Identification of miRNAs involved in Pathogen-Associated Molecular Pattern-triggered plant innate immunity

Running title: miRNAs in plant immunity

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Pathogen-associated molecular patterns (PAMPs) trigger plant defenses when perceived by surface-localized immune receptors. PAMP-triggered immunity (PTI) plays a vital role in the resistance of plants to numerous potential pathogens. microRNA(miRNA) biogenesis is known to be important for PTI, but miRNA species involved in this process have not been fully explored. Here we show that the miRNA effector protein, Argonuate1 (AGO1), is required for a number of PTI responses including PAMP-induced callose deposition, gene expression and seedling growth inhibition. Deep-sequencing of AGO1-bound small RNAs led to the identification of a number of miRNAs that are up- or down-regulated by flg22, a well-studied PAMP. Overexpression of selected miRNAs in stable transgenic plants demonstrated that miR160a positively regulate PAMP-induced callose deposition, whereas miR398b and miR773 negatively regulate PAMP-induced callose deposition and disease resistance to bacteria, suggesting a complexity of the miRNA regulation in plant innate immunity.

**Key words:** PAMPs, AGO1, microRNA, innate immunity
Plants are equipped to detect conserved molecular features of microbes, termed Pathogen-Associated Molecular Patterns (PAMPs), and trigger defenses (Zipfel and Felix, 2005). PAMP-triggered immunity (PTI) allows plants to fend off a large number of potential pathogens (Li et al., 2005). For example, flg22, a conserved peptide derived from *Pseudomonas syringae* flagellin (Felix et al., 1999), is perceived by the receptor FLS2 at the plasmamembrane (Gómez-Gómez and Boller, 2000; Chinchilla et al., 2007; Heese et al., 2007) and subsequently activates mitogen activated protein kinases (MPKs), a transient oxidative burst (ROS; Felix et al., 1999), callose (β-1,3-glucan) deposition at the cell wall (Gómez-Gómez et al., 1999; Brown et al., 1998), and the expression of defense-related genes (Zipfel et al., 2004; Zhang et al., 2007).

Many plant pathogens can deliver a variety of effector proteins into the host cell to inhibit PTI signaling (Zhou and Chai, 2008; Göhre and Robatzek, 2008). To counteract, plants have evolved resistance proteins to sense the activity of some of these effectors to activate a second layer of inducible defenses called effector-triggered immunity (ETI; Chisholm et al., 2006; Jones and Dangl, 2006).

In plants, small RNAs including microRNAs (miRNAs) and small interfering RNAs (siRNAs) regulate diverse processes including development (Mallory and Vaucheret, 2006; Jones-Rhoades et al., 2006), abiotic stress tolerance (Fujii et al., 2005; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004), and antiviral defenses (Mourrain et al., 2000; Dalmay et al., 2001; Morel et al., 2002). Several recent studies indicate that small RNAs also participate in plant disease resistance to bacterial pathogens. For example, flg22 induces the accumulation of miR393, which contributes to plant resistance against bacteria by negatively regulating the mRNA level of F-box auxin receptors TIR1, AFB2, and AFB3 (Navarro et al., 2006). Induced accumulation of a natural
antisense transcript-associated siRNA, nat-siRNAATGB2 (Katiyar-Agarwal et al., 2006), and a long siRNA, AtlsiRNA-1 (Katiyar-Agarwal et al., 2007), is required specifically for RPS2-mediated ETI, but not basal resistance to compatible *P. syringae* bacteria. Consistent with a role of these small RNAs in plant immunity, proteins required for small RNA biogenesis and function have been shown to be required for disease resistance to bacterial pathogens. For example, Dicer-like 1 (DCL1) and HEN1, which are required for the biogenesis of both miRNAs and long siRNAs, are required for PTI resistance (Navarro et al., 2008). Likewise, AGO7 is required for the accumulation of AtlsiRNA-1 and RPS2 resistance (Katiyar-Agarwal et al., 2007). In addition, AGO4, which is required for RNA-directed DNA methylation, contributes to resistance nonspecifically to both adapted and nonadapted *P. syringae* through an unknown mechanism (Agorio et al., 2007).

A key component in the miRNA pathway is Argonaute 1 (AGO1), which predominately binds mature miRNAs to form a RNA-induced silencing complex (RISC) in cytoplasm and cleaves the target mRNA through miRNA-mRNA base pairing (Okamura et al., 2004; Baumberger and Baulcombe, 2005; Qi et al., 2005) or represses translation through an association with polysomes (Lanet et al., 2009). AGO1 contains three characteristic domains: PAZ, MID and PIWI (Song and Joshua-Tor, 2006). PIWI domain adopts the structure of RNase H that contains the catalytic site formed by three residues (Asp, Asp and His), and provides a slicer activity that executes the miRNA-guided cleavage of target RNA (Liu et al., 2004; Song et al., 2004; Rivas et al., 2005). Several studies have shown the involvement of AGO1 in plant antiviral defense (Qu et al., 2008; Morel et al., 2002). However, the role for AGO1 in plant defenses to bacterial infection has not been fully explored. More importantly, miRNA species regulating plant disease resistance
remain largely unknown.

Here we show that AGO1 positively regulates PAMP-induced callose deposition, defense gene expression and seedling growth inhibition, and contributes to PAMP-induced disease resistance to *P. syringae*. Deep-sequencing of AGO1-bound small RNAs identified a small number of miRNA species whose accumulation was up- or down-regulated by flg22. Overexpression of selected miRNAs in stable transgenic plants indicated that *miR160a* positively regulates PAMP-induced callose deposition, whereas *miR398b* and *miR773* negatively regulate PAMP-induced callose deposition. Furthermore, *miR398b* and *miR773* overexpression plants showed enhanced susceptibility to both virulent and nonpathogenic strains of *P. syringae*, indicating an important role of these miRNAs in disease resistance.

**RESULTS**

**AGO1 contributes to flg22-induced disease resistance**

Flg22 treatment causes a strong reduction in seedling growth (Gómez-Gómez et al., 1999). The *ago1-25* and *ago1-27* mutants carrying a point mutation in PIWI domain are impaired in post-transcriptional gene silencing (PTGS) and viral resistance (Morel et al., 2002), but do not affect miRNA accumulation (Vaucheret et al., 2004). We tested the effect of *ago1-25* and *ago1-27* mutations on flg22–mediated growth inhibition. After growing in the 1/2 MS liquid medium containing 10 μM flg22 for 5 days, the WT seedlings displayed a significant reduction (60%) in fresh weight compared to control seedlings grown without flg22. The growth of *ago1-25* and *ago1-27* mutants was reduced only slightly (15%~20%) by flg22 treatment (Fig. 1A), indicating that AGO1 is required for flg22-mediated seedling growth inhibition. An examination of
flg22-induced callose deposition showed that the two mutants had significantly reduced callose deposition compared to WT (Fig. 1B). Similarly, callose deposition induced by a nonpathogenic *P. syringae* mutant *hrcC*, which lacks a functional type III secretion apparatus but contains a collection of PAMPs (Yuan and He, 1996), was also compromised in the *ago1* mutants. We examined the expression of *FRK1* and *WRKY29*, two PAMP-response genes (Asai et al., 2002), in *ago1-25* and *ago1-27* plants treated with flg22 and *hrcC* mutant bacteria. The two *ago1* mutants accumulated 50-70% less transcripts compared to that in WT (Figs. 1C-1F), indicating that AGO1 is partially required for flg22-induced gene expression. We also tested if MAP kinase activation and transient oxidative burst, two early events in PTI signaling, were affected in *ago1* mutants. Fig S1 shows that the *ago1-25* mutant had normal MAP kinase activation and oxidative burst in response to flg22 treatment, indicating that early and late PTI signaling events were differentially impacted by the *ago1* mutations. To determine whether AGO1 plays a role in plant resistance to bacteria, we conducted flg22-mediated protection assay on *ago1-27* and *ago1-25* (Zipfel et al., 2004). While pretreatment of WT plants with flg22 inhibited the growth of virulent DC3000 bacteria by approximately 100 fold two days after inoculation, it only slightly inhibited bacterial growth in the two *ago1* mutants (Fig. 1G), indicating that AGO1 plays an important role in flg22-induced resistance to bacteria.

AGO7 was reported to be required for RPS2-specified ETI resistance (Katiyar-Agarwal et al., 2007), but a potential role in PTI defenses has not been investigated. We therefore tested if the *ago7* mutant also impact PTI. The *ago7* mutant (*zip-1*; Hunter et al., 2003), a likely null allele, was completely normal in PTI responses when flg22-induced callose deposition, *FRK1* expression, seedling growth inhibition, and oxidative burst were measured (Fig. S2A-D). Consistent with the
normal PTI responses, \textit{hrcC} mutant bacteria multiplied normally in \textit{ago7} (Fig. S2E), and flg22-pretreatment provided similar protection against the virulent DC3000 bacteria in \textit{ago7} and WT plants (Fig. S2F). In contrast, \textit{ago7} plants showed reduced resistance to DC3000 (\textit{avrRpt2}), confirming previous report (Fig. S2G). Together these results are consistent with a specific role of AGO7 in RPS2 resistance to bacteria.

DCL1 is required for miRNA biogenesis. It has been shown that \textit{dcl1-9} mutant is compromised in resistance to \textit{hrcC} mutant bacteria (Navarro et al., 2008). An examination of PTI defenses showed that, like \textit{ago1} mutants, callose deposition was reduced by \textit{50-70\%} in \textit{dcl1-9} compared to WT control (Fig. S3A), and flg22-induced resistance to DC3000 bacteria was profoundly diminished in \textit{dcl1-9} plants (Fig. S3F). Unlike \textit{ago1} mutants, \textit{FRK1} and \textit{WRKY29} expression was not significantly altered in \textit{dcl1} (Figs. S3B-S3E). These results confirm an important role of DCL1 in PTI resistance. Like \textit{ago1} mutants, \textit{dcl1-9} was not affected in flg22-induced MAP kinase activation and transient oxidative burst (Figs. S4). Taken together, these data indicate that both AGO1 and DCL1 are required for flg22-induced plant resistance to \textit{P. syringae} bacteria, although their roles in specific PTI responses differ.

Characterization of AGO1-bound small RNAs during PTI defenses

As an effector protein, AGO1 must act through its bound small RNAs in PTI responses. We therefore examined AGO1-associated small RNA species in flg22- or H2O-treated plants by Illumina deepsequencing. Consistent with previous reports (Mi et al., 2008), AGO1-bound small RNAs displayed a strong bias for sequences beginning with a 5’-terminal uridine and a length of 21 nt (Figs. S5A and S5B), and this was not altered by flg22 treatment. Small RNAs mapped to
the Arabidopsis genome were categorized based on their genomic locations and functions (Fig. S5C; Table S1). 1477337 and 1385186 genome-matched small RNA reads were obtained from H$_2$O-treated and flg22-treated AGO1 complexes, respectively. These represent 46555 and 62387 unique small RNA sequences for H$_2$O and flg22 treatment, respectively, suggesting that flg22 treatment induces the biogenesis of significant number of unique AGO1-bound small RNAs. Most of the flg22-induced small RNA reads belong to non-microRNAs.

The majority of AGO1-bound small RNA reads are miRNAs in both treatments. Among them, 89 and 91 known miRNA sequences were identified in H$_2$O and flg22 treatments, respectively. Together these constitute most of the known miRNA species, indicating that our sequencing had a robust coverage of miRNAs (Table 1 and Supplemental Table S1). Most of the 67 miRNAs reported by a previous report (Mi et al., 2008) were identified in this study, except for a few low copy miRNA species.

In an effort to identify small RNA species important to PTI responses, the reads encompassing the defined miRNA sequence±2 nts on each side were calculated. We focused on flg22-regulated miRNAs in the current study. miRNAs with at least 100 reads and >30% increase or decrease in flg22 treatment were selected. In total, 16 up-regulated and 11 down-regulated miRNAs were identified (Table 1 and Table S2). RNA blot analyses were carried out for nine selected miRNAs (Fig. 2A). Flg22 treatment induced $miR_{393}$ accumulation to approximately 1.6 fold compared to the H$_2$O control in an RNA blot analysis, which is consistent with our sequencing data (~2 fold) and a previous report (Navorro et al., 2006). Likewise, $miR_{158a}$, $miR_{160a}$, $miR_{167}$, $miR_{169}$, $miR_{391}$ and $miR_{396}$ were induced by flg22 to 1.6, 2, 2, 1.6, 1.5 and 1.4 fold, respectively, compared to the H$_2$O control. These miRNAs accumulated similarly in
ago1-25 and WT plants, and this is in agreement with previous report that mutation in PIWI domain does not affect miRNA binding with AGO1 protein (Vaucheret et al., 2004). Contrary to above miRNAs, miR398b abundance was slightly reduced upon flg22 treatment. miR773 expressed at a level below the detection limit of RNA blot analysis.

Real-time RT PCR was used to determine if the flg22-regulated expression of miRNAs correlated with the expression of their putative target genes (protein-coding). miR167 targets ARF6 and ARF8 (Rhoades et al., 2002; Jones-Rhoades et al, 2004), which is supported by the down-regulation of ARF6 and ARF8 mRNA in the 35S:miR167 transgenic plants (Wu et al., 2006), whereas miR160a targets ARF10, ARF16 and ARF17 (Mallory et al., 2005). The ARF genes encode auxin response factors (Mallory et al., 2005). Flg22 treatment repressed the expression of ARF10, ARF16, and ARF17, but did not significantly alter ARF6 and ARF8 (Figs. 2B and 2C).

miR398 targets COX5b.1, CSD1 and CSD2 (Jones-Rhoades and Bartel, 2004), which respectively encode a cytochrome c oxidase and two copper superoxide dismutase. Flg22 treatment enhanced the accumulation of COX5b.1, CSD1 and CSD2 transcripts (Fig. 2D). Although miR773 RNA level was not detectable by RNA blot analysis, flg22 treatment resulted in greater expression of its target gene MET2 (Fig. 2E; Fahlgren et al., 2007), which encodes a DNA methyltransferase. The expression of the genes targeted by 6 other flg22-induced miRNAs was not significantly altered by flg22 treatment, with the exception of At3g03580, which was reduced by flg22 treatment (Fig. S6). Target genes for miR156, which was identified as down-regulated by flg22, showed reduced expression in response to flg22 for reasons unknown (Fig. S6).
**miR160a, miR398b, and miR773 regulate PTI defenses**

To further study the function of *miR160a, miR398b, miR773,* and *miR158a,* we generated stable transgenic plants overexpressing the four miRNAs. Three independent T2 transgenic lines overexpressing these miRNAs were identified by RNA blot analyses (Figs. 3A, 4A, 5A, and S7A).

We next determined if the overexpression of the miRNAs reduced their target transcripts. The expression of *ARF16* and *ARF17* was reduced to 10-20% in the three *miR160a* overexpression plants compared to the WT control, whereas *ARF10* was not significantly altered (Fig. 3B).

Consistent with previous reports that transgenic plants expressing *miR160*-resistant forms of *ARF10* and *ARF17* (*arf10* and *arf17*) display serrated leaves (Liu et al., 2007; Mallory et al., 2005), our 35S:*miR160a* plants exhibited a loss of leaf serration. We examined callose deposition induced by flg22 and the *hrcC* mutant bacteria. Fig. 3C shows that *miR160a* overexpression led to greater callose deposition in both treatments, indicating that *miR160a* positively regulates PAMP-induced callose deposition. However, *miR160a* overexpression plants were not significantly altered in basal resistance to DC3000 bacteria (Fig. 3D).

**MiR398b** transgenic plants displayed slightly yellowish leaves, but were otherwise normal in growth and development. *COX5b.1* mRNA level in two *miR398b* transgenic lines (#4 and #5) was reduced by 80% compared to WT control, whereas *CSD2* transcript was completely abolished in these transgenic lines (Fig. 4B). Callose deposition induced by flg22 and *hrcC* bacteria was decreased in the two transgenic lines (Fig. 4C). In agreement with a reduced PTI defenses, 35S:*miR398b* transgenic plants were significantly more susceptible to DC3000 bacteria and supported 3~5 fold more bacterial proliferation compared to WT (Fig. 4D). In addition, the 35S:*miR398b* transgenic plants also supported DC3000 *hrcC* bacteria by 7-10 fold (Fig. 4E).
indicating that miR398b negatively regulates PAMP-triggered disease resistance.

MiR773 transgenic plants were morphologically indistinguishable from WT plants. The three 35S:miR773 transgenic lines examined all showed greatly reduced MET2 mRNA level (~10-20% of WT control; Fig. 5B). The transgenic plants displayed reduced callose deposition (Fig. 5C) and enhanced disease susceptibility to P. syringae DC3000 (Fig. 5D) and DC3000 hrcC- bacteria (Fig. 5E), indicating that, like miR398, miR773 also negatively regulates PTI resistance to P. syringae.

The two 35S:miR158a lines examined showed reduced expression of the target gene At3g03580 (Fig. S7B). However, these plants were largely normal when PAMP-induced callose deposition was examined (Fig. S7C). Furthermore, these plants supported normal growth to P. syringae DC3000 bacteria. These results did not support a role of miR158a in PTI resistance.

DISCUSSION

In this study, we systematically examined the role of AGO1, AGO7, and DCL1 in various PTI responses. ago1 and dcl1 mutants are compromised in PTI responses and flg22 induced disease resistance, indicating that overall AGO1 and DCL1 positively regulate PTI. In contrast, the ago7 mutant was completely normal in PTI resistance, suggesting a more specific role of AGO7 in RPS2 resistance. Thus AGO1 and AGO7 likely control distinct immune pathways in Arabidopsis.

The ago1 and dcl1 mutants investigated in this study showed defects in one or more of the late responses induced by PAMPs. However, these mutants displayed normal MAPK activation and transient oxidative burst, two events that occur less than 5 min after flg22 treatment. The data are consistent with the possibility that AGO1 and DCL1 act in later stages of PTI signaling.
Because the agol mutants examined were partial loss of function alleles, we can not rule out the possibility that the remaining AGO1 activity is sufficient to mediate MAPK activation and oxidative burst. It is also possible that PAMP-induced gene expression and callose deposition occur independent of MAPK activation and oxidative burst, as recently suggested by Lu et al. (2009) and Tsuda et al. (2009).

To date, only one miRNA (miR393) is known to be involved in the regulation of PTI defenses (Navarro et al., 2006). By using deep-sequencing, we compared AGO1-bound small RNA in Arabidopsis plants after H2O and flg22 treatments. Our sequencing analyses led to the identification of 27 miRNAs that were either enriched or depleted in AGO1 upon flg22 treatment. Notably, miR160, miR167, miR393, miR396 and miR824 that were enriched in flg22-treated AGO1 had been shown to accumulate in plants treated with the DC3000 hrcC- bacteria (Fahlgren et al., 2007). RNA blot analysis confirmed that the increased presence of at least some of theses miRNAs in flg22-treated AGO1 was likely caused by increased abundance of miRNAs. By constructing stable transgenic plants overexpressing miRNA genes, we further demonstrated that miR160, miR398, and miR773 play important role in regulating PTI defenses.

Flg22 is known to induce miR393 accumulation, which specifically targets TIR/AFB transcripts. The repression of TIR/AFB transcripts consequently down-regulates auxin signaling pathway and enhances plant resistance to DC3000 bacteria (Navarro et al., 2006). Our results showed that flg22 also induces miR160a accumulation and represses its target genes ARF16 and ARF17. ARF proteins bind auxin-responsive elements (AuxREs) to activate or repress transcription of primary auxin-response genes (Hagen and Guilfoyle, 2002). Transgenic plants overexpressing miR160a exhibit enhanced callose deposition. Thus, multiple auxin pathway genes
may be regulated by miRNAs during PTI defenses.

Intriguingly, some of the AGO1-bound miRNA apparently play a negative role in PTI resistance, although AGO1 overall positively regulates PTI resistance. Flg22 suppressed \textit{miR398b} and \textit{miR773} accumulation. Consistent with this, flg22 treatment enhanced the expression of their target genes \textit{COX5b.1}, \textit{CSD2}, and \textit{MET1}. \textit{miR398b} and \textit{miR773} overexpression plants were compromised in PTI defenses exemplified by reduced callose deposition and supported greater DC3000 and DC3000 \textit{hrcC} proliferation, indicating that \textit{miR398b} and \textit{miR773} negatively regulate plant disease resistance.

It was reported that inoculation of plants with incompatible strains DC3000 (\textit{avrRpm1}) and DC3000 (\textit{avrRpt2}) but not the compatible strain DC3000 represses \textit{miR398} levels (Jagadeeswaran et al., 2009). It is possible that \textit{miR398} is involved in both PTI and ETI defenses.

The findings that \textit{miR160a}, \textit{miR398b}, and \textit{miR773} regulate PTI raise interesting questions concerning the potential role of their target genes in PTI. CSD1 and CSD2 are copper- and zinc-containing SOD (Cu/ZnSOD) enzymes which convert superoxide anion to hydrogen peroxide (Mori and Schroeder, 2004). It was shown previously that downregulation of \textit{miR398} by oxidative stresses leads to the accumulation of \textit{CSD1} and \textit{CSD2} and elevated tolerance to a variety of stresses (Sunkar et al., 2006). MET2 is one of the seven known DNA methyltransferases in plants. A previous report showed that Arabidopsis DNA methyltransferases MET1 and MET2 are required for optimum root transformation by \textit{Agrobacterium} (Crane and Gelvin, 2007). Future analyses of CSD1, CSD2 and MET2 functions may provide new insight into PTI regulation.

\textbf{MATERIALS AND METHODS}
Plants and Bacterial Strains

Arabidopsis plants used in this study include the wild type Col-0 and Ler, and ago1-27, ago1-25, ago7, and dcl1-9 (Ler background) mutants. Plants were grown in a growth room maintained at 23°C and 70% relative humidity with a 10/14 hr day/night light. Bacterial used in this study including Pseudomonas strains P. syringae pv tomato DC3000 and its nonpathogenic derivative hrcC.

Construction of miRNA Overexpression Plants

To make the 35S:miRNA construct, miR160a genomic sequence containing 291 bp upstream and 133 bp downstream sequences, miR398b genomic sequence containing 163 bp upstream and 158 bp downstream sequences, and miR773 genomic sequence containing 76 bp upstream and 84 bp downstream sequence were PCR-amplified from Col-0 genomic DNA. PCR products were cloned into 35S-pKANNIBAL vector between XhoI and KpnI. Constructs were transformed into Col-0 plants by Agrobacterium-mediated transformation. Transgenic plants were screened by spraying with 0.1% BASTA for 2 times.

Flg22-Protection Assay

5-6 week old plants were infiltrated with 1 μM flg22 or H2O 24 hrs before infiltrating 5×10⁵ CFU/ml DC3000 bacteria, and bacterial number was determined at indicated time points as described (Zipfel et al., 2004). Each data point consisted of at least four replicates.

Callose Staining
Five-week-old Arabidopsis leaves were infiltrated 1 μM flg22 for 12 hrs, and leaves were cleared, stained with 0.01% aniline blue for half an hour (Hauck et al., 2003). Callose deposition was captured with a fluorescence microscope and calculated by using the Image J software (Zhang et al., 2007). Each data consisted of at least six replicates.

Quantitative RT-PCR

RNA was extracted from leaves at indicated time points by Trizl reagent (Invitrogen) and reverse transcribed to obtain total cDNA using the SuperScript first-strand synthesis system (Invitrogen). SYBR Green Mix (TaKaRa) was used in real-time PCR to determine the abundance of mRNA. Gene expression level was normalized by using Actin 2 as a control. Primer used in real-time RT PCR were: 5’-GGTGTCATGGTTGTATGGGTC-3’ and 5’-CCTCTGTGAGTGAACCTGGTG-3’ for Actin2; 5’-TCTGAAGAATACGCTGAAGGC-3’ and 5’-TGTGGCTTCATCTCTGTG-3’ for FRK1; 5’-AAGGATCTCCATACCAAGGC-3’ and 5’-ATCCAAGCATCCATACCAAGGC-3’ for WRKY29; 5’-ATCCGCAGCTTTATCTGTG-3’ and 5’-TGATCTCAACACACAATGAGTGC-3’ for ARF6; 5’-AAAGGTGGTATGGGCTCGTG-3’ and 5’-TTCAAGGTCTGGGCTGAGGC-3’ for ARF8; 5’-ACTCCATTTGTTGTGGGATG-3’ and 5’-AATCCGTCTGGGCTGAGGC-3’ for ARF10; 5’-TGTCAGAGGATTGACTGCTG-3’ and 5’-AACCTCTCCAGCTTTGGGAG-3’ for ARF16; 5’-TTATCATGCTCAGTCAGTCG-3’ and 5’-TTATCATGCTCAGTCAGTCG-3’ for ARF17; 5’-TCCACATTTGTTGTGGGATG-3’ and 5’-TTTCCAGTAGGCCAGGCTGAG-3’ for COX5b; 5’-ACCTGCCGGGAGAATGTG-3’ and 5’-TTTCCAGTAGGCCAGGCTGAG-3’ for COX5b.
5’-TCGTAGCTATCCGGAAACCC-3’ for MET2; 5’-ACCTGCCGGACGAAAATGTG-3’ and
5’-TGCCTCTCATGATCATGCTG-3’ for AGL16; 5’-TTTGCTCCTGAACCCACTTC-3’ and
5’-ACCTGCCGGACGAAAATGTG-3’ for GRF3; 5’-TTTGCTCAGAGACTCGTTG-3’ and
5’-AAACATTTCATGTGATGATGGG-3’ for SPL15; 5’-TTTGCTCCCTGAACCCACTTC-3’ and
5’-AAACATTTCATGTGATGATGGG-3’ for SPL16; 5’-TTTGCTCAGAGACTCGTTG-3’ and
5’-AAACATTTCATGTGATGATGGG-3’ for SPL3; 5’-TTTCCTGGTTGAAGACTCC-3’ and
5’-AAACATTTCATGTGATGATGGG-3’ for AGL16; 5’-TTTGCTCAGAGACTCGTTG-3’ and
5’-AAACATTTCATGTGATGATGGG-3’ for SPL15; 5’-TTTGCTCCCTGAACCCACTTC-3’ and
5’-TTTGCTCAGAGACTCGTTG-3’ for SPL16; 5’-TTTGCTCCCTGAACCCACTTC-3’ and
5’-TTTGCTCAGAGACTCGTTG-3’ for SPL3.

**Growth Inhibition Assay**

Arabidopsis seedlings were germinated for 8 days on 1/2 MS agar plates and transferred to
liquid 1/2 MS medium containing 10 μM flg22 in a 24-well-plate. Fresh weight was determined 5
days later.

**Small RNA Gel Blot Analysis**

RNA blot analysis for small RNAs from total extracts was performed as described (Qi et al.,
2005; Mi et al., 2008). Leaves of 5-week-old plants were used for RNA extraction. miRNA probes
were end-labeled with $\gamma$-$^{32}$P-ATP and T4 polynucleotide kinase.

**Isolation of AGO1-Bound Small RNAs**

5-6 week old Arabidopsis leaves were infiltrated with 2 $\mu$M flg22 or H$_2$O and harvested 1 hr later. One ml Arabidopsis extract was incubated with Protein A-agarose for 60 min at 4°C. The precleared extracts were then incubated with 10 $\mu$l anti-AGO1 polyclonal antibodies at 4°C for 60 min, and 10 $\mu$l of Protein A agarose was added into the extracts and incubated for 2 hrs. Immunoprecipitates were washed three times (20 min each) in extraction buffer. AGO1-bound RNA was extracted from the Immunoprecipitates with Trizol (Invitrogen). Small RNA library preparation, and sequencing of small RNAs were performed as described (Qi et al., 2005; Mi et al., 2008).

**Bioinformatic Analysis of Small RNAs**

The small RNA reads with length of 19-27 nt were mapped to the Arabidopsis nuclear, chloroplast and mitochondrial genomes (http://www.arabidopsis.org/). The small RNAs with perfect genomic matches were used for further analysis. Annotation of small RNAs was performed using the following databases: TAIR7 annotations for coding sequences and non-coding RNAs (tRNAs, tRNAs, snoRNAs, snRNAs), and sequences from the intergenic regions (ftp://ftp.arabidopsis.org/Sequences/blast_datasets/TAIR7_blastsets/), Repbase (http://www.girinst.org) for transposons and repeats, ASRP for tasiRNA annotations (http://asrp.cgrb.oregonstate.edu/), and miRBase for miRNA annotations (http://microrna.sanger.ac.uk/sequences/). Annotations for the cis- or trans natural antisense genes
were extracted from published databases (Margulies et al., 2005; Wang et al., 2006). The abundance of small RNAs were calculated as reads per million.

**Oxidative Burst**

Leaves were sliced into 1 mm strips, and ~ 10 mm² leaf strips were incubated in 200 μl H2O in a 96 well plate for 8 hr prior to the addition of 1 μM flg22 in 200 μl buffer containing 20 mM luminol and 1 μg horseradish peroxidase (Sigma). Luminescence was determined with a Luminometer (Promega) for 30-40 min.

**MAPK activity Assay**

5-week-old plants were sprayed with 10 mM flg22 or H2O containing 0.02% Silwet L-77 for 10 min before protein extraction. 15μg total protein was electrophoresed on 10% SDS-PAGE gel, and the protein blot was reacted with anti-p-ERK antibody (Cell signalling) to detect determine phosphorylation state of MPK3, MPK4, MPK6. A duplicate blot was reacted with anti-MPK6 antibodies (Sigma) to determine the amount of total MPK6.

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Table 1. miRNAs up- or down-regulated by flg22. miRNAs with greater than 100 reads and showing >30% increase or decrease in flg22 treatment are selected.

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Figure Legends

Figure 1. AGO1 contributes to PTI. A, ago1 mutants are partially insensitive to flg22-induced seedling growth inhibition. WT and ago1 mutant seedlings were treated with or without 10 μM flg22 for 5 days, and fresh weight was measured. B, ago1 mutants are compromised in flg22- and hrcC-induced callose deposition. WT and ago1 plants were infiltrated with H2O, 1 μM flg22 or 2×10^7 CFU/ml hrcC bacteria for 12 hrs before stained for callose. C-F, ago1 mutants are compromised in PAMP-induced gene expression. WT and ago1 mutants were syringe-infiltrated with 2 μM flg22, 2×10^7 CFU/ml hrcC bacteria or H2O for 4 hrs, and RNA was extracted for quantitative RT-PCR analysis. mRNA level was normalized to that in H2O-treated WT plants. G, AGO1 is required for flg22-induced resistance to P. syringae DC3000. WT and ago1 mutants were infiltrated with 1 μM flg22 or H2O for 24 hrs before infiltrated with 5×10^5 CFU/ml DC3000 bacteria. Leaf bacterial population was determined at the indicated times. Error bars indicate standard deviation. Student’s t test was carried out to determine the significance of difference between WT and mutant plants following flg22 or hrcC treatment. * and ** indicate significant difference at a P value <0.05 and <0.01, respectively. The data shown are representative of 3 independent experiments.

Figure 2. Expression of selected miRNAs and their target genes in response to flg22 treatment. A, RNA blot analysis of miRNAs. WT and ago1-25 leaves were infiltrated with flg22 (F) or H2O (H) for 1 hr before RNA extraction. 15 μg small RNA was loaded. RNA blots were hybridized with DNA oligonucleotide probes complementary to the indicated miRNAs. U6 was used as loading control. Values below each panel represent relative abundance of miRNA normalized to U6
control. B-E, Quantitative RT-PCR analyses of mRNA levels for genes targeted by miR167 (ARF6 and ARF8), miR160 (ARF10, ARF16, and ARF17), miR398 (COX5b.1, CSD1, and CSD2), and miR773 (MET2). Error bars indicate standard deviation. Student’s t test was used to determine the significance of difference between H2O- and flg22-treatments. * and ** indicate significant difference at a P value <0.05 and <0.01, respectively. The experiments were repeated two times with similar results.

Figure 3. Overexpression of miR160a enhances PAMP-induced callose deposition. A, Accumulation of miR160a in 35S:miR160a plants. 20 μg small RNA was loaded for RNA blot analysis. B, Overexpression of miR160a down-regulates ARF16 and ARF17 mRNA in transgenic plants. RNA was extracted from T2 generation of 35S:miR160a plants for quantitative Real-Time RT-PCR Analyses. Error bars indicate standard deviation. C, Flg22- and hrcC-induced callose deposition in 35S:miR160a plants. D, 35S:miR160a plants were not affected in resistance to DC3000 bacteria. WT and 35S:miR160a transgenic plants were infiltrated with 5×10^5 CFU/ml DC3000 bacteria, and leaf bacterial population was determined at the indicated times. Error bars indicate standard deviation. The experiment was repeated three times with similar results. Student’s t test was carried out to determine the significance of difference between 35S:miR160a and WT plants within each treatment. * and ** indicate significant difference at a P value of <0.05 and <0.01, respectively.

Figure 4. miR398b negatively regulates PTI. A, RNA blot analysis of miR398b in transgenic plants. B, Quantitative RT-PCR analyses of CSD1, CSD2, COX5b.1 transcripts in 35S:miR398b
transgenic plants. C, miR398b overexpression represses flg22- and hrcC-induced callose deposition. D, miR398b overexpression enhances plant susceptibility to DC3000. WT and 35S:miR398b transgenic plants were infiltrated with 5×10^5 CFU/ml DC3000 bacteria, and leaf bacterial population was determined at the indicated times. E, miR398b overexpression plants support growth of nonpathogenic DC3000 hrcC^-mutant bacteria. Plants were spray-inoculated with 5×10^8 CFU/ml DC3000 hrcC^-mutant bacteria, and bacterial population in the leaf was determined at the indicated times. Error bars indicate standard deviation. Student’s t test was performed to determine the significance of difference between 35S:miR398b and WT plants within each treatment. * and ** indicate significant difference at a P value of <0.05 and <0.01, respectively. The experiments were repeated 2 (B, C and E) and 3 (D) times with similar results.

Figure 5. miR773 negatively regulates PTI. A, RNA blot analysis of miR773 in transgenic plants. B, Quantitative RT-PCR analyses of MET2 transcripts in 35S:miR773 transgenic plants. C, miR773 overexpression represses flg22- and hrcC^-induced callose deposition. D, miR773 overexpression enhances plant susceptibility to DC3000. WT and 35S:miR773 transgenic plants were infiltrated with 5×10^5 CFU/ml DC3000 bacteria. Leaf bacterial population was determined at the indicated times. E, miR773 overexpression plants support growth of nonpathogenic DC3000 hrcC^-mutant bacteria. Plants were spray-inoculated with 5×10^8 CFU/ml DC3000 hrcC^-mutant bacteria, and bacterial population in the leaf was determined at the indicated times. Error bars indicate standard deviation. Student’s t test was done to determine the significance of difference between 35S:miR773 and WT plants within each treatment. * and ** indicate significant difference at a P value of <0.05 and <0.01, respectively. The experiments were repeated two times.
with similar results.
Supplemental Data

The following materials are available in the online version of the article.

Supplemental Figure S1. ago1 mutants exhibit normal MAPK activation and transient oxidative burst.

Supplemental Figure S2. AGO7 differentially regulates PTI and RPS2 resistance.

Supplemental Figure S3. DCL1 contributed to PTI.

Supplemental Figure S4. dcl1-9 exhibits normal MAPK activation and transient oxidative burst.

Supplemental Figure S5. Flg22 treatment does not affect small RNA sorting to AGO1.

Supplemental Figure S6. Quantitative RT-PCR analysis of transcript levels of miRNA target genes in response to flg22.

Supplemental Figure S7. miR158 overexpression plants display normal PTI.

Supplemental Table S1. Category of AGO1-bound RNAs after flg22 treatment.

Supplemental Table S2. AGO1-bound miRNAs after flg22 treatment.
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