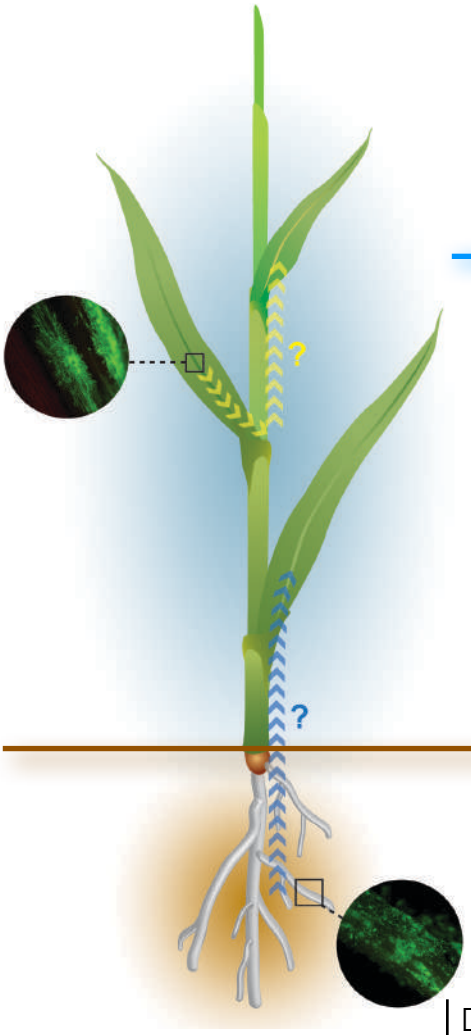

Above- and belowground antifungal resistance in maize:

aspects of organ-specific defense

Dissertation submitted to the University of Neuchâtel
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Summary

The hemibiotrophic fungus *Colletotrichum graminicola* causes devastating anthracnose on maize (*Zea mays*) and is responsible for annual losses of up to 1 billion dollars in the U.S. A key factor for its success is the capability to infect different plant organs. The predominant symptoms are leaf blight and stalk rot, but *C. graminicola* also infects roots. The vast majority of phytopathological studies were conducted on aerial disease stages, and only little is known about belowground defense responses. Moreover, most studies on antifungal resistance focus on either above- or belowground immune systems. Thus, this thesis investigated the local and systemic organ-specific interactions of maize and *C. graminicola*.

Firstly, a soil-free plant growth system was developed, allowing non-destructive *in vivo* observations of *C. graminicola* infection strategies on maize roots. This system consists of pouches containing nutrient-soaked filter paper which supplies the plants with nutrients adapted to the host.

Secondly, local and systemic molecular and chemical changes upon *C. graminicola* attack on maize leaves and roots were investigated. Distinct gene expression patterns in leaves and roots were found, in agreement with different dynamics of phytohormone induction. In roots defense-related genes were induced faster than in leaves, and roots also exhibited higher hormone levels upon infection. Local leaf and root infections triggered leaf-leaf and root-leaf systemic transcriptional and hormonal adaptations, including the induction of defense-related genes and hormones. Interestingly, local leaf and root infection also resulted in a systemic resistance against *C. graminicola* in distal leaves. Performing metabolomic fingerprinting, several local and systemic organ-specific compounds were identified, which could serve as chemical arsenal during antifungal immunity in maize.

Thirdly, the organ-specific microRNA (miRNA) transcriptome of maize during *C. graminicola* infection was examined. Several miRNAs were identified which are specifically induced or downregulated in fungal infected shoots or roots, but not upon challenge with the herbivore *Spodoptera frugiperda*. Some of those miRNAs target defense-related genes, thus miRNAs might play an important role in organ-specific antifungal defense.

In conclusion, exploiting organ-specific plant defense might be a prominent target for future crop enhancing programs.

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Chapter I
General introduction

IA. Thesis outline

Colletotrichum graminicola, the causal agent of maize anthracnose, is a highly adaptive fungus that is capable of infecting different plant organs, including shoots and roots. Generally, belowground antifungal defenses are barely investigated, mainly due to the limited accessibility of the root system. Moreover, crop model systems are less studied in regard to inducible systemic defenses. Hence, this thesis aims to shed light onto above- and belowground antifungal resistance in maize, one of the economically most important crop models. In anticipation of the current state of the art, this thesis focuses on three main scientific questions: 1) Which soil-free plant growth system enables the analysis of plant-pathogen interactions at the root level? 2) Do maize plants employ organ-specific defense responses against *C. graminicola*? And 3) what is the nature of systemic defense responses upon local *C. graminicola* leaf and root infection?

In **chapter I**, the prevailing knowledge of inducible resistance in crops, the role of phytohormones in plant defense and the contribution of micro RNAs in plant defense are introduced and reviewed. In **chapter II**, a soil-free plant growth system is presented, which facilitates the study of maize roots in interaction with both pathogenic and mutualistic microbes. In **chapter III**, organ-specific local and systemic defense responses of maize against *C. graminicola* are elucidated, and in **chapter IV**, changes of the maize small RNA transcriptome upon *C. graminicola* attack are described. In conclusion, this thesis aims to provide insights into a „*defense in depth*“, where the immune system of the entire maize plant, including different organs as well as local and systemic tissues, are examined at the molecular, chemical and physiological level.

Chapter I - General Introduction

IB: On the move: Induced resistance in monocots

adapted from:

Dirk Balmer*, Chantal Planchamp* and Brigitte Mauch-Mani. (2012) On the move: induced resistance in monocots. *Journal of Experimental Botany*, doi: 10.1093/jxb/ers248 (*contributed equally).

IB: On the move: Induced resistance in monocots

Abstract

Although plants possess an arsenal of constitutive defences such as structural barriers and preformed antimicrobial defences, many attackers are able to overcome the pre-existing defence layers. In response, a range of inducible plant defences is set up to battle these pathogens. These mechanisms, commonly integrated as induced resistance (IR), control pathogens and pests by the activation of specific defence pathways. IR mechanisms have been extensively studied in the Dicotyledoneae, whereas knowledge of IR in monocotyledonous plants, including the globally important graminaceous crop plants, is elusive. Considering the potential of IR for sustainable agriculture and the recent advances in monocot genomics and biotechnology, IR in monocots is an emerging research field. In the following, current facts and trends concerning basal immunity, and systemic acquired/induced systemic resistance in the defence of monocots against pathogens and herbivores will be summarized.

Introduction

Plants are continuously confronted with an armada of different pathogens and pests. These potential attackers utilize diverse tactics to clash with the plant defensive system. Bacteria can invade plants through natural openings such as stomata or wounds, pathogenic fungi can violently break cell walls to enter the host cell (Fig. 1), and insect herbivores employ enzymes to attenuate plant toxins. Moreover, pathogens are able to manipulate plant immunity by delivering effector molecules that are hijacking the defence pathways. Nonetheless, only a few pathogens successfully infect a specific plant species, although plants, unlike animals, do not possess specialized and mobile defender cells. Thus, the self- protection plants have developed throughout the evolutionary arms race with their attackers has to be highly intricate and efficient to help in surviving the diverse biological stress situations.

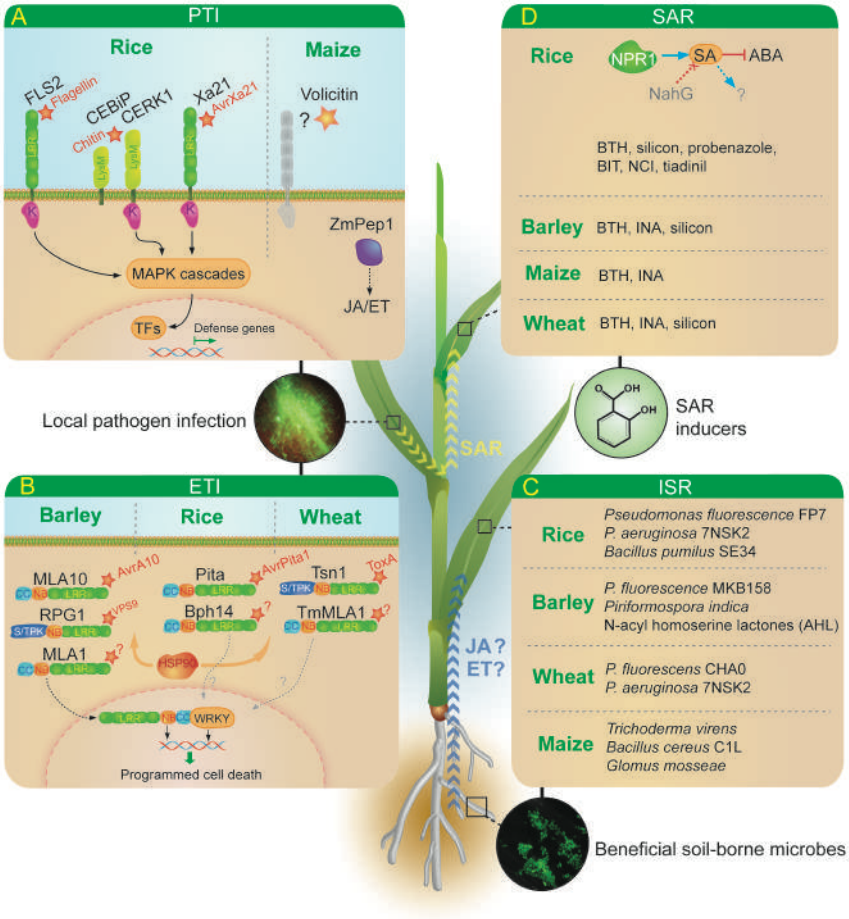

Figure 1. Snapshot of IR mechanisms in monocots **(**A) Molecular mechanisms of pattern-triggered immunity (PTI). The bacterial MAMP (microbe-associated molecular patterns) flagellin is recognized by FSL2, a PRR consisting of an extracellular LRR and cytoplasmic kinase (K) domain. The MAMP chitin is sensed by the LysM PRRs CEBiP and CERK1. MAMP-signaling activates MAPK cascades, which regulate transcription factors (TFs) driving the expression of defence genes. HAMPs (herbivore-associated molecular patterns) and damage-associated molecular patterns (DAMPs) are also triggering PTI. In maize, the HAMP volicitin is recognized by an unknown receptor, and the DAMP ZmPep1 functions as endogenous signal regulating jasmonic acid (JA)- and ethylene (ET)-dependent pathways during pathogen attack. (B) Effector-triggered immunity (ETI) mediated by NBS-LRR (nucleotide-binding leucine rich repeat) proteins. Pathogens employ effectors (represented by stars) to suppress PTI. Such effectors are contained by NBS-LRR proteins. Monocot NBS-LRR proteins usually have coiled-coil (CC) or serine/threonine protein kinase (S/TPK) domains and are localized in both the cytoplasm and nucleus. NBS-LRR proteins are folded into an active form by the heat shock protein 90 (HSP90). They interact directly with effectors and also regulate WRKY transcription factors. (C) Induced systemic resistance (ISR) following root infection by beneficial soil-borne microbes: examples of organisms triggering ISR in monocots. (D) Systemic acquired resistance (SAR). Mobile signals travel from attacked tissues to distant organs where systemic resistance responses are induced. In rice, the SAR-key player NPR1 down-regulates genes. SA suppresses the abscisic acid (ABA) pathway. Expressing the SA-degrading enzyme NahG in rice reduces pathogen resistance. SAR can also be triggered in monocots by application of SAR inducers such as BTH (S-methyl benzo-1,2,3-thiadiazole-7-carbothioate), INA (2,6-dichloroisonicotinic acid), BIT (1,2-benzisotziazole-1,1-dioxide) or NCI (N-cyanomethyl-2-chloroisonicotinamide). Image of the rhizobacteria *P. fluorescens* CHA0: courtesy of P. Kupferschmied and C. Keel, University of Lausanne.

In order to defend themselves, plants are armed with constitutive, pre-existing defences such as cell wall barriers or pre-formed and stored antimicrobial toxins. In such cases where attackers are able to overcome the constitutive defence layers, they face an arsenal of inducible defences (Fig. 1; Pieterse *et al*., 2009; Spoel and Dong, 2012). During an initial phase, plant cells exert a so-called 'innate immunity'. In a first branch of this immunity, pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) such as chitin or flagellin are recognized by membrane-localized pattern-recognition receptors (PRRs) (Zipfel, 2009). The perception of MAMPs by PRRs leads to the activation of multiple downstream defence signalling events. The second branch of the plant innate immune system acts mostly in the cytoplasm; NB-LRR (nucleotide-binding leucine rich repeat) proteins, which are encoded by plant resistance (*R*) genes, recognize pathogen-derived avirulence (Avr) proteins. These effector proteins help pathogens to overcome PAMP- or pattern-triggered immunity (PTI; Jones and Dangl, 2006). The recognition and attenuation of Avr proteins by plant R-proteins results in effector-triggered immunity (ETI), which is usually manifest in a hypersensitive response (HR; Greenberg and Yao, 2004).

PTI and ETI alleviate pathogen and pest attacks by inducing downstream responses that can result in a local and systemic induced resistance. Locally, these inducible defences consist of cell wall reinforcements through callose apposition and lignification, the production of secondary antimicrobial compounds, and the accumulation of pathogenesis-related (PR) proteins. Moreover, the attacked tissue is able to generate long-distance mobile alarm signals that are inducing systemic resistance in non-colonized organs (Shah, 2009). The systemic expression of defence in distal tissues can be observed upon infection with pathogens and is referred to as systemic acquired resistance (SAR). Resistance expressed following root colonization by non-pathogenic soil microbes is known as induced systemic resistance (ISR). SAR is predominantly effective against biotrophic pathogens (Vlot *et al*., 2008), whereas ISR is mainly counteracting necrotrophic pathogens and pests (Van Loon, 2007). Commonly, the inducible defence networks are regulated pivotally by phytohormones, which serve as specific chemical signals induced in response to particular attackers (Balmer and Mauch-Mani, 2012).

The vast majority of knowledge has been gathered from dicots such as cucumber, tobacco, and Arabidopsis. The knowledge about monocots remains elusive (Kogel and Langen, 2005). Monocots are a large group of about 59 300 species, amongst them the largest family is represented by orchids (*Orchidaceae*), followed by *Poaceae*, which include economically important plants such as rice, wheat, maize, sugarcane, and bamboo. Originating from a common angiosperm ancestor and going through an intimate co-evolution with plant pathogens, monocots and dicots are assumed to share most of the immune pathways. Here, we present the current knowledge of local and systemic IR mechanisms in monocots.

Pattern-triggered immunity (PTI): a stealth mission for pathogens?

Pathogens cannot sneak in: upon contact with invaders, plant cells use the first branch of their innate immune system by perceiving conserved microbial structures and peptides with the help of plasma membrane-localized PRRs (Fig. 1; Zipfel, 2009; Tsuda and Katagiri, 2010). In Arabidopsis, the best case study of this immune reaction is represented by the receptor-like kinase flagellin insensitive 2 (FLS2), which recognizes amino acids derived from bacterial flagellin. FLS2 interacts with BAK1, the brassinosteroid receptor BRI1-associated receptor kinase 1, to activate downstream defence responses (Chinchilla *et al*., 2007). Amongst monocots, various PRRs have been identified over the past few years (Table 1), notably in the model monocot rice (*Oryza sativa*; Chen and Ronald, 2011). FSL2 homologues are found in all higher plants, and the rice homologue OsFLS2 has been demonstrated to act as a functional flagellin receptor (Takai *et al*., 2008). Moreover, a variety of different MAMPs have been shown to be active in rice, including bacterial lipopolysaccharides (LPS; Desaki *et al*., 2006) and

Plant species	Protein name	Molecular pattern	Pathogen	Reference
Rice	CEBiP	Chitin	*Magnaporthe grisea*	Shimizu *et al*., 2010
	OsFLS2	Flagellin	*Pseudomonas avenae* *Acidovorax avenae*	Takai *et al*., 2008
	Pi-d2	Unknown	*M. grisea*	Chen *et al*., 2006
	XA21	Sulphated Ax21	*Xanthomonas* spp.	Lee *et al*., 2009
Barley	HvCEBiP	Chitin	*M. oryzae*	Tanaka *et al*., 2010
Wheat	WKS1 (Yr36)	Unknown	*Puccinia striiformis*	Fu *et al*., 2009
Maize	Unknown	ZmPep1	(Endogenous elicitor)	Huffaker *et al*., 2011

Table 1. Selected monocot sensors recognizing conserved molecular patterns.

chitin (Kishimoto *et al*., 2010). In rice, chitin is perceived by the plasma membrane glycoprotein CEBiP, which forms a dimer with the chitin elicitor receptor kinase 1 (CERK1, also known as Lys-M-RLK1; Shimizu *et al*., 2010). As for Arabidopsis, chitin reception in rice then triggers the generation of reactive oxygen species (ROS) and the expression of PR genes. The best-studied example of PTI in monocots is the *Xa21*-mediated disease resistance in rice. *Xa21* encodes a receptor exhibiting an extracellular LRR domain, as well as an intracellular non-RD (non-arginine–aspartate) domain. XA21 perceives the 194-amino acid bacterial protein Ax21, which is conserved in all known Xanthomonas strains (Lee *et al*., 2009). As for OsFLS2, XA21 induces downstream defence mechanisms by activating MAPK cascades, thereby actuating transcription factors, triggering the expression of PR genes and the development of HR (Tena *et al*., 2011). Xa21 homologues have been found in *Brachypodium*, sorghum, and maize (Tan et al., 2012). Several other non-RD receptor kinases have been identified in monocots. In rice, the B-lectin receptor kinase Pi-d2 confers resistance against *Magnaporthe grisea* (Chen *et al*., 2006). In addition to PAMPs and MAMPs, so-called damage-associated molecular patterns (DAMPs) are also recognized during pathogen attack. Known DAMPs are polysaccharides released from plant cell walls, or endogenous peptides such as the 23-amino acid peptide AtPep1 in Arabidopsis. Recently, the maize ZmPep1 peptide has been identified as an orthologue of AtPep1 (Huffaker *et al*., 2011), suggesting a similar role of DAMPs in monocots and dicots. In conclusion, PTI mechanisms are highly conserved in both monocots and dicots, although some PRRs such as EFR, the Arabidopsis receptor of bacterial EF-TU (elongation factor unstable), are not found in monocots (Boller and He, 2009). Nevertheless, the fact that rice encodes a higher variety of non-RD domain receptor kinases than Arabidopsis (Dardick and Ronald, 2006) indicates that, although PTI signalling is conserved in all angiosperms, both monocots and dicots underwent particular evolutionary adaptations.

Effector-triggered immunity (ETI): Special Forces striking back

Once detected by plant cells and facing PTI-triggered defences, successful pathogens are able to perturb the first inducible defence lines (Jones and Dangl, 2006). Bacteria, fungi, and

oomycetes are delivering effectors behind enemy lines to suppress PTI. There, these effectors manipulate host cellular mechanisms to favour subsequent invasion steps. Examples of such effectors are AvrPtoB and AvrPto, effectors from *Pseudomonas syringae* strains targeting the kinase domains of EFR, FLS2, and BAK1 (Boller and He, 2009). In contrast to bacterial effectors, eukaryotic pathogen effectors are less well studied. The oomycete *Hyaloperonospora arabidopsidis* produces ATR1 and ATR13 effectors (Sohn *et al*., 2007), and the fungus *Blumeria graminis* f.sp. *hordei* delivers AVRK and AVRA10 proteins into barley cells (Ellis *et al*., 2007). Pathogen effectors are able to render a plant susceptible, thus being a serious threat for plant survival. However, plants are promptly counterstriking by sending in recon troops that recognize effectors, thus triggering ETI (Fig. 1; Table 2). These recon troops are mostly NB-LRR proteins encoded by resistance (R) genes (Elmore *et al*., 2011). NB-LRR proteins usually exhibit an N-terminal TIR (Toll/Interleukin-1 Receptor) domain or coiled-coil (CC) motif. Activation of NB-LRRs induces local and systemic defence signalling involving hormonal networks, ROS-generation, and gene expression adaptations by WRKY and TGA transcription factors (Jones and Dangl, 2006). According to the guard hypothesis, some *R* genes can directly recognize pathogen molecules (effectors), while other *R* genes indirectly recognize metabolic perturbations due to the presence of the pathogen (Jones and Dangl 2006). In cereals, the prevailing situation seems to consist of direct surveillance as, in most

Plant Species	Protein name	Effector	Pathogen	Reference
Rice	Bph14	Unknown	Brown planthopper	Du *et al*., 2009
	Os11N3	AvrXA7	*Xanthomonas* spp.	Antony *et al*., 2010
	Pita	AvrPita1	*Magnaporthe grisea*	Jia *et al*., 2000
	Piz-t	AvrPiz-t	*M. grisea*	Li *et al*., 2009
	XA27	AvrXA27	*Xanthomonas* spp.	Gu *et al*. 2005
Barley	RDG2A	Unknown	*Pyrenophora graminea*	Bulgarelli *et al*., 2010
	RPG1	Urediniospore effectors (protein with a fibronectin type III susceptibility domain; vacuolar protein sorting associated protein 9)	*Puccinia graminis*	Brueggeman *et al*., 2002
Wheat	TmMla1	Unknown	*Blumeris graminis* f.sp. *hordei*	Jordan *et al*., 2011
	Tsn1	ToxA	*Stagonospora nodorum*	Faris et al. 2010
Sorghum	Cs1A & Cs2A	Unknown	*Colletotrichum sublineolum*	Biruma *et al*., 2012

Table 2. Selected monocot proteins recognizing pathogen effectors.

cases, a direct interaction between the resistance gene and the corresponding effector is the rule (Table 2).

NB-LRR encoding genes represent one of the largest and widely conserved gene families in plants, with over one-hundred family members for the majority of sequenced plants (Jones and Dangl, 2006), including monocots and dicots. Despite the extensive knowledge of NB-LRRs in monocots, their elucidation has been mainly limited to rice and, more recently, to wheat and sorghum. Compared with dicots, monocot genomes encode higher numbers of CC-NB-LRRs (Martin *et al*., 2011). Intriguingly, genes coding for TIR-NB-LRRs homologues are rare in monocots (Kim *et al*., 2012). The majority of described rice NB-LRRs is promoting resistance to *M. grisea*, such as Pita, Pib, Piz-t, Pikm, and Pit (reviewed in Chen and Ronald, 2011). Bph14 confers resistance to the brown planthopper (Du *et al*., 2009), and XA1 mediates resistance against *Xanthomonas oryzae* (Yoshimura *et al*., 1998). Despite the large number of rice NB-LRRs, most of their target effectors are unknown. Only four *M. grisea* effectors are described, AvrPiz-t (Shang *et al*., 2009), AvrPita (Jia *et al*., 2000), AvrPia and AvrPik/km/kp (Qu *et al*., 2006). AvrPita is recognized by the rice NBS-LRR protein Pita; direct binding of Pita to AvrPita induces cell death that retards the spread of M. grisea on rice (Jia *et al*., 2000). Other R-genes conferring resistance to *Xanthomonas oryzae* pv. *oryzae* in rice do not exhibit NBS or LRR domains, such as xa13 and Os11N3 (Antony *et al*., 2010). Xa13, a recessive allele belonging to the NODULIN3 (N3) gene family, triggers immunity by recognizing the *Xanthomonas* effectors AvrXA7. In turn, the type III effector AvrXa7 drives the expression of the rice susceptibility gene OS-8N3, which defeats Xa13 and induces effector-triggered susceptibility (ETS; Antony *et al*., 2010). The extensive synteny between the genomes of several major cereal species and the high colinearity between large portions of these genomes facilitates synteny-based positional cloning. The availability of detailed rice (International Rice Genome Sequencing Project, 2005) and, recently, barley genomic data as well (Mayer *et al*., 2011) will allow the identification of genes playing a crucial role in IR in major cereal species and, hopefully, lay the basis for genomics-based breeding strategies for defence in these plants.

In other monocot species, NB-LRRs are less explored. Nonetheless, in the genomes of *Brachypodium distachyon*, *Sorghum bicolor*, and *Zea mays*, conserved NB-LRR-encoding genes were identified (Kim *et al*., 2012). In sorghum, a CC-NB-LRR encoding gene cluster that confers resistance to *Setosphaeria turcica* has recently been discovered (Martin *et al*., 2011). The corresponding resistance gene has been found to be conserved in maize, rice, foxtail millet, and in *B. distachyon*. In addition, the NB-LRR encoding R genes *Cs1A* and *Cs2A* were shown to mediate the resistance of sorghum against *Colletotrichum sublineolum* (Biruma *et al*., 2012). In wheat, the recently identified CC-NB-LRR protein TmMla1 functions in resistance against *Blumeria graminis* f.sp. *hordei* (Jordan *et al*., 2011). Wild wheat (*Triticum turgidum* L. ssp. *dicoccoides*) possesses the *Yr36* gene, which encodes a kinase and putative START lipid-binding domain and confers resistance to *Puccinia striiformis* (Fu *et al*., 2009). In barley, the CC-NB-LRR-type gene *Rdg2a* has been discovered to confer resistance to *Pyrenophora graminea* (Bulgarelli *et al*., 2010). Another barley gene, *Rpg1*, regulates resistance against *Puccinia graminis* f.sp. *tritici* (Brueggeman *et al*., 2002). RPG1 interacts with two effector proteins from urediniospores, one of them is characterized as a vacuolar protein sorting-associated protein (VPS9). This leads to rapid phosphorylation followed by the degradation of RPG1. The resulting HR then confers resistance to the rust fungus (Nirmala *et al*., 2011). Thus far, ETI-mechanisms in monocots and dicots are highly conserved.

Systemic acquired resistance: a defence in depth in monocots?

Upon locally induced defence, plants employ an intricate defence mechanism that activates resistance responses in not-yet-attacked tissues. In the case of a local challenge by leaf pathogens, mobile alarm signals are sent to distal leaves to induce a systemic resistance against a broad range of subsequent attackers. This mechanism is known as SAR (Shah 2009). SAR has been extensively studied in the two dicot models tobacco and Arabidopsis, leading to the identification of specific molecular components and of a set of mobile defence signals (Vlot et al., 2008). Salicylic acid (SA) has been found to be the main chemical regulator of SAR. SA exerts its canonical action on NPR1 (non-expressor of *PR* genes, also known as NIM1).

Originally, npr1 was discovered as recessive mutation conferring a SAR^- phenotype (Cao *et al*., 1997). Now it is known that NPR1 is a transcription factor activator that is present in the cytosol in an oligomeric form. SA accumulation leads to its constitutive monomerization. As a monomer, NPR1 enters the nucleus to interact with transcription factors (Mou *et al*., 2003), triggering extensive changes in the defence gene transcriptome (Maleck *et al*., 2001). Novel evidence shows that two paralogues of NPR1, NPR3 and NPR4, are SA receptors with different binding affinities to SA. They regulate NPR1 stability and activity depending on the SA level in the cell. In unchallenged plants, NPR4 mediates the degradation of most of the NPR1. When a pathogen triggers ETI, a gradient of SA builds up from the local to the systemic part and the elevated SA levels trigger an HR. Further expansion of cell death is then restricted through NPR3/NPR1 interactions in the cells adjacent to the HR (Fu *et al*., 2012). Prior activation of defence genes in distal tissues renders them more resistant against future attacks. A common marker of SAR in dicots is the up-regulation of PR genes such as *PR1* and *PR5*.

For an effective SAR reaction, mobile alarm signal(s) have to be sent from locally infested leaves to distant tissues. In Arabidopsis, several mobile SAR signals have been discovered, such as glycerol-3-phospate (G3P; Chanda *et al*., 2011), azelaic acid (Jung *et al*., 2009), and the volatile methyl salicylate (MeSA) (Park *et al.*, 2007). Recent findings also propose dehydroabietinal (DA), a diterpenoid aldehyde, as the SAR-signal in Arabidopsis (Chaturvedi *et al*., 2012). The known SAR signals are generally controversial as they are highly conditional, depending on the experimental systems. This abundance of different signals could be considered as a safety mechanisms to prevent accidental activation of the cost-intensive immune response. Through cross-interaction between signals or even requirement of parallel activation, an appropriate induction of IR for a given specific situation might be achieved (Dempsey and Klessig, 2012). SAR can also be induced by the application of various synthetic chemical compounds such as INA (2,6-dichloroisonicotinic acid; Métraux *et al*., 1990), BTH (S-methyl benzo-1,2,3-thiadiazole-7-carbothioate; Görlach *et al*., 1996), probenazole (3-allyloxy-1,2-benziso-thiazole-1,1-dioxide; Nakashita *et al*., 2002 a), BIT (1,2-benzisothiazole-1,1-dioxide; Yoshioka *et al*., 2001) NCI (N-cyanomethyl-2-

chloroisonicotinamide; Nakashita *et al*. 2002b) or tiadinil (3'-chloro-4,4'-dimethyl-1,2,3-thiadiazole-5-carboxanilide; Yasuda *et al*., 2004).

Compared with dicots, the knowledge of SAR in monocots is scarce. NPR1, the master regulator of SAR in dicots, has been confirmed for all monocots where genomic data is available (Kogel and Langen, 2005). In rice, over-expression of both AtNPR1 (Chern *et al*., 2001) and the endogenous homologue OsNH1 (Chern *et al*., 2005) resulted in an enhanced resistance to *Xanthomonas oryzae* pv. *oryzae*. Transcriptomic analysis of OsNPR1 knockdown and over-expressing rice lines showed that OsNPR1 is dominantly involved in the down-regulation of genes, and in the SA-mediated suppression of abscisic acid (ABA)-responsive genes (Sugano *et al*., 2010). Chemical SAR inducers were also found to be active in monocots, such as BTH and INA in maize (Morris *et al*., 1998), BTH in wheat (Görlach *et al*., 1996), and INA in barley (Kogel *et al*., 1994). Similarly to Arabidopsis, BTH-treatment of maize triggers the expression of PR proteins such as PR1 and PR5 (Morris *et al*., 1998). Monocot and dicot PR protein sequences were found to share extensive similarities. However, when performing an unrooted phylogenetic tree analysis using PR1 homologues from different species, dicot PR1 genes grouped together in a cluster distant from monocot sequences (Lu *et al*., 2011a). Thus, PR1 probably underwent the main diversifications after the monocot–dicot separation. Other resistance inducers in addition are described for monocots, such as the effect of probenazole in rice (Umemura *et al*., 2009). Probenazole strongly up-regulates OsSGT1, which encodes an UDP-glucose:SA glucosyltransferase. OsSGT1 is believed to support rice defence mechanisms by converting free SA to conjugated SA-O-β-glucoside (SAG) which, in turn, can be converted back into SA when needed. SA-levels itself were not found to be altered upon probenazole-treatment, suggesting an exquisite role of SAG during SAR in rice (Umemura *et al*., 2009). In barley induced with INA, the situation presents itself differently: here, defence reactions against *Blumeria graminis* f.sp. *hordei* neither depend on, nor induce SA accumulation (Hückelhoven *et al*., 1999). In contrast to dicots, the role of SA during SAR in monocots has yet to be elucidated. Rice contains high endogenous levels of SA (Silverman *et al*., 1995), and pathogen infection does not up-regulate these levels. However, transgenic rice plants expressing the SA-degrading enzyme salicylate hydroxylase (NahG) exhibit a diminished resistance against

Magnaporthe grisea (Yang *et al*., 2004), although *PR* gene expression profiles were found to be unaltered. The role of SA in other monocot models is less studied. Some reports on wheat and barley showed a 'local acquired resistance" (LAR) where a first fungal inoculation on a leaf makes a second attack on the same leaf less efficient (Thordal-Christensen and Smedegaard-Petersen, 1988; Jørgensen *et al*., 1998). In both studies, SA levels were found to be unaffected. Nevertheless, a recent study of *P. syringae* pv. *tomato*-induced LAR in barley demonstrated similarities between gene expression profiles during LAR in barley and SAR in Arabidopsis (Colebrook *et al*., 2012).

Although general chemical and molecular SAR players such as NPR1, PR genes and transcription factors are conserved in monocots and dicots, only a few reports describe biological SAR phenomena in monocots. Infection of rice by *P. syringae* pv. *syringae* leads to a systemic resistance against *M. grisea* (Smith and Métraux, 1991). In wheat, SAR against stem and leaf rust has been noted (Barna *et al*., 1998). Nevertheless, these SAR phenomena are highly conditional, corroborated by the lack of reproducibility by other laboratories (Kogel and Langen, 2005). However, the intricate signalling process during SAR is highly conditional, depending on multiple factors such as type of attackers, age of plant, and growth conditions. In Arabidopsis, MeSA is not required for SAR when plants are exposed for more than 3.5h to light after a primary pathogen infection (Liu *et al*., 2011). Strong light conditions trigger SAR in Arabidopsis upon *P. syringae* pv. *maculicola* infection without the accumulation of either SA or PR1 in systemic leaves (Zeier *et al*., 2004). Hence, particular molecular or chemical SAR factors have to be specifically determined for a given pathosystem, which might, in turn, explain the discrepant mode of action of certain SAR regulators between dicots and monocots.

Induced systemic resistance: support from underground alliances

Colonization of plant roots by some soil microbes, such as plant growth-promoting rhizobacteria (PGPR) or endophytic fungi (PGPF), can directly stimulate plant growth by improving nutrient uptake or photosynthesis (Spaepen *et al*., 2009; Trillas and Segarra, 2009) or indirectly by suppressing soil-borne pathogens through the production of antibiotic

Plant species	Beneficial microorganisms	Plant attackers	References
Rice	*Pseudomonas fluorescens* PF1	*Cnaphalocrocis medinalis*	Radja Commarea *et al*., 2002
	P. fluorescens FP7		
	Pseudomonas fluorescens PF1	*Rhizoctonia solani*	Radjacommarea *et al*., 2004
	P. fluorescens Pf1, TDK1, PY15	*Cnaphalocrocis medinalis*	Saravanakumar *et al*., 2007
	P. fluorescens WCS374r	*Magnaporthe oryzae*	De Vleesschauwer *et al*., 2008
	P. fluorescens Aur6 *Chryseobacterium balustinum* Aur9	*Magnaporthe oryzae*	Lucas *et al*., 2009
	P. aeruginosa	*Rhizoctonia solani*	Saikia *et al*., 2006
	P. aeruginosa 7NSK2	*Magnaporthe oryzae*	De Vleesschauwer *et al*., 2006
		Rhizoctonia solani	
	Bacillus pumilus SE34 *Bacillus subtilis* GB03	*Xanthomonas oryzae* pv. *oryzae*	Chithrashree *et al*., 2011
	Serratia plymuthica IC1270	*Magnaporthe oryzae*	De Vleesschauwer *et al*., 2009
		Cochliobolus myiabeanus	
		Rhizoctonia solani	
Maize	*Trichoderma virens* T22	*Colletotrichum graminicola*	Djonovic *et al*., 2007
	Bacillus cereus C1L	*Cochliobolus heterostrophus*	Huang *et al*., 2010
	Glomus mosseae	*Rhizoctonia solani*	Song *et al*., 2011
Wheat	*P. fluorescens* CHA0	*Fusarium graminearum*	Henkes *et al*., 2011
	P. fluorescens CHA0	*Gaeumannomyces graminis* var. *tritici*	Sari *et al*., 2008
	P. fluorescens MKB158	*Fusarium graminearum*	Petti *et al*., 2008
	P. aeruginosa 7NSK2	*Blumeria graminis*	Muyanga *et al*., 2005
		Cochliobolus sativus	
	Chaetomium globosum	*Pyrenophora tritici-repentis*	Istifadah and McGee, 2006
	Fungal endophytes	*Puccinia recondite* f.sp. *tritici*	Dingle and McGee, 2003
Barley	*Piriformospora indica*	*Blumeria graminis* f.sp. *hordei*	Molitor *et al*., 2011
	P. fluorescens MKB158	*Fusarium graminearum*	Petti *et al*., 2010
	Fusarium oxysporum f.sp. *radicis-lycopersici*	*Blumeria graminis* f.sp. *hordei*	Nelson, 2005
Pearl millet	*B. pumilus* INR7	*Sclerospora graminicola*	Raj *et al*., 2003
	B. pumilus SE34		
	B. subtilis GB03		
	P. fluorescens UOM SAR 14	*Sclerospora graminicola*	Raj *et al*., 2004
Sorghum	*B. cereus* KBS2-6	*Pythium utlimum*	Itris *et al*., 2008
	B. cereus KFP9-A		
	Serratia marcescens KBS9-R		

Table 3. Examples of established cereal ISR pathosystems.

compounds (De Vleesschauwer and Höfte, 2009). Moreover, these beneficial microorganisms can also indirectly reduce plant disease through an induction of a systemic resistance, named ISR. ISR confers a resistance against a wide spectrum of attackers, mostly necrotrophic pathogens and pests (Van Wees *et al*., 2008; Pineda *et al*., 2010). Similarly, mycorrhizae have been reported to induce plant resistance in a way resembling that of ISR (reviewed by Pozo and Azcón-Aguilar, 2007). Various beneficial microorganisms are known to induce ISR in monocots. In cereals, endophytic fungi, PGPR or mycorrhizae are reported to induce resistance against pathogens and insect herbivores (Table 3). The potential resistance induced by PGPR in monocots depends on the host-PGPR combination and on the type of attacker. *P. aeruginosa* 7NSK2 and *Serratia plymuthica* IC1270 induce resistance against *Magnaporthe oryzae* in rice, but they enhance disease severity caused by *Rhizoctonia solani* (De Vleesschauwer *et al*., 2006, 2009). However, some pseudomonads induce resistance of rice against *R. solani* (Table 3). Induction of resistance by a specific strain of PGPR is not restricted to only one plant species: for example, *P. aeruginosa* 7NSK2 triggers ISR in rice (De Vleesschauwer *et al*., 2006) and wheat (Muyanga *et al*., 2005). Application of a PGPR mixture

enhances the efficacy of resistance induction compared with the use of individual strains in both dicots (De Boer *et al*., 2003) and monocots (Lucas *et al*., 2009).

Diverse microbial molecules have been identified as ISR elicitors in monocots. Exopolysaccharides produced by *Pantoea agglomerans* induce defence responses in wheat cells by triggering an increased accumulation of hydrogen peroxide and an augmented peroxidase activity (Ortmann and Moerschbacher, 2006). Siderophores and antibiotics produced by *Pseudomonas* strains, such as pseudobactins and pyocyanin, are important defence elicitors in rice against *M. oryzae* (De Vleesschauwer *et al*., 2008; De Vleesschauwer and Höfte, 2009). In contrast to tomato and bean, pyocyanin was shown to be the only component compulsory for triggering ISR in rice. Certain fungal endophytes have also been shown to trigger IR. A beneficial *Penicillium* primes Arabidopsis for defence against *P. syringae* (Hossain *et al*., 2008) and *Glomus mossae* protects tomatoes from infection by *Phytophthora* (Pozo *et al*., 2002). *Trichoderma virens*, an endophytic fungus that triggers ISR in maize, has been shown to facilitate resistance via the release of a proteinaceous elicitor (Djonovic *et al*., 2007). *Piriformospora indica* induces IR in both dicots and monocots but is probably best-known for this effect on barley. Here, it was shown to induce resistance without having to rely on the classical defence pathways involving SA, JA or ET (Waller *et al*., 2005). A barley leaf transcriptome and metabolite analysis revealed that *P. indica*-induced plants over-expressed a small set of defence-related genes including transcripts coding for PR and heat-shock proteins (Molitor *et al*., 2011). In creeping bentgrass (*Agrostis stolonifera*) which is closely related to cereals, treatment with (2R, 3R)-butanediol, a bacterial-derived volatile, induces resistance against *Microdochium nivale* (Cortes-Barco *et al*., 2010). Rhizobacteria can also produce hormones that manipulate phytohormone pathways. SA produced by *P. aeruginosa* strains triggers peroxidase accumulation in rice leading to an increase in resistance to *R. solani* (Saikia *et al*., 2006). Some N-acyl homoserine lactones (AHL) controlling quorum sensing in bacteria (Miller and Bassler, 2001) also have the capacity to induce resistance. AHLs from *Serratia liquefaciens* and *P. putida* induce resistance against *Alternaria* in tomato (Schuhegger *et al*., 2006). Intriguingly, *P. indica* is closely associated with an endobacterium, *Rhizobium radiobacter* (Sharma *et al*., 2008), that produces a series of AHLs. Application of these AHLs to

barley induces resistance against powdery mildew (Sharma *et al*., 2008). This raises the question as to whether the observed IR capacity of *P. indica* might not actually be due to the presence of the endophytic bacteria.

The efficacy of ISR in monocots against necrotrophic pathogens has been demonstrated repeatedly but only in a few cases, the involved defence signalling pathway has been investigated. ISR induced by *P. fluorescens* WCS374r against *M. oryzae* in rice depends on a jasmonic acid (JA)/ethylene (ET)-modulated signal but is independent from SA-signalling (De Vleesschauwer *et al*., 2008). Involvement of JA-signalling in ISR was also shown in maize (Djonovic et al., 2007; Song et al., 2011) and barley (Petti *et al*., 2010). Interestingly, ISR triggered by *T. virens* in maize also seems to be associated with the priming of genes involved in the production of volatile compounds called green leaf volatiles (GLV) (Djonovic *et al*., 2007). Several defence-related genes involved in SA- and JA-dependent pathways are strongly induced when mycorrhized maize plants are challenged with *R. solani* (Song *et al*., 2011). ISR in monocots is mostly linked to JA-dependent defences. However, some PGPR or PGPF induced an SA-dependent pathway effective against biotrophic pathogens (Muyanga *et al*., 2005; Molitor *et al*., 2011).

Overall, recent studies on ISR triggered by PGPR, PGPF or mycorrhiza in monocots and more specifically in cereals tend to point to common mechanisms with dicotyledonous plants

Induced resistance against insect herbivores: protection against air-borne assaults

Plants are confronted with a wide variety of insect herbivore attacks. To counteract these attacks promptly and specifically by inducing defence mechanisms, plants recognize molecules originating either from wounding damage or from compounds derived from the herbivore itself, such as oral secretions (OS) and oviposition fluids. These elicitors, called herbivore associated molecular patterns (HAMPs), have been found in several monocot

pathosystems. Volicitin, a hydroxyl fatty acid-amino acid conjugate found in *Spodoptera exigua* OS, induces volatile emission in maize (Alborn *et al*., 1997) and caeliferins from *Schistocerca americana* OS trigger IR in maize (Alborn *et al*., 2007). Plant perception of HAMPs is widely elusive, but similarities to MAMP-recognition have been proposed (Bonaventure *et al*., 2011). In maize, volicitin is perceived by a plasma membrane protein (Truitt *et al*., 2004), which is so far the only known HAMP-receptor in monocots.

Upon perception of an herbivore, IR mechanisms are mediated by different defence-related hormones. Plant-induced defences against phloem-feeding herbivores seem to share a common plant reaction to biotrophic pathogens by activating SA-dependent pathways associated with the production of PR proteins (Alagar *et al*., 2010) and callose deposition at the feeding site (Hao *et al*., 2008). In rice, defence induced by an attack of the phloem-feeding brown planthopper is mediated by a SA-related signalling and is associated with an accumulation of PR proteins and an HR (Zhou *et al*., 2009). In resistant wheat cultivars, but not in susceptible ones, infestation by gall insects induces changes in SA levels (Tooker and De Moraes, 2011). By contrast, plants induce JA and ET-dependent pathways against chewing herbivores. In maize, JA and ET are important in plant defence against *S. frugiperda* (Shivaji *et al*., 2010; Harfouche *et al*., 2006). JA was also shown to have an important role in IR of wheat against pests (El-Wakeil *et al*., 2010). In rice, the JA-dependent pathway induces resistance against insect herbivores and suppression of JA activity results in an improved larval performance of the striped stem borer and leaf folder (Zhou *et al*., 2009). Ethylene is another key player in fending off herbivores. ET emission induced by elicitors of *S. frugiperda* OS influences the expression of direct defences such as defence proteins and secondary metabolites (Harfouche *et al*., 2006). In rice, the ethylene responsive factor ERF3 mediates between SA, JA, and ET pathways and thus orchestrates the response to chewing or phloem-feeding insects (Lu *et al*., 2011b).

After herbivore attack, plants can induce defences that will directly act against insect herbivore. The maize insect resistance 1-cysteine protease (Mir1-CP) content increases in roots and leaves in response to larvae feeding on leaves, conferring a systemic induction of plant defence against herbivores (Lopez *et al*., 2007). Trypsin proteinase inhibitors are

important defence compounds against herbivores such as the striped stem borer and leaf folder in rice (Wang *et al*., 2011; Zhou *et al*., 2011). Secondary metabolites, such as the hydroxamic acids in cereals, can also have a direct negative effect on insect herbivores (Chen, 2008). Direct local defence can enhance direct plant defence systemically. Infestation of rice plants with *S. frugiperda*, for example, increases resistance against a subsequent attack by the rice water weevil (Hamm *et al*., 2010). Similarly, root infestation of maize by *Diabrotica virgifera virgifera* induces resistance in the leaves against *S. littoralis* and the necrotrophic pathogen *Setosphaeria turcica* (Erb *et al*., 2009). This illustrates that an induction of belowground defences can induce above-ground resistance in maize.

Many plants respond to insect herbivory or wounding by emitting blends of volatile organic compounds (VOCs). VOCs release is an important cue for systemic defence signalling within an attacked plant as well as for plant–plant communication. Exposure of a maize plant to VOCs from infested plants primes the defence response against the generalist *S. littoralis* (Ton *et al*., 2007). Green leaf volatiles (GLVs), specific VOCs emitted by plants upon wounding damages, can also activate defence mechanisms in neighbouring intact plants (Ruther and Furstenau, 2005).

Induced resistance (IR) in non-cereal monocots: the last bastion

Because of their economic importance, most of the research on IR in monocots has been conducted on cereals. Nevertheless, IR such as SAR and ISR can also be found in non-cereal monocots. In *Lilium formonasum*, a previous infection with *Botrytis elliptica* suppresses a secondary infection with the same pathogen in systemic tissues (Lu *et al*., 2007). Classical synthetic chemical SAR inducers have been reported in diverse non-cereal monocot systems. *L. formosanum* can be protected against *B. elliptica* by probenazole. Here, resistance is associated with a stomatal closure and increased callose deposition (Lu *et al*., 2007). SA-treatment primes callose accumulation in onion, which confers enhanced resistance to downy mildew (Polyakovskiy and Dmitriev, 2011). BTH enhances plant defence in banana against *Colletotrichum musae* via a higher chitinase defence gene expression (Ma *et al*., 2009).

Curcuma (Radhakrishnan *et al*., 2011) and sugarcane (Ramesh Sundar *et al*., 2006) were also protected by BTH treatment against *Pythium aphanidermatum* and *Colletotrichum falcatum*, respectively. Functional ISR has also been reported in non-cereal monocots, here mostly against necrotrophic fungal pathogens. For example, *Bacillus cereus* C1L was efficient in eliciting ISR in *Lilium formonasum* against *B. elliptica* (Liu *et al*., 2008). In banana plants, a combination of the rhizobacteria *Pseudomonas fluorescens* CHA0 and chitin induces systemic resistance against banana bunch top virus (Kavino *et al*., 2008). A mixture of several PGPRs seems to have an increased positive effect compared with a single strain use on resistance in gladiolus (Shanmugam *et al*., 2011) and in banana (Sangeetha *et al*., 2010). ISR induced by a hypoagressive isolate of *Fusarium oxysporum* in date palm against *Fusarium oxysporum* f.sp. *albedinis* is characterized by a primed reaction of the plant with a faster induction of peroxidase activity and a higher amount of phenolics (El Hassni *et al*., 2004).

Conclusion

Historically, the majority of research on IR has been performed in dicot model plants. Recent advances in monocot genomics, however, are helping to identify the key components of IR signalling. Further improvements in monocot biotechnology such as plant transformation methods will provide a more profound insight into IR mechanisms. Moreover, a variety of cereal and non-cereal IR model systems are now well established, making IR in monocots a research field ready to move forward. Novel insights into the functioning of IR in monocots are expected to have a positive impact on sustainability in modern agriculture.

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IC: Plant hormones as universal vocabulary in plant defense signaling

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IC: Plant hormones as universal vocabulary in plant defense signaling

Abstract

Plants are sessile organisms exposed to a highly dynamic environment and physiological flexibility including the rapid activation of suitable defense responses is crucial for their survival. Plants are confronted with an armada of pathogens and pests and throughout the ongoing evolutionary arms race with these attackers they have developed a sophisticated chemical signaling system, which allows them to activate highly specific and targeted defense responses. In this context, plant hormones and secondary metabolites play a pivotal role: they serve as signals in an intricate local and systemic communication network. This chapter presents recent insights into the vocabulary used by plants to fend off pathogens and pests.

Introduction

Despite a large variety of potential pathogens only few are capable to successfully infect a particular plant species. The intricate self-protection system plants have developed during co-evolution with their attackers makes disease the exception rather than the rule. Their defense barriers can only be overcome by specialized attackers. According to their lifestyle, plant pathogens are divided into biotrophs and necrotrophs. Biotrophic pathogens obtain nutrients from living host cells; in contrast, necrotrophs kill host cells to derive nourishment from dead tissue. Many pathogens, called hemibiotrophs, exhibit both stages during their life cycle. The defense system of plants is multilayered and typically consists of preformed physical and chemical barriers as well as of inducible defenses. Phytoanticipins constitute the first layer of defense. They are products of secondary plant metabolism, synthesized during regular development and stored in subcellular compartments (Morrissey and Osbourne 1999). Three main groups of such metabolites are known: phenolics, terpenes and nitrogen-containing organic compounds (Walters 2010). A number of those compounds are toxic to pathogens. By preventing initial pathogen or pest entry, phytoanticipins provide additional time for the plant to

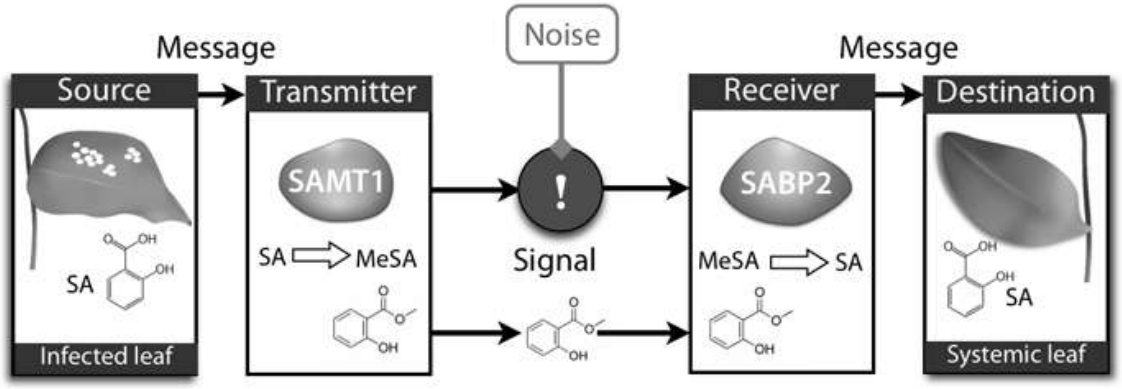

Figure 1. Plant defense signaling follows the communication model of Shannon and Weaver (1949). Shannon and Weaver's model embodies an information source, message, transmitter, signal, noise, receiver and destination. Methyl salicylate (MeSA)-triggered systemic defense is set up at a locally infected leaf that serves as source for the alarm message. Salicylic acid (SA) is induced and converted into MeSA by SA carboxyl methyltransferase 1 (SAMT1). SAMT1 acts as transmitter modifying the signal. MeSA then functions as mobile signal translocating to its destination, the non-infected systemic leaves. There, the message is perceived by salicylic acid binding protein 2 (SABP2), which converts MeSA back into SA. SA then exerts its defense signaling function to immunize the systemic leaves. Some pathogens are able to manipulate the signaling cascade, thus acting as "noise" interfering with the message.

set up inducible defenses. Another first layer of defense is induced upon recognition of conserved microbial features such as chitin, flagellin and lipopolysaccharides (Göhre and Robatzek 2008). During this "innate immunity" response, plants perceive pathogen-associated molecular patterns (PAMPs) with the help of pattern recognition receptors (PRRs), leading to a PAMP triggered immunity (PTI). Successful pathogens secrete effectors suppressing PTI, therefore promoting effector-triggered susceptibility (ETS). In turn, plants have resistance (R) proteins that recognize and attenuate pathogen-derived effectors, thus leading to an effector-triggered immunity (ETI; Jones and Dangl 2006). In induced plant defense, phytohormones and metabolites have a prominent role. Despite variations in quantity and blend between specific plants, tissues and attackers, they participate in the fine-tuning and translation of induced defense signaling (Pieterse *et al*. 2009). Moreover, plants utilize hormones as a vocabulary facilitating local and systemic communication during disease management. The action of plant hormones during disease management follows the principle of Shannon and Weaver's (1949) classic model of communication. They defined communication as an interplay of four main parts: a *source* which is the origin of a given message, a *transmitter* that modulates a signal for the transport through a defined channel, a *receiver* which accepts the signal and transforms it

to the message which is finally delivered to its *destination*. These four parts, namely source, transmitter, receiver and destination, can consistently be applied to phytohormone-mediated signaling, such as defense reactions triggered by methyl salicylate (MeSA; Fig. 1.) A locally infected plant part serves as source for a pathogen-specific alarm signal, which is often modified by co-factors and prepared for long-distance movement through the plant vascular system or in a volatile form through the air. The systemic tissue then perceives the alarm signal and decodes the message indicating the exact nature of the attack. This information allows the not yet-infected tissue to turn on a defense reaction specifically adapted to the given stress. Recent advances in understanding the role of phytohormones have unveiled an extensive interplay between various hormones (Pieterse *et al*. 2009). Here, we present highlights and recent advances on the ability of chemicals to function as information carrier in an intricate semiochemical communication network modulating plant defense responses.

Plant hormones involved in defense signaling

Phytohormones are generally defined as “chemical regulators” produced by plants to regulate not only growth and development but also in response to biotic and abiotic stress. Six major plant hormone groups are distinguished: auxins (AUX), cytokinins (CK), gibberellins (GA), abscisic acid (ABA), ethylene (ET) and brassinosteroids (BR). Additional compounds such as jasmonic acid (JA), salicylic acid (SA) and systemin have been identified as hormone-like regulators of plant defense and development. The fact that various pathogens possess the ability to interfere with phytohormone signaling supports their pivotal role for defense. Some strains of the hemibiotrophic bacterial pathogen *Pseudomonas syringae* produce a phytotoxin called coronatine (COR). *P. syringae* uses COR to mimic JA-signaling, thus down-regulating SA-dependent defenses (Spoel and Dong 2008). In a Shannon and Weaver-type communication model (Fig. 1), COR functions as “noise”, interfering with the signals and perturbing the messages sent by infected plant cells.

Hormonal signaling is based on key components such as receptors, protein interaction partners and transcription factors, which are mostly conserved throughout higher plants (Bari and Jones 2009). Despite the variety of signal sources, channels, destinations and signaling compounds, the hormones induced upon biotic stress share a common consequence of their action: they usually manipulate the expression of defense genes. For instance, out of 2375 selected *Arabidopsis* genes, 705 messenger RNAs were found to be substantially changed upon SA, ET, methyl jasmonate (MeJA) and *Alternaria brassicicola* treatment (Schenk *et al*. 2000).

Salicylic acid

SA belongs to the large group of phenolic plant compounds and plays a role not only in disease response but also in seed germination, cell growth, respiration, stomatal closure, senescence, thermo tolerance and flowering (Vlot *et al*. 2009). In *Arabidopsis thaliana* and *Nicotiana benthamiana*, the majority of pathogen-induced SA is synthesized by isochorismate synthase (ICS; Vlot *et al*. 2009). A SA-glucosyltransferase then converts most of the SA into *0*-β-glucoside (SAG; Dean and Delaney 2008). SAG is stocked in the vacuole, where it likely acts as storage form that can be converted back into SA when needed. SA is predominantly involved in defense against biotrophic pathogens. During defense communication, SA plays a role in both local and systemic resistance reactions. Locally, SA combats invading pathogens due to its natural antimicrobial properties (Murphy and Carr 2002). SA also functions as mediator of systemic acquired resistance (SAR). During SAR, a locally infected tissue emits phloem-mobile or airborne alarm signals to uninfected parts of the plants, thus rendering them more resistant against subsequent pathogen attack. Due to its presence in the phloem, SA was initially thought to be itself the signal mediating SAR. However, grafting experiments showed that SA is not required in the tissue *transmitting* the SAR signal, whereas it is indispensable in the systemic tissue *receiving* the SAR signal (Vernooji *et al*. 1994). In regard to the communication principle of Shannon and Weaver, SA seems therefore not to play a role as long-distance signal, it rather acts as a local communication mediator in infected cells and exerts a receiver-like function in non-infected tissue. The major role of SA during local disease

management is the modification of cellular signaling pathways, mainly through the interaction with NPR1 (nonexpressor of PR1; Cao *et a*l. 1997). NPR1 is present in the cytosol in a dimeric form. Accumulation of SA shifts the redox state inside the cell from oxidizing towards reducing conditions. Reduction of cysteine residues of NPR1 dimers leads to its monomerization. As a monomer, NPR1 translocates to the nucleus where it interacts with transcription factors such as TGAs and WRKYs to enhance defense gene expression (Vlot *et al*. 2009). Beside modification of NPR1 by shifting the redox state, SA also induces the expression of thioredoxins (TRX) that catalyze the monomerization of NPR1 (Tada *et al*. 2008). Therefore, NPR1 is the main "receiver" of the defense information delivered by SA, obtaining the signal via direct and indirect signal perception. Nevertheless, the true SA receptor is not yet known (Vlot *et al*. 2009).

Jasmonic acid

Jasmonates are oxygenated fatty acids produced by the octadeconoid pathway (Staswick 2008). They are important for a variety of processes including pollen maturation, fruit development, photosynthesis, senescence and root growth. Moreover, JA signaling is activated upon herbivore attack in a variety of different plant species and is crucial in regulating defense responses against necrotrophic pathogens and chewing insects (Pieterse *et al*. 2009). Furthermore, it also plays an important role during induced systemic resistance (ISR) mediated by non-pathogenic root colonizing bacteria (Pieterse *et al*. 2009). Recently, the COP9 signalosome has been shown to regulate JA-dependent insect defense (Hind *et al*. 2011). Intriguingly, JA acts as a negative regulator of SA-dependent defenses (Bari and Jones 2009, Pieterse *et al*. 2009). Upon wounding of plant tissues, linoleic acid is released from membrane lipids of chloroplasts and incorporated into the octadeconoid pathway, where it is transformed into JA (Staswick 2008). JA can further be metabolized into various products including volatile MeJA, and it can conjugate with amino acids and sugars (Wasternack 2007).

Referring to the Shannon and Weaver model (Fig. 1), the source of JA as chemical regulator signal are membrane-derived lipids that are metabolized into jasmonates, which are then perceived by a COI1/JAZ co-receptor. Furthermore, JA has also been shown to be

transmittable through the phloem into systemic tissues (Truman *et al*. 2007), therefore transporting a long-distance message to a destination tissue. Whether in its local or systemic destination, JA signaling drives the induction of defense-related genes. Further studies need to be undertaken to unveil how the products from JA-responsive genes contribute in detail in combatting disease.

Ethylene

The gaseous hormone ET is the major regulator of fruit ripening, seedling emergence, leaf and flower senescence and organ abscission but it contributes also to biotic stress signaling (van Loon *et al*. 2006). Both 1-Aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase, important enzymes in ET biosynthesis, are induced upon pathogen infection, wounding and light stress (Wang *et al*. 2002), ET moves by diffusion from its original site of synthesis to systemic tissues. There, it is perceived by a family of membrane-localized receptors. The role of ET during pathogen defense signaling is rather ambiguous. ET contributes to basal resistance in *Arabidopsis* against *Pseudomonas syringae* pv. *syringae* and *Xanthomonas campestris* pv. *vesicatoria* (Ton *et al*. 2006). In contrast, the proliferation of the bacterial leaf pathogen *Pseudomonas syringae* pv. *glycinea* is impaired on mutants lacking the capacity to produce ET (Weingart *et al*. 2001). Often, disease symptoms are enhanced after ET-treatment, probably due to the ET-triggered induction of senescence (van Loon *et al*. 2006). Recent findings suggest that ET plays a pivotal role during early defense reactions. Nitric oxide (NO) interacts with SA to regulate ET production mediating the hypersensitive response, a cell death phenomenon associated with rapid localized resistance to pathogens (Mur *et al*. 2008). ET signaling is also involved in ISR (Pieterse *et al*. 2009). Due to the ambiguous mode of action, it can be assumed that ET does not play a role as message carrier itself during defense communication, rather acting as fine-tuning mediator in the crosstalk of other major hormonal pathways (section 4). In fact, ET is known to interact synergistically with both the JA and SA signaling network (Pieterse *et al*. 2009). Unlike other chemical regulators such as SA and JA, ET affects all developmental stages; the fluctuating effect of ET during defense communication

therefore depends strongly on the age of the plant, the type of pathogen and the environmental conditions.

Auxins

Auxins are the main chemical regulators of growth and cell differentiation in plants. They are principally occurring as indole-3-acetic acid (IAA). IAA is synthesized from two distinct pathways, one using L-tryptophan as main precursor, and another tryptophan-independent pathway (Buchanan *et al*. 2002). The majority of IAA *in planta* is synthesized in meristems, young leaves and developing fruits and seeds. From its original site of biosynthesis IAA is transported by non-polar and polar transport mechanisms (Buchanan *et al*. 2002). Beside its crucial role in plant development, recent studies indicate that auxin also contributes to pathogen defense signaling in a rather ambivalent manner. The auxin-responsive gene GH3 has been shown to modulate SA and auxin signaling during *Pseudomonas syringae* infection in *Arabidopsis* (Zhang *et al*. 2007). *Arabidopsis* auxin-signaling mutants are more susceptible to the necrotrophic fungi *Plectosphaerella cucumerina* and *Botrytis cinerea* (Llorente *et al*. 2008). In contrast, treatment of *Arabidopsis* with a SA analogue resulted in the global repression of auxin-response genes, suggesting that the SA pathway inhibits auxin signaling to enhance pathogen resistance (Wang *et al*. 2007). Similarly, a plant microRNA (miRNA393) was discovered to contribute to antibacterial resistance in *Arabidopsis* by down-regulation of TIR1, thus repressing auxin-responsive genes (Navarro *et al*. 2006). Hence, auxins seem to attenuate plant defense responses rather than to act as defense mediating signaling compound. It is known that pathogens are able to manipulate auxin signaling to promote disease (Padmanabhan *et al*. 2008). Taken together, auxins are believed to act as either negative or positive modulators of defense responses by affecting the catabolism of other hormonal pathways and the plant physiology in general.

Abscisic acid

ABA is an isoprenoid phytohormone mainly involved in regulating seed germination, leaf senescence, stomatal aperture and plays a crucial role in response to water and salt stress (Wasilewska *et al*. 2008). ABA is a phloem-mobile and long-distance signal synthesized

primarily in vascular tissues (Nambara and Marion-Poll 2005). The role of ABA during pathogen defense is highly multifaceted and depends on the specific stage of defense and type of attacker (Ton *et al*. 2009). Generally, ABA is believed to act as a negative regulator of defense responses. ABA-deficient mutants or mutants impaired in ABA synthesis show increased resistance to different pathogens (Cao *et al*. 2011). Conversely, exogenous application of ABA can favor disease development (de Torres-Zabala *et al*. 2007). Different pathogens are known to produce ABA and thus interfere with host defense (Cao *et al*. 2011). However, ABA can also positively regulate defense responses (Mauch-Mani and Mauch 2005). The closure of stomata, which can serve as entry point for attacking bacteria, is triggered by ABA (Melotto *et al*. 2006). Moreover, ABA-treatment mediates resistance against some necrotrophs. This ABA-induced resistance is based on ABA-dependent priming for deposition of callose-containing cell wall reinforcement against penetration by pathogens (Ton and Mauch-Mani 2004). Taken together, ABA acts as positive and negative chemical regulator of plant defense. During the initial phase of invasion, ABA positively regulates resistance through mediation of stomatal closure. In the subsequent early stage of invasion, ABA enhances resistance against fungi and oomycetes by triggering callose deposition, but also diminishes resistance by inhibiting reactive oxygen species (ROS) generation and callose accumulation upon bacterial infection. Finally, during late defense reactions, ABA generally inhibits defense responses by suppressing JA, ET and SA-dependent signaling (Ton *et al*. 2009).

Brassinosteroids, cytokinins and gibberellin

Brassinosteroids, cytokinins and gibberellin play rather minor roles in defense responses; only few studies are providing evidence that these classical phytohormones contribute to plant immune reactions. BR, known for their involvement in seed germination, cell division, flowering and senescence, have been shown to enhance resistance of tobacco against TMV in a SA-independent manner (Nakashita *et al*. 2003). Similarly, exogenous application of BR on potato plants enhances their resistance against *Phytophthora infestans* (Krishna 2003). Components of BR signaling participate in early defense responses, as *Arabidopsis* mutants of the BR-receptor BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) exhibit higher susceptibility to

bacterial and fungal pathogens (Kemmerling *et al*. 2007), and BAK1 interacts with the flagellin-sensing transmembrane receptor kinase FLAGELLIN SENSITIVE2 (FLS2) to initiate PAMP-triggered immunity during early pathogen perception (Chinchilla *et al*. 2007). Taken together, BR seems to play an indirect role during defense responses by influencing other hormonal pathways and by PAMP-triggered immunity (Bari and Jones 2009).

In turn, the roles of CK during defense responses are less understood. Mainly involved in stem-cell control, vascular differentiation, chloroplast biogenesis, seed development, shoot and root growth, CK was recently shown to contribute to pathogen responses. Disease symptoms of *Arabidopsis* roots against *Plasmodiophora brassicae* were found to be increased by CK (Siemens *et al*. 2006), and *Agrobacterium tumefaciens* enhances CK production in *Arabidopsis* plastids to induce tumor formation (Sakakibara *et al*. 2005). Therefore, CK seems to have rather disease promoting effects, although its role in defense against different types of attackers is poorly understood.

In contrast, the growth-promoting hormone GA has been found to exert positive and negative effects on plant defense responses. GA stimulates plant growth by degradation of DELLA proteins, which negatively regulate plant growth. DELLA proteins regulate defense responses in *Arabidopsis* by altering SA- and JA-dependent immunity (Navarro *et al.* 2008). Hence, *Arabidopsis* DELLA mutants showed higher susceptibility to the necrotrophic pathogens *A. brassicicola* and *B. cinerea*, whereas the resistance against the biotrophs *Pst* DC3000 and *Hyaloperonospora arabidopsidis* was enhanced. Consequently, GA seems to be implicated in promoting resistance to biotrophs and susceptibility to necrotrophs. However, the mechanism of GA-regulated defense is still largely unexplored.

Systemin

Systemin is a plant peptide hormone playing an exclusive role following wounding in the *Solanaceae.* During herbivore attack, systemin is cleaved from its precursor prosystemin, and stored in the cytoplasm (Ryan and Pearce 2001). Its local and systemic induction triggers the activation of proteinase inhibitors (PI). PIs prevent the uptake of essential amino acids in the insect mid-gut, causing developmental defects (Chen *et al*. 2005). Following perception,

synthesis of JA and the expression of defense-related genes are activated (Kandoth *et al*. 2007). Grafting experiments have shown that neither systemin nor JA were required in the systemic tissue acquiring the signal, indicating that systemin acts at the local site of infection to facilitate the production of a long-distance and probably JA-derived signal (Li *et al*. 2002). Furthermore, over-expression of prosystemin resulted in an enhanced release of volatiles and synthesis of PIs upon herbivore attack in tomato, implicating that systemin and JA are regulating herbivore-induced systemic volatile emission (Degenhardt *et al.* 2010). So far, the exact role of peptide hormones in the regulation of plant defenses remains elusive.

Systemic defense signals

Following local events leading to the build-up of a defensive state, a signal has to be generated and transmitted to systemic plant parts. Induction of SAR follows PTI or ETI-mediated pathogen recognition and is associated with increased levels of SA and pathogen-related proteins (PR) in local and systemic tissues (Jones and Dangl 2006). At the root level, various microorganisms can trigger a systemic induction as observed for ISR, or rhizobacteria-and mycorrhiza induced resistance (Pieterse *et al.* 2009). Moreover, during systemic wound responses, herbivore-infected plants emit volatile signals to set up an indirect defense by attracting predatory insects (Heil and Silva Bueno 2007).

Systemic resistance represents an example of an intricate communication system, mediated by a series of mobile signals. Despite the major advances in recent years and the identification of multiple long-distance chemical signals, the exact nature of specific mobile signals remains elusive and controversial (Vlot *et al.* 2008). Recent studies proposed methyl salicylate (MeSA) as a critical SAR signal (Park et al. 2007). In TMV-infected tobacco leaves, SA carboxyl methyltransferase 1 (SAMT1) converts SA into MeSA, which is biologically inactive and volatile. MeSA can then act as a phloem-mobile or airborne signal immunizing non-infected systemic tissues. There, it is converted back to SA by salicylic acid binding protein 2 (SABP2) (Park et al. 2007). However, MeSA is not essential for SAR-expression in *Arabidopsis*

(Attaran *et al.* 2009). Jasmonates are also accepted as mobile defense signals. Volatile methyl jasmonate (MeJA) functions as phloem- and xylem-mobile signal during systemic wound responses (Thorpe *et al*. 2007). SAR is compromised in jasmonate-deficient *Arabidopsis* mutants, suggesting a signaling role for JA during SAR (Truman *et al*. 2007). Nonetheless, the role of JA during SAR is highly debatable and likely conditional, depending on the experimental system and the applied pathogen dose (Shah 2009).

Furthermore, azelaic acid has been identified as a SAR-eliciting factor (Jung *et al*. 2009). Elevated levels of azealic acid were found in petiole exudates of SAR-triggered plants, and locally applied radiolabelled azelaic acid was recovered in distant leaves, confirming its systemic nature. Its local application did not alter SA levels or SA-dependent gene expression (Jung *et al.* 2009). Recently, Glycerol-3-phosphate (G3P) was discovered to act as critical mobile signal for SAR in *Arabidopsis* and soybean (Chanda *et al*. 2011). *Arabidopsis* G3P biosynthesis mutants are unable to induce SAR, and G3P derivates are translocated to distal tissues with the help of the lipid transfer protein DIR1. Green leaf volatiles (GLVs) are also known to act as systemic defense signals, predominantly in response to wounding or herbivore attack (Heil and Silva Bueno 2007). They prime plants for enhanced induction of JA-dependent defenses during wounding and herbivore attack. Overall, recent studies suggest the presence of multiple mobile defense signals for systemic resistance. Beside MeSA, MeJA, azelaic acid, glycerol-3-phosphate and GLVs, a variety of additional chemical regulators such as ET, ABA, sugars and peptide hormones are likely to also contribute in systemic resistance. The nature of a specific signal strongly depends on the transport channel (vascular or airborne), on the plant species and its lifestyle, and on the type of attacker. Nevertheless, systemic defense highlights the plants capability to apply a complex communication network with distinct *signal sources*, *channels* and *signal receivers* according to Shannon and Weaver (1949).

Signal crosstalk

In contrast to animals, plants do not possess cells that are exclusively specialized in immune reactions. In order to adapt their defense to a continuously changing environment they fine-tune the crosstalk of the different chemical regulators involved in defense signaling (Pieterse *et al.* 2009). Genome-profiling experiments with *Arabidopsis* hormone mutants revealed the presence of an extensive and pliable network between the three main chemical regulators SA, JA and ET (Glazebrook *et al.* 2003). For instance, the interaction of SA and JA is normally antagonistic, due to trade-offs between SA-mediated resistance against biotrophs and JA-mediated resistance against necrotrophs. In *Arabidopsis*, JA-dependent defenses activated upon caterpillar feeding were suppressed by the SA-mediated defense reaction triggered by infection with the biotrophic pathogen *Hyaloperonospora arabidopsidis* (Koornneef *et al*. 2008). Similarly, exogenous application of SA diminishes the expression of JA-responsive genes such as *PDF1.2* and *VSP2*. However, the interaction between SA and JA is dose-dependent, as simultaneous treatment with low doses of SA and JA was shown to trigger synergistic effects on SA- and JA-responsive genes (Schenk *et al.* 2000). The suppression of the JA pathway is mediated by NPR1, the master regulator of the SA pathway. The SA-driven suppression of JA-responsive genes does not require nuclear localization of NPR1, indicating that cytosolic NPR1 is mediating negative effects on JA-signaling by a yet unknown mechanism (Spoel *et al*. 2003). ET modulates the NPR1-dependent JA-SA antagonism by potentiating the SA-dependent expression of *PR1* and rendering the JA-suppressing effects independent of NPR1 (Leon-Reyes *et al*. 2009). Often, ET interacts with JA in a synergistic manner (Pieterse *et al.* 2009). The expression of the JA-responsive gene *PDF1.2* requires the concomitant activity of JA and ET signaling cascades (Penninckx et al. 1998). Both JA and ET treatment induces the expression of the ET-responsive transcription factors ERF1 and ORA59, indicating that JA and ET signaling share nodes of convergence (Pré *et al*. 2008). ET also interacts with SA-dependent defenses. In tobacco, ET is indispensable for the activation of SAR upon TMV infection (Verberne *et al*. 2003). The extensive crosstalk between SA and ET has also been corroborated with the finding that the expression of SA-responsive genes was heavily affected in *Arabidopsis* mutants impaired in ET signaling (Glazebrook *et al*. 2003).

Beside the interaction of the major three defense hormones SA, JA and ET, it is also known that other chemical regulators participate in the defense crosstalk. ABA is known to generally attenuate SA- and JA/ET-dependent defense responses. In *Arabidopsis*, ABA inhibits the expression of JA and ET-responsive genes (Anderson *et al*. 2004). Moreover, ABA was demonstrated to interact antagonistically with SAR (Yasuda *et al*. 2008). Conversely, the activation of SAR inhibited the expression of ABA-responsive genes. Auxins are also known to affect the SA-JA-ET signaling network. The auxin responsive factors ARF6 and ARF8 have been demonstrated to promote jasmonic acid production (Nagpal *et al*. 2005), and auxin signaling promotes susceptibility of *Arabidopsis* to *P. syringae* (Navarro *et al*. 2006). Furthermore, both GA and brassinosteroids were shown to interact with the SA-JA-ET signaling network. DELLA proteins, the main regulators of GA signaling, were demonstrated to promote susceptibility to biotrophs and resistance to necrotrophs (Navarro *et al*. 2008). Similarly, brassinosteroids also interact with multiple hormones. They are known to affect ET biosynthesis, enhance auxin signaling and interact antagonistically with ABA (Zhang *et al*. 2009). In spite of the advances acquired over the past years, the majority of the mechanism underlying hormone crosstalk remains to be elucidated.

Concluding remarks

During the past years, much has been learned regarding the role of phytohormones during plant defense responses. Chemical regulators of plant growth were shown to be also orchestrating pathogen and pest defense. Although general roles of phytohormones in immune responses are known, the dissection of mechanisms triggering signal generation, transport and reception remains a challenge. Moreover, large-scale genomic analysis unveiled the presence of an intricate communication system driven by a multilayered crosstalk of phytohormones and metabolites. Advances in the field of metabolomics and system biology will help to dissect this extensive network and lead to the discovery of novel blends of alarm signals. A better understanding of the hormone- and metabolite-triggered plant defense communication will also impact the development of disease and pest resistance in crops.

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ID: Small yet mighty - microRNAs in plant-microbe interactions

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Abstract

Micro RNAs (miRNAs) are short non-coding RNAs of 20-24nt in length mediating RNA silencing, a eukaryotic, sequence-specific repressive gene regulation mechanism. In plants, miRNAs have a pivotal role during fundamental processes such as development, maintenance of genome integrity and abiotic stress responses. They originate from MIRNA genes that are transcribed by RNA polymerase II; MIRNA transcripts form imperfect fold-back structures that are further processed to miRNA duplexes. In Arabidopsis, over 180 MIRNA loci have been identified. Recent evidence shows that miRNAs are substantially implicated in regulating plant immunity. Pathogen attack triggers massive changes in the miRNA transcriptome; many of the altered miRNAs participate in controlling plant hormone pathways. Moreover, microorganisms are known to manipulate silencing pathways to counteract miRNA-mediated defenses. Thus far, miRNAs are believed to likely function as cardinal players in the concert of broad-spectrum disease resistance. Here, we summarize the highlights and latest findings of miRNAs as molecular regulators during plant-microbe interactions.

Introduction

Plants live in a fluctuant, unpredictable environment and being sessile, they are exposed to a large amount of potential stressors. Physiological flexibility is therefore a crucial attribute for plants when coping with biotic and abiotic stresses. Here, the regulation of gene expression is a key element in remaining adaptive to variable stresses. Such regulation mechanisms can impinge on all transcriptional levels including RNA processing, translation and posttranslational modifications. To facilitate genome integrity, plants employ an evolutionary conserved gene regulation mechanism called RNA silencing. This pan-eukaryotic sequence-specific mechanism is mediated by small RNAs (smRNAs), which are 19-30nt in length [1]. It affects

gene expression of cells by degradation of messenger RNA (mRNA), repression of RNA translation or modification of the chromatin state [2]. Generally, RNA silencing is mediated by perfect or nearly perfect double-stranded RNA (dsRNA), which are processed by the RNase III-like Dicer (DCL) proteins into pieces of 21 to 24nt in length. A single strand of such smRNA duplexes gets incorporated into slicer protein ARGONAUTE (AGO) and an RNA-induced silencing complex (RISC). There, a given smRNA functions as sequence-specific determinant guiding the degradation or translational repression of RNAs containing fully or partly complementary sequences.

Two main classes of smRNAs are known, microRNAs (miRNAs) and short interfering RNAs (siRNAs) [3]. Short interfering RNAs arise from long, perfect dsRNA derived from the transcription of inverted repeat sequences, convergent transcription of sense-antisense gene pairs, synthesis by RNA-dependent RNA polymerases (RDRs), or from virus-derived transcripts. In contrast, miRNAs originate from single-stranded RNAs transcribed from MIRNA loci. Most of the known MIR genes are transcribed by RNA polymerase II (Pol II), and form an imperfect fold-back structure that is further processed into a stem-loop precursor (pre-miRNA) [4]. The pre-miRNA is stabilized in nuclear processing centers called D-bodies. There, DCL1 generates a mature miRNA that is methylated by HEN1. Both pre-miRNAs and mature miRNAs are exported to the cytoplasm by the exportin-like protein HASTY and other co-factors. One strand of a mature miRNA duplex incorporates into a RISC to exert its molecular function, namely “slicing” of target mRNA, repression of translation or DNA cytosine and histone methylation. The other complementary miRNA strand, also called miRNA*, is usually degraded. AGO proteins are the central regulators in a RISC. In Arabidopsis, 10 AGO family members have been predicted, which fulfill variable roles during RNA silencing [5]. AGO1 and AGO4 are exerting a biochemical “slicing” activity, whereas AGO4 and AGO6 are guiding DNA cytosine methylation. Beside their cell-autonomous activity, miRNAs have recently been discovered to act as long-distance signal [6]. For instance, miR165/166 are known to regulate radial root patterning [7], as well as leaf polarity [8] and vascular specification through cell-to-cell transmission and therefore through a defined expression gradient. Moreover, some miRNAs such as miR399 are transmitted over a long distance through the phloem. Under phosphate

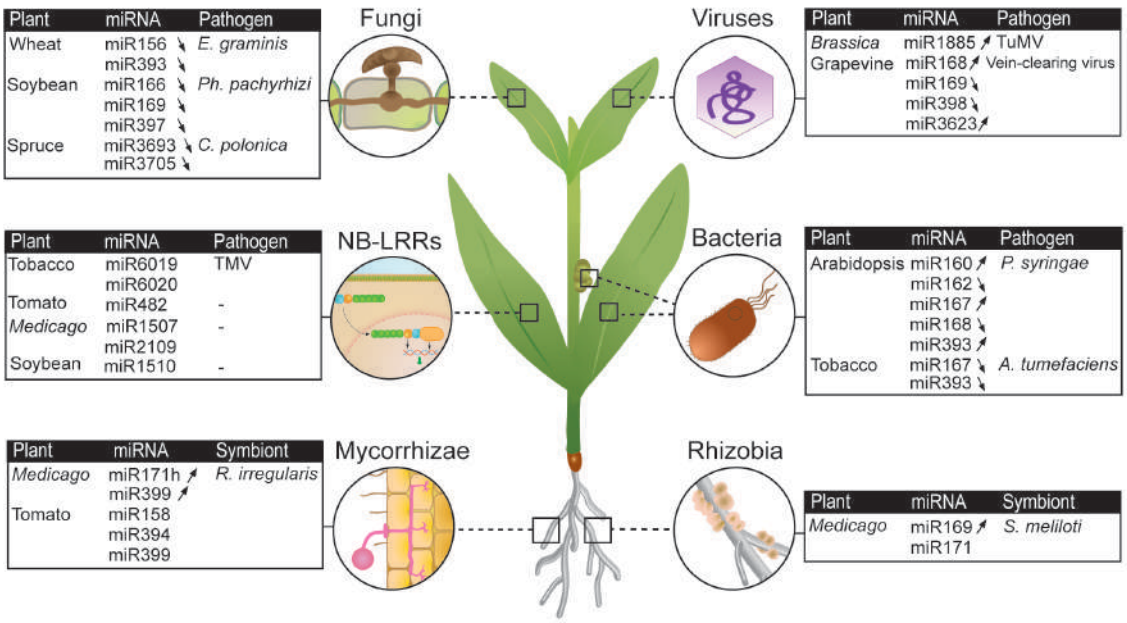

Figure 1. Overview of miRNAs contributing to plant-microbe interactions. In response to fungi, viruses, bacteria, rhizobia and mycorrhizae, various miRNAs show an altered expression level (upregulation indicated with ↗, downregulation with ↘). Microbes: *E. graminis* = *Erysiphe graminis*; *Ph. pachyrhizi* = *Phakopsora pachyrhizi*; *C. polonica* = *Ceratocystis polonica*; TMV = *Tobacco mosaic virus*; *R. irregularis* = *Rhizophagus irregularis*; TuMV = *Turnip mosaic virus*; *P. syringae* = *Pseudomonas syringae*; *A. tumefaciens* = *Agrobacterium tumefaciens*; *S. meliloti* = *Sinorhizobium meliloti*. miRNAs are also involved in regulating NB-LRR (nucleotide-binding site leucine-rich repeat protein)-mediated plant defense mechanisms.

deficiency, miR399 is induced in shoots and transported to the root where it downregulates PHO2 [9] which encodes an ubiquitin-conjugating E2 enzyme.

Despite the notable number of MIRNA loci in plants, only about 1% of all protein-coding genes are targeted by given miRNAs [10]. Nonetheless, miRNAs play a pivotal role in plant growth and development, as a vast number of them are regulating transcription factors that in turn are regulating versatile physiological processes. In the past few years, miRNAs were not only noted as crucial players in developmental processes, but also as regulators of plant abiotic and biotic stress responses. Over eighteen different miRNA families in various plant species are known to exert important roles during abiotic stress such as cold, heat, salt, drought, hypoxia and UV B [11]. Moreover, miRNAs are also crucial in response to nutrient deprivation, including phosphate, sulfate, copper and nitrogen deficiency. In addition, the role of smRNAs during antiviral defense is already well established [1]; however, it has been recognized recently that smRNAs including miRNAs are also widely involved in resistance

Plant	Interacting microbe	miRNA	Putative target genes	Ref.
Arabidopsis	*Pseudomonas syringae*	miR160	*ARF10, ARF16, ARF17* (AUXIN RESPONSIVE	[17]
		miR162	FACTORS)	[17]
		miR167	*DCL1*	[17]
		miR168	*ARF 6, ARF8* (AUXIN RESPONSIVE FACTORS)	[17]
		miR393	*AGO1*	[17]
			F-box proteins	
Brassica	*Turnip mosaic virus*	miR1885	TIR-NBS-LRR disease resistance protein	[25]
Grapevine	*Vein-clearing virus*	miR168	*AGO1*	[29]
		miR169	Zinc finger domain, NADP oxidoreductase, thaumatin	[29]
		miR398	Terpene synthase, cytochrome oxidase	[29]
		miR3623	TIR-NBS-LRR disease resistance protein	[29]
Medicago	*Rhizophagus irregularis*	miR171h	GRAS transcription factor *NSP2*	[50]
		miR399	Ubiquitin-conjugating E2 enzyme *PHO2*	[9, 47]
	Sinorhizobium meliloti	miR169	Transcription factor *HAP2-1*	[45]
		miR171	Transcription factors	[46]
Soybean	*Phakopsora pachyrhizi*	miR166	HD-ZIP transcription factor	[40]
		miR169	NUCLEAR FACTOR Y subunit	[40]
		miR397	Multicopper oxidase	[40]
Spruce	*Ceratocystis polonica*	miR3693	*LRR27* (TIR-NBS-LRR)	[42]
		miR3705	*LRR29* (TIR-NBS-LRR)	[42]
Tobacco	*Tobacco mosaic virus*	miR6019	Toll and Interleucin-1 receptor-NB-LRR *N*	[34]
		miR6020	Toll and Interleucin-1 receptor-NB-LRR *N*	[34]
	Agrobacterium tumefaciens	miR167	*ARF6, ARF8* (AUXIN RESPONSIVE FACTORS)	[23]
		miR393	F-box proteins	[23]
Tomato	*Rhizophagus irregularis*	miR158	*PPR* (PENTATRICOPEPTIDE REPEAT)	[48]
		miR394	F-box proteins	[48]
		miR399	*PHO2*	[48]

Table 1. Selected miRNAs from different plant species involved in plant-microbe interactions.

against bacteria, oomycetes, nematodes and herbivores [12]. Recent advances in RNA deep sequencing methods helped uncovering novel insights into entire miRNA transcriptomes during plant-pathogen interactions, corroborating the fact that miRNAs have a broad impact on plant defense mechanisms (Figure 1, Table 1). The multifaceted role of miRNAs during defense responses reaches from regulating pathogen effector recognition receptors, fine-tuning hormone pathways to coordinating defense gene expression. Here, we summarize the current knowledge of miRNAs as cardinal regulators of plant defense, focusing on plant-microbe interactions.

MiRNA-mediated defense against bacterial pathogens

The first indication of miRNAs as players during plant-bacteria interactions came with the finding that treatment of Arabidopsis with the bacterial peptide flg22 resulted in a rapid induction of miR393 [13]. This miRNA targets the mRNA of the F-box family genes *TIR1*, *AFB2*

and *AFB3*, thus resulting in a stabilization of Aux/IAA proteins, which repress auxin signaling. Overexpression of miR393 led to a reduced growth of *Pseudomonas syringae* pv. *tomato* DC3000. This observation is coherent with the finding that salicylic acid (SA) suppresses auxin signaling during bacterial defense [14], and that this suppression results in an enhanced resistance. Hence, the miRNA and SA pathway act synergistically during bacterial defense responses. Moreover, it has been recently discovered that the miR393*, the complementary strand of the mature miR393, also contributes to antibacterial immunity in Arabidopsis [15]. Upon bacterial infection, miR393* accumulates and promotes resistance by downregulating the *MEMB12* (SNARE) gene which encodes a vacuole-localized protein involved in membrane fusion. *MEMB12* deficient mutants exhibit an increased exocytosis of the pathogenesis-related protein PR1. Thus, downregulation of *MEMB12* by miR393* promotes the exocytosis of the antimicrobial PR1 protein. Further investigations showed that this mechanism is strongly regulated by AGO2, which binds miR393b* [15]. Intriguingly, bacteria seem to actively manipulate this miRNA-mediated resistance. The bacterial effector AvrPtoB downregulates the accumulation of the pri-miR393, the precursor of miR393 [16], probably through interference with factors required for the processing of miRNA precursors. The suppression of RNA silencing pathways by bacteria implicates an important role during antibacterial defense, as viruses also apply a similar strategy using suppressor proteins that interfere with the silencing machinery [1]. Using large-scale smRNA expression profiling, additional Arabidopsis miRNAs were found induced upon bacterial infection [17], such as miR167 and miR160, which both target auxin-related genes. Interestingly, bacterial infection also led to a downregulation of specific miRNAs, including miR168 and miR162 [17], which target *AGO1* and *DCL1*, indicating that setting up the miRNA pathway modulated by these proteins is an important feature during bacterial defense. Similarly, miR825, which targets genes that are not linked directly to plant defense, was downregulated upon bacterial infection [17].

Recent advances in RNA deep sequencing technologies have helped to uncover regulatory defense networks controlled by multiple miRNA families. Up to 20 miRNA families have now been described that are differentially expressed in Arabidopsis upon infection with different *Pseudomonas* strains [18]. Remarkably, the majority of the corresponding miRNA

target genes were linked to hormone biosynthesis and signaling pathways including SA, jasmonic acid (JA) and abscisic acid (ABA) pathways. SA usually mediates resistance to biotrophic pathogens, whereas JA is positively regulating defense responses against necrotrophs [19]. ABA can have both negative and positive effects on pathogen resistance [20]. Thus, miRNAs facilitate the fine-tuning of defense responses rather than targeting directly the plant immune system. Equivalently, *Xanthomonas axonopodis* pv. *manihotis* infection of cassava induces massive changes of the miRNA transcriptome [21]. Amongst upregulated miRNAs, some are targeting auxin-responsive factors, whereas some downregulated miRNAs are regulating disease resistance-associated genes. Nevertheless, deep sequencing of smRNAs that are bound to AGO1 resulted in the identification of miRNAs that were positively and negatively associated with direct defense responses [22]. Overexpression of miR160a enhances callose deposition and bacterial resistance, in contrast to miR398b and miR733 that act as negative regulators during the same process. Furthermore, miR393 and miR167 were found to be downregulated in tumors induced by *Agrobacterium tumefaciens* infections, and RNA silencing-deficient plants were hypersusceptible to the bacteria [23], suggesting that the miRNA pathways also contribute to defense responses against *A. tumefaciens*. In summary, miRNAs are important players in the chemical and molecular concert of antibacterial defense. Although the direct contribution of miRNAs and their specific targets remains unknown in most of the cases, it is clear that miRNAs contribute to the fine-tuning of defense responses by co-regulating physiological pathways that are linked to defense systems.

MiRNAs in plant virus-interactions

One of the main features of smRNAs in plants is their role in antiviral defense [1]. Antiviral silencing is exerted by siRNA pathways, which perceive virus-derived dsRNAs and process them further to smRNA, that trigger the silencing of viral RNAs. In turn, viruses evolved proteins that are able to suppress siRNA-mediated antiviral defense [24]. However, the role of miRNAs in antiviral defense remains elusive. Novel findings propose an ambiguous role of miRNAs in supporting antiviral resistance. In Brassica, bra-miR1885 was found induced upon *Turnip*

mosaic virus (TuMV) infection [25]; bra-miR1885 targets a TIR-NB-LRR (Toll/interleukin-1, nucleotide-binding site leucine-rich repeat) disease resistance gene. Probably, bra-miR1885 originates from inverted duplication events of TIR-NB-LRR coding genes. In Rice, RNA deep sequencing methods were applied to analyze miRNA profiles during infection with the *Rice dwarf virus* (RDV; dsRNA virus) and *Rice stripe virus* (RSV; negative sense and ambisense RNA virus) [26]. RSV infection triggered the accumulation of miRNA*s rather than the corresponding miRNAs, accompanied by the enhanced expression level of rice *DCL* and *AGO* genes. In contrast, RDV infection resulted in an upregulation of *OsRDR* genes. However, it is not known if the upregulation of *DCL*, *AGO* or *RDR* genes is linked to defense mechanisms. A recent study also investigated the Arabidopsis smRNA profile upon infection with the *Oilseed rape mosaic tobamovirus* (ORMV) [27]. Thereby, a size-specific enrichment of miRNAs was observed. As the corresponding mRNA targets did not exhibit a corresponding transcriptional change, it has been hypothesized that mature miRNAs only play minor roles during Arabidopsis:ORMV interactions. In a similar study, tomato plants challenged with the *Cucumber mosaic virus* (CMV) and the N5 strain of *Tomato mosaic virus* (ToMV) were subjected to a RNA deep sequencing study [28]. Over 85% of the analyzed miRNAs were found to be altered; however, the exact role if this phenomenon remains to be elucidated. In grapevine, infection with the *Grapevine vein-clearing virus* also triggers adaptations of the miRNA profile [29]. miR169 and miR398 were downregulated in response to viral infection, whereas miR168 and miR3623 were upregulated. However, it remains elusive whether the transcriptional change of these miRNAs has a direct or indirect effect on disease resistance. In summary, virus infections were shown to trigger changes in miRNA transcriptomes of several plant species. Nonetheless, for the majority of novel studies investigating the role of miRNAs in antiviral defense, the exact contribution to defense mechanisms is still unknown.

Resistance (*R*)-gene mediated innate immunity is regulated by miRNAs

Beside their role in fine-tuning hormonal pathways during defense responses, miRNAs have been recently demonstrated to play a pivotal role during early steps in plant immune

responses. The plant immune system is multilayered and consists of constitutive defense barriers such as cell walls or pre-formed toxins, as well as inducible local and systemic defenses [30]. Upon pathogen and pest perception, plants employ a so-called innate immunity which is mediated by two major receptor classes, namely pattern recognition receptors (PRRs, [31) and resistance (R) proteins which are generally intracellular nucleotide-binding site leucine-rich repeat (NB-LRR) proteins [32]. PRRs perceive conserved pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) such as flagellin or chitin. This triggers the activation of downstream defense pathways including the generation of reactive oxygen species (ROS) that are toxic for invaders, thus leading to a pattern-triggered immunity (PTI). However, successful pathogens are capable of suppressing PTI with the help of (Avr) proteins, pathogen effectors that either interact directly with PRRs or interfere with downstream factors [33]. These effectors are recognized and attenuated by NB-LRR proteins encoded by plant R-genes, thus leading to an effector-triggered immunity (ETI).

Over the past few years, novel findings uncovered a pivotal role of miRNAs during NB-LRR-mediated resistance. In tobacco, two miRNAs (nta-miR6019 and nta-miR6020) were discovered to cleave the mRNA of the tobacco *N*-gene that encodes a NB-LRR receptor [35]. *N* mediates resistance against *Tobacco mosaic virus* (TMV). Overexpression of both miRNAs resulted in an attenuated *N*-mediated resistance to TMV. Intriguingly, cleavage of *N* mRNA resulted in the accumulation of 21-nt siRNAs in phase with the cleavage site of nta-miR6019; the generation of these siRNAs requires RDR6 and DCL4. Hence, miR6019 triggers the generation of secondary siRNAs that act in concert to control innate immunity. In the same study, bioinformatic investigations also led to the identification of miRNAs targeting *R*-genes in tomato and potato. Similarly, the miRNA family miR1507, miR2109 and miR2118 were demonstrated to regulate NB-LRR encoding genes in legumes via phased siRNAs [35]. These findings were recently complemented by the demonstration that miR482/2118 targets NB-LRR encoding genes in tomato [36]. The miR482/2118 family was found highly abundant in the *Rutaceae*, *Solanaceae* and *Fabaceae*, suggesting a conserved regulatory role. Expression of miR482 was associated with the synthesis of secondary siRNAs, which also target several NB-

LRR encoding genes. Moreover, in bacteria- and virus-infected plants, the miR482-mediated suppression of NB-LRR was alleviated, resulting in enhanced levels of NB-LRR proteins.

In summary, miRNAs seem to be heavily implicated in regulating NB-LRR-mediated innate immunity. Normally, NB-LRRs are associated with race-specific immunity, thus specific NB-LRRs recognize race-specific effectors. A pathogen-mediated downregulation of miRNAs targeting NB-LRRs would lead to an overexpression of NB-LRRs in a non-race specific manner, thus resulting in a broader resistance. Therefore, low levels of NB-LRRs under miRNA control might reduce the plant defense costs, as multiple NB-LRRs can be rapidly induced upon pathogen stress.

MicroRNAs in defense responses against fungi and oomycetes

Besides playing an important role in innate immunity against bacteria, miRNAs are also involved in antifungal defense. Initially, the miRNA profile in loblolly pine (*Pinus taeda* L.) was investigated during gall development triggered by the rust fungus *Cronartium quercuum* f. sp. *fusiforme* [37]. This pathogen causes spindle-shaped galls on pine trees, being a serious threat for pine cultures. Transcriptional analysis of 26 identified pine tree miRNAs revealed a complex expression in and around galls. Some miRNAs were found to be significantly downregulated in galls, and others were upregulated around the infection site. The identified miRNAs were also shown to target defense-related genes including NB-LRR receptors, ubiquitin ligases, laccases and peroxidases. However, the concrete role of specific miRNA families remains elusive. In wheat (*Triticum aestivum* L.), the role of miRNAs upon infection with *Erysiphe graminis* f. sp. *tritici* (Egt) was investigated [38]. Twenty-four miRNAs were differently regulated in fungus-infected tissues. Interestingly, susceptible and near-isogenic lines carrying an *R*-gene showed a different miRNA profile. For instance, miR156 was repressed in the *R*-gene carrying wheat line compared to a susceptible one. Similarly, miR393 which targets auxin responsive factors was present in lower levels in resistant lines, suggesting that defense against *E. graminis* is linked to auxin signaling pathways. A similar approach was used for studying miRNA

expression profiles in soybean (*Glycine max* (L.) Merrill) upon infection with the oomycete *Phytophthora sojae* [39]. Three different soybean cultures, a susceptible and two resistant ones, were infected and subjected to a miRNA microarray analysis. The study demonstrated culture-specific alterations in the miRNA expression levels. Another recent work in soybean led to the discovery of miRNAs involved in the interaction with the rust fungus *Phakopsora pachyrhizi* [40]. A notable number of miRNAs including miR166, miR169, miR397 and miR1513 were found to be downregulated in response to fungal infection, whereas they were generally upregulated in during water stress responses. Intriguingly, the identified miRNAs were more downregulated in rust susceptible lines; several of those miRNAs target putative peroxidases that are contributing to ROS formation. Thus, a lower miRNA level in susceptible lines is not associated with an enhanced resistance based on an augmented ROS-mediated defense. A recent study also demonstrates a defensive role of miRNAs in belowground antifungal defense in cotton (*Gossypium hirsutum* and *G. barbadense*) [41]. Comparing smRNA libraries of tolerant and sensitive cultivars, a significant difference of the length distribution of smRNA sequence reads was observed. Moreover, over 65 miRNA families showed a distinctive expression profile in tolerant and sensitive cultivars. For instance, miR1917 and miR2118 were suppressed in response to fungal infection; miR1917 targets genes involved in ethylene (ET) signaling, and miR2118 regulates a putative NB-LRR protein, suggesting a concrete role of miRNAs in defense responses. However, as for the majority of miRNA-profiling studies, the specific molecular and chemical role of miRNAs remains elusive. A novel study also investigated the role of miRNAs in local and systemic defenses of Norway spruce (*Picea abies*) against the blue stain fungus *Ceratocystis polonica* [42]. Comparing miRNAs present in the secondary phloem of infected and control plants, it was shown that the expression levels of miR3693 and miR3705 were reduced in infected cells as well as in the phloem one day post inoculation. Nevertheless, the minor changes in predicted miRNA targets indicate that miRNAs are rather involved in the fine-tuning of defense responses than executing direct activity.

Recent miRNA profiling in plant-fungal interaction studies mainly focuses on plant smRNAs regulating endogenous genes. Nonetheless, novel work describes an additional interesting feature of smRNAs, namely the potential of host-derived dsRNA to trigger RNA

silencing in fungal cells [43]. Artificial dsRNA targeting transcripts of the powdery mildew fungus *Blumeria graminis* were expressed in barley (*Hordeum vulgare*) and resulted in an attenuated fungal development, demonstrating that host-derived dsRNA is able to silence genes in pathogen cells. Although it is not known if endogenous plant miRNAs are fulfilling a similar role, it is known in some cases that their targets share sequence similarities to pathogen-derived sequences. Thus, it cannot be excluded that plant miRNAs also exert a regulatory role in fungal cells.

MicroRNAs as regulators of symbiotic plant-microbe interactions

Beside their role in plant-pathogen interactions, miRNAs are also involved in regulating non-pathogenic plant-microbe interactions. For instance, the well-explored symbiotic interaction of nitrogen-fixing bacteria (rhizobia) and plants from the legume family is mediated by a miRNA regulatory system [45]. The interaction of rhizobia and plant roots initiates the formation of nodules, specific root organs in which rhizobia reduce nitrogen to ammonium. In *Medicago truncatula*, miR169 regulates the transcription factor *MtHAP2-1*, which is crucial for nodule development [45]. Interestingly, miR169 restricts the expression of *MtHAP2-1* to the meristematic zone of nodules, thus being crucial for their differentiation. Consequently, it was recently demonstrated in *M. truncatula* roots that miRNAs were selectively embodied to polyribosomes during infection with rhizobia [46]. In nodules, both miR169 and miR171, which target transcription factors, were shown to be associated with polyribosomes, which was in accordance with the expression levels of their target genes. In addition to their reported role in plant-rhizobia interactions, miRNAs were recently shown to participate in the symbiosis of plant roots with arbuscular mycorrhizae. In *M. truncatula*, a plant miRNA was shown to modulate the symbiosis with mycorrhiza by affecting phosphate signaling [47]. miR399 is upregulated in mycorrhized roots, thus downregulates *MtPHO2* expression. It has been hypothesized that *MtPHO2*-mediated high phosphate response could suppress the symbiosis with mycorrhizae; therefore, the repression of *MtPHO2* by miR399 ensures enhanced mycorrhizal colonization. Similarly, a global miRNA profiling study in tomato revealed of a set of

miRNAs regulated differentially in response to mycorrhization [48]. For instance, miR158, miR862-3p, miR319, miR394 and miR399 show all an altered expression pattern in tomato roots grown in different phosphate and mycorrhizal symbiosis conditions. Intriguingly, miR395, miR779, miR840 and miR867 showed an altered expression level in leaves upon mycorrhizal symbiosis. Thus, miRNAs seem to be in involved in local and systemic phosphate-depending signaling in plant-mycorrhiza interactions. Consistently, deep sequencing and RNA degradome analysis of mycorrhized *M. truncatula* roots uncovered a massive contribution of miRNAs during root symbiosis [49]. Over 20 miRNA families exhibited a different expression pattern in mycorrhized roots, which was consistent with the cleavage of their targets confirmed in the degradome analysis. The majority of identified target genes consist of transcription factors and genes involved in biotic stress and protein metabolism, indicating that miRNAs act as multilayered modulators during mycorrhizal symbiosis. Moreover, a novel study uncovered the role of a miRNA in negatively regulating mycorrhization thus preventing roots from being over-colonization by mycorrhizal fungi [50]. miR171h, which targets the GRAS transcription factor *NSP2* gene, was shown to be upregulated in the elongation zone of roots colonized by *Rhizophagus irregularis*. Plants over-expressing miR171h in roots exhibited a significantly reduced mycorrhizal colonization, similarly to *nsp2* mutants. In addition, in plants expressing a miR171h-resistant *NSP2* mRNA, fungal colonization was much enhanced up to the root elongation zone. These findings indicate that miR171h controls the extent of mycorrhizal colonization by regulating *NSP2*. In summary, miRNAs act as crucial regulators in both rhizobia- and mycorrhiza-triggered symbiosis, by either targeting nutritional pathways such as phosphate signaling, or by controlling the microbial colonization via the fine-tuning of transcription factors.

Conclusion and perspectives

The knowledge gathered over the past few years corroborates the fact that miRNAs exert a broad impact on regulatory mechanisms during plant-microbe interactions. However, the question remains to which extent miRNAs are actively modulating defense responses, or if they

are mainly fine-tuners of defensive systems by controlling hormonal networks and transcription factors. It is known that some smRNA mutants are more susceptible to pathogens [12], but the wide range of smRNA-controlled physiological processes makes it intricate to dissect direct links to defense mechanisms. Recent bioinformatic approaches uncovered large networks of miRNA families that show altered expression patterns upon infections by bacteria, viruses, fungi, oomycetes and even non-pathogenic soil-borne microbes (Figure 1, Table 1). These studies demonstrate that miRNAs are part of the regulatory networks orchestrating a multilayered plant defense. Nevertheless, the vast majority of studies demonstrate a stress-specific transcriptional change of miRNA transcriptomes; however, the exact role of given miRNAs during defense responses remains highly elusive. In this regard, an important future challenge will be to dissect these networks, and to address the role of specific miRNA families. Nevertheless, a few well-described cases demonstrate a direct role of miRNAs in plant-microbe interactions (Figure 1). Antibacterial defense is regulated by a miRNA targeting the auxin pathway [13]; plant *R*-genes are regulated by multiple miRNA families [36], fungal infection results in reduced levels of miRNAs targeting the ET pathway [41], and miRNAs control the colonization of roots by beneficial soil-borne microbes [50]. Overall, miRNAs likely provide an additional layer of flexibility to cope with diverse biotic stresses. As maintaining a defensive state is accompanied with high physiological costs, this flexibility provides an intricate cost minimizing system to plants, as proposed for miRNA-regulated NB-LRR genes [36]. In addition, the fact that pathogens employ suppressors that counteract miRNA pathways [16], and artificial host-derived dsRNAs are able to diminish the infection effectiveness of pathogens [43] implicates promising new ways for pathogen resistance programs in crops. In the near future, exploiting miRNA pathways in regard to pathogen resistance might provide a valuable option to control a broad range of pathogens and pests. Such an advance has been recently proposed in generating virus resistant plants [51]. It has been demonstrated that expression of modified miRNAs triggering the synthesis of artificial miRNAs (amiRNAs) that target viral RNA sequences can efficiently induce virus resistance. The advantage of this approach is the fact that artificial miRNAs can be generated in such a way that they are not targeting plant genes. Further advances in small RNA sequencing and their efficient control in

plant tissues will certainly provide the necessary tools to better understand the concrete molecular and chemical role of miRNAs during plant-microbe interactions, ultimately leading to miRNA-based improvements of biotic stress responses in important agricultural crops.

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Chapter II

A soil-free root observation system for the study of root-microorganism interactions in maize

II: A soil-free root observation system for the study of root-microorganism interactions in maize

adapted from:

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II: A soil-free root observation system for the study of root-microorganism interactions in maize

Abstract

Background and aims: The root surface of a plant usually exceeds the leaf area and is constantly exposed to a variety of soil-borne microorganisms. Root pathogens and pests, as well as belowground interactions with beneficial microbes, can significantly influence a plants' performance. Unfortunately, the analysis of these interactions is often limited because of the arduous task of accessing roots growing in soil. Here, we present a soil-free root observation system (SF-ROBS) designed to grow maize (*Zea mays*) plants and to study root interactions with either beneficial or pathogenic microbes.

Methods: The SF-ROBS consists of pouches lined with wet filter paper supplying nutrient solution.

Results: The aspect of maize grown in the SF-ROBS was similar to soil-grown maize; the plant growth was similar for the shoot but different for the roots (biomass and length increased in the SF-ROBS). SF-ROBS-grown roots were successfully inoculated with the hemi-biotrophic maize fungal pathogen *Colletotrichum graminicola* and the beneficial rhizobacteria *Pseudomonas putida* KT2440. Thus, the SF-ROBS is a system suitable to study two major belowground phenomena, namely root fungal defense reactions and interactions of roots with beneficial soil-borne bacteria.

Conclusions: This system contributes to a better understanding of belowground plant microbe interactions in maize and most likely also in other crops.

Introduction

Soil-borne pathogens are estimated to cause an annual monetary loss of US$4 billion in the US (Okubara *et al*., 2005). Root physiology under biotic and abiotic stress conditions is a field of increasing importance, specifically in view of improving crop yield and diminishing the possible negative environmental impact of agricultural practices (Gewin, 2010). However, most studies

on plant defense have been essentially focusing on aboveground plant parts. The root system plays a key role for the whole plant: roots are not only essential for nutrient and water uptake, they also contribute to adequately anchor the plant and have an important impact on its capacity to react to stress (Rasmann *et al*., 2008, Erb *et al*., 2009). In tobacco for example, nicotine is produced in the roots and translocated to the leaves in response to aboveground herbivore attack (Kaplan *et al*., 2008). *Colletotrichum graminicola* (Ces.) Wilson, the causal agent of corn anthracnose, infects both aboveground and belowground maize parts (Sukno *et al*., 2008). This pathogen is responsible for worldwide significant yield losses. Soil-borne pathogens, such as some species of *Fusarium*, *Phytophtora* or *Pythium,* have also an important economic impact. This stresses the importance of studies focussed on belowground plant interactions.

In addition to pathogenic interactions, beneficial interactions between microorganisms such as rhizobacteria or endophytic fungi and roots can have an impact on belowground stress reactions. The growing demand for sustainable alternatives to the massive input of pesticides in agriculture has led to an increase of interest concerning beneficial interactions betwwen plants and soil-borne microbes. Such beneficial microbes are able to stimulate plant growth and to induce aboveground systemic resistance against different types of stresses (Pineda *et al*., 2010). For example, filamentous fungi such as *Trichoderma virens* or *Piriformospora indica* induce resistance against biotrophic and necrotrophic pathogens in some cereals (Deshmukh *et al*., 2006, Djonovic *et al*., 2007). Various rhizobacteria such as some *Pseudomonas spp.* or *Bacillus spp.* also protect plants against above- or belowground stresses (reviewed in De Vleesschauwer and Höfte, 2009). Selected rhizobacteria have been tested for their capacity to enhance defense reactions against biotic and abiotic stress and to promote growth of maize plants (Huang *et al*., 2010, Nadeem *et al*., 2009). *Pseudomonas putida* KT2440 has been recently tested for its close interaction with maize seeds and roots (Neal *et al*., 2012) and for its capacity to induce resistance in Arabidopsis against the pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Matilla *et al*., 2010).

Research focusing on belowground plant defense is limited by the difficult accessibility of roots growing in soil. For some plants such as Arabidopsis (Gibeaut *et al*., 1997,

Hétu *et al*., 2005, Ishiga *et al*., 2011), tomato (Ahn *et al*., 2011), or rice (Kim *et al*., 2005) well-established growing systems circumventing the presence of soil are available as aeroponic, hydroponic, solid or semi-liquid cultures. A recently described aeroponic culture system allows even the study of root herbivory on Arabidopsis (Vaughan *et al*., 2011). For maize plants, most of the soil-free systems are based on a solid substrate like quartz sand (Hund *et al*., 2009a, Schulze and Pöschel, 2004) or glass beads (Boeuf-Tremblay *et al*., 1995). However, none of these maize systems allows an easy access to the root during all steps of development. Moreover, these substrates tend to stick to the roots and can interfere with some measurements and manipulations.

The aim of our study was to establish a growth system well adapted to maize. Moreover, this system should allow microorganism-root interactions and an easy access to the root system for *in vivo* observations, root inoculations with microbes and harvesting of material with the smallest possible damage to the roots. The soil-free root observation system (SF-ROBS) we finally established is adapted from the model of Hund *et al*. (2009b) created for maize root morphology analysis. The system has been used to study the response of maize roots to abiotic stresses, such as extreme temperature (Hund et al., 2012), low water potential induced by polyethylene glycol (Ruta *et al*., 2009) or aluminum toxicity (Trachsel *et al*., 2010) but was not tested for its suitability to study plant-microbe interactions.

In the following we report on the effect of the SF-ROBS on maize plants. Plants from standard soil-pot conditions were compared to plants grown in the SF-ROBS. Different parameters including the general aspect (habitus) of plants and measurements of different plant parts were assessed. Since one of the main reasons for amending the soil-free system was to obtain an easier access to the roots for specific studies in plant-microorganism interactions, we tested the suitability of the SF-ROBS for a pathogenic interaction with the hemibiotrophic fungus *C. graminicola* and a beneficial interaction with the rhizobacteria *P. putida* KT2440.

Materials and Methods

Growth of maize in the soil-free system

Figure 1. Fig. 1 Details of the SF-ROBS. (a) Elements of the pouch system with germinated maize seeds in the upper right corner. The SF-ROBS consists in pouches formed from black PE foil folded on itself and two humid filter papers. The layers are held together by paper clips. These pouches are attached to an aluminium rod with two foldback clips and placed in polypropylene containers that contain the maize nutrient solution (MNS). (b) View of the outside and the inside of the black PE foil pouch in which plants are growing. (c) Nine-day old maize plants. (d) Root systems of 5-day old plants in the SF-ROBS. Bar = 2 cm.

For sterilization, maize seeds (var. Golden Jubilee, West Coast Seeds, Canada) were rinsed in 70% ethanol, incubated for 5 minutes in 10% bleach and washed three times with sterile distilled water. The sterilized seeds were pre-germinated for 2-4 days between humid filter paper sheets (Filterkrepp Papier braun, 100 g m^{-2}, E. Weber & Cie AG, 8157 Dielsdorf, Switzerland) in a plant growth chamber (Percival AR-95L, CLF Plant Climatics GmbH, Wertingen, Germany) with the following conditions: 16 hours day at 26°C, 8 hours night at 22°C, 60% relative humidity and an irradiance of 400 µmol m^{-2} s^{-1}. They were then transferred into a soil-free root observation system (SF-ROBS). The SF-ROBS is based on the model of Hund et al. (2009b) with the following modifications: a larger size of pouches, the quality and quantity of the nutrient solution, the type of filter paper and an increased number of plants per pouch. In details, the SF-ROBS consists of a 34x64 cm polyethylene foil (PE-Teichfolie WA-1200, 0.5 mm, Walser AG, 8575 Bürglen, Switzerland), which was folded in half to form a 34x32 cm pouch (Figure 1). Each pouch had three 2x1 cm

slits, allowing the growth of the shoots; 3 cm below that aperture, a bulge made with a polyurethane bumper (3M Bumpon protective products, 12.7 mm diameter, 3.5 mm depth, 3M Europe, 1831 Diegem, Belgium) was pasted to one side of the pouch to keep the seedling in place. The seedling was fixed between two 33x33 cm filter papers (Filterkrepp) in the closed pouch by fixing two standard paper clips (43 mm long) on each side of the bulge. The pouch was attached to an aluminum rod with two foldback clips (Büroline, 51 mm, 69198 Schriesheim, Germany), one on each side of the upper edge of the pouch. The filter paper was humidified with maize nutrient solution (MNS). The MNS (a Ruakura solution adapted from Smith et al., 1983) consists of the following solutions: macronutrient stock solution A (2.31 g $MgSO_4.7H_2O$, 16.78 g $Ca(NO_3)_2.4H_2O$,8.48 g NH_4NO_3, 2.28 g KNO_3, 2.31 g $(NH_4)_2SO_4$ per liter); macronutrient stock solution B (2.67 g KH_2PO_4, 1.64 g K_2HPO_4, 6.62 g K_2SO_4, 0.60 g Na_2SO_4, 0.33 g NaCl per liter); micronutrient supplement (128.80 mg H_3BO_3, 4.48 mg $CuSO_4$, 81.10 mg $MnCl_2.4H_2O$, 0.68 mg MoO_3, 23.45 mg $ZnCl_2$, 809.84 mg $C_6H_5FeO_7$ per liter); each solution was sterilized by autoclaving; 200 mL of each of the macronutrient stock solutions was mixed with 100 mL of the micronutrient supplement and finally diluted with deionized water to a volume of 4.5 L. The pouches were put into polypropylene containers (60x40x32.5 cm, Rako, Migros, Switzerland), containing 4.5 L MNS, so that the bottom of the filter paper protruding from the pouches was constantly submerged in the nutrient solution. The filter paper was replaced every 3-4 days by new, moistened paper. The plastic containers with the growing maize plants were placed in the climate chamber at the same conditions as for seed germination. The SF-ROBS is also explained in the video available in the online supplementary material. To test an alternative nutrient solution, plants were grown as described above in Hoagland's No. 2 (Sigma-Aldrich, 3050 Spruce St., St. Louis, Missouri 63103, H2395) and compared with plants grown in MNS. Shoot and root fresh weights were measured for each plant (10 replicates per treatment) and possible symptoms of nutrient deficiencies were noted.

Plant growth in soil

For experiments in soil, maize plants were potted in polypropylene pots, 11 cm high, 4 cm in diameter (Semadeni, 3072 Ostermundingen, Switzerland). Seeds were sterilized and pre-

germinated as for the SF-ROBS. Germinated seeds were then transferred into a 50:50 (vol:vol) soil (25% compost, 12% sand and 63% peat; Ricoter Erdaufbereitung AG, 3270 Aarberg, Switzerland): sand (washed sand 0-4 mm, Jumbo, Switzerland) mixture. The soil:sand mixture was autoclaved one day before use. The plants were grown under the same conditions as for plants in the SF-ROBS.

Root infections with *Colletotrichum graminicola-gGFP*

To facilitate fungal detection and quantification a GFP-expressing strain of *C. graminicola* (gGFP; Erb *et al*., 2011) was used. *C. graminicola-gGFP* was maintained on potato dextrose agar under continuous light (70µmol m^{-2} sec^{-1}) and 25°C. Maize plants were removed from the SF-ROBS just before the inoculation process and the roots were inoculated by submerging the entire root system in a spore suspension (10^6 spores mL^{-1}, harvested from a 3 weeks old fungal culture) for 30 minutes in the dark at room temperature. Immediately afterwards, plants were put back in the SF-ROBS. Inoculations were performed at the end of a day period. Fungal root colonization was observed over a time period of 1-6 days post infection (dpi).

Plant inoculation with *Pseudomonas putida* KT2440

The rifampicin-resistant strain *Pseudomonas putida* KT2440 was grown on Luria-Bertani (LB; Difco LB, Becton, Dickinson and Company, 38800 Le Pont de Claix, France) agar supplemented with 100 µg mL^{-1} rifampicin (Fluka, Sigma-Aldrich, 9471 Buchs, Switzerland) at 25°C in the dark for 2-10 days. A single colony was picked and transferred to 100 mL of LB liquid medium with 100 µg mL^{-1} rifampicin for an overnight culture at 28°C, under continuous shaking at 150 rpm. The bacterial culture was divided in two, centrifuged at 3700 rpm, washed twice with sterile $MgSO_4$ 10 mM and the pellet was re-suspended in 25 mL of sterile M9 minimal medium (Sambrook and Russell, 2001). Maize seeds were first sterilized and pre-germinated for two days between Filterkrepp paper sheets as described above. The germinated seeds were then either mock-inoculated with sterile M9 minimal medium as control or with a fresh overnight bacterial suspension (1-3 x 10^{12} colony-forming units (CFU) mL^{-1} by shaking for 30 minutes at 35-40 rpm at room temperature. Bacterial root colonization

of such treated plants grown in the standard soil-pot system and in the SF-ROBS was then compared. Roots from 11-day old plants were harvested and cleaned from remaining soil under running tap water. Roots were then quickly dried and 100 mg of fresh weight per sample were ground in 600 µL sterile $MgSO_4$ 10 mM. For each plant (12 replicates), two root samples were collected: one near the seed, for the upper part of roots, and the other one in the primary root tip area, for the lower root parts. Serial dilutions of each sample were plated on solid King's medium B (Pseudomonas agar F, Merck KGaA, 64271 Darmstadt, Germany) supplemented with 100 µg mL^{-1} rifampicin to quantify the rifampicin-resistant *P. putida* KT2440 strain. After 18-20 hours of incubation at 25°C in the dark, the number of colony-forming units per gram of fresh root was determined.

Assessment of growth parameters

In order to compare plants from the SF-ROBS with plants grown in the standard soil system we measured shoot and root length as well as fresh weight of 12-day old plants. The part considered as shoot reached from the seed to the top of the longest leaf, whereas the root part was from the seed to the tip of the primary root. After these measurements, roots and shoots were dried separately in an oven at 70°C (Hybridisation oven/shaker SI20H, Stuart Scientific, UK) in coffee filter papers until sample weight remained constant. The dry weight of shoots and roots was then assessed.

Imaging

Microscopy of *C. graminicola-gGFP*-infected roots was performed using a (Eclipse E800, Nikon Corporation, Tokyo, Japan) microscope and a dissecting microscope (C-BD230, Nikon Corporation, Tokyo, Japan). Images were captured using a digital sight device (DS-L1, Nikon Corporation, Tokyo, Japan). GFP fluorescence of the fungal structures was excited with blue light (430-470 nm).

Statistical analysis

For the comparison of plants from the soil-free system with plants from the standard system, measurements were analyzed using a Student t-test, after passing a Shapiro-Wilk test as a normality test. All analyses were performed using the R software v2.12.1 (R Development Core Team (2011). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/). For comparison of plants grown in different nutrient solutions and for bacterial root colonization, the data were processed in SigmaPlot 11.0 (Systat Software, Inc., San Jose California USA, URL http://www.sigmaplot.com). Depending on the distribution of data, a Student t-test or a Mann-Whitney Rank Sum Test were performed.

Results

The SF-ROBS favors maize root development

To evaluate the effect of the SF-ROBS on maize growth in comparison to soil, we assessed the habitus of the plants (Fig. 2a and b) and measured parameters linked to plant fitness: the plant length (Fig. 3a), fresh and dry weight (Fig. 3b and 3c). Both planting methods yielded healthy plants with a similar habitus (Fig. 2a). This visual observation is supported by no significant differences for either shoot length or weight measurements between both systems (shoot length $p = 0.607$; shoot fresh weight $p = 0.415$; shoot dry weight $p = 0.106$). Interestingly,

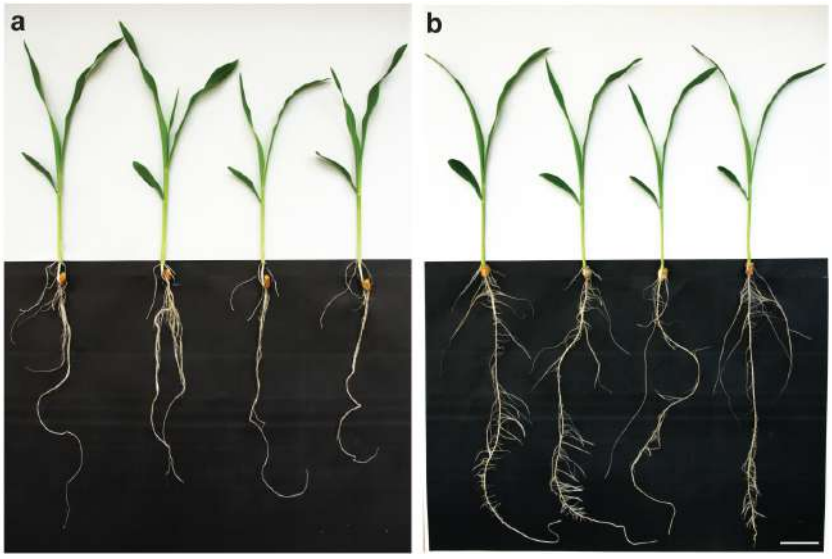

Fig. 2 Comparison between plants grown in standard soil conditions or in the SF-ROBS. (a) Nine-day old plants up-rooted from standard soil conditions. (b) Plants grown in the SF-ROBS. Bar = 4 cm.

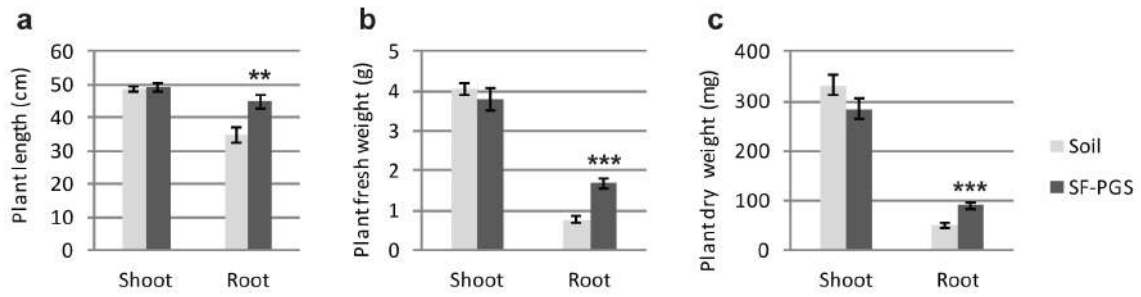

Fig. 3. Comparison of root and shoot length and weight. Shoot and root dry and fresh weight of 12-days old maize plants grown in standard soil conditions or in SF-ROBS were assessed: (a) shoot and root length (cm), (b) shoot and root fresh weight fresh weight (g), and (c) shoot and root dry weight (mg). Error bars indicate the standard errors for the average values of 18 replicates. Asterisks indicate a significant difference in a Student t-test (** = $p<0.01$, *** = $p<0.001$).

plants from the SF-ROBS had a better developed root system with enhanced branching compared to plants from the soil system (Fig. 3). This was reflected in root length (44.82 cm), root fresh weight (1.68 g) and root dry weight (90.26 mg) of SF-ROBS-grown plants, which were significantly higher compared to soil-grown plants (root length 34.68 cm, $p = 0.002$; root fresh weight 0.77 g, $p = 3.325 \times 10^{-6}$; root dry weight 50.38 mg, $p = 7.576 \times 10^{-5}$). Two different nutrient solutions were tested in the SF-ROBS: the MNS and the standard Hoagland's No. 2 solutions. Whereas plants with MNS were healthy, plants with Hoagland's No. 2 exhibited leaf chlorosis (Fig. 4).

Figure 4. Leaves of 12 days-old maize plants (var. Jubilee) grown in the SF-ROBS containing Hoagland's No. 2 (H) or MNS. Leaves grown in Hoagland's No. 2 exhibit a chlorotic phenotype. Bar = 1cm.

Moreover, plants grown in MNS had a significantly higher fresh weight of leaves (0.315 g; Mann-Whitney Rank Sum Test, $p = <0.001$) compared to plants grown in the standard solution (0.160 g). The root fresh weight of plants in MNS (0.496 g) was similar to plants in Hoagland's No. 2 (0.432 g; Student t-test, $p = 0.849$).

The SF-ROBS facilitates colonization of maize root by *Colletotrichum graminicola*

To investigate whether the SF-ROBS can be used for fungal infection assays, roots were inoculated with the GFP-expressing pathogenic fungus *C. graminicola-gGFP* (Fig. 5). By monitoring the colonization over time, we found a characteristic infection pattern: four days after inoculation, the appearance of acervuli was observed (Fig. 5a, c and d). In later infection stages, epidermal cells packed with falcate conidia were detected (Fig. 5e). Mature roots, root caps and the root elongation zones were rapidly and consistently colonized by *C. graminicola-gGFP* (data not shown), suggesting that there was no fungal penetration preference for the different zones of the roots. During advanced infection stages (>4 dpi), colonized roots showed a brown discoloration (not shown).

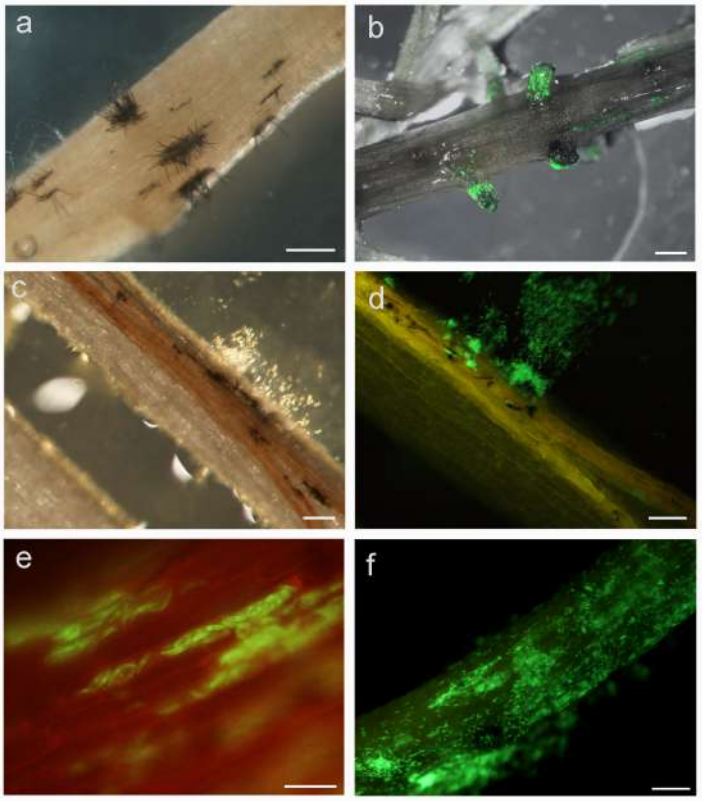

Fig. 5. *C. graminicola*-gGFP-infection of maize roots grown in the SF-ROBS. Roots of 10-days old plants were inoculated by dipping in a spore suspension and kept in the SF-ROBS for further development. Colonization was observed under epifluorescence (b, d, e and f) and bright field illumination (a, c). (a) Acervuli with characteristic setae on infected roots, 4 dpi. (b) Early infection stage on an inoculated root (1 dpi); the fluorescence-image is superimposed over the bright field picture. (c, d) The same infected root viewed under UV and visible light at 5 dpi, showing conidia flowing out of acervuli. (e) Root epidermal cell packed with falcate conidia, 5dpi. (f) Heavily infected root at late stage, 6dpi. Bars = 100 µm (a, b, c, d, f) and 30 µm (e).

The SF-ROBS provides accurate conditions for root colonization by rhizobacteria

In order to test whether the SF-ROBS can also be used in research with beneficial root colonizing bacteria, the plants were inoculated with *P. putida* KT2440. The number of colony-forming units of *P. putida* KT2440 extracted from such inoculated roots of 11-day old maize

plants was assessed to evaluate the capacity of the rhizobacteria to efficiently colonize roots in the SF-ROBS compared to the soil-pot standard system. There were no significant differences in root colonization of plants in the SF-ROBS (3,53 x 10^5 CFU g^{-1} of fresh roots) and in soil (2.5 x 10^5 CFU g^{-1} of fresh roots; Mann-Whitney Rank Sum Test, $p = 0.29$). The amount of bacteria differed between the upper andlower root parts. This difference was observed in both growing systems. There were more bacteria present in the upper parts (3.47 x 10^6 CFU g^{-1} of fresh roots in the SF-ROBS and 5.85 x 10^5 CFU g^{-1} of fresh roots in soil) than in the lower parts (3.60 x 10^4 CFU g^{-1} of fresh roots in the SF-ROBS and 8.7 x 10^4 CFU g^{-1} of fresh roots in soil; Mann-Whitney Rank Sum Test, $p = < 0.001$ for SF-ROBS and $p = 0.002$ for soil).

Discussion

The SF-ROBS facilitates the growth of young maize plants

Several culture methods have been described to allow growth of plants without soil. However, soil-free systems to study plants with higher biomass than Arabidopsis and demanding greenhouse care such as the monocotyledon model plant maize are less established. Gunning and Cahill (2008) described a method by which *Lupinus angustifolius* was cultivated in a system using blotting paper that was embedded between two plastic plates with a given spacing. They reported to have successfully tested maize in this system. Nevertheless, we found that this system was less viable in our hands for growing maize compared to our system. A major advance in this field was achieved with the development of a soil-free phenotyping platform for maize (Hund *et al*. 2009b). This system facilitates non-destructive digital assessments of the root morphology. Modification and adaptation of this method led to the development of the SF-ROBS presented here. Three critical elements of the SF-ROBS were identified: the pouches, in which the roots are growing, the filter paper providing the nutrients for the roots, and the nutrient solution itself. The pouches should shield the roots from light, and their surface should remain as aseptic as possible and not be toxic to plants. PE foil, which is commonly used to make garden ponds, was chosen since it satisfied these criteria. Choice of the right filter paper was found to be even more crucial. Standard filter or blotting paper inhibited growth (data not shown), therefore we used filter paper that had not been

treated with bleaching chemicals. Similarly, the nutrient solution had to be optimized for maize. Modified Ruakura solution (adapted from Smith *et al*. 1983) was identified as most convenient solution. Commonly used standard media such as Hoagland's No. 2 were found to be insufficient leading to visual chlorosis of the leaves. The leaf color and growth rate were similar in both SF-ROBS and soil conditions. Roots grown in the SF-ROBS were longer and exhibited enhanced branching. Consistently, the fresh weight and dry weight of roots was found to be higher for SF-ROBS- grown plants, indicating that the SF-ROBS favors root development. The reason for this may lie in the reduced contact between the roots and the substrate in the SF-ROBS compared to soil. To counteract this situation, the plant will enhance the root surface contact for nutrients uptake by inducing a higher production of roots. Taken together, we demonstrate that the SF-ROBS is a convenient system to cultivate young maize plants. The main advantage of the SF-ROBS is the easy root handling. Roots can be accessed during any early developmental stage, and they can easily be removed from the system to perform treatments such as inoculations or microscopic observations. Moreover, harvesting roots for further experiments such as gene expression analysis or metabolomic fingerprinting is simplified using the SF-ROBS. Removing soil residues from roots is time-consuming and often leads to tissue damage, which might interfere with downstream experiments. However, the SF-ROBS requires regular changes of filter papers and a growth environment to limit the risk of contamination favored by the presence of a constant humid filter paper. Another limiting factor of the SF-ROBS is the root growth and age of the plants. Limited by the size of the pouch in our system, we were able to keep the plants no longer than about 21 days in the SF-ROBS (corresponding to a maize plant with 4 developed leaves). The short growth period does not allow studies plant-microbe interactions which need a longer time to establish. An extended culture time would require a size modification of the pouches to accommodate a larger root system. Efforts to increase the size of such paper-based rhizotron systems are in progress.

The SF-ROBS is convenient to study fungal root infections

Thus far, described culture methods of soil-free systems in combination with pathogens are scarce and often limited to *in vitro* analysis. Here, we present a soil-free growth system

allowing *in vivo* observations of fungal root infections of maize. The *C. graminicola* infection assays performed in the SF-ROBS resulted in a colonization behavior and pattern which are similar to observations made for soil-grown plants (Sukno et al. 2008), indicating that the SF-ROBS does not alter the natural infection process. We observed an earlier development of acervuli on infected roots in the SF-ROBS compared to published data from a soil system (Sukno *et al*. 2008). This suggests that the infection process might be favored in the SF-ROBS, possibly through the constant humidity of the system but also by the enhanced infection efficiency or a decreased antiphytopathogenic potential in the paper compared to natural soil. Normally, maize roots are infected by either soaking seedlings (2 days after germination) in a spore suspension or by growing older seedlings in vermiculite mix containing agar plugs from *C. graminicola* cultures (Sukno *et al*., 2008). Especially the agar plug method makes it difficult to control the colonization of a specific root part, which results in a less efficient infection rate. The SF-ROBS allows the infection of specific root parts and enables easy sampling for downstream plant-pathogen interaction analysis such as gene expression profiling or hormone quantification.

The SF-ROBS allows the study of root-bacteria interactions

Despite the fact that plant-beneficial microbe interactions are an emerging research field, the molecular and chemical mechanisms underlying these interactions remain largely unknown. As for root-pathogen interactions, the arduous accessibility of roots is also an issue when studying beneficial root microbes.

The ease of root harvesting makes the SF-ROBS an advantageous system to study the interactions of roots and beneficial root-colonizing microbes. Instead of a bacterial inoculation of soil, it is possible to inoculate the roots directly without damaging them. Here, we show that the SF-ROBS is suitable for cultivating maize roots inoculated with the root beneficial bacterium *P. putida* KT2440. *P. putida* KT2440 was successfully recovered from 11-day old roots grown either in the SF-ROBS or in the soil, showing that the SF-ROBS does not inhibit bacterial development in plant roots. A similar amount of bacteria was found in roots of SF-ROBS-grown plants as well as in roots of soil-grown plants, indicating that growth

conditions do not affect the potential of bacterial root colonization. Bacterial colonization along the length of the root was similar in maize plants grown in the SF-ROBS as for plants grown in soil and as previously described in other systems (Simons *et al*., 1996) with a decreased gradient of bacteria from the root base towards the root tip. Furthermore, the density of *P. putida* KT2440 on roots is critical for bacterial contribution to plant defense. Raaijmakers et al. (1995) showed that a bacterial density of approximately 10^5 CFU per gram of root is required for direct disease suppression and induction of plant resistance. Hence, the SF-ROBS could be used for studies on maize resistance mechanisms induced by rhizobacteria. Since the availability of nutrients for the plant is controlled through the supply by the MNS growth medium in the SF-ROBS, it would be also easily possible to study the importance of selected nutrients or combinations thereof on root colonization, on direct bacterial inhibitory effects or on plant induced resistance mechanisms.

In summary, we have presented here a soil-free growth system that allows the non-destructive study of interactions of roots with pathogenic and beneficial microorganisms. Moreover, our system is suitable for crop plants such as maize and could therefore contribute to a better understanding, and finally management, of belowground stress situations of plants.

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Chapter III

Induced resistance in maize is based on organ-specific defense

III: Induced resistance in maize is based on organ-specific defense

adapted from:

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III: Induced resistance in maize is based on organ-specific defense

Summary

To get more insight into the intricate interplay between maize (*Zea mays*) and the fungal pathogen *Colletotrichum graminicola*, the local and systemic molecular and chemical defence responses of maize leaves and roots were simultaneously investigated and compared. Similar gene expression and hormonal patterns were detected in both above- and belowground organs; however, roots responded more rapidly and accumulated higher levels of defence-related hormones than leaves. Leaf and root infection with *C. graminicola* triggered systemic resistance in the foliage against the same fungus. This systemic defence response was associated with systemic transcriptional adaptations, and with elevated levels of salicylic acid and abscisic acid. Metabolomic profiling revealed significant differences in the composition of secondary metabolites in leaves and roots, corroborating the fact that both organs employ a distinct chemical defence system. In addition, higher basal levels of antimicrobial flavonoids suggest an enhanced basal defensive state of roots. Our findings uncover tissue-specific local and systemic antifungal defence mechanisms in maize.

INTRODUCTION

Members of the genus *Colletotrichum,* causing anthracnose and blights, but also devastating post-harvest rots, belong to the top 10 fungal plant pathogens attacking economically important crops (Dean *et al*., 2012). As a hemibiotrophic pathogen, *Colletotrichum* grows first biotrophically to soon switch to a necrotrophic lifestyle (Wharton *et al*., 2001; Mims and Vaillancourt, 2002). Due to the recently published genome sequences of *Colletotrichum* (O'Connell *et al*., 2012), a comparative genomic analysis of fungal lifestyle transitions is now possible.

Maize (*Zea mays*) anthracnose, caused by *C. graminicola,* has a worldwide impact on corn production, affecting all parts of the crop plant at every growth stage (Bergstrom and Nicholson, 1999) and leading to annual losses of up to 1 billion dollars in the U.S. (Frey *et al.*, 2011). Leaf blight and stalk rot are the predominant symptoms, but *C. graminicola* is also able to infect maize roots (Sukno *et al*. 2008). Root infections differ from leaf infections as both rhizodermal and cortical cells are infected in a mosaic pattern distinct from the typical cell-to-cell spread observed in leaves (Sukno *et al*. 2008). In contrast to leaf infections, where initial symptoms appear after a few days, roots can stay symptomless for up to 42 days post infection (dpi) (Sukno *et al*. 2008). Interestingly, root infections can also result in systemic infections of aerial parts (Sukno *et al*. 2008), suggesting an important role for root infections in the disease cycle.

Compared to aboveground defences, knowledge on root defences is more elusive, and comparative analyses of above- and belowground defences are scarce (Rasmann and Agrawal, 2008). *Arabidopsis* roots exposed to salicylic acid (SA) showed a significant difference in the SA-dependent transcriptome compared to SA-treated leaves (Badri *et al*., 2008). Similarly, roots and leaves are known to differ in the concentration of various defensive compounds. In *Nicotiana sylvestris*, the levels of alkaloids are up to 6 fold higher in roots than in leaves (Rasmann and Agrawal, 2008). During the past few years, extensive studies of root herbivore defences revealed a pronounced role for roots as part of local and systemic plant defensive systems (reviewed in Erb *et al*., 2009c). For example, roots can act as reservoir of secondary metabolites which are translocated to distal plant parts during biotic attacks. In tobacco plants, nicotine is predominantly synthesized in roots and transported to the shoots (Dawson and Solt, 1959) and belowground herbivory in maize triggers the systemic induction of the antibiotic compound DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) in aboveground parts (Erb *et al.*, 2009a).

Colletotrichum has a tradition as fungal model organism. *C. lagenarium* infection studies on cucumber contributed to the initial characterization of systemic acquired resistance (SAR; Hammerschmidt *et al*., 1982), usually induced upon local attack by necrotizing or

hypersensitive response triggering pathogens (Vlot *et al*., 2008). Such attacks lead to the generation of mobile alarm signals that are translocated through the vascular system or airborne to distal not-yet-infected plant parts, where they lead to SAR. SAR can also be triggered by application of SAR-inducing chemicals (Oostendorp *et al*., 2001). Induced resistance has been extensively investigated in the model plants *Arabidopsis* and tobacco, leading to the identification of a set of critical long-distance signals (Dempsey and Klessig 2012), such as glycerol-3-phosphate (G3P, Chanda *et al*., 2011), azelaic acid (Jung *et al*., 2009), the volatile methyl salicylate (MeSA, Park *et al*., 2007), or dehydroabietinal (DA; Chaturvedi *et al*., 2012). However, less is known about SAR mechanisms in monocots (Balmer *et al*., 2012). Although there is solid evidence that chemical SAR inducers such as BTH and probenazole (Görlach *et al.,* 1996; Nakashita *et al*., 2002) are active in monocots, data describing biologically-induced SAR are scarce. In rice, *Pseudomonas syringae* pv. *syringae* infections were shown to trigger SAR against *Magnaporthe grisea* (Smith and Métraux 1991). Similarly, SAR against leaf rust has been noted in wheat (Barna *et al*., 1998). It also remains to be shown whether the systemic signals described for dicots also play a role in monocots. Moreover, classical SAR research has mainly focussed on events in aboveground parts thereby neglecting the root system.

Little is known about local and systemic defence responses of maize upon above- and belowground fungal attack. In this study, we simultaneously investigated both local and systemic organ-specific above- and belowground maize defence responses during *C. graminicola* attack, providing an insight into a multi-layered defence system. Our studies on metabolomic responses to *C. graminicola* attack in shoots and roots show that both organs employ different chemical defence strategies. We also present evidence that the systemic transcriptional, hormonal and metabolomic adaptations occurring after local leaf and root infections are correlated to the expression of SAR against *C. graminicola* in systemic leaf tissues.

Results

Disease progress in leaves and roots

To compare both *C. graminicola* leaf and root infections under identical experimental conditions and time points, 12-day-old maize plants were inoculated with *C. graminicola*-gGFP (Erb *et al*., 2011), and fungal development was documented at 36, 48 and 96 hours post infection (hpi) using confocal microscopy (Figure S1). The chosen time points correspond to the known lifestyle transitions of *C. graminicola* during hemibiotrophic growth on leaves (Vargas *et al*., 2012). The disease progress differed between leaves and roots. In leaves, characteristic biotrophic structures such as bulbous biotrophic hyphae were present at 36 hpi (Figure S1a), whereas in roots, thin runner hyphae were observed at the surface (Figure S1b). At 48 hpi, the first necrotic symptoms on leaves marked the transition to necrotrophy. At this time, thin secondary hyphae grew without specific orientation inside leaf tissue (Figure S1c), while in the roots hyphae grew parallel to the longitudinal axis of epidermal cells (Figure S1d). At 96 hpi, leaf tissues were colonized by a dense hyphal network (Figure S1e). In contrast, roots were infected in a mosaic pattern consisting of a few colonized cells, which were often packed with falcate conidia, while neighbouring cells remained uninfected (Figure S1d). Thus, compared to the extensive colonization of leaves, fungal growth is restricted in roots. Selective inoculation of the different root types, namely primary, seminal and crown roots (Hochholdinger and Tuberosa 2009), showed that they were all susceptible to *C. graminicola* (Figure S2).

The transcriptional state of infected leaves and roots differs

The organ-specific defence responses at the molecular level were investigated by quantitative real-time PCR (qRT-PCR). Based on a marker system for biotic and abiotic stress responses (Erb *et al*. 2009a) primers for 44 defence- and stress-related genes were designed (Table S1). The local and systemic transcriptional response of leaves and roots at distinct time points including lifestyle transition points was compared (Figure 1). With a few exceptions, both leaves and roots showed a similar local expression pattern (Figure 1a). The pathogenesis related gene *PR2* was upregulated at later time points (96 hpi) only in infected roots. Similarly,

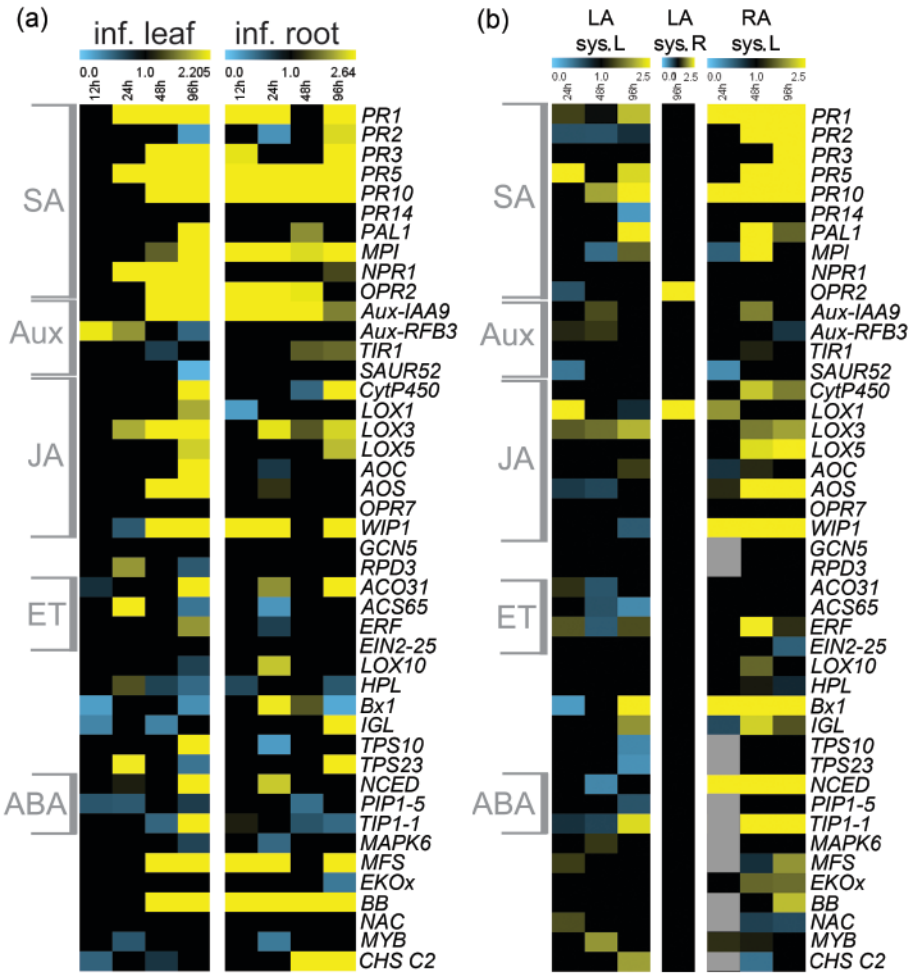

Fig. 1. Comparative analysis of defence genes. (a) *C. graminicola*-infected leaves (inf. leaf) and roots (inf. root) at 12, 24, 48 and 96 hpi. (b) Systemic gene expression profiles; systemic leaves of *C. graminicola*-leaf-infected plants (LA sys. L), systemic roots of leaf-infected plants (LA sys. R), and systemic leaves of root-infected plants (RA sys L) at 24, 48 and 96 hpi. Gene expression is indicated as fold induction compared to mock-treated plants; blue = downregulated genes, yellow = upregulated genes; SA (salicylic acid), Aux (auxin), JA (jasmonic acid), ET (ethylene), ABA (abscisic acid).

CHS C2, coding for a chalcone synthase important in flavonoid synthesis, was exclusively induced in infected roots. Moreover, the lipoxygenase (LOX) pathway was found to be involved more in leaf infection than in roots; *LOX1* for instance was only up-regulated in leaves. Although the transcriptional pattern was similar, roots responded faster than leaves. Already at 12 hpi, *PR* genes as well as *BB*, a Bowman-Birk serine trypsin inhibitor, a potential antimicrobial protein, were upregulated in the roots. In contrast, *Bx1* and *IGL*, both genes coding for enzymes involved in benzoxazinoid (BX) synthesis were downregulated at early leaf infection stages. Notably, in contrast to the rapid induction of defence genes in roots, leaves

responded generally later (for induction levels see Table S2). For example, there was a slight induction of *PR1* at early time points in roots. In contrast, *PR1* levels in leaves increased up to 800-fold at 48 and 96 hpi (Figure 2).

The transcriptional state of the different maize root types was assessed at 6 dpi (Figure S3) and showed the highest response in seminal roots, whereas a smaller number of genes was induced in crown roots. The general gene expression pattern, however, was similar with a few exceptions. *CHS C2* for instance was downregulated in crown roots, and *PR2* was only induced in seminal roots.

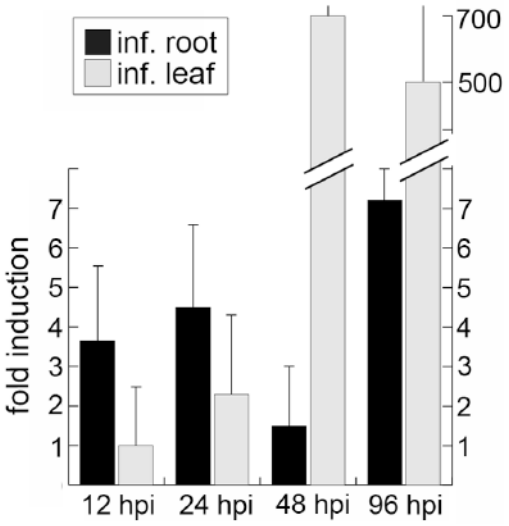

Fig. 2. Expression level of *PR1* in *C. graminicola*-infected roots (inf. root) and infected leaves (inf. leaf). Expression is indicated as fold change relative to mock-treated plants.

Leaf and root infections influence hormone levels

To elucidate the contribution of plant hormones to above- and belowground defence responses against *C. graminicola* SA, jasmonic acid (JA) and abscisic acid (ABA) were quantified by UHPLC-MS/MS (ultra-high pressure liquid chromatography-tandem mass spectrometry) during biotrophic (36 hpi) and necrotrophic (96 hpi) infection stages (Figure 3). While no significant differences in hormone levels between control and infected leaves were observed at 36 hpi (Figure 3a), levels of SA and ABA were significantly higher in roots at 36 hpi, whereas JA levels were lower in this organ (Figure 3b). However, a significant increase in levels of SA, JA and ABA was observed in both leaves and roots at 96 hpi (Figure 3c, d). Interestingly, the concentration of all three hormones was higher in infected roots compared to infected leaves.

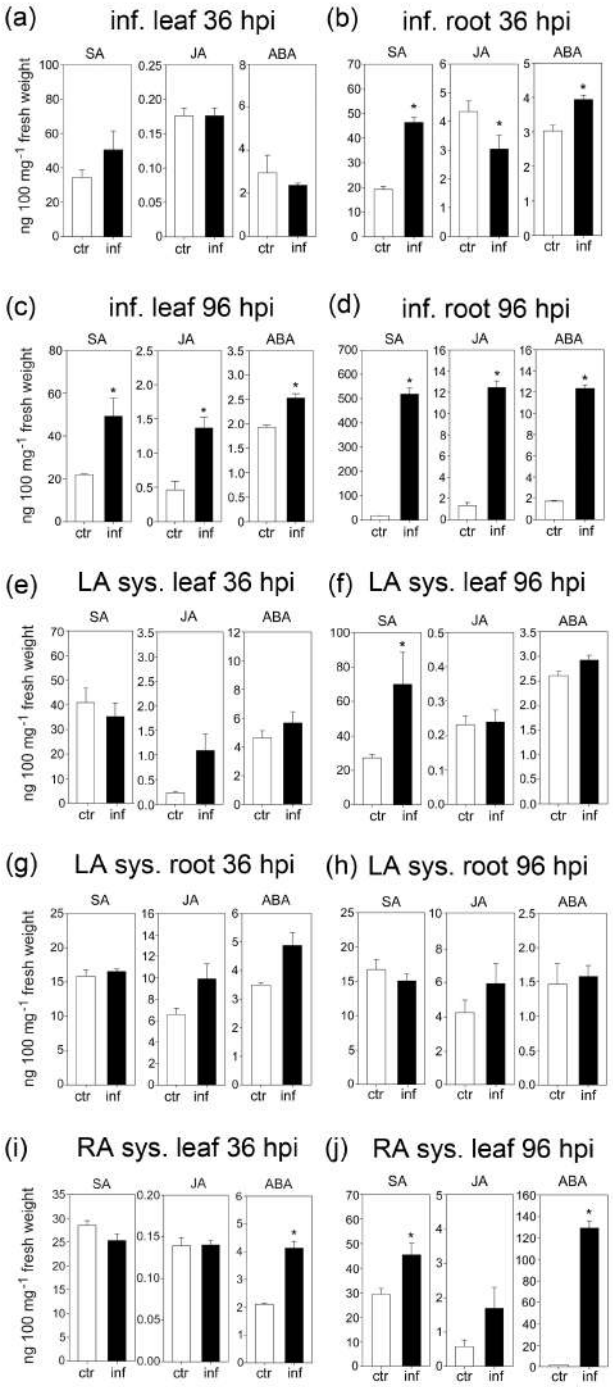

Fig. 3. SA (salicylic acid), JA (jasmonic acid) and ABA (abscisic acid) quantification of *C. graminicola* local and systemic infection assays. (a) Infected leaves 36 hpi, (b) infected roots 36 hpi. (c) Infected leaves 96 hpi, (d) infected roots 96 hpi. (e) Quantification of SA, JA and ABA in systemic tissues upon local *C. graminicola* leaf and root infection 36 hpi. (f) Systemic leaves of plants that received a local leaf infection 96hpi; (g) systemic roots 36hpi and (h) 96hpi from plants that received a local leaf infection; (i) systemic leaves of root-infected plants 36hpi and (j) 96hpi. LA = leaf assay; RA = root assay; sys. = systemic. Ctrl (control) and inf. (infected) plants were compared using Tukey's range test; significant differences ($P<0.05$) are indicated with (*).

Local infections induce systemic transcriptional responses

Systemic transcriptional changes were analysed by qRT-PCR using the same defence marker system as described above (Figure 1b). Changes were assessed in a leaf assay (LA), where a local leaf was infected and non-infected systemic leaf and root tissues were analysed.

Additionally, in a root assay (RA), the root system was infected and gene expression was quantified in systemic non-infected leaves. Systemic transcriptional adaptations were observed over a time course of 96 h starting at 24 hpi. In systemic leaves, the majority of upregulated genes such as *PR1*, *PR5*, *PR10* and *PAL1,* were SA-associated. *CHS C2*, *Bx1* and *IGL* were also induced, whereas terpene synthase genes such as *TPS10* and *TPS23* were downregulated at 96 hpi. In contrast to systemic leaves, only two genes with significantly different expression pattern were detected at 96 hpi in systemic roots, namely *OPR2* and *LOX1*. Interestingly, the transcriptional response of systemic leaves after root infection was much faster and stronger than after leaf infection. Already 24 hpi, pathogenesis-related genes like *PR1* and *PR10* were highly induced, in addition to *Bx1, LOX1* or *WIP1*. *NCED*, a key enzyme in ABA synthesis, was also rapidly induced, pointing to a contribution of ABA in defence responses of systemic leaves. In contrast to local leaf infections, root infections also triggered the upregulation of *PR3*, *CytP450, LOX5*, *AOS* and *BB*. To test for a possible root-to-root signalling, primary roots were infected and the expression of selected defence-related genes was analysed in systemic seminal and crown roots at 4 dpi (Figure S4). *PR* genes were induced in both systemic seminal and crown roots. However, seminal roots had a higher number of induced genes compared to systemic crown roots, where only *PR1* was induced. Nevertheless, this provides evidence of a systemic root-root signalling.

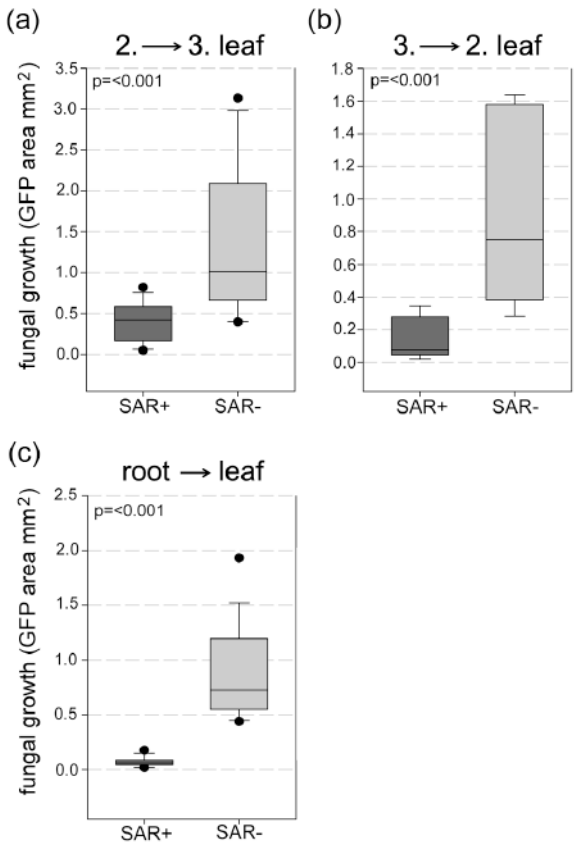

Fig. 4. Local *C. graminicola* leaf and root infection triggers systemic resistance against the same fungus. SAR$^+$ plants received an initial fungal infection, SAR$^-$ plants were mock-treated. (a) Pre-infected second leaves induce resistance in the younger, third leaves. (b) Pre-infection of third leaves induces resistance in older, second leaves. (c) Leaves of root-infected plants are more resistant against *C. graminicola* compared to SAR- plants that received a root mock treatment.

Local leaf and root infection with *C. graminicola* induces SAR in the systemic foliage

To determine the impact of local infection on the resistance of distal tissues, either one leaf per plant (leaf assay; LA) or the root system (root assay; RA), were subjected to an induction treatment by inoculation with *C. graminicola*. Six days later, systemic leaves were challenged with the same fungus. *C. graminicola*-induced plants were called SAR^+ and mock-treated plants SAR^-. In both LA and RA, fungal growth was quantified 3 days after challenge, and compared to infections on SAR^- plants. Fungal growth was significantly reduced in the systemic leaves of SAR^+ plants compared to SAR^- plants (Figure 4). Induced resistance was observed in both the leaf above (Figure 4a) and below (Figure 4b) the initially induced leaf, pointing to a bidirectional systemic response. Interestingly, root infection also induced foliar systemic resistance against *C. graminicola* (Figure 4c).

Plant hormones are implicated in systemic defence responses

To assess the contribution of plant hormones in the establishment of the resistant state, SA, JA and ABA were quantified in systemic tissues of both locally infected leaves (LA) and roots (RA) at 36hpi and 96hpi by UHPLC-MS/MS (Figure 3e-j). There was no significant change in hormone levels in the systemic foliage at 36hpi after local infection of leaves (Figure 3e), however at 96 hpi, SA had significantly accumulated in systemic leaves (Figure 3f). There were no statistically significant differences in SA, JA and ABA levels between roots from control and infected plants following infection at the aboveground level (LA; Figure 3g and 3h). Induction treatment of the root system (RA) led to a significant accumulation of ABA in the foliage at 36 hpi (Figure 3i) and to increased levels of both SA and ABA at

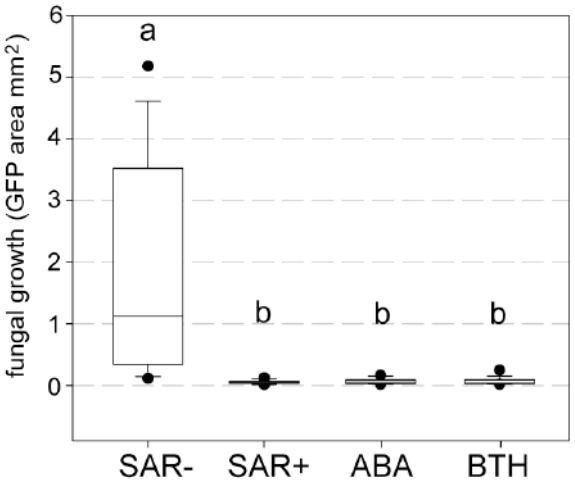

Fig. 5. ABA- and BTH-treatment of roots triggers systemic resistance against *C. graminicola* in the foliage similarly to the resistance on leaves of root-infected plants. Statistical differences are indicated with different letters (n= 20, 3 independent observations; one-way ANOVA, $P<0.05$)

96hpi (Figure 3j). Thus, while during leaf-to-leaf SAR only SA levels increased, during root-to-leaf SAR, both SA and predominantly ABA accumulated to a higher extent.

Belowground induced SAR against *C. graminicola* is mediated by ABA

The upregulation of ABA-related genes and the elevated ABA levels in systemic leaves following root inoculation suggested that ABA might act as chemical regulator of root-to-shoot SAR. To investigate this possibility, roots were treated with ABA and leaves were challenge-inoculated 6 days later with *C. graminicola* (Figure 5). ABA treatment of roots resulted in significantly reduced fungal growth on leaves compared to control plants. Thus, application of ABA to the root system of maize mimics biological SAR-induction and leads to the protection of the leaves against anthracnose. Moreover, root application of the functional SA-analogue

Compound	Mass	MF (M-H)-	Fragments	p[1]	ID	Leaf FI	Root FI
Naringenin chalcone	271.0609	C15H11O5	151.0031, 119.0500	-0.187914	std	57 **	9.6 **
Lysophosphatidic acid (18:2)	447.2508	C22H40O7P	279.2327	-0.176749	HRMS/MS	0.4	7.4***
Acylsugar	819.2335	C38H43O20	175.0397	-0.140926	HRMS/MS	8.5 **	2.9 ***
Eryodictiol	287.0559	C15H11O6	-	-0.135599	HRMS/MS	36.1 **	6.9 ***
3-caffeoyl-quinic acid	353.0872	C16H17O9	191.0559	-0.135461	std	3468.5 **	4.6 ***
N-p-coumaroyltryptamine	305.1293	C19H17N2O2	119.0500, 159.0924	-0.129356	HRMS/MS	101.2 **	120.5 **
Feruloyl-feruloyl-glycerol	443.1341	C23H23O9	193.0505, 134.0373	-0.126652	HRMS/MS	16.3 **	9 **
Citric acid	191.0197	C6H7O7	111.0082, 87.0081	-0.110255	std	3.4 *	2.8 *
Coumaroyl-tyramine	282.1133	C17H16NO3	119.0496, 162.0563	-0.110089	std	53.9 **	7.2 ***
3-caffeoyl-quinic acid (dimer)	707.1817			-0.0856772		154.1 **	nd
N-feruloyl-tryptamine	335.1397	C20H19N2O3	119.0498	-0.079998	HRMS/MS	68.1 **	6.5 ***
Apigenin	269.0455	C15H9O5	117.0340, 151.0032	-0.0665907	std	20.9 **	12.4 ***
Genkwanin	283.0607	C16H11O5	268.0373	-0.065281	std	78.2 **	6.1 ***
5-feruloyl-quinic acid	367.1029	C17H19O9	191.056	-0.0635136	HRMS/MS	28.9 **	5.8 ***
Coumaroyl-feruloyl-glycerol	413.1234	C22H21O8	193.0500, 163.0392	-0.0525341	HRMS/MS	3.4 *	7.4 **
Feruloyl-tyramine	312.1236	C18H18NO4	-	-0.0514736	HRMS/MS	89.7 **	4.8 **
Homoeryodictyol	301.0712	C16H13O6	117.0341	-0.0466089	HRMS/MS	12.7 **	7.6***

Table 1. Metabolites induced in maize leaves and roots upon *Colletotrichum graminicola* infection.P(1) = PCA ranking; ID = identification, std = standard, HRMS/MS = high resolution tandem mass spectra, FI = fold induction (infected vs control). P value (infected vs. control, Mann-Whitney U test) (*** <0.001, ** <0.01, *<0.05).

BTH resulted in a similar resistance level (Figure 5), suggesting an involvement of both ABA and SA in the induction of systemic resistance.

C. graminicola induces organ-specific local and systemic host metabolomic adaptations

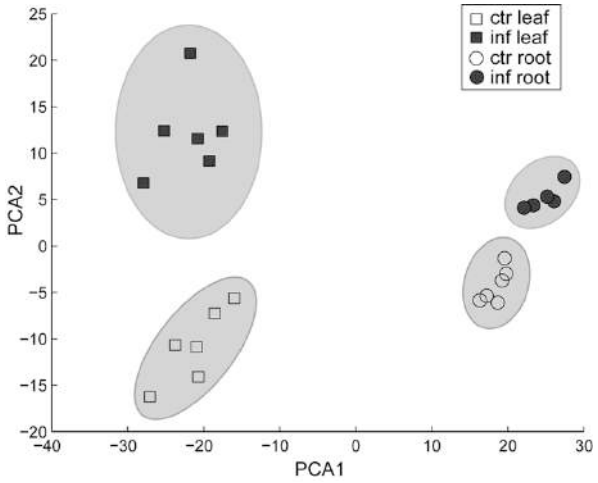

Fig. 6. Principal component analysis (PCA) score plot of *C. graminicola* infected and control leaves and roots. Ctr = control; inf = infected.

To determine the plant reactions at the metabolomic level, an UHPLC-QTOF (ultra-high pressure liquid chromatography-quadrupole time-of-flight mass spectrometry)-based analysis of secondary metabolites in local and systemic *C. graminicola*-infected maize roots and leaves was performed at 6 dpi. The goal was to obtain a general overview of metabolomic adaptations during maize-*C. graminicola* interactions, and to identify organ/tissue-specific markers of antifungal defence responses, using the identical experimental setup as for gene expression and hormone analysis.

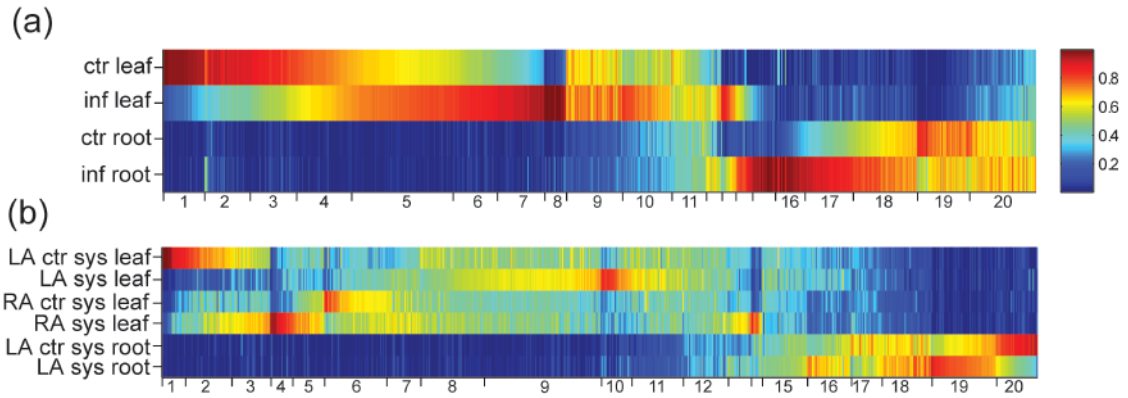

Fig. 7. Cluster plot of local (a) and systemic (b) metabolomic fingerprinting upon *C. graminicola* infection. (a) Control (ctr) and infected (inf) leaves and roots are compared in clusters 1-20. (b) During a leaf assay (LA), leaves from control plants (LA ctr sys leaf) are compared with systemic leaves from leaf-infected plants (LA sys leaf); systemic leaves from mock-treated (RA ctr sys leaf) and infected roots (RA sys leaf), systemic roots from mock-treated leaves (LA ctr sys root) and systemic roots from infected leaves (LA sys root) are analysed together.

In a first step, the metabolomic profile of local leaf and root infections was investigated. PCA score analysis allowed separation of control and infected leaves and roots into different groups (Figure 6), showing a distinct profile for both leaf and root control tissue as well as infected versus control tissues. From the 100 compounds most highly induced during local *C. graminicola* infection, only 25 were common to infected roots and leaves. Using HRMS/MS and/or chemical standards, 17 distinctive, significantly upregulated metabolites in either infected leaves or roots were identified (Table 1). In both organs, high amounts of flavonoids such as naringenin chalcone, apigenin or genkwanin were present. In contrast, lysophosphatidic acid was only induced in roots, whereas 3-caffeoyl-quinic acid (chlorogenic acid) was not detected in roots. In general, leaves responded to infection with an increased accumulation of the compounds compared to roots. However, the basal levels of flavonoids detected were higher in roots (Table S3). In contrast to the local metabolomic profiling, the profiles of systemic organs could not be separated by PCA. However, a supervised PLS-DA model (Figure S5) separated control and systemic parts, suggesting minor adaptations of the secondary metabolome in systemic tissues during local infection. Since the generated dataset was complex, the putative presence of co-regulated clusters was investigated by analysing the sets using the filtering and clustering tool MarVis (Kaever *et al*., 2009, 2012). Eight hundred and eighty six compounds were clustered for local conditions (Table S4) and 583 candidates for systemic conditions in negative mode (Table S5), identifying marker candidates with different intensity profiles (Figure 7). Clusters with higher intensities in control leaves, as well as large clusters that were induced in infected leaves were present (Figure 7a). Interestingly, these clusters showed a low intensity for both control and infected roots. Moreover, some clusters showed prominent induction in infected roots only. Some small clusters of markers induced in systemic leaves upon leaf infection could also be distinguished (Figure 7b). These clusters were distinct from the markers with high intensity in systemic leaves upon root infection. Amongst the clustered putative compounds induced in systemic leaves upon leaf infection, ascorbic acid (*m/z* 175.02407) was found to be accumulated. Moreover, the secondary metabolite maysin (*m/z* 575.1401) and the to our knowledge yet uncharacterized isoscoparin-rhamnoside (*m/z* 607.1663) also showed higher concentrations. In contrast, a different situation

was present in systemic leaves after root infection. The amino acids phenylalanine (*m/z* 166.0869), tryptophan (*m/z* 205.0979) and isoleucine/leucine (*m/z* 132.1034) as well as feruloyl-feruloyl-glycerol (*m/z* 443.1341) and a hexose (*m/z* 179.0556) were induced in systemic leaves after root infection. Interestingly, feruloyl-feruloyl-glycerol was also induced locally in infected roots and leaves (Table 1). In addition, selected markers showed a higher expression in systemic roots upon leaf infection, suggesting a leaf-to-root signalling.

Leaf and root metabolites contribute to antifungal defence

The role of inducible secondary metabolites identified during local *C. graminicola* leaf and root infection was assessed by testing the in *vitro* antifungal activity of selected compounds (Figure S7). Apigenin, genkwanin and chlorogenic acid all led to a dose-dependent reduction of radial growth of *C. graminicola* on medium amended with the corresponding chemicals, suggesting that the induction of these compounds is part of the inducible chemical arsenal maize uses to counteract *C. graminicola* infection.

Discussion

Breeding programs are generally focussed on yield improvement and mechanisms related to stress management by the plant are often neglected and not routinely selected for. Here, we investigated the capacity of maize to express local and induced resistance, both at the aboveground and belowground level against the maize anthracnose fungus *C. graminicola*. We assessed the reactions of the plants towards inducing and challenge treatments at the phenotypic, transcriptional and at the metabolomic level.

Local and systemic adaptations at the transcriptomic level upon infection

The interaction between maize and *C. graminicola* is characterized by a change in lifestyle of the fungus at a certain developmental stage as defined by Vargas *et al*. (2012). After an initial biotrophic phase in leaves, it becomes more invasive and switches to a necrotrophic lifestyle. In roots (Fig. S1), there was no obviously necrotrophic behaviour and growth was much

restricted compared to leaves. Most tested defence marker genes were similarly up- or downregulated in both organ types (Figure 1a). Generally, infection by *C. graminicola* seems to trigger the SA-dependent defence pathway. Remarkably though, the root system responded faster to fungal ingress than leaves (Figure 1a) but transcript levels of defence genes such as *PR1* reached up to 100 times higher levels in leaves than in roots (Figure 2 and Table S2). This might either be the consequence of a constitutively elevated basal defence state of the root system blocking pathogen ingress so rapidly that no major induction of defence transcripts is possible or/and the defence response might be actively suppressed by the pathogen in the roots. Similarly, *Magnaporthe grisea* infections in rice have a distinct disease progress in leaves and roots that is associated with a disparate transcriptional pattern (Marcel *et al*., 2010). The authors concluded that *M. grisea* utilizes a unique biotrophic lifestyle strategy during root infections. There was no downregulation of the defence gene transcriptome in *C. graminicola*-infected roots during at least six days following inoculation suggesting that *C. graminicola* employs an infection strategy that is different from the biotrophic one of *M. grisea*. Recent findings also suggest that *C. graminicola* does not suppress host defence mechanisms during biotrophic phases of leaf infections (Vargas *et al*., 2012) in contrast to other biotrophs (Doehlemann *et al*., 2008; Djamei *et al*. 2011). Thus, the biotrophic phase of *C. graminicola* is not comparable with the lifestyle of true biotrophs. Nonetheless, certain defence-related genes, for example *Bx1*, *IGL* or *CHS C2,* were downregulated during early leaf infection phases in the maize/*C. graminicola* interaction (Figure 1). Bx1 and IGL are both enzymes that convert indole-3-glycerole phosphate into indole, which is the first step in the synthesis of benzoxazinoids (BX; Glauser *et al*., 2011). Since BX play an important role in the immunity of maize plants against fungi and aphids and BX-deficient mutants are impaired in penetration resistance against a necrotrophic fungus (Ahmad *et al*., 2011), a downregulation of *Bx1* and *IGL* early in the interaction could facilitate the development of *C. graminicola* in leaves. In contrast, the early transient upregulation of *Bx1*in roots is in agreement with the higher basal resistance of this organ to *C. graminicola*.

Metabolomic above-and belowground changes in *C. graminicola* infected maize

Contrary to the minor differences at the transcriptomic level between *C. graminicola* infections of roots and leaves, respectively, the metabolomic fingerprinting yielded major organ-specific differences. This suggests that leaves and roots employ a distinct chemical arsenal of secondary metabolites during antifungal defence. Common to both organs was the increase in flavonoid levels in response to fungal infection. The constitutive levels of flavonoids were generally higher in control roots compared to control leaves. Interestingly, infection of leaf cells was accompanied by a strong autofluorescence including the aggregation of fluorescent vesicles around the penetration peg (Figure S6) early during infection. Since anthocyanins and hydroxycinnamic acid derivatives accumulate at the cell wall of *C. graminicola*-attacked maize leaf cells (Vargas *et al*. 2012), flavonoids and additional defence compounds might be directed towards the penetration site by vesicles (Kwon *et al*., 2008). Flavonoids play an important role in both above- and belowground plant-microbe interactions. In *Sorghum bicolour*, *C. sublineolum* foliar infection is associated with higher levels of phlobaphenes (Ibraheem *et al*., 2010). In the roots, flavonoids such as naringenin and quercetin are implicated in mycorrhization, nodulation, root development or nematode repulsion (Hassan and Mathesius 2012). Interestingly, *C. graminicola* encodes a putative quercetinase that can cleave quercetin (Krijger *et al*., 2008), indicating that the fungus has the potential to counteract flavonoid-based chemical defences. Flavonoids induced in both leaves and roots in response to *C. graminicola* attack also have an antagonistic effect on fungal growth (Figure S7).

The elevated basal level of flavonoids and other defensive compounds in roots (Table S3) suggests an enhanced defensive state of this organ, which is corroborated by the fact that roots adapt their transcriptome more rapidly to defence situations (Figure 1a) and also generate higher levels of defence-associated plant hormones (Figure 3). Maize crown roots are richer in defensive compounds compared to the other root types (Robert *et al*., 2012) and since they are vital in early development, this has been suggested to help the maize plants to defend them better against herbivory. Similarly, roots might employ an enhanced basal resistance

against fungal infections. In addition, roots could act as chemical arsenal. In Arabidopsis, flavonoids can be transported from roots to distal tissues (Buer *et al*., 2008). *C. graminicola* leaf infection might induce the translocation of flavonoids from root to shoot, which could explain why there was no significant up regulation of *CHS C2* in infected leaves although flavonoids were clearly present.

The role of plant hormones in maize defence against *C. graminicola*

Plant hormones and numerous other compounds with biological activity are of uttermost importance for the control of targeted reactions of plants during stress situations (Erb and Glauser 2010). The pattern of hormonal adaptations during leaf and root infections of maize with *C. graminicola* is in accordance with the transcriptome data: infected roots reacted faster than leaves and exhibited significant changes in SA, JA and ABA levels compared to control plants. The early downregulation of JA in infected roots is reminiscent of the observed interplay between SA and JA during biotrophic interactions (Pieterse *et al*., 2009) although, at later stages, SA, JA and ABA were all accumulating to higher levels. The hormone levels were higher in infected roots compared to infected leaves. This fits the attenuated and symptomless disease progress observed for *C. graminicola* in roots, provided that the hormones really contribute to a more effective defence. Only little is actually known about their importance in root defence (Gutjahr and Paszkowski 2009). JA inhibits nodule initiation by *Sinorhizobium meliloti* on *Medicago truncata* (Sun *et al*., 2006). Similarly, *Nicotiana tabacum* with reduced levels of SA showed elevated mycorrhizal colonization at early time points (Herrera Medina *et al*., 2003), indicating that SA is implicated in the control of biotrophic plant-microbe interactions at the root level. Thus, the strong upregulation of SA in *C. graminicola*-infected maize roots suggests a chemical defence strategy that is similar to the one found in biotrophic interactions. In rice, brassinosteroids (BR) were shown to suppress gibberellin- (GA) and SA-mediated root defences against *Pythium graminicola*, indicating that SA-mediated root resistance is strongly regulated by a hormonal network (De Vleesschauwer *et al*., 2012). Recently, ABA has been found to act as negative regulator of SA, JA and ET-mediated defence against root nematodes in rice (Nahar *et al*., 2012). ABA has also been demonstrated to be induced in maize roots upon

herbivore attack (Erb *et al*., 2009a). Since ABA has no negative effect on *C. graminicola in vitro* growth (Vargas *et al*., 2012), root-induced ABA might play a role in fine-tuning hormonal pathways as observed during foliar herbivore defence mechanisms in Arabidopsis (Bodenhausen and Reymond 2007). Furthermore, local ABA application on maize leaves led to an enhanced *C. graminicola* disease progress through a faster transition to necrotrophy (Vargas *et al.,* 2012). The controversy between our observations showing an increased resistance following ABA treatment and the results of Vargas and colleagues (Vargas *et al.,* 2012), who observed an augmented susceptibility of maize towards *C. graminicola* after ABA treatment might be due to the changes of the role of ABA during disease progress as described for several plant-pathogen interactions (Ton *et al.,* 2009). ABA induced or applied early in an interaction rather stimulates host resistance while during later phases, when the pathogen has penetrated the host tissue, ABA might interfere with ROS production and hence, cause an increase in susceptibility. As some pathogens are known to produce ABA (Mauch-Mani and Mauch, 2005), the presence of ABA on *C. graminicola* growing in liquid cultures was tested. ABA levels in these cultures were near detection limit (Figure S8) and supplementing the culture medium with crude plant extracts did not induce ABA accumulation, suggesting that *C. graminicola* does not produce ABA specifically during the interaction with its host.

SAR in maize

Both maize leaves and roots possess the ability to trigger systemic antifungal resistance in distal tissues (Figure 4). *C. graminicola* leaf infection led to systemic changes at the transcriptomic level, higher accumulation of SA and finally, elevated resistance against the same fungus. Similarly, root inoculation activated massive gene expression changes in systemic leaves, increased accumulation of SA and ABA in systemic leaves and systemic resistance against *C. graminicola* infection. SA seems to play a role in both leaf-leaf and root-leaf systemic resistance in maize against *C. graminicola*. In contrast, infection does not result in higher SA-levels in rice (Silverman *et al*. 1995) probably due to the already constitutively high basal levels of SA. However, resistance in general as also SAR mechanisms are highly conditional. This is for example illustrated by the fact that during infection of Arabidopsis with

P. syringae pv. *maculicola,* strong light triggers SAR in absence of either SA or PR1 induction in systemic leaves (Zeier *et al*., 2004).

SAR requires the generation of mobile alarm signals. The clustering of systemic metabolomic datasets (Figure 7b) indicates systemic metabolomic changes, which are, however, much less prominent than during local *C. graminicola* attack. At this point it is not possible to determine which compounds could act as mobile signals. Some of the systemically induced compounds identified in this study are known to play a role in plant defence. For instance, the C-glycosyl flavone maysin has been associated with resistance to the corn earworm *Helicoverpa zea* (Byrne *et al*. 1998), and sugars are known to act as priming molecules during plant immune responses (Bolouri Moghaddam and Van den Ende 2012). However, further implications of these compounds in SAR of maize remain to be investigated. Interestingly, SAR induced by a belowground infection resulted in a stronger resistance than SAR induced aboveground. This observation supports the idea that roots are better protected than leaves and can act as defence arsenal. Fungal root infections led to higher levels of ABA in leaves, similar to observations during root herbivory (Erb *et al*., 2009a). ABA application on maize roots triggers foliar resistance against *Spodoptera littoralis*, as well as the necrotrophic fungus *Setosphaeria turcica* (Erb *et al*., 2009a). As ABA root treatment also induces foliar resistance against *C. graminicola* (Figure 5), it could act as general root-shoot systemic resistance signal. Nonetheless, it has to be determined whether ABA translocates from roots to shoots during SAR or if ABA is also induced in systemic tissues. ABA likely also modulates other systemic defence pathways, since root treatment with ABA in maize primes the foliage for enhanced DIMBOA and chlorogenic acid content (Erb *et al*., 2009b).

Despite an absence of major systemic changes at the transcriptome and hormone level in roots following leaf infection, metabolomic fingerprinting uncovered clusters of metabolites that show higher intensities in systemic roots. This might be due to the higher defensive state of roots, which would dilute a systemic defence response. However, induction of SAR from the second to the third leaf and *vice versa* still suggests the possibility of bidirectional signalling as previously demonstrated or herbivore resistance. In caterpillar-

resistant maize, foliar caterpillar attack induces the accumulation of the cysteine protease Mir1-CP in roots (Lopez *et al*., 2007). Mir1-CP is highly toxic for caterpillars since it damages the insect midgut. Root-derived Mir1-CP is translocated to the shoot, corroborating the crucial role of roots in both local and systemic defence systems (Luthe *et al*., 2011).

In conclusion, this study demonstrates that both maize leaves and roots apply specific defence strategies in counteracting *C. graminicola* infection. Remarkably, *C. graminicola* is able to deal with these different strategies and is capable of infecting a variety of different tissues including different root types. This ability of *C. graminicola* might explain its success as serious maize pathogen. Recent advances in *C. graminicola* genomics (O'Connell *et al*., 2012) should be further applied to dissect tissue-specific physiological adaptations of the fungus. Maize roots employ higher basal levels of defensive compounds such as flavonoids, which might be a valuable target for future crop enhancement programs. Similarly, our study also shows that local *C. graminicola* infection triggers systemic resistance in yet uninfected tissues. Although both SA and ABA seem to be implicated in this defence mechanism, further studies are required to identify putative long-distance mobile signals. Considering climate change and growing demand of maize as high value crop, *C. graminicola* is likely going to be an emerging agricultural threat. Thus, understanding plant and fungal behaviour both on above- and belowground tissues is crucial to develop novel anthracnose disease control strategies.

Experimental procedures

Plants and fungi

Maize plants (*Zea mays*, variety Jubilee, West Coast Seeds, http://www.westcoastseeds.com) were grown in a soil-free system (Planchamp *et al*., 2012) in a growth chamber at 25°C day/ 22°C night temperature with 16 hours light (400 $\mu Em^{-2}sec^{-1}$) and 60% relative humidity. *C. graminicola* (M1.001, obtained from Lisa Vaillancourt, University of Kentucky, USA), and its transgenic GFP-expressing derivative *C. graminicola*-gGFP (Erb *et al*., 2009) were maintained

at 25°C on potato dextrose agar (Difco PDA, Becton, Dickinson and Co., http://www.bd.com/) under continuous illumination (70 $\mu Em^{-2}sec^{-1}$)

Fungal infections

Leaves were locally inoculated by spreading 20 µL of *C. graminicola* conidia suspension ($6x10^5$ spores mL^{-1} sterile water; 0.01% Silwet L-77, Lehle Seeds, http://www.arabidopsis.com) on the surface using a paintbrush. Inoculated plants were kept in the dark (100 % rel. humidity, 25°C, 16h), before transfer to the growth chamber. Roots were inoculated by dipping for 30 minutes in a similar spore suspension then transferred back to the soil-free growth system. Infections were consequently performed at the end of the day period.

Inhibition of fungal radial growth was tested by applying 3µL of conidia suspension ($3x10^5$ spores mL^{-1}) to the center of each well in 12-well culture plates (Millipore, http://www.millipore.com) containing PDA amended with the test compounds. Growth was measured after two days. Chlorogenic acid (R. Collins, University of Fribourg, Switzerland), apigenin and genkwanin (Extrasynthese, http://www.extrasynthese.com) were dissolved in EtOH (99.9%, Merck, ww.merckgroup.com) and further diluted in sterile water.

Microscopy

Light microscopy was performed using a Nikon Eclipse E800 microscope. *C. graminicola*-gGFP disease progress was observed using a TCS SP5 II confocal laser scanning microscope (Leica, http://www.leicabiosystems.com); digital images were acquired using LAS AF (version: 2.0.0 build 1934). To assess and quantify fungal colonization of plants, *C. graminicola*-gGFP infection spots were captured using the Nikon dissecting microscope C-BD230 with blue light excitation; the images were further processed as described (Erb *et al*, 2011).

RNA extraction and gene expression analysis

Plant RNA isolation was performed according to manufacturer's instructions using the RNeasy Plant Mini kit (Qiagen, http://www.qiagen.com). RNA was treated with DNase (Qiagen) and reverse transcribed into cDNA using SuperScript III RT (Invitrogen, http://www.invitrogen.com).

Primers for qRT-PCR were designed using the universal probe library assay design tool from Roche (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000). The primers used in this study are listed in Table S1. Primer efficiency was determined by performing a qRT-PCR with serial diluted cDNA; the minimal accepted efficiency for the primers was set to 0.8. The qRT-PCR was performed using the SensiMix SYBR kit (Bioline, http://www.bioline.com) on a Rotor Gene 6000 cycler (Qiagen). The reaction volume was 10 µL, consisting of 2.5 µL nuclease-free water, 5 µL SensiMix SYBR mastermix, 0.25 µL forward and reverse primer (each 10 µM) and 2 µL cDNA. PCRs were performed using 3 independent biological replicates per sample, each replicate consisting of a pool of 6 plants. PCR reactions were performed in technical duplicates as a three-step reaction (initial hold step, 95°C for 10 min; 40 cycles of amplification, 95°C for 15 s, 60°C for 20 s, 72°C for 20 s) with a final melting curve analysis (68°C-95°C). Melting curve and cycle threshold (Ct) analysis was performed on the Rotor-Gene 6000 software 1.7. Relative gene expression of infected tissue was calculated relative to control treated plants in regard to the two housekeeping genes *Zm-GAPc* and *Zm-Actin* and the specific primer efficiencies with the help of REST 2009 (Qiagen). The statistical outputs of the analysis using REST 2009 are summarized in supplementary document S5. The gene expression data was further visualized using the software MeV viewer (http://www.tm4.org).

Biological and chemical systemic resistance assays

To investigate SAR on the foliage, the second or third leaf, respectively, of 12-day-old plants was inoculated with *C. graminicola* as described above (n = 30 plants, minimum of three independent observations). Three 50 µL drops were applied and distributed over the whole leaf area (SAR^+ plants); in parallel, control plants were mock-treated (SAR^- plants). Challenge infection was performed at 6 dpi with a *C. graminicola*-gGFP spore suspension as described above. Infection was quantified at 3dpi as described (Erb *et al*., 2011). Fungal growth between treatments was compared using a Mann-Whitney *U* test. SAR induction via roots (12 days old plants; n = 30, 5 independent observations) was performed by dip-inoculation with a *C. graminicola* ($6x10^5$ conidia mL^{-1}) as described above. Challenge infections were performed 6

days after the inducing treatment on the second leaf of root-infected (SAR^+) and root mock treated (SAR^-) plants and quantified as described (Erb *et al*., 2011). ABA (300 µM ±-ABA; Sigma, http://www.sigmaaldrich.com/) was sprayed directly on roots (n = 30 plants, 3 replicates) of 12-days-old plants that were challenged 6 days later with *C. graminicola*-gGFP as described above. BTH (Bion, 1.5 mM, Syngenta, http://www.syngenta.com) was applied the same way.

Hormone quantification

SA, JA and ABA were quantified simultaneously in single samples using an optimized ultra-high pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method (Glauser *et al*., 2012). In brief, hormones from 100 mg fresh weight were extracted in EtOAc: formic acid, 99.5:0.5 (v/v). Before extraction, an internal standard solution containing isotopically labelled SA, JA and ABA (10 ng/mL) was added to the samples. The extracts were evaporated to dryness and resuspended in 100 µL of aqueous methanol (70%). After centrifugation, 5 µL of that solution were injected in UHPLC-MS/MS. The hormones were quantified by calculating a calibration equation obtained by linear regression from 5 calibration points for each analyte. Peak areas of the hormones measured in the samples were normalized to the internal standard before applying the calibration equation.

Metabolomic profiling

For metabolomic analysis, 12-days-old maize plants were infected with *C. graminicola* as described. At 6dpi metabolites were isolated from 100mg flash frozen and ground tissue using 500µL of extraction solvent (MeOH 80%, H_2O 19.5%, formic acid 0.5%). Three biological replicates (6 pooled plants per treatment) were analyzed in technical duplicates using UHPLC-QTOF. Separation was achieved on an Acquity UPLC (Waters, http://www.waters.com) at a flow rate of 400 $\mu L min^{-1}$ using an Acquity BEH C18 column (50x2.1 mm i.d., 1.7 µm particle size) thermostated at 30°C. Solvent A consisted of water and formic acid 0.05% and solvent B of acetonitrile and formic acid 0.05%. The following linear gradient was employed: 0-6 min 5-100% B, 6-8 min holding at 100% B, 8-10 min reequilibration at 5% B. The QTOF (Synapt G2, Waters) parameters were applied as in (Glauser *et al.*, 2011). The mass spectrometry data

were further processed using the MarkerLynx application of the MassLynx software (Waters). PCA and PLS-DA were generated in EZinfo (Umetrics, http://www.umetrics.com). In order to identify selected markers, co-elution with available reference standards and/or positive match with MS2 fragmentation spectra were requested. The toolbox MarVis (http://marvis.gobics.de33) was used for filtering and ranking the local and systemic metabolomic data. Raw data were converted into .CDF files that were extracted using the bioconductor packages "xcms" (Smith *et al*., 2006; Tautenhahn *et al*., 2008) and "multtest" (Pollard *et al*., 2010) in R (R development core team 2008). To filter a subset of high-quality markers, a Kruskal-Wallis one-way ANOVA ($P<0.01$) was applied. Clustering of the high-quality markers was based on m/z value ranking and visualized by a MarVis colormap.

Statistical analysis

Variances of quantified levels of metabolites and fungal growth for multiple groups were analysed with a one-way ANOVA; control and infected groups ($P<0.05$) were then compared using the Tukey's range test. The Mann-Whitney *U* test was used to compare significant differences between two sample groups. All statistical analysis was accomplished using Sigma Plot 11.0 (http://www.sigmaplot.com). The significance of gene expression data was calculated using the software REST 2009 (Qiagen), which applies the Pfaffl mathematical model for relative quantification of qRT-PCR data (Pfaffl 2001).

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Supporting information

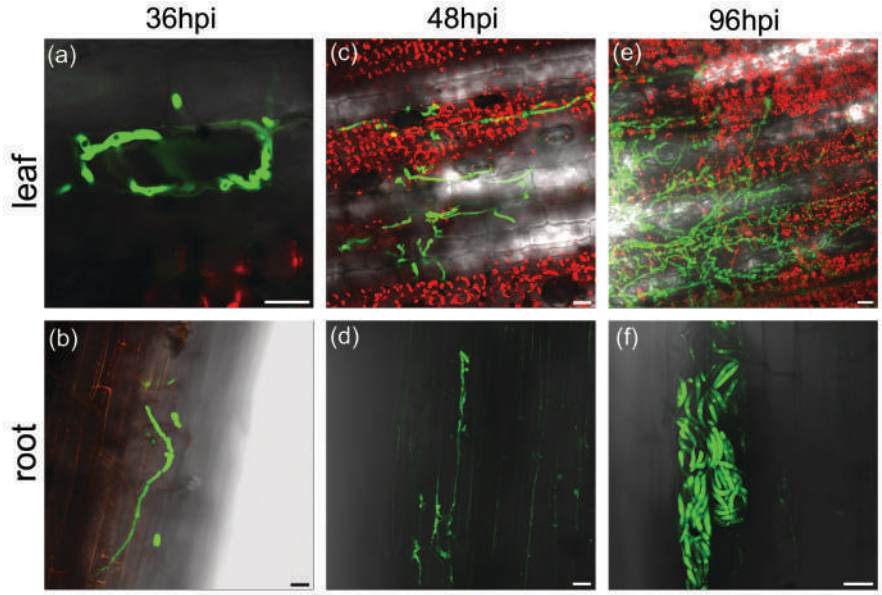


Figure S1. Confocal micrographs of maize leaf and root colonization by GFP-transformed *Colletotrichum graminicola*-gGFP. (a) Globular primary hyphae in leaf epidermal cell (36 hpi). (b) Runner hyphae on the root surface (36 hpi). (c) Thin secondary hyphae in leaf tissue (48 hpi). (d) Thin hyphae growing along the longitudinal axis of root cells (48 hpi). (e) Advanced colonization in leaves (96 hpi). (e) Advanced infection stages in roots (96 hpi). Epidermal cells packed with falcate conidia. Bars = 20 µM.

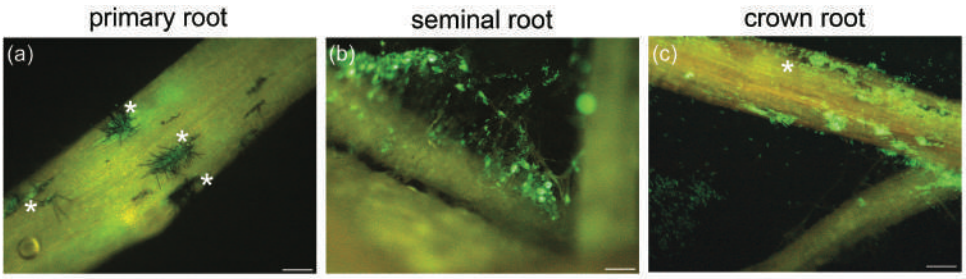


Figure S2. *C. graminicola* is able to infect all maize root types. Primary (a), seminal (b) and crown (c) roots 6 dpi with *C. graminicola*-gGFP. Acervuli are indicated with asterisks. Bar = 250 µM.

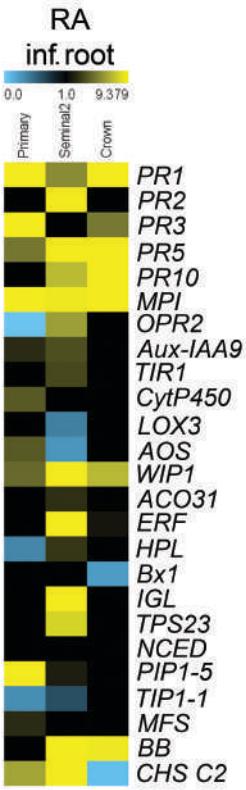


Figure S3. Gene expression profile of the different maize root types during *C. graminicola* infection. Primary, seminal and crown roots were analysed 6 dpi. Blue colour represent a downregulation, yellow indicates upregulated genes.

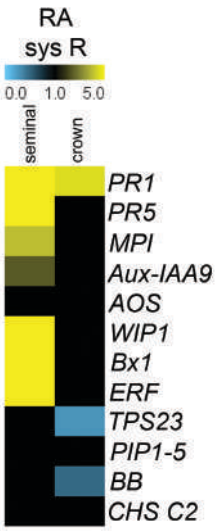


Figure S4. Systemic gene expression profile of seminal and crown roots 6 days following local infection of primary roots by *C. graminicola*. Yellow indicates upregulated genes, blue downregulated genes.

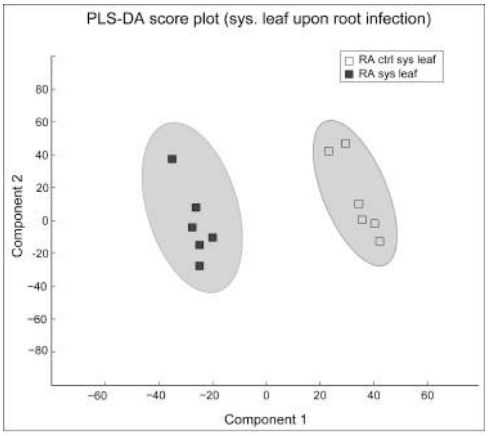


Figure S3. PLS-discriminant analysis (PLS-DA) of secondary metabolites in leaves of root-infected plants (RA sys leaf) and leaves of plants that received a mock treatment (RA ctrl sys leaf).

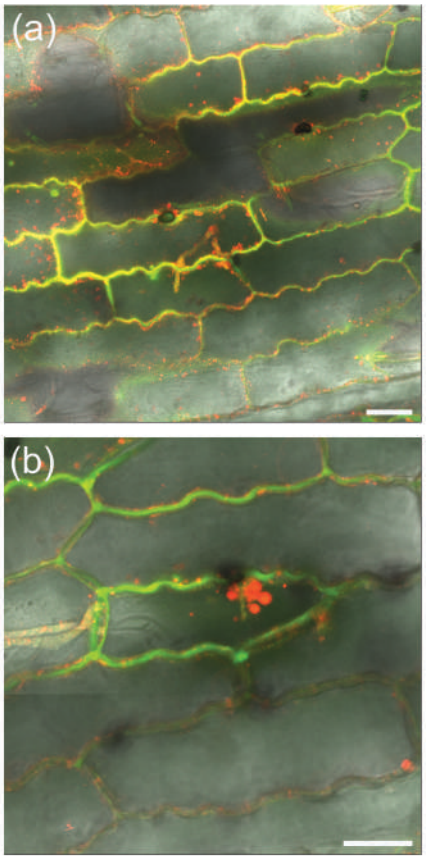


Figure S6. Confocal micrographs of infected maize leaf epidermal cells during early *C. graminicola* infection stages. (a) Strong autofluorescence observed around cell walls of attacked and adjacent cells. (b) Autofluorescent vesicles (red) accumulating around the penetration point of C. graminicola. Bars = 45 µM.

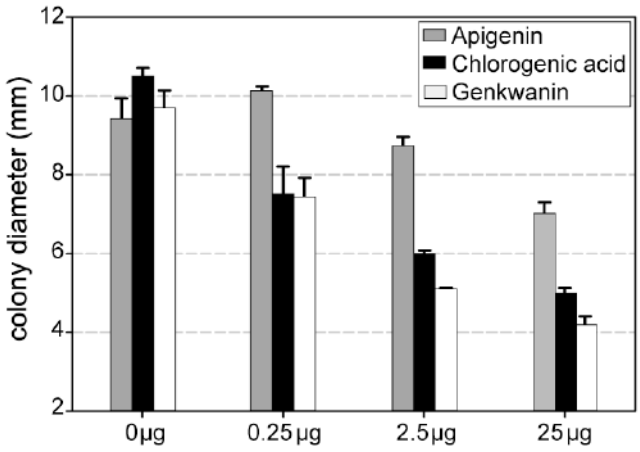
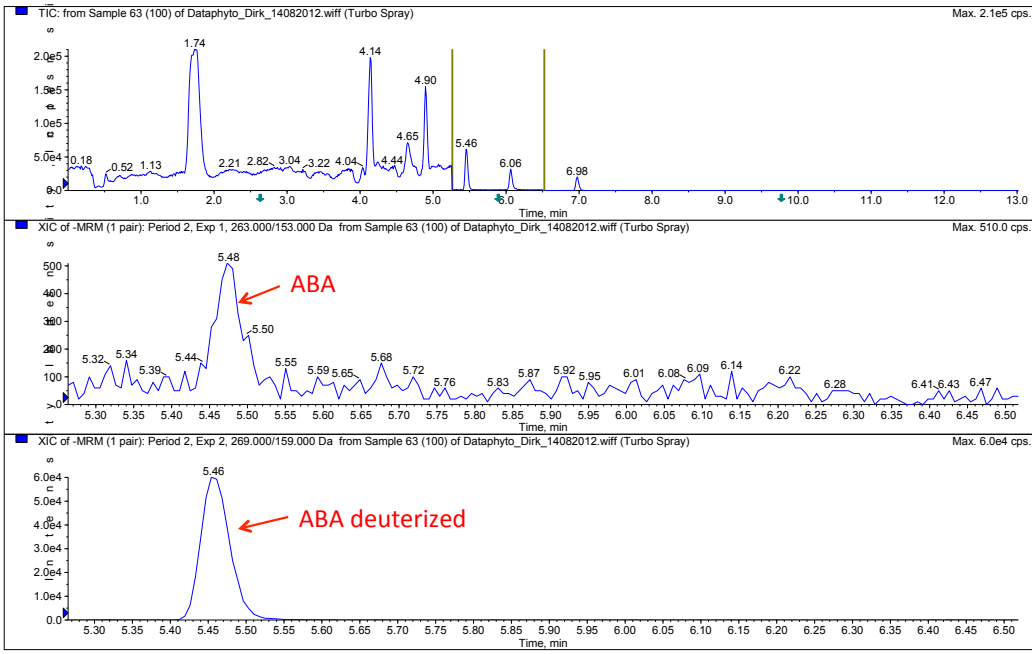


Figure S7. Chlorogenic acid, apigenin and genkwanin inhibit radial growth of *Colletotrichum graminicola* in a dose-dependent manner.

a) *Colletotrichum graminicola in vitro* culture



b) *Colletotrichum graminicola* + plant extracts

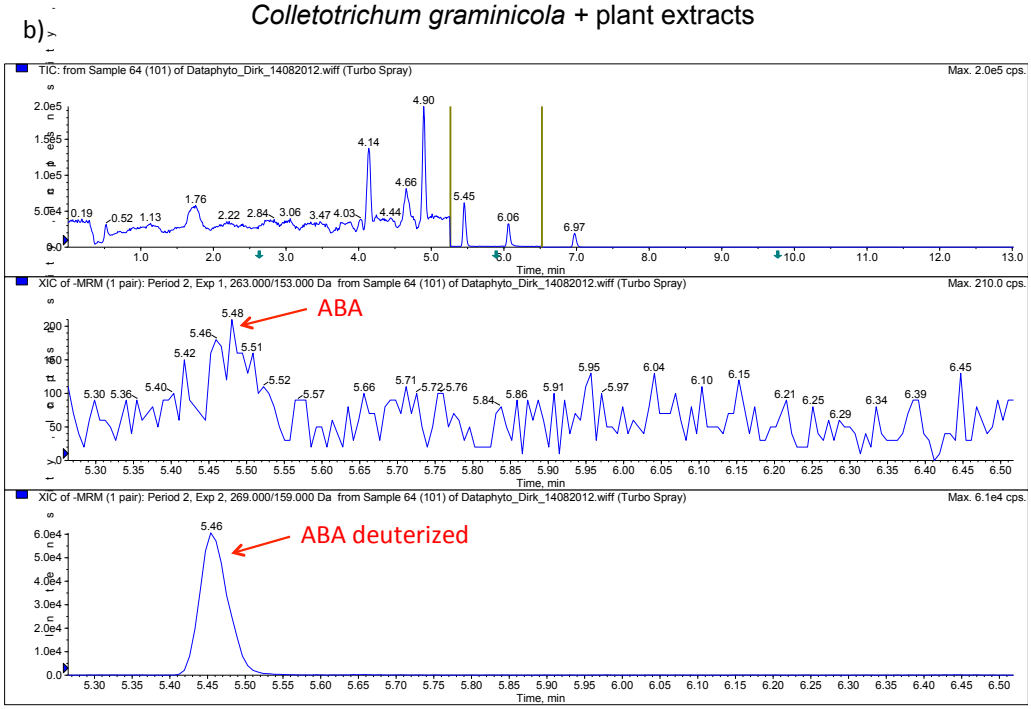


Figure S8. UHPLC-MS/MS chromatogram of in vitro *C. graminicola* cultures. Arrows show ABA and deuterized ABA detected in the chromatogram of *in vitro* fungal liquid cultures without (a) and with (b)s plant crude extracts. *C. graminicola* spores were cultured in Fries' medium in regular growth conditions as described in the experimental procedures.

Table S1. qRT-PCR primers used in this study.

Gene	Accession	Pathway	Description	Forward primer	Reverse primer
ACO31	AY359573.1	ET	B73 acc oxidase (ACO31)	gctcgtcttcgatcaattcc	cagcttggagaagtcgatca
ACS65	AY359571.1	ET	OH43 acc synthase (ACS6)	gtcaagggcgtgctcatc	aagtccaccagcatctccag
Actin	EU960271.1	normalizing gene	actin	taccatgttccctgggattg	gtggcgcaatcactttaacc
AOC	AY488136.1	JA	allene oxide cyclase	ccccttcaccaacaaggtgt	accgagatgtggccgtagtc
AOS	AY488135.1	JA	allene oxide synthase	acctgttcacgggcacctac	cgaggagcgaggagaagttg
Aux-IAA9	EU973552.1	auxin	auxin-responsive protein IAA9	aggaaatcagcagcaagtcc	ggtgaccaccacgtccat
Aux-RFB3	EU967025.1	auxin	auxin-responsive factor, transcription factor B3	gccctgatgtaggtttctgc	gtgttccaactggcagatca
BB	AY104926.2	Shoot-root signal	Bowman-Birk tryp inhbit.	tcttcttctgacgccatgc	agagcaagcaccgaacagag
Bx1	AY254103.1	DIMBOA	benzoxazinless1 / DIMBOA synthesis	cccgagcacgtaaagcagat	cttcatgcccctggcatact
CHS C2	BT061979.1	flavonoid biosynth.	chalcone synthase C2	caggaggcgatcaaggagt	cgacgtggtgcagaacacta
CytP450	EU956091.1	JA/stress	cytochrome P450 monooxygenase	gacaagtacctccgcgacat	ttcttggacaggagcatgaac
EIN2-25	AY359584.1	ET	ethylene insensitive 2	cctactctgtactgggcgaaa	tggaagaagcatggcttga
EKOx	EU966375.1	GA	ent-kaurene oxidase	tgcaacatgaacaaaaacgac	cttcaaacctcccgtccag
ERF	AY672654.1	ET	ethylene responsive factor	aaggtggaggcacagactca	taagggatgccgaggaagtt
GAPc	X07156	normalizing gene	glyceraldehyde-3-phosphate dehydrogenase	gcatcaggaaccctgaggaa	catgggtgcatctttgcttg
GCN5	AJ428540.1	chromatin modific.	histone acetyltransferase	aactctcaaccaagagctaggc	gtccagtccagtgagccaat
GID1L2	EU974409.1	GA	gibberellin receptor GID1L2	gggacagggcctactacga	aggaagaagacgtggtcctg
HPL	AY540745.1	green leaf volatiles	hydroperoxide lyase	ttcaccatcctggacatctg	cgggaaggagtggatgag
IGL	AF271383.1	IGL	indole-3-glycerol-phosphate lyase, DIMBOA synthesis	gcctcatagttcccgacctc	gaatcctcgtgaagctcgtg
LOX1	DQ335760.1	JA	lipoxgenase 1	cactcgagctcgtcaaggat	tccaacctgtcttgtcctctttt
LOX3	AF329371.1	JA	lipoxgenase 3	cgccaactcctgggtctac	tctggcttggcaggtacg
LOX5	DQ335763.1	JA	lipoxgenase 5	gcacccaacaaagaaagacc	cgggacgtagatgttcaggt
MAPK6	AY425817.1	env. stress	mitogen activated protein kinase 6 (cultivar WF9)	gccccctttctccaaagat	tgccttcttcttctggtcgt
MFS	BT042111.1	metabolites/sugars	Major Facilitator Superfamily	cactgtgggctgtgagcagt	gcaggccgaaatgtcttgat

MPI	X78988.2	SA/biotic stress	maize proteinase inhibitor (subtilisin-chymotrypsin inhibitor homolog1)	atgagctccacggagtgc	tcagccgatgtggggtgtc
MYB	EU951799.1	Shoot-root signal	Zea mays MYB family homol.	cagtcggatgcttttggttc	tctcagggacattattcccatc
NAC	BG458764.1	Shoot-root signal	Zea mays ANAC082 homol.	cagtgctaaaggcaaaagagg	ctcaacatcagttgggtgga
NCED	EU956583.1	ABA	viviparous-14 (9-cis-epoxycarotenoid dioxygenase)	aagtctcaccatccacacagg	tcaacggagccagctgat
NPR1	EU955884.1	SA/JA cross-talks	regulatory protein NPR1	ctccagaggggcacagccga	cgagaacacgctgccctccg
OPR2	AY921639.1	SA	12-oxo-phytodienoic acid reductase	gggcagttcgagctctctc	cgcgctgcgagtagtaca
PAL1	M95077.1	SA/biotic stress	phenylalanine ammonia lyase	aaggtgttcgtcggcatc	tcccactccttgaggcact
PIP1-5	AJ271796.1	ABA/salt stress	plasma membrane intrinsic protein1 (pip1e)	cacgtggtcatcatcaggg	cgtatgctgcatggttgct
PR1	U82200.1	SA/biotic stress	pathogenesis-related protein 1	cctacggcgagaacctctt	tcgtagtactgcttctcggaca
PR10.1	AY953127.1	SA/biotic stress	ribonuclease (Parsley PR1)	cagctggactgttgagatcg	gtgtgccagtccatcacg
PR14	AY105889	biotic stress	lipid transfer protein	ccgatcctgccctctaataa	atgaacgcctggagcttct
PR2	AY754698.1	SA/biotic stress	Bet v I allergen	atggcgtccaaggttgag	cttgtactgctcggggaaga
PR3	BT040514.1	SA/biotic stress	endochitinase A	ggtgcgaacgtggctaat	ccgggtgtagaagttcttgc
PR5	U82201.1	SA/biotic stress	zeamatin-like (thaumatin-like)	ctggccgagttcaccatc	gccatggcgaggttgtag
SAUR52	EU966039.1	auxin	auxin-responsive SAUR family member	gtgccttagcacccctgtct	ggctcctctcctgagcaaac
TIP1-1	AF037061.1	ABA/salt stress	tonoplast intrinsic protein1	cggcaacatcaccctgtt	agaagcggagcaggaagc
TIR1	EU957719.1	auxin	transport inhibitor response 1	ttgttgcaccaagaggtactgt	cttccgacacgaagataggg
TPS10	AY928079.1	volatiles	terpene synthase 10	acaatgcagcgtaccttagga	gcttcaagctggcgtctg
TPS23	EU259633.1	volatiles	E-beta-caryophyllene synthase	tctggatgatgggagtcttc	gcgttgccttcctctgtgg
WIP1	EU958996.1	JA	Bowman-Birk type wound-induced proteinase inhibitor WIP1 precursor	catgaagagcagcccacac	ggccttgctctcaaccag

Table S2. Statistical output of gene expression data analysed with REST 2009 (Qiagen); available online at *The Plant Journal* upon online publication.

Table S3. Comparison of basal and induced levels of metabolites identified in *C. graminicola*-infected maize leaves and roots (Table 1). Non-normalized peak areas were used to compare metabolites in control and infected conditions.

									leaf			root				
									inf	ctrl		inf	ctrl			
Compound	Mass	LA inf L	Ctrl LA inf L	FI	RA inf R	Ctrl RA inf R	FI		avg	avg	FI avg	avg	avg	FI avg		ctrl inf
Naringenin chalcone	271.0609	696.6354	0.346	2013.39711	1520.21	99	15.355657								Leaf	7.6381333 435.40679
		273.0165	1	273.0165	1630.86	97	16.81299								root	207.91478 1987.7055
		455.0892	6.74	67.52065282	3077.122	407.34	7.5541857									
		560.772	3.251	172.4921563	2343.88	372.82	6.2868945									
		331.05916	18.7817	17.62668768	1730.152	164.13	10.541351									
		295.8685	15.7101	18.83301188	1624.009	107.1987	15.149521		435.40679	7.6381333	57.004346	1987.7055	207.91478	9.5601932		
Lysophosphatidic acid (18:2)	447.2508	86.2479	960.34	0.089809755	1700.544	157.09	10.825285									830.33833 323.92904
		51.5136	576.92	0.089290716	1525.91	17.05	89.496188									166.025 1227.0775
		32.8209	1723.29	0.019045489	934.2	45.89	20.357376									
		991.3475	1180.26	0.839939928	1257.7	127.61	9.8558107									
		457.7153	82.7	5.534646917	1499.67	306.9	4.8865103									
		1	458.52	0.00218093	444.441	341.61	1.3010187		323.92904	830.33833	0.3901169	1227.0775	166.025	7.39092		
Acylsugar	819.2335	360.82	10.12	35.6541502	1546.28	547.908	2.8221526									
		220.6	11.76	18.7585034	1479.62	553.42	2.6735933									
		111.96	26.8899	4.163645086	1830.34	654.64	2.7959489									
		126.8085	29.54	4.292772512	1771.01	567.99	3.1180302									
		158.84	27.15	5.850460405	1878.13	606.13	3.0985597									
		129.1	24.117	5.353070448	1792.27	578.75	3.0967948		184.68808	21.59615	8.5518985	1716.275	584.80633	2.934775		21.59615 184.68808
Eryodictiol	287.0559	93.25	1	93.25	864.054	92.45	9.3461763									584.80633 1716.275
		20.322	1	20.322	955.488	92.993	10.274838									
		28.92	1	28.92	1058.46	213.98	4.9465371									
		20.71	1	20.71	823.84	215.51	3.822746									
		16.956	1	16.956	1075.5	147.61	7.2860917									
					980.77	64.43	15.222257		36.0316	1	36.0316	959.68533	137.82883	6.9628779		1 36.0316
3-caffeoyl-quinic acid	353.0872	4189.42	1	4189.42	1051.2047	355.4334	2.9575293									137.82883 959.68533
		3140.93	1	3140.93	1021.4	341.553	2.9904583									
		3499.6	1	3499.6	1881.7577	296.32	6.3504242									
		3272.41	1	3272.41	1756.857	294.12	5.973266									
		3496.566	1	3496.566	1174.026	233.41	5.0298873									
		3212.05	1	3212.05	1081.69	221.78	4.8773108		3468.496	1	3468.496	1327.8226	290.43607	4.571824		1 3468.496
N-p-coumaroyltryptamine	305.1293	374.8441	1	374.8441	906.46	112.24	8.076087									290.43607 1327.8226
		109.95	1	109.95	956.3044	89.23	10.717297									
		293.589	1.05	279.6085714	1051	166.56	6.3100384									
		303.8651	1	303.8651	806.01	145.73	5.5308447									
		315.5656	9.08	34.7539207	754.2	113.99	6.6163699									
		316.49	3.81	83.06824147	756.73	122.59	6.1728526		285.7173	2.8233333	101.19857	871.78407	7.2372482	120.45795		2.8233333 285.7173
Feruloyl-feruloyl-glycerol	443.1341	1080.1606	50.77	21.27556825	656.68	86.61	7.5820344									7.2372482 871.78407
		743.56	45.898	16.20027016	579.8	66.35	8.7385079									
		473.8	50.38	9.404525605	1242.78	93.91	13.233734									
		543.78	37.399	14.53996096	588.87	112.35	5.2413885									
		685.12	25.83	26.52419667	1076.67	129.08	8.3411063									
		594.13	42.08	14.11905894	903.45	72.0476	12.539627		686.75843	42.0595	16.32826	841.375	93.391267	9.00914		42.0595 686.75843
Citric acid	191.0197	3677.4958	318.119	11.56012624	1278.48	1485	0.8609293									93.391267 841.375
		1155.69	75.47	15.31323705	2524.44	618	4.0848544									
		1062.29	1	1062.29	1	1	1									
		959.0066	1702.37	0.563336173	1094.67	453.12	2.4158501									
		2104.44	1237	1.701244947	1262.521	1	1262.521									
		2283.68	1	2283.68	2836.72	604.05	4.6961675		1873.7671	555.8265	3.3711366	1499.6385	527.02833	2.8454609		555.8265 1873.7671
Coumaroyl-tyramine	282.1133	357.93	1	357.93	634.25	76.29	8.3136715									527.02833 1499.6385
		124.49	1	124.49	620.25	77.8822	7.9639507									
		295.001	6.59	44.76494689	1183.69	115.29	10.267066									
		278	10.5	26.47619048	897.8	144.89	6.1964249									
		412.225	13.99	29.46568978	579.72	82.9	6.9930036									
		372	1	372	496.81	117.77	4.2184767		306.60767	5.68	53.980223	306.60767	5.68	53.980223		5.68 306.60767
3-caffeoyl-quinic acid (dimer)	707.1817	3725.18	1	3725.18	0	0	#DIV/0!									102.5037 735.42
		2607.708	1	2607.708	0	0	#DIV/0!									
		2303.08	1	2303.08	0	0	#DIV/0!									
		2804.66	17.79	157.6537381	0	0	#DIV/0!									
		2600.26	69.02	37.67400753	0	0	#DIV/0!									
		2894.26	20.13	143.7784401	0	0	#DIV/0!		2822.5247	20.13	140.21484	ND	ND	ND		18.32333 2822.5247
N-feruloyl-tryptamine	335.1397	253.56	1	253.56	314.35	65.04	4.8331796									
		59.23	1	59.23	315.94	65.15	4.8494244									
		192.38	1	192.38	434.12	39.83	10.899322									
		194.06	1	194.06	343.64	56.3	6.10373									
		142.99	2.12	67.44811321	380.71	42.25	9.0108876									
		130.09	2.39	54.43096234	330.44	56.23	5.8765783		96.576257	2.39	40.408476	353.2	54.133333	6.5246305		1.418333 96.576257
Apigenin	269.0455	83.71	1.001	83.62637363	164.177	11.19	14.671761									54.133333 353.2
		30.68	0.127	241.5748031	411.11	18.54	22.174218									
		20.68	4.31	4.798143852	327.7	36.69	8.931589									
		40.33	2.648	15.23036254	234.06	34.2	6.8438596									
		22.54	1	22.54	234.51	12.89	18.193173									
		20.49	1.37	14.95620438	169.43	11.68	14.505993		36.405	1.7426667	20.890398	256.83117	14.220099	18.061138		1.7426667 36.405
Genkwanin	283.0607	287.117	1	287.117	271.05	31.51	8.6020311									20.865 256.83117
		143.86	1	143.86	269.771	37.68	7.1595276									
		99.64	3.6593	27.2292515	210.51	65.7	3.2041096									
		116.39	0.605	192.3801653	192.17	67.37	2.8524566									
		61.65	2.765	22.2965642	241.662	14.51	16.654859									
		57.62	0.773	74.54075032	220.37	15.8554	13.898735		127.71283	1.6337167	78.173184	234.2555	38.7709	6.0420444		1.6337167 127.71283
5-feruloyl-quinic acid	367.1029	1042.8	16.56	62.97101449	225.92	48.03	4.7037268									38.7709 234.2555
		576.116	16.89	34.10988751	209.43	35.47	5.9044263									
		558.42	1	558.42	464.56	36.9	12.589702									
		716.8	1	716.8	395.38	62.13	6.3637534									
		664.75	76.253	8.717689796	241.46	61.05	3.9551188									
		574.56	31.2	18.41538462	195.72	55.72	3.5125628		688.90767	23.817167	28.924837	288.745	49.883333	5.7884063		23.817167 688.90767
Coumaroyl-feruloyl-glycerol	413.1234	187.47	49.61	3.778875227	134.55	18.51	7.2690438									49.883333 288.745
		111.29	75.16	1.480707823	111.98	24.65	4.5427992									
		74.61	37.56	1.986421725	241.18	30.39	7.9361632									
		111.54	41.29	2.70138048	180.35	21.53	8.3766837									
		133.96	8.95	14.96759777	165.66	18.82	8.8023379									
		134.08	7.35	18.24217687	150.47	18.81	7.9994684		125.49167	36.653333	3.423745	164.03167	22.118333	7.4160952		36.653333 125.49167
Feruloyl-tyramine	312.1236	310.16	0.3	1033.866667	151.05	35.05	4.3095578									22.118333 164.03167
		92.65	0.5	185.3	144.23	31.41	4.5918497									
		219.04	1.35	162.2518519	245.05	40.88	5.9943738									
		189.59	2.87	66.05923345	233.96	38.52	6.0737279									
		252.544	8.5	29.71105882	175.41	46.55	3.7682062									
		238.27	1	238.27	157.76	36.35	4.3400275		217.04233	2.42	89.686915	184.57667	38.126667	4.8411436		2.42 217.04233
Homoeryodictyol	301.0712	36.39	1	36.39	101.76	20.46	4.973607									38.126667 184.57667
		1	1	1	149.72	29.78	5.0275353									
		15.31	1	15.31	136.89	50.19	2.7274357									
		20.76	1	20.76	144.13	4.32	33.363426									
		1	1	1	164.62	6.04	27.254967									
		1.64	1	1.64	154.38	1	154.38		12.683333	1	12.683333	141.91667	18.631667	7.6169604		1 12.683333
																18.631667 141.91667

Table S4. Clustering results of metabolomic markers in control and *C. graminicola*-infected maize leaves and roots. LAIL = leaf assay, infected leaf; LACIL = leaf assay, control leaf; RAIL = root assay, infected root; RACIL = root assay, control root. Available online at *The Plant Journal* upon online publication.

Table S5. Clustering results of metabolomic markers in systemic tissues during *C. graminicola* attack. LASL = leaf assay, systemic leaf; LACSL = leaf assay, control systemic leaf; LASR = leaf assay, systemic root; LACSR = leaf assay, control systemic root; RASL = root assay, systemic leaf; RACSL = root assay, control systemic leaf. Available online at *The Plant Journal* upon online publication.

Chapter IV

Local and systemic organ-specific changes in the small RNA transcriptome of maize upon *Colletotrichum graminicola* infection

IV: Local and systemic organ-specific changes in the small RNA transcriptome of maize upon *Colletotrichum graminicola* infection

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IV: The role of small RNAs during antifungal resistance in *Zea mays*

Summary

During fungal attack, plants employ highly adaptive and organ-specific defense responses, reaching from molecular to chemical and physiological adjustments of their defense machinery. MicroRNAs (miRNAs), 21-24 nt noncoding small RNAs (smRNAs) implicated in gene silencing, are well known play a role during abiotic and biotic stress responses. To examine the impact of miRNAs during the interaction of *Zea mays* with pathogen fungi, high-throughput deep sequencing of smRNAs of *Colletotrichum graminicola*-infected leaves and roots was carried out. *C. graminicola*, the causal agent of maize anthracnose, infects maize shoots and roots, facilitating the comparison of organ-specific smRNA profiles. Deep sequencing yielded smRNA libraries that are distinctive for infected shoots and roots. Fungal infection led to massive change in the miRNA transcriptome, which also differed from the miRNA expression pattern in leaves challenged with the herbivore *Spodoptera frugiperda*. In addition, local fungal infection of leaves also resulted in adaptations of the miRNA transcriptome in systemic un-infected leaves. Among the identified target genes of miRNAs with altered expression profiles, defense-related genes were found. In summary, this study indicates that miRNAs play a organ-specific role during local and systemic antifungal defense in maize.

Introduction

Plants are constantly exposed to numerous potential attackers and since they are lacking specialized defensive cells, they consequently depend on flexible resistance mechanisms. Such mechanisms operate at the molecular level, where they fine-tune defense responses which are tightly linked to the symphony of plant physiology including plant growth or nutrient distribution. In this regard, orchestrating defense mechanism is a delicate process that requires an intricate balance of cost and effectiveness. In recent years, small RNAs (smRNAs) have

been recognized as yet underrated but relevant regulators of both abiotic and biotic stress responses (Kruszka *et al*., 2012, Balmer and Mauch-Mani 2012). smRNAs are non-coding RNAs with a size of 19-26 nucleotides (nt), which regulate gene expression at the transcriptional and posttranscriptional level by either regulating DNA methylation, or triggering the degradation of mRNA as well as inhibiting the mRNA translation (Mallory and Vaucheret 2006). The two main classes of smRNAs, microRNAs (miRNAs) and short interfering RNAs (siRNAs), respectively, both originate from double-stranded RNAs; however, the two classes are characterized by a distinct biogenesis (Voinnet 2009). miRNAs are transcribed from endogenous *MIR* genes by RNA polymerase II, forming an imperfect stem-loop structure that is recognized by Dicer-like (DCL) proteins that cut the double-stranded region and therefore catalyze the formation of miRNA precursors. These precursors are further processed, and the mature miRNA is then methylated by HEN1 and exported to the cytoplasm. There, one of the miRNA duplex strands gets incorporated into an AGO protein, which guides the targeting and slicing of target mRNAs that possess a complementary binding site specific to given miRNAs (Vaucheret 2008). In contrast to miRNAs, siRNAs originate from long, perfect double-stranded (ds) RNA derived from inverted repeat sequences, transcription of sense-antisense gene pairs, virus-derived transcripts or artificially applied exogenous RNA (Ding and Voinnet 2007).

Besides their important role during plant development and maintenance of genome integrity, smRNAs are now also known to contribute to biotic stress responses of plants (Kruszka *et al*., 2012; Balmer and Mauch-Mani 2012). siRNAs fulfill a pivotal role in mediating plant antiviral immunity (Ding and Voinnet 2007). miRNAs are involved in a variety of biotic stress responses. For instance, they mediate antibacterial defense by regulating mRNAs coding for F-box proteins (Navarro *et al*., 2006). In addition, they also control innate immunity via the regulation of mRNAs encoding pattern recognition receptors (PRRs) (Li *et al*., 2012). Some studies also propose a role of miRNAs in antifungal defense. Pine trees infected with the rust fungus *Cronartium quercuum* f. sp. *fusiforme* show an altered miRNA transcriptome in response to fungal attack (Lu *et al*., 2007), and in wheat, *Erysiphe graminis* f. sp. *tritici* triggers a shift in the expression pattern of different miRNA families (Xin *et al*., 2010).

Recent advances in high-throughput sequencing technologies are helping to uncover the pivotal role of smRNAs during plant defense. smRNA deep sequencing using Illumina HiSeq platforms is a valuable technology that facilitates the analysis and comparison of whole smRNA transcriptomes under distinct biological conditions (Pais *et al*., 2011). Although this method generate massive datasets of siRNA or miRNA expression profiles, the biological meaning of such transcriptional adaptations often remains elusive. However, these datasets are a convenient starting point for further analysis such as miRNA target and target cleavage identification.

In this study, smRNA deep sequencing was used to elucidate the role of smRNAs during infection of maize with *C. graminicola*. This hemibiotrophic fungus causes maize anthracnose and leads to highly adaptive, organ-specific local and systemic immune responses in its host (Balmer *et al*., 2012). To explore putative molecular fine-tuning mechanisms, *C. graminicola*-infected maize leaves and roots were subjected to smRNA deep sequencing. To analyze the specificity of the smRNA expression in response to fungal attack, a library of maize plants challenged during 2 days with the herbivore *Spodoptera frugiperda* was generated for comparison. As *C. graminicola* employs a hemibiotrophic lifestyle when infecting leaves, both biotrophic (24 hours post inoculation (hpi)) and necrotrophic (96 hpi) stages were subjected to the deep sequencing of leaf tissues. Moreover, systemic changes in leaves 96hpi upon leaf and root infection were analyzed. For each biological treatment, a control treatment was sequenced in parallel. An overview of the different treatments is presented in Figure 1.

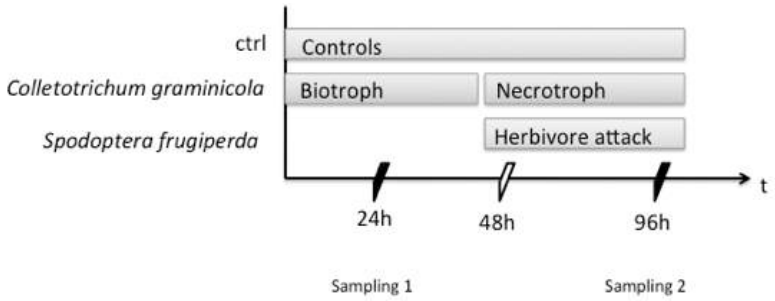

Figure 1. Overview of the experimental setup for the smRNA library construction. 12 days old maize plants were either control (ctrl) treated, or infected with *Colletotrichum graminicola*. Twenty-four hours post infection (hpi), ctrl and *C. graminicola*-infected plants were harvested, giving rise to libraries named GSB4 and GSB3, respectively. Similarly, 96 hpi, ctrl and *C. graminicola*-infected plants were sampled (libraries GSB2 and GSB1, respectively). In parallel, plants were challenged with *Spodoptera frugiperda* for 2 days (library GSB11); GSB2 was used as a control library. In addition, systemic defense responses were analyzed; systemic leaves upon leaf infection (96hpi) were subjected to deep sequencing (GSB7; GSB8 = ctrl), as well as systemic leaves upon root infection (96hpi) (GSB9, GSB10 = ctrl).

Results

Deep sequencing of maize smRNAs

Eleven smRNA libraries (GSB1-11) were prepared and subjected to high-throughput smRNA deep sequencing. Analysis of the sequence read statistics showed that the read length distribution differed between control and treated samples. For instance, *C. graminicola*-infected leaves exhibited a higher percentage of 21-22 nt read lengths compared to control samples, as well as a slightly higher number of 24 nt reads at 24 hpi (Figure 2a). In contrast, infected roots exhibit reduced levels of 21 and 24 nt reads (Figure 2b). Similarly, systemic leaves showed reduced amounts of 21-22 and 24 nt reads upon leaf infection (Figure 2c). Therefore, the read length analysis indicates a local and systemic adaptation of the smRNA transcriptome in response to *C. graminicola* infection.

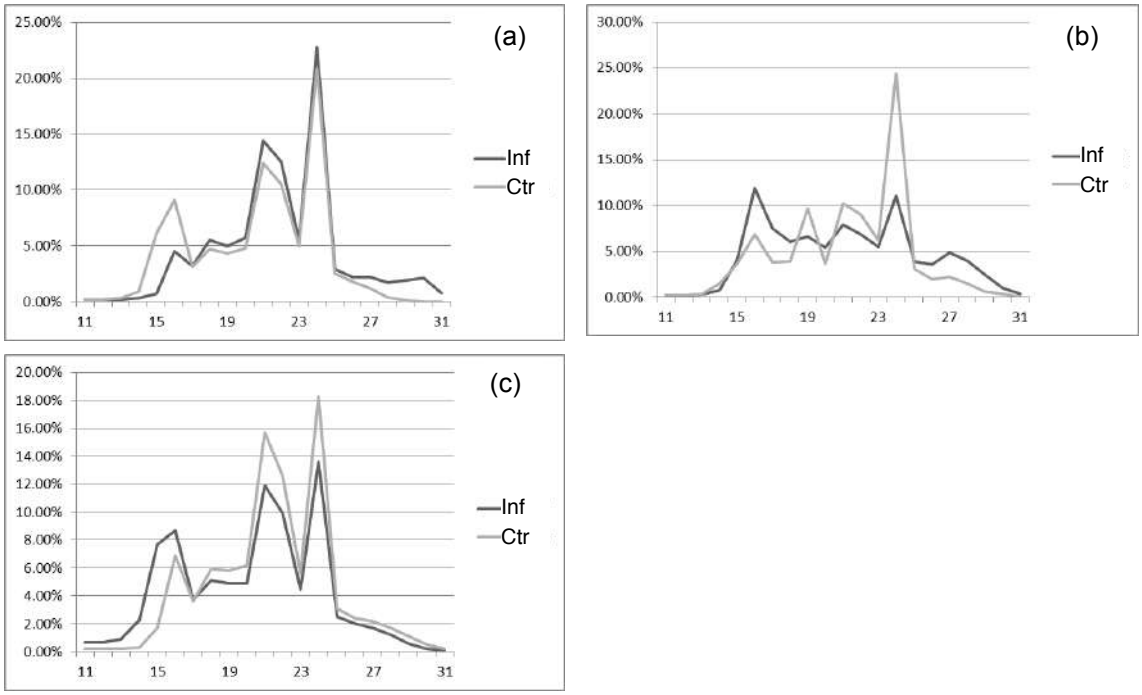

Figure 1. Profile of sequence read lengths in different smRNA libraries. (a) *C. graminciola*-infected (Inf) leaf, 24 hours post inoculation, Ctr = control leaf; y-axis indicates the percentage of a given read size compared to the total number of reads, the x-axis indiciates the read size in nucleotides. (b) *C. graminicola*-infected (Inf) and control (Ctr) roots. (c) Systemic leaves upon *C. graminicola* leaf infection (Inf) and control (Ctr) treatment. (d) Systemic leaves upon *C. graminicola* root infection (Inf) and control (Ctr) treatment.

Expression profiling and target identification of known miRNAs

Using annotated maize miRNAs, known miRNAs were identified in the different sequence libraries. To examine the expression of known miRNAs, the abundance of given miRNAs was noted as normalized read number. These data were summarized in a heatmap (Figure S1), and in Table S1, where the read counts of the 100 mostly expressed miRNAs are summarized. In

Library	miRNA	FI	Putative target genes
Inf L 24h	miR393	2.23	Calmodulin-binding protein MPCBP; cyclin-like F-box
	miR479	3	Unknown
	miR1120	-3	Unknown
	miR1432	2.3	Para-hydroxybenzoate-polyprenyltransferase (LOC100282174)
	miR2092	7	Unknown
Inf L 96h	miR168	2.7	Argonaute and Dicer protein; ubiquitin carboxyl-terminal hydrolase - *Oryza sativa*
	miR479	4	Unknown
	miR1432	18.3	Para-hydroxybenzoate-polyprenyltransferase (LOC100282174)
	miR2916	3.3	Quinone reductase 2 - *Triticum monococcum*; *Zea mays* 18S ribosomal RNA gene
	miR5205	-3.25	Unknown
Inf R 96h	miR166	-6.5	MFS14 protein precursor; basic-leucine zipper (bZIP) transcription factor; lipid-binding
	miR169	-3.8	RAPB protein - *Oryza sativa*; allene oxide synthase - *Zea mays*
	miR395	-15.5	ATP sulfurylase (LOC541653), mRNA
	miR909	5	Inhibin, beta B subunit; vinculin; heavy metal transport/detoxification protein
	miR1432	4.5	Para-hydroxybenzoate-polyprenyltransferase (LOC100282174)
	miR2092	2.6	Unknown
	miR2863	3.5	Unknown
Inf L sys L	miR397	2.2	Laccase; multicopper oxidase;
	miR916	3.3	Zein protein-body ER membrane protein
	miR5169	2.2	Unknown
Inf R sys L	miR395	-2.7	ATP sulfurylase (LOC541653), mRNA
	miR479	3	Unknown
	miR1877	-3	Putative protein binding protein
	miR2592	3	Unknown
Herbivore	miR164	4	Mutator transposable element MuA2 - *Zea mays*, cytochrome P450 ; No apical meristem (NAM) protein
	miR168	2	Argonaute and Dicer protein; ubiquitin carboxyl-terminal hydrolase - *Oryza sativa*
	miR393	2.6	Calmodulin-binding protein MPCBP, cyclin-like F-box
	miR394	11	Cyclin-like F-box; F-box associated type 1; galactose oxidase/kelch, orphan nuclear receptor-like
	miR474	5.5	Unknown
	miR2916	6.8	Unknown
	miR5072	-2.5	Unknown

Table 1. Differentially expressed miRNAs and their putative target genes. FI = fold induction compared to control libraries. Green letters = upregulation, red letters = downregulation. Inf = infected, L = leaf, R = root, sys = systemic.

both locally infected roots and leaves, various miRNAs showed an altered expression compared to control tissues (Table S1). Similarly, systemic tissues also exhibit a changed miRNA expression profile, as well as herbivore-challenged leaves.

In order to quantify the expression level of miRNA in the treated samples, the fold change expression was determined by calculating the relative difference of sequence reads in treated samples compared to the control libraries. Selected miRNAs showing a fold change >2 are summarized in Table 1. Comparing biotrophic and necrotrophic fungal infection stages, miR479, miR1318 and miR1432 were found to be upregulated; however, their fold induction was higher during the necrotrophic stage. Other miRNAs such as miR393, miR1120 and miR2092 showed an altered expression level exclusively during biotrophy. In contrast, the expression of miR168, miR2916 and miR5205 was altered only during necrotrophy. Interestingly, miR1432 and miR2092 were also upregulated in infected roots. However, infected roots showed also a distinct expression profile, with miR166, miR169 and miR395 that were downregulated, whereas miR909 and miR2863 were upregulated. In addition, a different situation was found in systemic leaves upon leaf infection; compared to local infected tissues, less miRNAs showed an altered expression. For instance, miR397, miR916 and miR5169 were upregulated. In systemic leaves upon root infection, miR1877 and miR2592 were down- and upregulated, respectively. Interestingly, mi395 was downregulated, and miR479 showed elevated expression levels; miR479 was also found to be upregulated in local leaf infections, whereas the downregulation of miR395 was also observed in infected roots. In contrast, leaves challenged with herbivores showed a distinct, massive change in the miRNA transcriptome. Amongst the altered miRNAs, miR164, miR168, mIR393, miR394, miR474 and miR2916 were upregualted, and miR5072 as well as miR5077 were downregulated. In summary, although some miRNAs were commonly regulated in both locally infected leaves and roots, the miRNA transcriptome was specific for a given biotic stress and in addition also highly organ-specific.

miR395 is downregulated in roots in response to *C. graminicola* infection and targets an ATP sulfurylase

To confirm the deep sequencing results, Northern blots of a selected miRNA were performed. Due to the relatively high expression level and the remarkable difference between control and treated samples, miR395 was selected. As expected, miR395 showed a reduced expression level upon fungal infections in roots (Figure 3). The signal intensity also corresponded to the sequence reads in the different libraries, with the highest number of reads (93) in control roots.

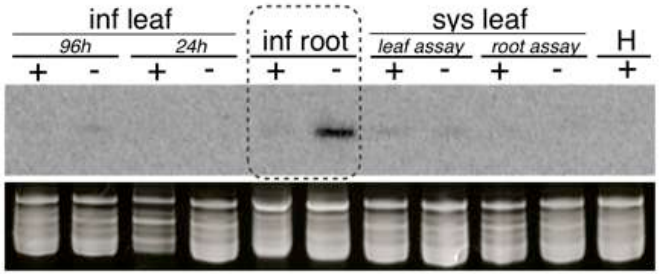

Figure 3. Northern blot analysis of miR395 expression. + indicates *C. graminicola* infection, - control tissue. H = herbivore (*S. frugiperda*).

To examine the putative role of miR395 during root infections, the maize genome was analyzed for putative target genes. Five known target genes were identified (Table 2). Two genes (dienelactone hydrolase and FMR1-interacting) exhibit two mismatch positions for miR395. The other genes (ATP sulfurylase on chromosome 1 and 5, sulphate anion transporter) matched perfectly to the miR395 sequence.

Target Site Alignment	Start	End	Chr	Mm	Gene	Gene Function
GAGTTCCTCCAAACACTTCA	274094195	274094214	1	0	GRMZM2G149952	ATP-sulfurylase; PUA-like
GAGTTTCCCCAAACACTTCA	24677524	24677543	1	0	GRMZM2G042171	Sulphate anion transporter; Sulphate transporter/antisigma-factor antagonist STAS; Sulphate transporter; Sulphate anion transporter, conserved site; Sulphate anion transporter; Sulphate transporter/antisigma-factor antagonist STAS; Sulphate transporter
GAGTTCCTCCAAGCACTTCA	7662886	7662905	5	0	GRMZM2G051270	ATP-sulfurylase; PUA-like; ATP-sulfurylase; PUA-like; ATP-sulfurylase; PUA-like
TAGTGCCCCCAAACACTTCA	226705918	226705937	3	2	GRMZM2G035620	Dienelactone hydrolase; Dienelactone hydrolase
GAGTTCGCCCAAATCCTTCA	9511424	9511443	1	2	GRMZM2G170567	Cytoplasmic FMR1-interacting

Table 2. Analysis of miR395 targets in the maize genome. Chr = chromosome; Mm = numbers of mismatches of the miRNA and target gene sequence.

Discussion

The goal of this study was the generation of maize smRNA libraries of *C. graminicola*-infected and *S. frugiperda*-challenged tissues, that could serve as basis for further analyses such as the expression analysis of known miRNAs. The deep sequencing data indicate a general up- or downregulation of 21 and 24 nt smRNAs in infected tissues compared to control samples, thus revealing a general transcriptional adaptation of certain smRNA families such as miRNAs. However, it remains to be determined which smRNA species are affected in the libraries generated in this study. Interestingly, infection of Arabidopsis with the *Oilseed rape mosaic tobamovirus* leads to a global enrichment of 21 nt smRNAs, including miRNAs and siRNAs (Hu *et al*., 2011). Nevertheless, it remains elusive whether this virus-induced smRNA enrichment or the fungal-induced alterations of smRNA levels respectively, play a role during plant immunity.

In addition, read count analysis of known miRNAs revealed an organ-specific transcriptional adaptation of the miRNA transcriptome upon fungal infections. Along with the finding that different plant miRNAs are induced or downregulated depending on the biotrophic or necrotrophic fungal lifestyle, the miRNA transcriptome also differed from the expression pattern in herbivore-challenged plants. Thus, the response of maize plants to given biotic stresses is highly specific. Considering the fact that the expression of miRNAs is known to be highly tissue- and organ-specific (Voinnet 2009), miRNAs might play an important role in mediating tissue-specific defense responses, as observed for antifungal defense responses in maize (Balmer *et al*., 2012).

Among the identified miRNAs that exhibited an altered expression upon fungal infection or herbivore challenge, few defense-related miRNAs were identified. For instance, miR1432 that targets a para-hydroxybenzoate-polyprenyltransferase, is upregulated during both biotrophic and necrotrophic fungal infection in leaves as well as in roots. The maize para-hydroxybenzoate-polyprenyltransferase is associated with three maize metabolic pathways, namely the KEGG (http://www.genome.jp/kegg) metabolic pathways zma00130 (ubiquinone biosynthesis), zma01100 (metabolic pathways), zma01110 (biosynthesis of secondary metabolites). The enzyme is important for the terpenoid-quinone biosynthesis. It is known that

flavonoids play an important role in defense against *C. graminicola* (Balmer *et al*., 2012), hence it could be speculated that the downregulation of the enzyme mRNA and therefore protein level could divert the secondary metabolism towards flavonoid biosynthesis. This hypothesis is supported by the fact that terpenoids are playing minor roles during *C. graminicola* infection in both leaves and roots (Balmer *et al*. 2012). Although the proposed role of miR1432 is highly speculative, the fact that it is upregulated during both fungal infection phases and in leaves as well as in roots makes it a prominent target for further investigations.

In contrast to miR1432, miR395 showed an organ-specific expression. Exhibiting only low levels in control and infected leaves, miR395 was 15-fold downregulated in *C. graminicola*-infected roots compared to control roots. miR395 targets a gene encoding an ATP sulfurylase (APS), a key enzyme in sulfate assimilation. In Arabidopsis, miR395 is known to be upregulated in roots in response to sulfate deficiency (Liang *et al.*, 2010; Matthewman *et al*., 2012). Arabidopsis overexpressing miR395 accumulate sulfate in the shoots but not in roots and exhibit a similar phenotype as *APS* mutants (Liang *et al*., 2010). Therefore it is assumed that the downregulation of *APS*-encoding genes by miR395 is mediating the accumulation of sulfate in the shoots likely by affecting root-to-shoot transport mechanisms (Matthewman *et al*., 2012). The situation presented in this study demonstrates a downregulation of miR395 in roots upon *C. graminicola* infection, as well as in systemic leaves upon *C. graminicola* root infection. Thus, this is reminiscent of an internal sulfate over-accumulation, which would lead to a miR395-mediated inhibition of root-shoot sulfate transport. A possible explanation would be that fungal infection leads to an accumulation of sulfate in the roots. On the other hand, APS play an important role in glutathione synthesis. Hence, downregulation of miR395 could promote the expression of *APS* mRNA and positively influence the glutathione synthesis pathway. Interestingly, treating Arabidopsis with buthionine sulfoximine, an inhibitor of glutathione synthesis also results in a suppression of miR395 expression levels (Matthewman *et al*. 2012), thus mimicking a fungal infection. Glutathione in its reduced chemical state (GSH) is known to be a pivotal regulator of the cellular redox state, thus being crucial for early defense signaling events such as the production of reactive oxygene species (ROS) (Dubreuil-Maurizi and Poinssot 2012). Nevertheless, the role of miR395 in antifungal defense in maize

has to be further elucidated, primarily by quantifying sulfate and GSH in local and systemic tissues, or by exposing plants to different sulfate conditions and analyzing the consequence on fungal resistance.

Interestingly, fungal root infections also results in the downregulation of miR169, a miRNA that putatively targets a gene encoding an allene oxide synthase (AOS). AOS is a crucial enzyme for jasmonic acid (JA) biosynthesis, thus playing an important role in hormone-mediated defense signaling (Park *et al*., 2002). Given the fact that *C. graminicola* infection results in elevated JA levels in roots, miR169 could act as suppressor of JA-signaling under normal conditions, whereas the downregulation of miR169 during infection could promote JA-synthesis.

Besides miRNAs with known targets, this study also showed altered expression patterns for miRNAs with yet unknown targets, such as miR479 or miR2092. An extensive degradome analysis of maize tissues during fungal attack would be required to further analyze putative smRNA targets. Interestingly, this study also showed that most of the altered miRNAs are targeting cellular and physiological processes rather than specific defense pathways such as resistance (*R*)-genes, which is for example the case in tomato infected with bacteria (Shivaprasad *et al*. 2012). However, this picture fits with the fact that defense mechanisms against *C. graminicola* are based on quantitative resistance rather than on race-specific *R*-genes (Bergstrom and Nicholson 1999).

Conclusions and outlook

In summary, this study shows that maize plants employ a tissue- and stress-specific adaptation of the smRNA transcriptome in response to fungal infection or herbivore challenge. Read count analysis of known miRNAs resulted in the identification of differently regulated miRNAs. The majority of these miRNAs putatively target genes involved in cellular or physiological processes; however, some altered miRNAs such as miR169, miR395 and miR1432 could also directly participate in defense signaling. This study extends the current

knowledge of smRNAs as players in the concert of plant immune responses to the high value crop model maize, providing a basis for further investigations of the intricate signaling network mediating antifungal defense.

Nonetheless, further work is needed to decipher smRNA-mediated defense mechanisms. For this reason, confirmation of miRNA target gene degradation by a RACE (Rapid Amplification of cDNA Ends) assay will be conducted, parallel to the expression analysis of target mRNAs. Moreover, exposing maize plants to different sulfate conditions and also quantifying GSH levels will clarify the role of miR395 during antifungal defense.

Materials and methods

Biological treatments

Maize plants (variety Jubilee, West Coast Seeds, www.westcoastseeds.com) were cultured in a soil-free system (Planchamp *et al.,* 2012). Leaf and root infection of 12-days old maize plants with *Colletotrichum graminicola* M1.001 (obtained from Lisa Vaillancourt, University of Kentucky) were executed as previously described (Balmer *et al*., 2012). Maize leaves were infested with *Spodoptera frugiperda* as described (Robert *et al*., 2012).

Deep sequencing of small RNAs

Total RNA was isolated using Trizol (Invitrogen, www.invitrogen.com). For each sample library, 6 biological replicates were pooled for further analysis. Ten µg total RNA was used for small RNA library construction; Illumina-Solexa sequencing was performed at FASTERIS (http://www.fasteris.com).

Identification and quantification of conserved miRNAs

For the identification of conserved miRNAs, sequences of 4,677 mature plant miRNAs were downloaded from miRBase (release 18.0, November 2011). Identical miRNA sequences identified in different species or duplicated loci in a genome were collapsed, resulting in a non-

redundant list consisting of 2,228 unique miRNAs. Sequences belonging to the same miRNA family were further analysed by multiple alignment using ClustalW (www.clustal.org) and classified in subgroups (S01, S02, S03, etc.) to distinguish *bona fide* mature miRNAs from misannotated miRNA* forms or sequences generated from diffqerent regions of the same precursor. This non-redundant library was then used to screen the small RNA libraries. All the small RNA reads in the range of 20 to 24 nt in size and represented by at least 2 reads in a library were aligned to the 1,772 unique miRNAs derived from miRBase. For the screening a maximum of 3 mismatches was allowed and up to 2 nt overhanging nucleotides at the 5' and/or 3' end. Alignments were performed using SeqMap (Jiang and Wong 2008). The output was filtered and reformatted with custom PERL scripts, classifying the identified miRNAs according to miRBase. Customized PERL scripts were used to create HTM heatmaps summarizing the information for all the miRNA families at once, showing either the sum of abundances of all the variants or the abundance of the most frequent variant for each miRNA family across the small RNA libraries. Heatmaps were also created for each miRNA family or subgroup, showing the abundance of each identified miRNA variant in the investigated samples. Abundances were normalized to the total size of each corresponding library.

Small RNA Northern blotting

Northern blotting of small RNAs was performed according to Si-Ammour *et al.* (2011).

Target prediction of maize miRNAs

Putative targets of maize miRNAs were identified using the psRNATarget web server (http://bioinfo3.noble.org/miRU2/) against *Zea mays* DFCI Gene index (version 19) and *Zea mays* PlantGDB genomic project. Default settings were applied.

Acknowledgements

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Supplemental materials

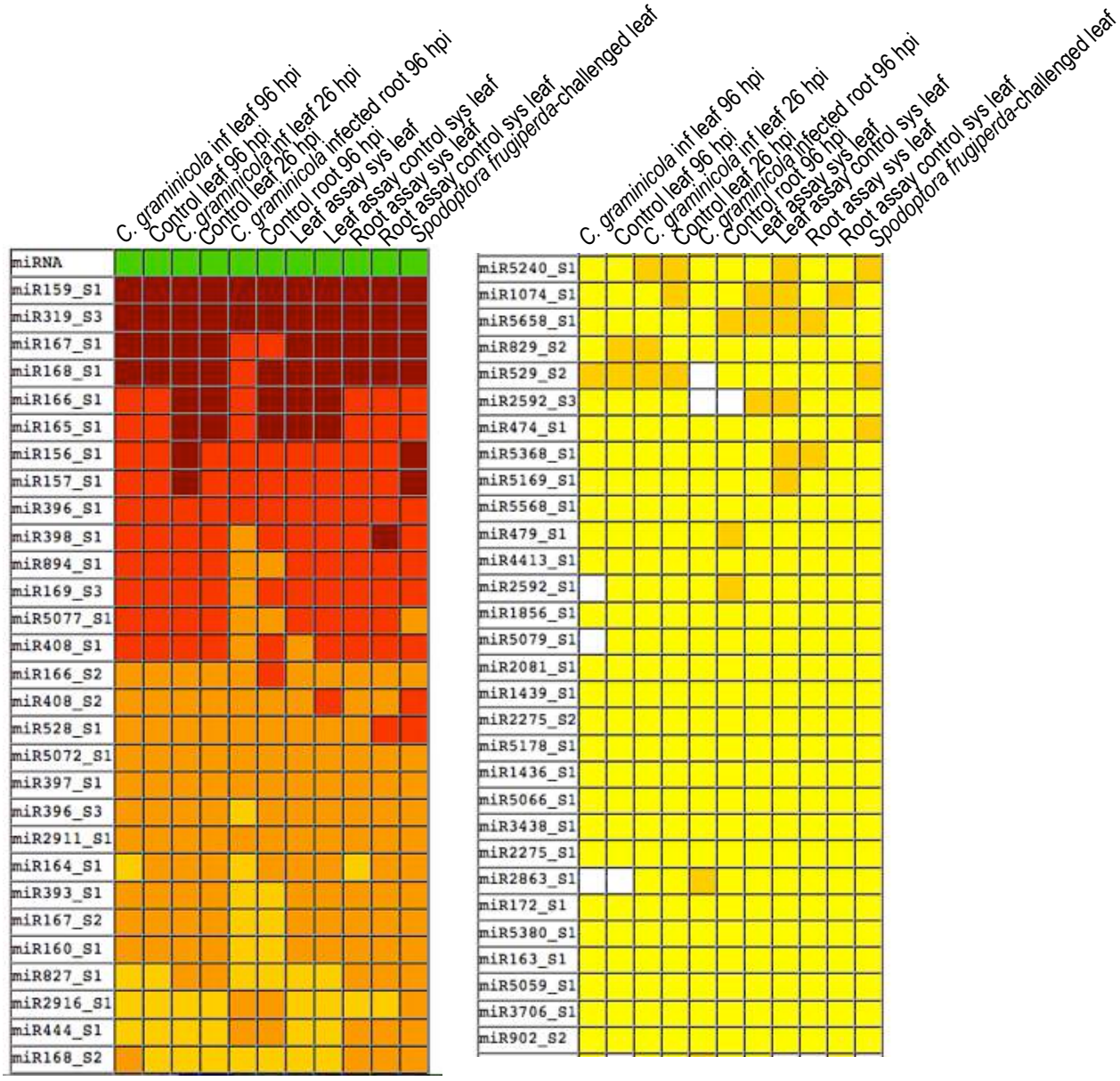

Figure S1. Heatmap of miRNA sequence reads in the different libraries. 1: *Colletotrichum graminicola*-infected leaf 96 hpi, 2: ctrl infected leaf 96 hpi. 3: infected leaf 26 hpi, 4: ctrl infected leaf 26 hpi. 5: infected root 96 hpi, 6: ctrl infected root 96 hpi. 7: leaf assay, systemic leaf, 8: ctrl leaf assay, systemic leaf. 9: root assay, systemic leaf, 10: ctrl root assay, systemic leaf. 11: *Spodoptera frugiperda*-challenged leaf.

Table S1. Read count of the 100 mostly expressed miRNAs in the different libraries.

	inf L 96	ctr L 96	inf L 24	ctr L 24	Inf R 96	ctr R 96	L sys L	ctr LsL	R sys L	ctrl RSL	ctr Herb	Herb
	GSB01	GSB02	GSB03	GSB04	GSB05	GSB06	GSB07	GSB08	GSB09	GSB10	GSB02	GSB11
miR159_S1	26662	35392	53486	62237	11568	33143	61887	66610	34223	61989	35392	76727
miR319_S3	26533	35182	52071	60684	11230	32518	60336	64844	33266	60443	35182	74864
miR167_S1	27919	22369	15789	14818	2566	3172	19402	18490	19065	23650	22369	24212
miR168_S1	34230	12340	11692	10833	9937	13752	11734	11301	15294	16974	12340	22664
miR166_S1	7361	7056	11505	11051	3337	15485	10442	12902	7198	9640	7056	8343
miR165_S1	7248	6911	11315	10880	3251	15288	10214	12603	7072	9491	6911	8192
miR156_S1	5566	8787	10166	9918	1127	2357	7987	7801	4217	7069	8787	11284
miR157_S1	5511	8715	10016	9753	1080	2311	7886	7686	4141	6963	8715	11120
miR396_S1	5059	6426	8470	7512	1538	4327	5715	7137	5433	6517	6426	6420
miR398_S1	5623	5573	2616	1869	507	1112	2079	3533	4603	12498	5573	4965
miR894_S1	1446	2296	2790	2660	533	689	3164	3568	3824	2986	2296	1162
miR169_S3	2291	3529	1772	1641	416	1585	2442	2363	1437	1948	3529	1791
miR5077_S1	1338	2049	2315	2070	346	457	2547	2878	2653	2213	2049	929
miR408_S1	1425	1637	1478	1454	603	1011	704	1162	1857	3436	1637	1613
miR166_S2	965	629	863	813	283	1861	763	754	634	957	629	821
miR408_S2	564	783	299	278	538	780	594	1125	502	769	783	1306
miR528_S1	349	580	393	282	366	671	474	742	566	1067	580	1475
miR5072_S1	249	455	330	360	161	173	432	450	882	532	455	181
miR397_S1	441	514	305	310	123	173	135	233	590	691	514	476
miR396_S3	437	418	407	433	83	118	354	412	341	425	418	433
miR2911_S1	161	203	131	102	476	350	169	213	157	105	203	116
miR164_S1	90	131	151	192	73	128	257	292	83	115	131	535
miR393_S1	170	215	371	166	44	87	149	172	238	127	215	113
miR167_S2	216	223	140	126	51	82	211	332	138	159	223	129
miR160_S1	102	122	131	126	54	92	158	181	132	177	122	213
miR827_S1	82	96	125	138	69	66	56	66	297	231	96	181
miR2916_S1	91	27	59	54	283	237	66	64	56	51	27	186
miR444_S1	56	51	97	70	124	231	80	100	119	101	51	125
miR168_S2	174	66	89	67	93	94	81	82	144	124	66	135
miR5139_S1	43	67	100	97	59	66	94	107	203	134	67	56
miR393_S2	41	43	155	143	15	39	73	94	135	126	43	112
miR171_S1	54	61	102	87	61	111	83	94	69	75	61	95
miR170_S1	49	54	84	74	54	102	67	79	60	63	54	84
miR5381_S1	34	34	82	85	39	65	60	85	58	64	34	66
miR5054_S1	14	38	61	56	23	29	67	80	117	96	38	28
miR159_S3	48	34	49	35	17	36	56	62	52	59	34	69
miR529_S1	45	49	48	78	8	4	24	22	21	18	49	65
miR162_S1	27	44	38	35	6	16	20	36	71	56	44	32
miR4995_S1	9	28	53	46	6	6	47	55	42	46	28	42
miR390_S1	36	41	54	51	5	14	44	38	24	32	41	40
miR399_S1	21	20	23	28	33	91	7	12	51	42	20	13
miR845_S1	21	30	39	41	26	44	21	37	23	27	30	24
miR395_S1	2	7	12	15	6	93	53	64	9	23	7	17
miR1511_S1	12	15	32	24	23	21	27	30	50	37	15	20
miR1088_S1	34	35	28	26	8	11	27	32	26	28	35	29
miR394_S1	3	5	41	30	9	23	33	29	19	30	5	55
miR5281_S1	11	15	30	35	11	18	27	34	23	26	15	43
miR5565_S1	16	19	30	26	16	19	25	35	24	31	19	27
miR444_S2	7	14	18	18	22	46	13	18	23	21	14	22
miR5205_S1	4	13	23	27	11	14	20	26	21	23	13	21
miR397_S2	17	17	18	16	7	13	7	15	31	40	17	20
miR444_S3	5	2	8	10	47	67	8	10	10	11	2	11
miR1432_S1	55	3	11	5	18	4	8	11	37	20	3	9
miR1318_S1	55	3	11	5	18	4	8	11	37	20	3	9
miR169_S6	12	16	18	13	2	5	26	22	11	20	16	26
miR1310_S1	8	11	21	9	16	15	22	23	24	13	11	7
miR5073_S1	10	9	16	12	1	3	15	20	22	20	9	21

miR1877_S1	6	8	10	9	2	5	31	26	5	15	8	23
miR916_S1	0	2	8	10	47	26	10	3	5	5	2	9
miR5240_S1	6	7	13	13	5	7	10	15	9	9	7	13
miR1074_S1	4	6	9	14	4	9	11	15	8	14	6	8
miR5658_S1	4	6	9	8	6	11	12	11	12	10	6	10
miR829_S2	10	11	11	9	8	10	5	9	7	9	11	8
miR529_S2	12	15	15	26	0	1	4	3	1	2	15	16
miR2592_S3	7	9	8	6	0	0	18	20	8	7	9	8
miR474_S1	3	4	6	2	10	7	7	9	7	8	4	22
miR5368_S1	2	4	10	10	1	1	10	13	16	10	4	7
miR5169_S1	6	3	8	9	2	2	5	11	9	6	3	8
miR5568_S1	4	6	5	4	3	6	4	9	6	7	6	9
miR479_S1	4	1	3	1	9	29	2	3	3	1	1	2
miR4413_S1	5	4	5	7	3	6	4	5	5	7	4	4
miR2592_S1	0	2	5	4	9	13	6	2	6	2	2	4
miR1856_S1	5	5	7	5	5	4	5	7	2	4	5	3
miR5079_S1	0	4	7	6	2	1	5	6	8	8	4	5
miR2081_S1	2	5	5	4	3	3	6	3	8	5	5	7
miR1439_S1	1	2	7	8	1	4	8	7	2	5	2	5
miR2275_S2	3	5	4	4	4	4	5	8	3	4	5	4
miR5178_S1	3	2	2	4	6	6	3	6	4	3	2	8
miR1436_S1	1	1	3	6	1	4	3	8	4	9	1	6
miR5066_S1	1	2	8	5	2	6	4	4	4	4	2	6
miR3438_S1	2	2	6	4	6	6	4	3	5	5	2	2
miR2275_S1	1	3	6	6	3	1	7	4	4	5	3	5
miR2863_S1	0	0	4	3	14	4	3	6	2	5	0	4
miR172_S1	2	2	3	3	4	4	3	5	6	6	2	6
miR5380_S1	2	3	4	6	3	3	3	5	5	3	3	6
miR163_S1	2	1	3	4	5	4	4	6	4	5	1	4
miR5059_S1	2	3	5	3	3	5	3	4	6	4	3	2
miR3706_S1	2	4	5	3	3	5	3	4	4	4	4	3
miR902_S2	2	2	4	4	3	5	4	4	4	2	2	5
miR2092_S1	10	0	7	1	13	5	1	0	0	1	0	0
miR1120_S1	1	4	2	6	2	3	3	6	3	4	4	4
miR833_S1	2	2	3	4	3	4	4	6	4	4	2	2
miR1884_S1	0	3	4	5	1	5	3	6	3	3	3	3
miR5532_S1	1	1	6	4	1	3	3	4	4	4	1	4
miR5149_S1	3	2	2	3	1	4	4	4	4	3	2	5
miR5083_S1	1	2	1	1	7	7	1	2	3	5	2	4
miR5013_S1	0	2	2	3	0	0	7	7	4	5	2	4
miR1520_S4	1	3	3	3	4	6	1	5	3	3	3	1
miR5641_S1	0	0	0	1	6	13	6	0	0	4	0	2

Chapter V

General conclusions and perspectives

V: General conclusions and perspectives

Conclusions

Conclusions

The results presented in this thesis provide novel insights into local and systemic above- and belowground defense responses of maize against the hemibiotrophic fungus *Colletotrichum graminicola*. In the following section, a synthesis of the different chapters is given, and future perspectives of the research in the field of maize-*C. graminicola* interactions are discussed.

Method development

Studies of belowground antifungal resistance are scarce, mainly due to the difficult accessibility of the root system. In this regard, development of a convenient system which facilitates root infection as well as observation was a crucial step in this thesis. Although several soil-free culture methods are known, systems applicable to crops are rare. In this thesis, a method previously developed to perform root phenotyping (Hund *et al*., 2009) was adapted for the study of root-microbe interactions. Two critical points were noted, namely the filter paper and the nutrient solution. Regular filter paper inhibited plants growth, thus filter paper that has not been chemically treated (for example bleached) has to be used. In addition, maize plants require a specifically adapted nutrient solution. Standard solutions such as Hoagland's No. 2 are insufficient for a proper development of maize seedlings.

The soil-free system presented in this thesis favors the analysis of both pathogenic and mutualistic plant-microbe interactions at the root level. Thus, this system is convenient to study different biological questions in the same experimental environment. Nevertheless, this system also has limiting factors, such as the restricted size of the pouches, which does not allow to grow the plants for longer than 20 days. Moreover, the method presented in this thesis is prone to contaminations, thus regular changing of filter papers and great care in handling the roots is required.

In conclusion, the soil-free plant growth system presented here allows a non-destructive analysis of maize-microbe interactions at the root level, which could contribute to a better understanding of belowground defense mechanisms.

Colletotrichum graminicola – Zea mays interactions: a multifaceted relationship

The results presented in this study show that maize plants employ both local and systemic defense responses during *C. graminicola* infections, and these defense responses are highly organ-specific. Roots respond faster than leaves in upregulating defense-related genes, and they also exhibit higher levels of defense-related phytohormones, as well as higher basal levels of potential antifungal metabolites. *C. graminicola* does not growth hemibiotrophically on maize roots; thus the distinctive lifestyle could be a result of fungal adaptations to the different defensive state of roots. The remarkably higher level of antifungal compounds such as flavonoids in roots suggests that roots could act as defensive storage organs. Flavonoids are known to be transported from roots to shoots, thus it is of future interest to study such potential transport pathways also in the model system presented in this thesis.

This study also demonstrates that both locally infected leaves and roots possess the ability to trigger systemic resistance in distal leaves against *C. graminicola*. The biological resistance demonstrated in this thesis is one of the few described cases of biologically-induced systemic resistance in monocots. It has been also noted in this study that roots are more efficient in triggering systemic resistance, which corroborates the hypothesis of roots as defensive organs. Moreover, root infections triggered systemic gene expression in distal leaves much faster than during leaf-leaf SAR. Hence, it can be proposed that targeting roots for enhancing inducible resistance mechanisms might be a valuable option for future crop enhancement programs.

New players in the game? miRNAs as potential defensive backups

The results presented here also describe the transcriptional adaptation of miRNAs in response to *C. graminicola* attack and *Spodoptera frugiperda* challenge. Consistent with the organ-specific defense responses described in chapter III, stress- and organ-specific down- or upregulation of maize miRNAs was detected. Although most of the target genes of the identified miRNAs are still unknown, some defense-related genes were found among the targets of miRNAs with altered expression patterns. However, these targets are not encoding genes directly involved in plant immunity, rather genes affecting the synthesis of secondary metabolites or plant hormones. Hence it is hypothesized that miRNAs play a subtle role in fine-tuning antifungal defense, rather than being crucial during defense signaling. Interestingly, this study also showed systemic changes in the miRNA transcriptome upon local fungal infections. Thus, miRNAs could also play a role in systemic resistance. As some miRNAs are known to be mobile from roots to shoots, miRNAs could possibly act as systemic defense signal.

Nevertheless, further work is required to gain better insights into miRNA-mediated antifungal defense. For this reason, the next planned steps in this study are to confirm and analyze the miRNA target genes, and to explore the molecular and chemical pathways regulated by the product of those genes.

Perspectives

In conclusion of this thesis, the following perspectives for future research have arisen:

- **Is the soil-free system applicable for other model crops?** Testing other crops, as well as other pathogens, would help to provide a deeper insight into belowground defense mechanisms.
- **Can organ-specific defenses be exploited for crop enhancement programs?** As the root tissue seems to possess an enhanced defensive capacity, it would be of great interest to screen crop varieties for higher contents of root-specific antimicrobial

compounds. Moreover, transgenic approaches could aim to transfer root-specific inducible defense mechanisms to aerial plant parts.

- **What are the mechanisms of systemic resistance in monocots?** Although this study demonstrates biologically-induced systemic resistance, along with transcriptional and metabolomic adaptations, the nature of the systemic signal remains elusive. Future research is required to identify those signals. This study demonstrates organ-specific systemic defense responses where roots have a great potential to generate this resistance. Hence, the quest for root-to-shoot signals could be of great agronomical interest.

- **How does *C. graminicola* deal with different defensive setups?** This thesis focuses on the different physiological adaptations of plants in response to *C. graminicola*. However, it is also of great future interest to dissect the adaptations of the fungus in response to organ-specific immune systems. The ability to infect different plant organs renders *C. graminicola* a fascinating model pathogen. Understanding the character of adapted fungal infection strategies would certainly help to develop novel disease control strategies. For instance, in the small RNA libraries generated during this thesis, we also detected fungal-derived sequences, and some maize miRNAs showed complementary to fungal genes. Future work has to be conducted to examine the nature of the intricate molecular crosstalk between both fungus and plant during infection and defense.

Appendix

A. List of Publications

Balmer D, Mauch-Mani B. (2012) Plant hormones and metabolites as universal vocabulary in plant defense signaling. In: Witzany G and Baluška F (eds.), *Biocommunication of Plants, Signaling and Communication in Plants* **14,** 37-50. Heidelberg: Springer.

Balmer D, Mauch-Mani B. (2012) Small yet Mighty - MicroRNAs in Plant-Microbe Interactions. *MicroRNA* (accepted).

Balmer D*, Planchamp C*, and Mauch-Mani B. (2012) On the move: Induced resistance in monocots. *J. Exp. Bot.*, doi:10.1093/jxb/ers248 (*contributed equally).

Balmer D, Villacres de Papajewski D, Planchamp C, Glauser G, Mauch-Mani B. (2013) Induced resistance in maize is based on organ-specific defense. *The Plant J.*, doi: 10.1111/tpj.12114.

Erb M, **Balmer D,** De Lange ES, Von Merey G, Planchamp C, Robert CA, Röder G, Sobhy I, Zwahlen C, Mauch-Mani B, Turlings TC. (2011) Synergies and trade-offs between insect and pathogen resistance in maize leaves and roots. *Plant Cell Environ*. 2011 **34,** 1088-103.

Glauser G, Vallat A, **Balmer D.** (2012) Hormone profiling. In Salinas J, Sanchez-Serrano JJ (eds.), *Arabidopsis Protocols*, 3th Edition (in press).

Planchamp C*, **Balmer D*,** Hund A, Mauch-Mani, B. (2012) A soil-free root observation system for the study of root-microorganism interactions in maize. *Plant & Soil*, DOI 10.1007/s11104-012-1497-8 (*contributed equally).

B. Awards

Best poster, second price: Balmer D, Planchamp C, Villacres de Papajewski D, Mauch-Mani B. Above- and belowground systemic resistance in *Zea mays*. NCCR Plant Survival annual PhD students conference 2011, Neuchâtel, Switzerland.

Best oral presentation award: Balmer D and Mauch-Mani B. The hemibiotrophic fungus *Colletotrichum graminicola* triggers above and belowground systemic resistance in *Zea mays*. PRIR 2011 conference, Neuchâtel, Switzerland.