The Absciscic Acid Pathway Has Multifaceted Effects on the Accumulation of *Bamboo mosaic virus*

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Absciscic acid (ABA) plays a key role in modulating plant responses to different biotic and abiotic stresses. However, the effect of ABA on virus infection is not fully understood. Here, we describe the effects of the ABA pathway on the accumulation of *Bamboo mosaic virus* (BaMV) and *Cucumber mosaic virus* (CMV) in two different hosts: *Arabidopsis thaliana* and *Nicotiana benthamiana*. We report that *ABA2* plays a critical role in the accumulation of BaMV and CMV. Mutants downstream of *ABA2* (*aao3*, *abi1-1*, *abi3-1*, and *abi4-1*) were susceptible to BaMV, indicating that the ABA pathway downstream of *ABA2* is essential for BaMV resistance. The *aba2-1* mutant decreased the accumulation of BaMV (+)RNA, (–)RNA, and coat protein, with the most dramatic effect being observed for (–)RNA. These findings were further validated by the use of virus-induced gene silencing and enzyme-linked immunosorbent assay in *N. benthamiana*. In addition, infecting *N. benthamiana* with BaMV or CMV increased ABA contents and activated the SA and ABA pathways, thereby disrupting the antagonism between these two cascades. Our findings uncover a novel role for *ABA2* in supporting BaMV and CMV accumulation, distinct from the opposing role of its downstream genes.

Plant hormones are small signaling molecules that regulate growth and development. Although they exist in small amounts, changes in their concentrations affect adaptive plant responses to biotic or abiotic stresses. Plant responses to biotic stress are controlled by the following hormone-regulated defense pathways: salicylic acid (SA), jasmonic acid (JA), and ethylene (Et) (Pieterse et al. 2009; Santner et al. 2009). These pathways antagonize one another and, thus, plants can fine-tune the levels of cross-talk to maintain effective defense while minimizing the metabolic cost. Plant resistance to viruses is usually mediated by the SA pathway. Upon activation, this pathway induces several defense responses, including reactive oxygen species (ROS) production, the hypersensitive response, and pathogenesis-related (PR) protein synthesis (Jovel et al. 2011; Lewsey et al. 2009; Soosaar et al. 2005).

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The key hormone in the response to abiotic stress is absciscic acid (ABA). ABA strongly antagonizes many hormone pathways, including SA (Soosaar et al. 2005; Yasuda et al. 2008), Et (Cheng et al. 2009; Ghassemian et al. 2000), and the synergistic Et/JA pathway (Broekaert et al. 2006). The role of ABA in plant defense was shown to be phase specific: ABA plays positive roles during the early stages of infection (for example, by inducing stomatal closure and callose deposition), and negative roles at later stages (which include suppressing ROS accumulation and SA induction) (Asselbergh et al. 2008; Munemasa et al. 2007; Ton et al. 2009).

Although the interactions between ABA and bacterial or fungal pathogens have been thoroughly studied, ABA–virus interactions are weakly addressed. Several studies showed that ABA is induced upon several viral infections but only a few works focused on how ABA is involved in plant defense to viruses. The earliest studies on ABA–virus interactions investigated the effect of *Tobacco mosaic virus* (TMV) infection on the accumulation of ABA in *Nicotiana tabacum* and tomato (Fraser and Whenham 1989; Whenham et al. 1986). ABA was found to mediate plant defense through increasing callose deposition and thereby limiting virus movement. Conversely, ABA content was not affected in potato cultivars infected with *Potato virus Y* (Kovac et al. 2009). Tomato plants harboring *Tm-1*, which confers resistance to TMV, exhibited elevated levels of ABA as compared with susceptible lines (Fraser and Whenham 1989). Furthermore, whereas ABA is not induced in response to *White clover mosaic virus* infection in *Phaseolus vulgaris* (Clarke et al. 1998), it has a crucial role in mediating callose deposition and restricting movement of *Tobacco necrosis virus* (TNV) in the same host; these latter effects were not evident in *P. vulgaris* treated with an ABA inhibitor (Iriti and Faoro 2008). Banana infected with *Banana bunchy top virus* exhibited significant accumulation of ABA at 35 days post-inoculation (dpi) (Zhang et al. 1997), and ABA levels were also increased in nonheading Chinese cabbage infected with *Turnip mosaic virus* (Wang et al. 2011). Infection with several RNA viruses, including *Cucumber mosaic virus* (CMV), *Brome mosaic virus*, and *Tobacco rattle virus* (TRV), confers improved host tolerance to drought, which is possibly a consequence of an increase in osmoprotectants and antioxidants (Xu et al. 2008). Despite the above observations, the role of ABA in plant defense against viruses requires further investigation.

Bamboo mosaic virus (BaMV) is a member of the *Potexvirus* genus, with a single-strand positive-sense RNA genome and a flexuous rod shape (Lin et al. 1994). The BaMV genome encodes five open reading frames (ORF) and produces genomic RNA (gRNA) (6.4 kb) and two major subgenomic RNAs

(sgRNA) (2.0 and 1.0 kb) during infection (Lin et al. 1992, 1994; Yang et al. 1997). BaMV moves systemically in *N. benthamiana*, in which it accumulates to high titers. It can also infect *Arabidopsis thaliana* but it does not undergo systemic movement; as such, it is present at lower titers and causes no visible symptoms (Lin et al. 2010).

Here, we investigate the requirement of the ABA pathway for BaMV accumulation in *A. thaliana* and *N. benthamiana*. Interestingly, BaMV titers were decreased in the ABA biosynthesis mutant *aba2-1*, and were affected to a lesser extent in a mutant of the upstream gene *NCED3*. However, plants with mutations in genes encoding proteins downstream of *ABA2* were more susceptible to infection. Thus, *ABA2* divides the ABA pathway into upstream genes required for BaMV accumulation and downstream genes required for plant resistance. These results were confirmed in *N. benthamiana*. We also report that infection with BaMV or CMV simultaneously induces the antagonistic ABA and SA pathways and results in significant accumulation of ABA. To our knowledge, this is the first study to describe the role of the ABA pathway in virus accumulation, thus uncovering a novel role for *ABA2* in mediating the accumulation of two different RNA viruses.

RESULTS

BaMV accumulation is differentially affected in mutants of ABA biosynthesis.

We first examined whether mutations of ABA biosynthesis affect the accumulation of BaMV in *A. thaliana*. To this end, we used mutants of the following genes: *NCED3*, *ABA2*, and *AAO3*. *NCED3* encodes a member of the 9-*cis*-epoxycarotenoid dioxygenase family, responsible for converting 9-*cis*-violaxanthin and 9-*cis*-neoxanthin into xanthoxin (Schwartz et al. 2003). The *nced3* mutant exhibits approximately 40% less ABA compared with wild-type (WT) plants, and fails to induce sufficient ABA under drought stress (Hwang et al. 2010; Wan and Li 2006). *ABA2* encodes a short-chain alcohol dehydrogenase, which catalyzes the conversion of xanthoxin into ABA-aldehyde; the *aba2-1* mutant contains as little as approximately 22% of the ABA content of the WT (Gonzalez-Guzman et al. 2002). *AAO3* catalyzes the conversion of ABA-aldehyde to ABA (Seo et al. 2000) and the *aao3* mutant exhibits approximately 45% of the ABA content of the WT (Seo et al. 2004). BaMV infection of these three mutants yielded surprising results: whereas BaMV titers were significantly reduced in *nced3* and *aba2-1* as compared with the WT (to approximately 60 and 25%, respectively), they were increased in *aao3* (approximately 400%) compared with the WT (Fig. 1A).

To determine whether the reduction in viral titer in the *nced3* mutant was a consequence of its decreased ABA content, we used an enzymatic inhibitor for the NCED family, named nordihydroguaiaretic acid (NDGA). NDGA is known to reduce the enzymatic activity of NCEDs to approximately 50% as compared with the mock line, thereby reducing ABA content greatly and leading to stomatal closure (Creelman et al. 1992; Han et al. 2004; Zhang et al. 2009). Low concentrations of NDGA (<1 mM) weakly reduced BaMV titers (data not shown) but higher concentrations (10, 20, and 30 mM) clearly suppressed the accumulation of BaMV (Supplementary Fig. S1).

We also confirmed the role of ABA by applying it exogenously to leaves. NDGA and ABA were dissolved in 0.2% ethanol to concentrations of 10 mM and 100 μ M, respectively. Plants sprayed with 0.2% ethanol mock solution are hereafter referred to as EtOH lines. As expected, 10 mM NDGA reduced BaMV titers to approximately 45%. Interestingly, ABA also significantly reduced BaMV titers (Fig. 1B). To confirm that ex-

ogenous ABA induced a response, we used quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) to measure the levels of ABA-responsive genes (*ABA2* and *RAB18*) in treated plants. *ABA2* and *RAB18* were significantly upregulated (by two- and 44-fold, respectively) in the mock (not inoculated) ABA-treated plants compared with the control (Fig. 1C and D). This increase was more evident in BaMV-infected plants, with a 4-fold increase in *ABA2* and an approximately 27-fold increase in *RAB18* in ABA-treated plants compared with the EtOH-infected plants (Fig. 1C and D). Therefore, exogenous ABA application induced expression of ABA-responsive genes.

ABA2 was significantly suppressed in BaMV-inoculated plants treated with NDGA, as compared with both EtOH- and ABA-treated lines (Fig. 1C). *RAB18* levels were generally unaffected by NDGA; levels were the same as those in EtOH-mock plants but significantly lower compared with those in the EtOH-infected line (Fig. 1D). We hypothesized that the level of *ABA2* may be affected in *nced3*, based on the reduced accumulation of BaMV in this mutant. Indeed, *ABA2* transcripts were significantly lower in infected *nced3* lines than in the WT control, which justifies the low level of BaMV in *nced3* (Supplementary Fig. S2).

Mutations in the ABA signaling pathway increase BaMV levels.

Based on the increased BaMV titers in *aao3* (Fig. 1A), we hypothesized that BaMV accumulation may be similarly affected by mutants in other downstream components of this signaling pathway. We tested this hypothesis using the following ABA-insensitive mutants: *abi1-1*, a gain-of-function dominant mutant of the negative regulator *ABI1* (Leung et al. 1997), and *abi3-1* and *abi4-1*, loss-of-function mutants of the positive regulators *ABI3* and *ABI4*, respectively (Finkelstein 1994; Koornneef et al. 1984). Levels of BaMV RNA were two- to threefold higher than the WT in all ABA-insensitive mutants examined (Fig. 2A). Enzyme-linked immunosorbent assay (ELISA) revealed that ABA was increased in *abi1-1* and *abi4-1* but not *abi3-1* compared with their respective controls (mock *Col-0* for *abi4-1* and *Ler* WT for *abi1-1* and *abi3-1*). BaMV infection resulted in a slight (and insignificant) increase in ABA in *abi3-1* (Fig. 2B). This suggests that disrupting the ABA signaling pathway enhances susceptibility to BaMV, and this is independent of the levels of background ABA.

We proceeded to investigate whether the increase in ABA or BaMV levels correlated with increased expression of ABA biosynthesis genes. Levels of *ABA2* and *AAO3* were significantly increased in mock and infected *abi4-1* lines as compared with *Col*-WT (Fig. 3B and C) but *NCED3* was induced only in the mock line (Fig. 3A). *Ler*-WT and *Col*-WT exhibited similar changes in gene expression in response to BaMV infection. Expression of *NCED3* was increased in mock and infected *abi1-1* but decreased in infected *abi3-1* (Fig. 3E). The level of *ABA2* was significantly increased only in infected *abi1-1* (Fig. 3F). *AAO3* was upregulated in both mock and infected *abi1-1* and *abi3-1* (Fig. 3G). Because ABA signaling was impaired in these lines, *RAB18* expression was reduced in both mock and infected plants (Fig. 3D and H). Taken together, these results indicate that disrupting the ABA signaling pathway downstream of *ABA2* enhances susceptibility to BaMV; moreover, these data suggest that the ABA pathway may at least partially be involved in the defense against BaMV, and this may involve transcriptional regulation by *ABI3* and *ABI4*.

ABA2 affects the accumulation of BaMV at different stages of replication.

Of the examined mutants of ABA biosynthesis and signaling, only *aba2-1* and, to a lesser extent, *nced3* exhibited reduced

accumulation of BaMV. To further elucidate the role of *ABA2*, we examined BaMV replication in *aba2-1* and a transgenic line that constitutively overexpresses *ABA2* (*ABA2-O/E*); ABA levels in this line are more than double that of the WT (Hwang et al. 2010; Lin et al. 2007). BaMV positive-strand (+)RNA was reduced 5-fold in *aba2-1* and increased 0.5-fold in the *ABA2-O/E* line compared with the WT (Fig. 4A). Similarly, negative-strand (–)RNA was reduced 20-fold in *aba2-1* as compared with the WT but was unaffected in *ABA2-O/E* (Fig. 4B). Coat protein (CP) levels were significantly affected by the

ABA2 level in a similar manner (Fig. 4C). The dramatic effect of *aba2-1* on the accumulation of (–)RNA BaMV implies that ABA may affect the early stages of replication. In addition, we observed similar effects on BaMV accumulation in ABA line protoplasts (data not shown), suggesting that the effect of *aba2-1* is exerted at the cellular level and not through cell-to-cell movement.

We subsequently measured the expression levels of *NCED3*, *ABA2*, *AAO3*, and *RAB18* in these lines. *NCED3* was significantly reduced in the *aba2-1* mock and infected lines; it was

Fig. 1. Accumulation of BaMV is affected by abscisic acid (ABA) treatment and mutants of ABA biosynthesis. Northern blots of positive-strand BaMV genomic RNA and subgenomic RNAs in **A**, ABA biosynthesis mutants (*nced3*, *aba2-1*, or *aao3*) or **B**, wild-type (WT) plants treated with mock (0.2% ethanol in H₂O), 10 mM nordihydroguaiaretic acid (NDGA), or 100 μM ABA. Plants were sprayed with the indicated chemical 12 h before inoculation with BaMV virions. Leaves were collected for RNA extraction and further processing at 10 days postinoculation (dpi) for the mutant lines and 6 dpi in the chemical treatment lines; 2 μg of total RNA per sample was used to run the Northern blot. M = mock and V = BaMV infected. Bar charts displaying the accumulation of BaMV genomic RNAs are shown on the right. Panels below the Northern blots indicate equal loading of total ribosomal RNA. Expression of **C**, *ABA2* and **D**, *RAB18* in plants treated with 0.2% ethanol (mock), NDGA, or ABA, as determined using quantitative reverse-transcriptase polymerase chain reaction. Data are the mean ± standard deviation from three biological replicates. One-sided *t* test was performed to determine the significance of regulation. Asterisks indicate the significant differences by comparing the values of treatments to that of the uninfected WT or mock lines; *, **, and *** represent *P* < 0.05, 0.01, and 0.005, respectively.

also reduced in the *ABA2-O/E* mock line but increased to WT levels upon viral infection (Fig. 4D). Interestingly, *ABA2* was not induced by BaMV infection in WT plants but was significantly reduced in the mock and infected *aba2-1* mutant, as expected. The *ABA2-O/E* line exhibited tremendous upregulation

of *ABA2*, with approximately 100- and 70-fold increases in mock and infected lines, respectively, as compared with the WT (Fig. 4E). *AAO3* expression was greater in mock *ABA2-O/E* than the WT, and increased significantly in all lines upon viral infection (Fig. 4F). *RAB18* expression was also greater in mock

Fig. 2. BaMV accumulation and abscisic acid (ABA) content in ABA signaling mutants. **A**, Levels of BaMV in the ABA-insensitive mutant *abi4-1* as compared with its wild-type (WT) background *Col-0*, and in *abi1-1* and *abi3-1* as compared with WT *Ler*. Leaves were collected at 10 days postinoculation (dpi), and 2 µg of total RNA was used for Northern blot. M = mock and V = BaMV infected. Lower panels indicate the levels of BaMV subgenomic RNA2. **B**, ABA content in mock or BaMV-infected *abi* mutants, as determined by enzyme-linked immunosorbent assay. Leaves were collected at 10 dpi for lyophilization and ABA extraction. ABA levels were measured in pmol per gram dry weight. One-sided *t* test was performed to determine the significance of regulation. Asterisks indicate the significant differences by comparing the values of treatments to that of mock WT lines; *, **, and *** represent $P < 0.05$, 0.01, and 0.005, respectively. Data are the mean $\pm$ standard deviation from three biological replicates. Data are the mean $\pm$ standard deviation from three biological replicates.

ABA2-O/E compared with the WT, and increased in the WT following viral infection; conversely, no change was observed in the *aba2-1* mutant (Fig. 4G).

Thus, *ABA2* and ABA seem to play different roles in plant defense. *ABA2* supports BaMV accumulation whereas ABA supports plant defense. To confirm this finding, we sprayed ABA onto the *aba2-1* mutant. We predicted that the absence of *ABA2* would enhance the effect of ABA on plant defense. As expected, ABA treatment enhanced the resistance of *aba2-1* to BaMV, because the viral titer was reduced to the lowest detectable level (Fig. 5). These findings support the conclusions that *ABA2* is required for BaMV to replicate but the hormone ABA is important for plant defense against BaMV infection.

***ABA2* is important for CMV accumulation.**

We next asked whether *ABA2* had similar effects upon infection with other viruses. We chose to use CMV, because it suppresses SA-mediated defense and antiviral small interfering RNA machinery through its 2b suppressor (Diaz-Pendon et al. 2007; Soosaar et al. 2005; Zhang et al. 2006). Surprisingly, the level of the CMV CP was reduced in *aba2-1* compared with the WT and was not increased in *ABA2-O/E* (Fig. 6). Thus, the effect of *aba2-1* on BaMV and CMV accumulation may be similar, and *ABA2* is required to support the accumulation of both viruses.

Virus-induced gene silencing knockdown of *EDS1* and *ABA2* in *N. benthamiana* has effects on viral infection similar to those of the *A. thaliana* mutants *eds1-2* and *aba2-1*.

BaMV does not move systemically in *A. thaliana*; consequently, accumulation of this virus is approximately 10-fold less in *A. thaliana* than in *N. benthamiana*, and it does not cause symptoms in *A. thaliana* (Lin et al. 2010). Furthermore, many RNA viruses, including BaMV, preferentially infect *N. benthamiana* (Goodin et al. 2008). Therefore, we examined whether *N. benthamiana* responded to BaMV infection in a similar manner as *A. thaliana*. To this end, we used virus-induced gene silencing (VIGS) to knockdown *NbABA2*. *NbEDS1* was knocked down as a control because BaMV levels

in the *Arabidopsis* SA mutant *eds1-2* were found to be increased as compared with WT plants (Supplementary Fig. S4).

In the National Center for Biotechnology Information database, the approximately 650-bp sequence available for xanthoxin dehydrogenase (*ABA2*) of *N. benthamiana* (accession number EH371197) was blasted to the *N. benthamiana* expressed sequence tag (EST) database. The bigger EST of approximately 1,150 bp was blasted against *Lycopersicon esculentum* and *A. thaliana* *ABA2* (accession numbers XM_004237780 and NM_104113, respectively). *NbABA2* showed approximately 82 and 69% identity with *L. esculentum* and *A. thaliana*, respectively (Supplementary Fig. S5; data not shown). Therefore, we used this sequence to choose a fragment for *NbABA2* knockdown.

N. benthamiana plants were agroinfiltrated with *Agrobacterium tumefaciens* harboring the binary vectors pTRV1 and pTRV2; the latter had a small insert of approximately 250 to 500 bp (either *NbEDS1* or *NbABA2*, respectively). At 7 days postinfiltration, three leaves were inoculated with 0.2 µg of BaMV RNA each, and inoculated leaves from two to three plants were collected at 7 dpi for RNA extraction. Although knocking down *EDS1* enhanced BaMV titers as compared with the empty vector (pEV), silencing *NbABA2* significantly decreased BaMV accumulation (Fig. 7A and D). VIGS reduced the transcript levels of *ABA2* and *EDS1-2* to approximately 40% of that of plants infiltrated with pEV (Fig. 7B and E), confirming the efficacy of knockdown. Therefore, decreases in *ABA2* had similar effects on BaMV infection in *Arabidopsis thaliana* and *N. benthamiana* (Figs. 1 and 7). We also took advantage of previous characterization of *ABA1* (a few earlier works termed it as *ABA2* in *N. tabacum* and *N. plumbaginifolia*, although it encodes zeaxanthin epoxidase, whose ortholog in *Arabidopsis* is *ABA1*) (Audran et al. 1998; Marin et al. 1996) and knocked down this transcript by agroinfiltrating pTRV2 carrying approximately 250 bp of *NbABA1*. Interestingly, knocking down *NbABA1* also reduced BaMV titer compared with pEV-infiltrated plants (Fig. 7A). The transcript level of *NbABA1* was reduced to 40% compared with the control plants (Fig. 7B). To investigate whether the levels of *NbABA2*

Fig. 3. Expression of abscisic acid (ABA) biosynthesis genes in *abi*-insensitive mutants following BaMV infection. Transcripts levels of **A** and **E**, *NCED3*; **B** and **F**, *ABA2*; **C** and **G**, *AAO3*; and **D** and **H**, *RAB18* in **A** to **D**, *abi4-1* compared with its Col wild-type (WT) background and **F** to **H**, *abi1-1* and *abi3-1* compared with their Ler WT background. Data are the mean $\pm$ standard deviation from three biological replicates. Data were normalized to the expression of each gene in mock-inoculated WT. One-sided *t* test was performed to determine the significance of regulation. Asterisks indicate the significant differences by comparing the values of treatments to that of the mock WT lines; *, **, and *** represent *P* < 0.05, 0.01, and 0.005, respectively.

Fig. 4. Accumulation of BaMV and abscisic acid (ABA) genes in ABA lines. Accumulation of **A**, BaMV positive-strand RNA; **B**, negative-strand RNA; and **C**, coat protein (CP) in wild-type (WT), *aba2-1*, and *ABA2*-overexpressing transgenic line (*ABA2-O/E*). Bar charts showing comparative accumulation are shown on the right. Total RNA and protein were extracted at 10 days postinoculation (dpi) from inoculated leaves, and 2 μ g of total RNA was used for Northern blot. **C**, Approximately 25 μ g of total protein were used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis; membranes were hybridized with BaMV anti-CP sera. Lower panels show Coomassie blue staining as a loading control. Expression levels of **D**, *NCED3*; **E**, *ABA2*; **F**, *AAO3*; and **G**, *RAB18* in WT, *aba2-1*, and *ABA2-O/E* lines, as determined using quantitative reverse-transcriptase polymerase chain reaction. Data are the mean $\pm$ standard deviation from three biological replicates. Data were normalized to the expression of each gene in mock-uninoculated WT. One-sided *t* test was performed to determine the significance of regulation. Asterisks indicate the significant differences by comparing the values of treatments to that of the mock WT lines; *, **, and *** represent $P < 0.05$, 0.01, and 0.005, respectively.

are related to the low BaMV titer in the *ABA1*-knockdown line, we used qRT-PCR to measure *NbABA2*. We found that *NbABA2* was also reduced approximately 48% in plants knocked down with *NbABA1* (Fig. 7C). This strengthens the finding that BaMV titers are positively correlated with the levels of *ABA2*. Therefore, we used *N. benthamiana* for further experiments.

CMV and BaMV infection increase ABA content and activate the ABA and SA pathways in *N. benthamiana*.

ABA accumulation was greater in both CMV- and BaMV-infected *N. benthamiana* than in mock plants (Fig. 8A). Furthermore, mRNA levels of all tested ABA genes (*ABA1*, *ABA2*, *AAO3*, *ABI3*, *ABI5*, and *RAB18*) as well as SA genes (*EDS1*, *SID2*, *NPRI*, *PR-1*, *PR-4*, and *PR-5*) were increased in CMV-infected *N. benthamiana* as compared with the WT (Fig. 8B, left panel; Supplementary Table S1). All examined SA and ABA genes were also upregulated in BaMV-infected plants but not *ABA1* (Fig. 8B, right panel). However, upregulation of the affected genes was greater in CMV-infected than in BaMV-infected plants. Therefore, CMV and BaMV infections both induce two antagonistic pathways: ABA and SA.

DISCUSSION

Previous studies have shown that exogenous application of ABA enhances the resistance of different plant hosts to viruses

(such as TMV and TNV) by increasing callose deposition and thereby limiting viral movement (Fraser and Whenham 1989; Iriti and Faoro 2008; Whenham et al. 1986). We analyzed the effects of the ABA pathway on BaMV infection in depth by using ABA biosynthesis and signaling mutants and exogenous application of ABA and ABA inhibitor. Through this study, we identified a novel role for *ABA2* in supporting the accumulation of two different RNA viruses, BaMV and CMV. Conversely, we found that *AAO3* and its downstream genes are important for plant defense, indicating that ABA signaling is required for defense against viruses. Therefore, the effect of *ABA2* on BaMV is distinct from the ABA pathway itself.

ABA2 protein is encoded by a single gene in the *Arabidopsis* genome. It converts xanthoxin to ABA-aldehyde, which is used by *AAO3* and the cofactor *ABA3* to produce ABA (Hauser et al. 2011; Nambara and Marion-Poll 2005). The recognition of ABA by the PYR/PYL/RCAR receptor results in the dissociation of the SnRK2/PP2C complex and the subsequent release of SnRK2s. The latter phosphorylate and activate downstream transcription factors, which initiate transcription at ABA-responsive promoter elements (Sheard and Zheng 2009).

The ABA biosynthesis pathway mutants *aba2-1* and, to a lesser extent, the upstream mutant *nced3* result in decreased BaMV titers, whereas *aao3* enhances viral titers (Fig. 1). All signaling pathway mutants responded similarly to *aao3*, by ex-

Fig. 5. Effect of exogenous application of abscisic acid (ABA) on BaMV accumulation in wild-type (WT) and *aba2-1* plants. Northern blot analysis of positive-strand BaMV genomic RNA and two subgenomic RNAs in WT and *aba2-1* plants. Plants were sprayed with ABA 12 h prior to BaMV infection, leaves were collected at 6 days postinoculation (dpi), and 2 µg of total RNA was used for Northern blot. Data are the mean ± standard deviation from three biological replicates. One-sided *t* test was performed to determine the significance of regulation. Asterisks indicate the significant differences by comparing the values of treatments to that of the uninfected WT-EtOH line, * and *** represent *P* < 0.05 and 0.005, respectively.

Fig. 6. CMV accumulation in the *aba2-1* mutant. *Arabidopsis thaliana* lines were infected with crude extracts of CMV-infected *Nicotiana benthamiana*. At 10 days postinoculation (dpi), inoculated leaves were collected for protein extraction; approximately 25 µg total protein were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and membranes were probed with anti-CMV coat protein (CP). The lower panel shows Coomassie blue staining as a loading control. The bar chart shows accumulation of CMV CP. Data are the mean ± standard deviation from three biological replicates. One-sided *t* test was performed to determine the significance of regulation. Asterisks indicate the significant differences by comparing the values of treatments to that of the WT line, * and *** represent *P* < 0.05 and 0.005, respectively.

hibiting enhanced BaMV titers (Fig. 2). These results suggest that genes downstream of *ABA2* are important for plant resistance to BaMV. The higher viral titer in the *aoa3* mutant (which fails to encode the protein required for the final step of ABA synthesis) strongly suggests that ABA is important for plant defense. The following lines of evidence support this conclusion: i) exogenous application of ABA reduced the BaMV titer in WT plants (Fig. 5) and ii) viral titers were increased in

signaling pathway mutants (*abi1-1*, *abi3-1*, and *abi4-1*) in a similar manner as in *aoa3* (Fig. 2A). Although ABA was induced in BaMV-infected *abi1-1* and *abi4-1* lines (Fig. 2B), disruption of the signaling pathway prevented ABA-mediated resistance to BaMV. The similar effects on viral accumulation observed in *abi1-1*, *abi3-1*, and *abi4-1* were not unexpected; *abi1-1* is a gain-of-function mutant of the negative regulator ABI1, in which the PP2C-SnRK2 complex is insensitive to the

Fig. 7. Effect of virus-induced gene silencing (VIGS) on BaMV accumulation in *Nicotiana benthamiana*. **A**, BaMV accumulation after VIGS knockdown of *NbEDS1* and *NbABA1* as revealed by Northern blot. Plants were infiltrated with pEV (pTRV1 + pTRV2), pEDS1 (pTRV1 + pTRV2::EDS1), or pABA1 (pTRV1 + pTRV2::ABA2), and inoculated with 0.2 µg of BaMV RNA at 7 days postinfiltration. Inoculated leaves were collected at 7 days postinoculation (dpi) for RNA extraction, and 2 µg of total RNA was used for Northern blot. The bar chart shows the relative accumulation of BaMV in *N. benthamiana*. **B**, Relative expression of *NbEDS1* and *NbABA1* in the indicated plants. **C**, Expression level of *NbABA2* in plants knocked down with *NbABA1* compared with pEV plants. **D**, BaMV accumulation after VIGS knockdown of *NbABA2*. The bar chart shows the relative accumulation of BaMV in *N. benthamiana*. **E**, Relative expression of *NbABA2* in the ABA2-silenced plants. Data are the mean ± standard deviation from three biological replicates. Data were normalized to the expression of each gene in pEV line. One-sided *t* test was performed to determine the significance of regulation. Asterisks indicate the significant differences by comparing the values of treatments to that of the uninfected pEV line. * and *** represent *P* < 0.05 and 0.005, respectively.

ABA; thus, SnRK2 will not be released from the complex, nor will it phosphorylate or be able to activate ABA-responsive elements (Sheard and Zheng 2009). Therefore, no ABA-defense mechanism will be activated in this mutant. Similarly, *abi3-1* and *abi4-1* are loss-of-function mutants of the positive regulators ABI3 and ABI4, in which the ABA signaling pathway is impaired and results, likewise, in no activation of ABA-related defense. However, although ABA levels were increased in *abi1-1* and *abi4-1* lines as compared with the WT, they were unaffected in *abi3-1*; this may be related to the observation that expression of *NCED3* and *ABA2* were decreased and unaffected, respectively, in this line as compared with the WT (Fig. 3E and F). We hypothesize that ABA-mediated defense is possibly transcriptionally regulated by *ABI3* and *ABI4*, because both encode transcription factors that regulate ABA-responsive genes (Finkelstein et al. 1998; Giraudat et al. 1992). Comparison of the *aba2-1* mutant and an *ABA2-O/E* transgenic line clearly demonstrated the importance of *ABA2* for BaMV accumulation; *aba2-1* negatively affects the accumulation of BaMV (+)RNA, (–)RNA, and CP, with the effect on (–)RNA being most profound (Fig. 4). This implies that lack of *ABA2* activity probably affects the early steps of BaMV replication (Fig. 9). We observed low titers of BaMV in lines with significantly reduced levels of *ABA2*, such as NDGA-treated and *nced3* lines (Fig. 1B). Although exogenous application of ABA significantly de-

creased BaMV titers (Fig. 1B), *ABA2* levels were also increased fourfold by viral infection (Fig. 1C). This may be why a clear-cut effect of exogenous ABA was not observed. The effects of ABA and *ABA2* on plant defense were demonstrated by treating *aba2-1* mutant plants with ABA, which reduced BaMV titers to almost undetectable levels (Fig. 5). This result also reveals that *ABA2* promotes BaMV accumulation in opposition to ABA, suggesting that ABA and *aba2-1* may affect different mechanisms. Knocking down *ABA2* in *N. benthamiana* had an effect on BaMV titers similar to that of the *aba2-1* mutant (Fig. 7). In fact, a recent study showed that ABA is important for plant resistance to TMVcg infection. However, this study found that the *aba2* mutant, like other downstream mutants, enhanced plant susceptibility to TMVcg infection, showing no distinct role of *ABA2* to other ABA mutants (Chen et al. 2013), contrary to our findings for BaMV and CMV. This strengthens the probability that *ABA2* is involved in replication or translation complexes for BaMV and CMV, and that such involvement is specific to some viruses and cannot be generalized. In fact, not all RNA viruses may respond similarly to the same mutant. Huang and associates (2005) found that CMV-Y and *Oil seed mosaic virus* were not affected by several SA mutants such as *eds1*, whereas BaMV accumulated more in this mutant.

Viral titers in the *ABA2-O/E* line were increased by 50% as compared with the WT (Fig. 4). In addition, expression of ABA

Fig. 8. Effect of CMV and BaMV infection on abscisic acid (ABA) content and defense gene expression in *Nicotiana benthamiana*. **A**, Effect of CMV (left panel) and BaMV (right panel) infection on ABA content in *N. benthamiana*. Systemic leaves of infected *N. benthamiana* were collected at 16 days postinoculation (dpi) and subjected to enzyme-linked immunosorbent assay to determine ABA content. ABA levels were measured in pmol per gram dry weight. One-sided *t* test was performed to determine the significance of regulation. Asterisks indicate the significant differences by comparing the values of treatments to that of the mock line, * represents $P < 0.05$. **B**, Changes in expression of the main defense pathway genes following CMV (left panel) or BaMV (right panel) infection at 16 dpi were determined using quantitative reverse-transcriptase polymerase chain reaction. Dashed lines mean putative negative effect or blockage; *NPR1* has a negative effect over ABA biosynthesis genes *ABA1* and *ABA2*, as well as over some ABA-responsive genes such as *RAB18*, while the *aa3* mutant allowed more *SID2* to accumulate (Yasuda et al. 2008). The upregulation of these transcripts implies that ABA-salicylic acid (SA) antagonism is disrupted by CMV or BaMV infection. Gray boxes indicate an increase of twofold or more and white boxes indicate no change. Values were normalized to actin.

genes (with the exception of *NCED3*) was increased in this line (Fig. 4). *ABA2-O/E* seems to have a dual effect on BaMV and plant defense. Overexpression of *ABA2* supports BaMV accumulation and enhanced levels of ABA support plant resistance to the virus. These opposing effects account for the weak increase in BaMV titer in *ABA2-O/E* (Figs. 4 and 9). These findings strongly suggest that i) either certain defense mechanisms are negatively regulated by ABA2, thereby affecting early events of the replication cycle, or BaMV-related proteins are positively regulated by *ABA2* directly or indirectly and ii) the ABA signaling pathway partially underlies plant resistance to BaMV infection. Another possibility is that ABA2 may have indirect effects through its substrates; in the absence of functional ABA2, metabolic flux through this pathway is disrupted; this may lead to the accumulation of precursor metabolites or compounds derived from alternative pathways, which inhibit virus replication.

We also used CMV infection to demonstrate the importance of ABA2 in CP accumulation. CMV CP levels were greatly reduced in the *aba2-1* mutant but unaffected in the *ABA2-O/E* line (Fig. 6); the latter finding may be due to the conflicting effects of increased ABA2 and ABA. Thus, ABA2 appears to be required for the accumulation of CMV, in a manner similar to BaMV.

In *N. benthamiana*, infection with CMV or BaMV induced ABA accumulation (Fig. 8A) and upregulated the mRNA levels of several genes in the SA and ABA pathways (Fig. 8B). This finding suggests that the induction of ABA and upregulation of the ABA and SA pathways may be common features of RNA virus infection. The disruption of ABA-SA antagonism was shown by the upregulation of almost all tested genes in each pathway. In fact, Yasuda and associates (2008) showed that

AAO3 has a suppressive effect on *SID2*, also *NPR1* has a suppressive effect on different ABA biosynthesis and downstream genes such as *ABA2* and *RAB18* (Yasuda et al. 2008). Our data showed that all of these genes, along with the rest of the tested ABA and SA genes (six per each pathway), were upregulated simultaneously under CMV or BaMV infection regardless of their antagonistic effects on the opposite pathway (Fig. 8B). This result implies that infection with these two RNA viruses disrupts the ABA-SA antagonism. Supportively, a recent study showed that the 2b silencing suppressor of CMV interferes with the ABA pathway and renders *A. thaliana* plants more tolerant to drought (Westwood et al. 2013). Another study reported that disruptions in ABA biosynthesis but not ABA signaling enhanced the nuclear localization of two resistance proteins, *SNC1* and *RPS4*, and negated the effect of these proteins on disease resistance at high temperature. We note that this work examined *aba1-6* and *aba2-1* mutants but not the downstream mutant *aoa3* (Mang et al. 2012). The positive effect of the mutant *aba2-1* on plant resistance to BaMV or CMV hypothesizes that there could be a defense mechanism which might be negatively regulated by ABA2, because low levels of ABA2 always lead to low BaMV or CMV titers. The other probability is that ABA2 could be an essential factor in the replication or translation complex so that, by reducing ABA2 levels, this affects the replication or translation efficiency and results in low-level viral replication forms, as shown for (+)RNA and (-)RNA of BaMV (Figs. 4 and 9). The nature of ABA2 as a dehydrogenase enzyme bears another probability, which is the associated NAD coenzyme (Gonzalez-Guzman et al. 2002) or other associated factors, and that by mutating or downregulating ABA2, NAD coenzyme will be more abundant and can affect the redox state in the cell. The redox state was

Fig. 9. Schematic representation of the effect of the abscisic acid (ABA) pathway on BaMV accumulation. The ABA pathway has two different effects on BaMV accumulation. Impairing ABA biosynthesis through the *nced3* and *aba2-1* mutants reduces BaMV titers in plants; *aba2-1* appears to affect BaMV replication primarily by decreasing (-)RNA and also by reducing (+)RNA and coat protein (CP) accumulation. Mutants downstream of *aba2-1* (such as *aao3*) or mutants in the ABA signaling pathway (such as *abi-1-1*, *abi3-1*, and *abi4-1*) also increased BaMV titers, indicating that ABA pathway components downstream of *aba2-1* are involved in the resistance against BaMV. ABA2 appears to be required to stabilize and support BaMV replication by acting on certain required factors or by negatively affecting an anti-BaMV mechanism.

found to be connected to plant basic immunity (Spoel and Dong 2012) and was also found to be involved in the regulation of RNA-protein bindings (Barnes and Mayfield 2003; Woloshen et al. 2011), which means there is a possibility of interference in the binding process between viral RNA and host proteins. Further studies on how ABA2 regulates BaMV replication are required to unveil any possible connection between ABA2 and defense systems against viruses.

To our knowledge, this is the first report to demonstrate that ABA2 promotes accumulation of two different RNA viruses, while downstream genes in ABA biosynthesis and the ABA signaling pathway enhance plant resistance to BaMV. It was also found that infection with BaMV or CMV can simultaneously induce two antagonistic pathways, SA and ABA.

MATERIALS AND METHODS

Plant materials and growth conditions.

WT *N. benthamiana* plants were grown under long-day conditions at 28°C with a relative humidity of approximately 70%. *A. thaliana* lines were grown under long-day conditions at 22°C with a relative humidity of approximately 60%.

Inoculation with BaMV.

Purified BaMV RNA was extracted from virions as previously described (Lin et al. 1992). Three leaves of each *A. thaliana* or *N. benthamiana* plant (approximately six replicate plants per line) were inoculated with 0.1 µg of BaMV RNA, unless otherwise stated. The inoculated leaves were collected from *A. thaliana* and *N. benthamiana* at 10 and 16 dpi, respectively, for further analyses.

Treatment with ABA and ABA inhibitor.

A. thaliana leaves were treated with ABA or an ABA biosynthesis inhibitor, NDGA, which targets 9-*cis*-epoxycarotenoid dioxygenase. Three concentrations of NDGA were prepared: 10, 20, and 30 mM. ABA was prepared at 100 µM and all solutions were dissolved in 0.2% EtOH-H₂O. Mock controls were treated with 0.2% EtOH-H₂O alone. All chemicals were from Sigma-Aldrich (St. Louis). Solutions were sprayed onto the adaxial and abaxial sides of leaves until they were completely coated (at which point, the solutions began to run off the leaves). After 12 h, leaves were mechanically infected with 1 µg of BaMV virions; at 6 dpi, RNA was extracted for use in subsequent analyses.

RNA analysis.

Total RNA was extracted from leaves by TRIzol (Invitrogen, Carlsbad, CA, U.S.A.), was purified using the phenol-chloroform method, and was precipitated in 0.1 volume of 3 M NaOAc and 2.5 volume of 100% ethanol.

Northern blot analysis: BaMV RNA was detected as follows: total RNA (2 µg) was glyoxylated and then separated by electrophoresis on a 1% agarose gel. The RNA was then transferred onto a Hybond-N⁺ membrane (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.), cross-linked under UV light, and hybridized against P³²-labeled (–)CP and (+)CP probes to detect (+)BaMV and (–)BaMV, respectively (Lin et al. 1993).

qRT-PCR. Each RNA sample (2 µg) was treated with RQ1-DNase (Promega, Madison, WI, U.S.A.) for 30 min at 37°C. RNA samples were then subjected to first-strand cDNA synthesis with Superscript III (Invitrogen). The resulting cDNA was diluted to a final concentration of 20 ng/µl. The qRT-PCR primers used in this study are shown in Supplementary Table S2. All qRT-PCR reactions were performed with SYBR Green Supermix (Applied Biosystems, Foster City, CA, U.S.A.), following the manufacturer's instructions. For the unannotated

gene *NbPDF1.2a*, primers were designed based on the *AtPDF1.2a* mRNA sequence and used to amplify a homologous fragment in *N. benthamiana* cDNA. Sequencing of the amplicon revealed 98% sequence identity between *NbPDF2.1a* and *AtPDF2.1a* (Supplementary Fig. S3); qRT-PCR primers were designed based on the amplicons. Actin was used as an internal control and experiments were carried out in triplicate.

Determining the density of RNA bands. A UVP BioSpectrum 600 Imaging System v.6.8 (Upland, CA, U.S.A.) was used to measure the density of BaMV gRNA bands (approximately 6.4 kb). Densities from at least three replicates were averaged and used as an indicator of viral RNA accumulation. In the *abi* experiment, the gRNA band was too weak to be detected in the WT. Overexposure resulted in the other *abi* bands becoming oversaturated, preventing comparison of their densities. Therefore, we used sgRNA2 for this purpose.

Protein analyses.

Leaves (approximately 0.1 g) were ground to fine powder in liquid nitrogen and homogenized by adding a similar volume (approximately 400 µl) of extraction buffer (0.1 M glycine-NaOH [pH 9.0], 0.1 M NaCl, 0.5 mM EDTA, 2% sodium dodecyl sulfate [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. Supernatants were then transferred to new tubes. For BaMV CP detection, crude protein extract (25 µg) was loaded with 2× dye (1 M Tris, 10% SDS, 100% glycerol, and 900 ml of β-mercaptoethanol in 50 ml of H₂O) onto 10% SDS-polyacrylamide gels for Western blot analysis, and membranes were hybridized with BaMV anti-CP sera (Lin et al. 1992).

ELISA.

ABA was measured as previously described (Hurng et al. 1994). Briefly, samples were collected at 10 and 16 dpi from *Arabidopsis* and *N. benthamiana*, respectively, then lyophilized and soaked in 1.5 ml of extraction buffer (80% MeOH and 2% glacial acetic acid [gAA]) for 24 h, then centrifuged at 5,000 rpm for 10 min. The resulting supernatant (1 ml) was speed vacuumed and the pellet was then dissolved in 1:1 MeOH and 0.2 M NH₄H₂PO₄, before being incubated at 4°C for 10 min. The samples were applied to individual polyvinyl-pyrrolidone columns, and the columns were then treated with 6 ml of double-distilled H₂O. A total of 100 µl gAA was added to a final volume of 7 ml and passed through C18 columns, which were then washed with washing buffer (20% MeOH and 2% gAA). Samples were collected with 4 ml of elution buffer (55% MeOH and 2% gAA), and 1 ml was speed-vacuumed (this last step was repeated twice, to obtain two mechanical replicates from the same sample). ABA was detected using the Agdia Phytodetek kit (catalog number PDK 09347/0096). Three biological replicates were performed for each line.

VIGS.

VIGS was performed as described (Dinesh-Kumar et al. 2003). In brief, an *NbABA1* fragment of approximately 250 kb was amplified from *N. benthamiana* cDNA using the primers GCAGAGTCTAGAACATGCCTTGC and CTTCCGAATTCTCGAGCTGC. A 200-kb *NbEDS1* fragment was amplified using GGATAAACTAGGGACGCAG and GTTTTCTTCCCTGAGTCTAC. An approximately 500-bp *NbABA2* fragment was amplified using CAAGAAGGAACCTGCCCATGGA and GCGCAAGGCCAGCAATAGCA, and the *NbPDS* fragment was amplified using GGTGGGAAGGTAGCTGCATGG and GGGCCGACAGGGTTCACAACC. Fragments were cloned separately into pTRV2 and used to transform *Agrobacterium tumefaciens* C85C1. The optical density at 600 nm was ad-

justed to 1, and *A. tumefaciens* carrying pTRV1 or pTRV2 (with the inserted fragment) was co-infiltrated into approximately 16-day-old *N. benthamiana* leaves (three mature leaves per treatment). Plants infiltrated with pTRV1 and pTRV2-PDS began to show a bleaching phenotype at 6 to 7 dpi, which indicated successful knockdown of phytoene desaturase. BaMV RNA (approximately 0.2 µg) was inoculated on three leaves above the infiltrated ones; inoculated leaves were collected for RNA analysis at 7 dpi. To determine the efficiency of VIGS, qRT-PCR was performed with primers targeting sites inside the cloned fragments and RNA extracted from upper leaves.

Infection of *N. benthamiana* with CMV and BaMV.

Three leaves of each *N. benthamiana* plant were inoculated with 1 µg of CMV virion or 0.1 µg of BaMV viral RNA. Severe symptoms were observed at 16 dpi. At this time point, two systemic leaves were collected from each plant for RNA extraction and ELISA. Three plants were examined for each line. Crude extracts from CMV-infected *N. benthamiana* were used to inoculate *Arabidopsis thaliana* lines. *A. thaliana* leaves were collected for CMV CP detection at 10 dpi by Western blot using anti CMV-CP sera (Chung 1988). Experiments were performed in triplicate, with at least three plants examined per line.

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