Arabidopsis Argonaute 2 Regulates Innate Immunity via miRNA393*-Mediated Silencing of a Golgi-Localized SNARE Gene, MEMB12

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SUMMARY

Argonaute (AGO) proteins are critical components of RNA silencing pathways that bind small RNAs and mediate gene silencing at their target sites. We found that Arabidopsis AGO2 is highly induced by the bacterial pathogen Pseudomonas syringae pv. tomato (Pst). Further genetic analysis demonstrated that AGO2 functions in antibacterial immunity. One abundant species of AGO2-bound small RNA is miR393b*, which targets a Golgi-localized SNARE gene, MEMB12. Pst infection downregulates MEMB12 in a miR393b*-dependent manner. Loss of function of MEMB12, but not SYP61, another intracellular SNARE, leads to increased exocytosis of an antimicrobial pathogenesis-related protein, PR1. Overexpression of miR393b* resembles memb12 mutant in resistance responses. Thus, AGO2 functions in antibacterial immunity by binding miR393b* to modulate exocytosis of antimicrobial PR proteins via MEMB12. Since miR393 also contributes to antibacterial responses, miR393/miR393 represent an example of a miRNA/miRNA pair that functions in immunity through two distinct AGOs: miR393* through AGO2 and miR393 through AGO1.

INTRODUCTION

Small RNA (sRNA)-mediated gene silencing has been recognized as an important regulatory mechanism in host immune responses of both plants and animals (Ding, 2010; Padmanaban et al., 2009; Wessner et al., 2010). Plants have evolved multiple levels of immune responses, including basal defense triggered by virulent pathogens in susceptible hosts and resistance (R) gene-mediated resistance activated by avirulent pathogens in resistant hosts. Conserved microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) are recognized by host pattern recognition receptors and activate PAMP-triggered immunity (PTI), whereas pathogen-derived effector proteins, which can attenuate PTI, are recognized by host R proteins and trigger effector-triggered immunity (ETI) (Chisholm et al., 2006; Jones and Dangl, 2006). In Arabidopsis, microRNA393 (miR393) negatively regulates auxin signaling pathways and contributes to PTI (Navarro et al., 2006). Endogenous small interfering RNAs (siRNAs), nat-siRNAATGB2 and AtlsiRNA-1, are induced by the bacterial pathogen Pseudomonas syringae pv. tomato (Pst) DC3000 carrying an effector gene, avrRpt2; these siRNAs play an important role in ETI by targeting negative regulators of the cognate R gene RPS2 signaling pathway (Katiyar-Agarwal et al., 2006, 2007). sRNAs are loaded into Argonaute proteins (AGOs) and silence targets with complementary sequences. Many eukaryotes have evolved functionally diversified AGOs that regulate many cellular processes. Many eukaryotes have evolved functionally diversified AGOs that regulate many cellular processes (Hutvagner and Simard, 2008; Mallory and Vaucheret, 2010). Specific AGOs from Drosophila, Arabidopsis, C. elegans, and Cryphonectria parasitica are essential for antiviral defenses by binding viral sRNAs and silencing viral genomes (Ding, 2010; Harvey et al., 2011). However, function of AGOs in antibacterial immunity is less clear. Arabidopsis encodes ten AGOs (Vaucheret, 2008). AGO1 is primarily associated with miRNAs and mainly regulates plant development and stress adaptations (Mallory and Vaucheret, 2010). AGO1 also contributes to PTI through several stress-related miRNAs (Li et al., 2010b). AGO7 mainly binds miR390 and triggers the generation of trans-acting siRNAs (Montgomery et al., 2008); it is required for the accumulation of AtlsiRNA-1 and contributes to ETI (Katiyar-Agarwal et al., 2007; Li et al., 2010b). AGO4, AGO6, and AGO9 predominantly bind heterochromatic siRNAs from different loci and function in RNA-directed DNA methylation (RdDM) (Havecker et al., 2010; Mallory and Vaucheret, 2010). AGO4 has been linked to antibacterial defense (Agorio and Vera, 2007). Whether these
activities of AGO4 involve sRNAs or RdDM, however, is unknown, because none of the other factors involved in the RdDM pathway has effects on antibacterial immunity (Agorio and Vera, 2007).

To understand the differentiated function of Arabidopsis AGOs in plant immunity, we examined the expression of all the AGO genes in response to bacterial infection. We show that AGO2 is highly induced by Pst and that AGO2 mutation attenuates antibacterial immunity. Profiling of AGO2-bound sRNAs by high-throughput sequencing revealed that one of the most abundant sRNAs is miR393b*, which targets a gene encoding a Golgi-localized, SDS-resistant, soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein, MEMB12. Mutation in MEMB12 but not SYP61, another intracellular SNARE gene, leads to increased exocytosis of the antimicrobial pathogenesis-related protein PR1. miR393 is known to contribute to PTI. Here, we show that miR393* also contributes to immunity, mainly ETI, by modulating secretion of PR1. Thus, we provide an example of a functional pair of miRNA* and miRNA, each of which targets different regulators in host innate immunity through two distinct AGOs: miR393* through AGO2 and miR393 through AGO1.

RESULTS

Arabidopsis AGO2 Is Highly Induced by Bacterial Infection and Plays an Important Role in Innate Immune Responses

To further our understanding of the differentiated function of AGOs in antibacterial immunity, we first examined the expression of all AGO genes in response to the infection of Pst DC3000. Transcripts of AGO2, but not other AGOs, were induced by the virulent strain of Pst carrying an empty vector (EV). This induction was even more pronounced in response to an avirulent strain, Pst (avrRpt2), at 6 and 14 hr postinoculation (hpi) (Figures 1A and S1A). Western blot analysis confirmed that AGO2 protein level was also highly induced by both Pst (EV) and Pst (avrRpt2) (Figure 1B).

To determine whether AGO2 regulates immune responses against bacterial infection, we carried out a bacterial growth assay on a loss-of-function mutant, ago2-1 (Lobbes et al., 2006). ago2-1 displayed enhanced susceptibility to Pst (avrRpt2) and supported 5- to 6-fold more bacterial growth than wild-type (WT) Col-0 at 3 days postinoculation (dpi) (Figure 1C). It also consistently showed moderately enhanced susceptibility to Pst (EV), permitting 3- to 4-fold more bacterial growth than WT (Figure 1C). Of the ten Arabidopsis AGOs, AGO2 is closely related to AGO3 and AGO7. Consistent with previous reports (Katiyar-Agarwal et al., 2007; Li et al., 2010b), an ago7 mutant (zip-1 [Hunter et al., 2003]) was more susceptible to Pst (avrRpt2) but not to Pst (EV) (Figure 1C). However, the effect of ago7 was weaker than that of ago2-1 (p < 0.05). No alteration in bacteria growth was observed in ago3-1 (Figure 1C) (Lobbes et al., 2006).

To test whether AGO2, AGO3, and AGO7 are partially redundant, double and triple mutants were generated. ago2ago7 was generated by crossing the relevant single mutants; ago2ago3 and ago2ago3ago7 were obtained by generating transgenic AGO3 RNAi lines in the background of ago2-1 and ago2ago7, respectively. Since AGO2 (At1g31280) and AGO3 (At1g31290)
are closely linked on chromosome 1, it is impossible to obtain an ago2 ago3 double mutant by crossing the single mutants. The transgenic AGO3 RNAi lines with the lowest AGO3 expression level were selected for functional analysis (Figures S1B and S1C). ago2 ago7 and ago2ago3 ago7 behaved very similarly and showed more susceptibility to Pst (avrRpt2) than ago2 or ago7 single mutants, allowing 9- to 10-fold more bacterial growth than WT plants (Figure 1C), confirming that both AGO2 and AGO7 contribute to ETI. Bacterial growth in ago2 ago3 was similar to that in ago2, which confirmed that ago3 has little effect on the resistance (Figure 1C). Pst (EV) grew to a similar level in the ago2 single, double, and triple mutants, suggesting that only AGO2 is involved in basal defense. The precise definition of basal defense is PTI plus weak ETI, minus effector-triggered susceptibility (Jones and Dangl, 2006). To determine whether AGO2 plays a role in PTI, we performed flg22-mediated protection assay (Zipfel et al., 2004). Inhibition of Pst (EV) growth by flg22 pretreatment was observed in both ago2 and WT plants (Figure S1D). Flg22 treatment also caused growth inhibition of the ago2 mutant to a similar level as with WT (Figure S1E). Furthermore, we still detected clear detection of AGO2 in response to Pst (EV) infection in bak1 mutant (Figure S1F). BAK1 is a common signaling component in PTI. These data suggest that AGO2 may play a minor role, if any, in PTI.

The bacterial effector avrRpt2 is recognized by its cognate R protein RPS2 in Col-0 and triggers local cell death, which is referred to as the hypersensitive response (HR). Under the conditions we used, visible HR occurred at 15 hpi in WT plants, but not in ago2 ago7 and ago2 ago3 ago7 (Figure S1G). A delayed HR (at 17–18 hpi) to Pst (avrRpt2) was observed in ago2 ago7 and ago2 ago3 ago7. Taken together, our results indicate that AGO2 plays an important role in plant innate immunity, especially in ETI against bacterial pathogens.

miR393b* Is Highly Enriched in AGO2

We hypothesized that, as a critical component of the RNAi pathway, AGO2 regulates plant immunity through the action of its bound sRNAs. Therefore, we used Illumina deep sequencing to analyze the AGO2-associated sRNA population after Pst (avrRpt2) and mock treatments using a transgenic AGO2::3HA-AGO2 line (Montgomery et al., 2008). The data set was deposited into NCBI (GSE26161). AGO1-associated sRNA libraries prepared under the same conditions were used as controls. Consistent with previous reports (Mi et al., 2008; Montgomery et al., 2008), AGO2-associated sRNAs were primarily 21 nt in length and had a bias for reads with the 5' -terminal A, whereas AGO1-bound sRNAs were mainly 21 nt miRNAs with 5'-terminal U. Strikingly, among the most abundant AGO2-bound sRNAs (Tables 1 and S1), several miRNA*s, including miR165a*, miR393b*, miR396b*, and miR472*, had more than 1000 reads per million genome-matched sequences (mgs) (Table 1). miRNA*s are sRNA species that pair with corresponding miRNAs within the hairpin structure of the miRNA precursors and are processed into miRNA:miRNA* duplexes by Dicer or Dicer-like proteins. They were often considered as nonfunctional byproducts because they are quickly degraded and are much less abundant than their corresponding miRNAs. However, we observed significant enrichment of several of these miRNA*s in the AGO2-immunoprecipitated (IP) fraction, suggesting that they may be biologically functional.

One of the most abundant miRNA*s that associated with AGO2 was miR393b*, which had more than 4500 reads/mgs in the AGO2-IP fraction after Pst (avrRpt2) treatment, but had only 24 reads/mgs in the AGO1-IP faction (Table 1). Interestingly, its corresponding miR393 was only present in the AGO1-IP but not the AGO2-IP fraction (Table 1). miR393 has been shown to contribute to PTI by silencing auxin receptors (Navarro et al., 2006). Therefore, we were very interested in testing whether miR393b* has a regulatory role like its miRNA partner. Mature miR393a and miR393b have the same sequence, but miR393a* and miR393b* differ in one nucleotide, and miR393b* is much more abundant than miR393a* (4546 versus 25 reads/mgs in the Pst (avrRpt2)-treated AGO2-IP libraries) (Table S1). To confirm that miR393b* and miR393 were incorporated into two different AGO proteins (AGO2 and AGO1, respectively), we performed sRNA northern blot analysis using total RNA from ago mutants and sRNA fractions from the AGO-IPs. Expression of miR393b* was almost abolished in ago2-1, while its corresponding miR393 was reduced in ago1-27, a relatively weak allele (Figure 2A) (Morel et al., 2002). miR393b* was detected only in AGO2- but not AGO1-IP fractions, whereas miR393 appeared only in AGO1- but not AGO2-IP fractions.

### Table 1. miRNA*s Are Highly Enriched in the AGO2-IP Fraction

<table>
<thead>
<tr>
<th>miRNA*</th>
<th>AGO2-IP</th>
<th>AGO1-IP</th>
<th>Total sRNA without IP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mock</td>
<td>Pst (avrRpt2)</td>
<td>Mock</td>
</tr>
<tr>
<td>miR393b*</td>
<td>2621.9</td>
<td>4546.6</td>
<td>0.3</td>
</tr>
<tr>
<td>miR393</td>
<td>0.4</td>
<td>0.5</td>
<td>358.1</td>
</tr>
<tr>
<td>miR165a*</td>
<td>7943.4</td>
<td>15529.2</td>
<td>1.0</td>
</tr>
<tr>
<td>miR165a</td>
<td>0.3</td>
<td>6.3</td>
<td>30890.4</td>
</tr>
<tr>
<td>miR396b*</td>
<td>1196.9</td>
<td>2866.4</td>
<td>188.2</td>
</tr>
<tr>
<td>miR396b</td>
<td>26.3</td>
<td>28.4</td>
<td>4121.9</td>
</tr>
<tr>
<td>miR472*</td>
<td>508.7</td>
<td>1273.7</td>
<td>28.1</td>
</tr>
<tr>
<td>miR472</td>
<td>0.9</td>
<td>2.0</td>
<td>246.9</td>
</tr>
</tbody>
</table>

Normalized miRNA* and miRNA (reads/mgs) from each sRNA library are listed. Total sRNAs without IP from our previous data set (Chellappan et al., 2010; Zhang et al., 2010, 2011) are included as controls. AGO2-associated miRNA*s with more than 1000 reads/mgs are shown. See Table S1 for the full list of miRNA and miRNA* from the AGO-IP deep sequencing data set.

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**Molecular Cell**

AGO2 Regulates Plant Immunity via a miRNA*
These results confirmed that miR393b* and miR393 were bound to distinct AGOs. The level of miR393b* associated with AGO2 was significantly increased after Pst (avrRpt2) infection (Figure 2B), most likely due to the induction of AGO2. These results strongly suggest that miR393b* is functional.

miR393b* Targets MEMB12 Encoding a SNARE Protein

Recent studies in animals suggested that some miRNA*s may have regulatory roles (Ghildiyal et al., 2010; Guo and Lu, 2010; Okamura et al., 2008). However, in vivo functional analyses of these miRNA*s have not been reported. We proposed that miR393b* regulates plant immunity by targeting genes involved in plant defense responses. Therefore, we predicted potential targets of miR393b* (Table S2). Three of the putative targets, At5g50440, At4g19490, and At3g09530, have predicted roles in protein trafficking and secretion. At5g50440 encodes MEMB12, a Golgi-localized SNARE protein (Uemura et al., 2004), but its biological function has not been determined. At4g19490 encodes a Golgi/post-Golgi compartment-localized protein, VPS54, which is the homolog of a subunit of yeast Golgi-associated retrograde protein (GARP)/Vps Fifty Three (VFT) complex involved in retrograde transport from the vacuolar/late endosome compartment to the Golgi apparatus (Conibear and Stevens, 2000; Guermonprez et al., 2008). At3g09530 encodes EXO70H3, which belongs to the large EXO70 gene family and is a subunit of the exocyst complex predicted to be responsible for exocytosis (Li et al., 2010a). The expression of EXO70H3 in leaves is below the detection limit (Li et al., 2010a). Several plasma membrane (PM)-associated proteins involved in cell surface trafficking have been shown to be essential in plant immune responses, which indicates the importance of protein trafficking in plant defense (Bednarek et al., 2010; Collins et al., 2003; Kalde et al., 2007; Kwon et al., 2008). However, proteins responsible for intracellular vesicle transport and the early steps of protein secretion have not been identified in host immune responses. Two of the putative targets, MEMB12 and VPS54, are localized to the Golgi apparatus and have a predicted function in vesicle transport. Mutations in VPS54 and other GARP subunits display a transmission defect through the male gametophyte and could not yield homozygous mutants (Guermonprez et al., 2008), making it
very difficult to test the role of VPS54 in plant defense. Therefore, in this study we focused on the characterization of \( \text{MEMB12} \) — a Golgi-localized SNARE protein — in plant immune responses.

To test experimentally whether \( \text{MEMB12} \) is a real target of \( \text{miR393b}^* \), we first performed \textit{Agrobacterium}-mediated transient coexpression assays in \textit{Nicotiana benthamiana}. A binary construct carrying the Flag-tagged \( \text{MEMB12} \) with the WT \( \text{miR393b}^* \) target site (\( \text{MEMB12-wt} \)) was coexpressed with \( \text{miR393b}^* \) or \( \text{miR395} \), a miRNA that cannot target \( \text{MEMB12} \). The \( \text{MEMB12} \) protein level was downregulated by \( \text{miR393b}^* \) but not \( \text{miR395} \) (Figure 3A). This downregulation was abolished when the \( \text{miR393b}^* \) target site in \( \text{MEMB12} \) was mutated (\( \text{MEMB12-mu} \), with no amino acid change) (Figures 3A and S2). These results indicate that \( \text{miR393b}^* \) is responsible for the downregulation of \( \text{MEMB12} \). The protein level of \( \text{MEMB12} \) was significantly reduced while its transcript level was reduced only slightly, suggesting that \( \text{miR393b}^* \) silences \( \text{MEMB12} \) mainly by translational inhibition. We also generated transgenic \textit{Arabidopsis} carrying \textit{YFP-MEMBER12-wt} or \textit{YFP-MEMBER12-mu} to test the regulatory role of \( \text{miR393b}^* \) in vivo. \( \text{MEMB12-wt} \) protein was downregulated after \textit{Pst (avrRpt2)} treatment, whereas \( \text{MEMB12-mu} \) was not (Figure 3B). The mRNA level of \( \text{MEMB12-wt} \) was not significantly reduced after infection of \textit{Pst (avrRpt2)} (Figure 3B), consistent with the notion that \( \text{miR393b}^* \) mainly mediates translational inhibition rather than mRNA degradation. If \( \text{MEMB12} \) is the target of \( \text{miR393b}^* \), \( \text{MEMB12} \) transcripts should also be associated with \textit{AGO2}, which is the AGO predominantly associated with \( \text{miR393b}^* \). Indeed, \( \text{MEMB12} \) mRNA was enriched in the AGO2-IP fraction but was not detected in the AGO1-IP fraction (Figure 3C). These results supported that \( \text{MEMB12} \) is a real target of \( \text{miR393b}^* \).

**Loss of Function of \text{MEMB12} Promotes Secretion of \text{PR1}**

To test whether \( \text{MEMB12} \) plays a role in antibacterial defense, we isolated the \( \text{MEMB12} \) knockout mutant \textit{memb12-1} (GT22391), which is a transposon-tagged line with an insertion in the first exon (Figures S3A–S3C) (Springer et al., 1995). \textit{memb12-1} had no obvious developmental defects, but displayed enhanced resistance to both avirulent and virulent strains of \textit{Pst}. Growth of \textit{Pst (avrRpt2)} in \textit{memb12-1} was reduced 5– to 6-fold compared with the WT control (Figure 4A). \textit{memb12-1} also showed enhanced resistance to \textit{Pst (EV)} at a greater degree (Figure 4A).

To understand the mechanism of function of \( \text{MEMB12} \) in innate immunity, we analyzed the localization of \( \text{MEMB12} \) by examining the transgenic \textit{YFP-MEMBER12-wt} plants. We observed punctate structures in the cytosol (Figure S3D), which is the characteristic feature of the plant Golgi apparatus (Chatre et al., 2005; Matheson et al., 2006). This observation is in accordance with previously reported \( \text{MEMB12} \) localization to the Golgi
Secretion of antimicrobial proteins or small molecules is essential for effective defense against pathogens (Bednarek et al., 2010; Collins et al., 2003; Kalde et al., 2007; Kwon et al., 2008; Nomura et al., 2006). To examine whether MEMB12 suppresses defense responses by affecting protein secretion, we examined the level of one of the major secreted antimicrobial pathogenesis-related proteins—PR1—in the intercellular wash fluid of memb12-1 before and after bacterial infection as described (Wang et al., 2005). After Pst (avrRpt2) infection, the level of intercellular secreted PR1 was more than 10-fold greater in memb12-1 than in WT (Figure 4B), while total PR1 protein only increased by about 3- to 4-fold in memb12-1 compared with that in WT (Figure 4B). These results indicate that the secretion of PR1 in memb12-1 is indeed increased. Secreted PR1 also accumulated in the mock-treated memb12-1 (Figure 4B). Furthermore, the secretion of PR1 upon Pst (avrRpt2) infection was significantly reduced and delayed in the ago2-1 mutant (Figures 4C, S3E, and S3F), which is consistent with the result that ago2 was more susceptible to Pst (avrRpt2) (Figure 1C).

On the contrary, mutation in the trans-Golgi network (TGN)/endosomal SNARE SYP61 gene did not alter the secretion level of PR1 (Figure 5A) (Robert et al., 2008; Sanderfoot et al., 2001; Sanmartín et al., 2007; Zhu et al., 2002). These results suggest that intracellular SNARE MEMB12 but not SYP61 specifically controls the exocytosis of vesicles containing antimicrobial protein PR1.

To determine whether suppression of MEMB12 interferes with general protein secretion, we also examined the level of another secreted protein: SYP121, a PM-associated syntaxin required for resistance to powdery mildew fungus (Collins et al., 2003). The level of SYP121 was slightly increased in the PM factions in memb12-1 as compared with WT plants (Figure 5B). Mutation in MEMB12 has a much smaller effect on SYP121 secretion than on PR1 secretion, suggesting that MEMB12 is responsible for trafficking of a specific group of proteins, such as PR1.

When massive accumulation and secretion of PR proteins occur during defense responses, a coordinated upregulation of the whole protein secretory machinery is often accompanied to ensure efficient transport (Wang et al., 2005). Indeed, we detected upregulation in memb12-1 of several secretion pathway genes that encode translocon complex Sec61α subunit, cochaperone Calnexin 1, a Clathrin-coat assembly protein, and a Vacuolar sorting receptor VSR6; upregulation occurred even without pathogen challenges (Figure 5C), which suggests that suppression of MEMB12 leads to an upregulation of the secretory pathway for transporting certain proteins. Taken together, these results indicate that MEMB12 controls the exocytosis of vesicles containing antimicrobial protein PR1.
together, our results suggest that miR393b* contributes to AGO2-regulated innate immunity by suppressing MEMB12 and subsequently promoting effective secretion of antimicrobial PR proteins.

miR393b* Overexpression Plants Resemble memb12 Mutant in Disease Resistance Responses

To determine whether overexpression of miR393b* results in a phenotype similar to the loss-of-function mutant of its target, MEMB12, we generated transgenic plants overexpressing the MIR393b gene controlled by the constitutive CaMV 35S promoter and selected the lines that expressed high level of miR393b* for bacterial growth assay and PR1 secretion analysis (Figure S4A). These plants showed enhanced disease resistance to Pst (avrRpt2) (Figure 6A) as well as increased PR1 protein accumulation and secretion (Figure 6B), which nicely resembled the phenotype of memb12-1 in resistance responses (Figure 4).

However, in these MIR393b overexpression plants, miR393 was also produced from the same MIR393b precursor (Figure S4A), which was evidenced by the curly leaf phenotype that is reminiscent of the double and triple mutants of the miR393 targets: tir1/afb2 and tir1/afb2/afb3 (Figures 6C, 5I, and 5J in Dharmasiri et al., 2005). Plants overexpressing MIR393a show enhanced resistance to virulent strain Pst DC3000 but have no obvious effect on Pst (avrRpt2)-triggered ETI (Navarro et al., 2006). Because MIR393a and MIR393b give rise to the same miR393, the positive regulatory effect of MIR393b on Pst (avrRpt2)-triggered ETI is due to the generation of miR393b*. However, it is impossible to tell whether the increased secretion of PR1 is due to the overexpression of miR393b* or miR393 in our MIR393b overexpression lines.

To distinguish the effects of miR393b* from that of miR393 on PR1 secretion, we used Web MicroRNA Designer and made an artificial miR393b* (amiR393b*) construct that produced miR393b* and the reverse complementary strand of miR393b* (referred to as miR393b**), which contains five different nucleotides from miR393 sequences (Figure 6D) (Schwab et al., 2006). The amiR393b* transgenic plants with high expression level of miR393b* were selected and no longer displayed the phenotype of altered auxin signaling (Figures 6C and S4B). Thus, we succeeded in uncoupling the effect of miR393b* and miR393 in these plants. We still observed increased PR1 secretion in these transgenic lines expressing artificial miR393b* (Figure 6E), which resembled the phenotype of memb12. These results support that MEMB12 is indeed one of the major targets of miR393b* and regulates PR1 secretion.

DISCUSSION

miRNA* was once considered as a useless by-product of miRNA biogenesis (Jones-Rhoades et al., 2006; Schwarz et al., 2003). Here, we identified a functional miRNA* that regulates plant immunity along with its cognate miRNA partner. Thus, miRNA genes have the potential to generate more than one functional sRNA under certain conditions. miRNA393* and miRNA393 represent an example of a pair of miRNA* and miRNA that function through different AGOs in vivo and regulate two different cellular pathways in innate immunity. miR393* is loaded into AGO2, targets genes involved in protein trafficking, and regulates plant immune responses, mainly ETI, by promoting exocytosis of PR proteins; miR393 is loaded into AGO1 and contributes to PTI by targeting auxin receptors and suppressing the auxin signaling pathway.
Strand selection of miRNA/miRNA* duplex within AGO complexes is largely determined by asymmetrical thermodynamic stability of the duplex termini (Khvorova et al., 2003; Schwarz et al., 2003). The strand with less stability at its 5’ terminus is incorporated as the guide strand (miRNA), while the other strand (so-called passenger strand or miRNA*) is mostly degraded. However, here we show that different AGO proteins select different guide strands within the same miRNA/miRNA* duplex regardless of their thermodynamic stability. sRNA loading into AGOs in plants is known to conform to the “5’ first nucleotide recognition” model (Mi et al., 2008; Montgomery et al., 2008), in which different AGOs preferentially associate with sRNAs with distinct 5’ first nucleotides. For example, AGO1 prefers uridine (U), AGO2 and AGO4 prefer adenosine (A), and AGO5 prefers cytosine (C). The fact that miR393 and miR393* start with 5’-terminal U and A, respectively, fits this model well. However, the other miRNA*s that were highly enriched in AGO2-IP fraction do not have 5’-terminal A (Table 1), such as miR165a* and miR396b*, which have 5’-terminal G. On the contrary, miR390, which features a 5’-terminal A, binds predominantly to AGO7 but not AGO2 (Montgomery et al., 2008). Thus, there must be other factors that help determine sRNA loading. Although all Arabidopsis AGO proteins contain the three conserved functional PAZ, MID, and PIWI domains, they have very different N-terminal regions. Even within the conserved domains, there are marked differences. For example, AGO2 and AGO3 are the only Arabidopsis AGOs that contain an Asp-Asp-Asp (DDD) motif instead of the conventional Asp-Asp-His (DDH) motif within the PIWI domain. This DDD motif is similar to that in bacterial RNaseH enzymes with cleavage activity (Nowotny et al., 2005; Vaucheret, 2008). It is likely that these sequence differences among AGO proteins may allow their interactions with different cofactors and direct distinct sRNA loading. The finding that the double-stranded RNA-binding protein DRB1 assists AGO1 loading of some miRNAs supports the idea that additional components are required for determination of sRNA binding (Eamens et al., 2009).

Protein secretory systems appear to play an important role in plant defense (Bednarek et al., 2010; Collins et al., 2003; Kalde et al., 2007; Kwon et al., 2008; Nomura et al., 2006). The SNARE proteins that have been identified to be involved in immune...
responses are mostly associated with the PM (Bednarek et al., 2010; Collins et al., 2003; Kalde et al., 2007; Kwon et al., 2008). Here, we identified a previously uncharacterized intracellular SNARE, MEMB12, as a miR393b* target. Upon bacterial infection, suppression of MEMB12 by miR393b* promotes the secretion and accumulation of PR1 protein and contributes to resistance. MEMB12 is mainly localized to the Golgi and mediates protein trafficking between the Golgi and the ER (Uemura et al., 2004). Our results suggest that MEMB12 may be responsible for the retrograde trafficking from the Golgi to the ER for protein recycling and balance maintenance. MEMB12 and its close homolog MEMB11 are homologous to Bos1 in yeast and GS27 or membrin in mammals and regulate retrograde protein transport between Golgi and endoplasmic reticulum (ER) (Bubeck et al., 2008; Chatre et al., 2005), which is consistent with our results that downregulation of MEMB12 promotes PR1 secretion, likely through inhibiting retrograde transport and protein recycling. Mutation in another intracellular SNARE protein, SYP61, had no effect on the secretion of PR1, indicating that not all the SNARE proteins affect secretion of PR proteins. Mutation in MEMB12 has a weak effect on the secretion of the PM-associated syntaxin SYP121, suggesting that different SNARE proteins may be responsible for transporting different sets of proteins. This specificity is also observed in other SNAREs. For example, VT12 but not its close homolog VT11 affects the transport of storage proteins (Sanmartín et al., 2007). These results suggest the specialization of MEMB12 in controlling the secretion of PR proteins for immune responses.

In addition to targeting MEMB12, miR393b* was predicted to target two other proteins involved in trafficking, VPS54 and EXO70H3. Like MEMB12, VPS54 is also Golgi localized and involved in the retrograde trafficking (Conibear and Stevens, 2000; Guermonprez et al., 2008). These results suggest that the immunity-specific secretory pathway is under regulation and fine-tuning by sRNA-mediated RNAi in response to pathogen attacks.

Our work suggests that Arabidopsis employs AGO2 as an important RNAi effector in plant antibacterial immunity. AGO2 also contributes to antiviral defenses against two viruses carrying silencing suppressors that target AGO1 (Harvey et al., 2011). Repression of AGO1 by viral suppressors leads to the downregulation of miR403 and subsequent induction of AGO2. It is unlikely that AGO2 induction by Psst is due to the same mechanism because strong induction of AGO2 by bacterial infection was still observed in ago1-27 mutant (Figure S1H). Furthermore, we did not observe any suppression of miR403 by these bacterial strains (Figure S1I). miR403 targets both AGO2 and AGO3 at their 3′ UTR, but only AGO2 was induced by Psst. We believe that induction of AGO2 by bacteria may mainly occur at the transcriptional level and is mechanistically different from the induction triggered by viruses. Several defense responsive cis-elements, including the W-box motif, elicitor-responsive element, and gibberellin-responsive element, are present in the AGO2 promoter, and future studies will elucidate their functions in AGO2 induction upon bacteria challenges.

AGO2 associates with a large array of sRNAs, among which miR393b* is one of the most abundant. It would be interesting to test whether other AGO2-associated sRNAs and their targets are also involved in plant immunity. We speculate that AGO2 may regulate and coordinate the expression of a group of genes involved in various pathways of plant immunity by binding to a group of functional sRNAs.
Molecular Cell

AGO2 Regulates Plant Immunity via a miRNA

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, four figures, and three tables and can be found with this article online at doi:10.1016/j.molcel.2011.04.010.

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