We are IntechOpen, the world's leading publisher of Open Access books
Built by scientists, for scientists

4,800
Open access books available

122,000
International authors and editors

135M
Downloads

154
Countries delivered to

Our authors are among the
TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Chapter 2

Signs of Silence: Small RNAs and Antifungal Responses in *Arabidopsis thaliana* and *Zea mays*

Andrea Balmer, Emanuele De Paoli,
Azeddine Si-Ammour, Brigitte Mauch-Mani and
Dirk Balmer

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.69795

Abstract

Plant small RNAs (sRNAs) are pivotal regulators of gene expression, which are crucial in maintaining genome integrity and flexibility during development, abiotic and biotic stress responses. Current evidence suggests that sRNAs might be inherent to the sophisticated plant innate immune system battling bacteria. However, the role of sRNAs during antifungal plant defences is less clear. Therefore, this chapter investigates the sRNA-mediated plant antifungal responses against the hemibiotrophic fungi *Colletotrichum higginsianum* and *Colletotrichum graminicola* in their respective compatible hosts *Arabidopsis thaliana* and Zea mays. A phenotypic and metabolomic analysis of *A. thaliana* sRNA mutants in response to *C. higginsianum* infection was performed, showing a hormonal and metabolic imbalance during fungal infection in these plants. To find whether fungal-induced sRNA could directly regulate defence genes in an agricultural important plant model, the expression of maize miRNAs in response to *C. graminicola* leaf and root infections was investigated. The results revealed the tissue-specific local and systemic adaptation of the miRNA transcriptome, where only a few miRNAs were targeting defence pathways. The general picture presented here points towards a role of sRNAs as fine-tuners of genetic and metabolomic defence response layers. This chapter also further discusses the potential of utilizing sRNA-based fungal control strategies.

Keywords: small RNA, antifungal plant defence, metabolomics, deep sequencing

1. Introduction

Small RNAs (sRNA) are small noncoding RNA segments of 19–30 nucleotides in length [1]. They mediate gene silencing, a gene regulation mechanism acting on a transcriptional

INTECH
open science | open minds

© 2017 The Author(s). Licensee InTech. This chapter is distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. cc BY

(transcriptional gene silencing (TGS)) and post-transcriptional level (post-transcriptional gene silencing (PTGS)). In general, sRNA molecules originate from the transcription of endogenous microRNA (miRNA genes), other genomic sRNA loci, aberrant RNA produced by transposons as well as invasive viral RNA [2]. Plants carry two main classes of sRNAs grouped according to their size, function and biogenesis, namely microRNAs (miRNA) and short-interfering RNAs (siRNA) [3]. Such sRNAs are generated through various mechanisms; within the miRNA biogenesis pathway, miRNA precursors derived from MIR genes are processed in the nucleus by Dicer-like protein 1 (DCL1) and exportin-like protein (HYL1) into mature miRNA duplexes of 20–22 nucleotides in length. Mature miRNAs are then methylated at the 3′ terminus by HEN1 (small RNA methyltransferase) and exported to the cytoplasm. One strand of the duplex is incorporated into an argonaute protein (AGO) protein to form an RNA-induced-silencing complex (RISC) [4]. The siRNAs, however, originate from long dsRNA that can be derived from transgenes, viruses, transposons and natural sense-antisense transcripts. Such long dsRNA is recognized and cleaved by a certain type of DCL proteins; thereby siRNA classes with different sizes are generated. Like miRNAs, siRNAs are loaded into an AGO protein-containing RISC that controls gene expression patterns through the degradation of mRNA or the repression of translation of fully/partly complementary sequences of mRNAs, as well through epigenetic changes via mediation of DNA and histone methylation [5, 6].

Gene silencing is not only important for the maintenance of genome integrity by silencing transposons or by degrading the viral RNA but also important during host immune responses of both plants and animals [7–9]. The recognition of pathogens by plants leads to the activation of a multi-layered immune system that comprises the establishment of a complex network of inducible defences including pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) [10, 11]. The entire signalling process involves the regulation of defence gene expression, the release of plant hormones and/or the induction of secondary metabolites [12]. Over the past few years, plant sRNA pathways were recognized as important players during PTI and ETI [13, 14]. In Arabidopsis, bacteria-induced miRNAs were identified to orchestrate components of plant hormone signalling, including auxin, abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) [15, 16]. A canonical example of an miRNA regulating plant defence is miR393. It is up-regulated upon treatment with a bacterial PAMP, and negatively regulates auxin signalling and therefore contributes to SA-mediated PTI responses in Arabidopsis [17].

Although the important role of sRNAs in plant defence against viruses and bacteria is documented [8, 13], their function as components of the plants' defence response against fungi is less clear. Advances in genome-wide studies revealed a massive adaptation of host miRNA expression patterns after infection by fungal pathogens such as *Fusarium virguliforme* [18], *Erysiphe graminis* [19], *Verticillium dahliae* [20], *Cronartium quercuum* [21], as well as the oomycete *Phytophthora sojae* [22]. The alterations in sRNA expression profiles upon fungal attack suggest that gene silencing also contributes to antifungal defence; however, up to date there are no putative mechanisms deciphered. Besides orchestrating plant defence, sRNA could also act as direct antifungal molecules, as some plant miRNAs could

http://dx.doi.org/10.5772/intechopen.69795

share complementarity to fungal genes. This possibility has already been demonstrated by engineering transgenic plants expressing dsRNA targeting fungal genes and exhibiting enhanced resistance to different fungi. For instance, this mechanism named host-induced gene silencing (HIGS) was successfully applied for various plant-fungi pathosystems such as silencing of the *Blumeria graminis* effector Avra10 [23], or *CYP51* genes of *F. graminearum* [24].

In this study, we aim to elucidate the role of sRNAs in regulating susceptibility to *Colletotrichum* spp.; hence we congregated results from two compatible pathosystems: *C. higginsianum*, which infects plants from the Brassicaceae family such as *Arabidopsis thaliana* (**Figure 1**) and *C. graminicola*, which is a devastating pathogen of the industrially important crop *Zea mays* (**Figure 2**). Both ascomycetes use a multistage hemibiotrophic strategy to infect their host and also share close genetic similarities making them tractable models to compare fungal pathogenicity in both dicot and monocot models [25–28]. In Arabidopsis, *C. higginsianum* employs first a biotrophic stage limited to a confined array of first invaded cells, from where the fungus develops secondary hyphae to switch to necrotrophic growth into surrounding cells. *C. graminicola* extends the biotrophic lifespan into many host cells, persisting biotrophic at the margins, whereas the centre of infection becomes necrotrophic. *C. graminicola* is a major worldwide threat for corn cultures, as it affects all parts of the plants, either as leaf blight or as stalk rot [29]. Depending on specific corn hybrids and culture conditions, *C. graminicola* can result in up to 40% yield loss where endemic.

During the first step, a selection of sRNA mutants and two fully and intermediate fungal susceptible accessions of *A. thaliana* was examined in order to dissect possible defence defects caused by mutations in sRNA biogenesis pathways. Thus, we analysed the accumulation of phytohormones that are known to mediate Arabidopsis resistance against *C. higginsianum* [30] and secondary metabolites that function as direct defences [31]. We show that some Arabidopsis sRNA mutants display an altered susceptibility against *C. higginsianum*,

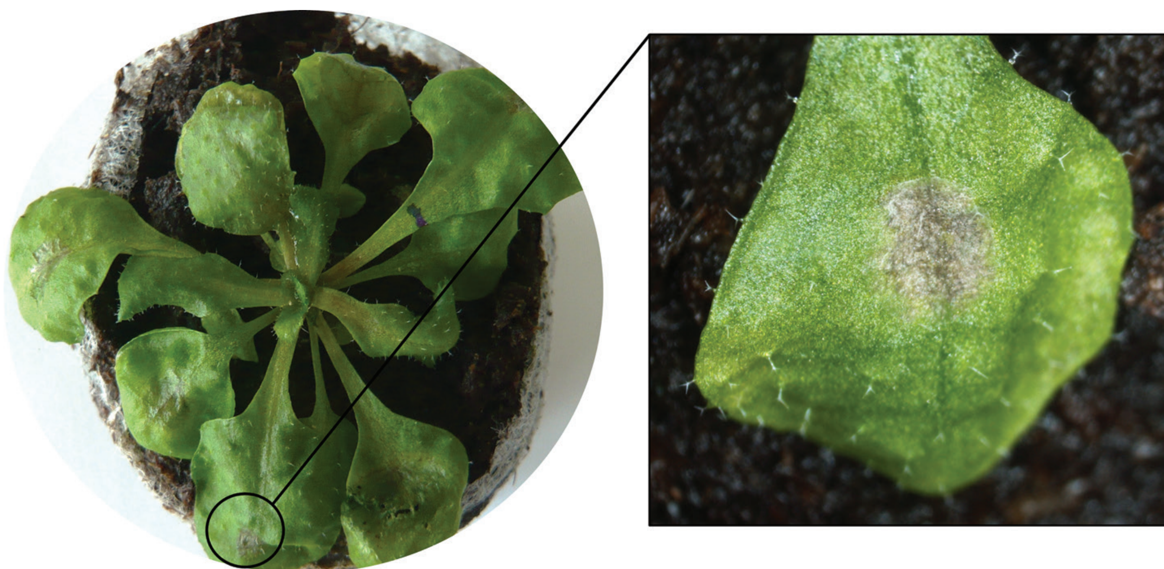

Figure 1. *Arabidopsis thaliana* leaves infected by *Colletotrichum higginsianum*, 6 days post inoculation.

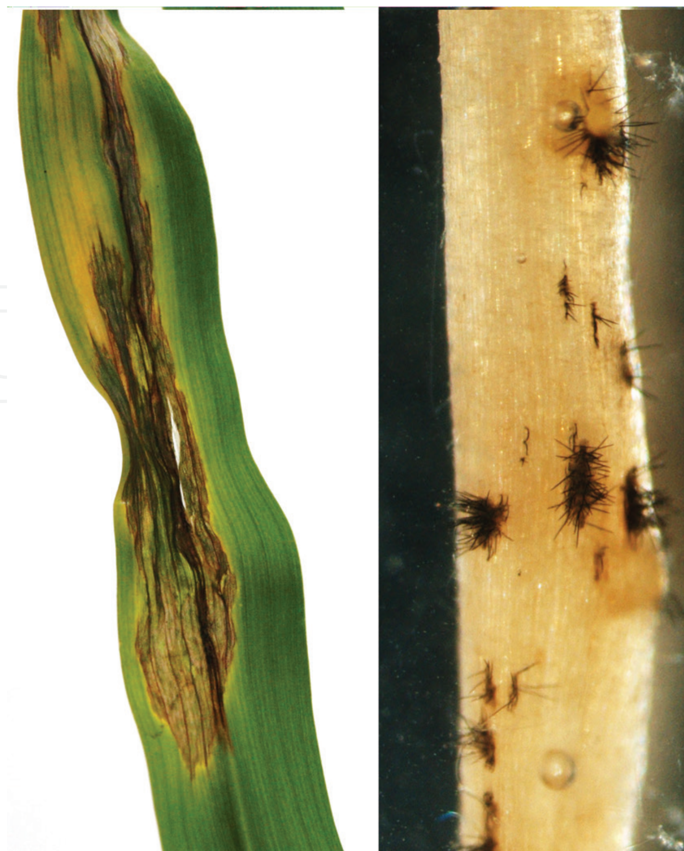

Figure 2. *Zea mays* leaf (left) and root (right) infected with *Colletotrichum graminicola*.

together with a defective setup of chemical defences. Moreover, to better understand the role of sRNA during infection with *Colletotrichum* spp., we performed an miRNA expression profiling to obtain a deeper insight into adaptations of the sRNA transcriptome in different *C. graminicola*-infected maize tissues. The miRNA profiling demonstrated that the vast majority of altered miRNAs were targeting genes that are not directly linked to antifungal-defence pathways, suggesting that antifungal-defence responses are not regulated by specifically induced miRNAs.

This chapter provides a multi-omics analysis of sRNA-mediated antifungal plant reactions on a phenotypic, metabolomic as well as transcriptomic point of view. Altogether, our data propose a rather indirect defensive role of sRNAs in calibrating metabolomic and transcriptomic balances during antifungal responses against *Colletotrichum* spp. Future putative applications of sRNA-based fungal control strategies will be commented.

2. Materials and methods

2.1. Plant material and growth conditions

A. thaliana genotypes (*hen1-1, hyl1-2, rdr6-15*, Col-0 and Ler-0) were germinated in soil maintained at 21°C day/20°C night, with 9 h of light (120 μE $m^{-2}s^{1}$) and 60% of relative humidity. Selected *A. thaliana* accession Ler-0 was described to be susceptible to *C. higginsianum* infection,

while Col-0 showed intermediate resistance [31]. Ler-0 is the wild-type genetic background of *hen1-1* mutants; all other mutants have a Col-0 genetic background. One week after germination, seedlings were individually transferred to 33-mL Jiffy pellets and kept in the same conditions until the infections. *Z. mays* (variety Jubilee, West Coast Seeds, www.westcoastseeds.com) was cultured in a soil-free plant growth system as described by Ref. [32].

2.2. Pathogen and pest cultivation and inoculation

C. higginsianum IMI34 349061-GFP [26] was cultured on potato dextrose agar (PDA) in a growth chamber under permanent light at 25°C. For infections, a fungal spore suspension of 10^6 spores mL^{-1} was prepared from 2-week-old cultures. Four- to five-week-old *A. thaliana* plants were drop-inoculated with 5 μL of the spore suspension. The plants were then incubated in darkness for 16 h at 25°C and 100% relative humidity. Post incubation, the growth condition of the plants was changed to long day (16 h/8h day/night cycle at 25°C). Control plants were treated only with sterile water. *C. graminicola* M1.001 was cultivated on PDA under permanent light at 25°C; infection assays were performed on 12-day-old maize plants as previously described [32].

2.3. Quantification of fungal growth

In planta fungal growth of *C. higginsianum* was measured every 24 h post infection for 4 days. The infection sites of the green fluorescent protein-expressing fungal strain were illuminated using a Nikon C-SHG1 UV lamp. Images were captured using a Nikon DS-L1 camera and the pictures were further analysed with the help of ImageJ (http://rsbweb.nih.gov/ij/) and Adobe Photoshop CS3 (http://labs.adobe.com). The area of fungal growth was measured in pixels and converted to mm^2.

2.4. Hormone quantification

For hormone analysis, salicylic acid, jasmonic acid and abscisic acid were quantified simultaneously from leaf material using UHPLC-MS/MS as described [32]. Hormone measurements were performed 4 days post *C. higginsianum* infection. To analyse each Arabidopsis accession, three independent biological replicates per sample were generated, each replicate a pool of five plants.

2.5. Metabolomic profiling

For metabolomic analysis, 4-week-old Arabidopsis plants were infected with *C. higginsianum*. Metabolites were isolated and analysed 4 dpi as described [32]. Six technical replicates for each treatment were analysed, and each replicate consisted of a pool of four plants.

2.6. Gene expression analysis

Confirmation of down-regulation of maize genes putatively targeted by miRNAs was conducted as described [32], using ZmGAPc as normalizing gene. Primer sequences are as follows: ZmATPS_fw: tcgtattaatgctggtgcaaac, ZmATPS_rev: ctctgtggggtggctcat; ZmSAT_fw: ttataaaaaccctgttcttctgctc, ZmSAT_rev: aggacaccttcctcaagaacc; ZmGAPc_fw: gcatcaggaaccctgaggaa, ZmGAPc_rev: catgggtgcatctttgcttg.

2.7. Deep sequencing and Northern blotting of maize sRNAs

For sRNA library preparation, six biological replicates were pooled and total RNA was isolated using Trizol (Invitrogen, www.invitrogen.com); 10 μg of total RNA was further processed using an Illumina-Solexa deep-sequencing approach at FASTERIS (http://www.fasteris.com). The expression of selected miRNAs was further analysed using sRNA Northern blotting techniques as described [33].

2.8. Identification and quantification of conserved miRNAs

To identify conserved maize miRNAs, sequences of 4677 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 2228 unique miRNAs. Sequences belonging to the same miRNA family were further analysed by multiple alignment using ClustalW (www.clustal.org) and classified in subgroups to distinguish bona fide mature miRNAs from misannotated miRNA* forms or sequences generated from different regions of the same precursor. This non-redundant library was then applied to screen the small RNA libraries. All the small RNA reads in the range of 20–24 nt in size, and which are represented and represented by at least two reads in a library were aligned to the 1772 unique miRNAs derived from miRBase. For the screening, a maximum of three mismatches was allowed and up to 2 nt overhanging nucleotides at the 5′ and/or 3′ end. Alignments were performed using SeqMap [34]. The output was filtered and reformatted with custom PERL scripts, classifying the identified miRNAs according to miRBase.

2.9. Target prediction of maize miRNAs

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

2.10. Statistical analysis

Variances of quantified levels of metabolites and fungal growth for multiple groups were analysed by a one-way analysis of variance (ANOVA); a P-value of <0.05 was considered significant. The Mann–Whitney U-test was used to compare significant differences between two sample groups. All statistical analysis was performed using Sigma Plot 11.0 (http://www.sigmaplot.com).

3. Results

3.1. Arabidopsis sRNA mutants show different levels of susceptibility to *C. higginsianum*

To test if a functional silencing machinery is required for a proper antifungal-defence response, *A. thaliana* wild types Ler-0 and Col-0 showing lower and intermediate resistance, respectively, and sRNA pathway mutants were subjected to fungal infection assays

to monitor the susceptibility to *C. higginsianum*. To cover important components of sRNA pathways, the loss of function mutants for the genes encoding *HYL1, HEN1*and *RDR6* was analysed. The sRNA pathway mutants were infected with *C. higginsianum*-GFP, and the disease progression was compared to the relative wild-type ecotype, for *hen1-1* namely *Landsberg erecta* (Ler-0), for all other mutants Columbia (Col-0). Fungal growth was monitored at 24, 48, 72 and 96 h postinfection (hpi) (**Figure 3**). These time points were chosen to cover all known infection stages of *C. higginsianum* during hemibiotrophic growth on leaves [25, 29]. The infection assays showed an altered susceptibility of mutants (**Figure 3**). For *hen1-1,* a significant higher susceptibility was only detected in late infection stages (96 hpi). Comparison of *hyl1-2* with Col-0 yielded statistically significant differences of fungal growth at all time points (**Figure 3(b)**). The RNA mutant was found to be more susceptible to *C. higginsianum* compared to the wild type. By contrast, *rdr6-15* was infected by *C. higginsianum* as efficiently as the wild type (**Figure 3(c)**). Altogether, a defective sRNA machinery seems to render plants more susceptible to fungal attack. However, mutations in *RDR6-15* did not alter the susceptibility against the *C. higginsianum*.

3.2. Arabidopsis sRNA mutants show an altered hormonal balance after *C. higginsianum* infection

Hormone signalling is a key process that regulates stress responses. To evaluate the implication of sRNA pathways in hormone-mediated plant defence against *C. higginsianum*, levels of salicylic acid, jasmonic acid and abscisic acid were quantified by HPLC-MS/MS. All selected mutants and wild-type accessions were analysed 4 days post *C. higginsianum* infection and hormone levels of both infected and mock were measured. In response to *C. higginsianum* attack, SA and JA were induced to different levels in all genotypes (**Figure 4**). Notably, SA and JA inductions were more pronounced in the mutants *hen1-1* and *hyl1-2* compared to their respective wild-type (Ler and Col-0) infected plants. For instance, in infected *hen1-1* plants, JA levels rose up to 589 ng/100 mg fresh weight, whereas in infected Ler plants, JA only reached 234 ng/100 mg fresh weight. However, *rdr6-15* did not appear to have significant differences of SA and JA levels compared to wild-type-infected plants (**Figure 4(a)(b)**). On the other hand, ABA levels were found to be induced during fungal infection in *hyl1-2* and *hen1-1* contrary to *rdr6-15* that show no significant changes in ABA quantity upon fungal infection (**Figure 4(c)**). These results suggest that the sRNA mutant *rdr6-15* is likely not implicated in the regulation of hormone levels during antifungal responses, whereas a functional HEN1 and HYL1 protein seems to be required to mount a full SA, JA and ABA response to fungal attack.

3.3. The metabolome of Arabidopsis sRNA mutants in responses to *C. higginsianum* infection

To compare the changes in the metabolomic profile of sRNA mutants and wild-type plants induced by *C. higginsianum* infection, an UHPLC-QTOF-based analysis of secondary metabolites was performed. The metabolomic fingerprinting provided a global view on the metabolic perturbations induced by *C. higginsianum* attack at 4 dpi. Comparison of the metabolome *hen1-1* and *hyl1-2* mutants by a principal component analysis (PCA) resulted in a clear separation of both control-treated and -infected mutants and their respective wild types (**Figure 5(a)** and **(b)**).

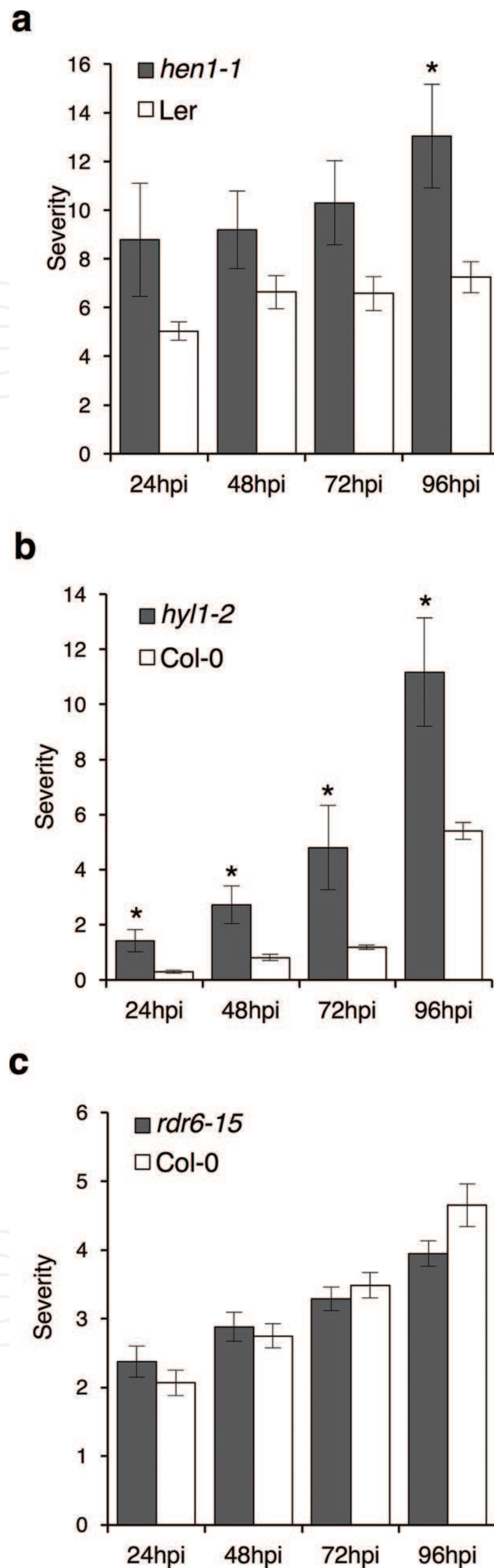

Figure 3. Disease severity of *C. higginsianum* in *A. thaliana* sRNA mutants and wild-type plants; (a). *hen1-1* mutant, (b). *hyl1-2* mutant, (c). *rdr6-15* mutant, compared to the respective wild-type background. Fungal growth was determined by quantifying the fluorescent area of *C. higginsianum*-GFP in mm^2 at different time points in all *A. thaliana* mutants compared to wild type. Severity was determined as percentage of leaf area affected. For statistical analysis, a one-way ANOVA was applied; asterisks indicate statistically significant differences ($P < 0.05$). Error bars indicate standard deviation (SD).

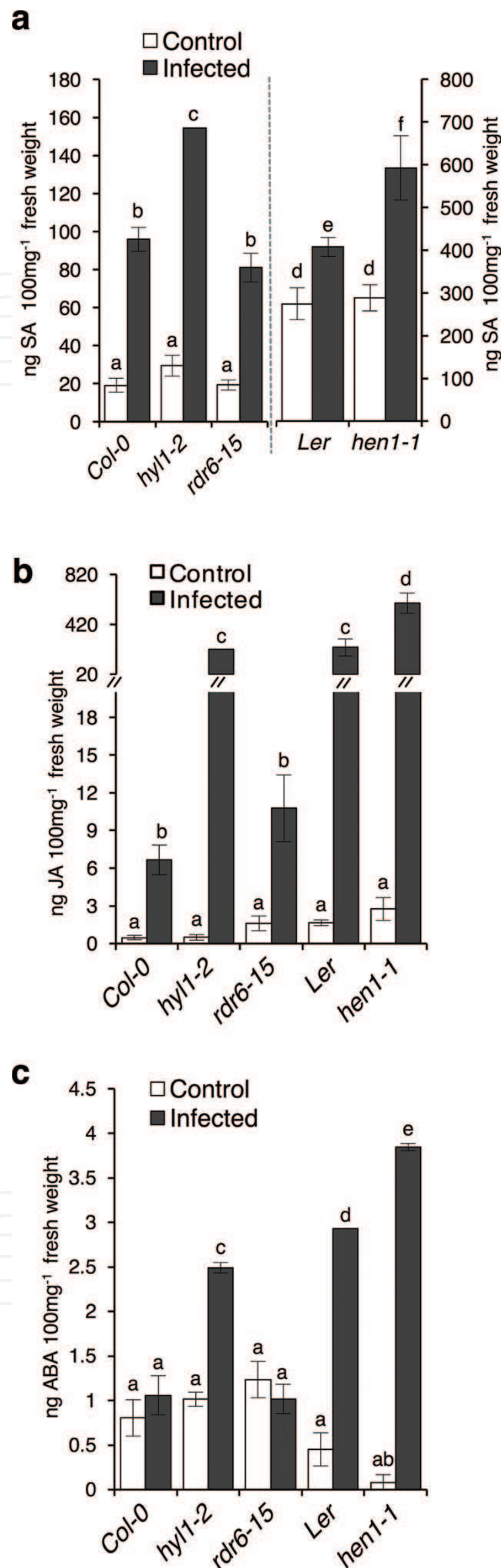

Figure 4. Quantification of phytohormones in *A. thaliana* sRNA mutants and wild-type plants after *C. higginsianum* infection. (a). salicylic acid (SA), (b). jasmonic acid (JA), and (c). abscisic acid (ABA) in *A. thaliana* sRNA mutants (*hen1-1*, *hyl1-2*, and *rdr6-15*) and wild-type plants (Col-0 and Ler) under two treatments: infected with *C. higginsianum* and control. Statistical significance was determined using one-way ANOVA. Letters indicate statistically significant differences ($P < 0.05$). Error bars indicate standard deviation (SD).

The PCA performed for *rdr6-15* grouped the mutants and wild type much closer (**Figure 5(c)**). The metabolomic fingerprinting allowed identifying groups of putative antifungal metabolites that were normally induced in the wild type, for which in turn the mutants showed an abnormal induction pattern. After PCA analysis, the compounds showing the greatest difference between wild type and mutants were selected for further identification (**Table 1**). Compounds were identified by exact mass, fragmentation spectrum and the retention time of the fragments using the online free databases Metlin, MassBank, Kegg and Aracyc and the in-house database from the chemical analytical service of the University of Neuchatel. The metabolomic analysis revealed a group of glucosinolates, flavonols, phenylpropanoids and the phytoalexine camalexine that were differentially induced in mutants and wild-type plants (**Table 1**). In response to *C. higginsianum*, *hen1-1* mutant showed lower fold induction of some glucosinolates like 7-methylthioheptyl glucosinolate, glucoerucin, glucoiberin, glucoiberverin and glucolesquerellin. Moreover, glucobrassicin was not induced after infection in *hen1-1* plants. Kaempferol 3-O-rhamnoside-7-O-rhamnoside (kaempferol 3-rha-7-rha) and kaempferol 3-O-rhamnoside-7-O-glucoside (kaempferol 3-rha-7-glu), flavonols which are well-described antifungal compounds [35], were down-regulated in *hen1-1* and Ler plants as well as the phenylpropanoids sinapoyl malate and 1-O-β-D-glucopyranosyl sinapate. The phytoalexin camalexin was the most induced compound after infection in *hen1-1* and Ler plants. Ler showed 84.4-fold induction of camalexin while infected *hen1-1* contained 10.7 more than mock-treated plants. The *hyl1-2* mutant exhibited lower fold induction in most of the glucosinolate levels compared to Col-0 (**Table 1**). Glucobrassicin, glucoiberin and glucoiberverin levels were higher in *hyl1-2* control and infected treatments than in wild type plants. Moreover, the induction of kaempferol 3-rha-7-rha and kaempferol 3-rha-7-glu was higher in Col-0 than *hyl1-2* plants. Levels of sinapoyl malate and 1-O-β-D-glucopyranosyl sinapate were also lower in *hyl1-2* control and infected plants compared to Col-0. Camalexin was 72.9-fold induced in Col-0 and 69.0-fold induced in *hyl1-2*. The *rdr6-15* mutant exhibited lower fold induction of all glucosinolates, flavonols and phenylpropanoids mentioned in **Table 1** compared to Col-0. The fold induction of camalexin was similar in *rdr6-15* mutant compared to Col-0 plants. In summary, sRNA mutant *hen1-1* exhibited lower levels of pathogen-induced camalexin, whereas the glucosinolates, flavonol and phenylpropanoid compounds were slightly less prominently induced in response to fungal infection in all the mutants compared to their respective wild-type plants.

3.4. *C. graminicola*-infected maize sets up a tissue-specific miRNA profile which is not directly linked to plant defence

Using annotated maize miRNAs (zma), known miRNAs were classified in the different maize sRNA libraries. In order to determine biostress-specific miRNAs and to quantify their expression level 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 of >2 are summarized in **Table 2**. Comparing biotrophic and necrotrophic fungal infection stages to mock, zma-miR479, zma-miR1318 and zma-miR1432 were found to be up-regulated; however, their fold induction was higher during the necrotrophic stage. Other miRNAs such as zma-miR393, zma-miR1120 and zma-miR2092 showed an altered expression level exclusively during the biotrophic stage. By contrast, the

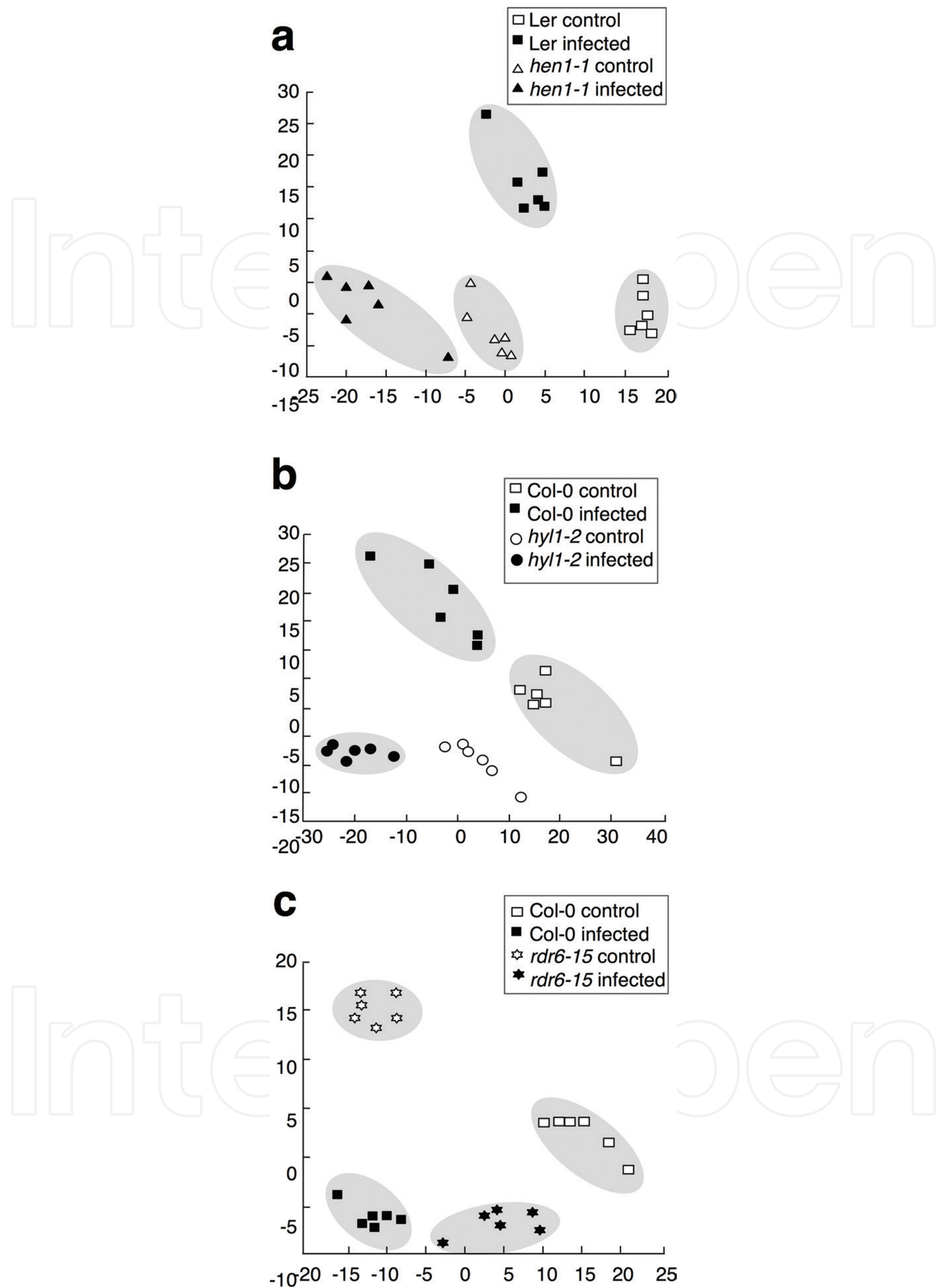

Figure 5. Metabolites distribution in sRNA mutants and wild-type plants upon *C. higginsianum* infection and control treatment. Principal component analysis (PCA) score plot of the metabolome. of the sRNA mutants *hen1-1* (a), *hyl1-2* (b), *rdr6-15* (c) and the wild-type Ler and Col-0 upon 4 dpi with *C. higginsianum* infection and control treatment. The PCA analyses were performed using Marvis Filter and Cluster packages, following a Kruskal-Wallis test ($P < 0.05$). Each data point represents one replicate of six independent biological replicates.

Compound	Mass	Fragments (M-H)-	*hen1-1* FI	*Ler FI*	*hyl1-2* FI	*rdr6-15* FI	Col-0 FI
Glucoberteroin	434.0612	96.9603, 95.9523	-	-	0.4	0.9	1.9
Glucobrassicin	447.0512	96.9601, 95.9523, 74.9914	0.8	1.7	2.0	0.6	2.4
Glucoerucin	420.0457	96.9628, 95.9551, 74.9943	1.0	1.6	0.2	0.8	1.7
Glucoiberin	422.0219	96.9619,95.9519, 74.9923	0.8	1.4	1.7	0.7	1.6
Glucoiberverin	406.0301	96.9619, 95.9494, 74.9920	1.1	2.1	1.0	0.7	1.4
Glucolesquerellin	448.0764	96.9590, 95.9513,74.9919	1.1	1.6	1.4	1.1	2.0
Gluconasturtiin	422.0578				0.8	0.8	2.1
Glucoraphanin	436.0406	372.0467, 178.0225	0.7	1.1	0.7	0.8	1.8
7-Methylthioheptyl glucosinolate	462.0958	95.9527, 74.9920	1.0	1.6	1.2	0.9	1.9
kaempferol 3-O-rhamnoside-7-O rhamnoside	578.1552	431.0942, 285.0399, 283.0236	0.6	1.0	1.4	0.8	1.7
kaempferol 3-rhamnoside-7-Glu	593.1534	447.0905, 285.0410, 283.0240	0.6	0.9	0.8	0.7	1.4
Sinapoyl malate	339.0745	223.0586, 164.0484, 149.0245	0.7	0.9	1.2	0.8	1.4
1-O-β-D-glucopyranosyl sinapate	385.1147	265.0794, 190.0267, 175.0030	0.8	1.0	0.9	0.5	1.2
Camalexin	199.0332		10.7	84.4	69.0	71.5	72.9

Fold induction of identified compounds from the metabolome of the sRNA mutants *hen1-1, hyl1-2, rdr6-15* and the wild-type Ler and Col-0 upon *C. higginsianum* infection (4 dpi).

Table 1. Fold induction of metabolites in sRNA mutants and controls upon *C. higginsianum* infection.

expression of zma-miR168, zma-miR2916 and zma-miR5205 was altered only during the necrotrophic stage. Notably, zma-miR1432 and zma-miR2092 were also up-regulated in infected roots, suggesting that some miRNAs are regulated organ independently. Notably, infected roots showed also a distinct expression profile with zma-miR166, zma-miR169 and zma-miR395 that were down-regulated, whereas zma-miR909 and zma-miR2863 were up-regulated. A different situation was found in systemic leaves upon leaf infection. Compared to local infected tissues, less miRNAs showed an altered expression. For instance, zma-miR397, zma-miR916 and zma-miR5169 were up-regulated. In systemic leaves upon root infection, zma-miR1877 and zma-miR2592 were down- and up-regulated, respectively. Interestingly, zma-mi395 was down-regulated, and zma-miR479 showed elevated expression levels; zma-miR479 was also found to be up-regulated in local leaf infections, whereas the down-regulation of zma-miR395 was also observed in infected roots. In summary, although some miRNAs were commonly regulated in both locally infected leaves and roots, the miRNA transcriptome was specific for a given infection stage and in addition also organ-specific (**Table 2**). 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, zma-miR395 was selected (**Figure 6**).

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

Library	miRNA	FI	Putative target genes
	miR169	−3.8	RAPB protein—*Oryza sativa*; allene oxide synthase—*Zea mays*
	miR395	−15.5	ATP sulphurylase (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 sulphurylase (LOC541653), mRNA
	miR479	3	Unknown
	miR1877	−3	Putative protein binding protein
	miR2592	3	Unknown

FI = fold induction compared to control libraries. Inf = infected, L = leaf, R = root, sys = systemic, zma = maize miRNAs.

Table 2. Maize miRNAs differently regulated upon *C. graminicola* infection.

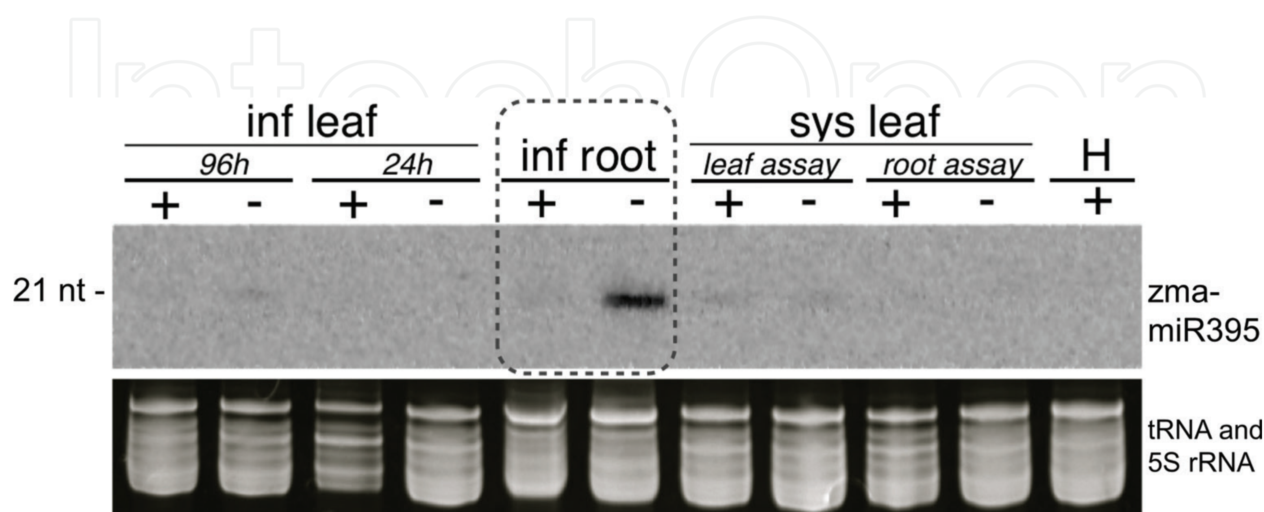

Figure 6. Northern blot analysis of miR395 expression. The signs + indicates *C. graminicola* infection, - control tissue. H= herbivore (*Spodoptera frugiperda*, non-fungus control). The tRNA and 5S rRNA are shown as a control for equal loading and were stained with ethidium bromide.

http://dx.doi.org/10.5772/intechopen.69795

As expected, zma-miR395 showed a reduced expression level upon fungal infections in roots. The signal intensity also corresponded to the sequence reads in the different libraries, with the highest number of reads (93) in control roots. To examine the putative role of zma-miR395 during root infections, the maize genome was analysed for putative target genes. Five known target genes were identified: two genes (dienelactone hydrolase and FMR1-interacting) exhibit two mismatch positions for zma-miR395. The other genes, ATP sulphurylase (APS) on chromosomes 1 and 5, and a sulphate anion transporter, perfectly matched to the zma-miR395 sequence. To confirm the genotype of a reduced expression level of zma-miR395 in infected maize roots, the gene expression of two zma-miR395 putative target genes (*ZmSAT* and *ZmATPS*) was analysed (**Figure 7**).

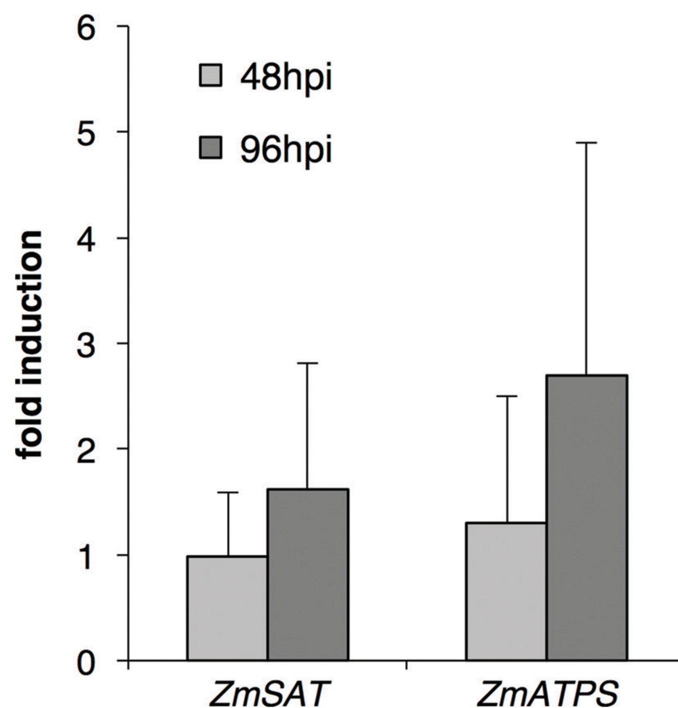

Figure 7. Expression profile of *ZmSAT* and *ZmATPS* genes in *C. graminicola*-infected roots.

4. Discussion

It has been documented that plant sRNAs can act as regulators of gene expression during plant-defence responses as reviewed in Ref. [36]. However, the mechanisms of sRNA-mediated immunity remain largely elusive, especially for host-fungi interactions. In rice cultivars that are susceptible to *Magnaporthe oryzae*, enhanced resistance could be achieved by overexpressing miR160 and miR398 [37]. MicroRNA160 targets auxin-responsive factor 16 (*ARF16*), and miR398 regulates superoxide dismutase 2 (*SOD2*), both known defence-related genes. In wheat, *B. graminis* infection was demonstrated to lead to massive adaptations of the miRNA expression profile, where miRNAs only induced in either resistant or susceptible cultivars where identified [19]. Various sRNA expression studies upon fungal infections point towards a role as fine-tuners in the concert of setting up efficient and targeted antifungal defences, rather

than having direct defensive impacts [38]. In this regard, it is not surprising that sRNAs were identified as regulators of basal immunity and *R*-gene-mediated resistance. In cotton, bioinformatic approaches revealed over 300 NBS-LRR genes potentially controlled by the miR482 family [39], which cleave NBS-LRR transcripts, resulting in the generation of secondary siRNAs that even enhance the silencing of multiple NBS-LRR genes. *V. dahliae* infection leads to a down-regulation of miR482, hence to a de-repression of *R*-genes.

Notably, plant RNAi pathway components were shown in specific cases to be important for mounting proper antifungal-defence responses. RDR6-deficient plants were found to be more susceptible to *Verticillium* spp. but not to *Botrytis cinerea, Alternaria brassicicola* or *Plectosphaerella cucumerina* [40]. Similarly, *ago4* mutants were discovered to be more susceptible to *B. cinerea* and *P. cucumerina* [41, 42], possibly due to the over-induction of the SA-defence pathway which leads to diminished JA-defence responses that are important in controlling necrotrophic pathogens.

The present study widens the understanding of the putative role of sRNAs in fine-tuning plant-hormonal pathways during fungal infection. First of all, Arabidopsis sRNA pathway components were demonstrated to be required for antifungal responses against *C. higginsianum*. HYL1- and HEN1-deficient plants were more susceptible than the wild type. The higher susceptibility was accompanied by a de-regulated hormonal response. The sRNA mutants *hen1-1* and *hyl1-2* exhibited higher SA-, JA- and ABA-induction levels. The hormonal imbalance might explain the altered susceptibility to *C. higginsianum*, as enhanced SA is known to be important during biotrophic infections, whereas high JA levels are typical for defence against necrotrophs [43]. On the other hand, mutation of *RDR6* did not affect the susceptibility against *C. higginsianum* suggesting that the tasiRNA (trans-acting siRNA) pathway is not involved in antifungal responses. Recent studies demonstrated that RDR6-deficient plants were more resistant to the hemibiotrophic pathogen *Pseudomonas syringae DC3000*, presumably by a constitutive activation of the SA-dependent-defence pathway. Hence, it was speculated that RDR6 acts as a negative regulator of PTI and basal defence in Arabidopsis [44]. Notably, the hormonal imbalance discovered in the sRNA mutants could only partially explain the altered susceptibility, as higher JA levels were found in *hyl1-2* mutants, which would lead to an enhanced resistance during the necrotrophic stage of *C. higginsianum*. This suggests that sRNAs act as putative-defence coordinators beyond hormonal pathways. Consequently, the metabolomic analysis uncovered additional layers of sRNA-regulated antifungal responses, namely the proper induction of defence-related secondary metabolites. Especially *hen1-1* and *hyl1-2* mutants were found to exert a massively altered defence metabolome, and to a lesser extent also the analysed *rdr6-15* mutants. sRNAs are known to be directly involved in the regulation of secondary metabolites; overexpression of miR393 for instance was shown to increase levels of glucosinolates and decreases camalexin [45], which indicates that miR393 is involved in the re-direction of the metabolic flows. Similarly, a possible link between miR163 and the biosynthesis of secondary metabolites was described [46]. Loss or overexpression of miR163 alters the transcription of target genes and the profiles of secondary metabolites after induction by the fungal elicitor alamethicin. On the other hand, *rdr6-15* mutants showed a wild-type-like metabolomic profile in both infected and control-treated conditions, despite the levels of some compounds being slightly different in the mutant after infection. The

minor differences in the metabolomic profile between *rdr6-15* and wild-type plants might be explained by the complex redundancies between the members of these protein families [3, 47]. Notably, some sRNA mutants such as *hen1-1* exhibit developmental defects, thus the genetic and metabolomic phenotype observed in response to fungi might be significantly affected by developmental pathways. However, this issue underlies all genetic studies using knock-out mutants with severe phenotypes. Using rigid statistical criteria for compound clustering, it is possible to partially differentiate developmental from antifungal responses, as shown in the PCA analysis of infected and control *hen1-1* mutants.

To extend the view on antifungal responses possibly linked to sRNA pathways, the miRNA transcriptome of the agricultural important model crop *Z. mays* infected with *C. graminicola* was analysed. During this interaction, maize was found to set-up an organ-specific miRNA profile. In the locally and systemically induced fungal-specific miRNAs, only a few were found to target defence genes. In particular, zma-miR1432, which targets a para-hydroxy-benzoate-polyprenyl transferase, was found to be up-regulated during both biotrophic and necrotrophic infection stages in maize leaves and roots. The miRNA target is essential in terpenoid-quinone synthesis. Hence, it could be speculated that its down-regulation could divert the flow of secondary metabolites from terpenoids towards flavonoid biosynthesis. This would be coherent with the fact that terpenoids play only minor roles during *C. graminicola* infection in maize [48]. The second identified miRNA linked to defence pathways was zma-miR169. This miRNA is down-regulated in response to fungal root infections, and it putatively targets a gene encoding an allene oxide synthase (*AOS*). AOS is a key enzyme in JA synthesis, thus it can be speculated that zma-miR169 acts as a suppressor of JA signalling under non-stressed conditions, whereas the down-regulation of zma-miR169 during fungal infection could promote JA synthesis. The enhanced JA levels in some Arabidopsis miRNA mutants support this hypothesis. Another yet intriguing altered miRNA was zma-miR395, which was down-regulated in *C. graminicola*-infected maize roots and systemic leaves upon root infection. The down-regulation of zma-miR395 was accompanied by the up-regulation of two of its putative targets in roots, one of them encoding an ATP sulphurylase. APS plays an important role in sulphate assimilation and glutathione synthesis; inhibiting glutathione synthesis in Arabidopsis was shown to trigger the suppression of miR395 [49], thus mimicking fungal infection. It can be speculated that the down-regulation of zma-miR395 positively regulates sulphate-mediated defence and/or the glutathione pathway. Intriguingly, miR168 that targets AGO1 was induced upon leaf infection, consistent with recent work demonstrating a similar fold induction of miR168 in Arabidopsis treated with elicitors of *F. oxysporum* [50]. Thereby, a majority of elicitor-responsive miRNAs were shown to be associated with development and miRNA homeostasis [50], corroborating the observation that sRNA pathways likely do not regulate direct-defence pathways.

Although some sRNA pathways components were shown here to be required for battling *C. higginsianum*, they seem to act as fine-tuner of defence schemes rather than to directly regulate defence genes and defensive compounds. A common picture found for sRNA mutants exhibiting higher susceptibility to *C. higginsianum* was rather a hormonal and metabolomic imbalance. Moreover, the absence of altered miRNAs targeting direct-defence genes in maize suggested an indirect defensive role of sRNAs against *Colletotrichum* spp.

5. RNA silencing and plant defence: an outlook

Altogether, the strategic outline of Arabidopsis and maize antifungal defence against *Colletotrichum* spp. points towards the concept that sRNAs are acting as fine-tuners mediating the balance of multiple genetic and metabolomic-defence layers. The sRNA-orchestrated fine-tuning of defensive layouts may provide a genetic flexibility allowing rapid and efficient adaptation of immune pathways. The question whether sRNA pathways are indispensable and pivotal antifungal-defence regulators remains debatable. Despite various studies showing the altered susceptibility of sRNA mutants, the general trend is that the outcome of sRNA-mediated defence strongly depends on a specific pathosystem. This chapter adds further highlights to this picture by showing that no miRNAs targeting classical defence pathways are de-regulated upon *C. graminicola* infection in maize, and Arabidopsis sRNA mutants under fungal attack appear to have altered metabolomic profile compared to the wild-type situation.

Nevertheless, considering the fact that sRNA pathways are also involved in setting up proper abiotic stress responses, it might represent a multi-valuable biotechnological approach to generate crops that are more efficient and variant in expressing their sRNA repertoire. Over the past years, a transgene-based approach where pathogen-targeting sRNAs are expressed in host species was repeatedly confirmed to efficiently control fungal diseases. This host-induced gene-silencing (HIGS) approach was successfully applied to a broader range of host-pathogen systems, thus bearing a valuable industrial potential. Significant drawbacks with this technology are the restrictive acceptance of genetically modified crops, and the yet elusive question of how fast pathogens evolve tolerance or resistance. For instance, *F. graminearum* sRNA mutants are showing normal virulence in wheat infection assays [51]. A yet elusive question is the role of plant endogenous sRNAs in targeting the genes of their fungal parasites. *B. cinerea* has been demonstrated to hijack plant genes using sRNA effectors [52]; thus it could be possible that plant sRNA effectors are able to infiltrate fungal cells to act as antimicrobial molecules.

Recent studies from two different research groups demonstrate fungal control by exogenous application of sRNAs to *F. graminearum* [53] and *B. cinerea* [54]. The so-called spray-induced gene silencing (SIGS) might provide novel biotechnological opportunities to control fungal diseases. Although the data from both studies are promising, it remains elusive how efficient sRNAs are compared to classical biologicals, and how broadly this technology can be applied. For instance, Botrytis is one of the few fungal species known to require a functional sRNA machinery for proper infection, hence possibly representing a special situation in sRNA-mediated plant-pathogen interaction. Moreover, exogenous control by sRNAs was efficient in controlling fungal growth on vegetables and fruits, and not demonstrated on leaves [54], suggesting efficacy only in a very specific infection condition. Altogether, it remains to be elucidated how efficient SIGS could work in field conditions, and in particular also the application spectrum of this technology. So far, this new technology lacks confirmation by additional independent studies to allow fully evaluating its industrial potential.

Prospective investigations will help in further elucidating of the full potential of sRNA-mediated antifungal defence. While the data presented here and in recent studies suggest

that sRNAs are subtle players in the concert of mounted antifungal defence, and new approaches using exogenously applied sRNAs are promising, there remains challenging basic research to be completed first in order to truly understand sRNA trafficking and signalling in plant-pathogen interactions.

Acknowledgements

We are grateful to Gaétan Glauser and Armelle Vallat-Michel (Chemical Analytical Service of the University of Neuchâtel) for their technical assistance in metabolomics and hormone analysis. Financial support from the National Centre of Competence in Research 'Plant Survival' and grant number 31003A-120197, both research programmes of the Swiss National Science Foundation, is gratefully acknowledged.

Author details

Andrea Balmer[1], Emanuele De Paoli[2], Azeddine Si-Ammour[3], Brigitte Mauch-Mani[1] and Dirk Balmer[1,4]*

*Address all correspondence to: dirk.balmer@syngenta.com

1 Institute of Biology, Laboratory of Molecular and Cell Biology, University of Neuchâtel, Neuchâtel, Switzerland

2 Dipartimento di Scienze Agrarie e Ambientali, Università degli Studi di Udine, Udine, Italy

3 Research and Innovation Centre—Fondazione Edmund Mach, Michele all'Adige, Trentino, Italy

4 Syngenta Crop Protection AG, Stein, Switzerland

References

[1] Ding SW, Voinnet O. Antiviral immunity directed by small RNAs. Cell. 2007;**130**:413-426

[2] Pumplin N, Voinnet O. RNA silencing suppression by plant pathogens: defence, counter-defence and counter-counter-defence. Nature Review Microbiology. 2013;**11**:745-760

[3] Borges F, Martienssen RA. The expanding world of small RNAs in plants. Nature Reviews Molecular Cell Biology. 2015;**16**:727-741

[4] Voinnet O. Origin, biogenesis, and activity of plant microRNAs. Cell. 2009;**136**:669-687

[5] Mallory AC, Vaucheret H. Functions of microRNAs and related small RNAs in plants. Nature Genetics. 2006;**38**(Suppl):S31-S36

[6] Kruszka K, Pieczynski M, Windels D, Bielewicz D, Jarmolowski A, Szweykowska-Kulinska Z, et al. Role of microRNAs and other sRNAs of plants in their changing environments. Journal of Plant Physiology. 2012;**169**:1664-1672

[7] Baulcombe D. RNA silencing in plants. Nature. 2004;**431**:356-363

[8] Ding SW. RNA-based antiviral immunity. Nature Reviews Immunology. 2010;**10**:632-644

[9] Li Y, Lu J, Han Y, Fan X, Ding SW. RNA interference functions as an antiviral immunity mechanism in mammals. Science. 2013;**342**:231-234

[10] Chisholm ST, Coaker G, Day B, Staskawicz BJ. Host-microbe interactions: Shaping the evolution of the plant immune response. Cell. 2006;**124**:803-814

[11] Jones JD, Dangl JL. The plant immune system. Nature. 2006;**444**:323-329

[12] Pastor V, Balmer A, Gamir J, Flors V, Mauch-Mani B. Preparing to fight back: generation and storage of priming compounds. Frontiers in Plant Science. 2014;**5**:295

[13] Jin H. Endogenous small RNAs and antibacterial immunity in plants. FEBS Letters. 2008;**582**:2679-2684

[14] Padmanabhan C, Zhang X, Jin H. Host small RNAs are big contributors to plant innate immunity. Current Opinion in Plant Biology. 2009;**12**:465-472

[15] Zhang W, Gao S, Zhou X, Chellappan P, Chen Z, Zhang X, et al. Bacteria-responsive microRNAs regulate plant innate immunity by modulating plant hormone networks. Plant Molecular Biology. 2011;**75**:93-105

[16] Boccara M, Sarazin A, Thiebeauld O, Jay F, Voinnet O, Navarro L, et al. The Arabidopsis miR472-RDR6 silencing pathway modulates PAMP- and effector-triggered immunity through the post-transcriptional control of disease resistance genes. PLoS Pathogens. 2014;**10**:e1003883

[17] Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, et al. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science. 2006;**312**:436-439

[18] Radwan O, Liu Y, Clough SJ. Transcriptional analysis of soybean root response to Fusarium virguliforme, the causal agent of sudden death syndrome. Molecular Plant-Microbe Interactions. 2011;**24**:958-72

[19] Xin M, Wang Y, Yao Y, Xie C, Peng H, Ni Z, et al. Diverse set of microRNAs are responsive to powdery mildew infection and heat stress in wheat (Triticum aestivum L.). BMC Plant Biology. 2010;**10**:123

[20] Yin Z, Li Y, Han X, Shen F. Genome-wide profiling of miRNAs and other small non-coding RNAs in the Verticillium dahliae-inoculated cotton roots. PLoS One. 2012;**7**:e35765

[21] Lu S, Sun YH, Amerson H, Chiang VL. MicroRNAs in loblolly pine (Pinus taeda L.) and their association with fusiform rust gall development. Plant Journal. 2007;**51**:1077-1098

[22] Guo N, Ye WW, Wu XL, Shen DY, Wang YC, Xing H, et al. Microarray profiling reveals microRNAs involving soybean resistance to Phytophthora sojae. Genome. 2011;**54**:954-958

[23] Nowara D, Gay A, Lacomme C, Shaw J, Ridout C, Douchkov D, et al. HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen Blumeria graminis. Plant Cell. 2010;**22**:3130-3141

[24] Koch A, Kumar N, Weber L, Keller H, Imani J, Kogel KH. Host-induced gene silencing of cytochrome P450 lanosterol C14alpha-demethylase-encoding genes confers strong resistance to Fusarium species. Proceedings of the National Academy of Sciences United States of America. 2013;**110**:19324-19329

[25] O'Connell RJ, Thon MR, Hacquard S, Amyotte SG, Kleemann J, Torres MF, et al. Lifestyle transitions in plant pathogenic Colletotrichum fungi deciphered by genome and transcriptome analyses. Nature Genetics. 2012;**44**:1060-1065

[26] O'Connell R, Herbert C, Sreenivasaprasad S, Khatib M, Esquerre-Tugaye MT, Dumas B. A novel Arabidopsis-Colletotrichum pathosystem for the molecular dissection of plant-fungal interactions. Molecular Plant-Microbe Interactions. 2004;**17**:272-282

[27] Huser A, Takahara H, Schmalenbach W, O'Connell R. Discovery of pathogenicity genes in the crucifer anthracnose fungus Colletotrichum higginsianum, using random insertional mutagenesis. Molecular Plant-Microbe Interactions. 2009;**22**:143-156

[28] Martins J, Branco F, Gonzalez Castro M, Cota M, Barroso J. A new concept of 3D DCS interface application for industrial production console operators. Universal Access in the Information Society. 2015;**14**:399-413

[29] Vargas WA, Martin JM, Rech GE, Rivera LP, Benito EP, Diaz-Minguez JM, et al. Plant defense mechanisms are activated during biotrophic and necrotrophic development of Colletotricum graminicola in maize. Plant Physiology. 2012;**158**:1342-1358

[30] Thomma BP, Penninckx IA, Broekaert WF, Cammue BP. The complexity of disease signaling in Arabidopsis. Current Opinion in Immunology. 2001;**13**:63-68

[31] Narusaka Y, Narusaka M, Park P, Kubo Y, Hirayama T, Seki M, et al. RCH1, a locus in Arabidopsis that confers resistance to the hemibiotrophic fungal pathogen Colletotrichum higginsianum. Molecular Plant-Microbe Interactions. 2004;**17**:749-62

[32] Balmer D, de Papajewski DV, Planchamp C, Glauser G, Mauch-Mani B. Induced resistance in maize is based on organ-specific defence responses. Plant Journal. 2013;**74**:213-225

[33] Si-Ammour A, Windels D, Arn-Bouldoires E, Kutter C, Ailhas J, Meins F, Jr., et al. miR393 and secondary siRNAs regulate expression of the TIR1/AFB2 auxin receptor clade and auxin-related development of Arabidopsis leaves. Plant Physiology. 2011;**157**:683-691

[34] Jiang H, Wong WH. SeqMap: mapping massive amount of oligonucleotides to the genome. Bioinformatics. 2008;**24**:2395-2396

[35] Balmer D, Flors V, Glauser G, Mauch-Mani B. Metabolomics of cereals under biotic stress: Current knowledge and techniques. Frontiers in Plant Science. 2013;**4**:82

[36] Weiberg A, Wang M, Bellinger M, Jin H. Small RNAs: A new paradigm in plant-microbe interactions. Annual Reviews in Phytopathology. 2014;**52**:495-516

[37] Li Y, Lu YG, Shi Y, Wu L, Xu YJ, Huang F, et al. Multiple rice microRNAs are involved in immunity against the blast fungus Magnaporthe oryzae. Plant Physiology. 2014; **164**:1077-1092

[38] Balmer D, Mauch-Mani B. Small yet mighty—microRNAs in plant-microbe interactions. Microrna. 2013;**2**:72-79

[39] Zhu QH, Fan L, Liu Y, Xu H, Llewellyn D, Wilson I. miR482 regulation of NBS-LRR defense genes during fungal pathogen infection in cotton. PLoS One. 2013;**8**:e84390

[40] Laender A, Goncalves M, Cota R, Barbosa F. Transitioning from the ecological fieldwork to an online repository: A digital library solution and evaluation. International Journal on Digital Libraries. 2007;**7**:109-112

[41] Lopez A, Ramirez V, Garcia-Andrade J, Flors V, Vera P. The RNA silencing enzyme RNA polymerase v is required for plant immunity. PLoS Genetics. 2011;**7**:e1002434

[42] Nonfiction-grades K-3. Library Talk. [Book Review]. **11**:29

[43] Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC. Networking by small-molecule hormones in plant immunity. Nature Chemical Biology. 2009;**5**:308-316

[44] Boccara M, Sarazin A, Thiebeauld O, Jay F, Voinnet O, Navarro L, et al. Correction: The Arabidopsis miR472-RDR6 silencing pathway modulates PAMP- and effector-triggered immunity through the post-transcriptional control of disease resistance genes. PLoS Pathogens. 2015;**11**:e1004814

[45] Robert-Seilaniantz A, MacLean D, Jikumaru Y, Hill L, Yamaguchi S, Kamiya Y, et al. The microRNA miR393 re-directs secondary metabolite biosynthesis away from camalexin and towards glucosinolates. Plant Journal. 2011;**67**:218-231

[46] Ng DW, Zhang C, Miller M, Palmer G, Whiteley M, Tholl D, et al. cis- and trans-Regulation of miR163 and target genes confers natural variation of secondary metabolites in two Arabidopsis species and their allopolyploids. Plant Cell. 2011;**23**:1729-1740

[47] Mallory A, Vaucheret H. Form, function, and regulation of ARGONAUTE proteins. Plant Cell. 2010;**22**:3879-3889

[48] Balmer D, Planchamp C, Mauch-Mani B. On the move: Induced resistance in monocots. Journal of Experimental Botany. 2013;**64**:1249-1261

[49] Nonfiction--Grades 1-3. Library Talk. [Book Review]. **11**:30

[50] Baldrich P, Kakar K, Sire C, Moreno AB, Berger A, Garcia-Chapa M, et al. Small RNA profiling reveals regulation of Arabidopsis miR168 and heterochromatic siRNA415 in response to fungal elicitors. BMC Genomics. 2014;**15**:1083

[51] Chen Y, Gao Q, Huang M, Liu Y, Liu Z, Liu X, et al. Characterization of RNA silencing components in the plant pathogenic fungus Fusarium graminearum. Scientific Reports. 2015;**5**:12500

[52] Weiberg A, Wang M, Lin FM, Zhao H, Zhang Z, Kaloshian I, et al. Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. Science. 2013;**342**:118-123

[53] Koch A, Biedenkopf D, Furch A, Weber L, Rossbach O, Abdellatef E, et al. An RNAi-based control of fusarium graminearum infections through spraying of long dsRNAs involves a plant passage and is controlled by the fungal silencing machinery. PLoS Pathogens. 2016;**12**:e1005901

[54] Wang M, Weiberg A, Lin FM, Thomma BP, Huang HD, Jin H. Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. Nature Plants. 2016;**2**:16151

IntechOpen

IntechOpen

IntechOpen