The function of *Arabidopsis* microRNAs in defense against the necrotrophic fungal pathogen *Alternaria brassicicola*

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Zusammenfassung

In ihrer natürlichen Umgebung müssen sich Pflanzen ständig mit abiotischen wie auch biotischen Stressfaktoren auseinandersetzen. Betrachtet man den Teil der mikrobiellen und pilzlichen Angriffe ist bisher die Interaktion zwischen *Arabidopsis thaliana* und dem (hemi-)biotrophen Pathogen *Pseudomonas syringae* sehr genau untersucht. Hierbei leisten microRNAs einen wichtigen Beitrag zur pflanzlichen Verteidigung. Das Verständnis wie *Arabidopsis thaliana* mit necrotrophen Pathogenen interagiert weist immer noch erhebliche Lücken auf. Infolgedessen, stellen wir uns die Frage inwiefern microRNAs in der Verteidigung von *Arabidopsis thaliana* gegen den necrotrophen Modelorganismus *Alternaria brassicicola*, welcher die Kohlschwärze an praktisch allen Arten innerhalb der *Brassicaceae* verursacht, beteiligt sind.

Um entscheidende microRNAs zu identifizieren, sequenzierte ich kleine RNAs. Hierbei zeigte sich, dass einige microRNAs auf eine Behandlung mit *Alternaria brassicicola* ansprechen. Die Funktion zweier gefundener microRNAs wurde hierauf im Detail untersucht. Die kürzlich evolvierte microRNA163 (miR163) ist deutlich hochreguliert nach einer Inokulation mit *Alternaria brassicicola* und zeigte eine Veränderung im Spleißverhältnis der intron-enthaltenden pri-miRNA. Infektionsassays mit dem Pilz *A. brassicicola* zeigten, dass *mir163* Mutanten resistenter sind. Dies weist daraufhin, dass miR163 ein negativer Regulator der pflanzlichen Abwehr ist, der möglicherweise externe Stimuli durch ein verändertes Spleißverhalten, das einen Einfluss auf die korrekte microRNA Biogenese hat, integriert.

Die untersuchte *mir827* Mutante zeigte keinen veränderten Phänotyp nach einer Behandlung mit *A. brassicicola*. Dennoch ist die Expression der miR827 deutlich erhöht und eine übermäßige Steigerung der Ziel-mRNA *VPT1* wird gemildert. Da miR827 wahrscheinlich in einem komplexen Netzwerk der Phosphatantwort eingebunden ist, könnten sowohl ein knock-out als auch eine Überexpression einer der Komponenten des Netzwerks unzureichend sind um das Netzwerk zu stören und die Resistenz verbleibt unverändert.

Eine sich ständig erhöhende Anzahl an Studien weist darauf hin, dass DNA Methylierung eine Schlüsselfunktion einnimmt um die pflanzliche Abwehr zu regulieren. Hierfür wurde ein induzierbares Vektorsystem etabliert, das Gene, die verantwortlich sind für DNA Methylierung, durch artifizielle miroRNAs herunterreguliert. Die Induzierbarkeit des Systems erlaubt eine gezielte Herunterregulierung von Genen zu einem bestimmten Stadium

des Infektionsprozesses und gibt die Möglichkeit Gene zu untersuchen, die im Embryo-Stadium letal sind oder starke Defekte in der Entwicklung aufweisen, wenn sie ausgeschaltet werden.

Summary

Plants constantly have to cope with abiotic and biotic stresses and they do that by a variety of regulatory mechanisms including adjustment of gene expression. Gene expression can be regulated by various means, including epigenetic regulation or post-transcriptional regulation by microRNAs (miRNAs). Concerning the part of microbial and fungal attacks, so far the interaction between *Arabidopsis thaliana* and (hemi-)biotrophic bacteria *Pseudomonas syringae* has been studied in detail, wherein microRNAs where shown to be an important factor in plant defense. The understanding how *Arabidopsis thaliana* interacts with necrotrophic pathogens still exhibits considerable gaps. Therefore, we asked the question in what way microRNAs are involved in defense of *Arabidopsis thaliana* against the necrotrophic model organism *Alternaria brassicicola*, which causes the black spot disease on virtually all plant species in the *Brassicaceae*.

In order to identify crucial microRNAs, I conducted a small RNA sequencing approach showing that several microRNAs are responsive to treatments with *Alternaria brassicicola*. I picked two of them and elucidated their function in more detail. The recently evolved microRNA163 (miR163) is clearly upregulated after *A. brassicicola* inoculations and showed a change in the splicing ratio of the intron-containing pri-miRNA. Infections assays with the *Alternaria brassicicola* fungi exhibited a more resistant phenotype of *mir163* mutants revealing that miR163 is a negative regulator of plant defense that putatively integrates external stimuli through its changed splicing ratio that affects proper miRNA processing.

The investigated *mir827* mutant did not show any changes phenotype when treated with *A. brassicicola* but miR827 is clearly upregulated and buffers the extensive increase of expression of its target mRNA *VPT1*. As miR827 is likely involved in the complex phosphate-response network, knock-out or overexpression of one component of the phosphate network could be insufficient to disturb the phosphate-starvation network and resistance remains unaltered.

An increasing amount of studies suggests that DNA methylation represent a key player in the regulation of plant defense. Therefore, I established an inducible vector system that knock-down DNA methylation genes by artificial microRNAs. The inducibility of this system allows to knock-down gene in a certain stage of the infection process and gives the opportunity to studies genes that are embryonic lethal or have strong developmental defects if knocked out.

1. Introduction

1.1 Plant defense response

As sessile organisms, plants are continuously challenged by abiotic stress as well as biotic stress and evolved strategies to cope with and defend against them. Biotic stress factors are for example herbivorous insects, nematodes, neighboring plants, pathogenic bacteria and fungi. In contrast to animals, plants do not have mobile defense cells or an adaptive immune system. They rely on their innate immunity defense system from each single cell (Nurnberger et al., 2004; Iriti and Faoro, 2007).

A model of the plant immune system was proposed by Jones and Dangl (2006): As a first line of defense, plants recognize pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs). PAMPs/MAMPs are conserved pathogenic structures. Most prominent examples are flg22, a conserved 22-amino acid peptide sequence of flagellin, and elf18, a peptide sequence of a bacterial elongation factor EF-Tu (Felix et al., 1999; Kunze et al., 2004). Those PAMPs/MAMPs are recognized by transmembrane pathogen recognition receptors (PRRs), like the flg22-sensing FLAGELLIN-SENSITIVE 2 (FLS2) (Chinchilla et al., 2006) and the elf18-sensing EF-TU RECEPTOR (EFR) (Zipfel et al., 2006). Recognition of a PAMP/MAMP results in downstream signaling and leads to pattern-triggered immunity (PTI) (Jones and Dangl, 2006). Typical downstream events of PTI are the production of reactive oxygen species, calcium ion influx, the activation of mitogen-activated protein kinases cascades, hormonal signaling, transcriptional reprogramming and the synthesis of antimicrobial metabolites (Ma and Berkowitz, 2007; Ahuja et al., 2012; Pieterse et al., 2012; Rasmussen et al., 2012; Baxter et al., 2014). All those reactions together are intended to prevent further spreading of the microbes. Successful pathogens in turn have evolved so-called effectors to overcome PTI leading to effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). Those effectors are essential for the virulence of the pathogen and for instance facilitate host penetration or suppress the plant immune response (Gohre and Robatzek, 2008).

During the arms race between plants and pathogens, plants evolved a subset of resistance genes (*R*-genes) against microbial effectors (McHale et al., 2006). *R*-genes encode for intracellular nucleotide-binding leucine rich repeat (NB-LRR) proteins that recognize pathogen-specific effectors directly or through the observation of indirect effects, called the "guard hypothesis" (Van der Biezen and Jones, 1998; Dangl and Jones, 2001; Jones and Dangl,

2006; McHale et al., 2006). The concept of the "guard hypothesis" implies that each R-protein monitors a cellular protein and whether the cellular protein is attacked. The recognition of a pathogen attack by NB-LRRs leads to effector-triggered immunity (ETI). ETI uses a common signaling network with PTI but ETI and PTI vary in robustness and duration and usually ETI includes hypersensitive response (HR) (Jones and Dangl, 2006; Tsuda and Katagiri, 2010).

Downstream of the activation of PTI and ETI, phytohormones display the key player of the defense signaling pathway. Dependent on the pathogen type different phytohormones (SA, JA, ET) and defense responses are activated (Pieterse et al., 2009; Pieterse et al., 2012).

Microbial plant pathogens can be generally divided in two different groups: Biotrophic pathogens that grow only on living host tissue and necrotrophic pathogens that kill the host tissue during infection process and feeds on the dead tissue (Glazebrook, 2005). Recognition of biotrophic pathogens like *Pseudomonas syringae* or *Hyaloperonospora arabidopsidis* activate the phytohormone salicylic acid (SA) and SA-mediated pathways, while jasmonic acid (JA) and ethylene (ET) are classical phytohormones for immunity against necrotrophs, like *Botrytis cinerea* or *Alternaria brassicicola* (reviewed in Pieterse et al. (2009; 2012)). Phytohormonal pathways of SA and JA/ET display extensive crosstalk between each other, as they defend against pathogens with completely different lifestyles and distinct defense genes and mechanisms have to be activated (reviewed in Pieterse et al. (2009; 2012)). While for example a hypersensitive response is an effective defense means to restrict infections with biotrophic pathogens, HR facilitates infections for necrotrophic pathogens (Govrin and Levine, 2000).

As plant-(hemi-)biotrophic pathogen model serves the interaction of *Arabidopsis thaliana* with the bacteria *Pseudomonas syringae* and depicts the most intensively studied plant-pathogen pair (reviewed in Katagiri et al. (2002)). *Pseudomonas syringae* pathovar *tomato* (*Pst*) DC3000 is the most commonly used bacteria since it is a virulent strain for *Arabidopsis thaliana*. *Pst* DC3000 introduces bacterial effectors via its type III secretion system. Modifications of *Pst* DC3000 by creating defects in the type III secretion system (e.g. *Pst* DC3000 *hrcC*-) or expressing avirulence factors (e.g. *avrRpt2*, *AvrPto*) that can be recognized by Arabidopsis through NB-LRRs provide a good tool to study plant interactions with nonpathogenic (*Pst* DC3000 *hrcC*-), virulent (*Pst* DC3000) and avirulent (e.g. *Pst* DC3000 *avrRpt2*) pathogens.

As necrotrophic model organisms serve *Alternaria brassicicola* and *Botrytis cinerea* amongst others, two pathogens with a broad host-range (van Kan, 2006; Cho, 2015). Important components for the immunity against necrotrophs are the phytohormone JA and phytoalexins

indicated by the enhanced susceptibility of mutants defective in biosynthesis of JA (*coi1* mutant) and the phytoalexin camalexin (*pad3* mutant) against *A. brassicicola* (Thomma et al., 1998; Thomma et al., 1999; Zhou et al., 1999). Additionally, BAK1, a co-receptor of PRRs, is required for resistance against *A. brassicicola* and *B. cinerea* (Chinchilla et al., 2007; Kemmerling et al., 2007; Halter et al., 2014). Another central role in the strategy of necrotrophs is the production of a variety of toxins and phytotoxic metabolites. For instance, *Alternaria* species produce Brassicicolin A and depudecin, an inhibitor of histone deacetylases, amongst other toxins (Kwon et al., 1998; Privalsky, 1998; Pedras et al., 2009). The concrete interactions of *A. brassicicola* with *Arabidopsis thaliana* are still enigmatic as so far only two virulence factors of *Alternaria brassicicola* (Cho et al., 2009) and only one NB-LRR encoding *R*-gene involved in immunity against *A. brassicicola* (Staal et al., 2008) were described. Likewise, no cellular targets for the majority of the phytotoxic metabolites and/or downstream signaling components were discovered.

1.2 Involvement of AGO proteins and their associated small RNAs in plant defense

In plants, several classes of endogenous small RNAs (sRNAs) have been described. Most are 21 - 24 nt long, are derived from a longer RNA precursor by DICER-LIKE (DCL) proteins and are methylated by HUA ENHANCER 1 (HEN1) (Borges and Martienssen, 2015). All sRNAs are loaded into the ARGONAUTE component of an RNA-induced silencing complex (RISC) that is directed towards its target by complementary base pairing between sRNA and target sequence (Borges and Martienssen, 2015).

The most abundant sRNAs in *Arabidopsis thaliana* are small-interfering RNAs (siRNAs) that are produced from a double-stranded RNA derived from hairpin-forming RNAs or the activity of RNA-dependent RNA-polymerases (RDRs) on single-stranded RNA or natural antisense transcripts (reviewed in Willmann et al. (2011), Borges and Martienssen (2015)).

Endogenous siRNAs have been described to function against viruses (Cao et al., 2014), herbivorous insects (Pandey et al., 2008), nematodes (Hewezi et al., 2008) and microbial pathogens (reviewed in Jin (2008) and Padmanabhan et al. (2009)).

Cao et al. (2014) showed, that viral infection of *Arabidopsis thaliana* leads to the production of vasiRNAs (virus-activated siRNAs) that act together with AGO2 in silencing of host genes and are proposed to confer broad-spectrum antiviral activity.

Plant infection with *Pst avrRpt2* leads to the production of a natural antisense transcripts that is further processed into siRNAATGB2 (Katiyar-Agarwal et al., 2006). siRNAATGB2 acts in *cis* and downregulates a pentatricopeptide repeats protein-like gene that is suggested to act as negative regulator of the RPS2-mediated resistance (Katiyar-Agarwal et al., 2006).

Some more *R*-genes have been proposed to be regulated by sRNAs. The *R*-gene cluster of the *RPP5 (recognition for Peronospora parasitica 5)* locus was shown to produce siRNAs that negatively regulate themselves (Yi and Richards, 2007).

Another class of recently discovered sRNAs, so-called long siRNAs (lsiRNA) with a size of 30 - 40 nt have been connected with plant pathogen defense (Katiyar-Agarwal et al., 2007). Derived from a natural antisense transcripts, clusters of one lsiRNAs (lsiRNA-1) are specifically upregulated upon *Pst avrRpt2* infection and repress *AtRAP* in *cis*, a gene that is putatively involved in basal defense (Katiyar-Agarwal et al., 2007).

1.3 Involvement of microRNAs in plant defense

Besides the previously described sRNAs, many sRNA of the microRNA class were observed to be responsive to biotic stress (Fahlgren et al., 2007; Li et al., 2010; Zhang et al., 2011a; Sunkar et al., 2012).

In *Arabidopsis thaliana*, more than 400 microRNAs are annotated (http://www.mirbase.org/; miRbase21). MicroRNAs are single-stranded, non-coding RNAs with a length of 21 - 24 nt and possess independent transcriptional units (Rogers and Chen, 2013; Szweykowska-Kulinska et al., 2013). The biogenesis of microRNAs is briefly depicted in Figure 1. Mature microRNAs are processed from a much longer primary transcript (pri-miRNA) produced by RNA polymerase II (Xie et al., 2005; Kim et al., 2011). The pri-miRNA is decorated with a 5'-cap as well as a 3'-polyadenylated tail (Xie et al., 2005; Zhang et al., 2005) and forms an imperfect stem-loop structure through partial sequence complementarity. Several proteins, the CAP-BINDING PROTEINS CBP20 and CBP80, the zinc-finger protein SERRATE (SE), HYPONASTIC LEAVES 1 (HYL1) and DICER-LIKE 1 (DCL1), process the pri-miRNA into the mature microRNA (Kurihara et al., 2006; Dong et al., 2008; Kim et al., 2008; Laubinger et al., 2008). The RNAse III-like enzyme DCL1 is the active dicing component and processes the pri-miRNA stem-loop structure into pre-miRNA and miRNA/miRNA* duplex with a two nucleotide overhang at the 3'-termini (Kurihara and Watanabe, 2004).

To protect the miRNA/miRNA* duplex against exonucleolytic activity, uridinylation and subsequent degradation, the methyltransferase HUA ENHANCER 1 (HEN1) 2'-*O*-methylates the duplex at its 3'-ends (Li et al., 2005; Yu et al., 2005; Yang et al., 2006b). Subsequently, the methylated miRNA duplex is exported from the nucleus into the cytoplasm inter alia by HASTY1 (Park et al., 2005) and thereafter mainly loaded into ARGONAUTE 1 (AGO1) (Baumberger and Baulcombe, 2005; Mi et al., 2008) and the miRNA* strand is removed (Rogers and Chen, 2013). Via complementary base pairing of the RISC-loaded microRNA with the target messenger RNA (mRNA), the target transcript is post-transcriptionally regulated by either cleavage through the AGO1 slicer activity followed by mRNA degradation or by translational inhibition (Baumberger and Baulcombe, 2005; Brodersen et al., 2008).

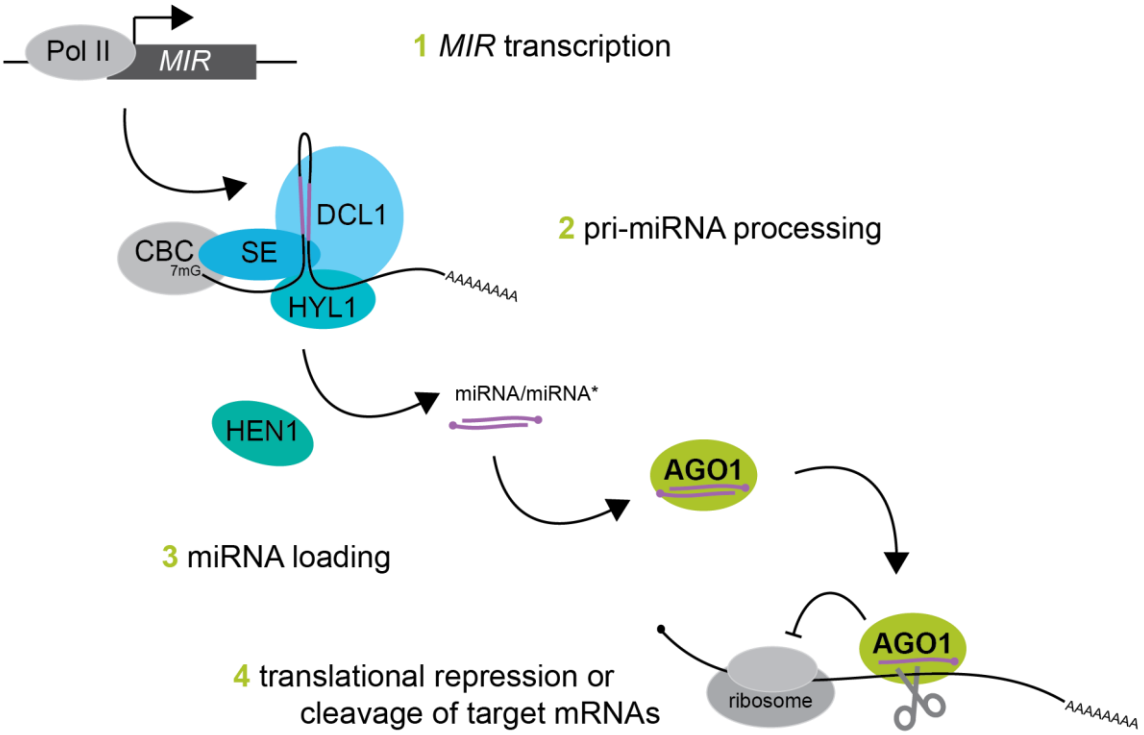


Figure 1: MicroRNA biogenesis and action. MIR genes are transcribed by RNA polymerase II (Pol II) into pri-miRNA. The cap-binding complex recruits the miRNA processing machinery consisting of SERRATE (SE), HYPONASTIC LEAVES 1 (HYL1) and DICER-LIKE 1 (DCL1) that produces the miRNA/miRNA* duplex. Subsequently, the miRNA/miRNA* duplex is methylated by HUA ENHANCER 1 (HEN1) and loaded into the microRNA effector protein ARGONAUTE 1 (AGO1). Sequence complementarity of the microRNA with the target mRNA leads to post-transcriptional silencing through cleavage of target mRNA or translational repression.

Several pathways have been known to control miRNA accumulation. The 3'-5'-exonucleases SMALL RNA DEGRADING NUCLEASE (SDN) and HEN 1 SUPPRESSOR 1 (HESO1)-mediated uridylation restrict the excessive accumulation of microRNAs (Ramachandran and Chen, 2008; Ren et al., 2012; Zhao et al., 2012). MiRNAs are also known to control the homeostasis of their biogenesis (Rhoades et al., 2002; Xie et al., 2003; Vaucheret et al., 2004; Meng et al., 2012). For instance, ARGONAUTE 1 is controlled via a negative feedback by miRNA168 (Rhoades et al., 2002; Vaucheret et al., 2004).

Functionally, many miRNAs were described to be crucial for developmental stages, most of them by controlling core proteins of regulatory networks (reviewed in Jones-Rhoades et al. (2006)) but also other functions of miRNAs have been described, for example miRNAs that are induced by biotic stress (Figure 2; Fahlgren et al. (2007), Li et al. (2010), Zhang et al. (2011a), Sunkar et al. (2012)).

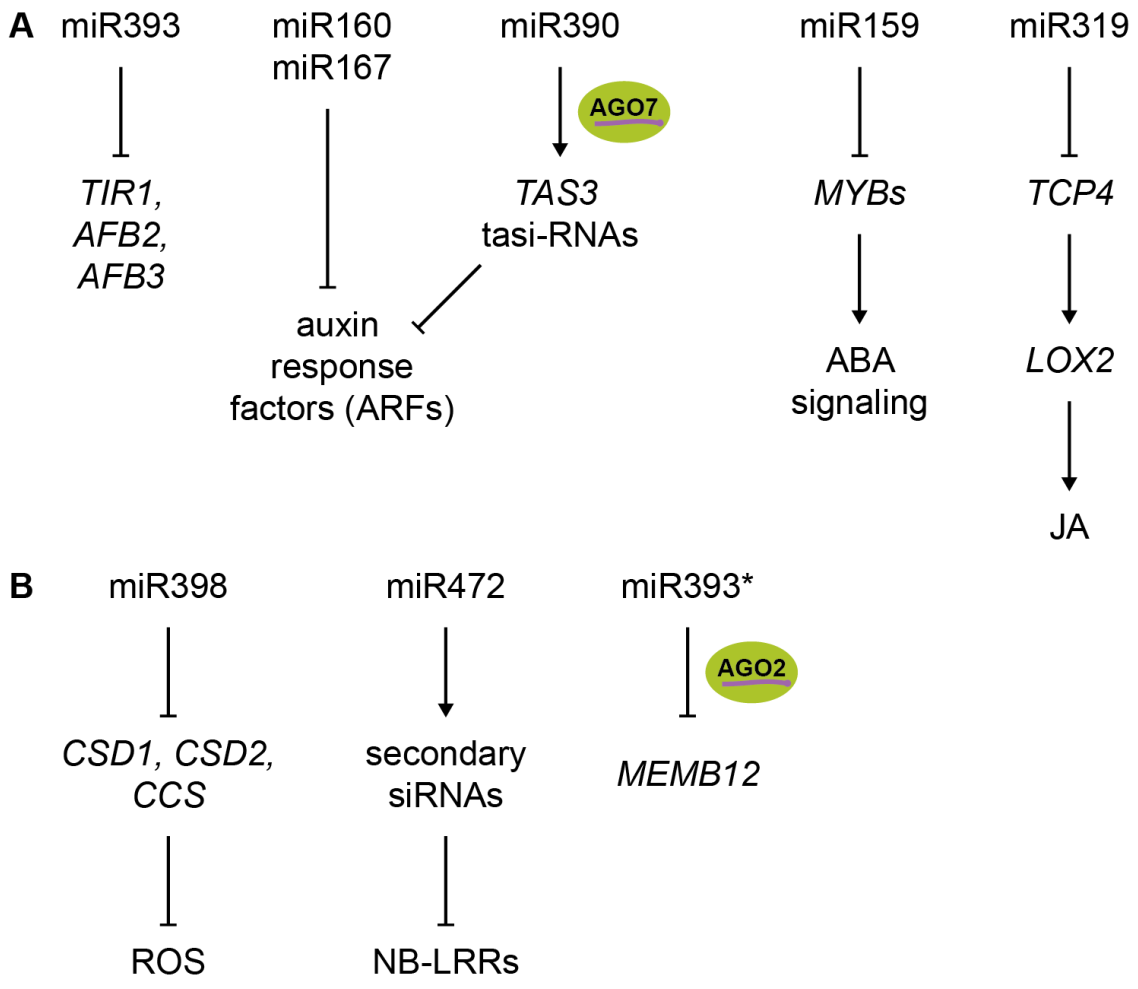


Figure 2: MicroRNAs described in defense against biotrophic pathogens and their targets. **A** MicroRNAs that influence hormonal pathways. **B** MicroRNAs induced by biotrophic pathogens regulate genes that are uninvolved in hormone signaling and pathways.

The first miRNA that has been reported to play a role in antibacterial PAMP-triggered immunity (PTI) was miR393 (Navarro et al., 2006). MiR393 is induced upon flg22 perception and post-transcriptionally downregulates its target genes *TRANSPORT INHIBITIOR RESPONSE 1 (TIR1), AUXIN SIGNALING F-BOX 2 (AFB2)* and *AFB3* encoding auxin-dependent F-box proteins (Gray et al., 1999; Dharmasiri et al., 2005; Kepinski and Leyser, 2005). The miRNA-mediated suppression of auxin signaling is implicated to promote resistance to the virulent bacteria *Pseudomonas syringae* pv. *tomato* DC3000 (Navarro et al., 2006).

Further connection between miRNAs and auxin signaling pathway has been established, as miR160 and miR167 were discovered to be induced upon flg22 and *Pst* (*hrcC*-, DC3000, *avrRpt2*) treatment (Fahlgren et al., 2007; Li et al., 2010; Zhang et al., 2011a). Both miRNAs

target transcription factors of the *AUXIN RESPONSE FACTOR* (*ARF*) family (Rhoades et al., 2002; Mallory et al., 2005; Wu et al., 2006; Li et al., 2010).

Two more miRNAs, miR159 and miR390, are downregulated 6 hours after inoculation with *Pst* DC3000 (Zhang et al., 2011a). miR159 targets *MYB* transcription factors, that are positive regulators of abscisic acid (ABA) signaling (Reyes and Chua, 2007). miR390 is required for the production of *TAS3* tasiRNAs by cleavage of *TAS3*, which targets *ARF2, ARF3* and *ARF4* (Fahlgren et al., 2006; Yoon et al., 2010). In addition, modulation of the JA biosynthesis is a target of the stress-induced miR319. *Pst hrcC-*, *Pst avrRpt2* and flg22 treatment induce expression of miR319 (Zhang et al., 2011a) that targets *TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR1* (*TCP*) transcription factors (Schommer et al., 2008). One of them, TCP4 regulates expression of *LIPOXYGENASE 2* (*LOX2*), encoding the first enzyme of the jasmonic acid biosynthesis pathway (Schommer et al., 2008).

However, not only bacteria-responsive microRNAs that modulate hormonal pathways were implicated to have a role in plant defense (Figure 2). For example, miR398 is downregulated upon flg22, nonpathogenic, virulent and avirulent *Pst* treatment (Jagadeeswaran et al., 2009; Li et al., 2010; Zhang et al., 2011a). miR398 targets *COPPER/ZINC SUPEROXIDE DISMUTASE (CSD)* genes that were suggested to detoxify reactive oxygen species (ROS) from the oxidative burst (Jagadeeswaran et al., 2009; Li et al., 2010; Baxter et al., 2014).

More recently, microRNAs have been described that regulate NB-LRR genes via production of secondary siRNAs in *Arabidopsis thaliana, Medicago truncatula* and Solanaceae (Zhai et al., 2011; Li et al., 2012; Shivaprasad et al., 2012; Boccara et al., 2014). miR472 for instance was described as a negative regulator of PTI and ETI through the production of secondary RDR6-dependent siRNAs and targeting of NB-LRR resistance genes (Boccara et al., 2014).

The fact that the majority of all miRNAs are loaded into an AGO1-RISC (Mi et al., 2008; Takeda et al., 2008; Zhang et al., 2014) and that several biotic stress responsive miRNAs have been transcribed (Fahlgren et al., 2007; Li et al., 2010; Zhang et al., 2011a; Sunkar et al., 2012) supports the role of AGO1 in plant immunity.

1.4 ARGONAUTE proteins in plant defense response

To accommodate the huge variety of small RNAs, the genome of *Arabidopsis thaliana* encodes for 10 ARGONAUTE (AGO) proteins (Mallory and Vaucheret, 2010). Both together, sRNA and AGO protein, form the functional RNA-induced silencing complex. Hence, it is not surprising that besides sRNAs also AGO proteins were shown to have crucial functions in plant defense.

The action of the above described lsi-RNA-1 is dependent on AGO7 as well as defense against turnip crinkle virus is mediated by AGO7 (Katiyar-Agarwal et al., 2007; Qu et al., 2008). AGO4 plays a role in plant defense against tobacco rattle virus (Ma et al., 2015) and *Pst* DC3000, *Pst avrRpm1* and *Pseudomonas syringae* pv. *tabaci* (Agorio and Vera, 2007).

A broader involvement has been shown for AGO2. As AGO2 is part of antiviral defense against cucumber mosaic virus, potato virus X, tobacco rattle virus, turnip crinkle virus, turnip mosaic virus and indicates that AGO2 has been specialized in antiviral defense throughout evolution (Qu et al., 2008; Harvey et al., 2011; Jaubert et al., 2011; Wang et al., 2011b; Carbonell et al., 2012; Zhang et al., 2012; Brosseau and Moffett, 2015; Ma et al., 2015). Additionally, the AGO2-targeting miR403 was repressed by *Pst hrcC-*, *Pst* DC3000 and *Pst avrRpt2* infections as well as AGO2 provides resistance together with miR393* against *Pseudomonas syringae* suggests an important role of AGO2 in plant immunity (Allen et al., 2005; Zhang et al., 2011a; Zhang et al., 2011b).

Especially the findings of Weiberg et al. (2013) has to be pointed out as they proved the first gene silencing event between kingdoms that occurs naturally. In their studies with *Arabidopsis thaliana* and *Solanum lycopersicum*, they showed that *Botrytis cinerea* releases fungal sRNAs into the plant host cell acting as effector molecules (Weiberg et al., 2013). Those fungal sRNAs are loaded into the host AGO1 protein and subsequently suppress host immunity genes and weaken plant immunity (Weiberg et al., 2013). This indicates that the pathogenicity of *B. cinerea* depends on the host AGO1 function.

As many different classes of endogenous and exogenous sRNA can be loaded into AGO1 and AGO1 is involved in defense against hemi-/biotrophic pathogens, necrotrophic pathogens and viruses, AGO1 is one of the central components of RNA-silencing-mediated host defense (Morel et al., 2002; Qu et al., 2008; Ellendorff et al., 2009; Azevedo et al., 2010; Weiberg et al., 2013).

1.5 MiRNA163 – a non-conserved microRNA with potential function in plant immunity

As a putative microRNA candidate that is involved in plant immunity network, miRNA163 was suggested in the literature due to its inducibility and target genes (Seo et al., 2001; Chen et al., 2003; Ng et al., 2011; Bielewicz et al., 2013). Furthermore, also in my studies miRNA163 showed up to play a role in plant immunity.

MIR163 is a non-conserved intron-containing miRNA that has recently evolved by inverted duplication of target gene sequences (Allen et al., 2004) and obtains several features that distinguishes *MIR163* from canonical miRNAs: It is 24 nt long (Kurihara and Watanabe, 2004) and is located in a co-evolved target gene cluster (Figure 3A; Allen et al. (2004)). Hence, it was assumed that *MIR163* evolved together with the expansion of the target gene family and was later converted into a miRNA (Allen et al., 2004). In addition, bioinformatic analysis revealed that the 5'-upstream sequence of *MIR163* resembles the promoter sequence of its targets genes (Wang et al., 2006) supporting the microRNA evolution model of Allen et al. (2004).

The promoter of *MIR163* possess a canonical TATA sequence and many predicted positive and negative regulating elements, inter alia elicitor responsive elements (Ng et al., 2011) and was shown to be responsive to phosphate starvation (Lundmark et al., 2010). It has been experimentally proven that miR163 levels are increased upon treatment with the fungal elicitor alamethicin and *Pst* DC3000 (Figure 3B; Ng et al. (2011), Bielewicz et al. (2013)).

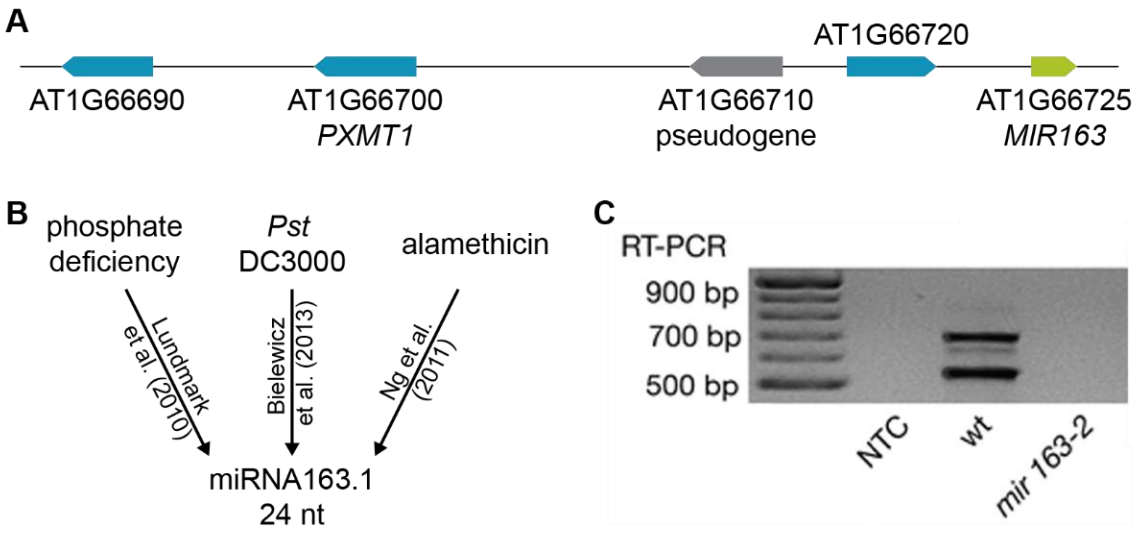


Figure 3: *MIR163* as several uncommon features. A *MIR163* (green) is located in a gene cluster together with putative target genes of the SABATH methyltransferase family (blue) on chromosome 1 (Allen et al., 2004). **B** Different external stimuli were shown to induce miRNA163.1 levels. **C** *MIR163* has two different splicing isoforms (No template control (NTC), wild type (wt), *mir163-2* (SALK_034556; in further experiments named *mir163-1*); from Bielewicz et al. (2013), Fig. 1).

MiR163 (AT1G66725) was suggested to post-transcriptionally target AT1G66690, AT1G66700 *(PXMT1)*, AT1G66720, AT3G44840, AT3G44860 *(FAMT)*, AT3G44870, that are members of the SABATH methyltransferase family (Zhao et al., 2008; Ng et al., 2011; Bielewicz et al., 2013; Chung et al., 2016). SABATH methyltransferase genes encode proteins involved in the methylation of small molecules like phytohormones and signaling molecules (Zhao et al., 2008) and were shown to play a role in plant defense (Seo et al., 2001; Chen et al., 2003). 5'-RACE revealed, that miR163 regulates its target genes *FAMT*, *PXMT1* and *AT1G66690* by mRNA cleavage (Ng et al., 2011; Bielewicz et al., 2013). Consistent with this observation, *PXMT1* and *FAMT* are constitutively upregulated in *mir163* mutants; *MIR163* overexpression lines showed downregulated *FAMT* and *PXMT1* transcript levels (Ng et al., 2011).

Target gene *FAMT* encodes for a FARNESOIC ACID METHYLTRANSFERASE, which methylates farnesoic acid (FA) to methyl farnesoate (MeFA) (Yang et al., 2006a), an unepoxidized precursor of the insect juvenile hormone III that interferes with insect development (Toong et al., 1988). PXMT1 is a putative 1,7-paraxanthine methyltransferase and the product of its enzymatic activity is a compound structurally related to the alkaloid caffeine

(Zhao et al., 2008; Ng et al., 2011). Like miR163, expression of *FAMT* and *PXMT1* are induced upon alamethicin treatment supporting their hypothetical role in plant defense (Ng et al., 2011).

In accordance with canonical 21 nt miRNAs, the biogenesis of miR163 is dependent on CBP20, CBP80, DCL1, HEN1, HYL1 and SE (Allen et al., 2004; Szarzynska et al., 2009; Bielewicz et al., 2013). In addition to that, also serine/arginine-rich splicing factors contribute to the biogenesis of miR163 (Bielewicz et al., 2013). Pri-miR163 can be spliced into two different isoforms with alternative poly(A) sites (Figure 3C), while the intron-containing isoform has a positive effect on accumulation of mature miR163 (Bielewicz et al., 2013; Schwab et al., 2013).

Additionally, two different miRNAs can be processed sequentially from the pri-miR163 precursor (miR163.1; miR163.2) (Kurihara and Watanabe, 2004) and dependent on their 5'-nucleotide they are loaded into different AGO proteins: MiR163.1 (5'-U) is loaded into AGO1, while miR163.2 (5'-A) is preferentially loaded into AGO2 (Mi et al., 2008; Takeda et al., 2008; Jeong et al., 2013).

1.6 MicroRNAs putatively connect phosphate availability to pathogen defense

Lately, a connection between the plant nutrition with phosphorus and the defense hormone JA as shown and therefore phosphorus is suggested to influence plant defense (Khan et al., 2016). Additionally, two microRNAs (miR827 and miR399) were already coupled to the phosphate network and thereby can putatively connect pathogen defense with phosphate availability (Bari et al., 2006; Hsieh et al., 2009; Pant et al., 2009).

Phosphorus is an essential macronutrient element and is absorbed as inorganic phosphate (P_i) from soil. Phosphorus is part of basic biological functions as structural element and regulatory component for enzymes and signal transduction cascades or in energy metabolism.

Recent studies provide evidence that couple phosphate stress and pathogenic stress (Wang et al., 2011a; Lu et al., 2014). Plants, mutant in the *PHOSPHATE TRANSPORTER 4.1 (PHT4.1),* showed increased susceptibility to virulent *Pseudomonas syringae* indicating that PHT4.1 plays a role in basal plant defense (Wang et al., 2011a). Additionally, a bacterial effector from Phytoplasma was connected to plant immunity and phosphate deficiency (Lu et al., 2014). Plants overexpressing the Phytoplasma effector inhibit plant defense response and triggers phosphate starvation response, including the upregulation of the phosphate-responsive miR399 (Lu et al., 2014).

Upregulation of miR399 was shown in a second study that explores the infection of citrus plant with the bacterial pathogen *Candidatus Liberibacter asiaticus* (Zhao et al., 2013). The exogenous application of phosphate was able to lower the disease symptom caused by *Candidatus Liberibacter asiaticus* indicating that phosphate availability is a factor for the development of disease symptoms (Zhao et al., 2013).

miR399 is a key regulator for phosphate homeostasis in plants, that is induced upon phosphate starvation and negatively regulates *PHOSPHATE 2 (PHO2)* encoding an E2 conjugase (Sunkar and Zhu, 2004; Allen et al., 2005; Fujii et al., 2005; Bari et al., 2006; Chiou et al., 2006; Lundmark et al., 2010). MiR399 was shown to be phloem-mobile and can travel from shoot to root and repress there *PHO2* (Pant et al., 2008). The repression of the E2 conjugase *PHO2* in roots results in an enhanced expression of phosphate uptake transporters (Bari et al., 2006). Hence, upon phosphate starvation, miR399 accumulates and increases P_i uptake.

Like miR399, miR827 is highly induced upon phosphate starvation in *A. thaliana* and *Brassica napus* and is highly abundant under phosphate limiting conditions in phloem sap of *Brassica napus* (Rajagopalan et al., 2006; Hsieh et al., 2009; Pant et al., 2009; Lundmark et al., 2010). Upon phosphate deficiency, induction of miR827 regulates its target gene *NITROGEN LIMITATION ADAPTATION (NLA)* post-transcriptionally (Hsieh et al., 2009; Pant et al., 2009; Kant et al., 2011). *NLA* encodes an E3 ubiquitin ligase that regulates directly and/or indirectly *PHOSPHATE TRANSPORTER 1.1 (PHT1.1), PHT1.4* and *PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR 1 (PHF1)* (Kant et al., 2011; Lin et al., 2013; Park et al., 2014). As second miR827 target, the tonoplast-localized *VACUOLAR PHOSPHATE TRANSPORTER 1 (VPT1)* was proposed, that transports P_i into the vacuole (Liu et al., 2015). Fitting to the concept, that phosphate and defense response are coupled, *nla* mutants display higher SA levels after infection with *Pst* DC3000 and bacterial growth of *Pst* DC3000 is restricted compared to wildtype (Figure 4A, B; Yaeno and Iba (2008)). Analogous to miR399, miR827 was suggested to be phloem-mobile and is highly enriched in *Brassica napus* phloem sap under phosphate deficient conditions (Pant et al., 2009). Furthermore, a direct connection has been proven between these two pathways. The E2 conjugase PHO2 acts together with the E3 ligase NLA in proteasomal degradation of PHT1.4 (Park et al., 2014).

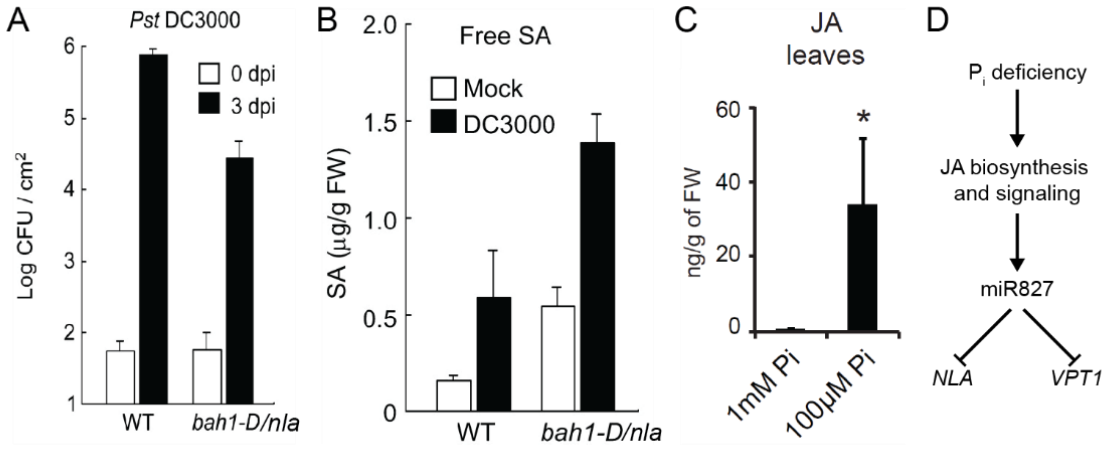


Figure 4: MiR827 putatively links phosphate deficiency with plant immunity. **A** *Bah1-D/nla* mutants are more resistant against *Pst* DC3000 (from Yaeno and Iba (2008), Fig. 2A). **B** *Bah1-D/nla* mutants accumulate more salicylic acid (SA) in mock- and *Pst* DC3000-treated leaves (from Yaeno and Iba (2008), Fig. 2B). **C** Plants grown under phosphate deficient conditions have a higher jasmonic acid (JA) level in leaves (from Khan et al. (2016), Fig. 2). **D** Model of phosphate-dependent miR827 activation and repression of its target genes.

Khan et al. (2016) lately showed a possible link that could explain the interconnection between phosphate deficiency and defense responses. Plants with a lack of phosphate displayed enhanced levels of the phytohormone JA and of JA signaling pathways (Figure 4C; Khan et al. (2016)) needed for defense against herbivorous insects and necrotrophic pathogens (reviewed in Pieterse et al. (2009; 2012)). Additionally, Hewezi et al. (2016) described the role of miR827 and its target gene *NLA* in the defense response against nematodes. Infections with the cyst nematode *Heterodera schachtii* activate miR827 in the syncytial feeding cells. Thus, *NLA*, a positive regulator of plant immunity, is downregulated and allows the nematode to establish the infection (Hewezi et al., 2016).

However, the mechanisms that connect P_i deficiency with JA response remains to be elucidated as the JA response is delayed but not completely abolished in *phr1* mutants. The transcription factor PHOSPHATE STARVATION RESPONSE 1 (PHR1) regulates the majority of P_i-starvation induced genes (Bustos et al., 2010), including miR399 (Bari et al., 2006). Interestingly, phosphate starvation does also shown a slight induction of miR163, that seems to be negatively controlled by PHR1 (Lundmark et al., 2010).

1.7 DNA methylation and its involvement of plant defense

Besides the post-transcriptional regulation of gene expression through microRNAs, gene expression can be regulated transcriptionally through epigenetic mechanisms, like DNA methylation. As there are many hints that epigenetic mechanisms and especially DNA methylation, that is coupled to sRNAs via the RNA-directed DNA methylation (RdDM) pathway, actively contribute to plant immune response, we decided to have a closer look how DNA methylation influences plant defense.

In general, epigenetics comprise all mechanisms that regulate gene expression without alterations in the DNA sequence. For example, the packaging state of DNA can epigenetically regulate gene expression. Further, chromatin remodeling factors can influence the packing state of DNA and move, remove, insert or modify nucleosomes (complex of DNA and histone octamers) and thereby influence access/affect for the transcription machinery. Histone modifying enzymes modify post-translationally the globular histone proteins at their N-terminal tail through methylation, acetylation, phosphorylation *et cetera* (Pfluger and Wagner, 2007). These modifications can tighten or loosen the interactions of DNA with histone proteins. Nucleosome density, histone modifications and DNA methylation pathways are interconnected, contribute together to the accessibility of the DNA sequence and therefore regulate gene expression at a transcriptional level. In general, loosely packed euchromatin is actively transcribed, while densely packed heterochromatic regions are transcriptionally inactive.

Directly, the DNA can be modified through the methylation of the base cytosine through DNA methyltransferases. Cytosine methylation is an epigenetic mark that silences transposable elements and repeats but is also involved in genomic imprinting and can occur in gene bodies with a so far unknown function (Law and Jacobsen, 2010).

In *Arabidopsis thaliana*, DNA methylation is catalyzed by the enzymes METHYLTRANSFERASE 1 (MET1), DOMAINS REARRANGED METHYLTRANSFERASE 1 (DRM1), DRM2 and CHROMOMETHYLASE 3 (CMT3) in all sequence contexts (reviewed in Law and Jacobsen (2010)). Cytosine methylation in the CG context is catalyzed and maintained by MET1 and around one third of all Arabidopsis genes have CG methylations in their coding region (Zhang et al., 2006). The plant-specific enzyme CMT3 is able to methylate DNA in CHG sequence context and is firmly integrated in a reinforcing loop with histone H3K9 methylation via histone methyltransferases. Especially, the asymmetric and *de novo* methylation in the CHH sequence context through DRM1 and DRM2

should be drawn attention to in respect to plant defense. This *de novo* methylation pathway in plants is mediated through a small RNA-directed DNA methylation (RdDM) pathway and is able to establish new DNA methylation marks and is therefore predestined to react dynamically to a pathogenic infection (Matzke and Mosher, 2014).

Epigenetic mechanisms have been described to be essential for adaptations and response towards abiotic and biotic stress factors (Pavet et al., 2006; Qi et al., 2006; Agorio and Vera, 2007; Lopez et al., 2011; Berr et al., 2012; Dowen et al., 2012; Kinoshita and Seki, 2014). Concerning biotic stress, Dowen et al. (2012) showed that the DNA methyltransferases of *Arabidopsis thaliana* MET1, DRM1, DRM2 and CMT3 directly contribute to bacterial resistance. Both *met1* mutant and the triple mutant *drm1drm2cmt3 (ddc)* show decreased bacterial growth after infections in non-pathogenic, virulent or avirulent strain of *Pst* and showed a disease resistance phenotype compared to wild type (Figure 5A; Dowen et al. (2012)). After analysis of differentially methylated cytosine residues, the authors suggested that the *Pst*-mediated changes of DNA methylation control transcription (Dowen et al., 2012).

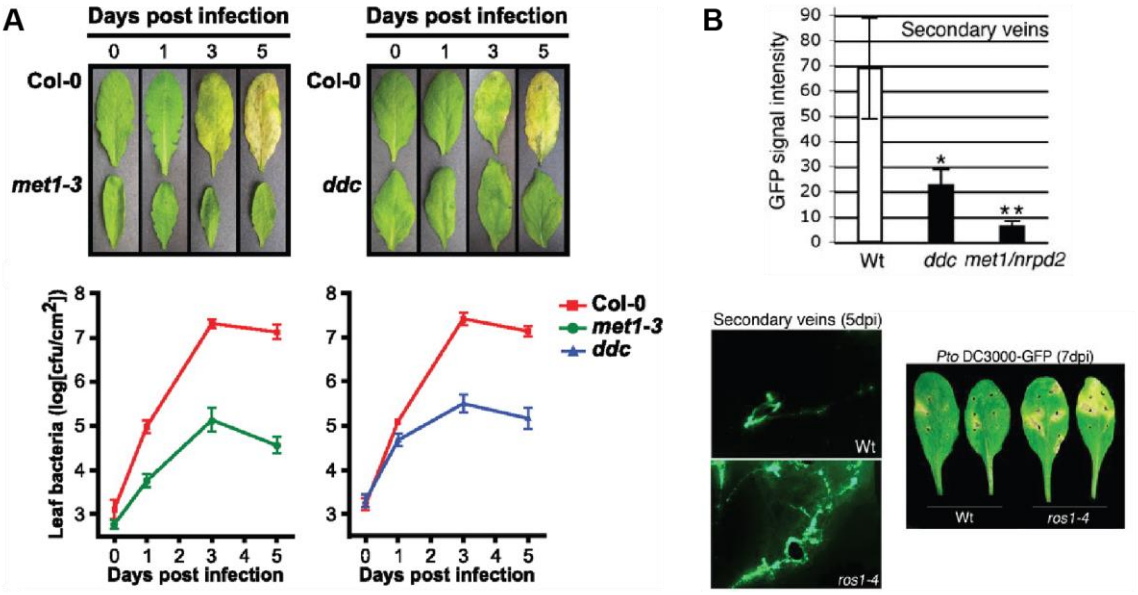


Figure 5: DNA methylation and DNA demethylation mutants have an altered resistance against *Pseudomonas syringae*. (Dowen et al., 2012; Yu et al., 2013). A Mutants defective in CG (*met1-3*) and non-CG methylation (*ddc*) infected with *Pst* DC3000 (from Dowen et al. (2012), Fig. 1). **B** Different DNA methylation mutants (upper panel) and the DNA demethylation mutant *ros1-4* (lower panel) infected with GFP-tagged *Pst* DC3000 (from Yu et al. (2013), Fig. 3).

Yu et al. (2013) infected leaves of the *ddc* triple mutants, double mutants *met1,nrdp2* (heterozygous for *met1*, homozygous for *nrpd2*) and *ros1* mutants with GFP-tagged *Pst* DC3000. The mutants defective in DNA methylation displayed reduced spread of the GFP-tagged bacteria, demethylation defective *ros1* mutant displayed increased bacterial spreading of GFP-tagged *Pst* and enlarged chlorotic and necrotic tissues surrounding the infection site compared to wild type (Figure 5B; Yu et al. (2013)), fitting to the study of Dowen et al. (2012).

Not only in *Arabidopsis thaliana*, but also in *Oryza sativa* the DNA methylation status contributes to plant immunity. Chemically mediated global demethylation of rice genome resulted in increased expression of the resistance gene *Xa21* and enhanced resistance against a pathogenic strain of *Xanthomonas oryzae* (Akimoto et al., 2007). Hypo- and hypermethylation was observed in all sequence contexts and indicates that cytosine can be actively and dynamically methylated and demethylated in response to pathogens (Dowen et al., 2012; Yu et al., 2013).

Apart from DNA methylation, other epigenetic components are also involved in plant immunity. The SWI/SNF chromatin remodeling ATPase SPLAYED regulates the activation of JA- and ET-activated defense genes and contributes to defense against *B. cinerea* (Walley et al., 2008). Moreover, histone H2B ubiquitination, histone acetylation levels and histone methylation were connected to biotic stress defense. Mutants defective in HISTONE MONOUBIQUITINATION 1 (HUB1), encoding an E3 ligase that monoubiquitinates histone H2B, are extremely sensitive towards infections with *A. brassicicola* and *B. cinerea* (Dhawan et al., 2009). HISTONE DEACETYLASE 19 (HDA19) is induced upon wounding, JA, ET precursor and *A. brassicicola* treatment and overexpressing lines and RNAi lines are more resistant and sensitive, respectively (Zhou et al., 2005). The histone methyltransferase SET DOMAIN GROUP 8 (SDG8) is required for defense against pathogenic and nonpathogenic strains of *Pseudomonas syringae* and necrotrophic fungi by activating JA/ET-pathway genes (Berr et al., 2010; Palma et al., 2010; De-La-Peña et al., 2012).

Taken together, different regulatory levels of epigenetic modifications were shown to be involved in plant immunity against biotrophic as well as necrotrophic pathogens.

1.8 Objectives on this work

As indicated in many examples, the interaction between *Pseudomonas syringae* pv. *tomato*, as biotrophic pathogen, and *Arabidopsis thaliana*, as plant model, is well studied. Quite unexplored is the link between necrotrophic pathogens and plants in plant immunity. Therefore, we wondered, which plant molecular components are involved in the defense against necrotrophic pathogens. Small RNAs represented striking candidates as different defense mechanisms are described wherein small RNAs, and especially microRNAs play an important role.

In order to investigate the role of small RNAs in plant defense against necrotrophic pathogens, *Alternaria brassicicola* was chosen as necrotrophic fungi.

(I) To get a first hint if small RNAs are crucial components in plant defense against *A. brassicicola*, we analyzed the microRNA pathway mutant *ago1*. Phenotypic analysis of *ago1* mutants infected with *A. brassicicola* led to the conclusion that small RNAs, and particularly microRNAs are part of the defense mechanism. Small RNA sequencing revealed several microRNA candidates.

(II) Putative miRNA candidates amongst others derived from small RNA sequencing were analyzed using phenotypic pathogen assays and molecular methods.

(III) Besides miRNAs, several studies provided evidence, that dynamic epigenetic modifications especially DNA methylation may be strongly connected with and contribute to defense against necrotrophic pathogens. Hence, we developed a modular, inducible vector system that is based on the Golden Gate cloning technique.

2. Results

2.1 AGO1 is a positive regulator of plant defense response against *Alternaria brassicicola*

The response of plants during an infection with biotrophic pathogens and viruses has been studied quite in detail so far and showed that AGO1 is part of that defense mechanism. AGO1 has been described to be involved in defense against attacks of hemi-/biotrophic pathogens, necrotrophic pathogen as well as viruses (Morel et al., 2002; Qu et al., 2008; Ellendorff et al., 2009; Azevedo et al., 2010; Weiberg et al., 2013). AGO1 was shown to be a positive regulator of defense against *Verticillium dahliae*, a hemibiotrophic fungal pathogen (Ellendorff et al., 2009) and AGO1 itself and AGO1 loaded microRNAs are players in the defense against *Pseudomonas syringae* (Navarro et al., 2006; Li et al., 2010; Zhang et al., 2011a). Controversial results were obtained concerning the involvement of AGO1 in antiviral defense. Morel et al. (2002) showed, that *ago1* mutants are hypersusceptible to infection with the cucumber mosaic virus (CMV). Contrary to these findings, Baumberger and Baulcombe (2005) could not reproduce the formerly shown hypersusceptibility of *ago1* as well as they could not detect any virus-specific siRNAs loaded in AGO1, which supports their observation of an unaltered disease phenotype of the *ago1* mutant. However, little is known about the mechanism during an infection with a necrotrophic pathogen. A first study proved, that AGO1 is involved in defense against necrotrophs as *ago1* mutants have a reduced disease susceptibility towards *Botrytis cinerea* (Weiberg et al., 2013). Therefore, we wondered if and which role AGO1 plays in the resistance against the necrotrophic pathogen *Alternaria brassicicola*.

Two characterized hypomorphic *ago1* mutants were used to analyze the role of AGO1 in defense against necrotrophs. Both *ago1* mutants (*ago1-26* and *ago1-27*) were obtained by ethyl methanesulfonate mutagenesis leading to amino acid exchanges at the C-terminus of the AGO1 protein and are deficient in post-transcriptional gene silencing (Morel et al., 2002). Leaves of wild type, *ago1-26* and *ago1-27* mutants were infected with *A. brassicicola* and the inoculated leaves were classified according to their symptom severity to characterize the progressive infection (Kemmerling et al., 2007).

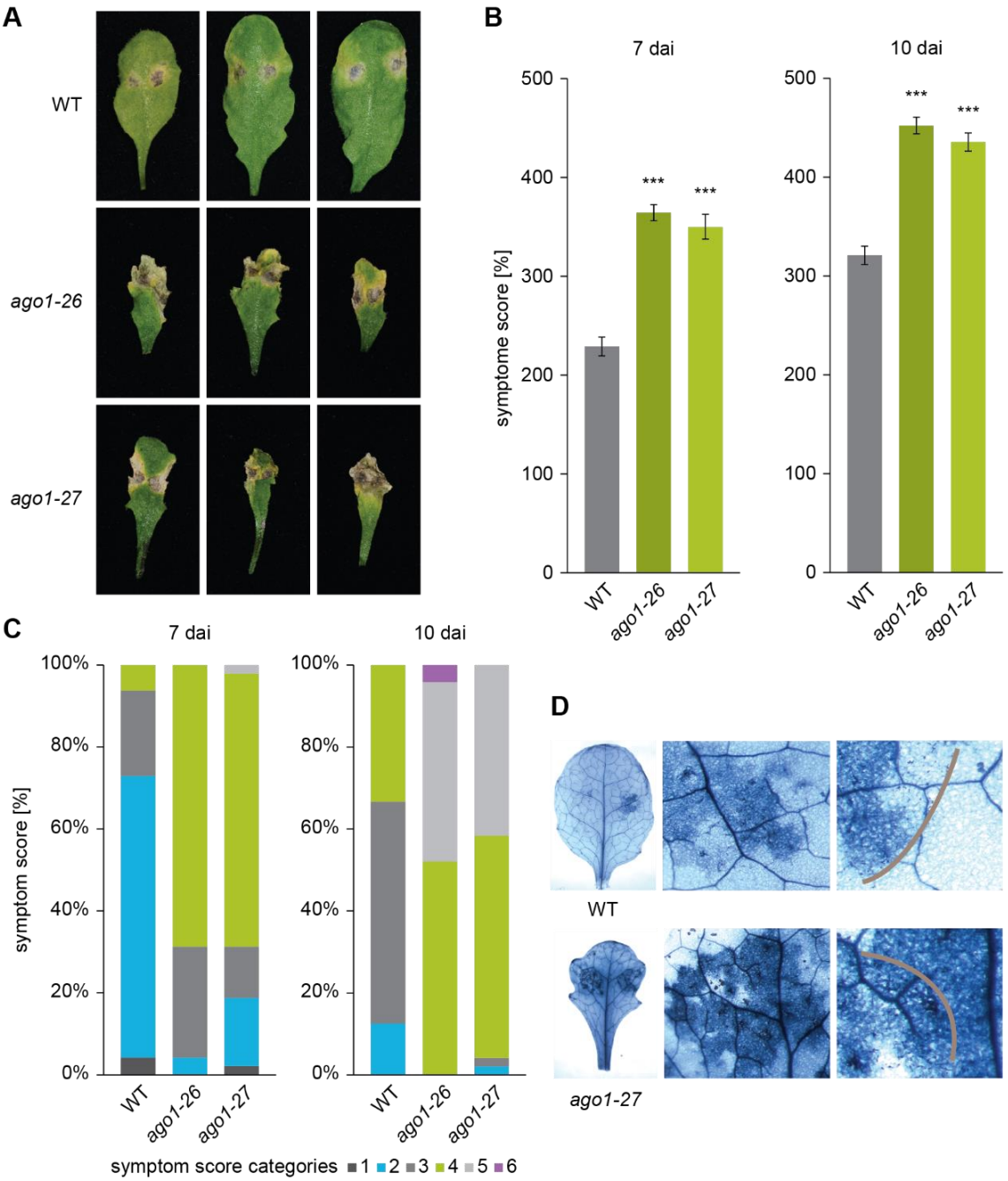


Figure 6: Disease phenotype of *ago1* mutants after *Alternaria brassicicola* inoculation. **A** Representative *A. brassicicola* infected leaves of wild type (WT), *ago1-26* and *ago1-27* are shown 10 days after inoculation. **B** Symptom score of *A. brassicicola* inoculated wild type (WT), *ago1-26* and *ago1-27* 7 and 10 days after inoculation (n = 48). Mann-Whitney U test was performed to show significant differences compared to the wildtype (significance level is indicated with asterisks: * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001). **C** Classification in percent of infected leaves of wild type (WT), and two *ago1* mutants after 7 and 10 days. **D** Trypan blue staining of infected WT and *ago1-27* mutants 4 days after infection.

Leaves of both investigated *ago1* mutants exhibited reduced susceptibility in response to *A. brassicicola* inoculations compared to the wild type. 10 days after inoculation, the *ago1* mutant leaves showed either clearly spreading necrosis or maceration, whereas the wild type displayed only locally infected tissues and beginning spreading events of necrosis (Figure 6A, C). Leaves of *ago1-26* and *ago1-27* inoculated with *Alternaria brassicicola* spores showed a significantly enhanced symptom score compared to wild type at both investigated time points (Figure 6B) indicating an involvement of AGO1 in defense against *A. brassicicola*.

As *ago1-26* and *ago1-27* mutants showed a very similar behavior in response to *A. brassicicola*, we decided to use the hypomorphic allele *ago1-27* for further analysis, which is assumed to be completely deficient in post-transcriptional gene silencing (Morel et al., 2002). To get a clearer picture of the enhanced disease phenotype, leaves of infected plants were stained with trypan blue, a method to visualize lesions of plant tissue and fungal mycelia. Observation of the lesion and the mycelial growth provide hints of either a broken disease resistance or a misregulation of cell death is causal for the susceptibility of *ago1-27* mutants. No difference could be determined in the mycelial growth between wild type and *ago1-27* (Figure 6D). Lesions of infected wild type leaves were restricted to the fungal infection site, whereas the *ago1-27* mutant showed lesions that are spreading beyond the local infection site (Figure 6D) suggesting that *ago1-27* has an altered cell death phenotype. Additionally, uninfected *ago1* mutant leaves showed in general a darker staining than the uninfected wild type tissues in concert with recently published data (Mason et al., 2016). Taken together, *ago1* mutant are clearly more sensitive towards *A. brassicicola* indicating that AGO1 serves as a positive regulator of cell death response.

As AGO1 is the major effector protein for microRNAs in plants (Baumberger and Baulcombe, 2005; Mi et al., 2008), we propose that specific microRNAs could be responsible for the more sensitive phenotype of the *ago1* mutants compared to the wild type. It is known that AGO1 and other microRNA biogenesis factors regulate itself by a negative feedback loop through microRNAs (Rhoades et al., 2002; Xie et al., 2003; Vaucheret et al., 2004; Vaucheret et al., 2006; Meng et al., 2012). To exclude that changes in the abundance of microRNAs were regulated through modification of the microRNA biogenesis machinery, protein levels of several microRNA biogenesis factors and AGO1 were analyzed by immunoblotting (Figure 7). Additionally, AGO2 levels were determined after *A. brassicicola* inoculation (Figure 7B) because AGO2 showed connections to biotic stress defense. AGO2 is induced by viral infections, involved in antiviral defense (Harvey et al., 2011; Wang et al., 2011b; Brosseau and Moffett, 2015) and provides resistance against *Pseudomonas syringae* (Zhang et al., 2011b).

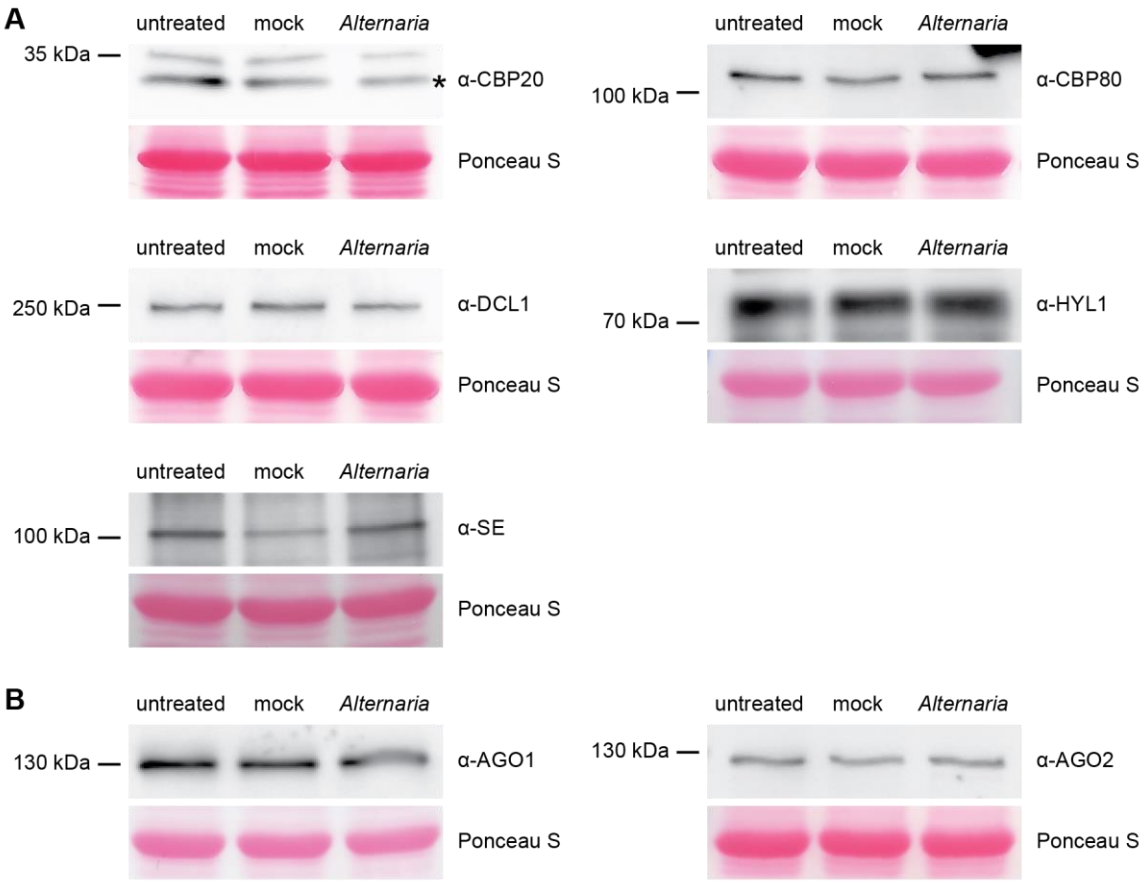


Figure 7: MiRNA biogenesis (A) and sRNA effector (B) factors are unchanged after *Alternaria brassicicola* treatment. Protein levels of various miRNA biogenesis and effector factors were detected by immunodetection of untreated, mock and *Alternaria brassicicola* infected plants after 3 days. One representative immunodetection out of three replicates is depicted. As loading control Ponceau S staining of Rubisco is shown. Asterisk depicts CBP20-specific signal.

None of the tested proteins showed altered protein levels comparing untreated, mock-treated and *A. brassicicola*-treated leaves (Figure 7). This indicated that microRNA biogenesis and effector proteins were unregulated during defense against *A. brassicicola.*

Taken together, *ago1* mutants exhibits a more sensitive phenotype and an altered cell death response towards *A. brassicicola* infection (Figure 6) indicating that AGO1 is a positive regulator of plant defense response and support the hypothesis that specific defense-associated microRNAs are crucial for the AGO1 defense phenotype.

2.2 Identification of *A. brassicicola*-induced microRNAs through RNA sequencing analysis

We proposed that microRNAs are causal for the sensitive phenotype of *ago1* mutants in response to *A. brassicicola* inoculations (Figure 6). For the identification of microRNAs involved in defense against *A. brassicicola*, a sRNA sequencing approach was chosen and two different kinds of sRNA libraries were created.

First, I performed sRNA sequencing to detect changes in microRNA abundance 3 days after *Alternaria brassicicola* inoculation compared to uninfected plants. Second, I cloned and sequenced sRNAs associated with AGO1 3 days after mock and pathogen treatments. For this, AGO1 was co-immunoprecipitated together with its bound RNAs and a small AGO1-immunoprecipitated fraction was controlled with immunoblotting for successful enrichment of AGO1 (Figure 8A). From the residual AGO1-immunoprecipitated fraction, AGO1-bound RNAs were extracted and used as starting material for sRNA library preparation. Final products of both sRNA library were loaded on a sRNA gel and bands of the correct size were extracted (Figure 8B) and prepared for sequencing.

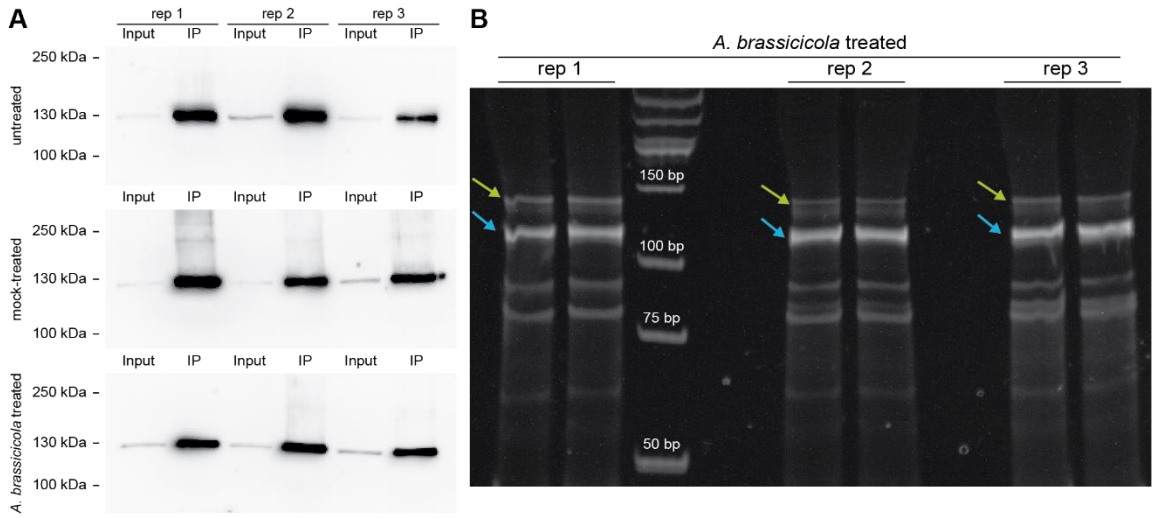


Figure 8: Preparation of the sRNA library from AGO1-coimmunoprecitated small RNAs after *A. brassicicola* treatment. A Immunoblot analysis of AGO1 protein levels of input and AGO1-immunoprecipitated (IP) samples for sRNA library preparation. **B** After adapter ligations and amplification, the final sRNA library was loaded on a TBE gel and stained with SyBr Gold® nucleic acid gel stain. Here, three replicates of AGO1-immunoprecipitated sRNA library from *A. brassicicola* exposed samples are exemplarily shown. DNA fragments of the correct size (140 - 160 bp; green arrows) were used for sequencing, blue arrows depict adapter dimer by-products.

This approach identifies AGO1-loaded endogenous microRNAs that could be causal for the *ago1* resistance phenotype. sRNA libraries were analyzed by Emese X. Szabó (Center for Plant Molecular Biology, University of Tübingen). The differentially expressed miRNAs of sRNA sequencing from total RNA or AGO1-immunoprecipiated RNAs are listed in Table 1 and Table 2, respectively.

Table 1: Differentially expressed microRNAs found in the sRNA library from total RNA (adjusted p-value < 0.1). *Alternaria brassicicola* treated samples were compared to untreated samples.

miRNA	log2 fold change	adjusted p-value
miR163	3.6	2.9E-03
miR169	-2.7	2.3E-06
miR172	1.0	6.9E-02
miR399	1.3	6.9E-02
miR827	2.5	4.1E-06
miR840	2.1	6.8E-02
miR846	2.2	6.8E-02
miR866	3.9	7.3E-03
miR6173	-2.8	2.2E-03
miR8175	-3.8	5.7E-02

Table 2: Differentially expressed microRNAs found in sRNA libraries from AGO1-loaded RNA (adjusted p-value < 0.1). *Alternaria brassicicola* treated samples were compared to untreated samples.

miRNA	log2 fold change	adjusted p-value
miR163	1.2	1.3E-03
miR164	-1.3	6.9E-02
miR399	1.3	1.0E-02
miR827	1.1	1.9E-02
miR846	1.0	3.2E-02
miR5651	1.5	1.4E-03

Analysis of both libraries showed several significant hits that were differentially regulated after *A. brassicicola* treatment (Table 1, Table 2). MiR163, miR399, miR827 and miR846 were significantly changed among the total as well as the AGO1 associated miRNAs. Three the differentially expressed miRNAs, miR163, miR399 and miR827, were upregulated 3 days after exposure to *A. brassicicola*. MiR846 showed a divergent regulation of its expression indicating that miRNA846 is putatively not loaded in AGO1 and is not contributing to the *ago1* mutant sensitivity phenotype.

To prove functional involvement of detected microRNAs, we analyzed in a transcriptome analysis approach, if targets of miR163, miR399 and miR827 were regulated in response to *A. brassicicola* inoculations.

Table 3: Differentially expression analysis of the transcripts corresponding to the identified microRNA hits. (adjusted p-value > 0.1 are marked as not significant (n. s.); NA - no answer).

AGI number	gene name	targeted by	log2 fold change	adjusted p-value
AT1G66690	-	miR163	0.011	n. s.
AT1G66700	*PXMT1*	miR163	5.614	9.9E-29
AT1G66720	-	miR163	-0.299	NA
AT3G44840	-	miR163	0.003	NA
AT3G44860	*FAMT*	miR163	3.131	3.9E-90
AT3G44870	-	miR163	-0.679	NA
AT2G33770	*PHO2*	miR399	0.087	n. s.
AT1G02860	*NLA*	miR827	-0.319	n. s.
AT1G63010	*VPT1*	miR827	0.599	1.9E-07

Analysis of the target gene of the microRNA hits exhibit that indeed also their targets show a different expression after plants were exposed to *A. brassicicola* (Table 3). Two known miRNA163 targets, *FAMT* and *PXMT1*, are induced in the *A. brassicicola* treated samples compared to untreated samples (Ng et al., 2011; Bielewicz et al., 2013). Just as for miR163, also *VPT1*, target of miR827, exhibited increased expression levels while *NLA* did not show a significant altered expression (Hsieh et al., 2009; Pant et al., 2009; Kant et al., 2011; Liu et al., 2015). *PHO2*, target of phosphate-starvation inducible miR399 did not exhibit a significant change in its expression (Sunkar and Zhu, 2004; Allen et al., 2005; Fujii et al., 2005; Bari et al., 2006; Chiou et al., 2006; Lundmark et al., 2010).

Based on the sRNA and mRNA library, two microRNAs (miR163, miR827) caught our interest as the microRNA itself and its targets display altered expression when plant are exposed to *A. brassicicola* and were chosen for subsequent analysis.

2.2.1 *Alternaria brassicicola* does not load sRNA in plant AGO1

It has been shown for *Botrytis cinerea* that during infection the fungus releases fungal sRNAs into the plant host cell acting as effector molecules (Weiberg et al., 2013). Those fungal sRNAs were loaded into the host AGO1 protein and thereby suppress host immunity (Weiberg et al., 2013).

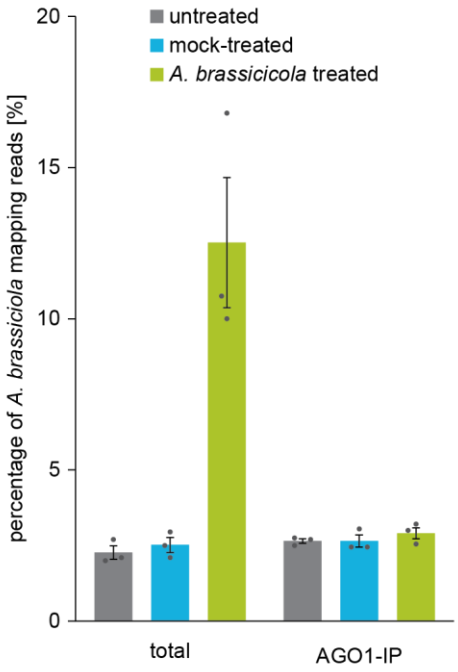


Figure 9: Percentage of reads that do not map to *Arabidopsis thaliana* but could be mapped to the *Alternaria brassicicola* genome. Error bars indicate standard error of biological replicates (n = 3). Single dots depict values of individual replicates.

Using comparative analysis of the two sRNA library, we analyzed if fungal sRNA could depict a commonly used mechanism or just an exception by necrotrophic fungi to overcome host resistance. Therefore, all sRNA reads that map to the *Arabidopsis thaliana* reference genome were excluded. On average, 99.1 % (values range from min. 97.9 - max. 99.6 %) of the sRNA read could be classified as *A. thaliana* derived (data not shown). The residual sRNA reads were then mapped to the *Alternaria brassicicola* genome. As shown in Figure 9, around 2.5 % of the residual reads in the untreated and mock samples map to the *A. brassicicola* genome, but as plants were completely untreated with *A. brassicicola* and treated with mock-solution, respectively, we assume that this percentage displays background levels. Most

likely, the background level is attributed to the analysis process or is derived from soil bacteria and fungi that have at least partially genome sequences similar to *A. brassicicola*.

For the *A. brassicicola* treated samples, a clear increase to ~ 12.5 % in the total sRNA libraries can be seen, what was expected as the harvested inoculated plant leaves were covered with *A. brassicicola* spores and mycelia. In the AGO1-immunoprecipitated *A. brassicicola*-treated libraries the percentage of *A. brassicicola* mapping reads drops to the background levels. This indicates that even if fungal sRNA could be putatively released into the plant host cell and interfere with plant defense, they are not loaded into AGO1 contrary to the situation after an infection with *B. cinerea* (Weiberg et al., 2013).

2.3 MiR163 contributes to resistance against *A. brassicicola*

Analysis of the sRNA sequencing data revealed miR163 to be upregulated after *Alternaria brassicicola* inoculations. MiR163 is a non-conserved microRNA that was suggested to regulate its co-evolved target genes of the SABATH family (Allen et al., 2004; Zhao et al., 2008). SABATH family enzymes were shown to play a role in plant defense (Seo et al., 2001; Chen et al., 2003). Therefore, miR163 is a promising candidate to be involved in plant defense response against *A. brassicicola* and we decided to investigate the function of miR163 in the *Arabidopsis thaliana*/*A. brassicicola* interaction in more detail.

2.3.1 *A. brassicicola* inoculation strongly induces miR163 and influences its splicing pattern

To verify the induction of miR163 shown in the sRNA library data, I analyzed miR163 levels after *A. brassicicola* inoculation by qPCR and small RNA blot analysis, respectively (Figure 10). As expected, the mature miR163 also showed an increase in abundance 3 days after *A. brassicicola* inoculation (Figure 10B). I also investigated the expression pattern of pri-miRNA163. Already one day after infection, I observed a clear induction of pri-miRNA163 expression after *A. brassicicola* treatment, which was further enhanced 3 days after infection (Figure 10A). This result suggests that miR163 could be involved in defense against *A. brassicicola*.

MIR163 is an intron-containing *MIRNA* gene and the intron plays a crucial role in proper miRNA production (Bielewicz et al., 2013; Schwab et al., 2013). Therefore, we tested changes in splicing patterns of pri-miRNA163 by semi-quantitative PCR. RT-PCR using intron-spanning oligonucleotides confirmed the existence of two previously reported splicing forms of pri-miRNA163. A change in the ratio of the two PCR products is clearly visible after *A. brassicicola* treatment (Figure 10C). Quantification of the splicing products with capillary electrophoresis (Agilent 2100 Bioanalyzer) revealed an two-fold increase of the unspliced pri-miR163 isoform after *Alternaria brassicicola* treatment (Figure 10D). It has been proven, that the unspliced form is more efficiently processed into the mature miRNA (Bielewicz et al., 2013; Schwab et al., 2013) and the change of the splicing ratio is thereby positively contributing to enhanced mature miR163 levels. Taken together, these results show that *A. brassicicola*

inoculation induces pri-miRNA163, changes the predominant splicing form and leads to increased levels of the mature miR163.

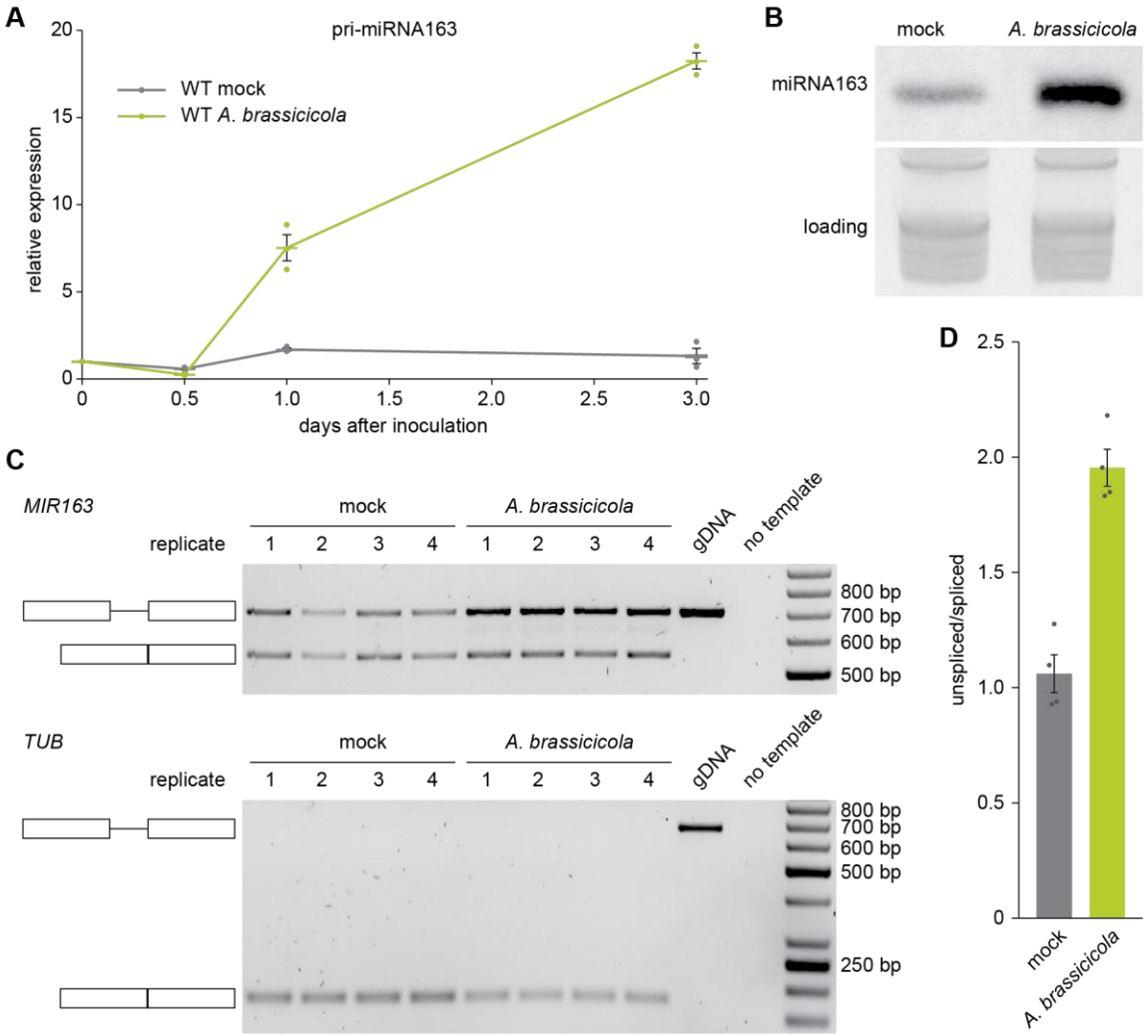


Figure 10: Changes in *MIR163* expression and splicing pattern after *A. brassicicola* inoculation. **A** Relative expression of pri-miRNA163 in a time series (0, 0.5, 1 and 3 dai) of mock (grey) and *A. brassicicola* (green) treated wild type plants. Relative expression values were normalized to actin. Error bars indicate standard error of three biological replicates. Horizontal lines depict mean average; single dots depict values of individual replicates. **B** Detection of mature miR163 by Northern Blotting in wild type in mock and *A. brassicicola* treated plants (3 dai). As loading control ethidium bromide stained gel is shown. **C** Semi-quantitative polymerase chain reaction detecting different splicing forms of MIR163 separated on an agarose gel are shown for four biological replicates 3 dai (upper panel). Lower panel shows detection of an intron-spanning tubulin amplicon. Genomic DNA (gDNA) and a no template reaction was included in the analysis and served as controls. **D** Quantification of the different splicing form shown in C by capillary electrophoresis. Error bars indicate the standard error of four biological replicates. Single dots depict values of individual replicates.

2.3.2 MiR163 buffers the excessive induction of *FAMT* and *PXMT1* in response to *A. brassicicola* inoculation

MiR163 putatively targets genes that encode members of the plant SABATH (salicylic acid/benzoic acid/theobromine) methyltransferase (Zhao et al., 2008). It has been experimentally proven that miR163 is able to negatively regulate *FAMT* and *PXMT1* (Ng et al., 2011; Chung et al., 2016). *FAMT* encodes a farnesoic acid carboxyl methyltransferase that catalyzes the reaction from farnesoic acid (FA) to methyl farnesoate (MeFA) (Yang et al., 2006a). MeFA is hypothesized to function in plant defense against attacks from herbivorous insects (Yang et al., 2006a). Biotic stresses, like herbivory, wounding, and phytohormone treatments induce *FAMT* expression (Chen et al., 2003; Yang et al., 2006a). *PXMT1* encodes for a putative paraxanthine methyltransferase that methylates paraxanthine and is inducible by herbivory (Chen et al., 2003).

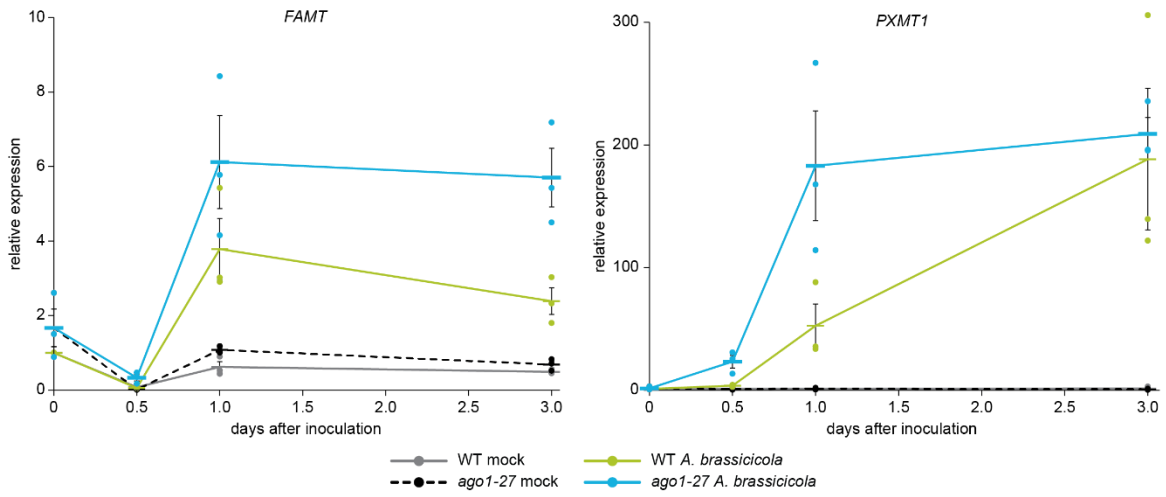


Figure 11: Relative expression level of miR163 targets *FAMT* and *PXMT1* in Col-0 and *ago1-27* mutant. Relative expression values were determined at the indicated time points after inoculation with *A. brassicicola* and were normalized to actin. Horizontal lines depict mean average; single dots depict values of individual replicates. Error bars indicate standard error of three biological replicates.

We tested if the expression of the targets genes *FAMT* and *PXMT1* were influenced by *A. brassicicola* inoculation. Mock treatment of wild type and *ago1*-27 showed no changes in expression (Figure 11). For wild type, an induction of *FAMT* is present already 1 day after infection and drops a little after 3 days (Figure 11). In *ago1-27*, *FAMT* showed an induction 1 dai and stayed at the same level also 3 dai (Figure 11). The second tested target gene *PXMT1*

also responded to *A. brassicicola* treatment. *PXMT1* mRNA levels increased 1 dai and showed a further increase at 3 dai in wild type (Figure 11). In *ago1-27* a slight induction of *PXMT1* can be seen already half a day after infection, after 1 and 3 days a strong increase can be seen in the relative expression level of *PXMT1* (Figure 11). In general, *ago1-27* mutants exhibit a higher (*FAMT*) or faster (*PXMT1*) increase of the miR163 target levels, suggesting that in wild type situation miR163 negatively regulates *FAMT* and *PXMT1* in response to *A. brassicicola* infection. The results obtained in qPCR analysis coincide with analysis of the transcriptome analysis (Table 3), wherein *FAMT* and *PXMT1* were induced upon stress treatment.

As *ago1* and *mir163* mutants have clearly opposite phenotype concerning their disease resistance against *A. brassicicola* (Figure 6, Figure 13), we asked if the target genes of miR163 *FAMT* and *PXMT1* show different responses in the different mutants.

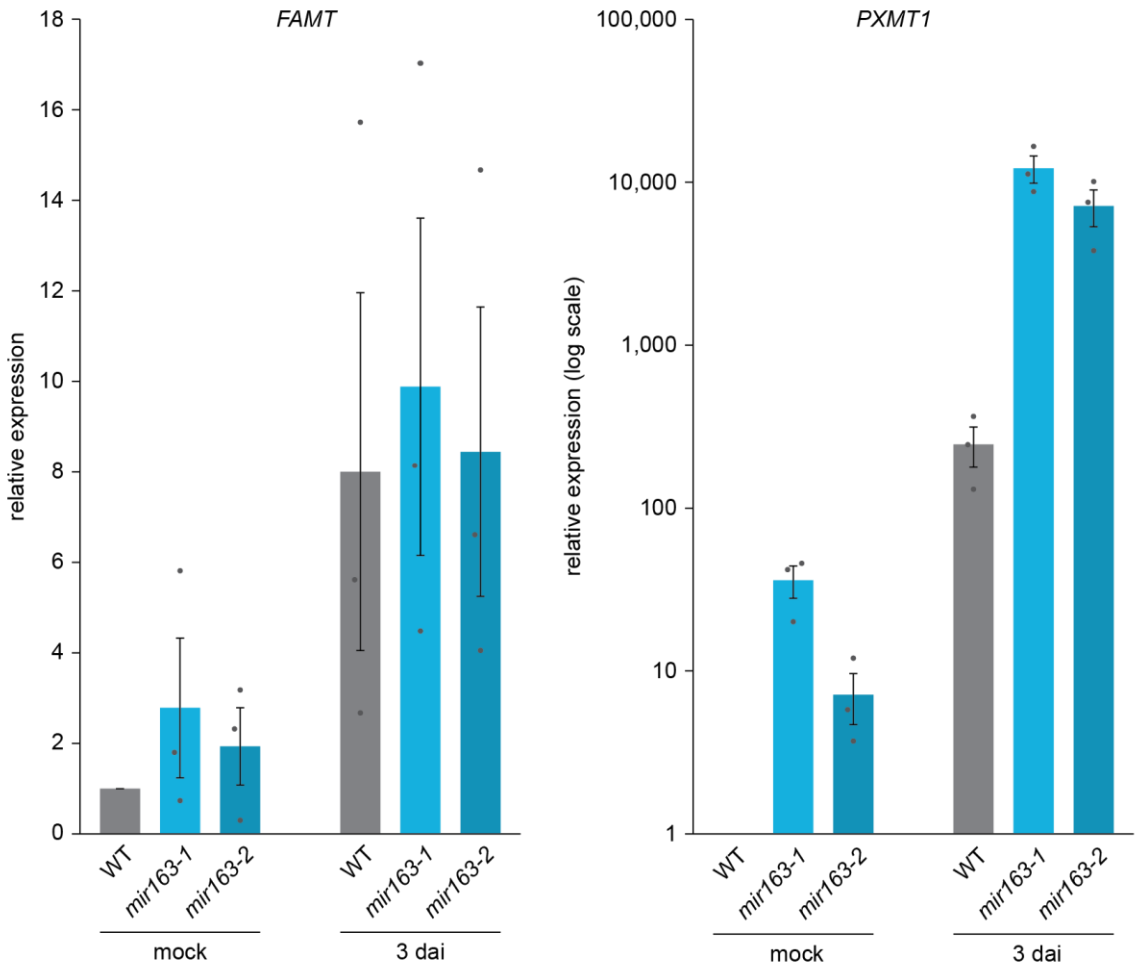


Figure 12: Relative expression level of miR163 targets *FAMT* and *PXMT1* in *mir163* mutants 3 days after inoculation with *A. brassicicola*. Relative expression values were normalized to actin. Error bars indicate standard error of three biological replicates. Single dots depict values of individual replicates.

In *mir163* mutants, *FAMT* was not or only slightly induced under mock-treated conditions compared to wild type (Figure 12). *A. brassicicola* inoculations increased the *FAMT* levels in the wild type in the *mir163* mutants to the same extend (Figure 12) but with a huge variability between the samples indicated by the error bars. *PXMT1* behaves slightly differently than *FAMT* after *A. brassicicola* inoculation. Mock-treated *mir163* mutants exhibits a derepression of *PXMT1* (Figure 12). Both wild type and *mir163* mutants showed an induction of *PXMT1* mRNA levels in response to *A. brassicicola* inoculation. In *mir163* mutants, relative *PXMT1* expression reached higher levels than the wild type after fungal inoculation (Figure 12). This result indicates that miR163 negatively regulates *PXMT1* and buffers its excessive induction as seen in *mir163*. The discrepancy of the behavior in *ago1* and *mir163* mutants after *A. brassicicola* inoculation could be explained by different courses of infections due to biological variance.

All in all miR163 cushions the excessive induction of *FAMT* and *PXMT1* in response to *A. brassicicola* inoculation.

2.3.3 MiR163 is a negative regulator of plant defense against *Alternaria brassicicola*

In order to identify the direct effect of miR163 on defense response, we inoculated two allelic *mir163* mutants with *A. brassicicola* spores under our standard growth conditions. Both mutants have no detectable mature miR163 (Ng et al., 2011; Bielewicz et al., 2013; Schwab et al., 2013) hence displaying null alleles.

7 days after inoculation, most of the inoculated *mir163* leaves had only local and weak infection symptoms compared to the wild type situation (Figure 13A). Comparison of the percentage of the individually scored leaves exhibits a lower score for the *mir163* mutants compared to wild type and 35S:MIR163 plants (Figure 13C). A significant reduction of the symptom score is present in both *mir163* alleles compared to the wild type and 35S:MIR163, showing that both *mir163* mutants are more resistant against the necrotrophic fungus *Alternaria brassicicola* (Figure 13B). This suggests that miR163 is a negative regulator of plant defense response against the necrotrophic pathogen *Alternaria brassicicola*. As miR163 was shown to be light-responsive (Chung et al., 2016), plants were grown under changed light conditions. Under these conditions, a different response to *A. brassicicola* infections was observed (Supplementary Figure SI5).

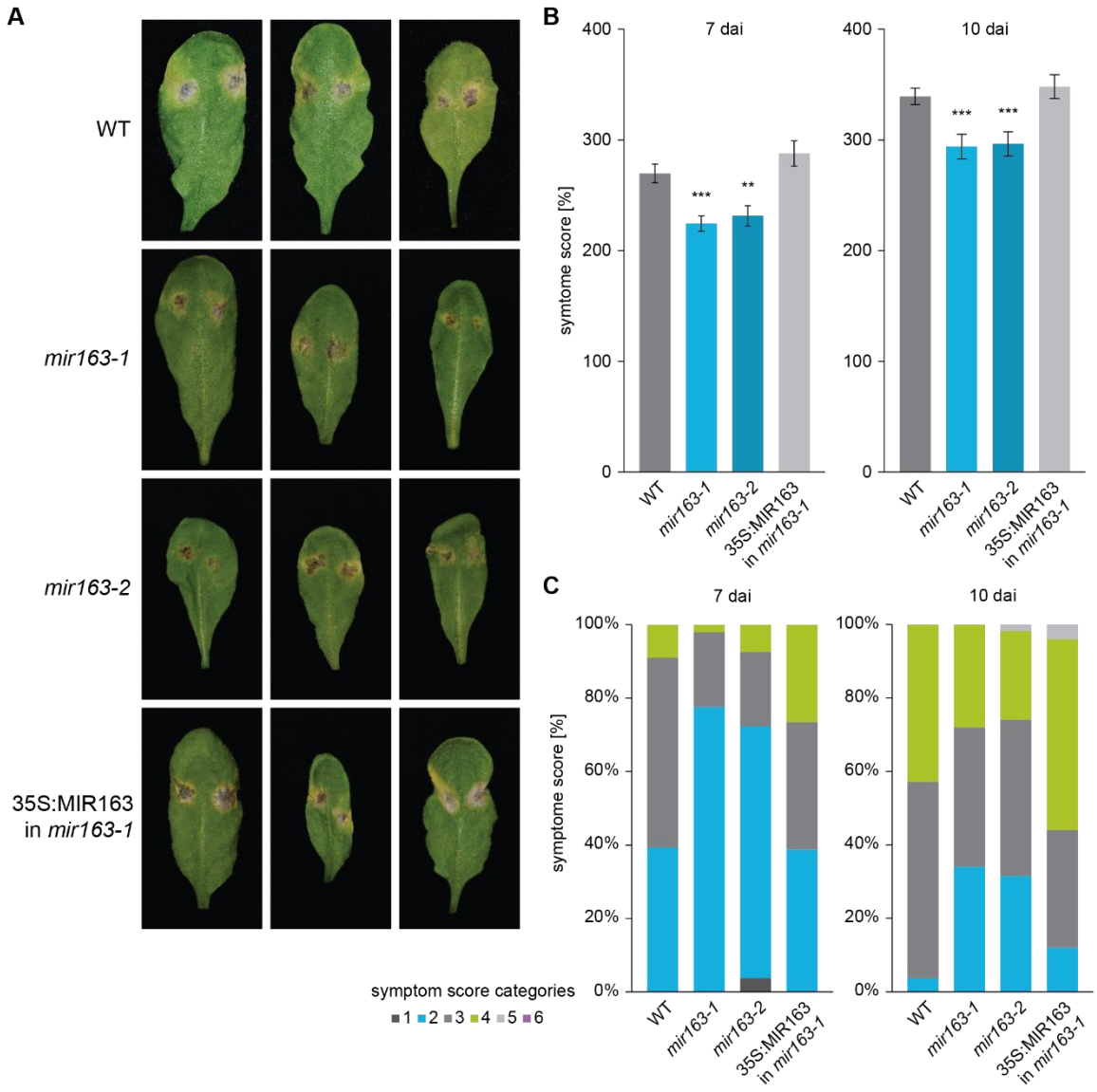


Figure 13: Disease phenotype of *mir163* mutants after *Alternaria brassicicola* inoculation. A Representative *A. brassicicola* infected leaves of wild type (WT), *mir163-1* and *mir163-2* and 35S:MIR163 are shown 10 days after inoculation. **B** Symptom score of *A. brassicicola* inoculated wild type (WT), *mir163-1*, *mir163-2* and 35S:MIR163 7 and 10 days after inoculation (n ≥ 49). Mann-Whitney U test was performed to show significant differences compared to wild type (significance level is indicated with asterisks: * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001). **C** Classification in percent of *A. brassicicola* infected leaves of wild type (WT), *mir163* mutants and 35S:MIR163 after 7 and 10 days (n ≥ 49).

2.3.4 MiR163 is not a general defense factor against necrotrophic pathogens

It has been already shown, that miR163 is induced by treatment with alamethicin, a fungal elicitor (Ng et al., 2011), as well as with the hemibiotrophic bacterium *Pseudomonas syringae* DC3000 (Bielewicz et al., 2013). Additionally, pri-miR163 and miR163 was clearly upregulated 3 days after inoculation with *Alternaria brassicicola* (Table 1, Table 2, Figure 10A, B) showing that various kind of biotic stresses induce *MIR163*. This is supported by *MIR163* promoter analysis that revealed several stress responsive elements (Ng et al., 2011).

To investigate if miR163 is specific for the defense against the necrotrophic fungi *Alternaria brassicicola* or is more broadly involved in defense against necrotrophs, *B. cinerea* infections of *mir163* mutants were examined. By qPCR, the transcript levels of miR163, *FAMT* and *PXTM1* were determined. All three depicted a clear induction 2 days after infection (Figure 14C, D) showing that all are responsive to infection with *B. cinerea*.

If fungal infections share a common set of plant defense genes and pathways that include miR163, we assumed that *mir163* mutants are also more resistant against *Botrytis cinerea*. To test this hypothesis, we infected leaves of *mir163* mutants with the necrotrophic fungus *Botrytis cinerea* and determined disease index on basis of the expanding lesion size. Surprisingly, inoculated leaves of wild type, *mir163* mutants and 35S:MIR163 showed the same level of disease symptoms 2 days after inoculation (Figure 14A, B) demonstrating that miRNA163 does not display a factor involved in defense against *B. cinerea*.

Despite the fact that miR163, *PXMT1* and *FAMT* were induced upon *B. cinerea* inoculations; they do not contribute to plant defense against the second tested necrotrophic pathogen (Figure 14). This suggested that miR163 is involved in defense against *A. brassicicola* but not against *Botrytis cinerea*.

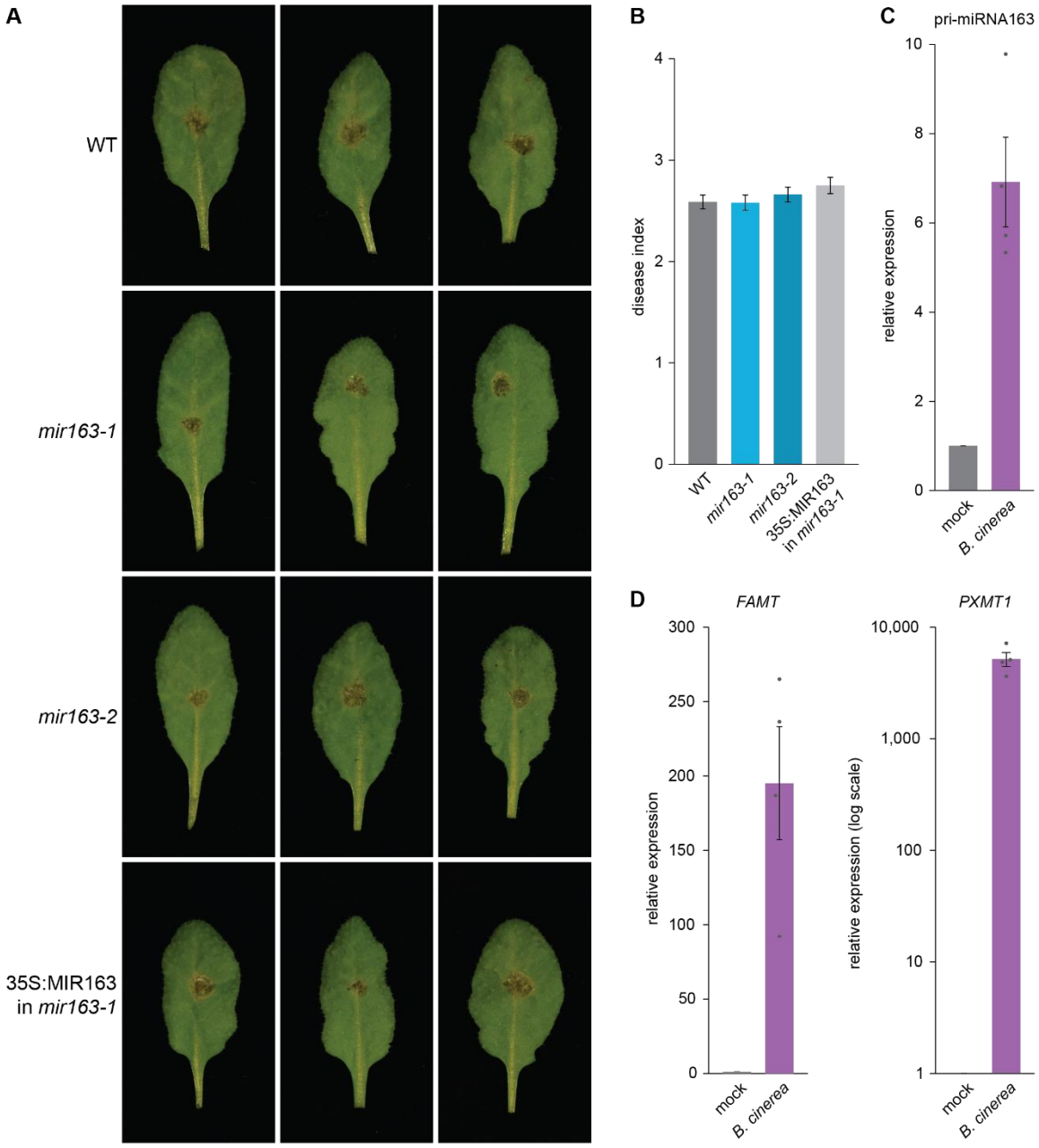


Figure 14: Function of miR163 in defense against *Botrytis cinerea*. A Representative *Botrytis cinerea* infected leaves of wild type (WT), *mir163-1*, *mir163-2* and 35S:MIR163 are shown 2 days after inoculation. **B** Symptom score of *Botrytis cinerea* inoculated wild type (WT), *mir163-1*, *mir163-2* and 35S:MIR163 2 days after inoculation. Mann-Whitney U test showed no significant difference between the different lines ($n \geq 100$). **C** Relative expression level of pri-miRNA163, **D** *FAMT* and *PXMT1* 2 days after inoculation with *B. cinerea* are shown. Relative expression values were normalized to actin. Error bars indicate standard error of four biological replicates. Single dots depict values of individual replicates.

2.4 Role of the phosphate-starvation induced miR827 in plant defense

Small RNA library analysis revealed that miR827 was upregulated after *A. brassicicola* inoculation. MiR827 is a microRNA discovered by a small RNA sequencing approach (Rajagopalan et al., 2006) and was described to be induced upon phosphate starvation and to negatively regulate its target gene *NITROGEN LIMITATION ADAPTATION (NLA)* (Hsieh et al., 2009; Pant et al., 2009; Lundmark et al., 2010; Kant et al., 2011). *NLA* encodes an E3 ubiquitin ligase that is involved in the regulation of phosphate transport through direct or indirect influence on *PHT1.1, PHT1.4* and *PHF1* (Kant et al., 2011; Lin et al., 2013; Park et al., 2014). As second target of miR827 *VPT1* was proposed, which seems to be not responsive to phosphate deprivation (Hsieh et al., 2009) but induced upon phosphate excess (Liu et al., 2015).

To verify sRNA library data, pri-miRNA827 levels were determined via qPCR showing an enhanced pri-miRNA827 expression 3 days after inoculation with *A. brassicicola* (Figure 15A). Under unstressed conditions, *NLA* and *VPT1* displayed unaltered expression levels in *ago1-27* mutants compared to wild type (Figure 15A). After *A. brassicicola* inoculation of wild type and *ago1-27*, *NLA* showed no induction, which suggests that *NLA* is not responsive to *A. brassicicola*. For *VPT1*, there was an increase of expression in wild type and an even higher increase of expression in the *ago1-27* mutant, suggesting that miR827 targets *VPT1* in wild type background after *A. brassicicola* inoculation and lowers its expression. Those findings of *NLA* and *VPT1* regulation match the transcriptome data, showing no induction for *NLA* and a slight but significant induction for *VPT1* (Table 3).

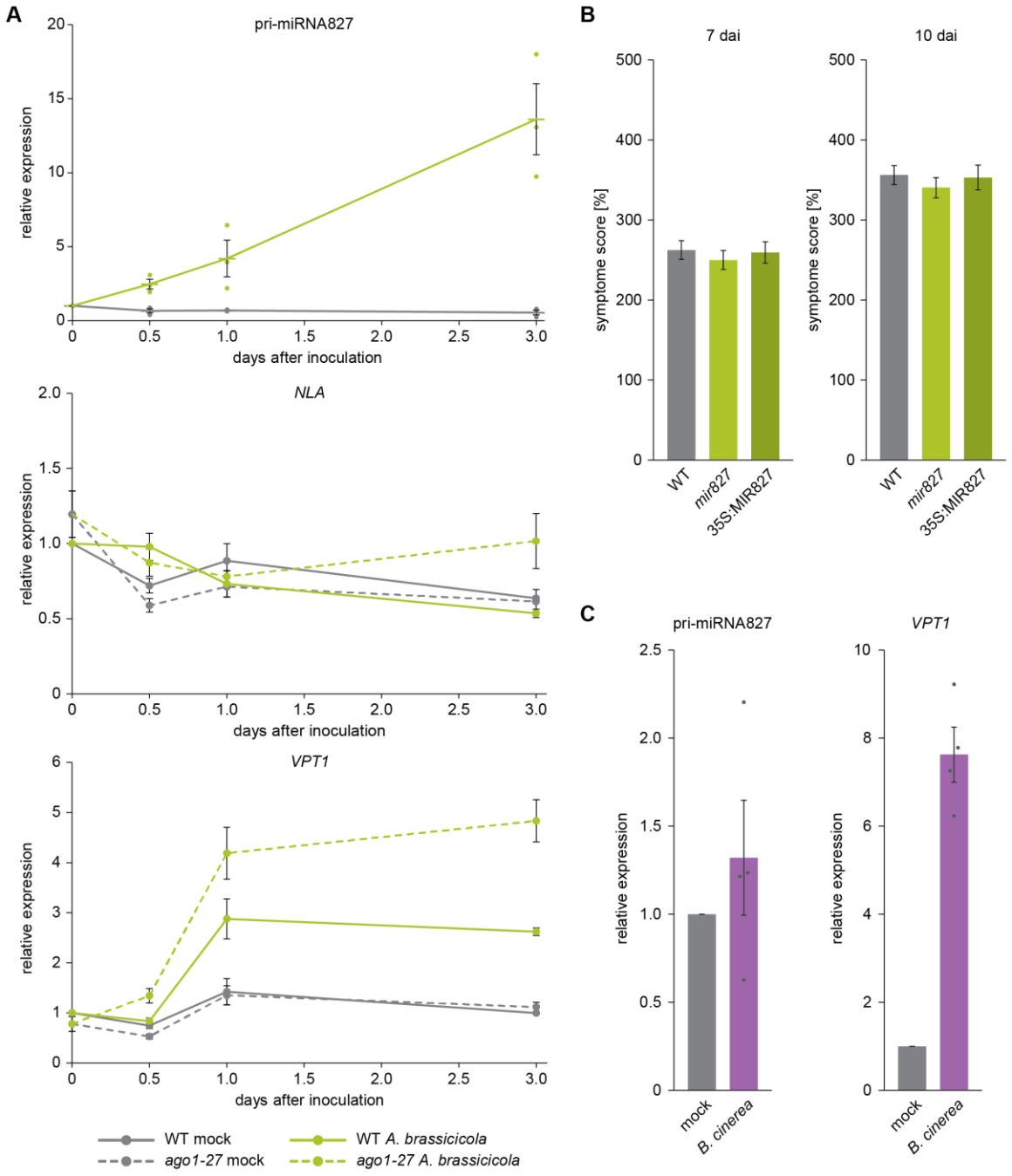


Figure 15: Involvement of miR872 in antifungal defense. A Relative expression level of pri-miRNA827 in wild type (WT) and the miRNA827 targets *NLA* and *VPT1* in wild type (WT) and *ago1-27* mutants 0, 0.5, 1 and 3 days after inoculation with *A. brassicicola*. Relative expression values were normalized to actin. Error bars indicate standard error of three biological replicates. For pri-miRNA827 horizontal lines depict mean average and single dots depict values of individual replicates. Individual replicates for *NLA* and *VPT1* are not depicted for clarity but are annotated in Supplementary Table SI11 and Supplementary Table SI12, respectively. **B** Symptom score of *A. brassicicola* inoculated wild type (WT), *mir827* mutant and overexpressing line 35S:MIR827 7 and 10 days after inoculation (n = 32). Mann-Whitney U test showed no significant difference between the different lines. **C** Relative expression level of pri-miRNA827 and *VPT1* in wild type 2 days after inoculation with *B. cinerea*. Relative expression values were normalized to actin. Error bars indicate standard error of four biological replicates. Single dots depict values of individual replicates.

To address the question, if miR827 actively contributes to plant resistance, a *mir827* mutant as well as a MIR827-overexpressing line were inoculated with *A. brassicicola* and disease symptoms were scored. Both lines behaved exactly like wild type 7 and 10 days after inoculation (Figure 15B). This suggested that miR827 does not contribute to disease resistance against *Alternaria brassicicola* or that the disturbed levels of miR827, which is tightly linked to a complex phosphate response network, are not sufficient to modify disease resistance.

As pri-miRNA827 and *VPT1* showed increased mRNA levels with *A. brassicicola*, we tested if they are also inducible by *B. cinerea*. Interestingly, pri-miRNA827 levels showed no increased expression levels 2 days after inoculation with *B. cinerea*, but *VPT1* was strongly induced (Figure 15C). This indicates that miR827 is specifically induced in antifungal defense against *A. brassicicola* but not *B. cinerea* whereas *VPT1* is induced by both fungi and could potentially contribute to general antifungal defense mechanisms.

2.5 MiR398 does not contribute to *A. brassicicola* resistance of *A. thaliana*

MiR398 was discovered in 2004 (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004) and is responsive to various kinds of abiotic and biotic stresses (Jagadeeswaran et al., 2009). MiR398 is down-regulated upon elicitor treatments (flg22) as well as upon treatments with non-pathogenic, virulent and avirulent *Pst* (Jagadeeswaran et al., 2009; Li et al., 2010; Zhang et al., 2011a). Plants overexpressing miR398 have an enhanced susceptibility to both *Pst* DC3000 and the non-pathogenic strain *hrcC*- and show defects in callose deposition in response to flg22 and *Pst hrcC*- treatment (Li et al., 2010). Furthermore, it was shown, that downregulation of miR398 in response to biotic stress leads to an increase of target mRNA levels (Jagadeeswaran et al., 2009; Li et al., 2010). Targets of miR398 are the *COPPER/ZINC DISMUTASE 1 (CSD1), CSD2, COPPER CHAPERONE OF SUPEROXIDE DISMUTASE (CCS)* (Sunkar et al., 2006; Beauclair et al., 2010). CSD1, CSD2 and CCS are enzymes putatively involved in the detoxification in reactive oxygen species (ROS), whose production is one of the first defense mechanisms that are activated while pathogen infection that leads inter alia to activation of the hypersensitive response (HR) (Zurbriggen et al., 2010; Baxter et al., 2014). ROS production and HR have been shown to enhance susceptibility to necrotrophic pathogens (Govrin and Levine, 2000; Torres et al., 2006; Heller and Tudzynski, 2011). As the *ago1-27* mutant inoculated with *A. brassicicola* showed an enhanced cell death response (Figure 6D) and *ago1-27* was described to develop lesions similar to HR-mediated cell death coupled with local ROS production (Mason et al., 2016), we wondered if miR398 as regulator of ROS detoxification plays a role on the defense regulation. Therefore, we tested miR398, *CSD1, CSD2* and *CCS* levels in *A. brassicicola* infected WT and *ago1-27* plants.

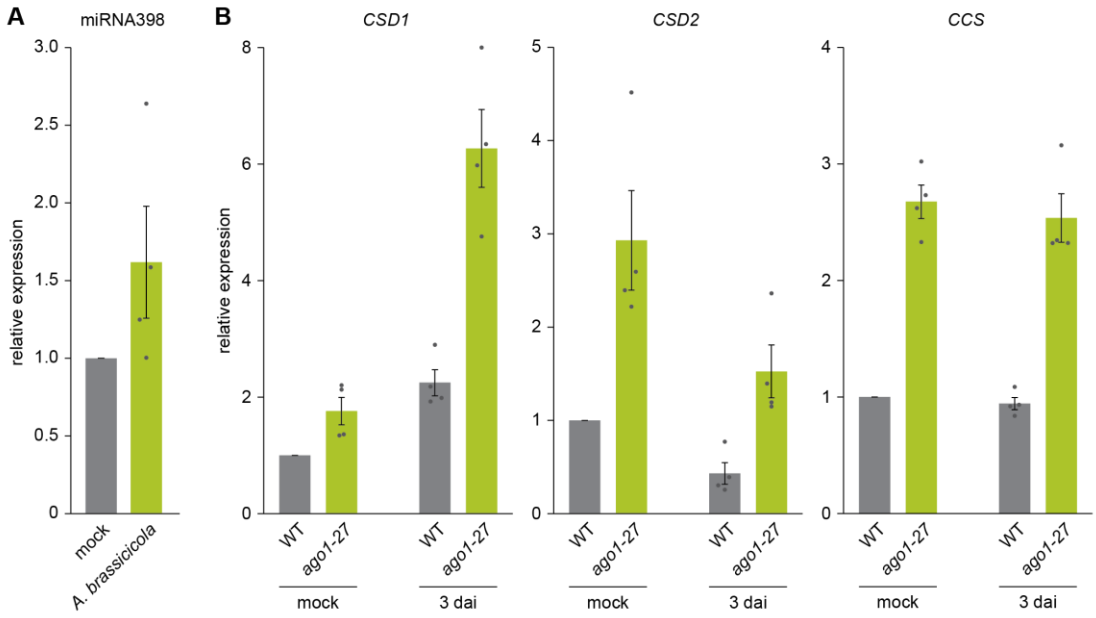


Figure 16: Relative expression levels of miR398 and its targets *CSD1, CSD2* and *CCS*. Relative expression levels of **A** miR398 in wild type (WT) and **B** the miR398 targets *CSD1*, *CSD2* and *CCS* in wild type (WT) and *ago1-27* mutants 3 days after inoculation with *A. brassicicola*. Relative expression values were analyzed were normalized to actin. Error bars indicate standard error of four biological replicates. Single dots depict values of individual replicates.

As shown in Figure 16A, miR398 levels were not changed in WT after *A. brassicicola* inoculation. The target mRNAs *CSD2* and *CCS* are upregulated, while *CSD1* mRNA is unchanged in the *ago1-27* mutant under unstressed conditions compared to the wild type. *A. brassicicola* inoculation did not alter the relative expression level of *CSD1*, *CSD2* and *CCS* in wild type (Figure 16B). Except for *CSD1*, no clear induction or repression of the miR398 targets were exhibited by the *A. brassicicola* treatment in the *ago1-27* mutants (Figure 16B).

Taken together, *A. brassicicola* inoculation did not significantly alter neither miR398 nor the targets *CSD1*, *CSD2* and *CCS*. This result indicates that miR398 is not involved in defense against *Alternaria brassicicola*.

2.6 Screen for microRNAs that contribute to plant defense using a short tandem target mimicry approach

To test our hypothesis that lack of microRNA action is crucial for the *ago1* disease phenotype, we tested putative candidate microRNAs by a short tandem target mimicry (STTM) approach; some of them have been described previously to be responsive to biotic stress (Fahlgren et al., 2007; Li et al., 2010; Zhang et al., 2011a; Sunkar et al., 2012). STTMs are characterized by two microRNA binding sites that are linked by an AT-rich spacer (Yan et al., 2012a). A trinucleotide bulge between the position 10 and 11 of the mature miRNA results in non-cleavable microRNA binding sites. A scheme of a STTM on the basis of miR393 is depicted in Figure 17.

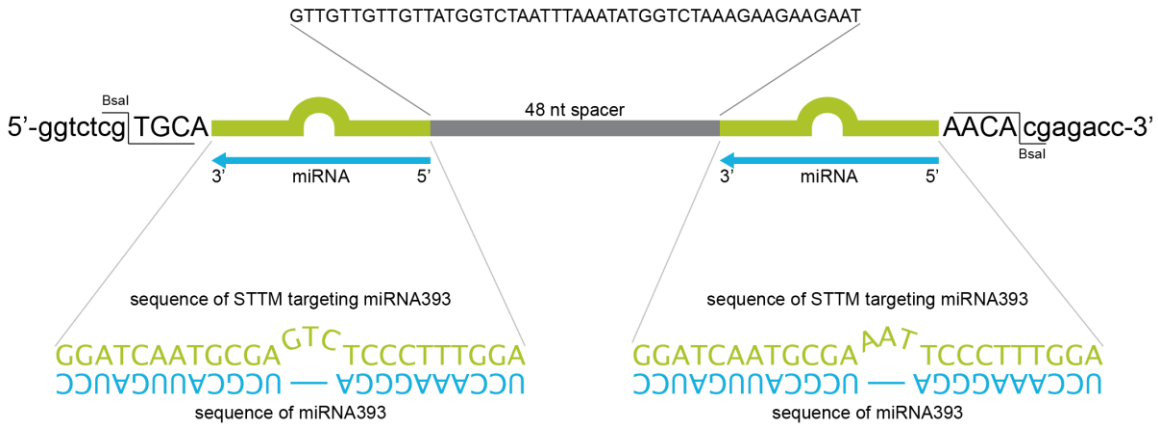


Figure 17: Scheme of STTM393 construct. The STTM393 contains two miRNA393 target sequences (green) that flank the 48 nucleotide spacer region (grey). The miRNA393 target sequences are complementary to miRNA393 sequence (blue) and are designed with a 3 nt bulge resulting in a non-cleavable microRNA binding site. Optionally, BsaI restriction sites (here compatible as module 6) can be added at the 5'- and 3'-end of the STTM construct.

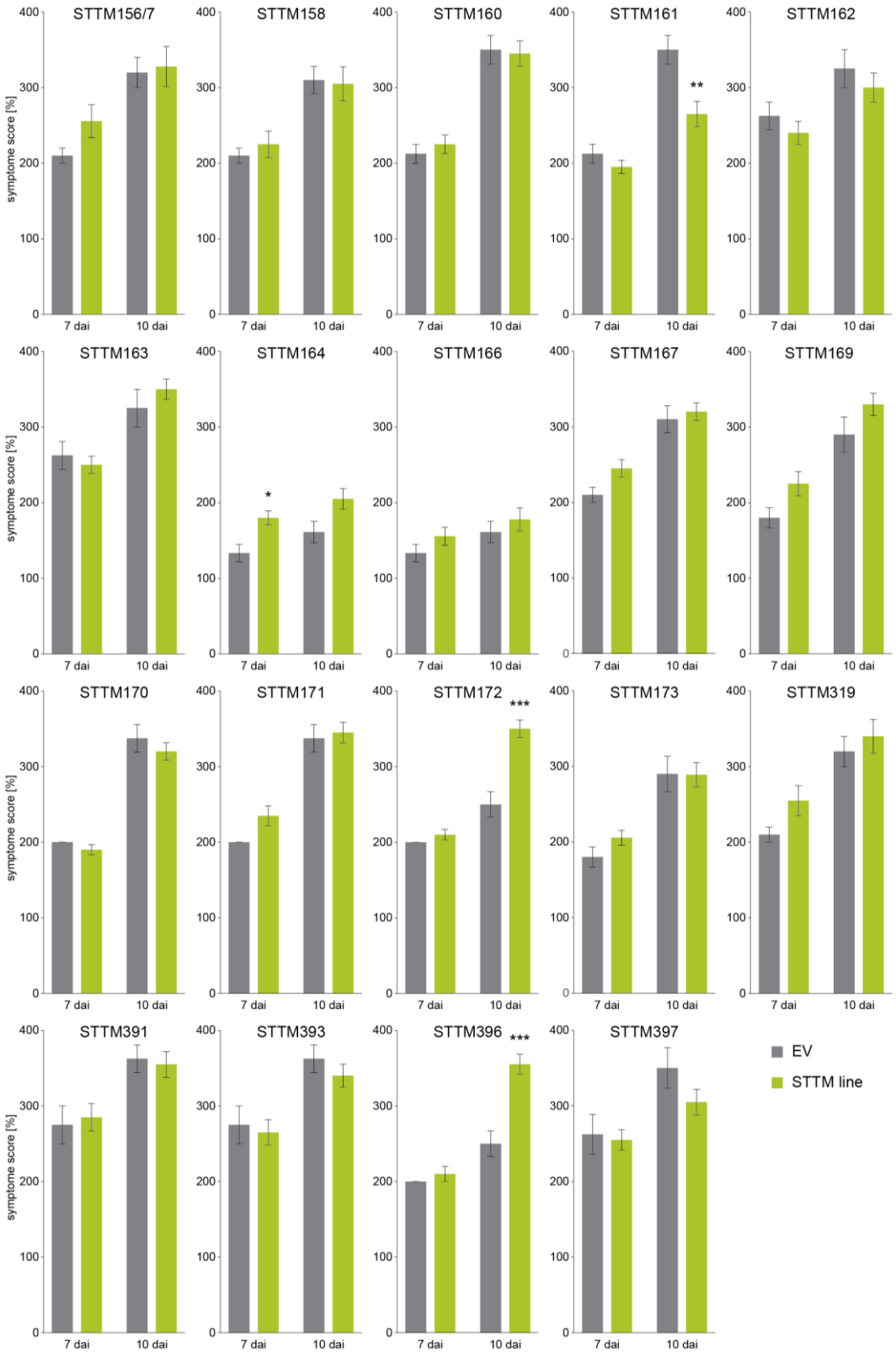


Figure 18: Symptom score of the STTM lines. *A. brassicicola* inoculated plants harboring an empty vector controls (EV) or the indicated STTM construct were scored 7 and 10 dai (n = 8 for EV, n = 18 for STTM). Mann-Whitney U test was performed to show significant differences (significance level is indicated with asterisks: * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001).

It is assumed, that STTMs leads mainly to degradation of the targeted miRNA through the exonucleases SMALL RNA DEGRADING NUCLEASE 1 (SDN1) and SDN2 (Yan et al., 2012a) leading to derepression of the microRNA target genes. Created STTM lines were screened in T1 generation for their resistance against *A. brassicicola*. Unexpectedly, none of the tested STTM lines showed a significant change in resistance 7 as well as 10 dai (Figure 18). In addition, none of the STTM lines in T1 generation showed expected developmental defects (Yan et al., 2012a) and the STTM163 line exhibited no altered resistance in response to *A. brassicicola*. As the STTM163 line is supposed to mimic a miR163 knockdown, a similar response after *A. brassicicola* inoculation of the STTM163 was suspected as described in Figure 13 for the *mir163* mutants.

Therefore, we tested if the STTM approach leads to a successful targeting of the corresponding microRNA what should lead to an upregulation of the microRNA target mRNA levels. By qPCR, the targeted mRNA levels were determined in pooled leaves of at least 7 independent T1 lines (Figure 19). For STTM160, *ARF16* relative expression level was unchanged while *ARF17* was clearly upregulated in the STTM160 lines (Figure 19). The *NAC* mRNA levels in the STTM164 line showed no difference compared to the empty vector control (Figure 19). For STTM156/7 and the STTM163 line, a slight downregulation of the target mRNAs were observed (Figure 19). These results indicate that some of the generated STTMs are not functional. This could explain the different behavior of the STTM163 line compared to the *mir163* mutants (Figure 13, Figure 19). In STTM167, STTM172 and STTM319, the target mRNA levels are upregulated suggesting that those STTMs are functional *in planta* (Figure 19).

Taken together, using the STTM approach to mimic miRNA mutants, no further microRNA that are potentially involved in *A. brassicicola* defense could be identified.

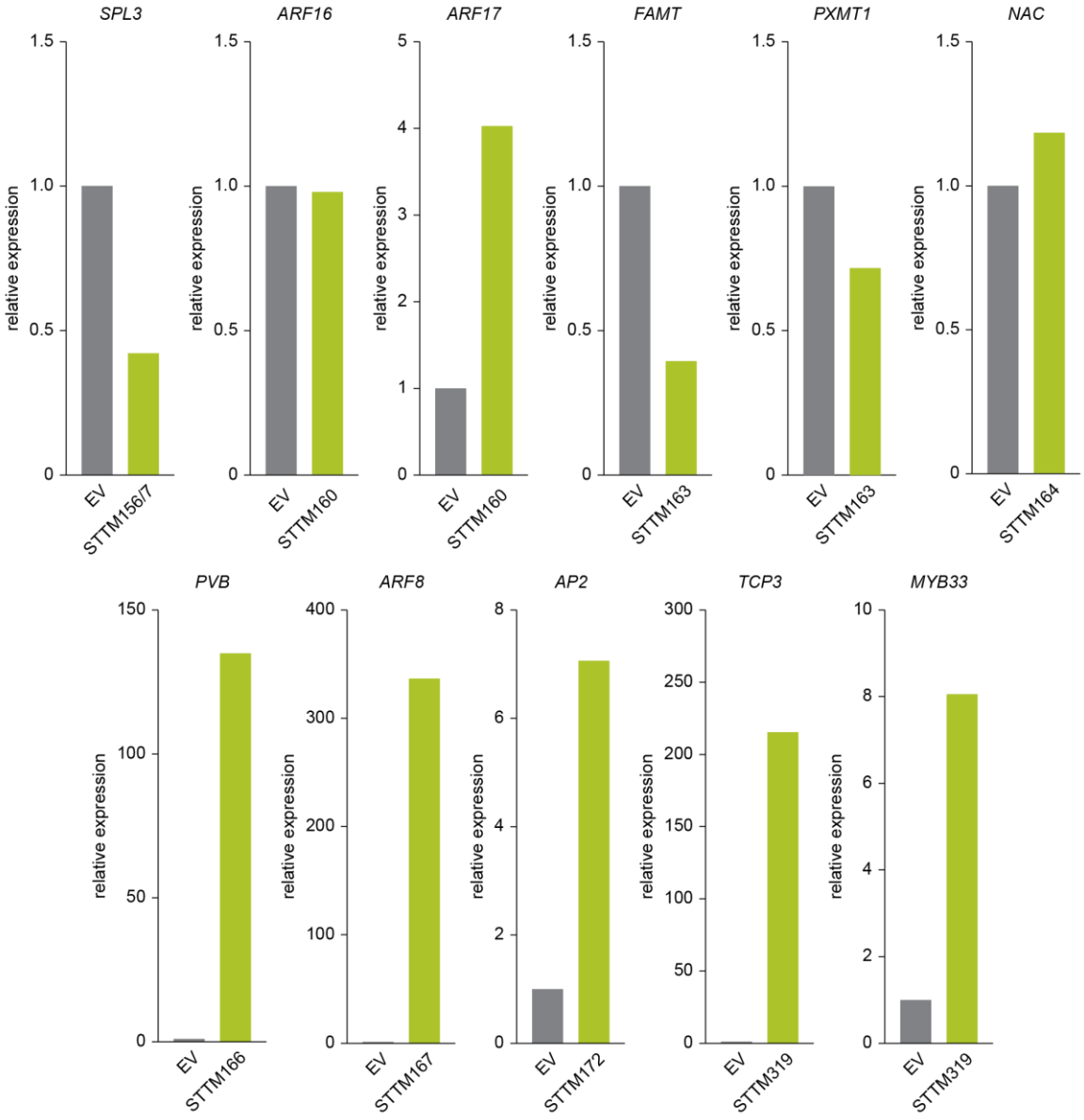


Figure 19: Several STTMs lines were tested to prove successful targeting of the corresponding microRNA by derepression of miRNA target mRNA. For the qPCR analysis, at least seven leaves of individual STTM lines in T1 were pooled.

2.7 Construction of an inducible and easy to clone tool to study involvement of DNA methylation in plant pathogen defense

Epigenetic mechanisms including DNA methylation has been suggested to play a role in plant defense response (Dowen et al., 2012; Yu et al., 2013; Ding and Wang, 2015). To study the role of DNA methylation in response to stress, an inducible vector system was developed to knock-down DNA methylation genes by amiRNAs.

Therefore, we used the pOp6/LhGR system (Craft et al., 2005; Samalova et al., 2005) and combined it with Golden Gate assembly (Engler et al., 2008; Engler et al., 2009). The pOp6/LhGR system consists of two modules: First, a chimeric LhGR composed of the ligand binding domain of a glucocorticoid receptor (GR) that was fused N-terminal to the synthetic transcription factor LhG4 (Moore et al., 1998; Craft et al., 2005). Hence, the LhG4 is controlled by the steroid-inducible, ligand binding GR domain. Second, an orientation-independent promoter pOp6 composed of 6 *lac* operators spaced by direct repeats flanked by two divergent 35S minimal promoters and translation enhancer sequence (Craft et al., 2005).

The pOp6/LhGR system was shown to fulfill several desired criteria for an inducible system: (I) It has an undetectable basal activity. (II) It is highly sensitive to dexamethasone treatment and therefore treatment does not interfere with plant development or physiology. (III) It allows spatial and temporal control through the combination of tissue-specific promoters and the application of dexamethasone. (IV) It can be induced locally or systemically. (V) It is effective in different plant species (Craft et al., 2005; Samalova et al., 2005).

The Golden Gate cloning system is based on type IIS restriction enzymes, that cleave DNA downstream of the recognition site and produce overhangs of 4 nucleotides (Engler et al., 2008; Engler et al., 2009). For the modular cloning, we used BsaI, the most commonly used type IIS restriction endonuclease. BsaI cleaves downstream of its recognition site and allows to determine the sequence of the 4 nt overhang as sticky ends for following cloning procedures and correct assembly of many modules in one reaction. Single module representing building blocks for the assembly are listed in Table 4.

Table 4: Single modules available for the dexamethasone inducible system.

Module	Vector	Insert
1	pCR®8/GW/TOPO®	35S promoter
2	pCR®8/GW/TOPO®	LhGR + terminator
3	pCR®8/GW/TOPO®	nos terminator
4	pCR®8/GW/TOPO®	amiR-GFP[1]
4	pCR®8/GW/TOPO®	amiR-Luciferase[1]
4	pCR®8/GW/TOPO®	eYFP
4	pCR®8/GW/TOPO®	GUS
4	pCR®8/GW/TOPO®	LUC
5	pUC57	pOp6 bidirectional promoter
6	pCR®8/GW/TOPO®	amiR-CMT3-1
6	pCR®8/GW/TOPO®	amiR-CMT3-2
6	pCR®8/GW/TOPO®	amiR-CMT3-endo
6	pCR®8/GW/TOPO®	amiR-DRM1-1
6	pCR®8/GW/TOPO®	amiR-DRM1-2
6	pCR®8/GW/TOPO®	amiR-DRM2-1
6	pCR®8/GW/TOPO®	amiR-DRM2-2
6	pCR®8/GW/TOPO®	amiR-ft-2
6	pCR®8/GW/TOPO®	amiR-GFP[1]
6	pCR®8/GW/TOPO®	amiR-lfy-1
6	pCR®8/GW/TOPO®	amiR-Luciferase[1]
6	pCR®8/GW/TOPO®	amiR-MET1-1
6	pCR®8/GW/TOPO®	amiR-MET1-2
6	pCR®8/GW/TOPO®	amiR-MET1-3
6	pCR®8/GW/TOPO®	amiR-trichome
6	pCR®8/GW/TOPO®	amiR-white-2
6	pCR®8/GW/TOPO®	eYFP
6	pCR®8/GW/TOPO®	GUS
6	pCR®8/GW/TOPO®	LUC
7	pCR®8/GW/TOPO®	ocs terminator
8	pENTR™3C	lacZ[2]

[1] cloned by Dr. C. Speth [2] cloned by M. Ohmer

A model of the Golden Gate assembled vector expression cassette is shown in Figure 20. The LhGR is constitutively expressed under the control of the 35S promoter. After dexamethasone treatment, LhGR is released from the complex with the HSP90 chaperone protein and is able to move from the cytosol into the nucleus. Nuclear LhGR binds to the pOp6 promoter and acts as transcriptional activator of both pOp6 driven genes.

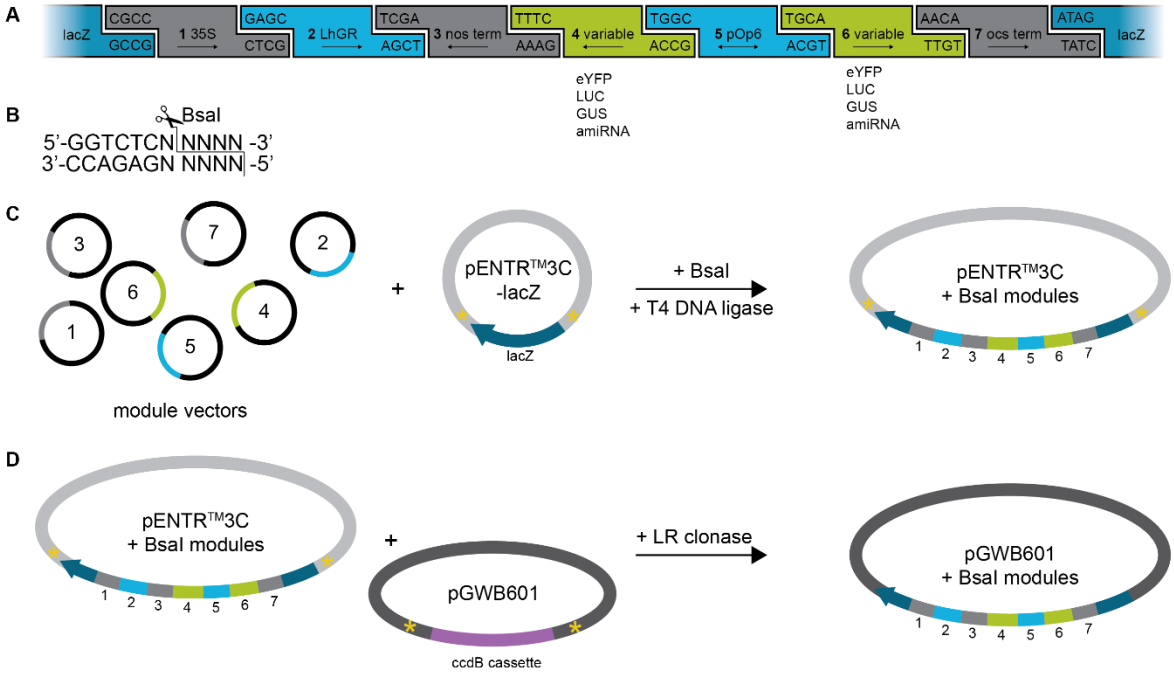


Figure 20: Schematic overview over Golden Gate assembly and modular structure of the dexamethasone-inducible system. A Assembled module of the designed system at its 4 nt overhangs. Insertion into the lacZ gene disrupting its functionality and allows negative blue-white selection. **B** Restriction sequence of BsaI; N stands for any base. **C** For the Golden Gate assembly, the desired module vectors were mixed with pENTR™3C-lacZ in a one-pot reaction with BsaI restriction enzyme and T4 DNA ligase resulting in pENTR™3C with the assembled BsaI modules. **D** Using Gateway recombination, the assembled BsaI-modules were transferred into Gateway binary vector pGWB601 (Nakamura et al., 2010). Attachment sites for Gateway recombination are marked with yellow stars.

We cloned constructs combining artificial miRNAs (amiRNAs) targeting DNA methyltransferase and controls genes (module 6) with eYFP and LUC reporter constructs (module 4), respectively (Table 5), and introduced them in *Arabidopsis thaliana*. Both reporters can be detected *in vivo* and analyzed non-invasively. Fluorescent proteins have the advantage that no substrate is necessary. Firefly luciferase enables a non-invasive analysis in real-time but the substrate needs to be supplied exogenously. It is highly sensitive and lacks post-translational modification.

For each DNA methyltransferase (CMT3, DRM1, DRM2, MET1), three amiRNAs were created. They were designed by the amiRNA designer WMD3 (http://wmd3.weigelworld.org/) and all have separate target sites on the mRNA. The already published amiRNAs (amiR-ft-2, amiR-lfy-1, amiR-trichome, amiR-white-2) should serve as control for the effectiveness of the system (Schwab et al., 2006). As negative controls, amiRNAs against firefly luciferase and GFP were planned and constructed (Manavella et al., 2012; Grant-Downton et al., 2013).

Table 5: Cloned combinations of reporters and artificial microRNA.

Module 4	Module 6
eYFP	amiR-CMT3-1
eYFP	amiR-CMT3-2
eYFP	amiR-CMT3-endo
eYFP	amiR-DRM1-1
eYFP	amiR-DRM1-2
eYFP	amiR-DRM2-1
eYFP	amiR-DRM2-2
eYFP	amiR-MET1-1
eYFP	amiR-MET1-2
eYFP	amiR-MET1-3
eYFP	amiR-ft-2
eYFP	amiR-lfy-1
eYFP	amiR-trichome
eYFP	amiR-white-2
LUC	amiR-CMT3-1
LUC	amiR-CMT3-2
LUC	amiR-CMT3-endo
LUC	amiR-DRM1-1
LUC	amiR-DRM1-2
LUC	amiR-DRM2-1
LUC	amiR-DRM2-2
LUC	amiR-MET1-1
LUC	amiR-MET1-2
LUC	amiR-MET1-3
LUC	amiR-ft-2
LUC	amiR-lfy-1
LUC	amiR-trichome
LUC	amiR-white-2

For successful activation of luciferase reporter gene activity two constructs were exemplarily tested (Figure 21). Luciferase activity could be detected in transiently transformed *Nicotiana benthamiana* leaves as well as stable transformed *Arabidopsis thaliana* in T1 generation after dexamethasone treatment.

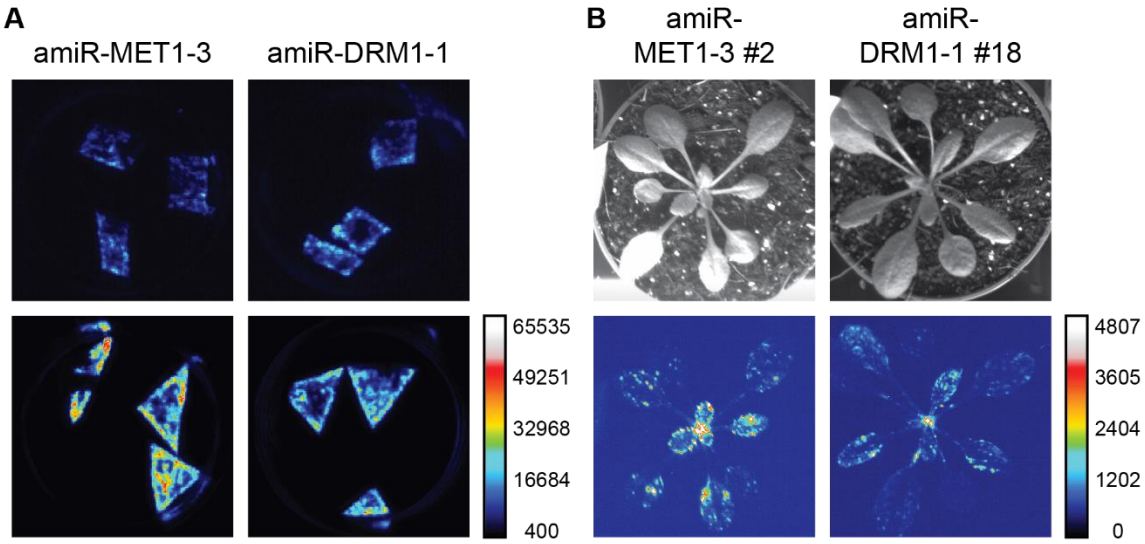


Figure 21: Pseudo-colored image of firefly luciferase reporter activity of two exemplarily shown dexamethasone-inducible constructs. (module 4: LUC, module 6: amiRNA) **A** Luciferase activity in transiently transformed Nicotiana benthamiana leaves (Upper panel: Co-infiltrated with dexamethasone; lower panel: One day after infiltration leaves were dexamethasone treated). **B** Stable transformed *Arabidopsis thaliana* with the indicated constructs (Upper panel: Image of plants before luciferase detection; lower panel: Pseudo-colored image of luminescence signal). Grayscale intensity values of the pseudo-colored images were mapped to the indicated color intensity.

A closing analysis of the cloned constructs including necessary controls in the stable transformed *A. thaliana* lines concerning properties like efficiency of amiRNAs and putative silencing effects in these lines, was not yet done because lines were not yet finally selected for homozygous, single insertion lines.

The here described tool is designed to investigate dynamic changes in the DNA methylation pattern that contribute to the plant immunity pathway.

3. Discussion

3.1 Role of AGO1 and identification of microRNAs involved in plant defense

In this study, the role of microRNAs in defense against necrotrophic pathogens was examined. As a first step, the microRNA effector mutants *ago1*-26 and *ago1-27* were investigated after exposure to *A. brassicicola*. Infections of *ago1* mutants revealed that AGO1 is a crucial factor in plant immunity response against necrotrophs. The hypomorphic *ago1* alleles were significantly reduced in their defense performance against *A. brassicicola* (Figure 6A - C). Furthermore, *ago1-27* mutants showed a disturbed cell death phenotype (Figure 6D; Mason et al. (2016)).

In several reports, either a direct or an indirect way was suggested or shown how AGO1 is part of pathogenic defense. AGO1 is actively involved in antiviral defense (Morel et al., 2002; Qu et al., 2008; Azevedo et al., 2010) and in defense against bacteria and fungi (Ellendorff et al., 2009; Li et al., 2010; Zhang et al., 2011a; Sunkar et al., 2012; Weiberg et al., 2013). In addition, studies showed that miR168, the microRNA that targets AGO1, is responsive to pathogen treatment and is actively targeting AGO1 thereafter (Várallyay et al., 2010; Baldrich et al., 2014). Based on our findings and the previously reported involvement of AGO1 and AGO1-loaded sRNAs in virus resistance and defense against biotrophs and necrotrophs, we conclude that AGO1 is an important intersection in pathways defeating pathogens.

The main class of endogenous sRNAs, which is loaded in AGO1, is depicted by microRNAs (Mi et al., 2008; Takeda et al., 2008; Zhang et al., 2014) and like other sRNAs, the expression of many microRNAs and their targets in plants can be induced upon external stimuli (Lewis et al., 2009; Zhang et al., 2011a; Sunkar et al., 2012). For instance, miR393 is responsive to bacterial attacks (Navarro et al., 2006), miR399 is induced upon phosphate starvation (Bari et al., 2006) and miR398 is coupled to oxidative stress (Sunkar et al., 2006). AGO1, which was described herein as positive regulator of necrotrophic defense, supports the potential of microRNAs in necrotrophic defense. Therefore, microRNAs are promising candidates to confer resistance. Hence, the focus of this study was the identification of crucial microRNAs in defense against necrotrophic pathogens.

A first candidate was depicted by miR398. This microRNA was previously described as responsive to flg22 and *Pst* (Jagadeeswaran et al., 2009; Li et al., 2010; Zhang et al., 2011a). The targets of miR398 are involved in detoxification of ROS. The production of ROS is one of

the first defense reaction activated after pathogen infections leading to HR (Zurbriggen et al., 2010; Baxter et al., 2014). Both ROS and HR promote susceptibility towards necrotrophic fungi (Govrin and Levine, 2000; Torres et al., 2006; Heller and Tudzynski, 2011). As an enhanced cell death response was observed in *ago1-27* (Figure 6D; Mason et al. (2016)), we the expression of miR398 and its target genes. Putative downregulation of miR398 and derepression of the ROS-detoxifying targets could increase resistance of *Arabidopsis thaliana* towards *A. brassicicola*. Quantitative PCR results of the *A. brassicicola*-infected plants, reveals unaltered miR398 levels and antagonistically regulated or unregulated targets. This result indicated that miR398 is not involved in plant immunity against *A. brassicicola*. Quantitative results only reveal pathogen responsiveness of microRNAs but this does not necessarily implicate a role in defense against *A. brassicicola*. Hence, two other different approaches were used to unmask pivotal microRNAs in antifungal defense against *A. brassicicola*.

First, several STTM constructs were cloned and introduced into plants. Therefore, many STTMs that targets putatively involved pathogen-responsive microRNAs (Fahlgren et al., 2007; Li et al., 2010; Zhang et al., 2011a; Sunkar et al., 2012) were constructed and introduced into *Arabidopsis thaliana*. Screening the T1 generation of the STTM lines did not exhibit crucial microRNAs, as none of the lines showed a constantly altered disease development 7 and 10 days after exposure to *A. brassicicola* (Figure 18). Putative silencing events in the STTM lines and partial ineffectiveness of the STTMs (Figure 19), respectively, hindered the successful identification of crucial microRNAs. More effort would have been necessary to improve STTM effectiveness and plant selection from transformation to stable lines with a single insertion is a time-consuming process. Additionally, this approach fails to include not yet described microRNAs putatively involved in biotic stress defense.

Second, a sequencing approach was chosen that overcomes above stated problems of the STTM approach. Therefore, sRNA libraries from total RNA and AGO1-immunoprecipiated RNA (no treatment, mock treatment, *A. brassicicola* treatment; 3 biological replicates) were sequenced and analyzed. They revealed several identical significant hits in both library settings. Two of them (miR163 and miR827) were examined in more detail as comparison of the microRNAs with target mRNAs from transcriptome sequencing data showed that the expression level of the microRNA targets were also altered in samples exposed to *A. brassicicola*.

3.2 MiR163 serves as negative regulator of plant defense

MiR163 was already suggested due to its alamethicin and *Pst* DC3000-mediated inducibility (Ng et al., 2011; Bielewicz et al., 2013) and its targets of the SABATH family (Seo et al., 2001; Chen et al., 2003) to be part of the plant immunity network. Fitting to previously published data, the results of this study revealed, that infections with *A. brassicicola* as well as *B. cinerea* increase pri-miRNA163, *FAMT* and *PXMT1* transcript levels (Table 1, Table 3, Figure 10 - Figure 14). Additionally, a change in the splicing ratio of miR163 was observed in the conducted experiments (Figure 10), indicating that the transcript processing machinery could be part of necrotrophic defense.

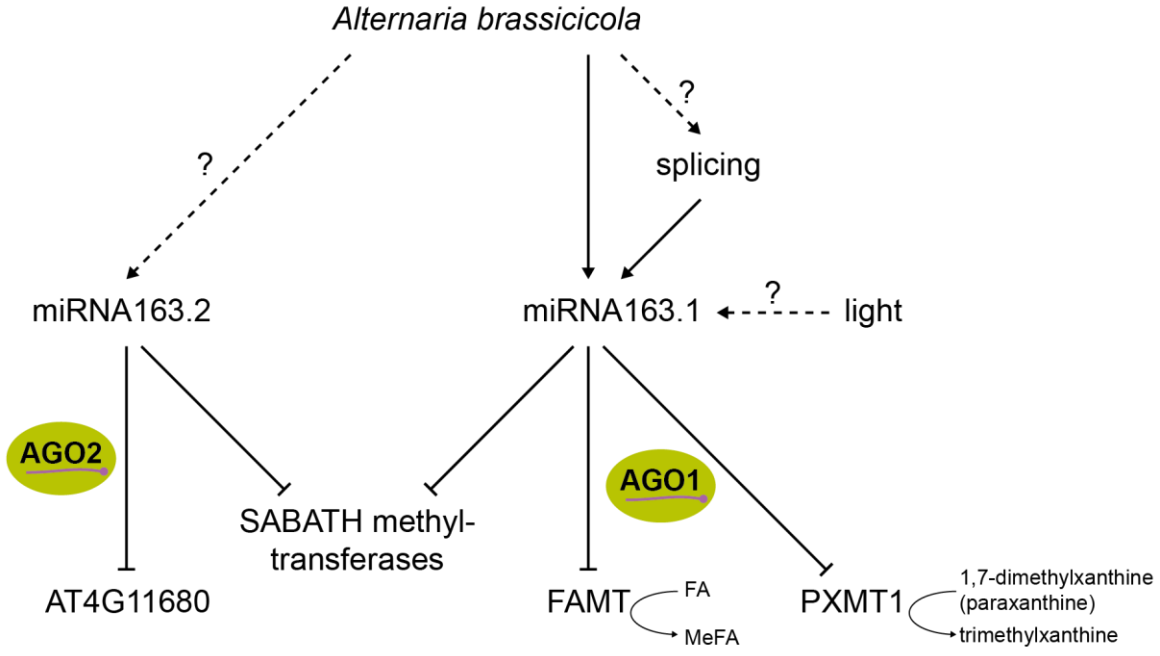


Figure 22: Model of miRNA163 in defense against *Alternaria brassicicola*.

Taken all results together, we propose a model for the miR163 regulation after *A. brassicicola* inoculations, which is depicted in Figure 22. The hypothesis is that *A. brassicicola* infections accumulate mature miR163.1 while both enhanced expression of *MIR163* and changes in the splicing ratio contribute to this. In parallel, SABATH methyltransferases, the targets of miR163, were induced as the promoter sequences of *MIR163* and its target clusters are evolutionary young and similar (Allen et al., 2004; Wang et al., 2006). Hence, the suggestion is that miR163.1 buffers the excessive induction of *FAMT* and *PXMT1*

after *A. brassicicola* infections. A direct prove that miR163 targets *FAMT* and *PXMT1* could be analyzed with microRNA-resistant forms of the target genes.

The two tested *mir163* mutants both displayed an enhanced basal expression level of *PXMT1* while *FAMT* levels were not clearly upregulated (Figure 12). This could explained by the general higher expression of *FAMT* in plants while *PXMT1* is nearly undetectable (qPCR experiments and Ng et al. (2011)). The increased constitutive and induced expression of *PXMT1* in *mir163* mutants (Figure 12) could be the reason for the enhanced resistance and indicates that PXMT1 activity is an important factor in plant immunity against *A. brassicicola*. This could be a hint that PXMT1 is a specialized protein in defense in addition to its recently discovered miR163-dependent function in early plant development (Chung et al., 2016). Chung et al. (2016) reported that miR163 represses *PXMT1* in a light-dependent manner and thereby promotes seed germination and seedling de-etiolation.

The most striking evidence, that miR163 is part of the defense mechanism in *Arabidopsis thaliana*, is the more resistant phenotype of *mir163* mutants compared to WT that could be complemented by introducing a 35S-driven *MIR163* construct (Figure 13). Unexpectedly, plants grown under different light conditions and humidity lose their resistant phenotype towards *A. brassicicola* (Supplementary Figure SI5). MiR163 and its target *PXMT1* was shown to contain several light-responsive promoter elements (Ng et al., 2011) and as the first microRNA, miR163 was shown to function in light-dependent processes (Chung et al., 2016). MiR163 levels increase during seed germination and seedling de-etiolation upon light perception; the highest induction of pri-miR163 is under white and blue light conditions in seedlings (Chung et al., 2016). Negatively correlated with the miR163 expression profile in light-perceiving seedlings is the abundance of the *PXMT1* transcript that is targeted by miR163. Pri-miR163 is also induced in 3-week old mature plants by light, but maturation of pri-miRNA163 to miR163 is unaffected. Therefore, miR163 levels are stable in mature plants, while *PXMT1* mRNA levels are low. However, it is not known, if different light qualities affect the miR163 levels in mature plants, but it could display an explanation for the altered defense phenotype of miR163 under different light conditions.

Commonly and herein used small RNA sequencing analysis using the miRBase-annotated microRNAs as reference precludes all herein unannotated microRNAs in the first place. For miR163 (miR163.1), a second microRNA isoform miR163.2 is present but not annotated in miRBase (Kurihara and Watanabe, 2004; Jeong et al., 2013). MiR163.2

contains 21 nt and is located immediately downstream of miR163.1 (Kurihara and Watanabe, 2004; Jeong et al., 2013). Parallel analysis of RNA ends (PARE) revealed that although miR163.2 is base-pairing with *PXMT1* mRNA, it is not cleaving its target mRNA like miR163.1 (Jeong et al., 2013). The same analysis proved that miR163.2 but not miR163.1 targets AT4G11680 encoding for a RING-domain containing protein with *in vitro* ubiquitin ligase activity (Stone et al., 2005; Jeong et al., 2013). PARE analysis only reveals post-translational regulation through cleavage of the target mRNAs. Therefore, it should be kept in mind that miR163 can target more genes of the SABATH family apart from *PXMT1* and that target mRNAs could be translationally inhibited instead of cleaved.

The two miR163 isoforms are loaded in different ARGONAUTE proteins according to their 5'-terminal nucleotide: miR163.1 (5'-U) into AGO1, miR163.2 (5'-A) preferentially into AGO2 (Mi et al., 2008; Takeda et al., 2008; Jeong et al., 2013). Surprisingly, miR163.2 levels loaded in AGO2 increased upon infection with *Pst avrRpt2* (Jeong et al., 2013) as well as AGO2 protein levels were induced upon *Pst* treatment (Zhang et al., 2011b). Together with miR393*, AGO2 contributes to defense against an avirulent *Pst* strain (Zhang et al., 2011b). These findings support the possibility that miR163.2 together with AGO2 is involved in defense against *A. brassicicola* due to the antagonistic signaling pathways of biotrophic and necrotrophic pathogens (Pieterse et al., 2009; Pieterse et al., 2012). In contrast to *Pst* infections, AGO2 levels were not increased after exposure to *A. brassicicola* (Figure 7) and so far levels of miR163.2 were not determined. Putatively, the splicing events in the precursor of miR163 also influences the accumulation of the miR163.2 isoform. If and to what extent miR163.2 affects pathogen defense needs further investigation and could provide an additional layer of control through the regulation of its unique target gene AT4G11680.

For the intron-containing miR163, we showed that the miR163.1 is differentially spliced after infection with *A. brassicicola* (Figure 10). Three proteins, CBP20, CBP80 and SE, were reported to function in both, microRNA processing and the splicing process (Laubinger et al., 2008). These are also essential for proper accumulation of mature miR163 (Bielewicz et al., 2013) and were furthermore proposed to display a starting point to recruit different factors, which can function in the pathways of microRNA biogenesis and splicing (Laubinger et al., 2008).

Beside the previously mentioned cap-binding complex and SE, other microRNA and splicing factors are involved in the biogenesis of the intron-containing *MIR163* (Allen et al., 2004; Szarzynska et al., 2009; Bielewicz et al., 2013). Mutants of microRNA biogenesis and

splicing factors showed an altered splicing ratio resulting in a disturbed accumulation of mature miR163 (Bielewicz et al., 2013) because the unspliced version of miR163 is favored over the spliced version to be diced into the mature miR163 (Bielewicz et al., 2013; Schwab et al., 2013). In case of miR163, it was shown that the 5'-splice site is essential for miRNA biogenesis and it was proposed that U1 small nuclear ribonucleoproteins (snRNPs) are involved in crosstalk between splicing and microRNA processing of *MIR163* (Bielewicz et al., 2013). In general for all microRNAs, it was postulated that U1 components interact with intron-containing pri-miRNAs and thereby lead to an increased efficiency of miRNA biogenesis (Szweykowska-Kulinska et al., 2013) as the 5'-splice site is bound by the U1 complex. Szweykowska-Kulinska et al. (2013) underpin their postulation by their unpublished observation that SE is able to interact with U1 snRNPs.

Also in mammals, a connection between U1 and the microRNAs biogenesis was shown for microRNAs, which reside in intronic sequences of protein-coding or non-coding genes. For two microRNA in mammals, it was shown that binding of U1 to the 5'-splice site precedes binding of further components of the microRNA biogenesis and the subsequent splicing of the intron (Janas et al., 2011).

Not only for miR163 differentially spliced isoforms are known (Kurihara and Watanabe, 2004; Bielewicz et al., 2013; Schwab et al., 2013) but also other microRNAs were shown to have differently spliced isoforms (Aukerman and Sakai, 2003; Hirsch et al., 2006). So for example, also the pri-miR399a transcript is alternatively spliced. For this microRNA, the 3'-splice site is selected differently in the two isoforms resulting in different sequences in the 3'-exon as well as different polyadenylation sites (Sobkowiak et al., 2012). The role and biological function of the two splicing isoform are still undiscovered. Further, the cistronic microRNA pair, *MIR846* and *MIR842*, are produced from the same transcript but from ABA-regulated alternatively spliced isoforms (Jia and Rock, 2013). In 2012, it was firstly proven, that accumulation of a certain microRNA is regulated through alternative splicing induced by environmental stress (Yan et al., 2012b). For miR163, Bielewicz et al. (2013) showed that the intron and its functional splice sites regulate miR163 biogenesis during bacterial infection. In addition, a changed splicing ratio of pri-miRNA163 can be seen after infection with *A. brassicicola* (Figure 10). In general, there is a big potential for alternative splicing to regulate microRNA biogenesis, as around 50 % of all microRNAs in Arabidopsis contain introns (Szweykowska-Kulinska et al., 2013). Hence, it could be a possible explanation, that alternative splicing events integrate biotic stress stimuli into the splicing process of miR163 and thereby regulate its accumulation post-transcriptionally.

MIR163 is a recently evolved gene in the plant lineage (Allen et al., 2004) and is highly expressed in all plant organs of *Arabidopsis thaliana* (Guo et al., 2005; Ng et al., 2011). In contrast to *Arabidopsis thaliana*, in *Arabidopsis arenosa*, which is closely related to *Arabidopsis thaliana* diverging about 5 million years ago (Koch et al., 2000), miR163 is nearly undetectable (Ng et al., 2011). Ng et al. (2011) showed that, *MIR163* of *Arabidopsis arenosa* has a higher similarity with its target At1G66700 than in *Arabidopsis thaliana*. This suggests that *MIR163* has undergone more mutation events in *Arabidopsis thaliana*. In general, the regulation of MIR163 and its targets are still quite similar in response to external stimuli (Figure 10A, B, Figure 12, Ng et al. (2011)). A reason for this could be displayed by the recent evolution of gene bodies and their promoter sequences (Allen et al., 2004; Wang et al., 2006).

Taken together, it is not yet clarified if miR163 and its targets or only its targets are contributing to defense in *Arabidopsis thaliana*. Overall, the regulation of *MIR163* and its targets seems to be not yet perfected and is still an evolutionary playground.

3.3 MIR827 couples phosphate stress and plant defense

Apart from miR163, I investigated miR827 concerning its role in plant immunity against necrotrophs. Sequencing data and qPCR analysis, respectively, revealed that mature miR827 and pri-miRNA827 is strongly induced after plant leaves were exposed to *A. brassicicola* (Table 1, Table 2, Figure 15A). Likewise, the miR827 target *VPT1*, a tonoplast-localized phosphate transporter ((Liu et al., 2015), is induced and down-regulated in an AGO1-dependent manner after infections with *A. brassicicola* (Figure 15A). The second described target *NLA* is neither directly nor indirectly responsive to *A. brassicicola* in leaves (Figure 15A).

MiR827 and its targets *NLA* and *VPT1* were described to be involved in the regulation of phosphate homeostasis (Kant et al., 2011; Liu et al., 2015). We therefore wondered why the expression of phosphate responsive genes was altered after inoculations with *A. brassicicola*. Recently, phosphate deficiency and the defense of herbivorous insects were linked together as both pathogens were shown to activate JA biosynthesis and signaling components (Khan et al., 2016). Beside the role of JA in defense against herbivory, JA is also necessary for the activation of defense responses against necrotrophic pathogens. We therefore propose that the JA pathway could be responsible for the activation of miR827 as it was found to be induced under phosphate limiting conditions (Hsieh et al., 2009; Pant et al., 2009; Lundmark et al., 2010) as well as *A. brassicicola* infection (Table 1, Table 2).

A putative regulatory pathway of miR827 after infection with *A. brassicicola* is depicted in Figure 23. MiR827 targets *VPT1* in leaves and therefore prevents storage of P_i in the vacuole and makes more P_i availably in the cells. Additionally, we propose that miR827 is loaded into the phloem and transported into roots based on the phloem mobility of miR827 in *Brassica napus* (Pant et al., 2009). Subsequently in roots, miR827 can degrade *VPT1* and *NLA* mRNA. Targeting of *NLA* ends in a derepression of phosphate transporters and enhanced phosphate uptake.

For *VPT1*, different scenarios or a combination of both are possible. Repression of *VPT1* in both roots and leaves could lead to reduced storage of cytosolic P_i into the vacuole and more available P_i in the cell. All effects together increase the phosphate levels in the plant and putatively contribute positively to defense against *A. brassicicola*. A second scenario for *VPT1* could be imagined as *VPT1* exhibited an unchanged expression level under phosphate deprivation (Hsieh et al., 2009) but increased upon phosphate excess and has a role in sequestration and detoxification of surplus P_i in the vacuole (Liu et al., 2015). Due to the

enhanced P_i uptake (degradation of *NLA* and derepression of phosphate transporters) and increasing P_i concentrations in the plant cells, *VPT1* is induced to sequester excessive P_i in the vacuole.

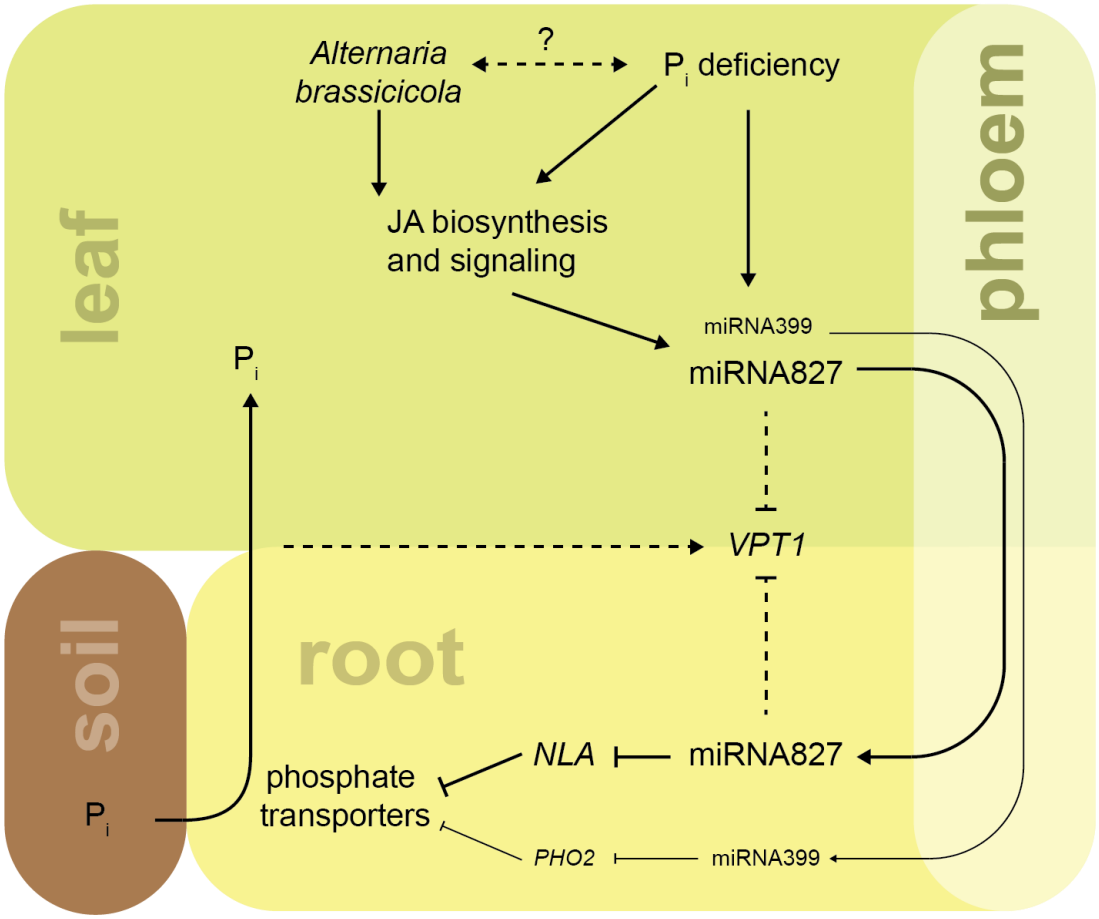


Figure 23: Model of miRNA827 action after *Alternaria brassicicola* infection.

The induction of phloem-mobile miR399 but unaltered target *PHO2* expression in leaves as indicated by sequencing data (Table 1 - Table 3) would fit into the phloem-mobile model having no effect of miR399 in the leaves. After transport to the roots, miR399 could target *PHO2*. The two targets of miR827 and miR399, *NLA* and *PHO2*, were shown to act together in degradation of a phosphate transporter (Park et al., 2014). The downregulation of *NLA* and *PHO2* results subsequently in derepression of phosphate transporters and hence augmented phosphate uptake. The fact that *NLA* is not downregulated in leaves could be explained by a tissue-specific non-overlapping expression pattern of miR827 and *NLA*.

In the *A. brassicicola* infection assay, miR827 and pri-miRNA827 were induced (Table 1, Table 2, Figure 15A). Surprisingly, infections with the necrotrophic pathogen *B. cinerea* did not influence pri-miRNA827 expression (Figure 15C).

In tomato, a special strategy from *B. cinerea* was described that could explain this different behavior. To overcome the host defense system in tomato, *Botrytis cinerea* produces an elicitor that activates the SA pathway and thereby antagonizing the essential JA signaling pathway for defense against *B. cinerea* (El Oirdi et al., 2011). As functional protein that mediates this phytohormonal crosstalk between SA and JA, *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1* (*NPR1*) was suggested in tomato and proven in *Arabidopsis thaliana*, respectively (Spoel et al., 2003; El Oirdi et al., 2011). Under the assumption that the phytohormonal crosstalk-manipulating mechanism of *B. cinerea* is functional in *Arabidopsis thaliana*, it provides an explanation for the discrepancy between *A. brassicicola* and *B. cinerea* infections on the activation of miR827.

A knock-out of miR827 did not display an altered defense phenotype against *A. brassicicola* as well as an overexpression of *MIR827* was not sufficient to manipulate response to a fungal infection (Figure 15B). As both miRNAs (miR399, miR827) are likely involved in the complex phosphate-response network, knock-out or overexpression of one component could be insufficient to disturb the complex phosphate-starvation network and resistance remains unaltered.

Several studies support the notion that the phosphate nutrition state and the plant immune defense are coupled (Zhao et al., 2013; Hewezi et al., 2016; Hiruma et al., 2016). So for example it has been shown that phosphate nutrient status is important for successful defense against the phloem-restricted bacteria *Candidatus Liberibacter asiaticus* in citrus (Zhao et al., 2013). Exogenous application of phosphate was able to reduce the severity of the disease symptoms (Zhao et al., 2013) indicating that sufficient phosphate availability is an important component for successful defense response. This study in citrus also proved, that the phosphate-starvation inducible miR399 is activated upon *Candidatus Liberibacter asiaticus* infection (Zhao et al., 2013). Up to date, it is not clearly understood, which phytohormonal pathway is responsible for the defense against *Candidatus Liberibacter asiaticus* since both SA and JA pathways are upregulated after infection (Martinelli et al., 2012).

In the model for miR827 action, I proposed that miR827 mainly acts in roots after its transport through the phloem (Figure 23) and is therefore putatively active against root pathogens or parasites. A vague connection might be seen as *Candidatus Liberibacter asiaticus* belongs to the family of the soil bacteria Rhizobiaceae, a diverse group containing plant

symbionts like *Rhizobium* as well as plant pathogens like *Agrobacterium* (Duan et al., 2009). Further, this is supported by the studies of Hiruma et al. (2016) und Hewezi et al. (2016), both working with root endophytes and root parasites, respectively.

Hiruma et al. (2016) described a beneficial interaction of Brassicaceae with the root endophyte *Colletotrichum tofieldiae* under phosphate starving conditions. Similar to the arbuscular-mycorrhizal symbiosis (Bucher, 2007), the fungal interaction partner provides insoluble and therefore for the plant inaccessible phosphate to the plant under phosphate deficient conditions and thus promotes plant growth and fertility (Hiruma et al., 2016). Interestingly, secondary metabolites from the group of indole glucosinolates, which are active compounds of the innate immune response, play the major role in this interaction. Indole glucosinolates are necessary to establish and control the beneficial interaction with the root endophyte *Colletotrichum tofieldiae*, while a lack of all tryptophan-derived metabolites leads to an overgrowing of the host roots by fungal mycelia and subsequent killing of the plant roots (Hiruma et al., 2016) like in a pathogenic relationship. More directly, Hewezi et al. (2016) connected miR827 with plant defense against a cyst nematode showing that miR827 and *NLA* regulate resistance against *Heterodera schachtii*. Taken together, those studies indicate that miR827 mainly acts in root tissue and one can speculate that miR827 plays its major role in defense against root pathogens and parasites rather than leaf pathogens.

However, it is not clear yet, how phosphate deficiency and defense response against necrotrophs are directly coupled. One could speculate that attacks from necrotrophs lead to a lack of P_i and therefore the increased uptake of P_i is of advantage to counteract against necrotrophic pathogens.

All in all, a direct prove is necessary that miR827 targets *VPT1* and *NLA* after infection with *A. brassicicola*. Therefore, microRNA resistant forms of *VPT* and *NLA* in combination with a co-modification of the miR827 displays a helpful tool. Additionally, tagged proteins or protein-specific antibodies are useful to examine downregulation on protein level and not only on RNA level. Also protein levels of phosphate transporters and the phosphate content should be investigated after plants were exposed to *A. brassicicola*.

MiR827 is a conserved microRNA and annotated in monocotyledons and dicotyledons (http://www.mirbase.org/; miRbase21). Therefore, the herein described and discussed principle of regulation and connection of phosphate stress and pathogen defense could be transferable in other plant species and modifications of its components could potentially confer increased resistance against pathogen and phosphate stress.

3.4 Dexamethasone-inducible system is a skillful and easy to clone system for the investigation of DNA methylation and its role in plant immunity

Several studies connected epigenetic modifications with plant immune response towards biotrophic and necrotrophic pathogens (Dowen et al., 2012; Yu et al., 2013; Ding and Wang, 2015).

For pathogen assays, an unaltered development and phenotype is of advantage to exclude influences on pathogen response. Knockout of several epigenetic components like MET1, or components of the chromatin remodeling complex are embryonic lethal, have strong developmental and/or transgenerational aggravating defects (Kakutani et al., 1996; Cao and Jacobsen, 2002; Wagner and Meyerowitz, 2002; Sarnowski et al., 2005; Chan et al., 2006; Hurtado et al., 2006; Mathieu et al., 2007). Hence, we chose an inducible system that suits best. Owing to these facts, a plastic tool based on Golden Gate cloning procedure combined with a steroid-inducible system was developed. The system was tested due to its reporter gene activity and is functional in transiently transformed tobacco and stable *A. thaliana* (Figure 21). A successful downregulation of the amiRNA targets after induction is still pending. Combining the inducible constructs introduced in *A. thaliana* with the *A. brassicicola* pathogen assay can reveal the importance and involvement of the DNA methyltransferases in the dynamic regulation of cytosine methylation and plant immunity.

Single candidate genes can be investigated with Chop-PCR based on methylation-sensitive or -dependent restrictions enzymes or bisulfite PCR. Both methods reveal differences in the methylation pattern of a single amplicon. A broader analysis could be done for CHH methylation through the sRNA libraries and transcriptome data. Analysis of altered siRNAs from the sRNA libraries (total RNA) can reveal putative siRNAs that can lead to different expression pattern in the targeted gene. Comparing the altered siRNA loci with the transcriptome data help to identify altered CHH methylation that leads to transcriptional silencing.

For global analysis of DNA methylation after pathogen attack bisulfite sequencing after is the method of choice. Therefore, the pOp6 system is induced firstly by dexamethasone treatment and downregulated the DNA methyltransferase of interested and secondly *A. brassicicola* infections were performed. Plant material from before and after pathogen treatment serve as material for bisulfite sequencing.

The inducible system itself is highly plastic and easy to clone. Its modular construction allows an easy adjustment for individual need. So it can be used for many different fields of applications beyond pathogen assays or epigenetic investigations.

4. Material and Methods

4.1 Cloning

In general, DNA fragments-of-interest were amplified with Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific). After purification (GeneJet™ PCR Purification Kit (Thermo Fisher Scientific)), PCR products were cloned into a gateway-compatible entry vector with pCR™8/GW/TOPO® TA Cloning Kit (Thermo Fisher Scientific). Entry vectors were controlled by sequencing after cloning at the Genome Center of the Max-Planck-Institute for Developmental Biology (Tübingen, Germany). Selection of *E. coli* (strain DH5α) was done on antibiotics-containing LB agar (ampicillin 100 µg/ml, kanamycin 25 µg/ml or spectinomycin 50 µg/ml) and if needed additional blue-white selection (top agar with 50 µl of X-Gal (20 mg/ml in dimethylformamide) and 50 µl of 100 µM IPTG).

LR reaction in the destination vectors pGWB602 (for STTM constructs) or pGWB601 (dexamethasone-inducible constructs) (Nakamura et al., 2010) was performed with the Gateway® LR Clonase® II Enzyme mix (Invitrogen).

Short tandem target mimicry constructs were separated with a 48-nt spacer (sequence 5'-GTTGTTGTTGTTATGGTCTAATTTAAATATGGTCTAAAGAAGAAGAAT-3') (Yan et al., 2012a) and named with numbers corresponding to the targeted microRNA. STTM constructs were amplified with overlapping primers by two consecutive extension steps. Sequence of the STTM constructs and used oligonucleotides are listed in Supplementary Table SI13. Template for the first STTM construct was the synthesized vector pUC57-STTM393 (GenScript). Most of the STTMs were designed to be compatible with the dexamethasone-inducible vector system as module 6 (optional 5'-end: 5'-ggtctcgTGCA-3'; optional 3'-end: 5'-ggtctcgTGTT-3'). Single module for the dexamethasone-inducible vector system were cloned by PCR amplification to add the necessary BsaI sites. Internal BsaI site in the 35S promoter sequence was removed by PCR-based mutagenesis. Oligonucleotides and templates for the single modules are listed in Supplementary Table SI15. The bidirectional promoter pOp6 for module 5 was synthesized and inserted into pUC57 by GenScript. Sequence of pOp6 with the according BsaI sites are indicated in Supplementary Information SI14.

Besides reporter genes, several artificial microRNAs were cloned. Artificial microRNA (amiRNA) constructs were designed using the amiRNA designer WMD3

(http://wmd3.weigelworld.org/) and amplified by PCR-based mutagenesis using pRS300 as template (Schwab et al., 2006). Used oligonucleotides are listed in Supplementary Table SI16.

For blue-white selection of successful assembly, lacZ gene was amplified from pFUS_A (Addgene plasmid # 31028; Cermak et al. (2011)) and NotI and SalI restriction site included in the oligonucleotides were added. Using NotI and SalI restriction enzymes (Thermo Scientific™ FastDigest™), the amplified lacZ gene was classically cloned into pENTR™3C (Invitrogen). The single modules of the dexamethasone inducible system were assembled based on the protocol of Engler et al. (2009). 150 ng of each module vector and 150 ng of pENTR™3C-lacZ was mixed with 1 µl BsaI (10 U/µl, New England Biolabs), 1 µl T4 DNA ligase (5 U/µl; Thermo Fisher Scientific) and 10x T4 DNA ligase buffer (Thermo Fisher Scientific) in a total volume of 20 µl. Reaction mix was incubated in a cycling program (10 cycles of incubation at 37 °C for 8 min (digestion) and at 16 °C for 15 min (ligation), followed by 50 °C for 5 min and 80 °C for 5 min). Afterward, 1 µl plasmid-safe™ ATP-dependent DNase (10 U/µl; Epicentre) and 1 µl ATP (25 mM; Epicentre) were optionally added and incubated at 37 °C for 1 h.

4.2 Plant transformation

Floral dip transformation by *Agrobacterium tumefaciens* (strain: GV3101 (pMP90)) was used to introduce the recombinant destination vectors into *Arabidopsis thaliana* Col-0 (Clough and Bent, 1998). Transformed plants were selected on Basta-watered soil (0.1 % (v/v)) and screened in T1 generation.

For transient gene expression, *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens*. Bacteria were adjusted to an optical density of 0.5 at a wavelength of 600 nm in infiltration solution (10 mM magnesium chloride, 10 mM 2-(*N*-morpholino)-ethanesulfonic acid pH 5.7, 100 µM acetosyringone; de Felippes and Weigel (2010)).

4.3 Plant Materials

Plants were grown under short day conditions (8 h light, 22 °C) in an Arabidopsis growth chamber (Percival Scientific, Model AR-66L3; incandescent and LED lightning) and were cultivated with a high humidity in GS90 soil for around 5 - 6 weeks before an experiment was conducted. *Ago1-26* and *ago1-27* mutants were derived from an ethyl methanesulfonate mutagenesis and previously described and characterized (Morel et al., 2002). *Mir163-1* (SALK_034556), *mir163-2* (SAIL_875_G02) and 35S:MIR163 (in *mir163-1* background) were described in (Ng et al., 2011; Bielewicz et al., 2013; Schwab et al., 2013) and kindly provided by Dr. Rebecca Schwab. *Mir827* (SALK_020837) and 35S:MIR827 were kindly provided by Prof. Dr. Steven J. Rothstein (Kant et al., 2011). All mutants used are in Col-0 background.

4.4 Dexamethasone treatment and luciferase assays

Infiltrated leaves of *Nicotiana benthamiana* were either co-infiltrated with Agrobacteria or their adaxial side was sprayed one day after infiltration with 10 - 30 µM dexamethasone solution. *Arabidopsis thaliana* lines were sprayed with dexamethasone solution 1.5 days before detection of luciferase activity.

To visualize luciferase activity in tobacco, small pieces of *Nicotiana benthamiana* leaves, were put with the abaxial side on top in 24-well culture plate 3 days after infiltration with *Agrobacterium tumefaciens*. Stably transformed *Arabidopsis thaliana* T1 lines were observed as whole plants. Leaf pieces of *Nicotiana benthamiana* and aerial parts of *Arabidopsis thaliana* plants were sprayed with luciferin solution (1 mM D-Luciferin Firefly, potassium salt (Biosynth) in 10 mM Tris-phosphate, pH 8) and luminescence signal was detected with a CCD camera (Peqlab). Pseudo-colored images of detected luminescence signals were created with ImageJ 1.46r (http://imagej.nih.gov/ij/).

4.5 Fungal pathogen assays

For symptom scoring, two leaves of each ~ 6-week old plant were infected with 2 drops of 5 µl *Alternaria brassicicola* (strain: MUCL 20297) spore solution (1 x 10^6 spores/ml) (Kemmerling et al., 2007). Disease symptoms were scored 7 and 10 days after inoculation. For gene expression analysis and library preparation, plants were sprayed with spore solution (1 x 10^6 spores/ml; 0.01 % Silwet L-77). Rosette leaves were harvested at indicated time points.

Inoculations with *Botrytis cinerea* (strain: B05.10) were done as described in Van Wees et al. (2013) with minor modifications. *B. cinerea* was grown under continuous light and harvested conidia were filtered through miracloth.

Pictures of representative infected leaves were taken with Nikon D5100 18-105 VR Kit (Nikon Instruments Europe B.V., Amsterdam, The Netherlands). Symptom scores were statistically analyzed with the Mann-Whitney U test using IBM® SPSS® Statistics (Version 23.0.0.0) using single leaves as biological replicates. P-values are listed in the Supplementary Information (Supplementary Table SI1 - Supplementary Table SI4). *Alternaria brassicicola* (strain MUCL 20297) and *Botrytis cinerea* (strain B05.10) were kindly provided by Dr. Birgit Kemmerling and Dr. Andrea Gust, respectively.

4.6 Trypan blue staining

To visualize dead plant cells, leaves were stained with trypan blue (Kemmerling et al., 2007). Briefly, leaves were covered in trypan blue staining solution (10 ml lactic acid, 10 ml glycerol, 10 ml phenol, 10 ml water, 80 ml ethanol, 2.5 mg/ml trypan blue), heated up in a water bath (100 °C) for 1 min and incubated for 5 min at room temperature. Afterwards, leaves were destained with chloral hydrate (1 mg/ml). Pictures were taken with the fluorescence stereomicroscope *Leica MZ FLIII*.

4.7 RNA isolation and RT-PCR analyses

Total RNA was extracted using the Direct-zol RNA MiniPrep Kit (ZymoResearch). Copy DNA (cDNA) was prepared from 250 ng - 1.5 mg of DNase-treated total RNA with RevertAid^{TM} first-strand cDNA synthesis kit (Thermo Fisher Scientific, http://www.thermoscientific.com). RNA was reverse transcribed using oligo(dT) primers and optionally with specific stem-loop oligonucleotides for miRNA detection (Varkonyi-Gasic et al., 2007). Quantitative real-time PCR was performed in a CFX384 system (Bio-Rad, http://www.bio-rad.com/) using SYBR Green (Thermo Fisher Scientific) as photometrical readout. *ACTIN* served as reference gene in all experiments.

Splicing analysis was performed on cDNA with DreamTaq DNA Polymerase (Thermo Fisher Scientific) for 25 (*TUB2*) or 35 cycles (*MIR163*) according to manufacturer's instruction and visualized on a 2 % agarose gel. For quantification, RT-PCR products were analyzed by capillary electrophoresis (Agilent 2100 Bioanalyzer with DNA1000 Chip) according to manufacturer's instructions. Peak areas were used to calculate the ratio between unspliced and spliced products. Genomic DNA was extracted with DNeasy® Plant Mini Kit (Qiagen, 69104) and concentrated with DNA Clean & Concentrator™-5 (Zymo research, D4014). Used oligonucleotides for RT-PCR analyses are listed in Supplementary Table SI17. Values of the individual replicates for time course experiments are listed in the Supplementary Information under "5.3 Relative expression values of each replicate in the time course experiment", Supplementary Table SI7 - Supplementary Table SI12.

4.8 Small RNA library preparation

For preparation of small RNA libraries from total RNA, sRNAs were isolated from 8 µg total RNA as described in (Gilbert et al., 2014).

For sRNA libraries from AGO1-loaded RNAs, an AGO1-Immunoprecipiation was done. For this, around 2.5 mg of plant material was ground in liquid nitrogen, resuspended in extraction buffer (50 mM Tris pH 7.5; 150 mM sodium chloride; 10 % glycerol; 0.05 % Nonidet P-40; Proteinase Inhibitor Roche cOmplete (EDTA-free); 1 mM phenylmethanesulfonyl fluoride), incubated at 4 °C for 20-30 min on a rotator and centrifuged at 4 °C at 3,200 g for 30 min. The protein extract was filtered (2x miracloth and 1x syringe

driven filter unit) and pre-cleared with 100 ml protein A agarose beads for 30 min on a rotator. After removal of the beads, supernatant was split in two fractions and incubated with/without 5 µl of AGO1 antibody (Agrisera; AS09 527) at 4 °C for 2 h on a rotator and an additional hour with 50 µl protein A agarose beads. Beads were collected by centrifugation and washed several times with extraction buffer. AGO1 complex was eluted from beads with AGO1 peptide (Agrisera; AS09 527P) as described in Qi and Mi (2010) with minor modifications. AGO1 complex was eluted in 100 µl peptide elution solution (50 µg/ml). RNA and protein from the complex was extracted by adding TRIzol® reagent and incubation at 50 °C for 5 min. Using chloroform, aqueous and organic phase were separated. The RNA containing, aqueous phase was further cleaned with chloroform and precipitated over night with one vol. isopropanol at -20 °C followed by centrifugation and washed with 80 % ethanol. The organic phase was precipitated with 3 volumes of acetone at -20 °C followed by centrifugation and washed with 80 % acetone. As control, AGO1 enrichment in the protein fraction was controlled by SDS-PAGE and immunodetection (see 4.11 Western Blot analysis). The extracted RNA was concentrated with RNA Clean and Concentrator^{TM}-5 (ZymoResearch).

Both sRNA libraries were prepared with Illumina-compatible Kits. The pooled libraries were sequenced as technical replicates on two lanes on Illumina HiSeq 2000. The sequencing data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and can be accessed through the GEO Series accession number GSE83488 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE83488). The subseries of the sRNA sequencing data are additionally available through GEO Series accession number GSE83487 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE83487).

4.9 mRNA library preparation

For transcriptome analysis, polyadenylated RNA were isolated from 3 µg total RNA with NEBNext® Poly(A) mRNA Magnetic Isolation Module (NEB) and used as input for the ScriptSeq™ v2 RNA-Seq Library Preparation Kit (Epicentre). Size selection (250 – 650 bp) was performed on the pooled libraries using BluePippin (Sage Science). The pooled library was sequenced on Illumina HiSeq 3000.

The sequencing data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and can be accessed through GEO Series accession number GSE83488 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE83488). The subseries of the mRNA sequencing data are additionally available through the GEO Series accession number GSE83478 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE83478).

4.10 Small RNA blot analysis

Total RNA (8 µg, concentrated with RNA Clean and Concentrator^{TM}-5 (ZymoResearch)) was separated on a 17 % urea acrylamide gel and stained with ethidium bromide to check equal loading. After that, RNA was transferred on Amersham Hybond-N+ membrane (GE Healthcare Life Science) and crosslinked to the membrane using UV light (Stratalinker; 120,000 µJ/cm^2). Membrane was pre-hybridized with PerfectHyb™ Plus Hybridization Buffer (Sigma) at 38 °C for 1 hour in permanent rotation, incubated with 5 pmol of denatured and DIG-labelled probe at 38 °C overnight under constant rotation and washed twice with 4x SSC (600 mM sodium chloride, 60 mM sodium citrate; pH 7,0) at 38 °C. Probe detecting mature miRNA163 (5'-ATCGAAGTTCCAAGTCCTCTTCAA-3') was labeled with DIG oligonucleotide tailing kit, 2nd generation (Roche), according to manufacturer´s instructions. For miRNA detection, membrane was incubated for 30 min in Blocking Reagent (Roche) with gentle agitation and for 1 h with the anti-Digoxigenin-AP antibody (Roche) (1:10,000 in Blocking Reagent). Afterwards, membrane was washed 4x in Washing Buffer (100 mM maleic acid, 150 mM sodium chloride, 0.3 % (v/v) Tween-20; pH 7.5) for 15 min and equilibrated for 5 min in Detection Buffer (100 mM Tris, 100 mM sodium chloride; pH 9.5). For detection, membrane was incubated with the chemiluminescent substrate CSPD ready-to-use (Roche) in dark at 37 °C for 10 min and light emission was detected with a CCD camera (Peqlab).

4.11 Western Blot analysis

For protein extraction, plant material was ground in liquid nitrogen and resuspended in protein extraction buffer (50 mM Tris pH 7.5, 150 mM sodium chloride, 10 % (v/v) glycerol, 1 mM ethylenediaminetetraacetic acid, and freshly added 1 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol, and Proteinase-Inhibitor Roche cOmplete (EDTA-free). Plants debris were removed by two centrifugation steps at 4°C (12,000 rpm, 10 min). Protein concentration was determined via Bradford assay (BioRad). 15-25 µg of protein extract was loaded in 5x Laemmli buffer (250 mM Tris pH 6.8, 50 % (v/v) glycerol, 10 % (v/v) sodium dodecyl sulfate, 1 mg/ml bromophenol blue and freshly added 500 mM dithiothreitol) on a denaturing polyacrylamide gel. Western Blot analysis was performed according to Speth et al. (2014). For Immunodetection, protein-specific antibodies against AGO1, CBP20, CBP80, DCL1, HYL1, SE (all Agrisera), AGO2 (Abiocode) and a HRP-conjugated goat-anti-rabbit IgG (Agrisera) were used. Signals were detected with a luminol-based chemiluminescent substrate (ECL reagent Kit; GE Healthcare) and a CCD camera (Peqlab).

5. Supplementary information

5.1 Significance level of symptom scoring

Supplementary Table SI1: P-values of Mann-Whitney U Test, shown in Figure 6B.

7 dai	**Col-0**	***ago1-26***	***ago1-27***
Col-0	-	**2.53E-13**	**1.232E-09**
ago1-26		-	0.742
ago1-27			-

10 dai	**Col-0**	***ago1-26***	***ago1-27***
Col-0	-	**1.49E-13**	**1.22E-11**
ago1-26		-	0.329
ago1-27			-

Supplementary Table SI2: P-values of Mann-Whitney U Test, shown in Figure 13B.

7 dai	**Col-0**	***mir163-1***	***mir163-2***	**35S:MIR163 in *mir163-1***
Col-0	-	**8.14E-05**	**1.04E-03**	0.287
mir163-1		-	0.690	**2.66E-05**
mir163-2			-	**2.62E-04**
35S:MIR163 in *mir163-1*				-

10 dai	**Col-0**	**mir163-1**	**mir163-2**	**35S:MIR163 in *mir163-1***
Col-0	-	**2.30E-03**	**1.96E-03**	0.347
mir163-1		-	0.928	**1.14E-03**
mir163-2			-	**1.06E-03**
35S:MIR163 in *mir163-1*				-

Supplementary Table SI3: P-values of Mann-Whitney U Test, shown in Figure 14B.

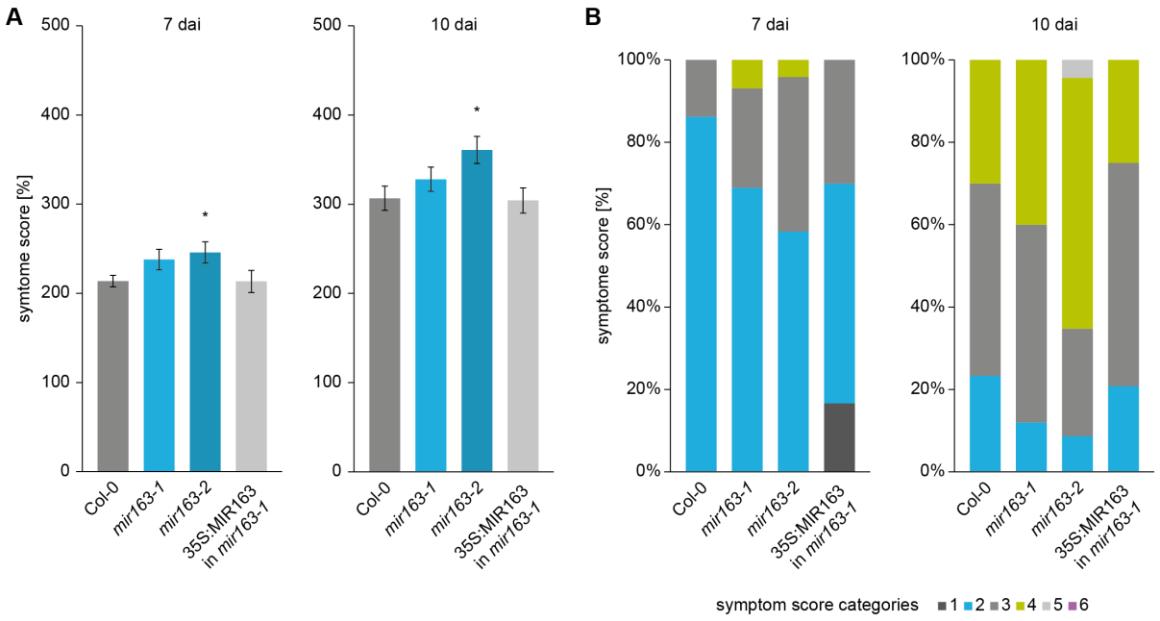
2 dai	**Col-0**	***mir163-1***	***mir163-2***	**35S:MIR163 in *mir163-1***
Col-0	-	0.930	0.571	0.147
mir163-1		-	0.523	0.141
mir163-2			-	0.382
35S:MIR163 in *mir163-1*				-

Supplementary Table SI4: P-values of Mann-Whitney U Test, shown in Figure 15B.

7 dai	**Col-0**	***mir827***	**35S:MIR827**
Col-0	-	0.383	0.693
mir827		-	0.676
35S:MIR827			-

10 dai	**Col-0**	***mir827***	**35S:MIR827**
Col-0	-	0.329	0.750
mir827		-	0.621
35S:MIR827			-

5.2 Different light conditions can influence the resistance phenotype of *mir163* mutants



Supplementary Figure SI5: *miR163* mutants behave differently dependent on growth conditions. A Symptom score of *A. brassicicola* inoculated wild type (WT), *mir163-1*, *mir163-2* and 35S:MIR163 7 and 10 days after inoculation (n ≥ 23). Mann-Whitney U test was performed to show significant differences compared to wild type (significance level is indicated with asterisks: * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001). **B** Classification in percent of *A. brassicicola* infected leaves of wild type (WT), *mir163* mutants and 35S:MIR163 after 7 and 10 days (n ≥ 23). Margaux Kaster (Center for Plant Molecular Biology, University of Tübingen) conducted this experiment.

MiR163 is the first microRNA that was shown to be influenced by light (Chung et al., 2016). Growing the plants under light conditions that differ from the standard conditions (short day, humidity of 50 %, 20 - 21 °C, illuminated by Osram L18W/77 Fluora and Osram L 18W/840 LUMILUX CoolWhite), the resistant phenotype of *mir163* mutants actually changed into an unaltered or slightly more sensitive phenotype compared with WT after *A. brassicicola* inoculation (Supplementary Figure SI5). This experiment was conducted by Margaux Kaster (Center for Plant Molecular Biology, University of Tübingen).

Supplementary Table SI6: P-values of Mann-Whitney U Test, shown in Supplementary Figure SI5.

7 dai	**Col-0**	***mir163-1***	***mir163-2***	**35S:MIR163 in *mir163-1***
Col-0	-	0.101	**0.021**	0.881
mir163-1		-	0.504	0.267
mir163-2			-	0.111
35S:MIR163 in *mir163-1*				-

10 dai	**Col-0**	***mir163-1***	***mir163-2***	**35S:MIR163 in *mir163-1***
Col-0	-	0.283	**0.010**	0.887
mir163-1		-	0.090	0.223
mir163-2			-	**0.008**
35S:MIR163 in *mir163-1*				-

5.3 Relative expression values of each replicate in the time course experiments

Supplementary Table SI7: Relative expression values of pri-miRNA163 for each replicate. Average and standard error are displayed in Figure 10A.

	Untreated	Mock			*Alternaria brassicicola*		
Replicate	0d	0.5 d	1 d	3 d	0.5 d	1 d	3 d
WT - 1	1.000	0.667	1.866	2.144	0.293	8.846	19.093
WT - 2	1.000	0.525	1.597	0.681	0.207	6.277	17.448
WT - 3	1.000	0.543	1.613	1.133	0.260	7.464	18.189
Average	1.000	0.578	1.692	1.319	0.253	7.529	18.244
Standard error	0.000	0.045	0.087	0.432	0.025	0.742	0.476

Supplementary Table SI8: Relative expression values of *FAMT* for each replicate. Average and standard error are displayed in Figure 11.

	Untreated	Mock			*Alternaria brassicicola*		
Replicate	0d	0.5 d	1 d	3 d	0.5 d	1 d	3 d
WT - 1	1.000	0.048	0.530	0.545	0.059	5.426	1.803
WT - 2	1.000	0.063	0.898	0.475	0.040	2.908	2.329
WT - 3	1.000	0.124	0.438	0.454	0.075	3.021	3.031
Average	1.000	0.078	0.622	0.491	0.058	3.785	2.388
Standard error	0.000	0.023	0.140	0.028	0.010	0.821	0.356

	Untreated	Mock			*Alternaria brassicicola*		
Replicate	0d	0.5 d	1 d	3 d	0.5 d	1 d	3 d
ago1-27 - 1	2.612	0.010	1.057	0.521	0.173	4.155	5.426
ago1-27 - 2	0.889	0.015	1.003	0.710	0.471	8.427	7.185
ago1-27 - 3	1.505	0.024	1.181	0.826	0.356	5.776	4.500
Average	1.669	0.016	1.080	0.686	0.334	6.119	5.704
Standard error	0.504	0.004	0.053	0.089	0.087	1.245	0.787

Supplementary Table SI9: Relative expression values of *PXMT1* for each replicate. Average and standard error are displayed in Figure 11.

	Untreated	Mock			*Alternaria brassicicola*		
Replicate	0d	0.5 d	1 d	3 d	0.5 d	1 d	3 d
WT - 1	1.000	0.365	0.847	2.888	2.742	88.035	139.585
WT - 2	1.000	1.815	1.659	0.719	4.272	33.708	121.938
WT - 3	1.000	1.125	0.611	0.557	3.694	35.629	303.384
Average	1.000	1.102	1.039	1.388	3.569	52.457	188.302
Standard error	0.000	0.419	0.317	0.751	0.446	17.797	57.766

	Untreated	Mock			*Alternaria brassicicola*		
Replicate	0d	0.5 d	1 d	3 d	0.5 d	1 d	3 d
ago1-27 - 1	3.063	0.949	1.248	0.318	30.803	167.730	195.361
ago1-27 - 2	0.671	0.441	1.919	1.202	13.454	266.871	196.039
ago1-27 - 3	0.693	1.469	0.574	0.584	25.457	114.167	235.568
Average	1.476	0.953	1.247	0.701	23.238	182.923	208.990
Standard error	0.794	0.297	0.388	0.262	5.130	44.732	13.291

Supplementary Table SI10: Relative expression values of pri-miRNA827 for each replicate. Average and standard error are displayed in Figure 15A.

	Untreated	Mock			*Alternaria brassicicola*		
Replicate	0d	0.5 d	1 d	3 d	0.5 d	1 d	3 d
WT - 1	1.000	0.722	0.693	0.787	2.370	3.959	13.086
WT - 2	1.000	0.398	0.629	0.238	1.945	2.189	9.747
WT - 3	1.000	0.877	0.753	0.618	3.084	6.453	18.001
Average	1.000	0.665	0.691	0.548	2.467	4.200	13.612
Standard error	0.000	0.141	0.036	0.162	0.332	1.237	2.397

Supplementary Table SI11: Relative expression values of *NLA* for each replicate. Average and standard error are displayed in Figure 15A.

	Untreated	Mock			*Alternaria brassicicola*		
Replicate	0d	0.5 d	1 d	3 d	0.5 d	1 d	3 d
WT - 1	1.000	0.815	0.695	0.644	0.821	0.622	0.500
WT - 2	1.000	0.685	1.091	0.648	1.133	0.669	0.518
WT - 3	1.000	0.660	0.871	0.618	0.983	0.904	0.590
Average	1.000	0.720	0.885	0.637	0.979	0.732	0.536
Standard error	0.000	0.048	0.114	0.010	0.090	0.087	0.028

	Untreated	Mock			*Alternaria brassicicola*		
Replicate	0d	0.5 d	1 d	3 d	0.5 d	1 d	3 d
ago1-27 - 1	1.505	0.595	0.631	0.457	0.782	0.705	1.185
ago1-27 - 2	1.053	0.509	0.657	0.697	1.053	0.841	1.214
ago1-27 - 3	1.028	0.664	0.853	0.693	0.785	0.796	0.651
Average	1.196	0.589	0.714	0.616	0.873	0.780	1.017
Standard error	0.155	0.045	0.070	0.079	0.090	0.040	0.183

Supplementary Table SI12: Relative expression values of *VPT1* for each replicate. Average and standard error are displayed in Figure 15A.

	Untreated	Mock			*Alternaria brassicicola*		
Replicate	0d	0.5 d	1 d	3 d	0.5 d	1 d	3 d
WT - 1	1.000	0.826	1.185	0.976	0.959	3.605	2.612
WT - 2	1.000	0.785	1.945	1.017	0.737	2.799	2.497
WT - 3	1.000	0.631	1.137	0.997	0.807	2.227	2.751
Average	1.000	0.747	1.422	0.997	0.834	2.877	2.620
Standard error	0.000	0.060	0.262	0.012	0.066	0.400	0.074

	Untreated	Mock			*Alternaria brassicicola*		
Replicate	0d	0.5 d	1 d	3 d	0.5 d	1 d	3 d
ago1-27 - 1	1.003	0.481	1.042	1.098	1.266	4.579	5.521
ago1-27 - 2	0.497	0.473	1.324	0.963	1.141	4.823	4.908
ago1-27 - 3	0.844	0.635	1.693	1.292	1.619	3.160	4.070
Average	0.781	0.530	1.353	1.118	1.342	4.187	4.833
Standard error	0.150	0.053	0.189	0.096	0.143	0.518	0.421

5.4 Oligonucleotides used for cloning and qPCR analysis

Supplementary Table SI13: Oligonucleotides used for cloning of the short tandem target mimicry constructs.

Name	Sequence 5'-to-3'	miRNA target
STTM156/157 F1	TCACTCCTGTCTTCTGTCAGTTGTTGTTGTTATGGTC	miR156/157
STTM156/157 R1	AGAAGACTTGAGTGAGCACATTCTTCTTCTTTAGAC	miR156/157
STTM156/157 F2	GTGCTCACTCCTGTCTTCTG	miR156/157
STTM156/157 R2	TGACAGAAGACTTGAGTGAGCA	miR156/157
STTM158 F1	TGTCTCAGACATTTGGGGGTTGTTGTTGTTATGGTC	miR158
STTM158 R1	ATTGTGACAGACAAAGCAATTCTTCTTCTTTAGACCA	miR158
STTM158 F2	TGCTTTGTCTCAGACATTTG	miR158
STTM158 R2	CCCCAAATGTGACAGACAAAG	miR158
STTM160 F1	ATACAGGAGAGAGCCAGGCAGTTGTTGTTGTTATGGTC	miR160
STTM160 R1	TGGCTCGGACCTGTATGCCAATTCTTCTTCTTTAGACCA	miR160
STTM160 F2	GGTCTCGTGCATGGCATACAGGAGAGAGCCAG	miR160
STTM160 R2	GGTCTCGTGTTTGCCTGGCTCGGACCTGT	miR160
STTM161 F1	GATGTATCGGTCACTTTCAGTTGTTGTTGTTATGGTC	miR161
STTM161 R1	GTGACGATTACATCGGGGTATTCTTCTTCTTTAGACCA	miR161
STTM161 F2	GGTCTCGTGCAACCCCGATGTATCGGTCACT	miR161
STTM161 R2	GGTCTCGTGTTTGAAAGTGACGATTACATC	miR161
STTM162 F1	TGCAGATACGGTTTATCGAGTTGTTGTTGTTATGGTC	miR162
STTM162 R1	ACCCATTCTGCATCCAGATTCTTCTTCTTTAGACCA	miR162
STTM162 F2	GGTCTCGTGCACTGGATGCAGATACGGTTTATC	miR162
STTM162 R2	GGTCTCGTGTTTCGATAAACCCATTCTGCATC	miR162
STTM163 F1	AGTTCCAAGCGATCCTCTTCAAGTTGTTGTTGTTATGGTC	miR163
STTM163 R1	GAATCCTTGGAACTTCGATATTCTTCTTCTTTAGACCA	miR163
STTM163 F2	ATCGAAGTTCCAAGCGATCCTC	miR163
STTM163 R2	TTGAAGAGGAATCCTTGGAACT	miR163
STTM164 F1	GTGCCCGGATGCTTCTCCAGTTGTTGTTGTTATGGTC	miR164
STTM164 R1	AAGCAAGCGGGCACGTGCAATTCTTCTTCTTTAGACCA	miR164
STTM164 F2	GGTCTCGTGCATGCACGTGCCCGGATGCT	miR164
STTM164 R2	GGTCTCGTGTTTGGAGAAGCAAGCGGGCACGT	miR164
STTM166 F1	GAGCCAGCTTACAACAGTCCGTTGTTGTTGTTATG	miR166
STTM166 R1	TGTGTATCCTGGCTCGAGGATTCTTCTTCTTTAGA	miR166
STTM166 F2	CCTCGAGCCAGCTTACAACAG	miR166
STTM166 R2	GGACTGTTGTATCCTGGCT	miR166
STTM166 F2	GGTCTCGTGCACCTCGAGCCAGCTTACAACAG	miR166
STTM166 R2	GGTCTCGTGTTGGACTGTTGTATCCTGGCT	miR166
STTM167 F1	TCATGCTATCGGCAGCTTCAGTTGTTGTTGTTATGGTC	miR167
STTM167 R1	CTGCCCTAAGCATGATCTAATTCTTCTTCTTTAGACCA	miR167
STTM167 F2	GGTCTCGTGCATAGATCATGCTATCGGCAG	miR167
STTM167 R2	GGTCTCGTGTTTGAAGCTGCCCTAAGCATGA	miR167

Supplementary Table SI13 (continued): Oligonucleotides used for cloning of the short tandem target mimicry constructs.

Name	Sequence 5'-to-3'	miRNA target
STTM169 F1	CAAGTCACTCTCCTTGGCTAGTTGTTGTTGTTATG	miR169
STTM169 R1	CAAGGAGTCTGACTTGCCTGATTCTTCTTCTTTAGA	miR169
STTM169 F2	GGTCTCGTGCACAGGCAAGTCACTCTCCTTG	miR169
STTM169 R2	GGTCTCGTGTTTAGCCAAGGAGTCTGACTTGCCT	miR169
STTM170 F1	TGACACAGTGGCTCAATCAGTTGTTGTTGTTATGGTC	miR170
STTM170 R1	GAGCCTGAGTGTCAATATCATTCTTCTTCTTTAGACCA	miR170
STTM170 F2	GGTCTCGTGCAGATATTGACACAGTGGCTC	miR170
STTM170 R2	GGTCTCGTGTTTGATTGAGCCTGAGTGTCA	miR170
STTM171 F1	TGGCGCACTGGCTCAATCAGTTGTTGTTGTTATGGTC	miR171
STTM171 R1	TGAGCCTAAGCGCCAATATCATTCTTCTTCTTTAGACCA	miR171
STTM171 F2	GGTCTCGTGCAGATATTGGCGCACTGGCTC	miR171
STTM171 R2	GGTCTCGTGTTTGATTGAGCCTAAGCGCCA	miR171
STTM172 F1	AGCATCAGGGTCAAGATTCTGTTGTTGTTGTTATG	miR172
STTM172 R1	TCTTGAAACTGATGCTGCAGATTCTTCTTCTTTAGA	miR172
STTM172 F2	GGTCTCGTGCACTGCAGCATCAGGGTCAAGATTC	miR172
STTM172 R2	GGTCTCGTGTTAGAATCTTGAAACTGATGCTGCA	miR172
STTM173 F1	CTTACAACGCAGAGAATCAGTTGTTGTTGTTATGGTC	miR173
STTM173 R1	TCTGGTATGTAAGCGAAAATTCTTCTTCTTTAGACCA	miR173
STTM173 F2	GGTCTCGTGCATTTCGCTTACAACGCAGAG	miR173
STTM173 R2	GGTCTCGTGTTTGATTCTCTGGTATGTAAGCGA	miR173
STTM319 F1	AGCTCCCGCATTCAGTCCAAGTTGTTGTTGTTATGGTC	miR319
STTM319 R1	ACTGAAAATGGGAGCTCCCTATTCTTCTTCTTTAGAC	miR319
STTM319 F2	GGTCTCGTGCAAGGGAGCTCCCGCATTCAGTC	miR319
STTM319 R2	GGTCTCGTGTTTTGGACTGAAAATGGGAGCT	miR319
STTM391 F1	GCTATCTTACCTCCTGCGAAGTTGTTGTTGTTATGGTC	miR391
STTM391 R1	AGGAGCATAGATAGCGCCAATTCTTCTTCTTTAGACCA	miR391
STTM391 F2	GGTCTCGTGCATGGCGCTATCTTACCTCCTG	miR391
STTM391 R2	CAGAGCACAATTCGCAGGAGCATAGATAG	miR391
STTM393 F	GGTCTCGTGCAGGATCAATGCGAGTCTCCCT	miR393
STTM393 R	GGTCTCGTGTTTCCAAAGGGAATTTCGCATTG	miR393
STTM396 F1	CAAGAATCTAGCTGTGGAAGTTGTTGTTGTTATGGTC	miR396
STTM396 R1	AGCTACATTCTTGAACTTATTCTTCTTCTTTAGACCA	miR396
STTM396 F2	GGTCTCGTGCACAGTTCAAGAATCTAGCTGTG	miR396
STTM396 R2	GGTCTCGTGTTTTCCACAGCTACATTCTTG	miR396
STTM397 F1	ACGCTGTGACACTCAATGAGTTGTTGTTGTTATGGTC	miR397
STTM397 R1	GAGTGAGTCAGCGTTGATGATTCTTCTTCTTTAGACCA	miR397
STTM397 F2	GGTCTCGTGCACATCAACGCTGTGACACTC	miR397
STTM397 R2	GGTCTCGTGTTTCATTGAGTGAGTCAGCG	miR397

Supplementary Information SI14: Synthesized sequence of pOp6.

5’-GGTCTCGTGCACTAGCTGTAGTTGTAGAATGTAAAATGTAATGTTGTTGTTGTTTGTTG
TTGTTGTTGGTAATTGTTGTAAAAATACGCGCGTCTAGCTTCAGCGTGTCCTCTCCAAAT
GAAATGAACTTCCTTATATAGAGGAAGGGTCTTGCGAAGATCGATCCACTAGTCTTTCAA
TTGTGAGCGCTCACAATTCTTTCTCTTCCCTTTCTTCTTTCTAGTCTAGTCTTTCAATTGT
GAGCGCTCACAATTCTTTCTCTTCCCTTTCTTCTTTCTAGTCTAGTCTTTCAATTGTGAGC
GCTCACAATTCTTTCTCTTCCCTTTCTTCTTTCTAGTCTAGTCTTTCAATTGTGAGCGCTC
ACAATTCTTTCTCTTCCCTTTCTTCTTTCTAGTCTAGTCTTTCAATTGTGAGCGCTCACAA
TTCTTTCTCTTCCCTTTCTTCTTTCTAGTCTAGTCTTTCAATTGTGAGCGCTCACAATTCTT
TCTCTTCCCTTTCTTCTTTCTAGTCTTTCAATTGTGAGCGCTCACAATTCTTTCTCTTCCC
TTTCTTCTTTCTAGCTCCACCGCGGTGGCGGCCGGCCGCTCTAGTGGATCGATCTTCGC
AAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACACGCTGAAGCTAGA
CGCGCGTATTTTTACAACAATTACCAACAACAACAACAAACAACAACAACATTGCCACGA
GACC-3’

Supplementary Table SI15: Oligonucleotides used for cloning of inducible vector system.

Name	Sequence 5'-to-3'	Template vector
1 35S-F1	tggttagagaggcttacgcagcaggtttcatcaagacgatc	pGWB641 (Nakamura et al., 2010)
1 35S-F2	aaagaatgctaacccacagatggttagagaggcttacgcag	
1 35S-F3	attagcctttcaatttcagaaagaatgctaacccacagatg	
1 35S-F4	ctgcaggtcccagattagccttttcaatttcag	
1 35S-F5	ggtctctcgccgaatatatatatattcctgcaggtcccagattagcc	
1 35S-R	ggtctcggctcagtcccccgtgttctctccaaatg	
2 LhGR-Ter-R	ggtctcgtcgagaatatatatatattctaatagtggcaaatttcttgtg	pOpOff2(hyg) (Wielopolska et al., 2005)
2 LhGR-Ter-F	ggtctcggagcatggctagtgaagctcgaaaaac	
3 nos terminator-F	ggtctcggaaagagctcgaatttcccgatc	pGWB641 (Nakamura et al., 2010)
3 nos terminator-R	ggtctcgtcgagaatatatatattcagttagctcactcattaggcac	
4 eYFP-F	ggtctcggccaatggtgagcaagggcgagg	pGWB641 (Nakamura et al., 2010)
4 eYFP-R	ggtctcgtttcctaagccttgtacagctcgtc	
4 GUS-F	ggtctcggccaatggtacgtcctgtagaaac	pGWB633 Nakamura et al., 2010)
4 GUS-R	ggtctcgtttctcattgtttgcctccctgctg	
4 LUC-F	ggtctcggccaatggaagacgccaaaaacataaag	Manavella et al. (2012)
4 LUC-R	ggtctcgtttcctacaatttggactttccgc	
4 universal amiRNA A + BsaI-F	ggtctcggccacaaacacacgctcggacgcatattac	pRS3000 (Schwab et al., 2006)
4 universal amiRNA B + BsaI-R	ggtctcgtttccatggcgatgccttaaataaagataaa	

Supplementary Table SI15 (continued): Oligonucleotides used for cloning of inducible vector system**.**

Name	Sequence 5'-to-3'	Template vector
6 eYFP-F	ggtctcgtgcaatggtgagcaagggcgagg	pGWB641 (Nakamura et al., 2010)
6 eYFP-R	ggtctcgtgttctaagccttgtacagctcgtc	
6 GUS-F	ggtctcgtgcaatggtacgtcctgtagaaac	pGWB641 (Nakamura et al., 2010)
6 GUS-R	ggtctcgtgtttcattgtttgcctccctgctg	
6 universal amiRNA A + BsaI-F	ggtctcgtgcacaaacacacgctcggacgcatattac	pRS3000 (Schwab et al., 2006)
6 universal amiRNA B + BsaI-R	ggtctcgtgttcatggcgatgccttaaataaagataaa	
6 LUC-F	ggtctcgtgcaatggaagacgccaaaaacataaag	Manavella et al. (2012)
6 LUC-R	ggtctcgtgttctacaatttggactttccgc	
7 ocs terminator-F	ggtctcgaacactagtccctagagtcctgct	pEarleyGate102 (Earley et al., 2006)
7 ocs terminator-R	ggtctcgctatgaatatatatattctcctgctgagcctcgacatgt	
8 lacZ-NotI	agtgcggccgcttgccgccagagaccaccggtg	pFUS_A; Addgene plasmid #31028 (Cermak et al., 2011)
8 lacZ-SalI	tcagtcgacgtggctatcgagaccggcgccg	

Supplementary Table SI16: Oligonucleotides used for cloning of artificial microRNAs.

Name	Sequence 5'-to-3'	Target gene	amiRNA sequence 5'-to-3'	Reference
universal amiRNA A + BsaI-F Modul 6	ggtctcgTGCAcaaacacacgctcg			Modified with BsaI sites; Schwab et al. (2006)
universal amiRNA B + BsaI-R Modul 6	gacgcatattac ggtctcgTGTTcatggcgatgcctta aataaagataaa			
amiR-MET1-1 I miR-s	gaTAAAATCGATGATTGCC GCGCtctctctttgtattcc			
amiR-MET1-1 II miR-a	gaGCGCGGCAATCATCGAT TTTAtcaaagagaatcaatga	MET1	TAAAATCGATGATTGCCGCGC	
amiR-MET1-1 III miR*s	gaGCACGGCAATCATGGAT TTTTtcacaggtcgtgatatg			
amiR-MET1-1 IV miR*a	gaAAAAATCCATGATTGCC GTGCtctacatatatattcct			
amiR-MET1-2 I miR-s	gaTATCATTCGCCTGTAAC CCCGtctctctttgtattcc			
amiR-MET1-2 II miR-a	gaCGGGGGTTACAGGCGAA TGATAtcaaagagaatcaatga	MET1	TATCATTCGCCTGTAACCCCG	
amiR-MET1-2 III miR*s	gaCGAGGTTACAGGCCAAT GATTtcacaggtcgtgatatg			
amiR-MET1-2 IV miR*a	gaAATCATTGGCCTGTAAC CTCGtctacatatatattcct			
amiR-MET1-3 I miR-s	gaTCAGTAATACTCTGTTG GCTAtctctctttgtattcc			
amiR-MET1-3 II miR-a	gaTAGCCAACAGAGTATTA CTGAtcaaagagaatcaatga	MET1	TCAGTAATACTCTGTTGGCTA	
amiR-MET1-3 III miR*s	gaTAACCAACAGAGTTTTA CTGTtcacaggtcgtgatatg			
amiR-MET1-3 IV miR*a	gaACAGTAAAACTCTGTTG GTTAtctacatatatattcct			

Supplementary Table SI16 (continued): Oligonucleotides used for cloning of artificial microRNAs.

Name	Sequence 5'-to-3'	Target gene	amiRNA sequence 5'-to-3'	Reference
amiR-DRM1-1 I miR-s	gaTAATTGGAAATTGTCAG TCGCtctctctttgtattcc	DRM1	TAATTGGAAATTGTCAGTCGC	
amiR-DRM1-1 II miR-a	gaGCGACTGACAATTTCCA ATTAtcaaagagaatcaatga			
amiR-DRM1-1 III miR*s	gaGCAACTGACAATTACCA ATTTtcacaggtcgtgatatg			
amiR-DRM1-1 IV miR*a	gaAAATTGGTAATTGTCAG TTGCtctacatatattcct			
amiR-DRM1-2 I miR-s	gaTGATTATGGATATACCC GCTCtctctctttgtattcc	DRM1	TGATTATGGATATACCCGCTC	
amiR-DRM1-2 II miR-a	gaGAGCGGGTATATCCATA ATCAtcaaagagaatcaatga			
amiR-DRM1-2 III miR*s	gaGAACGGGTATATCGATA ATCTtcacaggtcgtgatatg			
amiR-DRM1-2 IV miR*a	gaAGATTATCGATATACCC GTTCtctacatatattcct			
amiR-DRM2-1 I miR-s	gaTAGATTTTCGACCCTAC GCTGtctctctttgtattcc	DRM2	TAGATTTTCGACCCTACGGCTG	
amiR-DRM2-1 II miR-a	gaCAGCGTAGGGTCGAAAA TCTAtcaaagagaatcaatga			
amiR-DRM2-1 III miR*s	gaCAACGTAGGGTCGTAAA TCTTtcacaggtcgtgatatg			
amiR-DRM2-1 IV miR*a	gaAAGATTTACGACCCTAC GTTGtctacatatattcct			

Supplementary Table SI16 (continued): Oligonucleotides used for cloning of artificial microRNAs.

Name	Sequence 5‘-to-3‘	Target gene	amiRNA sequence 5‘-to-3‘	Reference
amiR-DRM2-2 I miR-s	gaTTCTATGTGTAATGAGT GCGGtctctctttttgtattcc	DRM2	TTCTATGTGTAATGAGTGCGG	
amiR-DRM2-2 II miR-a	gaCCGCACTCATTACACAT AGAAtcaaagagaatcaatga			
amiR-DRM2-2 III miR*s	gaCCACACTCATTACTCAT AGATtcacaggtcgtgatatg			
amiR-DRM2-2 IV miR*a	gaATCTATGAGTAATGAGT GTGGtctacatatattcct			
amiR-CMT3-endo I miR-s	gaTGGGTGGTGATCATATA AGATtctctctttttgtattcc	CMT3	TGGGTGGTGATCATATAAGAT (endogenous miRNA823)	
amiR-CMT3-endo II miR-a	gaATCTTATATGATCACCAC CCAtcaaagagaatcaatga			
amiR-CMT3-endo III miR*s	gaATATTATATGATCTCCAC CCTtcacaggtcgtgatatg			
amiR-CMT3-endo IV miR*a	gaAGGGTGGAGATCATATA ATATTctacatatattcct			
amiR-CMT3-1 I miR-s	gaTAAGGTCTATAAAACCG CCGAtctctctttttgtattcc	CMT3	TAAGGTCTATAAAACCGCCGA	
amiR-CMT3-1 II miR-a	gaTCGGCGCGGTTTTATAGAC CTTAtcaaagagaatcaatga			
amiR-CMT3-1 III miR*s	gaTCAGCGGTTTTATTGAC CTTTtcacaggtcgtgatatg			
amiR-CMT3-1 IV miR*a	gaAAAGGTCAATAAAACCG CTGAtctacatatattcct			

Supplementary Table SI16 (continued): Oligonucleotides used for cloning of artificial microRNAs.

Name	Sequence 5‘-to-3‘	Target gene	amiRNA sequence 5‘-to-3‘	Reference
amiR-CMT3-2 I miR-s	gaTATAACTTTTTTAGGGAC CCCGtctctctttgtattcc	CMT3	TATAACTTTTTTAGGGACCCCG	
amiR-CMT3-2 II miR-a	gaCGGGGTCCCTAAAAAGT TATAtcaaagaaatcaatga			
amiR-CMT3-2 III miR*s	gaCGAGGTCCCTAAATAGT TATTtcacaggtcgtgatatg			
amiR-CMT3-2 IV miR*a	gaAATAACTATTTTAGGGAC CTCGtctacatatatattcct			
amiR-trichome I miR-s	gaTCCCATTCGATACTGCT CGCCtctctctttgtattcc	TRY, CPC, ETC2	TCCCATTCGATACTGCTCGCC	Schwab et al. (2006)
amiR-trichome II miR-a	gaGGCGAGCAGTATCGAAT GGGAtcaaagagaatcaatga			
amiR-trichome III miR*s	gaGGAGAGCAGTATCCAAT GGGTtcacaggtcgtgatatg			
amiR-trichome IV miR*a	gaACCCATTGGATACTGCT CTCCtctacatatatattcct			
amiR-lfy-1 I miR-s	gaTAACAGTGAACGTACTG TCGCtctctctttgtattcc	LFY	TAACAGTGAACGTACTGTCGC	Schwab et al. (2006)
amiR-lfy-1 II miR-a	gaGCGACAGTACGTTCACT GTTAtcaaagagaatcaatga			
amiR-lfy-1 III miR*s	gaGCAACAGTACGTTGACT GTTTtcacaggtcgtgatatg			
amiR-lfy-1 IV miR*a	gaAAACAGTCAACGTACTG TTGCtctacatatatattcct			

Supplementary Table SI16 (continued): Oligonucleotides used for cloning of artificial microRNAs.

Name	Sequence 5‘-to-3‘	Target gene	amiRNA sequence 5‘-to-3‘	Reference
amiR-ft-2 I miR-s	gaTTGGTTATAAAGGAAGA GGCCtctctctttgtattcc	FT	TTGGTTATAAAGGAAGAGGCC	Schwab et al. (2006)
amiR-ft-2 II miR-a	gaGGCCTCTTCCTTTATAA CCAAtcaaagagaatcaatga			
amiR-ft-2 III miR*s	gaGGACTCTTCCTTTTTAAC CATtcacaggtcgtgatatg			
amiR-ft-2 IV miR*a	gaATGGTTAAAAAGGAAGA GTCCtctacatatattcct			
amiR-white-2 I miR-s	gaTTTAACCAGATTTTGCGT CGCtctctctttgtattcc	GUN4	TTTAACCAGATTTTGCGTCGC	Schwab et al. (2006)
amiR-white-2 II miR-a	gaGCGACGCAAAATCTGGT TAAAtcaaagagaatcaatga			
amiR-white-2 III miR*s	gaGCAACGCAAAATCAGGT TAATtcacaggtcgtgatatg			
amiR-white-2 IV miR*a	gaATTAACCTGATTTTGCGT TGCtctacatatattcct			
amiR-GFP I miR-s	gaTATTTGTATAGTTCGTCC GTGtctctctttgtattcc	GFP	TATTTGTATAGTTCGTCCGTG	Grant-Downton et al. (2013)
amiR-GFP II miR-a	gaCACGGACGAACTATACA AATAtcaaagagaatcaatga			
amiR-GFP III miR*s	gaCAAGGACGAACTAAACA AATTtcacaggtcgtgatatg			
amiR-GFP IV miR*a	gaAATTTGTTTAGTTCGTCC TTGtctacatatattcct			

Supplementary Table SI16 (continued): Oligonucleotides used for cloning of artificial microRNAs.

Name	Sequence 5‘-to-3‘	Target gene	amiRNA sequence 5‘-to-3‘	Reference
amiR-LUC I miR-s	gaTTAACGCCCAGCGTTTT CCCGtctctctttgtattcc	LUC	TTAACGCCCAGCGTTTTCCCG	Manavella et al. (2012)
amiR-LUC II miR-a	gaCGGGAAAACGCTGGGC GTTAAtcaaagagaatcaatga			
amiR-LUC III miR*s	gaCGAGAAAACGCTGCGC GTTATtcacaggtcgtgatatg			
amiR-LUC IV miR*a	gaATAACGCGCAGCGTTTT CTCGtctacatatatattcct			

Supplementary Table SI17: Oligonucleotides used for quantitative and semi-quantitative PCR analysis.

Name	Sequence 5'-to-3'	Reference
ACT2-F	CTTGCACCAAGCAGCATGAA	Czechowski et al. (2005)
ACT2-R	CCGATCCAGACACTGTACTTCCTT	Czechowski et al. (2005)
AP2-F	ATACTCCCAATTCAAACCACC	
AP2-R	TCAAGAAGGTCTCATGAGAGG	
ARF16-F	GATCAATTCGATTCCAGTACCT	
ARF16-R	CAAACCTGATGCATCATGAAC	
ARF17-F	AGCACCTGATCCAAGTCCTTCTATG	
ARF17-R	TGGTGAATAGCTGGGGAGGATTTC	
ARF8-F	ATTGGACTCCTTGCTGCTGCTG	
ARF8-R	GTACCTGCGGACACTCGACTCT	
CCS-F	CCACAGCTGGTGTATCAACG	
CCS-R	CATCGGTCTTGTACACCACG	
CSD1-F	CTCAAGCACTTGATTCTTTCC	
CSD1-R	AGACATGCAACCGTTAGTGG	
CSD2-F	CTAACAACATGACACACGGAG	
CSD2-R	GAGGTCATCCTTAAGCTCGTG	
FAMT-F	TCCTCTGGACCGAACACTTTCAC	Ng et al. (2011)
FAMT-R	GTCTTGAAGAGAGTGTTAAAATCGTTGTTTG AAG	Ng et al. (2011)
HAP2C-F	AACTCCGATAAACCGAGCC	
HAP2C-R	TCTTGGTCGTTCTTGTGATGTC	
miR universal-R	GTGCAGGGTCCGAGGT	designed after Varkonyi-Gasic et al. (2007)
miR398 stemloop RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACTTGGGG	designed after Varkonyi-Gasic et al. (2007)
miR398-F	GGGCAGTGTGTTCTCAGGTCA	designed after Varkonyi-Gasic et al. (2007)
MYB33-F	TCCCTTCATTCCAATATTCAG	
MYB33-R	GAGTTTCATCTGCATTTTGTGTG	
NAC2-F	GTACAAAGGTTCCAATGTCA	
NAC2-R	GGACTCGTGGACAAGTCTTT	
NLA-F	ACAATTGTTCTCGTGAATGCCC	Liang et al. (2015)
NLA-R	GAGCATGCTCGTTAAACCATCC	Liang et al. (2015)
PHV-F	ATCAGCATCCTCAGCGTGAT	
PHV-R	TTCCACTGCAGTTGCGTGAA	

Supplementary Table SI17 (continued): Oligonucleotides used for quantitative and semi-quantitative PCR analysis.

Name	Sequence 5'-to-3'	Reference
pri-miR163-F	CCTCTTCAACGACAACGATTTCAACAC	
pri-miR163-R	TGATATGGACTCACTCTCAGGAACCG	
pri-miR163-F splicing	GAGAGTGAGAAAAATAAAGAG	Bielewicz et al. (2013)
pri-miR163-R splicing	AGGATGTTGACACGTGTAAAC	Bielewicz et al. (2013)
pri-miR827-F	CACATGTTGATCATCCTTGTG	
pri-miR827-R	CGAGATTCCAAGAAGCGATG	
PXMT1-F	GATTGGAGGAGACGGTCCTGAGA	Ng et al. (2011)
PXMT1-R	GGCTGAGATCGCCTTGGTCAT	Ng et al. (2011)
SPL3-F	ACGCTTAGCTGGACACAACGAGAGAAG	
SPL3-R	TGGAGAAACAGACAGAGACACAGAGGA	
TCP3-F	CATCCAGTTTATAGCCAAAG	
TCP3-R	ATGGCGAGAATCGGATGAAG	
TUB2-F	GAGCCTTACAACGCTACTCTGTCTGTC	
TUB2-R	ACACCAGACATAGTAGCAGAAATCAAG	
VPT1-F	GTTGGGATCAGCAAGAGCTG	
VPT1-R	AGCCATTCCAAGAGCACTTG	

Author contributions

If not stated explicitly here, I, Stephanie Rausch, performed all experiments and wrote the manuscript with contributions from Dr. Sascha Laubinger.

pCR®8/GW/TOPO® with amiRNA against GFP and luciferase for module 4 and module 6 were cloned by Dr. Corinna Speth (Center for Plant Molecular Biology, University of Tübingen). pENTR™3C-lacZ was constructed by Marilena Ohmer (Center for Plant Molecular Biology, University of Tübingen).

Emese X. Szabó (Center for Plant Molecular Biology, University of Tübingen) analyzed the sRNA as well as the mRNA library and contributed to the uploading of the sequencing data to NCBI/GEO.

Trypan blue staining (Figure 6D) and Northern blotting (Figure 10B) experiments were performed by Henriette Stoy (Center for Plant Molecular Biology, University of Tübingen) and Svenja Saile (Center for Plant Molecular Biology, University of Tübingen), respectively, under my supervision. Margaux Kaster (Center for Plant Molecular Biology, University of Tübingen) performed the experiment shown in Supplementary Figure SI5.

Bibliography

Agorio, A., and Vera, P. (2007). ARGONAUTE4 is required for resistance to Pseudomonas syringae in Arabidopsis. The Plant cell **19,** 3778-3790.

Ahuja, I., Kissen, R., and Bones, A.M. (2012). Phytoalexins in defense against pathogens. Trends in plant science **17,** 73-90.

Akimoto, K., Katakami, H., Kim, H.-J., Ogawa, E., Sano, C.M., Wada, Y., and Sano, H. (2007). Epigenetic inheritance in rice plants. Annals of Botany **100,** 205-217.

Allen, E., Xie, Z., Gustafson, A.M., and Carrington, J.C. (2005). microRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell **121,** 207-221.

Allen, E., Xie, Z., Gustafson, A.M., Sung, G.H., Spatafora, J.W., and Carrington, J.C. (2004). Evolution of microRNA genes by inverted duplication of target gene sequences in Arabidopsis thaliana. Nature genetics **36,** 1282-1290.

Aukerman, M.J., and Sakai, H. (2003). Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. The Plant cell **15,** 2730-2741.

Azevedo, J., Garcia, D., Pontier, D., Ohnesorge, S., Yu, A., Garcia, S., Braun, L., Bergdoll, M., Hakimi, M.A., Lagrange, T., and Voinnet, O. (2010). Argonaute quenching and global changes in Dicer homeostasis caused by a pathogen-encoded GW repeat protein. Genes & development **24,** 904-915.

Baldrich, P., Kakar, K., Siré, C., Moreno, A.B., Berger, A., García-Chapa, M., López-Moya, J.J., Riechmann, J.L., and San Segundo, B. (2014). Small RNA profiling reveals regulation of Arabidopsis miR168 and heterochromatic siRNA415 in response to fungal elicitors. BMC genomics **15,** 1083–1098.

Bari, R., Datt Pant, B., Stitt, M., and Scheible, W.R. (2006). PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. Plant physiology **141,** 988-999.

Baumberger, N., and Baulcombe, D.C. (2005). Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. Proceedings of the National Academy of Sciences of the United States of America **102,** 11928-11933.

Baxter, A., Mittler, R., and Suzuki, N. (2014). ROS as key players in plant stress signalling. Journal of experimental botany **65,** 1229-1240.

Beauclair, L., Yu, A., and Bouche, N. (2010). microRNA-directed cleavage and translational repression of the copper chaperone for superoxide dismutase mRNA in Arabidopsis. The Plant journal : for cell and molecular biology **62,** 454-462.

Berr, A., Menard, R., Heitz, T., and Shen, W.H. (2012). Chromatin modification and remodelling: a regulatory landscape for the control of Arabidopsis defence responses upon pathogen attack. Cellular microbiology **14,** 829-839.

Berr, A., McCallum, E.J., Alioua, A., Heintz, D., Heitz, T., and Shen, W.-H. (2010). Arabidopsis histone methyltransferase SET DOMAIN GROUP8 mediates induction of the jasmonate/ethylene pathway genes in plant defense response to necrotrophic fungi. Plant physiology **154,** 1403-1414.

Bielewicz, D., Kalak, M., Kalyna, M., Windels, D., Barta, A., Vazquez, F., Szweykowska-Kulinska, Z., and Jarmolowski, A. (2013). Introns of plant pri-miRNAs enhance miRNA biogenesis. EMBO reports **14,** 622-628.

Boccara, M., Sarazin, A., Thiebeauld, O., Jay, F., Voinnet, O., Navarro, L., and Colot, V. (2014). The Arabidopsis miR472-RDR6 Silencing Pathway Modulates PAMP- and Effector-Triggered Immunity through the Post-transcriptional Control of Disease Resistance Genes. PLoS pathogens **10,** e1003883.

Borges, F., and Martienssen, R.A. (2015). The expanding world of small RNAs in plants. Nature Reviews Molecular Cell Biology **16,** 727-741.

Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P., Yamamoto, Y.Y., Sieburth, L., and Voinnet, O. (2008). Widespread translational inhibition by plant miRNAs and siRNAs. Science **320,** 1185-1190.

Brosseau, C., and Moffett, P. (2015). Functional and Genetic Analysis Identify a Role for Arabidopsis ARGONAUTE5 in Antiviral RNA Silencing. The Plant cell **27,** 1742-1754.

Bucher, M. (2007). Functional biology of plant phosphate uptake at root and mycorrhiza interfaces. The New phytologist **173,** 11-26.

Bustos, R., Castrillo, G., Linhares, F., Puga, M.I., Rubio, V., Pérez-Pérez, J., Solano, R., Leyva, A., and Paz-Ares, J. (2010). A central regulatory system largely controls transcriptional activation and repression responses to phosphate starvation in Arabidopsis. PLoS genetics **6,** e1001102.

Cao, M., Du, P., Wang, X., Yu, Y.-Q., Qiu, Y.-H., Li, W., Gal-On, A., Zhou, C., Li, Y., and Ding, S.-W. (2014). Virus infection triggers widespread silencing of host genes by a distinct class of endogenous siRNAs in Arabidopsis. Proceedings of the National Academy of Sciences **111,** 14613-14618.

Cao, X., and Jacobsen, S.E. (2002). Locus-specific control of asymmetric and CpNpG methylation by the DRM and CMT3 methyltransferase genes. Proceedings of the National Academy of Sciences **99,** 16491-16498.

Carbonell, A., Fahlgren, N., Garcia-Ruiz, H., Gilbert, K.B., Montgomery, T.A., Nguyen, T., Cuperus, J.T., and Carrington, J.C. (2012). Functional analysis of three Arabidopsis ARGONAUTES using slicer-defective mutants. The Plant cell **24,** 3613-3629.

Cermak, T., Doyle, E.L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J.A., Somia, N.V., Bogdanove, A.J., and Voytas, D.F. (2011). Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic acids research **39,** e82.

Chan, S.W., Henderson, I.R., Zhang, X., Shah, G., Chien, J.S., and Jacobsen, S.E. (2006). RNAi, DRD1, and histone methylation actively target developmentally important non-CG DNA methylation in arabidopsis. PLoS genetics **2,** e83.

Chen, F., D'Auria, J.C., Tholl, D., Ross, J.R., Gershenzon, J., Noel, J.P., and Pichersky, E. (2003). An Arabidopsis thaliana gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. The Plant journal : for cell and molecular biology **36,** 577-588.

Chinchilla, D., Bauer, Z., Regenass, M., Boller, T., and Felix, G. (2006). The Arabidopsis receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. The Plant cell **18,** 465-476.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J.D., Felix, G., and Boller, T. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. Nature **448,** 497-500.

Chiou, T.J., Aung, K., Lin, S.I., Wu, C.C., Chiang, S.F., and Su, C.L. (2006). Regulation of phosphate homeostasis by MicroRNA in Arabidopsis. The Plant cell **18,** 412-421.

Cho, Y. (2015). How the necrotrophic fungus Alternaria brassicicola kills plant cells remains an enigma. Eukaryotic cell **14,** 335-344.

Cho, Y., Kim, K.H., La Rota, M., Scott, D., Santopietro, G., Callihan, M., Mitchell, T.K., and Lawrence, C.B. (2009). Identification of novel virulence factors associated with signal transduction pathways in Alternaria brassicicola. Molecular microbiology **72,** 1316-1333.

Chung, P.J., Park, B.S., Wang, H., Liu, J., Jang, I.C., and Chua, N.H. (2016). Light-Inducible MiR163 Targets PXMT1 Transcripts to Promote Seed Germination and Primary Root Elongation in Arabidopsis. Plant physiology **170,** 1772-1782.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. The Plant journal : for cell and molecular biology **16,** 735-743.

Craft, J., Samalova, M., Baroux, C., Townley, H., Martinez, A., Jepson, I., Tsiantis, M., and Moore, I. (2005). New pOp/LhG4 vectors for stringent glucocorticoid-dependent transgene expression in Arabidopsis. The Plant journal : for cell and molecular biology **41,** 899-918.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., and Scheible, W.R. (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant physiology **139,** 5-17.

Dangl, J.L., and Jones, J.D. (2001). Plant pathogens and integrated defence responses to infection. Nature **411,** 826-833.

De-La-Peña, C., Rangel-Cano, A., and Alvarez-Venegas, R. (2012). Regulation of disease-responsive genes mediated by epigenetic factors: interaction of Arabidopsis-Pseudomonas. Molecular plant pathology **13,** 388-398.

de Felippes, F.F., and Weigel, D. (2010). Transient assays for the analysis of miRNA processing and function. Plant MicroRNAs: Methods and Protocols**,** 255-264.

Dharmasiri, N., Dharmasiri, S., and Estelle, M. (2005). The F-box protein TIR1 is an auxin receptor. Nature **435,** 441-445.

Dhawan, R., Luo, H., Foerster, A.M., Abuqamar, S., Du, H.N., Briggs, S.D., Mittelsten Scheid, O., and Mengiste, T. (2009). HISTONE MONOUBIQUITINATION1 interacts with a subunit of the mediator complex and regulates defense against necrotrophic fungal pathogens in Arabidopsis. The Plant cell **21,** 1000-1019.

Ding, B., and Wang, G.-L. (2015). Chromatin versus pathogens: the function of epigenetics in plant immunity. Frontiers in plant science **6**.

Dong, Z., Han, M.H., and Fedoroff, N. (2008). The RNA-binding proteins HYL1 and SE promote accurate in vitro processing of pri-miRNA by DCL1. Proceedings of the National Academy of Sciences of the United States of America **105,** 9970-9975.

Dowen, R.H., Pelizzola, M., Schmitz, R.J., Lister, R., Dowen, J.M., Nery, J.R., Dixon, J.E., and Ecker, J.R. (2012). Widespread dynamic DNA methylation in response to biotic

stress. Proceedings of the National Academy of Sciences of the United States of America **109,** E2183-2191.

Duan, Y., Zhou, L., Hall, D.G., Li, W., Doddapaneni, H., Lin, H., Liu, L., Vahling, C.M., Gabriel, D.W., Williams, K.P., Dickerman, A., Sun, Y., and Gottwald, T. (2009). Complete genome sequence of citrus huanglongbing bacterium, 'Candidatus Liberibacter asiaticus' obtained through metagenomics. Molecular plant-microbe interactions : MPMI **22,** 1011-1020.

Earley, K.W., Haag, J.R., Pontes, O., Opper, K., Juehne, T., Song, K., and Pikaard, C.S. (2006). Gateway-compatible vectors for plant functional genomics and proteomics. The Plant journal : for cell and molecular biology **45,** 616-629.

Edgar, R., Domrachev, M., and Lash, A.E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic acids research **30,** 207-210.

El Oirdi, M., El Rahman, T.A., Rigano, L., El Hadrami, A., Rodriguez, M.C., Daayf, F., Vojnov, A., and Bouarab, K. (2011). Botrytis cinerea manipulates the antagonistic effects between immune pathways to promote disease development in tomato. The Plant cell **23,** 2405-2421.

Ellendorff, U., Fradin, E.F., de Jonge, R., and Thomma, B.P. (2009). RNA silencing is required for Arabidopsis defence against Verticillium wilt disease. Journal of experimental botany **60,** 591-602.

Engler, C., Kandzia, R., and Marillonnet, S. (2008). A one pot, one step, precision cloning method with high throughput capability. PloS one **3,** e3647.

Engler, C., Gruetzner, R., Kandzia, R., and Marillonnet, S. (2009). Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. PloS one **4,** e5553.

Fahlgren, N., Montgomery, T.A., Howell, M.D., Allen, E., Dvorak, S.K., Alexander, A.L., and Carrington, J.C. (2006). Regulation of AUXIN RESPONSE FACTOR3 by TAS3 ta-siRNA affects developmental timing and patterning in Arabidopsis. Current biology : CB **16,** 939-944.

Fahlgren, N., Howell, M.D., Kasschau, K.D., Chapman, E.J., Sullivan, C.M., Cumbie, J.S., Givan, S.A., Law, T.F., Grant, S.R., Dangl, J.L., and Carrington, J.C. (2007). High-throughput sequencing of Arabidopsis microRNAs: evidence for frequent birth and death of MIRNA genes. PloS one **2,** e219.

Felix, G., Duran, J.D., Volko, S., and Boller, T. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. The Plant Journal **18,** 265-276.

Fujii, H., Chiou, T.J., Lin, S.I., Aung, K., and Zhu, J.K. (2005). A miRNA involved in phosphate-starvation response in Arabidopsis. Current biology : CB **15,** 2038-2043.

Gilbert, K.B., Fahlgren, N., Kasschau, K.D., Chapman, E.J., Carrington, J.C., and Carbonell, A. (2014). Preparation of Multiplexed Small RNA Libraries From Plants. Bio-protocol **4,** e1275.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annual review of phytopathology **43,** 205-227.

Gohre, V., and Robatzek, S. (2008). Breaking the barriers: microbial effector molecules subvert plant immunity. Annual review of phytopathology **46,** 189-215.

Govrin, E.M., and Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen Botrytis cinerea. Current biology : CB **10,** 751-757.

Grant-Downton, R., Kourmpetli, S., Hafidh, S., Khatab, H., Le Trionnaire, G., Dickinson, H., and Twell, D. (2013). Artificial microRNAs reveal cell-specific differences in small RNA activity in pollen. Current biology : CB **23,** R599-601.

Gray, W.M., del Pozo, J.C., Walker, L., Hobbie, L., Risseeuw, E., Banks, T., Crosby, W.L., Yang, M., Ma, H., and Estelle, M. (1999). Identification of an SCF ubiquitin-ligase complex required for auxin response in Arabidopsis thaliana. Genes & development **13,** 1678-1691.

Guo, H.S., Xie, Q., Fei, J.F., and Chua, N.H. (2005). MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for arabidopsis lateral root development. The Plant cell **17,** 1376-1386.

Halter, T., Imkampe, J., Mazzotta, S., Wierzba, M., Postel, S., Bücherl, C., Kiefer, C., Stahl, M., Chinchilla, D., and Wang, X. (2014). The leucine-rich repeat receptor kinase BIR2 is a negative regulator of BAK1 in plant immunity. Current Biology **24,** 134-143.

Harvey, J.J., Lewsey, M.G., Patel, K., Westwood, J., Heimstadt, S., Carr, J.P., and Baulcombe, D.C. (2011). An antiviral defense role of AGO2 in plants. PloS one **6,** e14639.

Heller, J., and Tudzynski, P. (2011). Reactive oxygen species in phytopathogenic fungi: signaling, development, and disease. Annual review of phytopathology **49,** 369-390.

Hewezi, T., Howe, P., Maier, T.R., and Baum, T.J. (2008). Arabidopsis small RNAs and their targets during cyst nematode parasitism. Molecular plant-microbe interactions : MPMI **21,** 1622-1634.

Hewezi, T., Piya, S., Qi, M., Balasubramaniam, M., Rice, J.H., and Baum, T.J. (2016). Arabidopsis miR827 mediates post-transcriptional gene silencing of its ubiquitin E3 ligase target gene in the syncytium of the cyst nematode Heterodera schachtii to enhance susceptibility. The Plant journal : for cell and molecular biology **88,** 179-192.

Hirsch, J., Lefort, V., Vankersschaver, M., Boualem, A., Lucas, A., Thermes, C., d'Aubenton-Carafa, Y., and Crespi, M. (2006). Characterization of 43 non-protein-coding mRNA genes in Arabidopsis, including the MIR162a-derived transcripts. Plant physiology **140,** 1192-1204.

Hiruma, K., Gerlach, N., Sacristan, S., Nakano, R.T., Hacquard, S., Kracher, B., Neumann, U., Ramirez, D., Bucher, M., O'Connell, R.J., and Schulze-Lefert, P. (2016). Root Endophyte Colletotrichum tofieldiae Confers Plant Fitness Benefits that Are Phosphate Status Dependent. Cell **165,** 464-474.

Hsieh, L.C., Lin, S.I., Shih, A.C., Chen, J.W., Lin, W.Y., Tseng, C.Y., Li, W.H., and Chiou, T.J. (2009). Uncovering small RNA-mediated responses to phosphate deficiency in Arabidopsis by deep sequencing. Plant physiology **151,** 2120-2132.

Hurtado, L., Farrona, S., and Reyes, J.C. (2006). The putative SWI/SNF complex subunit BRAHMA activates flower homeotic genes in Arabidopsis thaliana. Plant molecular biology **62,** 291-304.

Iriti, M., and Faoro, F. (2007). Review of innate and specific immunity in plants and animals. Mycopathologia **164,** 57-64.

Jagadeeswaran, G., Saini, A., and Sunkar, R. (2009). Biotic and abiotic stress down-regulate miR398 expression in Arabidopsis. Planta **229,** 1009-1014.

Janas, M.M., Khaled, M., Schubert, S., Bernstein, J.G., Golan, D., Veguilla, R.A., Fisher, D.E., Shomron, N., Levy, C., and Novina, C.D. (2011). Feed-forward microprocessing and splicing activities at a microRNA-containing intron. PLoS genetics **7,** e1002330.

Jaubert, M., Bhattacharjee, S., Mello, A.F., Perry, K.L., and Moffett, P. (2011). ARGONAUTE2 mediates RNA-silencing antiviral defenses against Potato virus X in Arabidopsis. Plant physiology **156,** 1556-1564.

Jeong, D.H., Thatcher, S.R., Brown, R.S., Zhai, J., Park, S., Rymarquis, L.A., Meyers, B.C., and Green, P.J. (2013). Comprehensive investigation of microRNAs enhanced

by analysis of sequence variants, expression patterns, ARGONAUTE loading, and target cleavage. Plant physiology **162,** 1225-1245.

Jia, F., and Rock, C.D. (2013). MIR846 and MIR842 comprise a cistronic MIRNA pair that is regulated by abscisic acid by alternative splicing in roots of Arabidopsis. Plant molecular biology **81,** 447-460.

Jin, H. (2008). Endogenous small RNAs and antibacterial immunity in plants. FEBS letters **582,** 2679-2684.

Jones-Rhoades, M.W., and Bartel, D.P. (2004). Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Molecular cell **14,** 787-799.

Jones-Rhoades, M.W., Bartel, D.P., and Bartel, B. (2006). MicroRNAS and their regulatory roles in plants. Annual review of plant biology **57,** 19-53.

Jones, J.D., and Dangl, J.L. (2006). The plant immune system. Nature **444,** 323-329.

Kakutani, T., Jeddeloh, J.A., Flowers, S.K., Munakata, K., and Richards, E.J. (1996). Developmental abnormalities and epimutations associated with DNA hypomethylation mutations. Proceedings of the National Academy of Sciences **93,** 12406-12411.

Kant, S., Peng, M., and Rothstein, S.J. (2011). Genetic regulation by NLA and microRNA827 for maintaining nitrate-dependent phosphate homeostasis in arabidopsis. PLoS genetics **7,** e1002021.

Katagiri, F., Thilmony, R., and He, S.Y. (2002). The Arabidopsis thaliana-Pseudomonas syringae interaction. The Arabidopsis Book**,** e0039.

Katiyar-Agarwal, S., Gao, S., Vivian-Smith, A., and Jin, H. (2007). A novel class of bacteria-induced small RNAs in Arabidopsis. Genes & development **21,** 3123-3134.

Katiyar-Agarwal, S., Morgan, R., Dahlbeck, D., Borsani, O., Villegas, A., Jr., Zhu, J.K., Staskawicz, B.J., and Jin, H. (2006). A pathogen-inducible endogenous siRNA in plant immunity. Proceedings of the National Academy of Sciences of the United States of America **103,** 18002-18007.

Kemmerling, B., Schwedt, A., Rodriguez, P., Mazzotta, S., Frank, M., Qamar, S.A., Mengiste, T., Betsuyaku, S., Parker, J.E., Mussig, C., Thomma, B.P., Albrecht, C., de Vries, S.C., Hirt, H., and Nurnberger, T. (2007). The BRI1-associated kinase 1, BAK1, has a brassinolide-independent role in plant cell-death control. Current biology : CB **17,** 1116-1122.

Kepinski, S., and Leyser, O. (2005). The Arabidopsis F-box protein TIR1 is an auxin receptor. Nature **435,** 446-451.

Khan, G.A., Vogiatzaki, E., Glauser, G., and Poirier, Y. (2016). Phosphate deficiency induces the jasmonate pathway and enhances resistance to insect herbivory. Plant physiology **171,** 632-644.

Kim, S., Yang, J.Y., Xu, J., Jang, I.C., Prigge, M.J., and Chua, N.H. (2008). Two cap-binding proteins CBP20 and CBP80 are involved in processing primary MicroRNAs. Plant & cell physiology **49,** 1634-1644.

Kim, Y.J., Zheng, B., Yu, Y., Won, S.Y., Mo, B., and Chen, X. (2011). The role of Mediator in small and long noncoding RNA production in Arabidopsis thaliana. The EMBO journal **30,** 814-822.

Kinoshita, T., and Seki, M. (2014). Epigenetic memory for stress response and adaptation in plants. Plant and Cell Physiology **55,** 1859-1863.

Koch, M.A., Haubold, B., and Mitchell-Olds, T. (2000). Comparative evolutionary analysis of chalcone synthase and alcohol dehydrogenase loci in Arabidopsis, Arabis, and related genera (Brassicaceae). Molecular biology and evolution **17,** 1483-1498.

Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T., and Felix, G. (2004). The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. The Plant cell **16,** 3496-3507.

Kurihara, Y., and Watanabe, Y. (2004). Arabidopsis micro-RNA biogenesis through Dicer-like 1 protein functions. Proceedings of the National Academy of Sciences of the United States of America **101,** 12753-12758.

Kurihara, Y., Takashi, Y., and Watanabe, Y. (2006). The interaction between DCL1 and HYL1 is important for efficient and precise processing of pri-miRNA in plant microRNA biogenesis. RNA **12,** 206-212.

Kwon, H.J., Owa, T., Hassig, C.A., Shimada, J., and Schreiber, S.L. (1998). Depudecin induces morphological reversion of transformed fibroblasts via the inhibition of histone deacetylase. Proceedings of the National Academy of Sciences **95,** 3356-3361.

Laubinger, S., Sachsenberg, T., Zeller, G., Busch, W., Lohmann, J.U., Ratsch, G., and Weigel, D. (2008). Dual roles of the nuclear cap-binding complex and SERRATE in pre-mRNA splicing and microRNA processing in Arabidopsis thaliana. Proceedings of the National Academy of Sciences of the United States of America **105,** 8795-8800.

Law, J.A., and Jacobsen, S.E. (2010). Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nature reviews. Genetics **11,** 204-220.

Lewis, R., Mendu, V., Mcnear, D., and Tang, G. (2009). Roles of microRNAs in plant abiotic stress. In Molecular techniques in crop improvement, S.M. Jain and D.S. Brar, eds (Springer Netherlands), pp. 357-372.

Li, F., Pignatta, D., Bendix, C., Brunkard, J.O., Cohn, M.M., Tung, J., Sun, H., Kumar, P., and Baker, B. (2012). MicroRNA regulation of plant innate immune receptors. Proceedings of the National Academy of Sciences of the United States of America **109,** 1790-1795.

Li, J., Yang, Z., Yu, B., Liu, J., and Chen, X. (2005). Methylation protects miRNAs and siRNAs from a 3'-end uridylation activity in Arabidopsis. Current biology : CB **15,** 1501-1507.

Li, Y., Zhang, Q., Zhang, J., Wu, L., Qi, Y., and Zhou, J.M. (2010). Identification of microRNAs involved in pathogen-associated molecular pattern-triggered plant innate immunity. Plant physiology **152,** 2222-2231.

Liang, G., Ai, Q., and Yu, D. (2015). Uncovering miRNAs involved in crosstalk between nutrient deficiencies in Arabidopsis. Scientific reports **5,** 11813.

Lin, W.Y., Huang, T.K., and Chiou, T.J. (2013). Nitrogen limitation adaptation, a target of microRNA827, mediates degradation of plasma membrane-localized phosphate transporters to maintain phosphate homeostasis in Arabidopsis. The Plant cell **25,** 4061-4074.

Liu, J., Yang, L., Luan, M., Wang, Y., Zhang, C., Zhang, B., Shi, J., Zhao, F.G., Lan, W., and Luan, S. (2015). A vacuolar phosphate transporter essential for phosphate homeostasis in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America **112,** E6571-6578.

Lopez, A., Ramirez, V., Garcia-Andrade, J., Flors, V., and Vera, P. (2011). The RNA silencing enzyme RNA polymerase v is required for plant immunity. PLoS genetics **7,** e1002434.

Lu, Y.T., Li, M.Y., Cheng, K.T., Tan, C.M., Su, L.W., Lin, W.Y., Shih, H.T., Chiou, T.J., and Yang, J.Y. (2014). Transgenic plants that express the phytoplasma effector SAP11 show altered phosphate starvation and defense responses. Plant physiology **164,** 1456-1469.

Lundmark, M., Korner, C.J., and Nielsen, T.H. (2010). Global analysis of microRNA in Arabidopsis in response to phosphate starvation as studied by locked nucleic acid-based microarrays. Physiologia plantarum **140,** 57-68.

Ma, W., and Berkowitz, G.A. (2007). The grateful dead: calcium and cell death in plant innate immunity. Cellular microbiology **9,** 2571-2585.

Ma, X., Nicole, M.C., Meteignier, L.V., Hong, N., Wang, G., and Moffett, P. (2015). Different roles for RNA silencing and RNA processing components in virus recovery and virus-induced gene silencing in plants. Journal of experimental botany **66,** 919-932.

Mallory, A., and Vaucheret, H. (2010). Form, function, and regulation of ARGONAUTE proteins. The Plant cell **22,** 3879-3889.

Mallory, A.C., Bartel, D.P., and Bartel, B. (2005). MicroRNA-directed regulation of Arabidopsis AUXIN RESPONSE FACTOR17 is essential for proper development and modulates expression of early auxin response genes. The Plant cell **17,** 1360-1375.

Manavella, P.A., Hagmann, J., Ott, F., Laubinger, S., Franz, M., Macek, B., and Weigel, D. (2012). Fast-forward genetics identifies plant CPL phosphatases as regulators of miRNA processing factor HYL1. Cell **151,** 859-870.

Martinelli, F., Uratsu, S.L., Albrecht, U., Reagan, R.L., Phu, M.L., Britton, M., Buffalo, V., Fass, J., Leicht, E., and Zhao, W. (2012). Transcriptome profiling of citrus fruit response to huanglongbing disease. PloS one **7,** e38039.

Mason, G.A., Vergara, T.L., and Queitsch, C. (2016). The Mechanistic Underpinnings of an ago1-Mediated, Environmentally Dependent, and Stochastic Phenotype. Plant physiology **170,** 2420-2431.

Mathieu, O., Reinders, J., Čaikovski, M., Smathajitt, C., and Paszkowski, J. (2007). Transgenerational stability of the Arabidopsis epigenome is coordinated by CG methylation. Cell **130,** 851-862.

Matzke, M.A., and Mosher, R.A. (2014). RNA-directed DNA methylation: an epigenetic pathway of increasing complexity. Nature reviews. Genetics **15,** 394-408.

McHale, L., Tan, X., Koehl, P., and Michelmore, R.W. (2006). Plant NBS-LRR proteins: adaptable guards. Genome biology **7,** 212.

Meng, Y., Shao, C., Ma, X., Wang, H., and Chen, M. (2012). Expression-based functional investigation of the organ-specific microRNAs in Arabidopsis. PloS one **7,** e50870.

Mi, S., Cai, T., Hu, Y., Chen, Y., Hodges, E., Ni, F., Wu, L., Li, S., Zhou, H., Long, C., Chen, S., Hannon, G.J., and Qi, Y. (2008). Sorting of small RNAs into Arabidopsis argonaute complexes is directed by the 5' terminal nucleotide. Cell **133,** 116-127.

Moore, I., Galweiler, L., Grosskopf, D., Schell, J., and Palme, K. (1998). A transcription activation system for regulated gene expression in transgenic plants. Proceedings of the National Academy of Sciences of the United States of America **95,** 376-381.

Morel, J.B., Godon, C., Mourrain, P., Beclin, C., Boutet, S., Feuerbach, F., Proux, F., and Vaucheret, H. (2002). Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. The Plant cell **14,** 629-639.

Nakamura, S., Mano, S., Tanaka, Y., Ohnishi, M., Nakamori, C., Araki, M., Niwa, T., Nishimura, M., Kaminaka, H., Nakagawa, T., Sato, Y., and Ishiguro, S. (2010). Gateway binary vectors with the bialaphos resistance gene, bar, as a selection marker for plant transformation. Bioscience, biotechnology, and biochemistry **74,** 1315-1319.

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., and Jones, J.D. (2006). A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science **312,** 436-439.

Ng, D.W., Zhang, C., Miller, M., Palmer, G., Whiteley, M., Tholl, D., and Chen, Z.J. (2011). cis- and trans-Regulation of miR163 and target genes confers natural variation of secondary metabolites in two Arabidopsis species and their allopolyploids. The Plant cell **23,** 1729-1740.

Nurnberger, T., Brunner, F., Kemmerling, B., and Piater, L. (2004). Innate immunity in plants and animals: striking similarities and obvious differences. Immunological reviews **198,** 249-266.

Padmanabhan, C., Zhang, X., and Jin, H. (2009). Host small RNAs are big contributors to plant innate immunity. Current opinion in plant biology **12,** 465-472.

Palma, K., Thorgrimsen, S., Malinovsky, F.G., Fiil, B.K., Nielsen, H.B., Brodersen, P., Hofius, D., Petersen, M., and Mundy, J. (2010). Autoimmunity in Arabidopsis acd11 is mediated by epigenetic regulation of an immune receptor. PLoS pathogens **6,** e1001137.

Pandey, S.P., Shahi, P., Gase, K., and Baldwin, I.T. (2008). Herbivory-induced changes in the small-RNA transcriptome and phytohormone signaling in Nicotiana attenuata. Proceedings of the National Academy of Sciences of the United States of America **105,** 4559-4564.

Pant, B.D., Buhtz, A., Kehr, J., and Scheible, W.R. (2008). MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. The Plant journal : for cell and molecular biology **53,** 731-738.

Pant, B.D., Musialak-Lange, M., Nuc, P., May, P., Buhtz, A., Kehr, J., Walther, D., and Scheible, W.R. (2009). Identification of nutrient-responsive Arabidopsis and rapeseed microRNAs by comprehensive real-time polymerase chain reaction profiling and small RNA sequencing. Plant physiology **150,** 1541-1555.

Park, B.S., Seo, J.S., and Chua, N.H. (2014). NITROGEN LIMITATION ADAPTATION recruits PHOSPHATE2 to target the phosphate transporter PT2 for degradation during the regulation of Arabidopsis phosphate homeostasis. The Plant cell **26,** 454-464.

Park, M.Y., Wu, G., Gonzalez-Sulser, A., Vaucheret, H., and Poethig, R.S. (2005). Nuclear processing and export of microRNAs in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America **102,** 3691-3696.

Pavet, V., Quintero, C., Cecchini, N.M., Rosa, A.L., and Alvarez, M.E. (2006). Arabidopsis displays centromeric DNA hypomethylation and cytological alterations of heterochromatin upon attack by pseudomonas syringae. Molecular plant-microbe interactions : MPMI **19,** 577-587.

Pedras, M.S.C., Chumala, P.B., Jin, W., Islam, M.S., and Hauck, D.W. (2009). The phytopathogenic fungus Alternaria brassicicola: phytotoxin production and phytoalexin elicitation. Phytochemistry **70,** 394-402.

Pfluger, J., and Wagner, D. (2007). Histone modifications and dynamic regulation of genome accessibility in plants. Current opinion in plant biology **10,** 645-652.

Pieterse, C.M., Leon-Reyes, A., Van der Ent, S., and Van Wees, S.C. (2009). Networking by small-molecule hormones in plant immunity. Nature chemical biology **5,** 308-316.

Pieterse, C.M., Van der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S.C. (2012). Hormonal modulation of plant immunity. Annual review of cell and developmental biology **28,** 489-521.

Privalsky, M.L. (1998). Depudecin makes a debut. Proceedings of the National Academy of Sciences **95,** 3335-3337.

Qi, Y., and Mi, S. (2010). Purification of Arabidopsis argonaute complexes and associated small RNAs. Methods Mol Biol **592,** 243-254.

Qi, Y., He, X., Wang, X.J., Kohany, O., Jurka, J., and Hannon, G.J. (2006). Distinct catalytic and non-catalytic roles of ARGONAUTE4 in RNA-directed DNA methylation. Nature **443,** 1008-1012.

Qu, F., Ye, X., and Morris, T.J. (2008). Arabidopsis DRB4, AGO1, AGO7, and RDR6 participate in a DCL4-initiated antiviral RNA silencing pathway negatively regulated by DCL1. Proceedings of the National Academy of Sciences of the United States of America **105,** 14732-14737.

Rajagopalan, R., Vaucheret, H., Trejo, J., and Bartel, D.P. (2006). A diverse and evolutionarily fluid set of microRNAs in Arabidopsis thaliana. Genes & development **20,** 3407-3425.

Ramachandran, V., and Chen, X. (2008). Small RNA metabolism in Arabidopsis. Trends in plant science **13,** 368-374.

Rasmussen, M.W., Roux, M., Petersen, M., and Mundy, J. (2012). MAP Kinase Cascades in Arabidopsis Innate Immunity. Frontiers in plant science **3,** 169.

Ren, G., Chen, X., and Yu, B. (2012). Uridylation of miRNAs by hen1 suppressor1 in Arabidopsis. Current biology : CB **22,** 695-700.

Reyes, J.L., and Chua, N.H. (2007). ABA induction of miR159 controls transcript levels of two MYB factors during Arabidopsis seed germination. The Plant journal : for cell and molecular biology **49,** 592-606.

Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B., and Bartel, D.P. (2002). Prediction of plant microRNA targets. Cell **110,** 513-520.

Rogers, K., and Chen, X. (2013). Biogenesis, turnover, and mode of action of plant microRNAs. The Plant cell **25,** 2383-2399.

Samalova, M., Brzobohaty, B., and Moore, I. (2005). pOp6/LhGR: a stringently regulated and highly responsive dexamethasone-inducible gene expression system for tobacco. The Plant journal : for cell and molecular biology **41,** 919-935.

Sarnowski, T.J., Ríos, G., Jásik, J., Świeżewski, S., Kaczanowski, S., Li, Y., Kwiatkowska, A., Pawlikowska, K., Koźbiał, M., and Koźbiał, P. (2005). SWI3 subunits of putative SWI/SNF chromatin-remodeling complexes play distinct roles during Arabidopsis development. The Plant cell **17,** 2454-2472.

Schommer, C., Palatnik, J.F., Aggarwal, P., Chetelat, A., Cubas, P., Farmer, E.E., Nath, U., and Weigel, D. (2008). Control of jasmonate biosynthesis and senescence by miR319 targets. PLoS biology **6,** e230.

Schwab, R., Speth, C., Laubinger, S., and Voinnet, O. (2013). Enhanced microRNA accumulation through stemloop-adjacent introns. EMBO reports **14,** 615-621.

Schwab, R., Ossowski, S., Riester, M., Warthmann, N., and Weigel, D. (2006). Highly specific gene silencing by artificial microRNAs in Arabidopsis. The Plant cell **18,** 1121-1133.

Seo, H.S., Song, J.T., Cheong, J.J., Lee, Y.H., Lee, Y.W., Hwang, I., Lee, J.S., and Choi, Y.D. (2001). Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. Proceedings of the National Academy of Sciences of the United States of America **98,** 4788-4793.

Shivaprasad, P.V., Chen, H.M., Patel, K., Bond, D.M., Santos, B.A., and Baulcombe, D.C. (2012). A microRNA superfamily regulates nucleotide binding site-leucine-rich repeats and other mRNAs. The Plant cell **24,** 859-874.

Sobkowiak, L., Bielewicz, D., Malecka, E.M., Jakobsen, I., Albrechtsen, M., Szweykowska-Kulinska, Z., and Pacak, A. (2012). The Role of the P1BS Element Containing Promoter-Driven Genes in Pi Transport and Homeostasis in Plants. Frontiers in plant science **3,** 58.

Speth, C., Toledo-Filho, L.A., and Laubinger, S. (2014). Immunoprecipitation-based analysis of protein-protein interactions. Methods Mol Biol **1158,** 175-185.

Spoel, S.H., Koornneef, A., Claessens, S.M., Korzelius, J.P., Van Pelt, J.A., Mueller, M.J., Buchala, A.J., Métraux, J.-P., Brown, R., and Kazan, K. (2003). NPR1 modulates cross-talk between salicylate-and jasmonate-dependent defense pathways through a novel function in the cytosol. The Plant cell **15,** 760-770.

Staal, J., Kaliff, M., Dewaele, E., Persson, M., and Dixelius, C. (2008). RLM3, a TIR domain encoding gene involved in broad-range immunity of Arabidopsis to necrotrophic fungal pathogens. The Plant Journal **55,** 188-200.

Stone, S.L., Hauksdóttir, H., Troy, A., Herschleb, J., Kraft, E., and Callis, J. (2005). Functional analysis of the RING-type ubiquitin ligase family of Arabidopsis. Plant physiology **137,** 13-30.

Sunkar, R., and Zhu, J.K. (2004). Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. The Plant cell **16,** 2001-2019.

Sunkar, R., Kapoor, A., and Zhu, J.K. (2006). Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance. The Plant cell **18,** 2051-2065.

Sunkar, R., Li, Y.F., and Jagadeeswaran, G. (2012). Functions of microRNAs in plant stress responses. Trends in plant science **17,** 196-203.

Szarzynska, B., Sobkowiak, L., Pant, B.D., Balazadeh, S., Scheible, W.R., Mueller-Roeber, B., Jarmolowski, A., and Szweykowska-Kulinska, Z. (2009). Gene structures and processing of Arabidopsis thaliana HYL1-dependent pri-miRNAs. Nucleic acids research **37,** 3083-3093.

Szweykowska-Kulinska, Z., Jarmolowski, A., and Vazquez, F. (2013). The crosstalk between plant microRNA biogenesis factors and the spliceosome. Plant signaling & behavior **8,** e26955.

Takeda, A., Iwasaki, S., Watanabe, T., Utsumi, M., and Watanabe, Y. (2008). The mechanism selecting the guide strand from small RNA duplexes is different among argonaute proteins. Plant & cell physiology **49,** 493-500.

Thomma, B.P., Nelissen, I., Eggermont, K., and Broekaert, W.F. (1999). Deficiency in phytoalexin production causes enhanced susceptibility of Arabidopsis thaliana to the fungus Alternaria brassicicola. The Plant Journal **19,** 163-171.

Thomma, B.P., Eggermont, K., Penninckx, I.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P., and Broekaert, W.F. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. Proceedings of the National Academy of Sciences **95,** 15107-15111.

Toong, Y.C., Schooley, D.A., and Baker, F.C. (1988). Isolation of insect juvenile hormone III from a plant. Nature **333,** 170-171.

Torres, M.A., Jones, J.D., and Dangl, J.L. (2006). Reactive oxygen species signaling in response to pathogens. Plant physiology **141,** 373-378.

Tsuda, K., and Katagiri, F. (2010). Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. Current opinion in plant biology **13,** 459-465.

Van der Biezen, E.A., and Jones, J.D. (1998). Plant disease-resistance proteins and the gene-for-gene concept. Trends in biochemical sciences **23,** 454-456.

van Kan, J.A. (2006). Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends in plant science **11,** 247-253.

Van Wees, S.C., Van Pelt, J.A., Bakker, P.A., and Pieterse, C.M. (2013). Bioassays for assessing jasmonate-dependent defenses triggered by pathogens, herbivorous insects, or beneficial rhizobacteria. Methods Mol Biol **1011,** 35-49.

Várallyay, É., Válóczi, A., Ágyi, Á., Burgyán, J., and Havelda, Z. (2010). Plant virus-mediated induction of miR168 is associated with repression of ARGONAUTE1 accumulation. The EMBO journal **29,** 3507-3519.

Varkonyi-Gasic, E., Wu, R., Wood, M., Walton, E.F., and Hellens, R.P. (2007). Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. Plant methods **3,** 12.

Vaucheret, H., Mallory, A.C., and Bartel, D.P. (2006). AGO1 homeostasis entails coexpression of MIR168 and AGO1 and preferential stabilization of miR168 by AGO1. Molecular cell **22,** 129-136.

Vaucheret, H., Vazquez, F., Crete, P., and Bartel, D.P. (2004). The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. Genes & development **18,** 1187-1197.

Wagner, D., and Meyerowitz, E.M. (2002). SPLAYED, a novel SWI/SNF ATPase homolog, controls reproductive development in Arabidopsis. Current Biology **12,** 85-94.

Walley, J.W., Rowe, H.C., Xiao, Y., Chehab, E.W., Kliebenstein, D.J., Wagner, D., and Dehesh, K. (2008). The chromatin remodeler SPLAYED regulates specific stress signaling pathways. PLoS pathogens **4,** e1000237.

Wang, G.Y., Shi, J.L., Ng, G., Battle, S.L., Zhang, C., and Lu, H. (2011a). Circadian clock-regulated phosphate transporter PHT4;1 plays an important role in Arabidopsis defense. Molecular plant **4,** 516-526.

Wang, X.B., Jovel, J., Udomporn, P., Wang, Y., Wu, Q., Li, W.X., Gasciolli, V., Vaucheret, H., and Ding, S.W. (2011b). The 21-nucleotide, but not 22-nucleotide, viral secondary small interfering RNAs direct potent antiviral defense by two cooperative argonautes in Arabidopsis thaliana. The Plant cell **23,** 1625-1638.

Wang, Y., Hindemitt, T., and Mayer, K.F. (2006). Significant sequence similarities in promoters and precursors of Arabidopsis thaliana non-conserved microRNAs. Bioinformatics **22,** 2585-2589.

Weiberg, A., Wang, M., Lin, F.M., Zhao, H., Zhang, Z., Kaloshian, I., Huang, H.D., and Jin, H. (2013). Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. Science **342,** 118-123.

Willmann, M.R., Endres, M.W., Cook, R.T., and Gregory, B.D. (2011). The functions of RNA-dependent RNA polymerases in Arabidopsis. The Arabidopsis Book **9,** e0146.

Wu, M.F., Tian, Q., and Reed, J.W. (2006). Arabidopsis microRNA167 controls patterns of ARF6 and ARF8 expression, and regulates both female and male reproduction. Development **133,** 4211-4218.

Xie, Z., Kasschau, K.D., and Carrington, J.C. (2003). Negative feedback regulation of Dicer-Like1 in Arabidopsis by microRNA-guided mRNA degradation. Current biology : CB **13,** 784-789.

Xie, Z., Allen, E., Fahlgren, N., Calamar, A., Givan, S.A., and Carrington, J.C. (2005). Expression of Arabidopsis MIRNA genes. Plant physiology **138,** 2145-2154.

Yaeno, T., and Iba, K. (2008). BAH1/NLA, a RING-type ubiquitin E3 ligase, regulates the accumulation of salicylic acid and immune responses to Pseudomonas syringae DC3000. Plant physiology **148,** 1032-1041.

Yan, J., Gu, Y., Jia, X., Kang, W., Pan, S., Tang, X., Chen, X., and Tang, G. (2012a). Effective small RNA destruction by the expression of a short tandem target mimic in Arabidopsis. The Plant cell **24,** 415-427.

Yan, K., Liu, P., Wu, C.-A., Yang, G.-D., Xu, R., Guo, Q.-H., Huang, J.-G., and Zheng, C.-C. (2012b). Stress-induced alternative splicing provides a mechanism for the regulation of microRNA processing in Arabidopsis thaliana. Molecular cell **48,** 521-531.

Yang, Y., Yuan, J.S., Ross, J., Noel, J.P., Pichersky, E., and Chen, F. (2006a). An Arabidopsis thaliana methyltransferase capable of methylating farnesoic acid. Archives of biochemistry and biophysics **448,** 123-132.

Yang, Z., Ebright, Y.W., Yu, B., and Chen, X. (2006b). HEN1 recognizes 21-24 nt small RNA duplexes and deposits a methyl group onto the 2' OH of the 3' terminal nucleotide. Nucleic acids research **34,** 667-675.

Yi, H., and Richards, E.J. (2007). A cluster of disease resistance genes in Arabidopsis is coordinately regulated by transcriptional activation and RNA silencing. The Plant cell **19,** 2929-2939.

Yoon, E.K., Yang, J.H., Lim, J., Kim, S.H., Kim, S.K., and Lee, W.S. (2010). Auxin regulation of the microRNA390-dependent transacting small interfering RNA pathway in Arabidopsis lateral root development. Nucleic acids research **38,** 1382-1391.

Yu, A., Lepere, G., Jay, F., Wang, J., Bapaume, L., Wang, Y., Abraham, A.L., Penterman, J., Fischer, R.L., Voinnet, O., and Navarro, L. (2013). Dynamics and biological relevance of DNA demethylation in Arabidopsis antibacterial defense. Proceedings of the National Academy of Sciences of the United States of America **110,** 2389-2394.

Yu, B., Yang, Z., Li, J., Minakhina, S., Yang, M., Padgett, R.W., Steward, R., and Chen, X. (2005). Methylation as a crucial step in plant microRNA biogenesis. Science **307,** 932-935.

Zhai, J., Jeong, D.H., De Paoli, E., Park, S., Rosen, B.D., Li, Y., Gonzalez, A.J., Yan, Z., Kitto, S.L., Grusak, M.A., Jackson, S.A., Stacey, G., Cook, D.R., Green, P.J., Sherrier, D.J., and Meyers, B.C. (2011). MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, trans-acting siRNAs. Genes & development **25,** 2540-2553.

Zhang, B.H., Pan, X.P., Wang, Q.L., Cobb, G.P., and Anderson, T.A. (2005). Identification and characterization of new plant microRNAs using EST analysis. Cell research **15,** 336-360.

Zhang, W., Gao, S., Zhou, X., Chellappan, P., Chen, Z., Zhang, X., Fromuth, N., Coutino, G., Coffey, M., and Jin, H. (2011a). Bacteria-responsive microRNAs regulate plant innate immunity by modulating plant hormone networks. Plant molecular biology **75,** 93-105.

Zhang, X., Zhao, H., Gao, S., Wang, W.C., Katiyar-Agarwal, S., Huang, H.D., Raikhel, N., and Jin, H. (2011b). Arabidopsis Argonaute 2 regulates innate immunity via miRNA393*-mediated silencing of a Golgi-localized SNARE gene, MEMB12. Molecular cell **42,** 356-366.

Zhang, X., Niu, D., Carbonell, A., Wang, A., Lee, A., Tun, V., Wang, Z., Carrington, J.C., Chang, C.E., and Jin, H. (2014). ARGONAUTE PIWI domain and microRNA duplex structure regulate small RNA sorting in Arabidopsis. Nature communications **5,** 5468.

Zhang, X., Yazaki, J., Sundaresan, A., Cokus, S., Chan, S.W., Chen, H., Henderson, I.R., Shinn, P., Pellegrini, M., Jacobsen, S.E., and Ecker, J.R. (2006). Genome-wide high-resolution mapping and functional analysis of DNA methylation in arabidopsis. Cell **126,** 1189-1201.

Zhang, X.C., Zhang, X.F., Singh, J., Li, D.W., and Qu, F. (2012). Temperature-Dependent Survival of Turnip Crinkle Virus-Infected Arabidopsis Plants Relies on an RNA Silencing-Based Defense That Requires DCL2, AG02, and HEN1. J Virol **86,** 6847-6854.

Zhao, H., Sun, R., Albrecht, U., Padmanabhan, C., Wang, A., Coffey, M.D., Girke, T., Wang, Z., Close, T.J., Roose, M., Yokomi, R.K., Folimonova, S., Vidalakis, G., Rouse, R., Bowman, K.D., and Jin, H. (2013). Small RNA profiling reveals phosphorus deficiency as a contributing factor in symptom expression for citrus huanglongbing disease. Molecular plant **6,** 301-310.

Zhao, N., Ferrer, J.L., Ross, J., Guan, J., Yang, Y., Pichersky, E., Noel, J.P., and Chen, F. (2008). Structural, biochemical, and phylogenetic analyses suggest that indole-3-acetic acid methyltransferase is an evolutionarily ancient member of the SABATH family. Plant physiology **146,** 455-467.

Zhao, Y., Yu, Y., Zhai, J., Ramachandran, V., Dinh, T.T., Meyers, B.C., Mo, B., and Chen, X. (2012). The Arabidopsis nucleotidyl transferase HESO1 uridylates unmethylated small RNAs to trigger their degradation. Current biology : CB **22,** 689-694.

Zhou, C., Zhang, L., Duan, J., Miki, B., and Wu, K. (2005). HISTONE DEACETYLASE19 is involved in jasmonic acid and ethylene signaling of pathogen response in Arabidopsis. The Plant cell **17,** 1196-1204.

Zhou, N., Tootle, T.L., and Glazebrook, J. (1999). Arabidopsis PAD3, a gene required for camalexin biosynthesis, encodes a putative cytochrome P450 monooxygenase. The Plant cell **11,** 2419-2428.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T., and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell **125,** 749-760.

Zurbriggen, M.D., Carrillo, N., and Hajirezaei, M.R. (2010). ROS signaling in the hypersensitive response: when, where and what for? Plant signaling & behavior **5,** 393-396.

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