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

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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 crucifier strain in Arabidopsis, and several mutants of the ABA pathway exhibit enhanced accumulation of tobacco mosaic virus crucifier 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).

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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 sRNA 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 *gsl8-RNAi* (*gsl8i*) line (Chen et al., 2009) in Arabidopsis, in which induction significantly reduces the transcript level of *Gsl8*, the callose synthase-like gene that functions in callose deposition at cell walls and plasmodesmata (Thiele et al., 2009; Guseman et al., 2010). Treatment of ~22-d-old *gsl8i* plants with dexamethasone (hereafter referred as dexa-*gsl8i* 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 dexa-*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 ~35% and 52%, respectively. Interestingly, dexa-*gsl8i* plants also exhibited reduced BaMV levels upon ABA treatment, to ~55% compared with the corresponding mock line (Fig. 1B, first gel). As a parallel control, ABA treatment induced the expression of *HAI1* (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 100% and *P* value of ~0.0002 (Fig. 2A). This finding suggests that ABA-related factors regulate the expression of these AGOs. In addition, several binding motifs for MYB and MYC 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* (~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 few 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 ~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 ~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 ~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

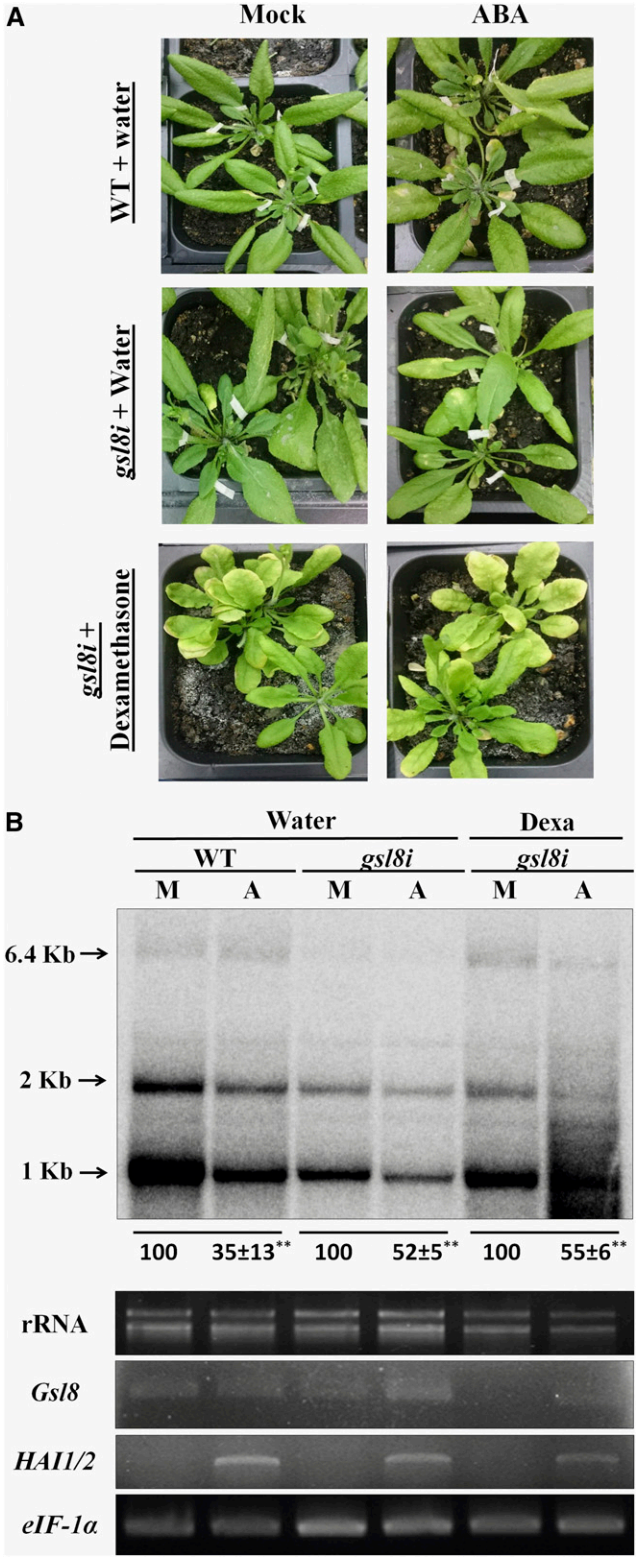

Figure 1. Effects of ABA on plant resistance to BaMV in the *gsl8i* line. A, Plant responses to induction of the *gsl8-RNAi* line by treatment with dexamethasone. Wild-type (WT) and *gsl8i* plants treated with water showed similar phenotypes with or without ABA. At age ~24 d, selected regions of *gsl8i* plants were watered with dexamethasone (25 μm in 1 L per watering) for 6 d, then all plants received double ABA treatments (100 μM): 1 d before BaMV infection and at 3 d postinfection (dpi). Leaves were inoculated with BaMV virions (1 μg in 5 μL of diethyl pyrocarbonate water per leaf) and collected at 4 dpi for further analysis. Photographs were taken when plants were 35 d old. B, BaMV RNA levels measured at 4 dpi by northern blotting in wild-type and *gsl8i*-induced plants with or without ABA. Average densities of the second subgenomic BaMV RNA were calculated from three different replicates, and the results were normalized to those in mock-treated lines. Statistical analysis was carried out using one-sided Student's *t* test to determine the significance of regulation (**, $P < 0.05$). rRNA was used as an internal control. Reverse transcriptase (RT)-PCR was used to measure transcripts of *Gsl8*, *HAI1/2* (an ABA-responsive gene), and *eIF-1α* (an internal control). Experiments were repeated three times with similar results. A, ABA-treated plants; M, mock-treated plants.

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 (Fig. 2B) 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

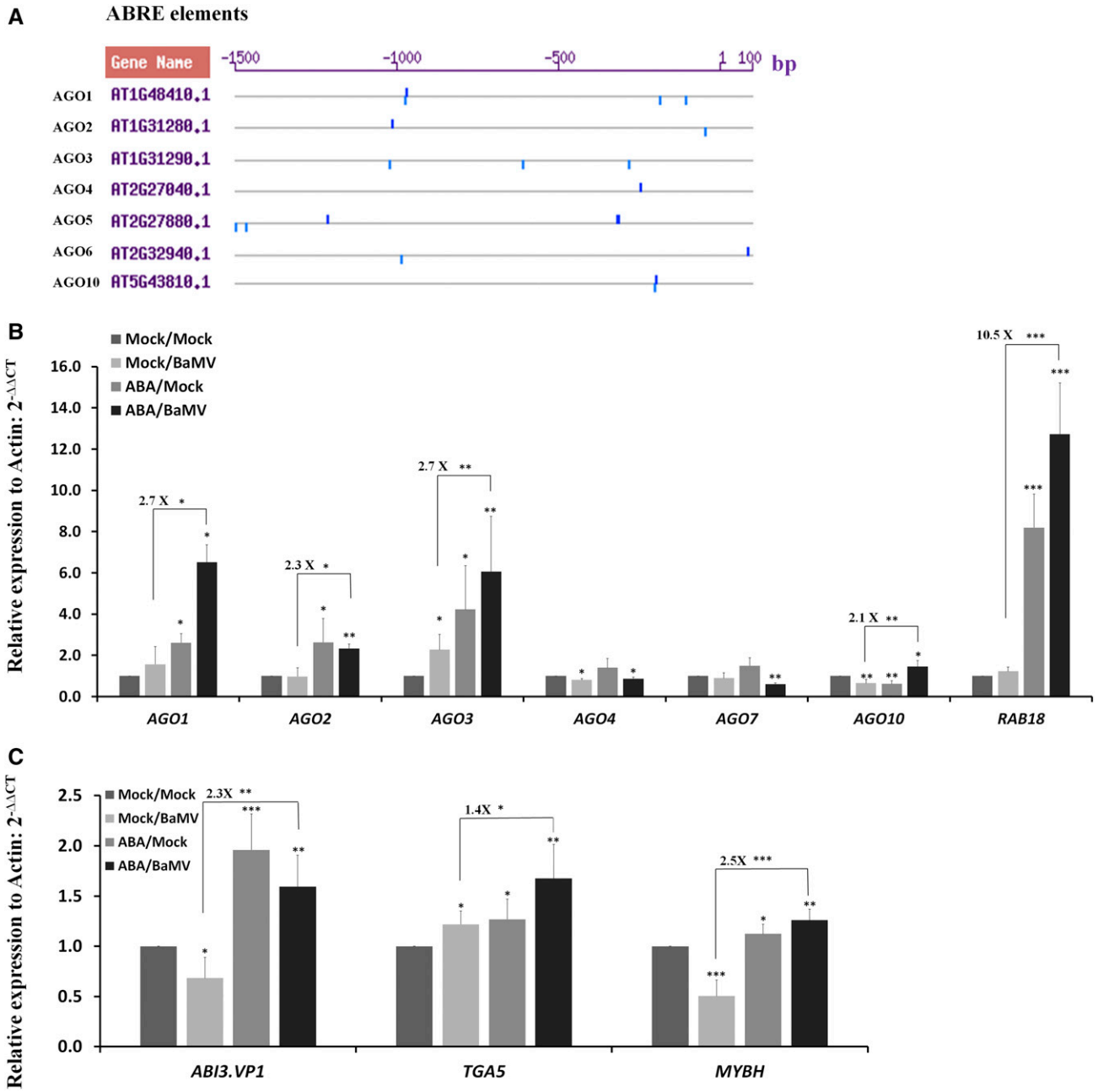

Figure 2. Identification of ABREs in promoters of AGO genes, and the effect of ABA and BaMV infection on the expression of AGOs and ABA-related transcription factors (TFs). A, ABREs in AGO promoters. Promoter fragments located between −1,500 and +100 bp of the start codon were examined for the presence 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).

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

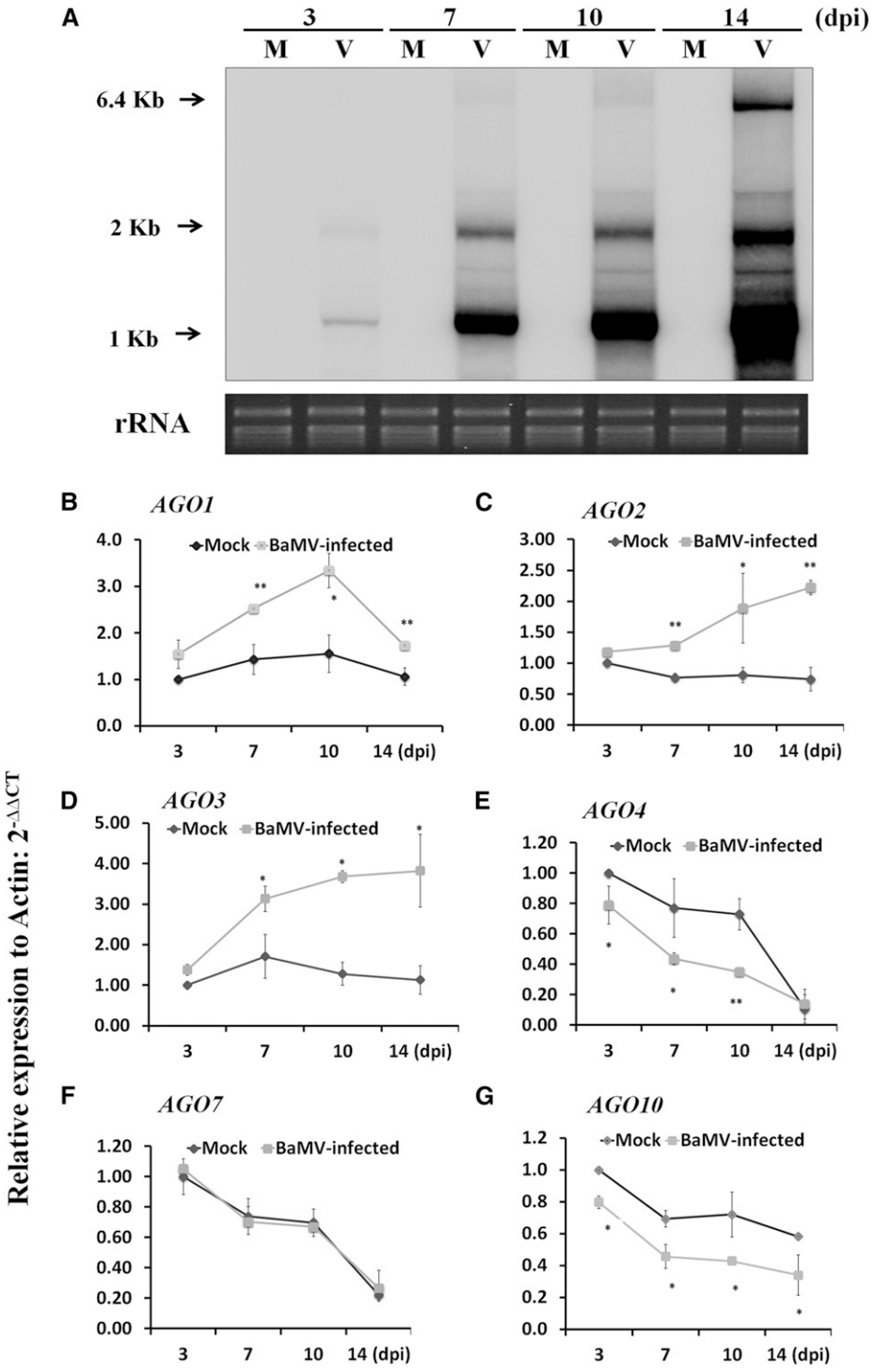

Figure 3. Progression of BaMV accumulation in Arabidopsis and its effect on *AGO* gene expression. Approximately 25-d-old Arabidopsis plants were inoculated with 1 μg of BaMV virions, while mock-inoculated plants were inoculated with water. A, Northern-blot analysis of BaMV RNA level in inoculated leaves at 3, 7, 10, and 14 d after BaMV infection. The rRNA level was examined as a loading control. M, Mock-treated plants; V, BaMV-infected plants. B to G, RTqPCR of the relative dynamics of AGO expression during BaMV progression: *AGO1* (B), *AGO2* (C), *AGO3* (D), *AGO4* (E), *AGO7* (F), and *AGO10* (G). Data are means ± SD of three biological replicates. Statistical analyses were carried out as described in Figure 1: *, $P < 0.05$ and **, $P < 0.01$, by Student *t* test.

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

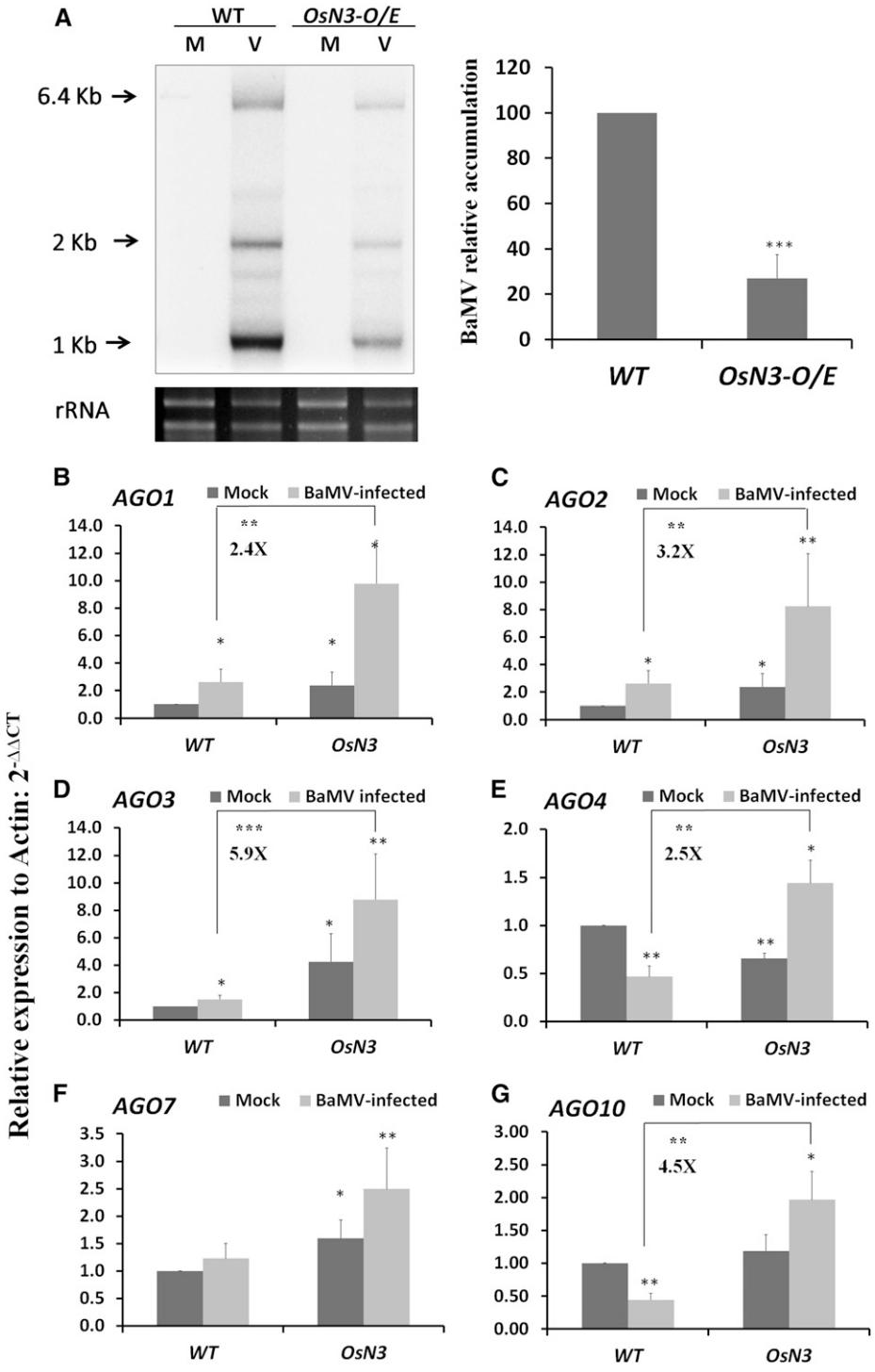

Figure 4. Effects of ABA on BaMV and AGO levels in the *OsN3-O/E* transgenic line. A, Northern-blot analysis (left) and quantitative analysis (right) of BaMV RNA level in wild-type (WT) and *OsN3-O/E* leaves at 10 dpi. M, Mock infected; V, BaMV infected. B to G, RTqPCR analysis of AGO gene levels in 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.

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

producing more ABA (Alazem et al., 2014), the several-fold 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 down-regulated 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

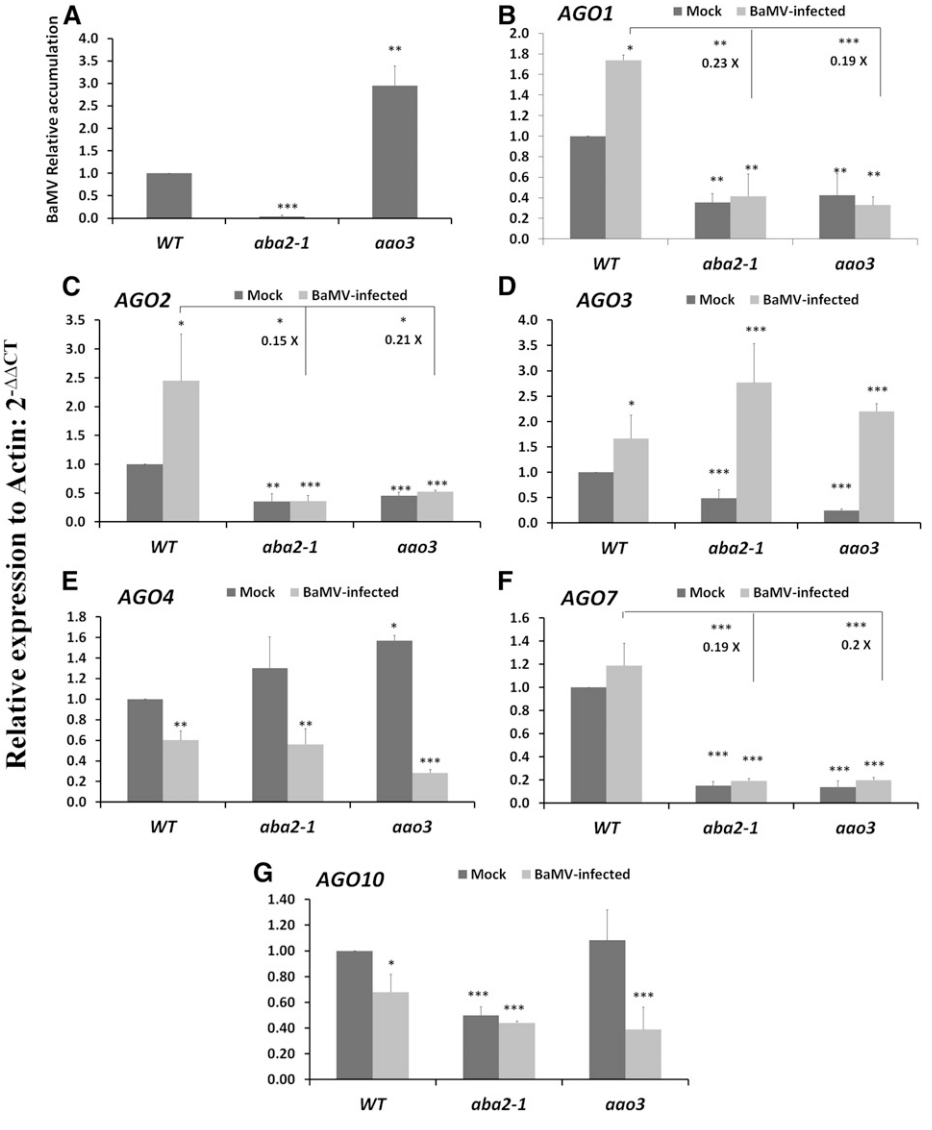

Figure 5. BaMV accumulation and AGO transcript expression in ABA-deficient mutants. RTqPCR analysis is shown for the levels of BaMV *CP* gene (A), *AGO1* (B), *AGO2* (C), *AGO3* (D), *AGO4* (E), *AGO7* (F), and *AGO10* (G) in mock and infected lines of *aba2-1* and *aao3* ABA-deficient mutants. Plants 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-2*, 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 *AGO*s

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

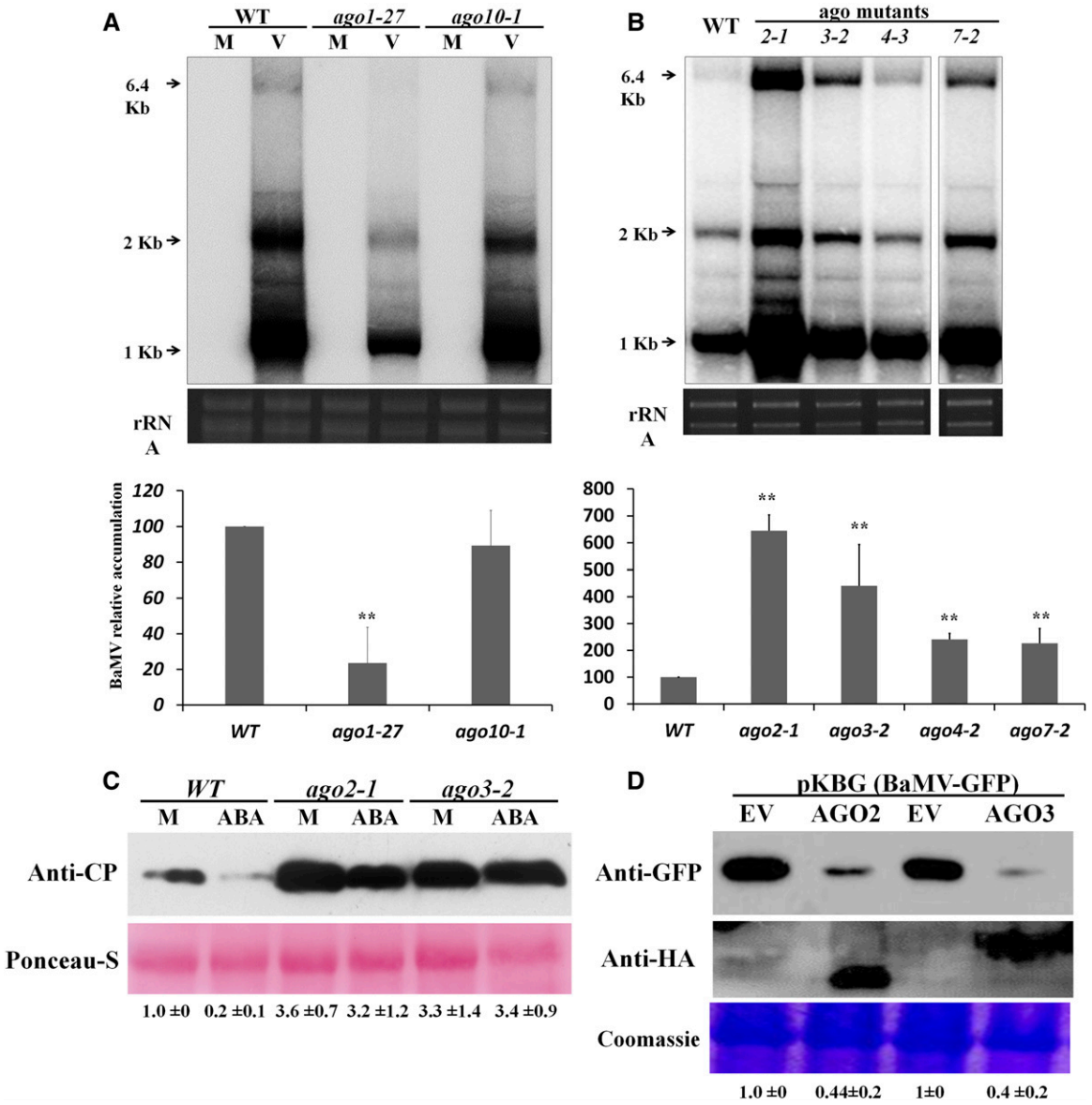

Figure 6. Effects of *AGO* mutants on the accumulation of BaMV in Arabidopsis. A and B, Northern-blot analyses of genomic and two subgenomic BaMV RNAs in *ago1-27* and *ago10-1* (A) and *ago2-1*, *ago3-2*, *ago4-3*, and *ago7-2* (B) at 10 dpi. The relative accumulation of BaMV in the wild-type (WT) and mutants is shown below each blot. The northern blot in B was not exposed for as long as that in A to prevent saturation of the bands and allow for the measurement of BaMV density in *ago2-1* and other backgrounds. M, Mock infected; V, BaMV infected. C, Protein blots for BaMV CP in the wild type and *ago2-1* and *ago3-2* mutants pretreated with 100 μM ABA 1 d before BaMV infection and then given another treatment at 3 dpi. The bottom gel represents Ponceau S staining, and data are means ± SD of three independent replicates. D, Protein blots of BaMV-GFP (top) and AGO-HA (middle). Commassie Blue staining (bottom) data are means ± SD of three independent replicates. Statistical analysis was carried out as described in Figure 3: **, $P < 0.01$.

example, it binds *miR403* and posttranscriptionally downregulates *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

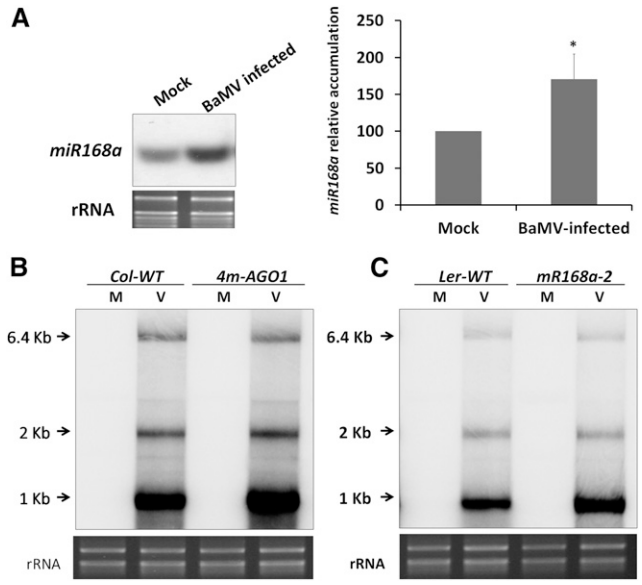

Figure 7. Effects of impaired *AGO1* or *miR168a* on the accumulation of BaMV in Arabidopsis. A, Northern-blot analysis of *miR168a* levels in wild-type mock- and BaMV-infected lines. Relative accumulation of *miR168a* is shown at right. Data are means ± SD of three biological replicates. Statistical analysis was carried out as described in Figure 3: *, $P < 0.05$. B and C, Northern blots of BaMV RNAs in the transgenic line *4mAGO1* (B) and the mutant line *mR168a-2*. Plants were infected with 1 μg of BaMV virions, and leaves were collected at 10 dpi for further analysis. Col, Columbia-0; Ler, Landsberg *erecta*; M, mock infected; V, BaMV infected; WT, wild type.

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 RNA-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 4F), its expression was reduced significantly in *aba2-1* and *aao3* mutants, indicating that its basal expression still requires

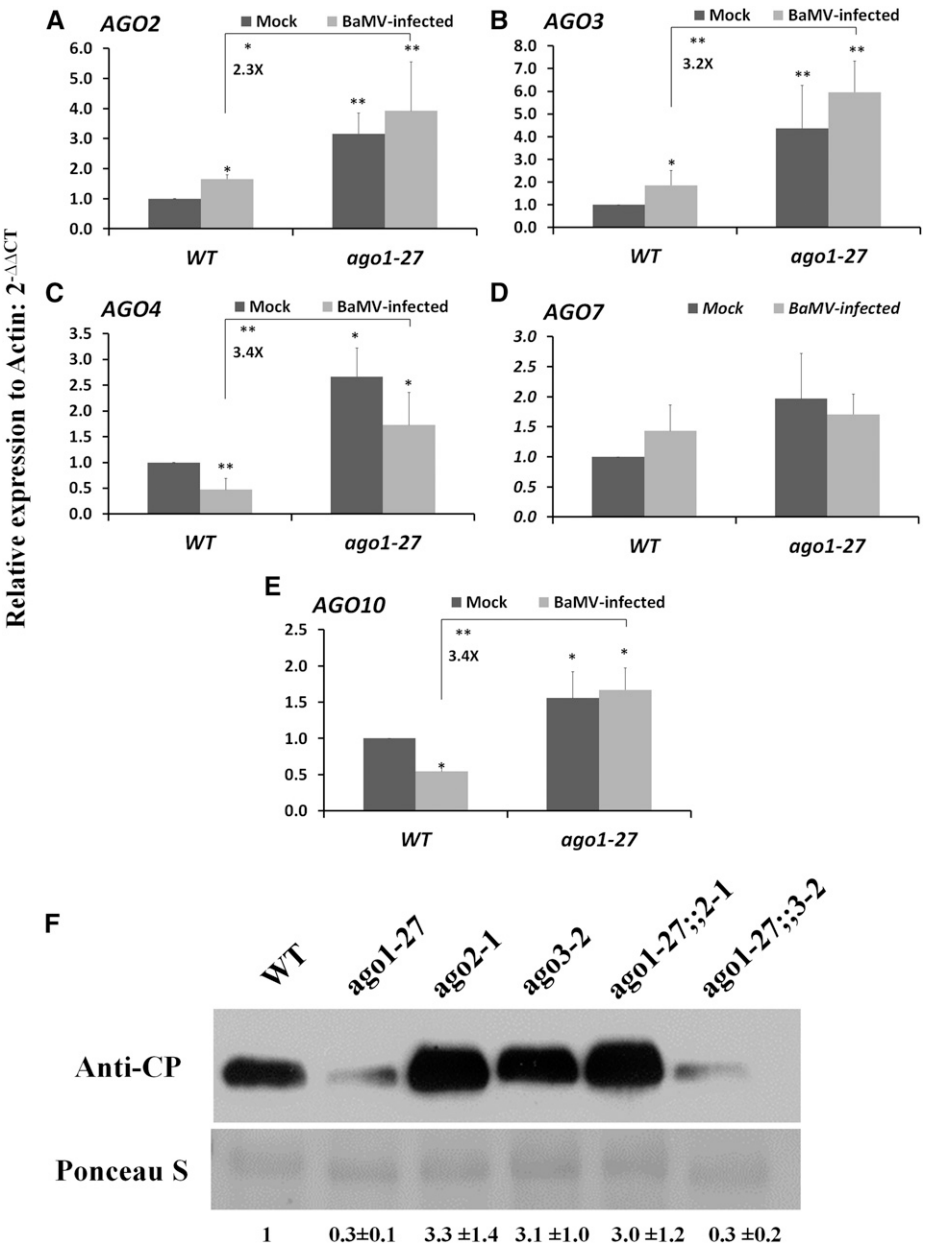

Figure 8. Effects of the *ago1-27* mutant on the expression of *AGO2*, *AGO3*, *AGO4*, *AGO7*, and *AGO10* and the susceptibility of *ago2-1* and *ago3-2* mutants. A to E, RTqPCR analysis of the levels of *AGO2* (A), *AGO3* (B), *AGO4* (C), *AGO7* (D), and *AGO10* (E) in mock and infected lines of *ago1-27* mutants at 10 dpi. Data are means ± SD 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 ± SD of three replicates.

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 ΔP25-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

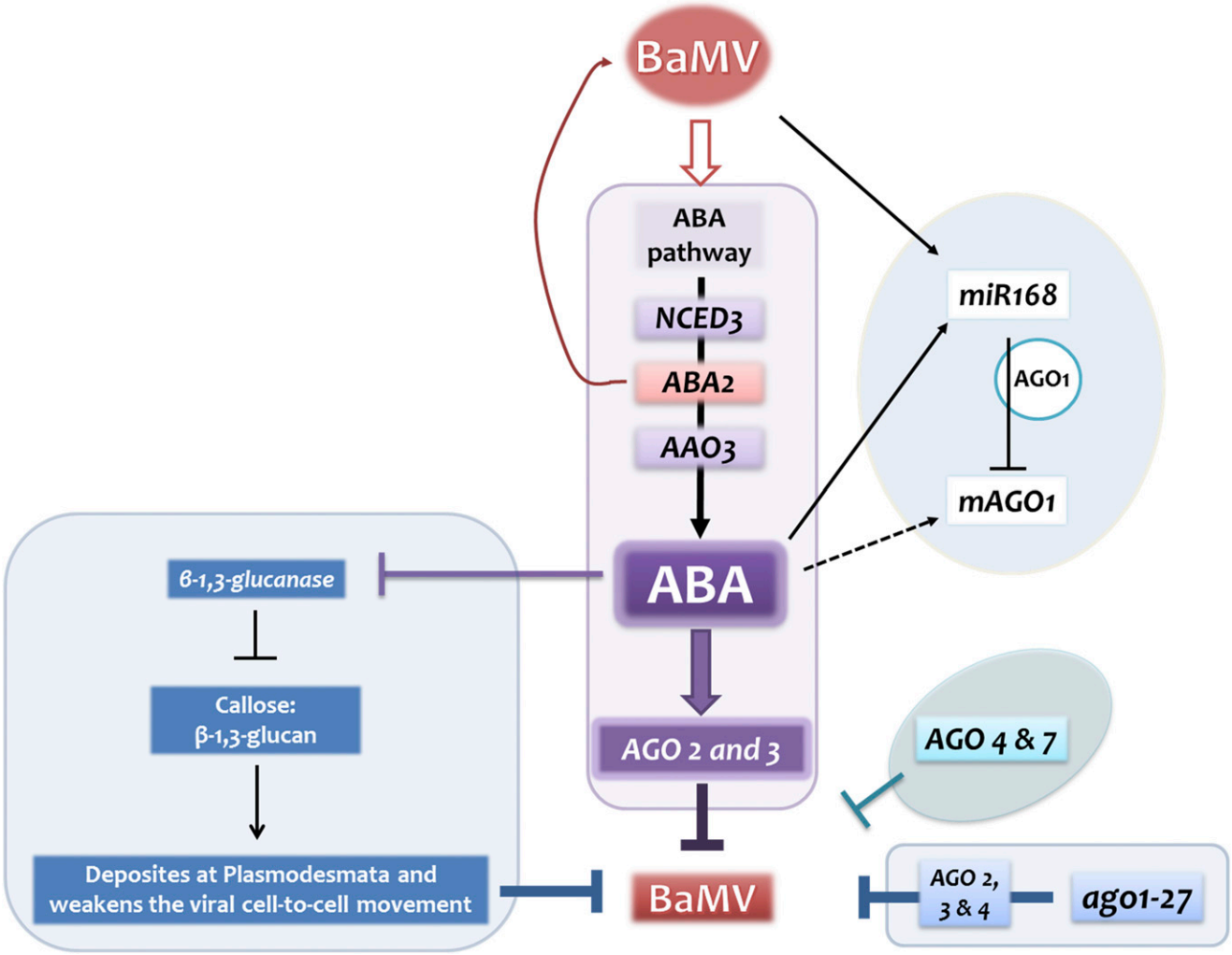

Figure 9. Roles of ABA in modulating plant antiviral defenses. The schematic representation shows the central roles of ABA in modulating the plant response to BaMV infection. The ABA pathway is induced by BaMV infection (Alazem et al., 2014), which regulates several defense responses. However, BaMV benefits 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 *miR168a* 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 *miR168a* 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).

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

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 a 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 (Garcia-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 *AGO*s (*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 *RdR6* (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 *gsl8i* plants (Chen et al., 2009) were grown until they reached the age of ~24 d. At this time, some of the *gsl8i* plants were watered with dexamethasone (Sigma; D4902) 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 *4mAGO1* and *miR168a-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 ^{32}P-labeled CP probe (Lin et al., 1993). *miR168a* 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 ^{32}P (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 *gsl8i* 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 RQ1-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 GFP171.1 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 mM EDTA, 2% SDS, and 1% sodium laurosarcosine; Várallyay et al., 2010). Samples were boiled for 5 min and subsequently centrifuged at 13,000 rpm for 5 min. For GFP171.1 and PVX-GFP detection, crude protein extract (75 μg) was loaded with 2× dye (1 M Tris, 10% SDS, 100% glycerol, and 900 mL 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 OD_{600} = 0.2 for BaMV and OD_{600} = 0.4 for AGO2-HA, AGO3-HA, and EV and then mixed equally. The combinations pKBG+AGO2-HA and pKBG+AGO3-HA were used for infiltration on the same *N. benthamiana* leaf on one side, and pKB+EV was used on the other side. At 3 dpi, tissues were collected to detect CP and AGO-HA. For AGO-HA detection, 1 g of fresh tissue was ground in 2 mL of RISC extraction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 M $MgCl_2$, 5 mM DTT, and 5% Nonidet P-40) with protease inhibitor cocktail (Roche). Immunoprecipitation was carried out with 2 mL of supernatant with 50 μL of HA-agarose beads (Sigma) for 2 h at 4°C on a rotary shaker, followed by several washes with RISC buffer. Beads were resuspended in 100 μL of RISC buffer, and 30 μL was proportionally mixed with 1.5× Laemmli 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 *OsN3-O/E*.

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

Supplemental Figure S4. Levels of GFP171.1 and PVX-GFP in the *aba2-1* mutant.

Supplemental Table S1. Primers used in the study.

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