Identification of MicroRNAs Involved in Pathogen-Associated Molecular Pattern-Triggered Plant Innate 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 Arabidopsis (Arabidopsis thaliana) miRNA effector protein, Argonaute1 (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.

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 plasma membrane (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 (reactive oxygen species; Felix et al., 1999), callose (β-1,3-glucan) deposition at the cell wall (Brown et al., 1998; Gómez-Gómez et al., 1999), 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 (Göhre and Robatzek, 2008; Zhou and Chai, 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 (Jones-Rhoades et al., 2006; Mallory and Vaucheret, 2006), abiotic stress tolerance (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Fujii et al., 2005), 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-Like1 (DCL1) and Hua Enhancer1, 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 resis-
A key component in the miRNA pathway is Argonaute1 (AGO1), which predominately binds mature miRNAs to form a RNA-induced silencing complex 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 (Morel et al., 2002; Qu et al., 2008). 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 Arabidopsis (*Arabidopsis thaliana*) seedling growth (Gómez-Gómez et al., 1999). The *ago1-25* and *ago1-27* mutants carrying a point mutation in PIWI domain are impaired in posttranscriptional gene silencing 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 one-half Murashige and Skoog liquid medium containing 10 μM flg22 for 5 d, the wild-type 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% to approximately 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 wild type (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% to 70% less transcripts compared to that in wild type (Fig. 1C–F), 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. Supplemental Figure 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 wild-type plants with flg22 inhibited the growth of virulent DC3000 bacteria by approximately 100-fold 2 d 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 impacted 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 (Supplemental Fig. S2, A–D). Consistent with the normal PTI responses, *hrcC−* mutant bacteria multiplied normally in *ago7* (Supplemental Fig. S2E), and flg22 pretreatment provided similar protection against the virulent DC3000 bacteria in *ago7* and wild-type plants (Supplemental Fig. S2F). In contrast, *ago7* plants showed reduced resistance to DC3000 (*avrRpt2*), confirming previous report (Supplemental 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 *dcl1-9* mutant is compromised in resistance to *hrcC−* mutant bacteria (Navarro et al., 2008). An examination of PTI defenses showed that, like *ago1* mutants, callose deposition was reduced by approximately 50% to 70% in *dcl1-9* compared to wild-type control (Supplemental Fig. S3A), and flg22-induced resistance to bacteria.
resistance to DC3000 bacteria was profoundly diminished in dcl1-9 plants (Supplemental Fig. S3F). Unlike ago1 mutants, FRK1 and WRKY29 expression was not significantly altered in dcl1 (Supplemental Fig. S3, B–E). These results confirm an important role of DCL1 in PTI resistance. Like ago1 mutants, dcl1-9 was not affected in flg22-induced MAP kinase activation and transient oxidative burst (Supplemental Fig. S4). Taken together, these data indicate that both AGO1 and DCL1 are required for flg22-induced plant resistance to 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 water-treated plants by Illumina deep sequencing. 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 (Supplemental Fig. S5, A and B), and this was not altered by flg22 treatment. Small RNAs mapped to the Arabidopsis genome were categorized based on their genomic locations and functions (Supplemental Fig. S5C; Supplemental Table S1). A total of 1,477,337 and 1,385,186 genome-matched small RNA reads were obtained from water-treated and flg22-treated AGO1 complexes, respectively. These represent 46,555 and 62,387 unique small RNA sequences for water 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-miRNAs.

The majority of AGO1-bound small RNA reads are miRNAs in both treatments. Among them, 89 and 91 known miRNA sequences were identified in water and flg22 treatments, respectively. Together these constitute most of the known miRNA species, indicating that our sequencing had a robust coverage of miRNAs (Table I; 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 this 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 I; Supplemental Table S2). RNA-blot analyses were carried out for nine selected miRNAs (Fig. 2A). Flg22 treatment induced miR393 accumulation to approximately 1.6-fold compared to the water control in an RNA-blot analysis, which is consistent with our sequencing data (approximately 2-fold) and a previous report (Navarro et al., 2006). Likewise, miR158a, miR160a, miR167, miR169, miR391, and miR396 were induced by flg22 to 1.6-, 2-, 1.6-, 1.5-, and 1.4-fold, respectively, compared to the water control. These miRNAs accumulated similarly in ago1-25 and wild-type 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 reverse transcription (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 auxin response factors ARF6 and ARF8 (Rhoades et al., 2002; Jones-Rhoades and Bartel, 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 (Fig. 2, B and C). 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 mRNA 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 six other flg22-induced miRNAs was not significantly altered by flg22 treatment, with the exception of At3g03580, which was reduced by flg22 treatment (Supplemental Fig. S6). Target genes for miR156, which was identified as down-regulated by flg22, showed reduced expression in response to flg22 for reasons unknown (Supplemental Fig. S6).

### miR160a, miR398b, and miR773 Regulate PTI Defenses

To further study the function of miR160a, miR398b, and 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–5A; Supplemental Fig. S7A). We next determined if the overexpression of the miRNAs reduced their target transcripts. The expression of ARF16 expression was reduced to 10% to 20% in the three miR160a overexpression plants compared to the wild-type 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 (Mallory et al., 2005; Liu et al., 2007), our 35S:miR160a

### Table 1. miRNAs up- or down-regulated by flg22

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plants exhibited a loss of leaf serration. We examined callose deposition induced by flg22 and the *hrcC*− mutant bacteria. Figure 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 (nos. 4 and 5) was reduced by 80% compared to wild-type 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 reduced PTI defenses, 35S:*miR398b* transgenic plants were significantly more susceptible to DC3000 bacteria and supported 3- to approximately 5-fold more bacterial proliferation compared to wild type (Fig. 4D). In addition, the 35S:*miR398b* transgenic plants also supported DC3000 *hrcC*− bacteria by 7- to 10-fold (Fig. 4E), indicating that *miR398b* negatively regulates PAMP-triggered disease resistance.

*MiR773* transgenic plants were morphologically indistinguishable from wild-type plants. The three 35S:*miR773* transgenic lines examined all showed greatly reduced *MET2* mRNA level (approximately 10%–20% of wild-type 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 (Supplemental Fig. S7B). However, these plants were largely normal when PAMP-induced callose deposition was examined (Supplemental 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.

Figure 3. Overexpression of miR160a enhances PAMP-induced callose deposition. A, Accumulation of miR160a in 35S:miR160a plants. Twenty micrograms 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 so. C, Flg22- and hrcC2-induced callose deposition in 35S:miR160a plants. D, 35S:miR160a plants were not affected in resistance to DC3000 bacteria. Wild-type (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 so. 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 wild-type plants within each treatment. Asterisks (* 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, and COXSB. C, miR398b overexpression represses flg22- and hrcC2-induced callose deposition. D, miR398b overexpression enhances plant susceptibility to DC3000. Wild-type (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 hrcC2 mutant bacteria. Plants were spray inoculated with 5 × 10^5 cfu/mL DC3000 hrcC2 mutant bacteria, and bacterial population in the leaf was determined at the indicated times. Error bars indicate so. Student’s t test was performed to determine the significance of difference between 35S:miR398b and wild-type plants within each treatment. Asterisks (* and **) indicate significant difference at a P value of <0.05 and <0.01, respectively. The experiments were repeated two (B, C, and E) and three (D) times with similar results.
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 ago1 mutants examined were partial loss-of-function alleles, we cannot 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 water 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− mutant bacteria (Fahlgren et al., 2007). RNA-blot analysis confirmed that the increased presence of at least some of these miRNAs in flg22-treated AGO1 was likely caused by increased abundance of miRNAs. By constructing stable transgenic plants overexpressing miR160, miR398, and miR773, we further demonstrated that miR160, miR398, and miR773 play important roles 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 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.

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. Wild-type (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 SD. Student’s t test was done to determine the significance of difference between 35S:miR773 and wild-type plants within each treatment. Asterisks (*) indicate significant difference at a P value of <0.05 and <0.01, respectively. The experiments were repeated two times with similar results.
Intriguingly, some of the AGO1-bound miRNA apparently play a negative role in PTI resistance, although AGO1 overall positively regulates PTI resistance. Flg22 suppressed miR398b and miR773 accumulation. Consistent with this, flg22 treatment enhanced the expression of their target genes COX5b.1, CSD2, and MET1. miR398b and miR773 overexpression plants were compromised in PTI defenses exemplified by reduced callose deposition and supported greater plants were compromised in PTI defenses exemplified wild-type Columbia-0 (Col-0) and Landsberg CSD1 and CSD2 are copper- and zinc-containing superoxide dismutase enzymes that convert superoxide anion to hydrogen peroxide (Mori and Schroeder, 2004). It was shown previously that down-regulation of miR398 by oxidative stresses leads to the accumulation of CSD1 and 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 Agrobacterium (Crane and Gelvin, 2007). Future analyses of CSD1, CSD2, and MET2 functions may provide new insight into PTI regulation.

MATERIALS AND METHODS

Plants and Bacterial Strains

Arabidopsis (Arabidopsis thaliana) plants used in this study include the wild-type Columbia-0 (Col-0) and Landsberg erecta, and ago1-27, ago1-25, ago7, and del1-9 (Landsberg erecta background) mutants. Plants were grown in a growth room maintained at 23°C and 70% relative humidity with a 10/14 h day/night light. Bacteria used in this study include Pseudomonas syringae and hrcC by reduced callose deposition and supported greater bacterial number was determined at indicated time points as described (Zipfel et al., 2004). Each data point consisted of at least four replicates.

Callase Staining

Five-week-old Arabidopsis leaves were infiltrated 1 μl flg22 for 12 h, 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 at Trizol 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 miRNA. Gene expression level was normalized by using Actin 2 as a control. Primers used in real-time RT-PCR were 5′-GCTGTCAAGTTGATGGTGC-3′ and 5′-CCTCTTCTTAAACCTTCCG-3′ for Actin2; 5′-TGTGAAATTAGCACTATGG-3′ and 5′-ATCCGAGCTTGATGAGTGG-3′ for WKR2Y9; 5′-ATCCCGAGTTTATCTGTGAG-3′ and 5′-TATACGCGCACTGAGCAG-3′ for AVR3; 5′-AAGGTGTATGGGCTCTGG-3′ and 5′-TCCCGGAACAAACACTATGC-3′ for ARF10; 5′-CTGGAAGCCATTAGATGTGC-3′ and 5′-AATCCTCTTCACCCTTGG-3′ for ARF3; 5′-TTATACGAGAGCCGCTACAT-3′ and 5′-TATGCTCCTTCCCTTAATG-3′ for ARF17; 5′-TCCATCTAACCACCAGTAC-3′ and 5′-TTTTCATGACCAAGCTGAG-3′ for CSD1; 5′-TCTCTACACTGTAATGTC-3′ and 5′-AGGATCTCATTATGCTG-3′ for CSD2; 5′-AGACCTGCTGTTCCTATC-3′ and 5′-TTCGTCGACAAGGTCCGAG-3′ for DC3000; 5′-ACGCCCTGAGGAAATCGT-3′ and 5′-TCTGACATTCGGGGAGAT-3′ and 5′-TTGAGAGCCGAAACAGTGC-3′ for Actin2; 5′-ACGCTGGAGAAAATGCTG-3′ and 5′-TGGGCTCAGATGTGAGTTAT-3′ for miR398b overexpression plants.

Construction of miRNA Overexpression Plants

To make the 35S:mRNA 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 Xhol and KpnI. Constructs were transformed into Col-0 plants by Agrobacterium-mediated transformation. Transgenic plants were screened by spraying with 0.1% BASTA for two times.

Flg22-Protection Assay

Five- to 6-week-old plants were infiltrated with 1 μl flg22 or water 24 h before infiltrating 5 × 10⁵ colony forming units (cfu)/ml. DC3000 bacteria, and


2229

MicroRNAs in Plant Immunity
Bioinformatic Analysis of Small RNAs

The small RNA reads with length of 19 to 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 noncoding RNAs (rRNAs, tRNAs, snRNAs, ssnRNAs), and sequences from the intergenic regions (ftp://ftp.arabidopsis.org/Sequences/blast_databases/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 approximately 10 mm² leaf strips were incubated in 200 µL water in a 96-well plate for 8 h prior to the addition of 1 µL flg22 in 200 µL buffer containing 20 mM luminol and 1 µg horseradish peroxidase (Sigma). Luminescence was determined with a Luminometer (Promega) for 30 to 40 min.

MAPK Activity Assay

Five-week-old plants were sprayed with 10 mM flg22 or water containing 0.02% Silwet L-77 for 10 min before protein extraction. Fifteen micrograms of total protein was electrophoresed on 10% SDS-PAGE gel, and the protein blot was incubated in 200 µL water in a 96-well plate for 8 h prior to the addition of 1 µL water in 200 µL buffer containing 20 mM luminol and 1 µg horseradish peroxidase (Sigma). MAPK Activity Assay

LITERATURE CITED

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MicroRNAs in Plant Immunity

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