Abscisic Acid Induces Resistance against *Bamboo Mosaic Virus* through Argonaute 2 and 3[^1][OPEN]

Mazen Alazem, Meng-Hsun He, Peter Moffett, and Na-Sheng Lin*

Institute of Plant and Microbial Biology, Academia Sinica, Nankang, Taipei 11529, Taiwan, Republic of China (M.A., M.-H.H., N.-S.L.); and Département de Biologie, Université de Sherbrooke, Sherbrooke, Québec, Canada J1K 2R1 (P.M.)

ORCID IDs: 0000-0003-3690-7459 (M.A.); 0000-0001-8026-1400 (M.-H.H.); 0000-0003-1148-6256 (N.-S.L.).

Plant resistance to pathogens is tuned by defense-related hormones. Of these, abscisic acid (ABA) is well documented to moderate resistance against fungi and bacteria. However, ABA’s contribution to resistance against viruses is pleiotropic. ABA affects callose deposition at plasmodesmata (therefore hindering the viral cell-to-cell movement), but here, we show that when callose synthase is down-regulated, ABA still induces resistance against infection with *Bamboo mosaic virus* (BaMV). By examining the potential connections between the ABA and RNA-silencing pathways in *Arabidopsis* (*Arabidopsis thaliana*), we showed that ABA regulates the expression of almost the whole ARGONAUTE (AGO) gene family, of which some are required for plant resistance against BaMV. Our data show that BaMV infection and ABA treatment regulate the same set of AGOs, with positive effects on AGO1, AGO2, and AGO3, no effect on AGO7, and negative effects on AGO4 and AGO10. The BaMV-mediated regulation of AGO1, AGO2, and AGO3 is ABA dependent, because the accumulation of these AGOs in BaMV-infected ABA mutants did not reach the levels observed in infected wild-type plants. In addition, the AGO1-miR168a complex is dispensable for BaMV resistance, while AGO2 and AGO3 were important for ABA-mediated resistance. While most ago mutants showed increased susceptibility to BaMV infection (except ago10), ago1-27 showed reduced BaMV titers, which was attributed to the up-regulated levels of AGO2, AGO3, and AGO4. We have established that ABA regulates the expression of several members of the AGO family, and this regulation partially contributes to ABA-mediated resistance against BaMV. These findings reveal another role for ABA in plants.

RNA silencing provides plants with broad resistance against virus infection through small RNA (sRNA)-directed degradation (Ding, 2010). While it is well established that salicylic acid (SA) controls plant resistance to virus infection via R gene resistance (Baebler et al., 2014), it also has been suggested that SA regulates a few genes in the RNA-silencing pathway (Alamillo et al., 2006; Hunter et al., 2013). In fact, the regulation of defense responses has been demonstrated to be a hormone-tuned process in many cases. Other hormones, such as jasmonic acid and ethylene, also are involved in the plant-virus interaction but exhibit specificity in terms of the infecting virus and the type of resistance triggered (Alazem and Lin, 2015).

Abscisic acid (ABA) also is involved in modulating plant resistance against various pathogens, but the timing of its activation is critical for determining plant susceptibility or resistance; therefore, it has been labeled as a phase-specific modulator of defense responses (Mauch-Mani and Mauch, 2005). Nevertheless, ABA does not seem to be phase specific against viruses, and few reports have shown that ABA improves plant resistance to viruses. We previously found that ABA treatment decreases titers of *Bamboo mosaic virus* (BaMV) in inoculated leaves of *Arabidopsis* (*Arabidopsis thaliana*) and that ABA-mediated resistance may be transcriptionally regulated by (at least) ABI3 and ABI4 (Alazem et al., 2014). However, not all genes in the ABA pathway affect BaMV similarly: the biosynthesis mutant aba2-1 displays a resistance phenotype to BaMV, while mutants of genes downstream of aba2-1 (such as aao3, abi1, abi3, and abi4) have the opposite effect, causing severalfold increases in BaMV titers (Alazem et al., 2014). Our results strongly suggest that ABA2 is required for BaMV accumulation and that the ABA pathway downstream of ABA2 is required for resistance. In addition, there is no specific ABA gene required for the infection of the tobacco mosaic virus crucifer strain in *Arabidopsis*, and several mutants of the ABA pathway exhibit enhanced accumulation of tobacco mosaic virus crucifer strain in the upper systemic leaves of *Arabidopsis* but not in inoculated leaves; this study also showed that ABA resistance is *ABI4* dependent (Chen et al., 2013).

[^1]: This work was supported by an Academia Sinica Investigator Award and the Ministry of Science and Technology (grant no. 104-2321-B-001-055), Taipei, Taiwan.

* Address correspondence to nslin@sinica.edu.tw.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Na-Sheng Lin (nslin@sinica.edu.tw).

M.A., P.M., and N.-S.L. designed the research plan; M.A. performed most experiments with help from M.-H.H.; M.A., P.M., and N.-S.L. analyzed the data; M.A. and N.-S.L. wrote the article, which was approved by all authors.

[^OPEN]: Articles can be viewed without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.16.00015
Previous studies suggested that ABA-mediated defense is mediated indirectly via the induction of callose (β-1,3-glucan) accumulation at plasmodesmata, thereby hindering viral cell-to-cell movement (Fraser and Whenham, 1989; Iriti and Faoro, 2008). Callose accumulation is the outcome of the ABA-mediated transcriptional down-regulation of β-1,3-glucanase, which degrades callose (Oide et al., 2013). The effect on cell-to-cell movement is apparent by the observed restriction of viruses at infection sites, which hampers their movement locally and systemically (Iriti and Faoro, 2008; Chen et al., 2013). However, a number of works have suggested connections between the ABA and small RNA pathways, which imply possible effects of ABA on resistance to viruses via the silencing pathway. For instance, miR168a, which posttranscriptionally regulates the ARGONAUTE (AGO) gene AGO1 and is specifically induced in virus-infected tissues, possesses a few abscisic acid-responsive elements (ABREs) in the promoter region that make it regulated by ABA (Liu et al., 2008; Laubinger et al., 2010; Li et al., 2012). RNA-dependent RNA polymerase (RdRP) is positively regulated by ABA as well (Hunter et al., 2013). Furthermore, abiotic stresses, of which some, such as severe drought, are controlled by ABA, also showed altered expression of some genes involved in the siRNA pathway, such as AGO1, AGO4, and AGO7, in Medicago truncatula (Capitao et al., 2011). However, the characterization of ABA-sRNA interrelations remains in the early stages, and the possible effect of ABA on other members of the AGO family is still largely unexplored.

The RNA-silencing pathway is triggered once the plant senses highly structured or double-stranded viral RNAs. These structures are recognized by dicer-like proteins (DCLs); DCL2 and DCL4 are the main players against RNA viruses and process these double-stranded viral RNAs into primary virus-derived small interfering RNAs (siRNAs; Blevins et al., 2006; Du et al., 2007). RdRP1 and RdRP6 amplify these virus-derived siRNAs (Garcia-Ruiz et al., 2010; Willmann et al., 2011), and they are subsequently loaded into RNA-induced silencing complexes (RISCs), which cleave viral RNA in a sequence-specific manner by AGO protein (Qu et al., 2008; Vaucheret, 2008; Mallory and Vaucheret, 2010; Alvarado and Scholthof, 2012).

In Arabidopsis, the AGO protein family consists of 10 members involved in several regulatory pathways in addition to defense pathways (Vaucheret, 2008; Ding, 2010). According to their functions in regulating plant growth and development, the AGO family is divided into three clades: clade 1, composed of AGO1, AGO5, and AGO10 and involved in posttranscriptional gene silencing; clade 2, composed of AGO2, AGO3, and AGO7, with unverified functions; and clade 3, composed of AGO4, AGO6, AGO8, and AGO9 and involved in transcriptional gene silencing (Vaucheret, 2008; Mallory and Vaucheret, 2010; Fang and Qi, 2016). With the exception of ago1, AGO mutants do not show obvious phenotypes, which suggests a functional redundancy of these AGOs. Moreover, AGO8 is believed to be a pseudogene, because it has no detectable transcripts (Takeda et al., 2008; Mallory and Vaucheret, 2010).

To date, antiviral activity has been reported for several members of the AGO family, including AGO1, AGO2, AGO4, AGO5, and AGO7, which are involved in the defense against RNA viruses. These AGO proteins possess specificity against certain viruses. For example, AGO1 is critical for resistance against Turnip crinkle virus (TCV) and Cucumber mosaic virus (CMV) and is considered a key player in the siRNA machinery (Qu et al., 2008; Ruiz-Ferrer and Voinnet, 2009; Harvey et al., 2011). AGO2 is active against Potato virus X (PVX), TCV, and CMV in Arabidopsis and against Tomato bushy stunt virus in Nicotiana benthamiana (Scholthof et al., 2011).

Furthermore, AGO2 has a major contribution to the nonhost resistance of Arabidopsis against PVX (Jaubert et al., 2011). Loss of AGO4 contributes to sensitivity to Tobacco rattle virus (Ma et al., 2015), and AtAGO5 is involved in resistance against PVX in N. benthamiana and in Arabidopsis (Brosseau and Moffett, 2015). Finally, AGO7 is involved in resistance against TCV in Arabidopsis (Qu et al., 2008). However, no reports have indicated that AGO3, AGO6, or AGO9 is involved in the antiviral defense.

In this study, we used BaMV to assess the interrelation between ABA and AGOs. BaMV is a single-stranded positive-sense RNA virus with a genome of 6.4 kb. BaMV belongs to the Potexvirus genus (Lin et al., 1994) with flexible filamentous morphology, whose new atomic model was disclosed recently (DiMaio et al., 2015). BaMV infects Arabidopsis but cannot move systemically or induce symptoms; however, in N. benthamiana, which is a preferable host, high titers of BaMV accumulate and necrotic mosaic symptoms emerge (Lin et al., 2010). Here, we show that ABA is required for the expression of AGO1, AGO2, and AGO3 and negatively regulates the expression of AGO4 and AGO10. Wild-type plants treated with ABA or the NCED3 transgenic line showed enhanced resistance to BaMV, and the regulation of these AGOs became stronger after BaMV infection. In addition, mutations in ABA-regulated AGO genes enhanced susceptibility, with critical effects observed in ago2 and ago3 mutants.

RESULTS

Callose-Deficient Plants Showed Reduced BaMV Levels upon Treatment with ABA

ABA-mediated resistance to viruses is achieved by enhancing callose deposition on plasmodesmata and, therefore, hindering viral cell-to-cell movement. However, evidence linking ABA with the sRNA pathway suggests that the former also may induce resistance via the RNA-silencing pathway. To test this, we used the dexamethasone-inducible gsi8-RNAi (gsi8i) line (Chen et al., 2009) in Arabidopsis, in which induction significantly reduces the transcript level of Gsi8, the callose synthase-like gene that functions in callose deposition at cell walls and plasmodesmata (Thieie et al., 2009; Guseman et al., 2010). Treatment of ~22-d-old gsi8i plants with dexamethasone (hereafter referred as dexa-gsi8i plants) for 10 d significantly reduced leaf expansion, softened
tissues, and changed the color of leaves to light green, while water-treated gsl8i plants retained a similar appearance to wild-type plants (Fig. 1A). The Gsl8 transcript level was lower in dexta-gsl8i plants than in wild-type or gsl8i plants irrigated with water (Fig. 1B, third gel). We next examined whether ABA treatment affected plant resistance to BaMV. As expected, ABA reduced BaMV levels in wild-type and noninduced gsl8i plants to \( \sim 35\% \) and \( \sim 52\% \), respectively. Interestingly, dexta-gsl8i plants also exhibited reduced BaMV levels upon ABA treatment, to \( \sim 55\% \) compared with the corresponding mock line (Fig. 1B, first gel). As a parallel control, ABA treatment induced the expression of HAIL (Fig. 1B, fourth gel), which is known to be a sensitive ABA marker gene (Bhaskara et al., 2012). These results suggest that ABA may control other defense mechanisms in addition to callose deposition in Arabidopsis.

Promoters of AGO Genes Contain Several ABREs, and Their Expression Levels Are Affected by Exogenous ABA

Some reports indicated that ABA regulates several components of the sRNA pathway, such as AGO1 and its regulator miR168 as well as RdR1 (Li et al., 2012; Hunter et al., 2013; Alazem and Lin, 2015). We hypothesized that ABA-mediated resistance is achieved by positive regulation of the RNA-silencing pathway and that such regulation also may affect members of the AGO family because several of these proteins have antiviral properties (Carbonell and Carrington, 2015). To test this, we first examined the promoter regions of AGO genes for the presence of ABREs and MYC and MYB motifs. Fragments located between \(-1,500\) and \(+100\) bp of the start codons were scanned for ABREs using the PlantPAN server (Chang et al., 2008). Several ABREs were found in the promoters of AGO1, AGO2, AGO3, AGO4, AGO5, AGO6, and AGO10, with a confidence level of \( \sim 100\% \) and \( P \) value of \( \sim 0.0002 \) (Fig. 2A). This finding suggests that ABA-related factors regulate the expression of these AGOs. In addition, several binding motifs for MYC and MYB TFs were found in the promoter regions of all of these AGOs (Supplemental Fig. S1). It is known that several members of these two families regulate responses to various abiotic stresses, and of these responses, many involve the ABA pathway (Abe et al., 1997, 2003). To confirm that ABA affects the expression of these genes, we used real-time quantitative PCR (RTqPCR) to measure AGO transcripts in ABA-treated wild-type plants. ABA treatment (twice during a 4-d period) significantly increased the expression of AGO1, AGO2, and AGO3, while AGO10 was negatively affected and AGO4 and AGO7 were unaffected. In plants infected with BaMV for 4 d, the expression of AGO1, AGO2, and AGO7 was not changed significantly and that of AGO3 was increased slightly. However, the levels of AGO4 and AGO10 were decreased significantly (Fig. 2B). ABA treatment of infected plants significantly enhanced the up-regulation of AGO1 (2.7-fold), AGO2 (2.3-fold), and AGO3 (\( \sim 2.7\)-fold) compared with BaMV-infected mock-treated plants (Fig. 2B) and decreased levels of BaMV (Fig. 1B). While ABA treatment did not significantly affect the down-regulation of AGO4 and AGO7, it unexpectedly increased the level of AGO10 (by 2.1-fold) in infected plants. RAB18, another ABA-responsive gene used as a control, was up-regulated severalfold in ABA-sprayed plants (both mock-inoculated and BaMV-infected plants; Fig. 2B). Of note, levels of AGO5, AGO6, and AGO9 in leaves were too low to be measured, and this finding was not changed by BaMV infection or ABA treatments.

We next tested the expression of a few ABA-related TFs in response to BaMV infection with/without ABA treatment. Since the number of ABA TFs is huge, we used a database that was established on a large-scale in vitro study that queries the entire Arabidopsis genome against TFs (O’Malley et al., 2016). Indeed, the query results showed a small number of ABA TFs that bind to the promoter regions of most of the AGOs. Among these TFs are ABI3/VIVAPAROUS1 (VP1), a member of a large group of AP2/B3-like TFs that act as intermediaries in regulating ABA-responsive genes (Suzuki et al., 2003), TGA5, a member in the bZIP TF family that is induced by ABA (Banerjee and Roychoudhury, 2017), and MYBH (or KUA1), which also is involved in ABA signaling and response (Huang et al., 2015). Interestingly, BaMV infection significantly reduced the expression of ABI3/VP1 and MYBH, while TGA5 was increased slightly (Fig. 2C). As expected, ABA treatment induced the expression of these TFs \( \sim 2\), \( 1.3\), and \( 1.1\)-fold for ABI3/VP1, TGA5, and MYBH, respectively (Fig. 2C). With BaMV infection, ABA was still able to increase the levels of these TFs \( \sim 2.3\), \( 1.4\), and \( 2.5\)-fold for ABI3/VP1, TGA5, and MYBH, respectively, compared with BaMV-infected mock-treated plants (Fig. 2C). These results indicate that BaMV infection interferes with some ABA responses in plants. However, the exogenous application of ABA compensates the negative effects of BaMV exerted on ABA-responsive genes and increases their levels significantly.

Progression of BaMV Accumulation Affects the Expression of AGO Genes

The interaction between BaMV and Arabidopsis is compatible, allowing the infection to progress with no signs of resistance (Fig. 3A; Lin et al., 2010). However, Arabidopsis is not a preferred host for BaMV; therefore, the accumulation of BaMV titers is low compared with preferable hosts such as *N. benthamiana* (Lin et al., 2010). To accurately quantify the difference among several infection time points, we used RTqPCR to measure the levels of AGO genes in infected tissues over time. This experiment also revealed how the expression of AGOs is affected by increasing titers of BaMV. Four to five leaves of \( \sim 25\)-d-old Arabidopsis plants infected with BaMV were collected at 3, 7, 10, and 14 dpi, and BaMV level increased over time, showing a severalfold increase at later stages of infection (10 and 14 dpi; Fig. 3A). We next investigated whether AGO expression
was correlated with BaMV titers and/or plant age. We found that AGO1 was not affected significantly by age in mock-inoculated plants, with similar levels across the four tested time points (Fig. 3B). However, the expression of AGO1 in infected plants was increased significantly after BaMV infection: the expression peaked at 10 dpi and started to decline thereafter, remaining slightly higher than that in mock-inoculated plants at day 14 (Fig. 3B). The levels of AGO2 and AGO3 in mock-inoculated plants did not change over 14 d (Fig. 3, C and D), and levels of AGO4, AGO7, and AGO10 decreased over time, particularly at 14 dpi (Fig. 3, E–G). AGO2 and AGO3 expression correlated positively with BaMV titers, with expression 2- to 3-fold greater than those in mock-inoculated plants (Fig. 3, C and D). In contrast, BaMV infection enhanced the reduction of AGO4 and AGO10 during the investigated time periods (Fig. 3, E and G), indicating that BaMV negatively affected the accumulation of these AGOs. While aging reduces the expression of AGO7, BaMV did not affect its expression, with similar levels observed between infected and mock-inoculated plants at all four tested time points (Fig. 3F). These results indicate that plants respond to BaMV infection by inducing the expression of AGO1, AGO2, and AGO3 and reducing that of AGO4 and AGO10, with no change in the level of AGO7. It can be noted that the AGO3 response to infection was clearer at 4 dpi than at 3 dpi (Fig. 3D), probably because of the rapid increase in BaMV titers. Because the expression of many of these AGOs showed significant differences at 10 dpi, we chose this time point for further investigation of the effects of ABA on AGO levels.

ABA Overproduction Enhances Plant Resistance to BaMV and Induces the Expression of Several AGO Genes

To examine the long-term effect of ABA on the levels of AGOs, we used a transgenic line in which the rice (Oryza sativa) ABA biosynthesis gene OsNCED3 is constitutively overexpressed, thereby enhancing ABA levels by more than 2-fold compared with wild-type

![Figure 1. Effects of ABA on plant resistance to BaMV in the g58i line.](https://www.plantphysiol.org)
plants (OsN3-O/E; Hwang et al., 2010). This line provides a consistent effect of ABA in that exogenous spraying of ABA may result in uncontrolled variation. OsN3-O/E plants showed enhanced resistance to BaMV infection, with BaMV titers less than 30% compared with that in wild-type infected plants (Fig. 4A). Because the biosynthetic ABA2 gene is specifically required for BaMV accumulation and ABA induces ABA2, AAO3, and

**Figure 2.** Identification of ABREs in promoters of AGO genes, and the effect of ABA and BaMV infection on the expression of AGOs and ABA-related transcription factors (TFs). A, ABREs in AGO promoters. Promoter fragments located between –1,500 and +100 bp of the start codon were examined for the presence of ABREs, as described by Chang et al. (2008). Several ABREs were identified, with a confidence level of 100% and P value of −0.0002, in AGO1, AGO2, AGO3, AGO4, AGO5, AGO6, and AGO10. B and C, Relative expression of AGOs (B) and ABA-related TFs (C) upon BaMV infection with or without ABA treatment. Plants were first treated with ABA at 24 h before BaMV infection and then were given a second dose of ABA at 3 dpi. Leaves were collected at 4 dpi for RNA extraction and the determination of expression levels of members of the AGO family. Expression levels of AGO5, AGO6, and AGO9 were undetectable. RAB18 is an ABA-responsive gene. Actin was used as an internal control. Data are means ± SD from three biological replicates. Statistical analysis was carried as described in Figure 1: *, P < 0.05; **, P < 0.01; and ***, P < 0.005, by Student t test. Additional statistical analyses were carried out to compare levels between mock-sprayed and ABA-sprayed plants (both infected with BaMV).
other biosynthesis genes (Alazem et al., 2014), we measured the level of ABA2, AAO3, and NCED3 in OsN3-O/E leaves. The increase in ABA2 or NCED3 level was not as great as that of AAO3 after infection (less than 2-fold for ABA2 and ~4-fold for AAO3; Supplemental Fig. S2) nor as great as that of ABA2 in the ABA2-O/E-infected line (70-fold; Alazem et al., 2014). Therefore, the ABA2 level may not be enough to support BaMV accumulation in the OsN3-O/E line as in the ABA2-O/E line (~70-fold), and its effect on BaMV accumulation could be marginal.

We next measured the expression levels of AGOs in wild-type and OsN3-O/E plants. In wild-type infected plants, the transcript levels of AGO1, AGO2, and AGO3 were increased significantly, with AGO7 not affected significantly and AGO4 and AGO10 regulated negatively at 10 dpi (Fig. 4, B–G). A comparison
of the mock-inoculated OsN3-O/E line with infected wild-type plants revealed a similar regulation of most of the AGOs (i.e. up-regulation of AGO1, AGO2, and AGO3 and negative regulation of AGO4 but no effect on AGO7 and AGO10; Fig. 4). The synergistic effects of BaMV infection and ABA overproduction in the infected OsN3-O/E line largely enhanced the expression of these AGOs, with ~2.4-, 3.2-, and 5.9-fold up-regulation of AGO1, AGO2, and AGO3, respectively, compared with BaMV-infected wild-type plants (Fig. 4, B–D). However, AGO4 and AGO10 showed 2.5- and 4.5-fold up-regulation, respectively, in infected OsN3-O/E plants compared with infected wild-type plants (Fig. 4, E and G), with the same observation when comparing mock and infected OsN3 lines in that all AGOs were up-regulated after infection. Since plants respond to BaMV infection by

Figure 4. Effects of ABA on BaMV and AGO levels in the OsN3-O/E transgenic line. A, Northern-blot analysis (left) and quantitative analysis (right) of BaMV RNA level in wild-type (WT) and OsN3-O/E leaves at 10 dpi. M, Mock infected; V, BaMV infected. B to G, RTqPCR analysis of AGO gene levels in the OsN3-O/E line: AGO1 (B), AGO2 (C), AGO3 (D), AGO4 (E), AGO7 (F), and AGO10 (G). Data are means ± SD of three biological replicates. Statistical analysis was carried out as described in Figures 1 and 2: *, P < 0.05; **, P < 0.01; and ***, P < 0.005, by Student t test. Additional statistical analyses were performed to compare levels between wild-type infected and OsN3-O/E-infected lines.
producing more ABA (Alazem et al., 2014), the severalfold up-regulation of AGO1, AGO2, AGO3, AGO4, and AGO10 may be attributed to the enhanced ABA effect caused by BaMV infection and account for the tolerance phenotype in the OsN3-O/E line.

**Impairing the ABA Biosynthesis Pathway Reduces the Expression of Several AGOs**

To confirm these results, we next measured AGO expression in two ABA biosynthesis mutants: aba2-1 and aao3. We previously showed that each mutant responded differently to BaMV infection; aba2-1 showed a marked reduction in BaMV titers, with aao3 and several downstream mutants showing increased BaMV titers (Fig. 5A; Alazem et al., 2014). The differences in the resistance phenotypes of aba2-1 and aao3 are not related to the SA defense pathway, because levels of the SA-related genes EDS1 and SID2 were higher in both mutants compared with wild-type plants (Supplemental Fig. S3, A and B). Also, mutants in these SA genes accumulated higher levels of BaMV compared with the levels in wild-type plants (Supplemental Fig. S3C; Alazem et al., 2014). Therefore, the difference in susceptibility between aba2-1 and aao3 may not be related to the levels of SA genes in their backgrounds.

Interestingly, the AGOs identified previously to be positively regulated (AGO1 and AGO2) were downregulated in the infected mutants compared with wild-type infected plants (Fig. 5, B and C). However, the expression of AGO3 in both infected mutants was restored to levels similar to or a little more than those in wild-type infected plants (Fig. 5D). In mock-inoculated ABA mutants, AGO4 levels were not affected in aba2-1 but were increased slightly in aao3 (Fig. 5E). Nevertheless, BaMV infection reduced AGO4 levels in both mutants to levels comparable to those in wild-type infected plants (Fig. 5E). The level of AGO10 was reduced in the

**Figure 5.** BaMV accumulation and AGO transcript expression in ABA-deficient mutants. RTqPCR analysis is shown for the levels of BaMV CP gene (A), AGO1 (B), AGO2 (C), AGO3 (D), AGO4 (E), AGO7 (F), and AGO10 (G) in mock and infected lines of aba2-1 and aao3 ABA-deficient mutants. Plants were infected with 1 μg of BaMV virions and then collected at 10 dpi for analysis as described previously (Alazem et al., 2014). Data are means ± SD of three biological replicates. Statistical analysis was carried out as described in Figure 1: *, P < 0.05; **, P < 0.01; and ***, P < 0.005, by Student t test. WT, Wild type.
mock-inoculated aba2-1 line but was unaffected in the aao3 line; however, AGO10 expression was reduced after infection in both mutants (Fig. 5G). The AGO7 level showed a slight increase in the OsN3-O/E mock line, which was enhanced after BaMV infection (Fig. 4F), and complemented the findings of aba2-1 and aao3 mutants, where AGO7 levels were reduced significantly even after BaMV infection compared with wild-type infected plants (Fig. 5F). Hence, AGO7 requires an intact ABA pathway for basic expression. In addition, RdR1 is induced by ABA in Arabidopsis (Hunter et al., 2013). Consistent with these findings, we also found that ABA deficiency reduced RdR6 levels, and BaMV infection enhanced this reduction in the wild-type and the two ABA mutants (Supplemental Fig. S3D). Collectively, these results indicate that ABA positively regulates the expression of AGO1, AGO2, and AGO3 and negatively regulates that of AGO4 and AGO10; furthermore, AGO7 requires a minimum threshold of ABA for its expression. BaMV infection has a similar effect, which is probably due to the increased ABA content in plants infected with BaMV (Alazem et al., 2014). Following BaMV infection, ABA deficiency failed to restore AGO1, AGO2, and AGO7 levels to the wild-type level, which indicates that their full expression requires the ABA pathway.

With the levels of AGO1 reduced in the aba2-1 mutant, we next tested if aba2-1 has effective remaining AGO1 by transiently expressing the GFP171.1 protein (a reporting construct [35S:GFP171.1] that expresses GFP with a binding site for miR171.1 [Brodersen et al., 2008] and is targeted by miR171.1 loaded into AGO1 [Wang et al., 2011]). Following the AGROBEST method (Wu et al., 2014), we transiently expressed GFP171.1 in wild-type and aba2-1 plants. While the normal levels of AGO1 in wild-type plants restricted GFP levels, aba2-1 allowed GFP to accumulate significantly because of the low AGO1 level (Supplemental Fig. S4A). Similarly, with the low level of AGO2 in aba2-1, PVX is expected to replicate and move systemically in Arabidopsis, because AGO2 is responsible for the nonhost resistance against PVX (Jaubert et al., 2011). Indeed, we found PVX-GFP able to accumulate locally and move systemically in aba2-1 but not in wild-type plants (Supplemental Fig. S4B). Collectively, these results support the earlier findings of the effect of ABA deficiency on the levels of AGOs.

Effects of AGO Mutants on BaMV Accumulation

To determine which member of the AGO family is important for defense against BaMV, we infected AGO mutants (ago1-27, ago2-1, ago3-2, ago4-3, ago7-2, and ago10-1) with BaMV. As noted previously, we excluded ago5, ago6, and ago9 from the mutant collection because the expression of these genes is almost undetectable using RTqPCR in Arabidopsis rosette leaves after ABA treatment or BaMV infection (data not shown). In three independent experiments, BaMV titers were reduced by 3-fold in ago1-27 compared with the wild type (Fig. 6A). Other mutants showed enhanced susceptibility to infection: ago2-1 and ago3-2 were the most susceptible lines, with BaMV titers increased by 6- and 4-fold, respectively, compared with the wild type (Fig. 6B). The mutants ago4-3 and ago7-2 showed moderate susceptibility, with titers increased ~2- to 3-fold compared with the wild-type plants (Fig. 6B). Only ago10-1 did not exhibit an altered BaMV level (Fig. 6A). These results highlight the critical roles of AGO2 and AGO3 in defense against BaMV and indicate that AGO4 and AGO7 partially contribute to the defense against BaMV.

We next tested whether ABA could still induce resistance in the absence of AGO2 or AGO3. We sprayed ABA on ago2-1 and ago3-2 mutants and then infected them with BaMV. Wild-type plants showed the expected low level of BaMV coat protein (CP) after ABA treatment, with ~5-fold less protein than the wild-type mock line, whereas ago2-1 showed a slight decrease in CP level and ago3-2 showed almost no change in CP level (Fig. 6C). In a complementary assay, overexpressing AGO2-HA and AGO3-HA with BaMV in N. benthamiana leaves showed that both proteins could reduce CP level significantly (Fig. 6D). Hence, the ABA-mediated effect on BaMV was achieved mainly via AGO2 and AGO3.

The AGO1-miR168 Complex Has No Effect on BaMV Accumulation

AGO1 is posttranslationally regulated by miR168 via the AGO1 protein (Vaucheret et al., 2006; Mallory and Vaucheret, 2009), and miR168 is induced by ABA (Li et al., 2012) and viral infection (Várallyay et al., 2010). BaMV infection induced miR168a by ~70% in wild-type plants (Fig. 7A). Because AGO1 was up-regulated in the OsN3-O/E mock-inoculated line and the up-regulation was enhanced further by BaMV infection (Fig. 4A), we examined whether the increase in miR168a may reduce the AGO1 depletion-mediated reduction of BaMV. To test this hypothesis, we used two different lines: 4m-AGO1, an AGO1 transgenic line with four mismatches that prevent miR168 binding and miR168-mediated regulation; and an miR168a-mutant, miR168a-t2, that possesses four mismatches to prevent binding to wild-type AGO1 (Vaucheret et al., 2004). Surprisingly, the levels of BaMV in both lines were comparable to those in their corresponding wild-type plants (Fig. 7, B and C). These results suggest that miR168 and AGO1 do not contribute to ABA-mediated resistance and that the resistance phenotype of the ago1-27 mutant may be attributable to the regulation of other genes by AGO1.

The ago1-27 Mutant Exhibits Higher Levels of Several AGOs

AGO1 plays a central role in sRNA pathways, including siRNA and microRNA pathways, and regulates the expression of many genes involved in growth and development (Vaucheret et al., 2004; Shao et al., 2014). For
example, it binds miR403 and posttranslationally down-regulates AGO2 and AGO3 (Allen et al., 2005). Therefore, we hypothesized that, in the absence of AGO1, the expression of AGO2 and AGO3, and possibly other AGOs, will increase significantly. Indeed, the mock-inoculated ago1-27 mutant showed significant increases in AGO2, AGO3, AGO4, and AGO10 levels (3.2-, 4.4-, 2.7, and 1.6-fold increases, respectively, compared with the wild type), and these increases remained significant after BaMV infection (Fig. 8, A–C and E). AGO7 was not increased significantly in mock-inoculated or BaMV-infected ago1-27 (Fig. 8D). To confirm that the reduced BaMV CP level in ago1-27 resulted from the increase in AGO2 and AGO3 levels, we infected the double mutants ago1-27;ago2-1 and ago1-27;ago3-2 with BaMV. Indeed, the CP level in ago1-27;ago2-1 was as high as
that in the ago2-1 mutant, which confirms the effect of high levels of AGO2 on BaMV accumulation in ago1-27 background (Fig. 8C). However, ago3-2 showed ~3-fold BaMV CP accumulation, whereas the double mutant ago1-27 ago3-2 showed reduced CP level, similar to that found in ago1-27. Certain factors required for AGO3 function might be down-regulated in the ago1-27 mutant (Fig. 8F). These results and those in Figure 6 collectively suggest that the high levels of AGO2, AGO3, and AGO4 in the ago1-27 background may account for the reduced BaMV levels in ago1-27 and imply that AGO1 is involved in the transcriptional regulation of AGO2, AGO3, AGO4, and possibly AGO10.

DISCUSSION

The importance of ABA in plant resistance against viruses has been attributed to its role in increasing callose deposition at plasmodesmata and hindering viral cell-to-cell-movement (Iriti and Faoro, 2008; Oide et al., 2013). However, several members in the DCL, AGO, or RDR families also are regulated by ABA or by abiotic stimuli in which ABA plays major roles (Yang et al., 2008; Capitao et al., 2011; Bai et al., 2012; Li et al., 2012; Hunter et al., 2013). Therefore, it was suggested that ABA may exert a large regulatory effect on the sRNA pathway and that ABA-mediated resistance to viruses may partially be exerted through this pathway.

The effect of ABA on defense under conditions in which Gsl8 was down-regulated (Fig. 1) prompted us to search for a link between the ABA and sRNA-silencing pathways. After the identification of several ABREs in the promoter regions of AGO genes, we subsequently focused on the AGO family (Fig. 2A). In this study, we characterized the responses of several AGO members to ABA treatment and BaMV infection and showed how ABA modulates the expression of AGO genes, some of which are important for resistance to BaMV (Fig. 9).

Elevated levels of exogenous or endogenous ABA and BaMV infection have similar effects on the expression of AGO genes, but to different extents. The infected wild-type plants and mock-inoculated OsN3-O/E plants exhibit increased expression of AGO1, AGO2, and AGO3 (Figs. 3 and 4), probably due to the prolonged effects of ABA compared with plants infected for 4 d (Fig. 2). Moreover, the synergistic effects of BaMV with ABA in the infected OsN3-O/E or ABA-treated plants boosted the expression of these AGOs remarkably (Figs. 2 and 4), which implies that the BaMV-mediated increase of AGO expression is similar to, or dependent on, the ABA pathway. The resemblance between the effects of BaMV and ABA is most likely due to the increased ABA content in BaMV-infected plants. Indeed, BaMV infection increases ABA content in Arabidopsis and N. benthamiana (Alazem et al., 2014). Of the TFs able to bind AGO promoters in vitro, ABI3/VP1 and MYBH were negatively affected by BaMV infection, unlike TGA5, which was increased slightly (Fig. 2C). This implies that BaMV partially interferes with several ABA responses. However, ABA treatment restored their levels to more than wild-type levels. Since ABA-related TFs are numerous and belong to different families, further work is required to validate the involvement of TF candidates in the transcription of AGO genes.

ABA-deficient mutants showed reduced levels of certain AGOs compared with the infected wild-type plants (Fig. 5), indicating that the BaMV-mediated increase of AGO levels is dependent on the ABA pathway. In the aba2-1 and aao3 lines, infection restored only AGO3 expression to levels comparable to that in infected wild-type plants (Fig. 5D), probably because of the increase in levels of other ABA-antagonistic hormones/factors. For example, the deficiency of ABA in these mutants enhances other antagonistic pathways such as the SA pathway, which also may regulate AGO3 (Yasuda et al., 2008; de Torres Zabala et al., 2009). On the other hand, AGO4 and AGO10 were negatively affected by both treatments and responded similarly in the infected OsN3-O/E line, showing significant increases compared with the infected wild type (Fig. 4, E and G). It remains to be determined how or why both genes were unexpectedly up-regulated in the OsN3-O/E line. In ABA-deficient mutants, the expression of both genes remained significantly lower than that in the infected wild-type plants (Fig. 5, E and G), which implies that ABA partially tunes their expression within a certain range. Although AGO7 was not induced by ABA or BaMV infection (Figs. 3F and 4P), its expression was reduced significantly in aba2-1 and aao3 mutants, indicating that its basal expression still requires
an intact ABA pathway (Fig. 5F). Some factors might be down-regulated in ABA mutants, thereby reducing the transcription of AGO7 under such conditions.

It has been suggested that AGO members in the same clade, such as the AGO1/5/10 clade (Mallory et al., 2009; Fang and Qi, 2016) or the AGO4/6/9 clade (Havecker et al., 2010; Fang and Qi, 2016), may be functionally redundant. However, our results suggest that members of the AGO2/3/7 clade may not be redundant for resistance against BaMV infection, because the mutants ago2-1 and ago3-2 showed 6- and 4-fold increases in BaMV titers compared with the wild type, whereas other mutants showed an ~2-fold increase in titers (Fig. 6). It seems that this clade may be more important than others in resisting BaMV and other potexviruses (Brosseau et al., 2016). It was reported previously that P25, the viral suppressor of RNA (VSR) in PVX, affects AGO3 activity, and when P25 is deleted, AGO3 had a significant effect on resistance to DP25-PVX (Brosseau and Moffett, 2015). In addition, PVX levels were strongly reduced when AGO2 and AGO5 were overexpressed in N. benthamiana (Brosseau and Moffett, 2015). Interestingly, the superior effects of AGO2 and AGO5 were lost when plants were infected with a PVX variant that lacks its VSR, because all AGOs performed similarly (Brosseau and Moffett, 2015). Of note, AGO3 does not play a major role in resisting PVX infection (Brosseau and Moffett, 2015). While AGO2 exerts antiviral

Figure 8. Effects of the ago1-27 mutant on the expression of AGO2, AGO3, AGO4, AGO7, and AGO10 and the susceptibility of ago2-1 and ago3-2 mutants. A to E, RT-qPCR analysis of the levels of AGO2 (A), AGO3 (B), AGO4 (C), AGO7 (D), and AGO10 (E) in mock and infected lines of ago1-27 mutants at 10 dpi. Data are means ± so of three biological replicates. Statistical analysis was carried out as described in Figure 1: *, P < 0.05 and **, P < 0.01. Additional statistical analysis was carried out to compare transcript levels in the infected wild-type (WT) and ago1-27 lines. F, Protein blot for BaMV-CP in ago1-27, ago2-1, and ago3-2 and the double mutants ago1-27;ago2-1 and ago1-27;ago3-2. Data are means ± so of three replicates.
slicing activity and is efficient against several target viruses (Harvey et al., 2011; Scholthof et al., 2011; Minoia et al., 2014), there is less evidence that AGO3 has such effects. In fact, an in vitro assay suggested that AGO3 does not possess antiviral slicing activity, has no preference for the 21- or 22-nucleotide siRNA, and has no bias for the 5' terminus-associated nucleotide (Schuck et al., 2013). Our data showed that overexpressing both AGO2 and AGO3 down-regulated BaMV-CP levels (Fig. 6, C and D), but their exact role against BaMV infection has yet to be determined.

Recently, viroid-derived siRNAs from Potato spindle tuber viroid were found incorporated into AGO3 and AGO9 but not as strongly as in AGO1, AGO2, or AGO4 (Minoia et al., 2014). Thus, how AGO3 actually functions within the context of the siRNA machinery remains to be

Figure 9. Roles of ABA in modulating plant antiviral defenses. The schematic representation shows the central roles of ABA in modulating the plant response to BaMV infection. The ABA pathway is induced by BaMV infection (Alazem et al., 2014), which regulates several defense responses. However, BaMV benefits from this stimulation by inducing ABA2, which specifically supports the accumulation of BaMV (Alazem et al., 2014). In response to BaMV infection, AGO1, AGO2, and AGO3 are all up-regulated in an ABA-dependent manner, but only AGO2 and AGO3 have critical effects on resistance to BaMV (Figs. 3 and 5). AGO1 seems to play a different role in plant resistance to BaMV despite being up-regulated by ABA, as the mutant showed a resistance phenotype (Fig. 6A). The AGO1 regulator miR166a is also up-regulated in response to ABA or BaMV infection, and this up-regulation maintains mAGO1 RNA within certain levels (Vaucheret et al., 2006; Mallory and Vaucheret, 2009). When miR166a is impaired, AGO1 does not affect plant resistance to BaMV (Fig. 6). However, the mutant ago1-27 exhibits a resistance phenotype due to the increased levels of AGO2, AGO3, and AGO4 (Fig. 8). AGO4 is down-regulated by ABA and BaMV infection (Fig. 2). However, ABA overexpression and BaMV infection together increase AGO4 level (Fig. 4). Nevertheless, AGO4 still contributes to anti-BaMV defense (Fig. 5). ABA also was reported to be responsible for the enhanced callose deposition on plasmodesmata, which hinders viral cell-to-cell movement (Fraser and Whenham, 1989; Iriti and Faoro, 2008). However, induction of the dexamethasone-inducible RNAi line gsl8i, which inhibits callose synthase, did not affect the defensive role of ABA in plants (Fig. 1).
established. Does it act directly by slicing viral genes or indirectly by regulating plant genes employed by the viral replication complex? For example, in the effects of AGOs against PVX infection, all AGOs (AGO1–AGO10) seem to have antiviral effects but differ in their contribution to resistance, whereas such effects are observed only when the VSR of PVX is impaired (Brosseau and Moffett, 2015). This functional redundancy enriches the plant defense options against viruses, especially those with various VSRs that may target different AGOs (Hamera et al., 2012). If a VSR blocks any of these AGOs, the plant still has other players to fully compensate for the loss of function of the virally impaired AGOs. For instance, AGO1 plays an important role in the silencing pathway against several viruses, such as TCV or TuMV (Turnip Mosaic Virus; Qu et al., 2008; Ruiz-Ferrer and Voinnet, 2009; Harvey et al., 2011). When AGO1 is targeted by the VSR of TCV (Qu et al., 2008) or CMV (Zhang et al., 2006), AGO2 appears to be the major backup player, because the ago2-1 mutant shows enhanced susceptibility to TCV or CMV infection (Harvey et al., 2011). In our case, AGO2 and AGO3 appear to be the major players against BaMV, and the redundancy of AGO4 and AGO7 can partially compensate for the loss of function of AGO2 or AGO3.

Although AGO1 is important for defense against some viruses, we did not observe increased resistance against BaMV in the mir168a-resistant line 4mAGO1 or in the mutant mir168a-2, in which AGO1 avoids mir168a regulation (Fig. 8). AGO1 is feedback regulated by mir168a, which means that the more AGO1 protein available to mir168a, the less AGO1 transcripts remain, as a consequence of mir168a regulation (Mallory and Vaucheret, 2009, 2010). Our results showed that AGO1 increased after BaMV infection, which implies that the AGO1 protein might be impaired by a BaMV-related suppressor, probably TGBp1, thereby allowing more AGO1 transcripts to accumulate. The VSR of PVX, P25, interacts with AGO1 and leads to its degradation (Chiu et al., 2010). In contrast, the mutant ago1-27 showed a reduced level of BaMV, ~4-fold reduction compared with the wild-type plants (Fig. 6A). These reduced levels were also observed for PVX and TuMV (García-Ruiz et al., 2015; Brosseau et al., 2016). AGO1 regulates several genes, including AGO2 and AGO3 (Allen et al., 2005), through the microRNA pathway, and we confirmed up-regulated levels of these genes in the ago1-27 mutant (Fig. 8). More interestingly, we noticed that levels of AGO4 and AGO10 also were up-regulated significantly (Fig. 8). Therefore, the increase in expression of these AGOs (AGO2, AGO3, and AGO4) and the observation that their mutants showed enhanced susceptibility to BaMV may underlie the reduced BaMV CP level in the ago1-27 mutant. In fact, this was confirmed by the use of the ago1-27,ago2-1 double mutant. Our data clearly show that protecting AGO1 from down-regulation by impairing the binding of the AGO1-mir168a complex did not affect the BaMV level, indicating that AGO1 may not be required for ABA-mediated resistance (Fig. 7).

We found AGO4 to be negatively regulated by ABA or BaMV infection (Figs. 2 and 4); however, the ago4 mutant showed increased susceptibility compared with the wild type (Fig. 6B). This indicates that AGO4-mediated resistance is independent of ABA and suggests that AGO4 may be regulated by other ABA-antagonizing hormones, such as SA, or by TFs that are negatively regulated by ABA. Such regulation increases the ability of plants to activate alternative AGOs if a pathogen is able to evade plant defenses or induce pathways that antagonize defense systems. Similarly, ago4 mutant showed increased susceptibility to another potexvirus “Plantago asiatica mosaic virus” (Brosseau et al., 2016), and this means that all plant responses to potexviruses are similar but with different degrees of AGO involvement. For instance, AGO3 is more important for resistance to BaMV than PVX infection.

In summary, our data tightly connect ABA activity with that of several AGO members and indicate that ABA-mediated defense against BaMV infection is achieved via two major AGOs that belong to the same clade: AGO2 and AGO3. Other AGOs, such as AGO4 and AGO7, also are involved in resistance but act independently of ABA (Fig. 9). In addition, AGO1 and its regulator mir168a were found to be dispensable for ABA-mediated resistance, and their loss did not even affect plant susceptibility to BaMV (Fig. 9). In contrast, ago1-27 showed enhanced resistance due to increased background levels of AGO2, AGO3, and AGO4 (Fig. 8). It is also possible that ABA regulates other genes in the sRNA pathway in addition to Dicer (Supplemental Fig. S3D). Elucidating the role of ABA in the sRNA pathway would provide a better understanding of its role in virus resistance. For example, it remains to be revealed how ABA affects the DCL and RDR families so that a bigger picture of ABA’s roles in the RNA silencing pathway can be drawn.

MATERIALS AND METHODS

Promoter Analysis of AGO Genes

To analyze promoter regions of AGO genes, we obtained fragments located between −1,500 and +100 bp of each start codon from TAIR and subjected these to promoter scanning to search for ABREs and MYC- and MYB-binding motifs. The online server PlantPAN (http://plantpan.mbc.nctu.edu.tw) was used (Chang et al., 2008). Analysis was performed with a confidence level of 100% and P of ~0.0002.

Plant Materials and Growth Conditions

Wild-type and mutant Arabidopsis (Arabidopsis thaliana) lines were grown under long-day conditions at 22°C with relative humidity of ~60%. Arabidopsis wild-type (Columbia-0) and gdl8i plants (Chen et al., 2009) were grown until they reached the age of ~24 d. At this time, some of the gdl8i plants were watered with dexamethasone (Sigma; D9802) prepared in a final concentration of 25 μM in water. Each pot was watered with ~25 mL of dexamethasone solution every other day for 6 d. All plants were sprayed with ABA (Sigma; A1049) at a final concentration of 100 μM, once on the day before BaMV infection and then at 3 dpi. ABA was sprayed on adaxial and abaxial sides until the solution began to run off the leaves. Leaves were collected at 4 dpi for further analysis.

Inoculation with BaMV

Four to five leaves of ~25-d-old Arabidopsis wild-type plants, the 4mAGO1 transgenic line, and the mir168a-2 mutant (Vaucheret, 2009) were inoculated...
with 1 µg of BaMV-S virions (prepared in a concentration of 1 µg per 5 µL of diethyl pyrocarbonate water), which were purified from *Nicotiana benthamiana* plants inoculated with an infectious cDNA clone of BaMV-S, pCB (Lin et al., 2004). For time-course assays, the inoculated leaves of wild-type plants were collected at 3, 7, 10, and 14 dpi for further analyses. For baMV-1 and mir166b-2 lines, leaves were collected at 10 dpi for further assays. The BaMV infectivity assays for the ABA overexpression line OsN3-O/E (Hwang et al., 2010) and the ABA-deficient mutants aba2-1 and aao3 were performed as described previously (Alazem et al., 2014). Approximately eight to 10 leaves from three infected plants were collected for further RNA extraction, PCR, and northern-blot assays.

**RNA Analysis**

Total RNA was extracted from leaves by the TRizol method (Invitrogen), purified using the phenol-chloroform method, and precipitated in 0.1 volume of 3 M sodium acetate and 2.5 volumes of 100% (v/v) ethanol.

**Northern-Blot Analysis**

BaMV RNA was detected as described previously (Alazem et al., 2014). An amount of 2 µg of total RNA was glyoxylated and then separated by electrophoresis on a 1% agarose gel. The RNA was then transferred onto a Hybond-N+ membrane (Life Technology), cross-linked under UV light, and hybridized against 32P-labeled CP probe (Lin et al., 1993). mirR168a was detected by separating 5 µg of total RNA on a 19% acrylamide/7 M urea gel and then hybridizing the RNA with (~)miR168a probe labeled with 32P (Chen et al., 2007).

**Measuring the Density of BaMV RNA Bands**

The UVP BioSpectrum 600 Imaging System version 6.8 (www.uvb.com) was used to measure the density of BaMV genomic RNA bands (~6.4 kb), except for Figure 1B data, for which genomic RNA in the golihi line was too weak to be measured correctly. Thus, subgenomic RNA2 (which encodes BaMV CP) was used instead for the quantification. Densities from three replicates were averaged and used as an indicator of viral RNA accumulation, and statistical analysis was carried out using Student’s t test.

**RTqPCR**

An amount of 2 µg of each RNA sample was treated with RQI-DNase (Promega) for 30 min at 37°C. RNA samples were then subjected to first-strand cDNA synthesis with SuperScript III in accordance with the manufacturer’s instructions (Invitrogen). The resulting cDNA was diluted to a final concentration of 20 ng µL−1. The RTqPCR primers used in this study are shown in Supplemental Table S1. All RTqPCRs were performed with SYBR Green Supermix (Applied Biosystems) following the manufacturer’s instructions. ACTIN2 was used as an internal control, and experiments were carried out in triplicate.

**Protein Analyses**

Transient expression of GFPPl7.1 was carried out on 6-d-old Arabidopsis seedlings as described by Wu et al. (2014). PVX-GFP was transiently expressed in *N. benthamiana* plants, systemic leaves were collected at 6 dpi, and leaf crude extract was used as inoculum on Arabidopsis plants (~25 d old). Arabidopsis inoculated and systemic leaves were collected at 10 and 20 dpi, respectively. Leaves (~0.5 g) were ground to a fine powder in liquid nitrogen and homogenized by adding a similar volume of extraction buffer (0.1 M Gly-NaOH [pH 9], 0.1 M NaCl, 0.5 M EDTA, 2% SDS, and 1% sodium laurosarcosine; Vårällay et al., 2010). Samples were boiled for 5 min and subsequently centrifuged at 13,000 rpm for 5 min. For GFPPl7.1 and PVX-GFP detection, crude protein extract (75 µg) was loaded with 2× dye (1 M Tris, 10% SDS, 100% glycerol, and 900 µL of β-mercaptoethanol in 50 mL of water) onto 10% SDS-polyacrylamide gels for western-blot analysis, and membranes were hybridized with anti-GFP antibody (GeneTex). For BaMV-CP detection, ~10 µg of total protein was used for western-blot analysis. Overexpressing AGO2-HA and AGO3-HA (in pBICp35 vector) in *N. benthamiana* leaves was carried out as described previously (Brosseau and Moffett, 2015; Brosseau et al., 2016). Briefly, the Agrobacterium tumefaciens strain C58C1 harboring the BaMV clone (pKBG; Prasanth et al., 2011), AGO2-HA, AGO3-HA, and pBicP35 empty vector (EV; Takeda et al., 2008) were grown to 100 µL of RISC buffer, and 30 µL was proportionally mixed with 1.5× Laemml loading buffer and loaded onto an SDS-PAGE gel. HA was detected using anti-HA horseradish peroxidase-conjugated antibodies (clone 3F10; Roche).

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Identification of MYB and MYC motifs in the promoters of AGO genes.

**Supplemental Figure S2.** Quantitative analysis of ABA biosynthesis transcripts in the Arabidopsis transgenic line OsnJ-O/E.

**Supplemental Figure S3.** Relative expression of marker genes in the SA and RNA pathways in aba2-1 and aao3 mutants.

**Supplemental Figure S4.** Levels of GFPPl7.1 and PVX-GFP in the aba2-1 mutant.

**Supplemental Table S1.** Primers used in the study.

**ACKNOWLEDGMENTS**

We thank Dr. Jae-Yean Kim for the golihi mutant; Dr. Hervé Vaucerer (Institut National de la Recherche Agronomique) and Dr. James Carrington (Donald Danforth Plant Science Center) for providing several of the mutants used in this study; Chantal Brosseau and Geneviève Giroux (University of Sherbrooke) for technical assistance; Dr. W.-H. Cheng (Institute of Plant and Microbial Biology, Academia Sinica) for support and critique of the study, and the Sequencing Core Laboratory at the Institute of Plant and Microbial Biology, Academia Sinica, for technical assistance. We extend our special thanks to the late Prof. Biao Ding, whose valuable suggestions on the project made this research article possible.

Received January 6, 2017; accepted March 3, 2017; published March 7, 2017.

**LITERATURE CITED**


Downloaded from on November 11, 2017 - Published by www.plantphysiol.org

Copyright © 2017 American Society of Plant Biologists. All rights reserved.


Copyright © 2017 American Society of Plant Biologists. All rights reserved.