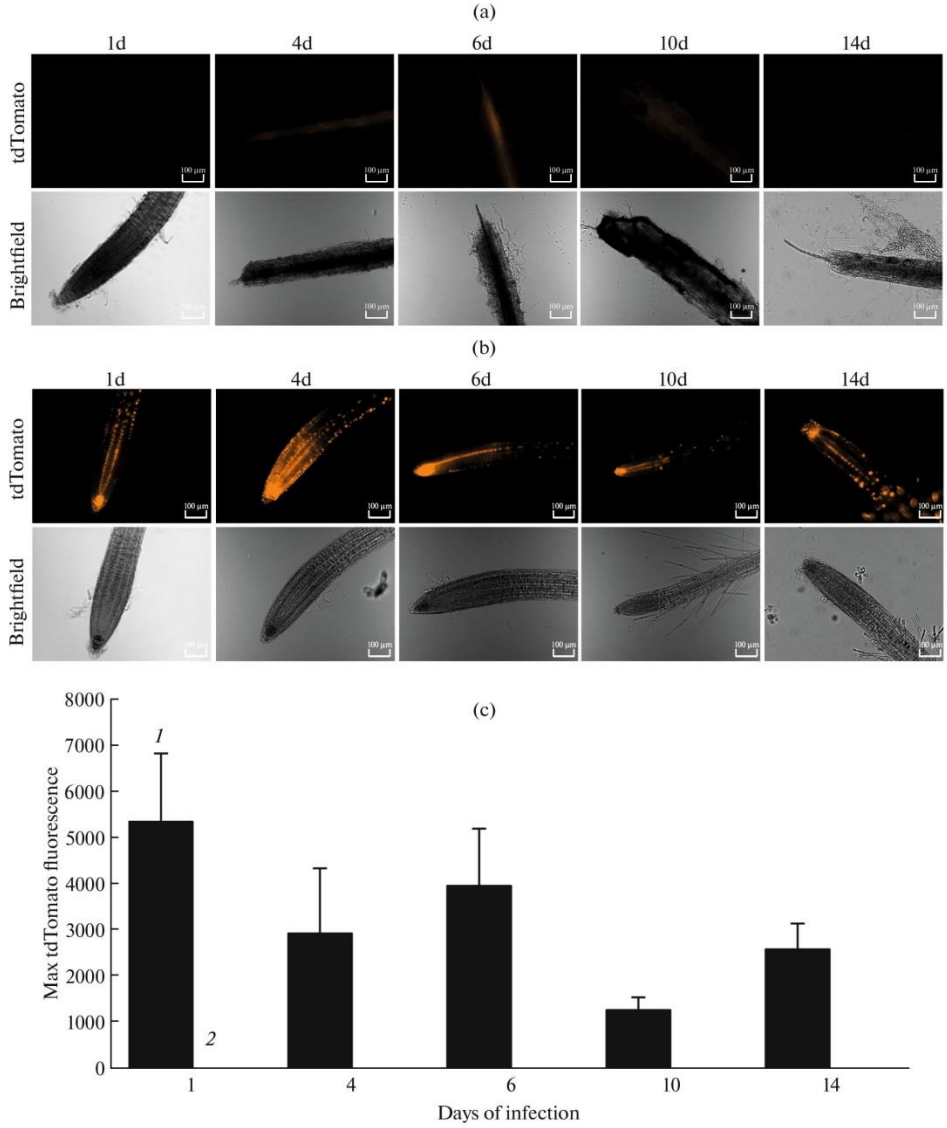

Fig. 5. Visualization of auxin maxima using the reporter construct *DR5::EGFP-DR5v2::tdTomato* during inoculation with the necrotrophic fungus *Verticillium dahliae*. (a, b) Fluorescence images of the *Arabidopsis* root tip after 1 up to 14 days of co-cultivation. Shown are the brightfield image and the tdTomato fluorescence in presence of a *V. dahliae* plaque (a) and a plaque with PDA medium only as the respective control (b). Scale bar = 100 μm; (c) graphic presentation of the maximum tdTomato fluorescence intensity of the mock treatment (plaque without mycelium, black bar) and in the presence of *V. dahliae* (with fungal mycelium, gray bar). No fluorescence was detectable in the *V. dahliae* treatment due to the rapid infection during the plant–fungus interaction ($n = 5$). (*1*) Mock; (*2*) *V. dahliae*.

was hardly visible compared to the tdTomato during fluorescence microscopy. Therefore, we focused on the tdTomato fluorescence to obtain a better resolution. When low dosages of IAA were applied to *Arabidopsis* root tips, it could be observed that the reporter gene expression extended throughout the entire root tip with the fluorescence extending shootwards into the elongation zone (Fig. 1). This expression profile is consistent with the polar auxin transport which is known to be involved during root growth [25]. Hence, the construct was further used to study auxin distribution patterns in *Arabidopsis* roots in response to different root-interacting fungi.

Phytohormones play a crucial role in growth and development of plants especially in the framework of plant–fungus interactions [12]. IAA being the most common form of auxin possesses an important role in regulating growth and plant development, especially in response to microbial signals [12]. It is postulated that the elevated auxin levels in roots colonized by beneficial microbes are a major stimulator for root growth [12]. The endophytic fungus *P. indica* is known to affect root colonization and root hair morphology in *Arabidopsis* [23] and also in Chinese cabbage. Several reports show that *P. indica* stimulates auxin production and signaling in various hosts, thereby enabling enhanced root growth [12]. Co-culture experiments showed that *P. indica*-colonization significantly induces higher fluorescence within 24 h in the entire root tip (Figs. 2a, 2c). However, the increased fluorescence signal was not persistent and returned after 4 days to a basal level matching the control treatment (Fig. 2). Later time points (6, 10 and 14 days after infection) showed a similar effect (Fig. 2). Hilbert et al. [26] showed a 3-fold elevation of free IAA for barley roots co-cultured with *P. indica* within the first 3 days, whereas at later time points (5 and 14 days after infection), this effect disappeared. This result is in congruence with our results, although the timeframe of response appears to be shorter for *Arabidopsis*. This effect might be due to the fact that every plant responds in a species-specific manner to the co-cultivation treatments and conditions [27]. Moreover, closer inspection showed that the fluorescence induced by *P. indica* increased within the first 3 h of co-cultivation with the fluorescence intensity being 10 times higher in the entire root compared to the uncolonized control [5]. These results indicate that the *P. indica*-induced fluorescence from the reporter construct is caused by the presence of higher levels of IAA in the root tissue. So far it is not clearly identifiable where the detected IAA originates from. The IAA could be provided by *P. indica* or originated from the plant itself [26]. Auxin or auxin derivatives can be of fungal origin, playing an essential role in the fungus-plant interactions [12, 26] as shown for *P. indica*. Hilbert et al. [26] performed biochemical analyses of the biosynthetic pathways for auxin production and showed that, feeding on tryptophan, *P. indica* can produce IAA and indole-3-lactate (ILA) through the intermediate indole-3-pyruvic acid (IPA). Time course transcriptional analyses after exposure to tryptophan designated the fungal *piTam1* gene as a key player. A green fluorescence protein reporter study and transcriptional analysis of colonized barley roots showed that piTam1 is induced during the biotrophic phase. *P. indica* strains in which the *piTam1* gene was silenced via an RNA interference approach were compromised in IAA and ILA production and displayed reduced colonization of barley roots in the biotrophic phase, but the elicitation of growth promotion was not affected compared with the wild-type situation. The results from Hilbert et al. [26] suggest that IAA is involved in the establishment of biotrophy in the *P. indica*–barley symbiosis and might represent a compatibility factor in this system. Our results suggest that in the early phase (within 24 h) of the interaction with *Arabidopsis* roots, *P. indica* increases auxin levels in the plant—irrespective of the origin of IAA—and this might be vital for root development and enhanced growth.

The fluorescence signal obtained from the microscopic images with the beneficial fungus *M. hyalina* did not show meaningful differences in comparison with uncolonized roots during all time points (Fig. 3). This result showed that *M. hyalina* did not induce an auxin response in *Arabidopsis* roots. Further studies using live-imaging of *Arabidopsis* roots infected with *M. hyalina* spores demonstrated that the fluorescence was slightly elevated within the first 5 h—however the overall response did not significantly differ from the control treatment [5]. Interestingly, the increased fluorescence during the first interaction phase between the plant and the fungus might be influenced by other processes such as the presence of other phytohormones, e.g., jasmonates. Meents et al. [5] demonstrated that although there is a significantly increased amount of IAA present in the root tissue upon co-cultivation, no reporter activation could be observed opposed to the *P. indica* treatment (Figs. 2 and 3). This effect might be based on an auxin-jasmonate crosstalk in the roots, since the simultaneous accumulation of jasmonates can have an inhibitory effect on the activation of the auxin machinery in the plant. Additionally, either the available amount of active IAA might be reduced or physiologically inactive during these co-cultivation periods. These observations are consistent with results published by Johnson et al. [16] demonstrating that *M. hyalina*-colonized *Arabidopsis* seedlings did not show any significant increase in the root biomass while the aerial parts of the colonized seedlings became bigger.

When the two pathogenic fungi *A. brassicicola* and *V. dahliae* were applied to *Arabidopsis* roots, a comparable tdTomato response of the construct could be detected: after 1 day, the fluorescence entirely disappeared from the fungus-colonized roots (Figs. 4 and 5). This observation was quite surprising since *V. dahliae*

is known to be a biotroph during early stages of infection [20]. Therefore at least during early time points, a beneficial interaction would result in the stimulation of auxin functions with a subsequent necrotrophic phase; however, the beneficial effect could not be observed in this case (Fig. 5).

Overall, fluorescence microscopy in this study showed chain-like conidia spore formation of *A. brassicicola* on the root tip (Fig. 4) after 24 h. In the following days, the spores covered the root and destroyed the root tissue (Fig. 4a). Roots treated with *A. brassicicola* spores were also evaluated using light sheet microscopy, and showed a complete loss of tdTomato fluorescence within 3 h [5]. A previous study [28] suggested that *A. brassicicola* spores produce AB toxins which are severely attacking the host plant. This toxic effect might explain the observations made in this study: the fluorescence from the auxin reporter construct might be terminated due to cell damage or cell death caused by these toxins.

V. dahliae treatment also resulted in the disappearance of the *DR5v2* expression in the root within 24 h (Figs. 5a, 5c). Brightfield images taken after 4 days showed severe damaged of the plant tissue and root tip (Fig. 5a). Meents et al. [5] specified that the loss of fluorescence occurs within 6 h of spore infection. Interestingly, the observed damage to the plant here appears to be much more severe compared to the previous study, which can be explained by the plug treatment which transfers a higher amount of viable fungal mycelium to the root tissue compared to spore application. Hence, *V. dahliae* colonizes the roots rapidly by covering it in conidia within 6 h of infection [20]. Regarding the mode of action, it is well known that *V. dahliae*, similar to *A. brassicicola*, produces different phytotoxins and other molecules that cause the death of host cells by degrading its tissue [29].

Overall, this study highlights the different mode of actions of beneficial and pathogenic fungi during plant–fungus interaction and the importance of auxin regulation especially in the root tissue which is constantly exposed to symbionts and enemies. Additionally, the construct *DR5::EGFP-DR5v2::tdTomato* proved to be a highly sensitive and stable reporter to monitor and visualize auxin maxima in different plant species and tissues which can be used to gain deeper insights into symbiotic interactions.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants as objects of research.

AUTHOR CONTRIBUTIONS

AKM, ACUF, and RO designed the experiments. AKM, ACUF, and SÖ performed the experiments. AKM, ACUF and SÖ analyzed the data. AKM, ACUF, SÖ, and RO wrote the manuscript with contributions from all authors.

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3. Discussion

Plants are key components within a complex, diverse, and resource-abundant environment. In order to adapt to these constantly changing conditions, comprising biotic and abiotic influences, each individual needs to perceive, decode, translate, and transduce the respective stimuli into responses appropriate to the given scenario. A variety of available chemical messenger, such as Ca^{2+}, phytohormones, volatiles, and peptides serve as fast and specific signals linking the perception of external stimuli to a tailor-made response within the plant itself or the surrounding community (Zebelo and Maffei, 2015). This study aimed to disentangle the aforementioned chemical signals involved during biotic interactions with herbivores and fungi in the model organism *Arabidopsis thaliana* and the crop plant *Ipomoea batatas* and the respective physiological response in each species.

3.1 Plant signaling molecules involved in wounding- and herbivory-induced defense

Wounding and herbivory are known to be among the most common stressors within a plant's life cycle. Damage to the tissue alone has already been shown to be sufficient to trigger a vast range of physiological responses (Schilmiller and Howe, 2005;Hou et al., 2019). Independent from the presence of herbivores and the concomitant host races, these wound signaling cascades can be regarded as the most ancient form of tissue-derived danger or alarm signals that initiate cellular signaling cascades, which often initiate defined defense responses (Manuscript 2). Nevertheless, current pest management mainly targets mechanisms involved in plant-herbivore interactions, trying to identify and exploit the key players affecting plant performance and herbivore resistance. Although the introduction of the respective attacking organisms and their inherent herbivore- or pathogen-associated molecular patterns (HAMPs & PAMPs) adds another layer of complexity, it also provides additional targets for novel pest management strategies. Plants alone possess powerful defense strategies, which can directly affect herbivore survival and reproductive success or indirectly by attracting natural enemies of the insect pest (War et al., 2012;Pérez-Hedo et al., 2021). Direct defense mechanisms comprise the production of toxins and feeding deterrents (proteinase inhibitors (PIs), terpenoids, alkaloids) (Pérez-Hedo et al., 2021) or fortification with mechanical barriers (War et al., 2012). Indirect defenses, e.g. damage- or herbivore-induced plant volatiles (DIVs & HIPVs), guide the herbivore's natural predators to protect the plant from infestation (Paré and Tumlinson, 1999;Mithöfer and Boland, 2012;War et al., 2012).

Prior to the activation of all aforementioned protective mechanisms, the adequate perception and elicitation of stimulus-specific outputs is crucial. Therefore, the following chapters will focus on herbivory-induced signaling components and their interplay in two different species, e.g. the model plant *A. thaliana* and the crop *I. batatas.*

3.1.1 Annexin1 – a regulator of calcium-mediated systemic herbivore defense

As one of the first signaling relays after wounding or herbivory, calcium has been extensively studied concerning its activation of a variety of downstream elements (Arimura et al., 2011), such as the induction of defense-related genes (Maffei et al., 2007), and the accumulation of jasmonates (Wang et al., 2019). These calcium-mediated responses occur locally as well as systemically, forwarding information from the site of damage through the vascular tissue by following a distinct pattern (Kiep et al., 2015).However, the generation of such rapid and transient $[Ca^{2+}]_{cyt}$ signatures requires the involvement of stress-inducible calcium channels in the first place. Within the last decade, a broad range of studies identified multiple wounding or herbivory-induced candidates and demonstrated their involvement in cytosolic calcium influx, e. g. glutamate receptor-like channels (GLRs) (Mousavi et al., 2013), Two Pore Channel 1 (TPC1) (Kiep et al., 2015), and more recently cyclic nucleotide-gated channel 19 (CNGC19) (Meena et al., 2019). Apart from these conventional channels, ubiquitous proteins such as annexins gained increasing attention as potential key elements in the formation of Ca^{2+}-permeable transport pathways (Davies, 2014). Especially Annexin 1 was shown to mediate the elevation of $[Ca^{2+}]_{cyt}$ in response to extracellular hydroxyl radicals and salt stress (Lee et al., 2004;Laohavisit et al., 2012;Richards et al., 2014) as well as cold-triggered Ca^{2+} influx resulting in enhanced freezing tolerance in *Arabidopsis thaliana* (Liu et al., 2021). Interestingly, the majority of available annexin-related reports mainly focused on the investigation of its involvement during abiotic stresses, comprising heat, salt, drought, cold, and osmotic stresses in various plant species (Yadav et al., 2018). Considering the involvement of biotic factors and a potential impact on plant annexins, some studies confirmed that the host plant's annexin expression was induced upon infection with viruses, bacteria, fungi, and phytohormonal treatments in general (Yadav et al., 2018). However, the role of annexins within wounding and especially biotic herbivore interactions has only been scarcely explored up to now.

We found the expression of *ANN1* in *A. thaliana* Col-0 plants to be significantly induced after 90 minutes of wounding or herbivory treatment (Manuscript 1). Interestingly, the observed upregulated *ANN1* transcript levels did not deviate depending on the presence of OS-derived HAMPS or water only,

indicating the presence of annexins as a universal wounding response. This hypothesis is supported by the occurrence of annexins in a multitude of other plants, including crops such as alfalfa, tomato, or wheat (Xu et al., 2016). Within these species it was shown that annexins are not exclusively transcriptionally activated after wounding but as a result of drought, salinity, cold, heat, phytohormones, or heavy metal stress (Xu et al., 2016). Although not stimulus-specific, our observations update previous studies conducted by Konopka-Postupolska et al. (2009) demonstrating upregulated *ANN1* mRNA levels after wounding with forceps, after longer time periods (24 and 48 h). Therefore we could highlight that the activation of annexins upon mechanical damage and herbivory can be regarded as a rapid but rather general stress response.

In order to further unravel the potential role of ANN1 in the downstream signaling cascade following actual and simulated herbivory, we investigated the cytosolic calcium elevations in Col-0 and *ann1-1* Arabidopsis plants (Manuscript 1). Using the bioluminescent $[Ca^{2+}]_{cyt}$ reporter (apo)aequorin, we found that - although slightly weaker compared to Col-0 plants – a rapid local accumulation of $[Ca^{2+}]_{cyt}$ occurred also in the absence of functional ANN1 after wounding with the addition of oral secretion from *Spodoptera littoralis* (Manuscript 1). Although the local calcium signal was clearly initiated in both wild-type and *ann1-1* plants, neither water nor OS could induce a systemic calcium signal in the connected leaves within *ann1-1* mutants. This crucial observation highlights the importance of ANN1 during systemic wounding signal propagation and how its absence impedes connected leaves from receiving information necessary for survival, comparable to GLRs (Nguyen et al., 2018;Toyota et al., 2018). Similar observations were made within mutants of the vacuolar cation channel, Two Pore Channel 1 (tpc1), where mechanical wounding-triggered systemic calcium waves were undetectable in contrast to the local response (Kiep et al., 2015).

Considering the lack of systemic calcium signal propagation in *ann1-1* mutants, our study aimed to investigate whether this impacts defensive downstream components, e.g. phytohormone accumulation and gene regulation in local and systemic leaves. As a readout for defense-related phytohormones, JA and its active form JA-Ile were investigated after 30 and 90 minutes of feeding by *S. littoralis* in locally treated leaves. Although increased jasmonate accumulation was detectable in all lines, *ann1* mutants displayed significantly lowered concentrations of JA and JA-Ile compared to Col-0 plants (Manuscript 1), whereas especially JA-Ile levels were significantly upregulated in the *ANN1* overexpression line. This finding supports the hypothesis, that a lack of *ann1* reduces downstream jasmonate responses and hinders the full activation of jasmonate-defense mechanisms, comparable to findings for CNGC19

(Meena et al., 2019). To investigate whether the reduced jasmonate accumulation is also visible on a systemic level, mechanical wounding using a pattern wheel with or without the addition of OS or water was performed followed by the quantification of jasmonates in locally treated and connected systemic leaves (leaves 8 and 13, respectively) (Dengler, 2006;Farmer et al., 2013) after 90 minutes (Manuscript 1). Similar to the omitted calcium elevations after mechanical wounding ± water/OS, JA and JA-Ile contents were not induced after mechanical wounding with or without OS in leaves 8 and 13 – strictly in contrast to the observed response in wild-type plants. Analyses of jasmonate-responsive genes (*JAZ10* and *VSP2*) after larval feeding showed a clear local as well as systemic upregulation of transcript levels in Col-0 plants, whereas *ann1-1* once more did not display such an increase (neither local nor systemic). These findings are comparable to observations made by Meena et al. (2019), who also found reduced expression levels of *VSP2*, *LOX2,* and *PLANT DEFENSIN1.2* conjoined with reduced jasmonate levels in *cngc19* mutants upon herbivory.

Feeding assays of *S. littoralis* on two annexin knockout and overexpression lines showed that impaired *ann1* expression results in reduced herbivore resistance, being closely tied together with the lack of systemic $[Ca^{2+}]_{cyt}$ signaling and jasmonate levels (Manuscript 1). Interestingly, although annexins are ubiquitously expressed in a variety of plant species, studies by Carella et al. (2016) outlined that ANN1 is not necessarily required for systemic signaling upon bacterial infection. Therefore, the Ca^{2+}-dependent recruitment of ANN1 might be a specific stress response to herbivory and can vary with regards to the applied stress. The exact recruiting mechanism for annexins during biotic or abiotic stress still remains elusive. As ANN1 can occur as a mobile (cytoplasmic) protein transported via the phloem (Guelette et al., 2012) or rather static as an integral part of the plasma membrane (Alexandersson et al., 2004;Marmagne et al., 2007), a variety of options leading to its channel-like activity is possible. One option would be transport *via* vesicles or direct recruitment to the membranes during stress (Laohavisit and Davies, 2011;Laohavisit et al., 2012;Davies, 2014) - also in combination with additional channels and transporters, e.g. by selective delivery or retraction from membranes similar to potassium channels in response to phytohormones (Sutter et al., 2007).

Our finding that genetic modifications of a single annexin already reduced $[Ca^{2+}]_{cyt}$ -mediated defense responses leads to the question whether there is a possibility to rescue the impaired systemic signaling upon activation of additional genetic interacting partners. As local calcium elevations are attenuated but still occurring, we deduce that ANN1 is important for the generation of systemic Ca^{2+} responses but not for local elevations itself comparable to TPC1 (Kiep et al., 2015). Nevertheless, calcium bursts in *ann1*

mutants might be insufficient to trigger additional signaling mechanisms as resistance against *S. littoralis* was significantly impaired. However, plants possess a multitude of additional signaling components, e.g. ROS, electropotential waves, or communication via hydraulic pressure changes. The induction of annexins under heat stress and its proposed interaction with ROS and Ca^{2+}, heat stress response transcription factors, and calcium-dependent protein kinases (Wang et al., 2015;Liao et al., 2017a;Dvorak et al., 2020) highlight new possibilities for stimulus-specific complex formations of annexin with other signaling components. Overall, we conclude that the lack of ANN1 hinders the transmission of wounding- and feeding-related signals to distal but connected leaves, resulting in the absence of calcium elevation and systemic jasmonate responses. The drastic impact of these crucial signals not reaching the necessary plant structures is clearly displayed in a reduced overall defense of ann1-1 mutants against *S. littoralis*.

3.1.2 Anti-herbivore defense signaling in sweet potato (*I. batatas*)

Apart from strictly calcium-mediated intraplant signaling, the power of volatiles as signaling components involved in intra- and interplant defense has been demonstrated for a broad range of species, ranging from trees, shrubs, grasses, and model plants to agricultural crops (Manuscript 2) with the list of investigated species constantly being updated. Driven by the need of finding eco-friendly alternatives to conventional pesticides, the focus of plant volatile research has been increasingly shifted towards plant-herbivore interactions in commercially relevant crop species, e.g. maize, tomato, potato, cabbages, beans, and many more (Manuscript 2). In order to test the availability of a potential target for volatile-mediated defense activation, the inherent protection mechanisms of each plant species have to be understood in the first place. Therefore, this thesis aimed to 1) identify and disentangle local and systemic defense mechanisms in sweet potato, being one of the most important tuber crops worldwide, followed by 2) the investigation of emitted DIVs and HIPVs regarding their defensive signaling potential (Manuscript 3).

Our initial finding that the trypsin-inhibitor sporamin is predominantly expressed in unwounded systemic leaves upon local wounding or herbivory by *Spodoptera littoralis,* confirmed previous studies (Yeh et al., 1997a;Yeh et al., 1997b;Chen et al., 2016) and underlines the necessity of a signaling component, triggering this systemic anti-herbivore protection (Manuscript 3). The identification of the homoterpene DMNT as the most abundant VOC emitted after mechanical damage or herbivory - although predominantly in the insect-resistant cultivar Tainong 57 – was not surprising based on the fact

that DMNT is a common DIV and HIPV found in a variety of plant species (Holopainen, 2004). However, airborne contact with DMNT alone proved to be sufficient to upregulate *sporamin* transcripts and trypsin inhibitor activity in unwounded TN57 plants, resulting in enhanced resistance against the herbivore *Spodoptera litura* (Manuscript 3). Our novel finding in sweet potato corroborates groundbreaking studies by Arimura et al. (2000), which supplied first evidence that DMNT alone can induce defense-related genes in lima bean and resistance against spider mites. Interestingly, in our case DMNT harbored no direct toxicity against the lepidopteran caterpillar *Spodoptera litura,* whereas in other cases DMNT severely damaged the peritrophic matrix of the insect pest *Plutella xylostella* while serving as a repellant (Chen et al., 2021). By disrupting the physical barrier separating food and pathogens from the insect's midgut epithelial cells, DMNT promotes inflammation and overall gut microbiota imbalance, leading to the pest's death (Chen et al., 2021). Although the gut microbiome and integrity also plays a vital role in our model organism *Spodoptera littoralis* as well as in *Spodoptera litura* (Xia et al., 2020;Mazumdar et al., 2021), its larger body size might protect it from DMNT-induced poisoning. Another option might be that although DMNT is a rather universal HIPV, toxicity only occurs in an herbivore-specific manner. Apart from a direct toxic effect in the herbivore, DMNT was reported to also suppress orientation of *S. littoralis* to host plants and mates by interfering with pheromone and host plant attractant components (Hatano et al., 2015). Although our study did not address the effect of being located by herbivore attackers we could demonstrate that upon insect feeding, DMNT-induced defense significantly decreased weight gain in *S. littoralis,* underlining its versatility as a defensive compound.

In contrast to systemically induced *sporamin* transcripts, jasmonates accumulated only locally during wounding or feeding treatment and showed no response to DMNT exposure. Thus, we concluded that within our setup, the direct anti-herbivore defense occurs in a jasmonate-independent manner. This effect appears to be rather species-specific as DMNT induced JA elevations in tea plants, promoting resistance of neighboring plants against insect attack (Jing et al., 2021). As demonstrated within this thesis, systemic sweet potato signaling is not only species- but also cultivar-dependent, as TN66 did not respond to DMNT treatment. The lacking trypsin inhibitor upregulation during DMNT exposure leads to the question whether other signaling and defense mechanisms exist in this cultivar, e.g. *via* other HIPVs and/or the generation of other defensive compounds. These are questions which will be addressed in the future.

3.2 Plant signaling during root-microbe interaction

As previously highlighted, efficient anti-herbivore defense mechanisms are a crucial aspect within a plant's life cycle. However, before being able to build up defenses, the plant needs to establish itself in unpredictable environmental conditions in the first place, therefore having to prioritize growth rather than defense (Figueroa-Macías et al., 2021). During seed germination and the following stages of root formation and leaf development, phytohormones play a major role determining the plant's success in adapting to surrounding abiotic and biotic stressors. Being the most influential signaling molecules during this initial growth period, they also pose as the most vulnerable target to manipulation for surrounding organisms (Xu et al., 2018). As plants do not live in a sterile environment, they are constantly shaped by a plethora of microbial communities (Eichmann et al., 2021) with the plant root as the first point of contact with soil microbiota present. In the first stages of plant-microbe interaction, there is no distinction between beneficial or pathogenic microbes as all foreign organisms are considered to be an intruder triggering immune responses by releasing bacterial flagellin or chitin (Eichmann et al., 2021;Figueroa-Macías et al., 2021). Interestingly, hormones provide a bridge and common chemical language between both organism types, therefore allowing microbes to alter the plants' hormone homeostasis and 1) mediate the interaction with beneficial symbionts (Egamberdieva et al., 2017;Eichmann et al., 2021) or 2) promoting pathogenicity and virulence (Kunkel and Harper, 2018;Han and Kahmann, 2019). The resulting interactions are highly complex and can determine the success or downfall of involved parties depending on whether the interaction is beneficial or pathogenic. As the majority of land plants successfully established symbiotic relations with fungi (Bidartondo et al., 2011), this study aimed to decipher the impact of beneficial and pathogenic fungal species on the plant's hormone regulatory network. As it is known that especially during beneficial interactions, auxin plays a central role interfering with biosynthesis and regulation of other hormones such as JA and SA (Sirrenberg et al., 2007;Ludwig-Müller, 2015), we were particularly interested in how fungus-microbe pairings alter hormonal outputs in the model organism *Arabidopsis thaliana.*

3.2.1 *Piriformospora indica* reprograms *Arabidopsis thaliana* root development during early recognition phases

Within the past two decades, *Piriformospora indica* (also currently known as *Serendipita indica*) emerged as one of the best-studied endophytic fungi of the Sebacinaceae family (Opitz et al., 2021). Originally isolated from the Indian Thar Desert (Verma et al., 1998) it gained increasing popularity –

especially in the light of progressing climate change – due to its ability to colonize the root of many plant species whilst promoting plant growth and conferring drought resistance (Sherameti et al., 2008). Upon host contact, *P. indica* elicits mild defense responses in the plant *via* cellotriose-induced $[Ca^{2+}]_{cyt}$ elevations in the root (Vadassery et al., 2008;Johnson et al., 2018;Oelmüller, 2018).

In addition to calcium responses, we found that colonization with *P. indica* activated the auxin-responsive DR5v2 promoter in Arabidopsis roots (Manuscript 4) within 3 h of co-cultivation. Strikingly, the fluorescence levels continuously increased within the next 24 h (Manuscript 4) up to 4 days (Manuscript 5) before returning to a basal level. In contrast to interaction with the beneficial fungus *M. hyalina,* 24 h of contact with *P. indica* initiated the formation of lateral root primordia, highlighting reprogramming of root development during an early recognition phase (Manuscript 4; discussion section 3.2.4 Fig. 8). *P. indica* co-cultivation experiments with Chinese cabbage confirmed our findings, reporting enhanced lateral root development, however after a prolonged treatment period of 7 d (Lee et al., 2011). Taken together, these morphological changes combined with the ~10-fold upregulated fluorescence levels indicate a rapidly increased availability of free IAA in the root tissue. Interestingly, the impact of *P. indica* treatment on the availability of auxin occurs in a highly time-dependent manner, as co-cultivation with barley lead to increased IAA levels after 3 d of exposure which was subsequently omitted after 5 and 14 d (Hilbert et al., 2012). Similar observations were made in Arabidopsis by Vadassery et al. (2008) who did not detect any differences in IAA levels between *P. indica*-treated and control seedlings after 7, 10, and 14 days. Within our setup, we found IAA levels significantly increased after 24 h (Manuscript 4 & 5). However, we could not distinguish whether the auxin originates from the endophyte itself - as the mycel of *P. indica* contains high amounts of IAA in the absence of the plant (Manuscript 4) - or is produced by the plant during fungal infection. Among others, studies by e.g. Contreras-Cornejo et al. (2009) and Liao et al. (2017b) confirmed that plant growth promotion can be mediated by fungal auxin metabolites which are converted into auxin in the host. Interestingly, the presence of newly produced indole derivatives by *P. indica* is not always required to directly promote growth but rather facilitate root colonization (Hilbert et al., 2012). However, auxin-dependent mechanisms are species-specific, depending on the host plant and whether auxin signaling or auxin production is targeted as shown for Arabidopsis and Chinese cabbage (Lee et al., 2011). This highlights the necessity to further investigate the mechanisms of plant-fungus symbiosis in order to unravel signaling and biosynthetic circuits involved.

Investigation of additional phytohormone profiles after 24 h of *P. indica* infection revealed increased SA levels in the absence of jasmonate upregulation (Manuscript 4). As an antagonistic crosstalk between SA and IAA has been suggested (Xu et al., 2018), we did not anticipate simultaneously increased IAA and SA levels in *P. indica* -colonized roots. However, this effect was also reported in Sun et al. (2014) and Vahabi et al. (2018) with the potential explanation that SA alters auxin-dependent signaling and not the auxin concentration *per se* (Wang et al., 2007).

3.2.2 Auxin -jasmonate antagonism during *Arabidopsis-Mortierella hyalina* interaction

Apart from *P. indica*, increasing numbers of plant growth promoting fungi have been newly discovered, among them various *Mortierella* species (Ozimek and Hanaka, 2021). As the most abundant filamentous soil fungi worldwide (Ozimek and Hanaka, 2021), *Mortierella* strains occur in a wide range of adverse environmental conditions ranging from nutrient-poor caves to constantly evolving rivers and lakes at each latitude (Ozimek and Hanaka, 2021). Due to their ability to thrive in unfavorable habitats and their saprotrophic lifestyle providing alternative nutrient resources, *Mortierella* inoculates gained increasing popularity as agriculturally valuable decomposers. Additionally, their beneficial growth-promoting effect has been successfully demonstrated in a variety of crops, e.g. castor bean, corn, avocado, and watermelon (Ozimek and Hanaka, 2021).

Studies by Johnson et al. (2019) demonstrated that, in addition to promoting increased shoot biomass production in *A. thaliana*, the early root colonizer *Mortierella hyalina* is able to reduce *Alternaria brassicicae* infection *via* activation of calcium-dependent defense mechanisms. These findings highlight the versatility of *M. hyalina* as a mediator of pathogen resistance as well as a growth promotor. Interestingly, co-cultivation of *A. thaliana* with *M. hyalina* did not promote root growth (Johnson et al., 2019) confirming our findings that in contrast to *P. indica, M. hyalina* does not initiate the formation of lateral root primordia (Manuscript 4; discussion section 3.2.4 Fig. 8). The lack of root growth seemingly contradicts our results showing a significantly increased amount of IAA within the roots after 24 h of incubation (Manuscript 4) compared to control samples. Induction of IAA (and ABA) concentrations in the roots was also observed during corn – *M. elongata*- interaction (Li et al., 2018) corroborating previous findings by Wani et al. (2017) stating that co-cultivation of *Crocus sativus* with *M. alpina* leads to increased IAA accumulation. However, they. did not differentiate between root and shoot and

investigated later stages of infection in contrast to our study presented here. Due to our experimental setup, it is not possible to distinguish whether the detected IAA levels originate from the plant upon contact with the fungus or from *M. hyalina* itself. We demonstrated that *M. hyalina* mycel contains a detectable amount of free IAA, however much lower compared to *P. indica* (Manuscript 4). It has been shown previously that *Mortierella* strains are able to produce up to 70 mg IAA per liter depending on the species, strain, incubation temperature, and tryptophan availability (Wani et al., 2017;Ozimek et al., 2018;Ozimek and Hanaka, 2021). Therefore, it is possible that the detected IAA either originates from the fungus itself or is produced by *M. hyalina* and/or *A. thaliana* during the early stages of infection.

An indicator that other signaling components might antagonistically prevent IAA-induced root growth promotion is the expression pattern of the auxin reporter gene observed during co-cultivation for 24 h up to 14 d (Manuscript 4 & 5). Fluorescence levels of auxin maxima remained on a constant level during the 24 h measuring period, supporting our hypothesis that although IAA is present, no auxin-mediated reporter activation takes place (Manuscript 4). This effect could also be seen during our long-term experiments where no change in reporter expression could be detected within 2 weeks of measurements (Manuscript 5). Considering that SA levels remained at a basal level compared to control plants (Manuscript 4) based on a proposed IAA- and SA antagonism (Kazan and Manners, 2009), jasmonates provide a valid explanation for the absence of root growth promotion. We found JA and its active form JA-Ile as well as the precursor OPDA significantly accumulated within 24 h, suggesting that jasmonates play an important role during the early infection stages with *M. hyalina* (Manuscript 4). As jasmonates are mainly induced as defense-related signaling molecules during wounding or herbivore- and necrotrophic pathogen attack (Thaler et al., 2004;Kachroo and Kachroo, 2009), our findings implicate that during the first contact phase *M. hyalina* is perceived as a potential intruder. During later time points this effects seem to be diminished as *M. hyalina*-colonized roots did not display elevated jasmonate levels or JA-responsive gene expression within 4 d of treatment (Johnson et al., 2019).

Although no data is available investigating the expression of auxin-related genes during early stages of infection with *M. hyalina*, we could demonstrate that addition of increasing concentrations of JA impaired DR5::EGFP-DR5v2::tdTomato fluorescence in a dose-dependent manner (Manuscript 4). Our findings confirm previous experiments conducted by Ishimaru et al. (2018) showing that JA treatment of Arabidopsis seedlings inhibits lateral rooting and accumulation of the auxin reporter DR5::GUS. Interestingly, this auxin-jasmonate interaction occurred independent of the JA-receptor complex COI1,

highlighting that an interaction is possible by skipping certain mechanistic components of the JA signaling pathway.

Taken together, our results confirm a crosstalk between auxins and jasmonates, which has been the center of vivid discussions throughout the past years. As JA and IAA are commonly attributed to be defense- or growth related hormones perceived to function antagonistically, mounting evidence can be found demonstrating that JA mediates induction of auxin biosynthesis genes in Arabidopsis (Dombrecht et al., 2007), whereas auxin can *vice versa* induce JA biosynthesis (Tiryaki and Staswick, 2002). As described by Hoffmann et al. (2011), the auxin and jasmonate signaling pathways share an intriguing variety of common perception and signal transduction components which are not yet well understood - especially in the light of plant/fungus interactions. Thus, further evidence needs to be gathered shedding more light on the plant's and fungus' contributions to hormone regulatory processes.

3.2.3 *Alternaria brassicicola* infection suppresses auxin responses in *A. thaliana* roots

Opposing the beneficial effects of growth-promoting microbes, plant pathogens are causing massive crop yield losses on a daily bases. Therefore, extensive research has been conducted, attempting to unravel underlying infection mechanisms to enhance plant immunity and prevent rot and wilt diseases. Depending on whether the pathogen derives its nutrients from living host tissues or dying cells, plant pathogens can be further subdivided into biotrophs and necrotrophs (Glazebrook, 2005). Taking a closer look at the impact on the host plant's phytohormone distribution, it is widely accepted that SA mediates resistance against biotrophs and hemi-biotrophs whereas JA promotes defense against necrotrophs (Qi et al., 2012).

One of the most prominent examples causing black leaf spot disease in crucifers and a variety of other species is the necrotrophic fungus *Alternaria brassicicola* (Glazebrook, 2005). Due to its broad host range and ability to infect leaves and roots, *A. brassicicola* provides an excellent pathogen to study infection mechanisms, especially in model organisms such as *A. thaliana.* From its mode of action, the fungus penetrates the plant tissue with a subsequent production of hydrolytic enzymes and toxins resulting in black leaf spot disease and ultimately cell death (Otani et al., 1998;Glazebrook, 2005).

Within our studies, we observed that 24 h- *A. brassicicola* spore inoculations diminished DR5-reporter fluorescence levels in the root within 3 h (Manuscript 4 & 5; discussion section 3.2.4 Fig. 8) indicating

suppression of auxin responsive genes. In contrast to the lack of tdTomato-derived fluorescence, we found significantly increased concentrations of IAA (Manuscript 4) in the treated roots confirming observations by Qi et al. (2012) showing upregulated expression levels of several auxin biosynthetic genes in a time-dependent manner as well significantly increased free IAA concentrations after 24 h of fungal treatment. This again highlights the importance of auxins in mediating pathogen resistance, confirming observations showing that plants defective in auxin biosynthesis are more susceptible to *A. brassicicola* infection (Bari and Jones, 2009;Kazan and Manners, 2009;Qi et al., 2012).

Jasmonates are widely accepted defense regulators against necrotrophs, therefore it is not surprising that *coi1-2*- jasmonate-receptor mutants are more prone to *Alternaria* infection comparable to aforementioned auxin ones (Qi et al., 2012). Upregulation of jasmonates generally occurs during infection with necrotrophic fungi, however we surprisingly did not find any induction of JA, JA-Ile, or OPDA levels (Manuscript 4). A similar effect has been observed by Scholz et al. (2018) for the pathogenic fungus *Verticillium dahliae.* Due to the short treatment time, it is highly probable that although gene expression patterns started to be altered, penetration of the root has not yet occurred (Scholz et al., 2018). In order to fully perceive the fungal attack and unfold jasmonate-dependent defense responses, pathogen-induced cell disruption (comparable to wounding) needs to be triggered first to activate JA and JA-Ile accumulation. Overall, upon 1 d- co-cultivation with fungal plaques, no auxin reporter fluorescence could be detected, highlighting the severity and rapid infection progression in the presence of increased spore amounts (Manuscript 5) independent on infection or penetration rates. Similar to our observations, Johnson et al. (2019) found *A. brassicicola*- treated seedlings dead within a few days.

3.2.4 Manipulation of auxin signaling in the root occurs during early stages of *Verticillium dahliae* infection

Aside from the aforementioned purely pathogenic *Alternaria*, hemi-biotrophic fungi present an intriguing but economically devastating threat to a wide range of agricultural crops (Deketelaere et al., 2017). During the initial stages of colonization, hemi-biotrophic fungi penetrate and systemically spread through the plant host's root xylem without any display of disease symptoms or reduced plant performance (Scholz et al., 2018;Dhar et al., 2020). However, during later stages of disease progression, these fungi shift from a biotrophic to a necrotrophic interaction once the hyphae extend to the host's aerial plant tissue (Fradin and Thomma, 2006). Upon this change, a variety of responses is triggered within the plants, e.g.: 1) blocked xylem transport impairing vascular transportation promoting leaf

wilting (Klosterman et al., 2011), 2) synthesis and release of fungal toxins (Fradin and Thomma, 2006), 3) reprogramming of phytohormone metabolism (Veronese et al., 2003;Thaler et al., 2004;Tjamos et al., 2005), and 4) induction of additional signaling molecules such as nitric oxide (NO) and hydrogen peroxide (H_2O_2) (Yao et al., 2011;Yao et al., 2012). Although the initial stages of infection remain largely undetected allowing the fungal intruder to thrive, ultimately the host plant activates defense genes to induce cell death (Reusche et al., 2014;Zhang et al., 2016a).

The genus *Verticillium* with *V. dahliae* as one of its most prominent and destructive members (Inderbitzin et al., 2011;Inderbitzin and Subbarao, 2014), causes extensive yield losses in a plethora of crop species worldwide comprising cotton, tomato, spinach, potato, but also trees and shrubs (Dhar et al., 2020). As a soilborne pathogen mainly attacking plant roots, *V. dahliae* produces spores during growth phases within the xylem in addition to formation of microsclerotia, allowing long-term survival in soil for up to 10 years (Scholz et al., 2018).

Our study aimed to take a closer look at the hormone reprogramming during early stages of infection. As *V. dahliae* displays a specific combination of growth phases followed by attacking the host, growth-related auxin as well as defensive jasmonates were of main interest.

We could not detect any increase in free IAA, SA, or jasmonate (JA & JA-Ile) contents after 24 h of co-cultivation with *V. dahliae* (Manuscript 4). With regards to free IAA, our observations confirm previous reports stating that *V. dahliae* or *V. longisporum*- treated Arabidopsis show IAA levels similar to control plants - however measured at later timepoints ranging from 2 to 14 dpi (Iven et al., 2012;Fousia et al., 2018). Taken together, these findings indicate that co-cultivation with *V. dahliae* does not affect auxin biosynthesis neither during early nor later stages of infection. The lack of JA accumulation during 24 h of *V. dahliae* treatment was also observed by Scholz et al. (2018) highlighting that elevation of JA and JA-Ile rather occurs during later timepoints (~21 dpi) as reported by Sun et al. (2014) and due to the hemibiotrophic nature of the fungus. Interestingly we found the JA-precursor OPDA to be significantly accumulated within 1 d (Sun et al., 2014), however as we cannot distinguish between active *cis*- and inactive *trans*-OPDA, further measurements need to be performed to provide a more accurate statement.

Opposed to basal IAA levels during early timepoints of pathogen treatment, we found the auxin reporter gene fluorescence levels in the root tip completely diminished after 6 h (Manuscript 4) strengthening our findings observed during fungal plaque treatments (Manuscript 5). Based on these results, signals

released by *Verticillium* likely inhibit the activation of our reporter construct *DR5::EGFP-DR5v2::tdTomato* and promote infection by manipulating auxin signaling within the roots instead of the actual IAA production. On a gene regulatory level, it is known that expression of growth-associated genes like the auxin-responsive genes (e.g. *ARF5* and several genes belonging to the *GH3*-family (e.g., *GH3.17, GH3.4, DFL2, WES1)* decreased during the 24 h of *Verticillium* treatment as reported by Scholz et al. (2018). Additional findings by Fousia et al. (2018) showed that *V. dahliae* infected roots displayed induced expression levels of auxin receptor genes, e.g. transport inhibitor response 1 (*TIR1*), auxin signaling F box protein 1 (*AFB1*), auxin signaling F box protein 3 (*AFB3*), and auxin transporter gene auxin resistant 4 (*AXR4*) during prolonged inoculation periods. Studies by Kidd et al. (2011) and Lyons et al. (2015) have made similar observations, showing that a number of auxin signaling genes in *A. thaliana* were differentially expressed in roots and shoots after *Fusarium oxysporum* inoculation. As the investigated timepoints from the latter studies (3 dpi – 14 dpi) mainly focused on later responses in 20 d- old potted plants, it is evident that expression patterns of auxin- related genes strongly depend on the observed measuring timeframe. Overall, it becomes evident that *V. dahliae* is able to influence auxin-dependent signaling pathways in Arabidopsis from early stages of infection until the actual display of disease symptoms.

Consequently, a key element to unravel specific events during *Verticillium* treatment is the determination of the infection stage and its progression. Scholz et al. (2018) documented that within their 24 h- setup the fungus rapidly colonized and penetrated the root surface, however without invading the vascular tissue yet. As the fungus is still growing in its pre-vascular phase, these conditions provided an optimal setup to investigate early interaction events, which we could implement for the experiments described in this thesis. Although phytohormone levels did not show any significant changes during 24 h of contact with *Verticillium,* a rapid colonization could be observed (Manuscript 5) conjoined with an inhibition of the auxin reporter construct (Manuscript 4; discussion section 3.2.4 Fig. 8). Both results indicate that on a signaling level, growth and defense-related pathways are already affected early on by spore treatment independent on fungal- induced phytohormone changes. As JA pathway- and auxin signaling mutants displayed increased disease resistance to *Verticillium* infection (Scholz et al., 2018;Dhar et al., 2020), *V. dahliae* most likely employs components of jasmonate and auxin signaling pathways to promote disease progression to overcome the plant's defense mechanisms. As well-known antagonists of SA (Dhar et al., 2020), the presence of IAA and JA would putatively diminish protective SA pathway components, favoring establishment and disease progression by *V. dahliae.*

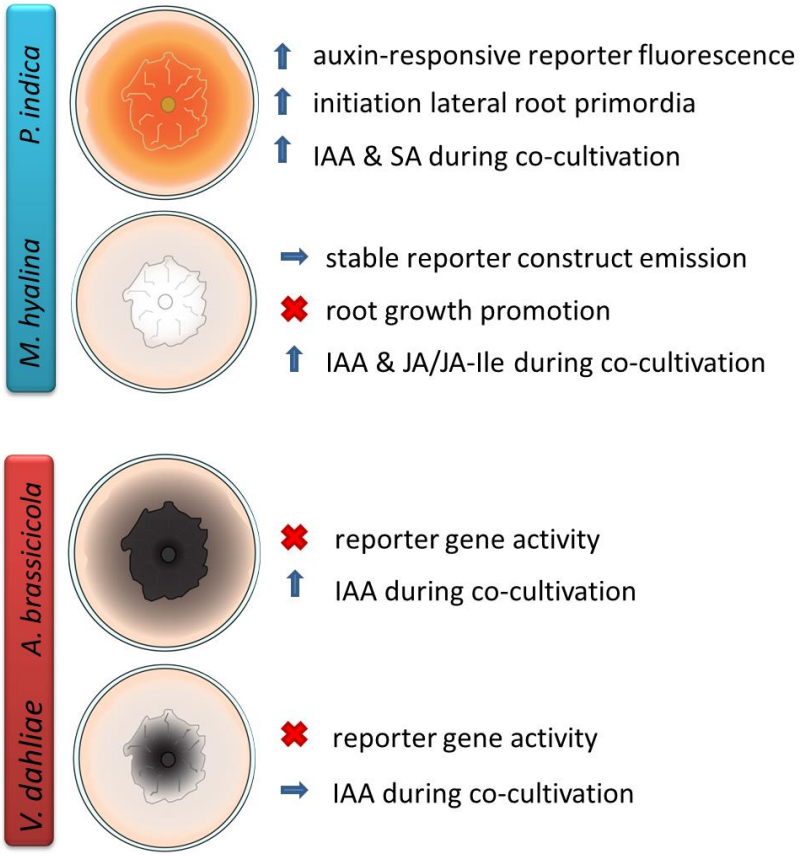

Fig. 8 Overview of investigated responses in transgenic *Arabidopsis thaliana* auxin reporter lines upon infection with the fungi *Piriformospora indica, Mortierella hyalina, Alternaria brassicicola,* and *Verticillium dahliae*. ↑upregulation; → no detectable effect compared to control treatment; X inhibition.

4. Outlook

As can be seen throughout the sections above, plant signaling mechanisms during biotic interactions provide a beautiful and intriguing maze, which I attempted to further explore within this thesis. The involvement of the unconventional calcium channel protein ANNEXIN1 as a regulator of systemic signaling during herbivory was discovered. However, the underlying mechanism of its distribution and channel formation during plant-insect interaction ultimately promoting Ca^{2+} -conductance, still requires further investigation. As ANN1 is only a single member of the annexin's diverse multigene family, future studies will elucidate the role of single and multiple annexins during herbivore attack and their involvement and interaction with other signaling components, e.g. calcium binding proteins and channels.

In contrast to well-investigated calcium signaling in model plants, the interplay of intra- and interplant signaling components in genetically complex crops, e.g. the hexaploid species *I. batatas*, remains largely obscure. The findings emphasize the potency of DMNT alone to induce protective mechanisms for resistance against herbivores, however in a cultivar-specific manner. As the emitted VOC bouquet consists of additional compounds apart from DMNT, further studies are necessary to investigate the effect of other volatile on the defense and signaling output in sweet potato – especially on a community level. The plant's ability to communicate with conspecific neighbors while omitting long-distance signaling within the same individual provides an efficient protective mechanism against herbivores. However, it would be intriguing to gather further information on whether the plant solely relies on VOCs for systemic responses. As shown by Chen et al. (2008), signaling peptides such as *Ib*HypSys4 provide a valid additional opportunity for systemic signaling *via* the vascular system. Combined with our current knowledge, experiments have to be conducted showing the order of events leading to herbivore resistance, e.g. how jasmonate burst, VOC release, peptide induction, and defense output are intertwined. The mounting identification of downstream signaling components leads to the subsequent question about the initial perception mechanism during wounding, insect feeding, and after VOC release in sweet potato. In order to better understand the hierarchy of events following the various aforementioned stimuli, additional experiments are required to identify VOC and peptide receptors as well as their biological functions.

It has been shown that VOCs are not exclusively implemented by herbivore-infested plants during defense but also by the beneficial fungus *M. hyalina*, ultimately promoting shoot growth in the host

(Johnson et al., 2019). This distinct mode of action highlights the necessity to better understand plant-microbe interaction mechanisms, consolidating volatile and phytohormone signaling in the light of growth- and defense responses. The importance of IAA during beneficial and pathogenic plant-fungus co-cultivations was verified, resulting in a variety of results depending on the availability of jasmonates and salicylic acid. The performed study mainly focused on the expression of a single auxin-responsive promotor and the accumulation of phytohormones. As early signaling events upon fungal infection are often visible by reprogramming of distinct hormonal signaling pathways, further studies investigating the expression levels of JA- and or SA-related genes would be advisable. Additionally, the used experimental setup only detected IAA in the mycel and in the plant (with fungal residues) after 24 h of co-cultivation. As auxin derivatives can also play a crucial role in plant-microbe interactions, the involvement of alternatives to IAA should be addressed. On that note, further knowledge is needed on whether phytohormones are formed and supplied by the fungus or the plant upon contact to distinguish the contributions of each interaction partner.

5. Summary

In the course of evolution, plants have developed a broad toolbox of protective mechanisms to specifically tailor their physiological reactions to their surrounding impulses and current needs. As environmental factors and the presence of friends and foes in the shape of microbes and herbivorous attackers constantly changes, the necessity for tightly regulated constitutive and inducible response mechanisms gained increasing importance. In order to respond efficiently to a given stimulus, plants evolved distinct perception and signal transduction pathways, comprising a large variety of components serving as messenger molecules.

Calcium (Ca^{2+}) serves as a crucial secondary messenger during early signaling transduction processes in plants. Upon perception of environmental stresses, e.g. wounding and herbivory, the rapid elevation of free cytosolic calcium levels induces local and systemic signaling cascades as well as distinct defense responses. Although a variety of channels and calcium-binding proteins contributing to this early signaling events have been identified, the mode of action of unconventional calcium channels such as annexins still remains unknown. As members of a diverse multigene family, annexin proteins have been shown to bind anionic phospholipids, ultimately promoting Ca^{2+} conductance. In this study we investigated the role of ANNEXIN1 during wounding and herbivory of *Arabidopsis thaliana* and found systemic cytosolic calcium elevation significantly impaired in *ann1* loss-of-function mutants. Subsequent bioassays revealed that the generalist herbivore *Spodoptera littoralis* performed better in the absence of ANNEXIN1 (and *vice versa* in ANN1-oeverexpressing lines), therefore highlighting its importance as a positive defense regulator. As especially downstream phytohormones, e.g. jasmonates, mediate the onset of Ca^{2+}-elicited signaling events, we investigated whether *ann1* mutants display a comparable induction of defense-related responses locally and systemically. Conjoined with the aforementioned reduced calcium elevation in systemic leaves, expression of defense genes and jasmonate accumulation remained local in *ann1* plants. Taken together, our findings provide evidence for the importance of ANN1 in the activation of systemic calcium signaling events, ultimately enhancing resistance against attacking herbivorous insects.

From a broader perspective, a central aspect of cellular signaling cascades initiating plant defense responses is the presence of damage-associated molecular patterns (DAMPs). Indicated by its given name, DAMPs are released upon tissue damage, providing information on wounding events or herbivore

attack in the plant. Apart from cell wall components, peptides, eATP, and a variety of other compounds, volatile organic compounds (VOCs) were also classified as airborne DAMPs and provide an intriguing signaling component for intra- and interplant communication. In this thesis, we show that sweet potato plants are able to release, perceive, and respond to VOCs in a highly species-specific manner, underlining the importance of plant-plant communication during herbivore defense. We identified a distinct blend of herbivory-induced VOCs with the homoterpene *(E)*-4,8–dimethyl–1,3,7-nonatriene (DMNT) as the most prominent compound. This single compound systemically induced the trypsin inhibitor sporamin, resulting in increased resistance against *Spodoptera* larvae in neighboring plants. Intriguingly, this effect was only observed in an allegedly more herbivore-resistant cultivar Tainong 57, whereas a second cultivar Tainong 66 displayed reduced DMNT emission and increased susceptibility during insect attack. As jasmonates were only locally upregulated during feeding and simulated herbivory, we propose that the systemic defense activation in sweet potato is jasmonate-independent and inducible without any previous priming incident.

Aside from DMNT-mediated signaling, previous findings in sweet potato provided evidence that wound- and jasmonate-elicited hydroxyproline-rich glycopeptides (HypSys peptides) could act as an additional systemic intraplant messenger. Investigations of the defense-related peptide *Ib*HypSys4 demonstrated its ability to activate *sporamin* expression and DMNT release, highlighting its importance within the DMNT regulatory signaling pathway. Although no receptor for *Ib*HypSys4 perception has been identified, the discovery of the novel peptide-receptor pairing *Ib*PepI-*Ib*LRR-RLK1 provides a new angle to study the involvement of peptides in sweet potato stress responses. We suggest that *Ib*HypSys4 and *Ib*PepI possess divergent biological functions; however, additional information has to be gathered to cement this hypothesis.

In addition to signaling events during plant-herbivore interactions, a plethora of plant species is known to be associated with microbes, especially root-interacting fungi. Upon colonization, these fungi are able to alter phytohormone levels during early stages of infection. The phytohormone auxin (indole-3-acetic acid, IAA) is a key regulator in root growth in development, therefore providing an easy target for manipulation by microbial intruders. Available auxin reporters (DR5::EGFP-DR5v2::tdTomato) expressed in the model plant *A. thaliana* allow visualization of auxin distribution patterns in fungi-colonized Arabidopsis roots. Live-imaging fluorescence microscopy techniques combined with LC-MS revealed that the beneficial endophytes *Mortierella hyalina* and *Piriformospora indica* produce IAA in their mycelia

whilst the latter stimulates expression of auxin-responsive reporter genes. Co-cultivation of 24 h already resulted in significantly higher auxin levels, promoting elongation of lateral root primordia – however only during *P. indica* treatment. These responses were strictly time-dependent as induction of auxin maxima disappeared during longer treatment periods. In addition to IAA accumulation, we found jasmonate levels strongly increased in *M. hyalina*-colonized roots, pointing out an inhibitory effect of jasmonates on downstream auxin signaling events. In contrast to observed auxin stimulatory effects during beneficial interactions, we demonstrated that the necrotrophic fungus *Alternaria brassicicola* and the hemi-biotroph *Verticillium dahliae* diminish fluorescence levels of auxin-responsive reporter within 3-6 h.

Overall, this thesis provides new insights into the complex interplay of signaling mechanisms involved in plant-herbivore and -microbe interactions, demonstrated in a model and a crop plant species.

6. Zusammenfassung

Im Verlauf der Evolution entwickelten Pflanzen ein breites Spektrum an Schutzmechanismen, um ihre physiologischen Reaktionen gezielt auf die sie umgebenden Reize und daraus resultierenden Bedürfnisse abzustimmen. Aufgrund der sich konstant verändernden Umweltfaktoren, wie beispielsweise die Präsenz von vorteilhaften und pathogenen Mikroben sowie Pflanzenfressern, gewann die Notwendigkeit von streng regulierten konstitutiven und induzierbaren Reaktionsmechanismen zunehmend an Bedeutung. Um eine effiziente Reizweiterleitung sowie adäquate Reaktion zu garantieren, bildeten sich zunehmend spezifischere Wahrnehmungs- und Signalübertragungswege in diversen Pflanzenspezies aus. Diese strikt umweltinduzierten Signalwege werden von einer Vielzahl an Botenmolekülen reguliert.

Calcium (Ca^{2+}) ist ein wichtiger sekundärer Botenstoff während früher Signalübertragungsprozesse in Pflanzen. Bei der Wahrnehmung von umweltbedingten Stressoren, z. B. Verwundung und Herbivorie, führt der rasche Anstieg des freien cytosolischen Calciumspiegels zu lokalen und systemischen Signalkaskaden sowie diversen Abwehrreaktionen. Eine Vielzahl von Kanälen und calciumbindenden Proteinen, die zu diesen frühen Signalereignissen beitragen, wurden bereits identifiziert. Die Wirkungsweise von insbesondere unkonventionellen Calciumkanälen wie den Annexinen ist jedoch weiterhin unbekannt. Annexin-Proteine gehören zu einer vielfältigen Familie von Multigenen und binden nachweislich anionische Phospholipide, was schlussendlich die Förderung der Ca^{2+}-Leitfähigkeit bewirkt. In dieser Studie untersuchten wir die Rolle von ANNEXIN1 während der mechanischen Verwundung und Herbivorie von *Arabidopsis thaliana.* Unser Ergebnisse zeigten eine erhebliche Beeinträchtigung des systemischen cytosolischen Calciumanstiegs in *ann1*-„Loss-of-Function“-Mutanten. Anschließende Bioassays zeigten, dass der generalistische Herbivor *Spodoptera littoralis* in Abwesenheit von ANNEXIN1 ein höheres Körpergewicht und damit eine bessere Performance aufzeigte mit einem gegenteiligen Effekt in ANN1-überexprimierenden Linien. Diese Beobachtungen zeigen die Bedeutung von ANNEXIN1 als positiver Abwehrregulator während der Pflanzenabwehr auf. Da insbesondere nachgeschaltete Phytohormone, z. B. Jasmonate, den Beginn von Ca^{2+}-ausgelösten Signalereignissen vermitteln, untersuchten wir in anschließenden Experimenten, ob *ann1*-Mutanten lokal und systemisch eine vergleichbare Induktion von abwehrbezogenen Reaktionen zeigen. In Verbindung mit der oben erwähnten reduzierten Calciumerhöhung in systemischen Blättern, konnten wir eine Expression von Abwehrgenen und die Jasmonatakkumulation in *ann1*-Pflanzen ausschließlich lokal verzeichnen.

Insgesamt belegen die Ergebnisse die Bedeutung von ANN1 für die Aktivierung systemischer Calcium-Signale, die letztlich die Resistenz gegen angreifende pflanzenfressende Insekten erhöhen.

Ein zentraler Aspekt der zellulären Signalkaskaden, die Pflanzenabwehrreaktionen auslösen, ist das Vorhandensein von schadensassoziierten molekularen Mustern (DAMPs). Wie bereits im Namen indiziert, werden DAMPs bei Gewebeschäden freigesetzt und liefern der geschädigten Pflanze intern Informationen über Verwundungen oder Angriffe von Pflanzenfressern. Neben Zellwandbestandteilen, Peptiden, eATP und einer Vielzahl anderer Verbindungen, wurden auch volatile organische Verbindungen (VOCs) als spezielle über die Luft übertragene DAMPs klassifiziert und stellen eine interessante Signalkomponente für die Kommunikation innerhalb und zwischen Pflanzen dar. In dieser Arbeit zeigen wir, dass Süßkartoffelpflanzen in der Lage sind, VOCs in einer sehr artspezifischen Weise freizusetzen, wahrzunehmen und darauf zu reagieren. Dies unterstreicht die Wichtigkeit der Pflanze-zu-Pflanze-Kommunikation innerhalb einer Population während der Abwehr von Pflanzenfressern. Wir identifizierten eine spezifische Mischung von VOCs, die von Pflanzenfressern freigesetzt werden, wobei das Homoterpen *(E)*-4,8-Dimethyl-1,3,7-Nonatrien (DMNT) die wichtigste Verbindung darstellte. Diese einzelne Verbindung induzierte systemisch den Trypsin-Inhibitor Sporamin, was zu einer erhöhten Resistenz gegen *Spodoptera*-Larven in benachbarten Pflanzen führte. Interessanterweise wurde dieser Effekt nur bei der Herbivoren-resistenteren Sorte Tainong 57 beobachtet, während eine zweite Sorte, Tainong 66, eine geringere DMNT-Emission und eine erhöhte Anfälligkeit für Insektenbefall aufwies. Da Jasmonate nur lokal während Herbivorie und mechanischer Verwundung hochreguliert wurden, gehen wir davon aus, dass die systemische Abwehraktivierung in der Süßkartoffel jasmonatunabhängig und ohne vorheriges Priming-Ereignis induzierbar ist.

Neben der DMNT-getragenen Signalübertragung lieferten frühere Ergebnisse in Süßkartoffelstudien bereits Hinweise darauf, dass durch Verwundung und Jasmonate ausgelöste hydroxyprolinreiche Glykopeptide (HypSys-Peptide) als zusätzlicher systemischer Botenstoff innerhalb einer Pflanze fungieren könnten. Untersuchungen des verteidigungsrelevanten Peptids *Ib*HypSys4 zeigten, dass es die *Sporamin*-Expression und die Freisetzung von DMNT aktivieren kann, was seine Bedeutung innerhalb des DMNT-Signalwegs unterstreicht. Obwohl bis dato kein Rezeptor für die *Ib*HypSys4-Wahrnehmung identifiziert wurde, bietet die Entdeckung der neuartigen Peptid-Rezeptor-Paarung *Ib*PepI-*Ib*LRR-RLK1 einen neuen Ansatzpunkt zur Untersuchung der Beteiligung von Peptiden an Stressreaktionen innerhalb

Süßkartoffeln. Wir vermuten, dass *Ib*HypSys4 und *Ib*PepI unterschiedliche biologische Funktionen innehaben; zur Bestätigung dieser Hypothese müssen jedoch weitreichendere Informationen gesammelt werden.

Abgesehen von der Signalübertragung bei der Interaktion zwischen Pflanzen und Herbivoren ist bekannt, dass eine Vielzahl von Pflanzenarten mit Mikroben –insbesondere Pilzen- assoziiert ist, die mit den Wurzeln interagieren können. Nach ihrer Kolonisierung sind diese Pilze in der Lage, den Phytohormonspiegel in den frühen Phasen der Infektion zu verändern. Das Phytohormon Auxin (Indol-3-Essigsäure, IAA) ist ein wichtiger Regulator des Wurzelwachstums in der Entwicklung und daher ein leicht angreifbares Ziel für die Manipulation durch mikrobielle Eindringlinge. Verfügbare Auxin-Reporter (DR5::EGFP-DR5v2::tdTomato), die in der Modellpflanze *A. thaliana* exprimiert werden, ermöglichen die Visualisierung von Auxin-Verteilungsmustern in pilzbesiedelten Arabidopsis-Wurzeln. Live-imaging Fluoreszenzmikroskopie-Techniken in Kombination mit LC-MS zeigten, dass die nützlichen Endophyten *Mortierella hyalina* und *Piriformospora indica* IAA in ihren Myzelien produzieren, während letzterer die Expression von Auxin-responsiven Reportergenen stimuliert. Die Co-Kultivierung von 24 Stunden führte bereits zu signifikant erhöhten Auxinspiegeln und förderte die Bildung von Seitenwurzelprimordien - allerdings nur während der Behandlung mit *P. indica*. Diese Reaktionen waren streng zeitabhängig, da die Induktion von Auxinmaxima bei längerer Behandlungsdauer nicht mehr sichtbar war. Zusätzlich zur IAA-Akkumulation stellten wir fest, dass der Jasmonatspiegel in den von *M. hyalina* besiedelten Wurzeln stark anstieg, was auf eine hemmende Wirkung von Jasmonaten auf nachgeschaltete Auxin-Signale hinweist. Im Gegensatz zu den beobachteten Auxin-stimulierenden Effekten während gutartiger Interaktionen konnten wir zeigen, dass der nekrotrophe Pilz *Alternaria brassicicola* und der hemi-biotrophe Pilz *Verticillium dahliae* die Fluoreszenzwerte des auf Auxin reagierenden Reporters innerhalb von 3-6 Stunden vermindern.

Insgesamt bietet diese Arbeit - , demonstriert an einer Modell- sowie einer Nutzpflanze - neue Einblicke in das komplexe Zusammenspiel von Signalmechanismen, die an der Interaktion zwischen Herbivoren und Mikroben beteiligt sind.

7. References

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8. Übersicht Eigenanteile

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Kurzreferenz: Meents et al (2020), Russ. J. Plant Physiol.

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Abbildung(en) # 1	☐	100 % (die in dieser Abbildung wiedergegebenen Daten entstammen vollständig experimentellen Arbeiten, die der Kandidat/die Kandidatin durchgeführt hat)
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9. Eigenständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Fakultät für Biowissenschaften der Friedrich-Schiller-Universität Jena erkläre ich, dass ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die bei der Durchführung der Experimente, Auswertung sowie der Fertigstellung der Manuskripte mitgewirkt haben sind vor den jeweiligen Publikationen sowie im Anhang aufgeführt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht. Weiterhin wurde keine ähnliche oder andere Abhandlung als Dissertation anderswo eingereicht.

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Anja Katharina Meents

10. Curriculum vitae

Anja Katharina Meents

Personal information

Address	Hafenstraße 3c, 04179 Leipzig
Phone	+49 163 4893930
E-Mail	ameents@ice.mpg.de; anjameents91@gmail.com
Date of birth	27.06.1991, Gießen, Germany

Academic career

Since 04/2021 Sales Operations Associate at Azenta Life Sciences

01/2017-04/2021 PhD student in the Research Group Plant Defense Physiology at the Max Planck Institute for Chemical Ecology in Jena (in cooperation with the Friedrich Schiller University, Jena)

Topic: Induced intra- and interplant signaling mechanisms upon biotic interaction in *Ipomoea batatas* and *Arabidopsis thaliana*

08/2016 Master of Science (M.Sc.) degree in Biological Sciences, University of Konstanz (in cooperation with the National Taiwan University, Taipei, Taiwan, and the Max Planck Institute for Chemical Ecology, Jena)

Topic: Analysis of defense-related reactions upon herbivore attack in *Ipomoea batatas*

09/2013 Bachelor of Science (B.Sc.) degree in Biological Sciences, University of Konstanz (conducted at the Animal Research Facility at the University of Konstanz, Konstanz)

Topic: Auswirkungen einer Minerallösung auf die frühe Entwicklung von "*danio rerio*"(Zebrafisch)

Publications

2021 Malabarba, J., **Meents, A**., Reichelt, M., Scholz, S., Peiter, E., Rachowka, J., Konopka-Postupolska, D., Wilkins, K. A., Davies, J. M., Oelmüller, R., Mithöfer, A. (in press). ANNEXIN1 mediates calcium-dependent systemic defense in Arabidopsis plants upon herbivory and wounding. New Phytologist., doi:10.1111/nph.17277.

2020 **Meents, A.**, Mithöfer, A. Plant-plant communication: Is there a role for volatile damage- associated molecular patterns? Frontiers in Plant Science, 11: 583275.

doi:10.3389/fpls.2020.583275.

Ritter, M., Oetama, V. S., Schulze, D., Muetzlaff, K., **Meents, A.,** Seidel, R. A., Görls, H., Westerhausen, M., Boland, W., Pohnert, G. Pyrrolic and dipyrrolic chlorophyll degradation products in plants and herbivores. Chemistry – A European Journal, 26(28), 6205-6213. doi:10.1002/chem.201905236.

2019 **Meents, A.**, Chen, S.-P., Reichelt, M., Lu, H.-H., Bartram, S., Yeh, K.-W., Mithöfer, A. Volatile DMNT systemically induces jasmonate-independent direct anti-herbivore defense in leaves of sweet potato (*Ipomoea batatas*) plants. Scientific Reports, 9: 17431. doi:10.1038/s41598-019-53946-0.

Meents, A., Furch, A. C., Almeida-Trapp, M., Özyürek, S., Scholz, S., Kirbis, A., Lenser, T., Theißen, G., Grabe, V., Hansson, B. S., Mithöfer, A., Oelmüller, R. Beneficial and pathogenic Arabidopsis root-interacting fungi differently affect auxin levels and responsive genes during early infection. Frontiers in Microbiology, 10: 380. doi:10.3389/fmicb.2019.00380.

Meents, A., Özyürek, S., Oelmüller, R., Furch, A. C. U. Fungal-induced formation of auxin maxima in *Arabidopsis thaliana* roots. Russian Journal of Plant Physiology, 66(6), 872-883. doi:10.1134/S102144371907001X.

Awards

2019 IMPRS travel award for the best talk at the 18th IMPRS Symposium, Dornburg, Germany

2018 IMPRS travel award for the best poster at the 17th IMPRS Symposium, Dornburg, Germany

2017 IMPRS travel award for the best poster at the 16th IMPRS Symposium, Dornburg, Germany

Conference presentations

2019 "From sweet potato to tomato - peptide signaling in *Ipomoea batatas*". Talk presented at Institute Symposium, Max Planck Institute for Chemical Ecology, Jena, Germany

"Plants vs. fungi? – How fungal infection alters auxin levels in Arabidopsis roots". Talk presented at 18th IMPRS Symposium, International Max Planck Research School, Dornburg, Germany

"A single volatile induces systemic herbivore resistance in

leaves of sweet potato (*Ipomoea batatas*)". Poster presented at the 35th ISCE Meeting, International Society of Chemical Ecology, ISCE, Atlanta, USA

"Good vs Evil: Monitoring fungal-induced auxin maxima in Arabidopsis roots". Talk presented at 17th Mitteldeutsche Pflanzenphysiologie Tagung, Leipzig, Germany

"Plants vs Fungi: Monitoring jasmonate – auxin crosstalk in Arabidopsis roots upon fungal infection". Poster presented at the Regulatory Oxylipins Meeting, Gent, Belgium

2018 "Good vs Evil: Monitoring fungal-induced auxin in Arabidopsis roots". Poster presented at Institute Symposium, Max Planck Institute for Chemical Ecology, Jena, Germany

"Could you be the One? – Identification of calcium signaling mutants". Poster presented at 17th IMPRS Symposium, International Max Planck Research School, Dornburg, Germany

"Identification of Arabidopsis mutants using calcium-based screening approaches". Talk presented at 16th Mitteldeutsche Pflanzenphysiologie-Tagung, Dresden, Germany

2017 "DMNT-induced upregulation of defensive sporamin in sweet potato". Poster presented at 16th IMPRS Symposium, International Max Planck Research School, Dornburg, Germany

"DMNT-induced upregulation of defensive sporamin in sweet potato". Poster presented at 30th Tagung Molekularbiologie der Pflanzen, Dabringhausen, Germany

2016 "DMNT-induced upregulation of defensive Sporamin in sweet potato". Poster presented at SAB Meeting, Max Planck Institute for Chemical Ecology, Jena, Germany

Work experience abroad

05/2016 – 06/2016
11/2015 – 12/2015 National Taiwan University (NTU), Institute of Plant Biology, Taipei, Taiwan

Committee work

2017-2020 PhD representative of the Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Jena, Germany

Advanced training and workshops

09/2020	Project management, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany
08/2020	BWL kompakt, Graduate Academy Friedrich Schiller University, Jena, Germany
05/2020	MINI-LECTURE SERIES on Natural Products Biochemistry, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany
05/2020	How to find your job on the non-academic labour market, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany
04/2020	Die schriftliche Bewerbung für den außeruniversitären Arbeitsmarkt, Graduate Academy Friedrich Schiller University, Jena, Germany
11/2019	Excursion to Wacker Biotech, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany
11/2019	Plant Transformation Workshop, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany
02/2019	Bioinformatics tools for mass spectrometry, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany
02/2019	Excursion to Jena BioScience, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany
12/2018	Data visualization Workshop, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany
11/2018	Guided Tour Bayer Crop Science, IMPRS Max Planck Institute for Chemical Ecology, Jen, Germany a
09/2018	Digital Tools in Science Project Management, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany
09/2018	Guided tour Zeiss Jena, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany
06/2018	Guided tour Analytik Jena, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany
04-05/2018	Introduction in basic statistic and R, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany
02/2018	The basics of light and fluorescence microscopy, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany
11/2017	Introductory R Course, IMPRS Max Planck Institute for Chemical

Ecology, Jena, Germany

11/2017 Leadership Skills, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany

09/2017 Speed Informing with MPI-CE Alumni, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany

06/2017 Fundamentals of Mass Spectrometry, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany

06/2017 How to Plan your Science Career in Germany and Abroad - Career Planning for Scientists, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany

04/2017 Adobe Illustrator, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany

03/2017 Academic Writing: How to Create Good Text, IMPRS Max Planck Institute for Chemical Ecology, Jena, Germany

Teaching and PR activities

10/2020-04/2021 Supervision Master thesis "Defensive role of peptide signals in the leaves of *Ipomoea batatas*", Anitta Joseph, Friedrich Schiller University, Jena, Germany

03/2021 Presented "Chemical Ecology of Plant Insect Interactions" at the UFSCAr Summer School on Chemical Ecology, Sao Carlos, Brazil, (online)

02/2020 "Light sheet fluorescence microscopy": Preparation, mounting, and imaging of transgenic Arabidopsis seedlings, Carl Zeiss Microscopy Jena, Jena, Germany

01/2020 Featured in Zeiss User Story: "Observing Root Interacting Fungi with in vivo Plant Microscopy", Carl Zeiss Microscopy Jena, Jena, Germany

2020 Supervision Master thesis "Influence of beneficial fungi on NRT2.4 expression in *Arabidopsis thaliana* grown under stress condition", Anindya Majumder, Friedrich Schiller University, Jena, Germany

12/2019 Presented at "Application specialists training in Light sheet fluorescence microscopy": "Plants vs Fungi? - How fungal infection alters auxin levels in *Arabidopsis thaliana*", Carl Zeiss Microscopy Jena, Jena, Germany

12/2019 Attendance, demonstration, and training of Application Specialists in root sample preparation during "Application

specialists training for Light sheet fluorescence microscopy" at Carl Zeiss Microscopy Jena, Jena, Germany

09/2019 Supervised at IMPRS Recruitment 2019: candidate parenting, Max Planck Institute for Chemical Ecology, Jena, Germany

06/2019 Presented „Lichtblatt-Mikroskopie zur Visualisierung von Interaktionen zwischen Pflanzenwurzeln und Pilzen“ at visit of „weiterführende Schule, Institutsbesuch im Rahmen der 14. Jenaer Sommerschule zum Thema Biophotonik“, Max Planck Institute for Chemical Ecology, Jena, Germany

08/2018 Supervised at IMPRS Recruitment 2018: candidate parenting, Max Planck Institute for Chemical Ecology, Jena, Germany

2018 Supervision Master thesis “Analysis of fungal-induced formation of auxin maxima in *Arabidopsis thaliana* roots”, Sedef Özyürek, Friedrich Schiller University, Jena, Germany

11/2017 Presentation “Fleischfressende Pflanzen - Fakten und Mythen“ at 6th Long Night of Science, Max Planck Institute for Chemical Ecology, Jena, Germany

11/2017 Presentation “Back to the roots - Systemische Calcium Signale in *Arabidopsis thaliana*”, board of trustees Max Planck Society, Max Planck Institute for Chemical Ecology, Jena, Germany

06/2017 Assistant for guided tours and experiments for children during the 20th anniversary of the MPI-CE, Max Planck Institute for Chemical Ecology, Jena, Germany

06/2017 Assistant of guest program during the „Max-Planck-Hauptversammlung in Weimar”, Max Planck Institute for Chemical Ecology, Jena, Germany

Skills

Languages	German	■■■■■
	English	■■■■□
	Spanish	■■■□□
	French	■■■□□
IT	MS-Office	■■■■□
	R, Adobe Illustrator, Origin	■■■□□
	Endnote, EDUKON DB	■■■■□
Others	Laboratory Animal Sciences (FELASA, Category B)	
	driver’s license class B	

11. Acknowledgement

Last but not least, I would like to dedicate the following pages to all the people who helped in the making of this thesis and supported me throughout the last four years; making it such an amazing experience.

First of all I would like to thank my supervisor PD Dr. Axel Mithöfer for giving me the opportunity to work in his group at the Max Planck Institute and ultimately reviewing this thesis. Although there was no space for a practical student back in the days, you took me in anyway and let me become a "fully grown" PhD. You always gave me all the freedom I needed to develop my own project ideas, experiments, and thoughts, while always being there when help and advice were much needed. During that time I learned a lot about science, people, different cultures, and myself as well. Thank you for your support, motivation, patience, and all the fun over the last years! I will definitely miss the BBQs, group lunches, wine retreats,... (and the list goes on and on!). You were the best mentor I could wish for.

I would also like to thank my second supervisor Prof. Ralf Oelmüller. It was a great opportunity to become a part of your lab, join you on your travels to Gent and Madrid, dive deeper into the university structures and of course – into the world of calcium, colorful fungi, and auxins. I always enjoyed our discussions and was extremely grateful for all of your ideas and input throughout the projects. Being (hopefully) finally able to answer your daily question "Working hard or hardly working?" - I guess I can finally say that I worked hard enough ;-) .

I would also like to express my gratitude to Prof. Stephan Pollmann and Prof. Nicole van Dam for sacrificing their time reviewing and evaluating this thesis. Thank you so much for your effort.

Many heartfelt thanks also to Dr. Claudia Voelckel, Prof. Jonathan Gershenzon, Angela Kiesewetter, Alexandra Meier, and the IMPRS as a whole for your constant moral as well as financial support throughout my PhD. Right from the beginning you made me feel welcome and being a part of the team – enabling me to fully identify myself with the graduate school and the program itself (Still do)!

Additionally I want to thank Prof. Wilhelm Boland for hosting me in his department and Prof. David Heckel for the financial support.

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I would like to thank Prof. Kai Wun Yeh for his inspiration and perseverance during the sweet potato project. Thank you for all of the fruitful discussions here in Jena and in Taipei and of course for your outstanding hospitality. On that note I also want to send a big hug and thanks to the other people involved in this project: Dr. Shi Peng Chen & Dr. Yun Wei Kuo as well as Hsueh Han Lu. Finally I want to thank the current and former lab members being part of this amazing experience: Cherry, Jao Zhong & the little noodle(s), Xiao Ying, Chao Wei, and Andy. Thank you for your friendship over the years.

Many, many thanks also to Prof. Edgar Peiter for his guidance, help, and his ability to put things into the right perspective during the annexin project. Thank you for hosting me and letting me learn many new things while spending time in your lab. I also want to thank the whole lab but especially Tina Peiter-Volk, Bastian, and Nico for all the help and the great atmosphere and entertainment (in- and outside) the dark basement.

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On that note, I want to express my deepest gratitude to my mentor, friend, and motivator Alex (Frau Fuuuuurch!!). Thank you for adopting, teaching, supporting, and pushing me throughout all these times and for overall saving me ;-). I couldn’t have done it without you and wouldn’t miss out on our fun times ;-) Ei Gude wie!!!!

Ultimately I want to thank my parents and my whole family as well as my parents in law for their endless support, motivation, prayers, and love.

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