Acibenzolar-S-Methyl Restricts Infection of *Nicotiana benthamiana* by Plantago Asiatica Mosaic Virus at Two Distinct Stages

Yuki Matsuo,¹ Fawzia Novianti,¹ Miki Takehara,¹ Toshiyuki Fukuhara,² Tsutomu Arie,¹ and Ken Komatsu^{1,†}

¹ Plant Pathology Laboratory, Graduate School of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwaicho, Fuchu, Tokyo 183-8509, Japan

² Molecular and Cellular Biology Laboratory, Graduate School of Agriculture, Tokyo University of Agriculture and Technology

Accepted 11 July 2019.

Plant activators, including acibenzolar-S-methyl (ASM), are chemical compounds that stimulate plant defense responses to pathogens. ASM treatment inhibits infection by a variety of plant viruses, however, the mechanisms of this broad-spectrum and strong effect remain poorly understood. We employed green fluorescent protein (GFP)-expressing viruses and *Nicotiana benthamiana* plants to identify the infection stages that are restricted by ASM. ASM suppressed infection by three viral species, plantago asiatica mosaic virus (PIAMV), potato virus X (PVX), and turnip mosaic virus (TuMV), in inoculated cells. Furthermore, ASM delayed the long-distance movement of PIAMV and PVX, and the cell-to-cell (short range) movement of TuMV. The ASM-mediated delay of long-distance movement of PIAMV was not due to the suppression of viral accumulation in the inoculated leaves, indicating that ASM restricts PIAMV infection in at least two independent steps. We used *Arabidopsis thaliana* mutants to show that the ASM-mediated restriction of PIAMV infection requires the *NPR1* gene but was independent of the dicer-like genes essential for RNA silencing. Furthermore, experiments using protoplasts showed that ASM treatment inhibited PIAMV replication without cell death. Our approach, using GFP-expressing viruses, will be useful for the analysis of mechanisms underlying plant activator-mediated virus restriction.

Keywords: acibenzolar-S-methyl, NPR1, plant immunity, systemic acquired resistance

Plant viruses widely infect agricultural crops and damage world food production. In host plants, they have three major

infection stages, replication, cell-to-cell movement, and long-distance movement. Due to their biotrophic features, pesticides that act directly on viruses have not yet been developed. For disease control, we usually employ genetically resistant varieties or exclude insect vectors using agrochemicals. However, genetic resistance is not durable owing to viral genomic plasticity, and vector eradication has a significant environmental impact (Faoro and Gozzo 2015). Therefore, we need further studies focusing on resistance mechanisms to viral diseases, which remain poorly understood, for the development of new resistant cultivars and control methods.

Previous studies have shown that multiple phytohormones are involved in induced antiviral resistance of plants (Palukaitis et al. 2017). Among them, much research has focused on salicylic acid (SA), which plays an important role in defense signaling in general. SA induces resistance against a broad range of biotrophic pathogens through systemic acquired resistance (SAR) accompanied by pathogenesis-related (PR) gene expression and the hypersensitive response (Faoro and Gozzo 2015; Glazebrook 2005; Singh et al. 2004). Establishment of SAR through SA signaling requires NPR1, a positive regulatory hub in the SA-mediated defense responses (Yan and Dong 2014). In *Arabidopsis thaliana*, SA treatment inhibited infection by the oomycete pathogen *Peronospora parasitica* and the bacterial pathogen *Pseudomonas syringae* by inducing SAR (Glazebrook 2005). SA also suppresses various viral infections by interfering with different infection stages, depending on the virus-host combinations. For instance, in the tobacco mosaic virus (TMV)-tobacco pathosystem, SA inhibits both replication and cell-to-cell movement (Chivasa et al. 1997; Murphy and Carr 2002).

Given the importance of SA in plant resistance, agrochemicals called plant defense activators (PAs), which boost SA-related defense responses in plants, have been used for crop protection (Noutoshi et al. 2012). PAs are effective against various fungal and bacterial plant diseases and are also reported to suppress viral infection. For example, the PA probenazole promotes SA signaling upstream of SA accumulation (Bektas and Eulgem 2015) and enhances resistance to TMV in tobacco carrying the *N* gene, which confers resistance to TMV (Nakashita et al. 2002). Another PA, acibenzolar-S-methyl (ASM) (Bektas and Eulgem 2015; Nakashita et al. 2002), acts downstream of SA accumulation and limits infection by a wide range of viruses in their susceptible host plants and enhances resistance in their resistant hosts. ASM treatment reduces the accumulation of cucurbit chlorotic yellows

[†]Corresponding author: K. Komatsu; akomatsu@cc.tuat.ac.jp

Funding: This work was supported by a grant-in-aid for scientific research (A) from the Japan Society for the Promotion of Sciences (JSPS) (number 16H02536) to T. Arie and the Pesticide Science Society of Japan to K. Komatsu.

*The e-Xtra logo stands for “electronic extra” and indicates that nine supplementary figures and two supplementary tables are published online.

The author(s) declare no conflict of interest.

virus RNA in melon plants (Takeshita et al. 2013), suppresses the symptoms of TMV in tobacco plants without the *N* gene, and inhibits turnip crinkle virus infection in *A. thaliana* (Friedrich et al. 1996; Lawton et al. 1996). ASM also induced SAR in a tobacco carrying the *N* gene against TMV (Tripathi et al. 2010). Thus, PAs generally suppress viral infection similarly to SA. However, our understanding of the effects of PAs on viral infection remains fragmented. Because their effects vary depending on the virus species and the host plant species, we cannot draw general conclusions about how SA and PAs effectively inhibit infection by a variety of viruses (Palukaitis et al. 2017).

One possible mechanism for PA-mediated virus restriction is programmed cell death, which has been mainly studied in the context of the resistance gene-mediated hypersensitive response (Palukaitis et al. 2017). However, the available data suggest that programmed cell death may not explain all virus resistance mechanisms in plants (Greenberg and Yao 2004). Another candidate mechanism is antiviral RNA silencing, in which three Dicer-like (DCL) proteins, DCL2, DCL3, and DCL4, play critical roles (Deleris et al. 2006). Indeed, several studies have shown a link between SA signaling and antiviral RNA silencing. SA induces the activity of RNA-dependent RNA polymerase, which is necessary for the amplification of RNA silencing (Alamillo et al. 2006; Xie et al. 2001). Alamillo and associates (2006) also demonstrated that RNA silencing activity against plum pox virus decreases in transgenic tobacco plants that cannot accumulate SA. In contrast, SA treatment

decreases the accumulation of TMV and cucumber mosaic virus in the *Arabidopsis dcl2/dcl3/dcl4* triple mutant, which completely lacks antiviral RNA silencing activity (Lewsey and Carr 2009). Therefore, the relevance of RNA silencing in SA- and PA-induced viral resistance remains unclear.

Our overall goal is to clarify how PA-induced resistance works against viral infection, in order to develop new methods for the control of plant viruses. In this study we used *Nicotiana benthamiana*, which is susceptible to a variety of plant viruses, with three green fluorescent protein (GFP)-expressing plant viruses, plantago asiatica mosaic virus (PIAMV), potato virus X (PVX), and turnip mosaic virus (TuMV). PIAMV and PVX are in the genus *Potexvirus* and TuMV is in a different genus, *Potyvirus*. Using these three viruses, we can compare the effect of PA on different viruses in the same genus as well as on those in different families. This system allows us to quantitatively evaluate the progression of viral infection at each of the three major infection stages. Our approach will be useful for the analysis of mechanisms underlying PA-mediated virus restriction.

RESULTS

ASM treatment restricts PIAMV-GFP infection in the initially infected cells and delays its long-distance movement.

PIAMV-GFP is a modified PIAMV that carries the GFP gene (Minato et al. 2014). It is based on a Li1 isolate and

Fig. 1. Effects of acibenzolar-*S*-methyl (ASM) and salicylic acid (SA) treatments on green fluorescent protein-expressing plantago asiatica mosaic virus (PIAMV-GFP) infection of inoculated *Nicotiana benthamiana* leaves. **A**, Representative fluorescence microscopy images of inoculated leaves showing GFP fluorescence in a single cell (left; bar = 100 μm) at 2 days postinoculation (dpi) and in multiple cells (right; bar = 500 μm) at 5 dpi. **B**, Numbers of fluorescent spots per inoculated leaf in control plants and plants treated with 1 mM ASM or 1 mM SA at 5 dpi ($n = 5$). The data are representative of three independent experiments with similar results. The box plots represent the median, interquartile range, maximum, and minimum numbers. Different letters indicate significant differences among treatments ($P < 0.05$) by Duncan's multiple range test. **C**, Representative fluorescence microscopy images of PIAMV-GFP infection sites on inoculated leaves of control plants and *N. benthamiana* plants treated with 1 mM ASM or 1 mM SA, observed at 2 dpi. Arrowheads represent single cell spots. Bars = 500 μm.

systemically infects *N. benthamiana* with mild mosaic symptoms. When we mechanically inoculate *N. benthamiana* with the PIAMV-GFP virus, fluorescence can be seen in individual cells after about 1 day (Fig. 1A, left panel). This indicates that the virus has replicated in the initially infected cells. Following this, the GFP-expressing virus spreads to adjacent cells via the plasmodesmata, resulting in the appearance of fluorescent spots containing multiple cells (Fig. 1A, right panel). Eventually the virus spreads via long-distance movement to uninoculated upper leaves and can be detected by GFP fluorescence in the uninoculated leaves (Minato et al. 2014). This pathosystem allows us to use GFP fluorescence to quantify the progress of viral infection at each step and to examine the effect of PAs on the progression of viral infection.

In order to examine the effect of ASM on viral infection, we first measured expression of the PA- and SA-inducible *PR-1* gene, to determine the appropriate timing for viral inoculation after ASM treatment. Both SA and ASM (applied by foliar spraying at 1 mM concentration) caused relatively low levels of *PR-1* induction at 1 and 2 days after treatment. At 3 days after treatment with either compound, the *PR-1* transcript increased to much higher levels (Supplementary Fig. S1). This suggested

that the SA-dependent defense pathway was activated by the treatments at this timepoint. It should also be noted that a 1 mM concentration of both SA and ASM does not show any toxicity to *N. benthamiana* plants (Supplementary Fig. S2).

We treated *N. benthamiana* plants by spraying a single leaf on each plant with 1 mM ASM or SA or water (as a control) and then inoculated the same leaf with PIAMV-GFP at 3 days after treatment. At 5 days postinoculation (dpi), we counted the number of fluorescent spots on the inoculated leaves. Both treatments reduced the number of GFP spots when compared with the controls, and there was a trend for a lower number of spots in the ASM-treated leaves compared with the SA-treated leaves, although not statistically significant (Fig. 1B). In parallel, when PIAMV-GFP was inoculated to the upper, non-sprayed leaf of the ASM-treated plants, the number of fluorescent spots was reduced compared with control plants (Supplementary Fig. S3). This result indicates that ASM can also restrict virus infection in a nontreated upper leaf, which reminds us of the SAR.

When we analyzed the effect of ASM and SA in more detail by fluorescence microscopy, we found no clear differences in the mean percentages of single cell spots versus total spots between

Fig. 2. Effects of acibenzolar-*S*-methyl (ASM) treatment on cell-to-cell movement of green fluorescent protein-expressing plantago asiatica mosaic virus (PIAMV-GFP) in inoculated *Nicotiana benthamiana* leaves. **A**, Representative photographs of control leaves and leaves treated with 1 mM ASM and inoculated with PIAMV-GFP, taken under UV light at 8 days postinoculation (dpi). Bars = 1 cm. **B**, Number of fluorescent spots on inoculated leaves at 8 dpi. Data are means $\pm$ standard error ($n = 9$). The asterisk indicates a significant difference between treatments ($P < 0.05$, Student's *t* test). **C**, Reverse transcription-quantitative-PCR analysis of PIAMV-GFP RNA accumulation in control leaves and leaves treated with 1 mM ASM at 8 dpi. Data are means $\pm$ standard error of three biological replicates, normalized to the *N. benthamiana* 18S ribosomal RNA and expressed relative to the control mean. The asterisk indicates a significant difference between treatments ($P < 0.05$, Student's *t* test). **D**, Average size of PIAMV-GFP spots at 8 dpi. Data are means $\pm$ standard error of spot sizes calculated from those observed in a total of six leaves relative to the control mean.

the leaves treated with ASM or SA and those treated with water at 2 dpi, although ASM significantly reduced the number of total spots by one third (Fig. 1C; Supplementary Fig. S4). In leaves treated with ASM or SA, 14.0 or 16.9% of the spots were single cells, respectively, and in the controls, 14.8% of the spots represented single cells, which was not significantly different with ASM- or SA-treated leaves (Supplementary Table S1). Similar results were obtained for three independent experiments. This result suggested that ASM treatment restricts PIAMV-GFP infection in the initially infected cells but does not restrict the cell-to-cell movement of the virus via the plasmodesmata. A similar trend was observed when we visually examined the number and size of fluorescent spots in inoculated leaves at 8 dpi. ASM treatment reduced spot number in the inoculated leaves to about one third of the number found in controls (Fig. 2A and B). In accordance with this, ASM treatment reduced viral RNA accumulation to approximately one eighth of the levels found in controls (Fig. 2C). In contrast, there were no obvious differences in the sizes of the fluorescent spots between ASM-treated and control leaves (Fig. 2D).

To determine whether ASM treatment suppresses the long-distance movement of PIAMV-GFP, we followed the time course of its long-distance movement in ASM-treated and control plants. As in the experiments we performed above, we treated a single leaf on each plant with 1 mM ASM and inoculated PIAMV-GFP in the treated leaf. This method was justified by the fact that spraying the whole plant with ASM

did not increase its inhibitory effect on PIAMV infection (Supplementary Fig. S5). Based on the appearance of GFP fluorescence in uninoculated leaves, long-distance movement was detected in one control plant at 6 dpi and had occurred in all eight control plants by 8 dpi. On the other hand, long-distance movement was not detected in any ASM-treated plants until 11 dpi and, by 15 dpi, only five of the eight plants had shown long-distance movement of the virus (Fig. 3A and B). This result suggests that ASM treatment delays long-distance movement of PIAMV-GFP.

The suppression of PIAMV-GFP infection in the inoculated leaves and the delay of its long-distance movement are independent effects of ASM treatment.

The results shown in Figures 1 to 3 indicated that ASM treatment restricts infection of PIAMV-GFP in the initially infected cells and delays its long-distance movement. However, we could not exclude the possibility that the apparent delay in long-distance movement was due to lower amounts of the virus in the inoculated leaves. To test this possibility, we prepared a 1/4 dilution of PIAMV-GFP inoculum and performed a similar experiment to the one shown in Figure 3. The control plants treated with diluted inoculum (labeled 1/4 control plants) had similar numbers of spots and similar levels of viral RNA accumulation to those observed in ASM-treated plants inoculated with undiluted PIAMV-GFP (Fig. 4A; Supplementary Fig. S6). Based on the appearance of GFP fluorescence in the upper leaves,

Fig. 3. Effects of acibenzolar-*S*-methyl (ASM) treatment on long-distance movement of green fluorescent protein-expressing plantago asiatica mosaic virus (PIAMV-GFP). **A**, Representative photographs of PIAMV-GFP fluorescence in control plants and plants treated with 1 mM ASM at 9, 11, and 15 days postinoculation (dpi), showing the long-distance spread of PIAMV-GFP to uninoculated, upper leaves of the plants. Pictures were taken under UV light. Circles indicate the first uninoculated leaves that showed GFP fluorescence. **B**, Kaplan-Meier curves of the time course of PIAMV-GFP movement in seven control plants (continuous line) and seven plants treated with 1 mM ASM (dotted line). The appearance of GFP fluorescence in uninoculated leaves was recorded for each plant and the cumulative data was plotted. Statistical differences between the two curves were analyzed using the log-rank test ($P = 0.00019$).

long-distance movement in the 1/4 control plants was slightly delayed when compared with the control plants inoculated with undiluted inoculum. By 8 dpi, all the undiluted control plants and four of the five 1/4 control plants showed evidence of long-distance movement (Fig. 4C). None of the ASM-treated plants showed evidence of long-distance movement until 11 dpi, and, by 12 dpi, only three ASM-treated plants showed GFP in uninoculated upper leaves (Fig. 4B). This result suggested that the delay in long-distance movement by ASM treatment was not entirely caused by the lower amounts of virus in the inoculated leaves. Therefore, ASM treatment suppressed the primary infection stage in inoculated leaves and then delayed the long-distance movement of PIAMV-GFP in two independent steps.

ASM treatment restricts PVX-GFP infection in the initially infected cells and delays its long-distance movement.

To determine whether ASM treatment restricts other viral infections, we next conducted similar experiments using PVX, which belongs to the same genus (*Potexvirus*) as PIAMV. In this

case, we treated *N. benthamiana* plants by spraying single leaves with water or 0.25 mM ASM rather than 1 mM, because PVX is more sensitive to ASM than PIAMV (data not shown). We then inoculated the same leaves with a modified PVX virus (PVX-GFP) in which GFP is expressed as a fusion protein with the coat protein, separated by the foot-and-mouth disease virus 2A peptide (Cruz et al. 1996). As for the plants inoculated with PVX-GFP, the number of GFP fluorescent spots per leaf was greatly reduced by ASM treatment, but size of the spots was not significantly affected (Fig. 5A to C). ASM treatment also caused a delay in the long-distance movement of PVX-GFP (Fig. 5D and E). Overall, these results suggest that ASM treatment inhibits infection in the initially infected cells and delays long-distance movement of PVX as it does for PIAMV.

ASM treatment restricts TuMV-GFP infection and cell-to-cell movement in the initially inoculated leaves.

Next, we investigated whether ASM treatment affects infection by TuMV, which belongs to the genus *Potyvirus*. We treated *N. benthamiana* plants by foliar spraying with water or

Fig. 4. Effects of inoculum dilution on long-distance movement of green fluorescent protein-expressing plantago asiatica mosaic virus (PIAMV-GFP). **A**, Representative photographs taken under UV light showing PIAMV-GFP infection on inoculated leaves at 8 days postinoculation (dpi) (upper panels) and on whole plants at 11 dpi (lower panels). Left, control plants inoculated with PIAMV-GFP using the normal inoculum concentration; middle, control plants inoculated with 1/4 of the normal concentration (1/4 control); right, plants treated with 1 mM acibenzolar-S-methyl (ASM) inoculated with the normal concentration. **B**, Kaplan-Meier curves of the time course of PIAMV-GFP movement in control (continuous black line), 1/4 control (continuous gray line), and plants treated with 1 mM ASM (dotted line). Five plants were used for each treatment. The appearance of green fluorescent protein fluorescence in uninoculated leaves was recorded for each plant and the cumulative data were plotted. Statistical differences between the ASM and 1/4 control curves were analyzed using the log-rank test ($P = 0.039$).

1 mM ASM and then inoculated the sprayed leaves with a GFP-expressing TuMV (TuMV-GFP). In this modified virus, the GFP cistron was inserted between the RNA-dependent RNA polymerase (N1b) and coat protein cistrons (Takeshita et al. 2012). In this case, both the number and size of GFP fluorescent spots were reduced in the ASM-treated plants compared with the control plants (Fig. 6A to C). Furthermore, ASM treatment did not appear to affect the long-distance movement of TuMV-GFP, since fluorescent spots were detected in uninoculated leaves in both control and ASM-treated plants at 3 dpi and disease symptoms clearly appeared in both control and ASM-treated plants between 4 and 8 dpi (Fig. 6D). These data suggest that, unlike the potexviruses, ASM treatment restricts TuMV-GFP infection and cell-to-cell movement in the initially inoculated leaves but does not delay its long-distance movement.

ASM treatment restricts PIAMV-GFP replication without cell death.

The results described above showed that, for all three viruses tested, ASM treatment reduced the numbers of fluorescent spots in the inoculated leaves. To determine whether this reduction in the viral infection rate was manifested at the single-cell level, we prepared protoplasts from *N. benthamiana* leaves 3 days after treatment with 1 mM ASM or water and inoculated them with PIAMV-GFP. We first measured expression of the *PR-1*

gene in the uninoculated protoplasts and found that the levels of *PR-1* transcript were greatly increased in the ASM-treated protoplasts compared with the control protoplasts (Supplementary Fig. S7). This indicated that the SA signaling pathway was activated in the treated protoplasts. We inoculated the protoplasts with RNA transcripts of an infectious PIAMV-GFP cDNA clone and investigated the rates of infection and viral RNA accumulation at 24 h postinoculation (hpi). In each of five experiments, the infection rate was reduced in ASM-treated protoplasts compared with controls (Fig. 7A; Supplementary Table S2). Viral RNA accumulation in the ASM-treated protoplasts was about one fifth of the accumulation in controls (Fig. 7B). To examine whether cell death occurs at the time of virus infection, specifically after ASM treatment, we determined the percentages of viable protoplasts in uninoculated, water-inoculated, and viral RNA-inoculated protoplasts, using fluorescein diacetate staining, at 24 hpi. The percentages of viable control protoplasts in all treatments were between 68 and 75%, indicating that neither the mock inoculation nor the viral RNA inoculation had significant effects on protoplast viability (Fig. 7C). Viability among the ASM-treated protoplasts was around 60% for all treatments, but there were no statistically significant differences between the controls and the ASM-treated protoplasts (Fig. 7C). Therefore, neither the ASM treatment nor the viral inoculation had significant

Fig. 5. Effects of acibenzolar-*S*-methyl (ASM) treatment on green fluorescent protein-expressing potato virus X (PVX-GFP) infection. **A**, Representative photographs of control leaves and leaves treated with 0.25 mM ASM inoculated with PVX-GFP, taken under UV light at 7 days postinoculation (dpi). Bars = 1 cm. **B**, Numbers of PVX-GFP fluorescent spots on the inoculated leaves. Data are means $\pm$ standard error ($n = 9$). The asterisk indicates a significant difference between treatments ($P < 0.05$; Student's *t* test). **C**, Average sizes of PVX-GFP spots formed at 7 dpi. Data are means $\pm$ standard error of spot sizes relative to the control mean ($n = 7$). **D**, Representative photographs of PVX-GFP fluorescence in uninoculated leaves of control plants and plants treated with 0.25 mM ASM at 14 dpi. **E**, Kaplan-Meier curves of the time course of PVX-GFP movement in five control (continuous line) and five plants treated with 0.25 mM ASM (dotted line). The appearance of GFP fluorescence in uninoculated leaves was recorded for each plant and the cumulative data were plotted. Statistical differences between the two curves were analyzed using the log-rank test ($P = 0.0023$).

effects on protoplast viability at 24 hpi. In support of these results, we did not detect any necrotic tissues in PIAMV-GFP-infected *N. benthamiana* leaves stained with trypan blue, which can detect cell death in leaves infected with necrosis-inducing viruses (Komatsu et al. 2010) (Supplementary Fig. S8). No cell death was observed in the infected leaves, whether or not they were pretreated with ASM. Reverse transcription-quantitative (RT-q)PCR analysis of *Nbhsr203J*, a marker gene of cell death, indicated that *Nbhsr203J* expression was not significantly increased upon ASM treatment combined with PIAMV-GFP inoculation, which supports the above finding that cell death was not associated with the ASM-mediated virus restriction (Supplementary Fig. S9). These results indicate that ASM treatment suppresses the replication of PIAMV at the single-cell level without inducing cell death.

ASM-mediated restriction of PIAMV-GFP infection in *Arabidopsis thaliana* requires *NPR1* but not the *DCL* genes involved in antiviral RNA silencing.

To investigate the mechanism by which ASM treatment restricts virus infection, we took advantage of the availability of a variety of mutant lines of *A. thaliana*, which is

susceptible to PIAMV infection (Minato et al. 2014). We first demonstrated that 1 mM ASM treatment 3 days before inoculation reduced the number of fluorescent spots in wild type (Col-0) *A. thaliana* leaves at 2 dpi after inoculation with PIAMV (Fig. 8A, left panels). Therefore, ASM treatment suppresses PIAMV infection in *A. thaliana*, as it does in *N. benthamiana*. We then tested the effect of ASM treatment on PIAMV-GFP infection rates in several *Arabidopsis* defense-related mutants. However, none of the mutants other than the *npr1* mutant, which is deficient in NPR1, a critical signaling component of the SA-mediated defense pathway, failed to restrict PIAMV-GFP infection upon ASM treatment (data not shown). Figure 8 shows the data on the *dcl2/dcl3/dcl4* (*dcl2/3/4*) mutant, which is deficient in antiviral RNA-silencing, and the *npr1* mutant. We found that ASM treatment reduced the number of fluorescent spots in PIAMV-GFP-inoculated *dcl2/3/4* leaves to less than half the number found in untreated leaves, as it did in the wild-type plants (Fig. 8A and B). In contrast, ASM treatment did not significantly reduce the number of fluorescent spots in the *npr1* mutant (Fig. 8). These results indicated that the ASM-mediated restriction of PIAMV-GFP infection requires

Fig. 6. Effects of acibenzolar-*S*-methyl (ASM) treatment on green fluorescent protein-expressing turnip mosaic virus (TuMV-GFP) infection. **A**, Representative fluorescence microscope images of TuMV-GFP infection sites on inoculated leaves of control *Nicotiana benthamiana* plants and plants treated with 1 mM ASM observed at 2 days postinoculation (dpi). Bars = 500 μ m. **B**, Number of TuMV-GFP fluorescent spots on the inoculated leaves. Data are means $\pm$ standard error ($n = 8$). The asterisk indicates a significant difference between treatments ($P < 0.05$, Student's *t* test). **C**, Average size of TuMV-GFP spots formed at 2 dpi. Data are means $\pm$ standard error of spot sizes relative to the control mean ($n = 17$). The asterisk indicates a significant difference between treatments ($P < 0.05$, Student's *t* test). **D**, Left, representative images of TuMV-GFP fluorescence in uninoculated upper leaves at 3 dpi, taken using a LAS-3000 luminescent image analyzer. The red circles show areas containing spots of GFP fluorescence. Right, photographs of TuMV-GFP-infected *N. benthamiana* plants at 4, 8, and 20 dpi.

NPRI but does not require the *DCL* genes, which encode essential RNA silencing components.

DISCUSSION

PAs, including the SA analog ASM, are compounds that stimulate defense mechanisms against pathogens in plants and are widely used for crop protection. However, our knowledge of their mechanisms of action are fragmented. In this study, we developed a system for analyzing the mode of action of plant activators by tracking the progression of viral infection, viral propagation in the initially infected cells, cell-to-cell movement via the plasmodesmata, and long-distance movement. We employed GFP-expressing viruses combined with *N. benthamiana* plants to examine these stages of infection in untreated plants and in plants treated with ASM. This system enables us to examine the effects of ASM on each distinct stage of infection and thus provides insights into the mode of action of this PA in protecting plants against viral infection.

Indeed, using the system developed in this study, we found that ASM, which is one of the most studied PAs, consistently suppressed infection by three viruses (PIAMV, PVX, and TuMV) in the initially inoculated cells of *N. benthamiana* leaves. This result may seem unexpected because ASM, which activates the SA signaling pathway, was considered as an

inducer of SAR. However, our results corroborate the findings of a previous study showing that the primary antiviral target of SA is replication (Singh et al. 2004). Additionally, we found that ASM inhibited the cell-to-cell movement of TuMV and delayed the long-distance movement of PIAMV and PVX. Consistent with this, previous studies on the effect of SA on TMV and cucumber mosaic virus have demonstrated that SA induces at least two distinct antiviral responses, depending on the virus (Murphy et al. 2001). However, these previous studies did not examine whether the SA-mediated inhibition of virus replication and subsequent cell-to-cell or long-distance movement were independent effects. A notable finding in our study is that the effect of ASM on long-distance movement is independent of its effect on viral propagation in the initially infected cells. We were able to demonstrate this by diluting the inoculum used to infect control plants so that the numbers of infected sites on the inoculated leaves were similar to those on ASM-treated leaves. This multistep inhibitory effect of ASM may explain its high effectiveness against various plant viruses (Palukaitis et al. 2017). Because our method for evaluating the effect of ASM can be applied to other PAs and viruses, it will be interesting to examine whether other PAs can also inhibit multiple steps of viral infection.

ASM treatment did not appear to affect the cell-to-cell movement of PIAMV-GFP or PVX-GFP, but it did appear to

Fig. 7. Effects of acibenzolar-*S*-methyl (ASM) treatment on replication of green fluorescent protein-expressing plantago asiatica mosaic virus (PIAMV-GFP) in *Nicotiana benthamiana* protoplasts. **A**, Representative fluorescence microscope images of protoplasts inoculated with PIAMV-GFP RNA at 24 h post-inoculation (hpi). Bars = 100 µm. **B**, Reverse transcription-quantitative PCR analysis of PIAMV-GFP RNA accumulation in protoplasts prepared from control leaves and leaves treated with 1 mM ASM at 24 hpi. Values represent means $\pm$ standard error of seven biologically independent replicates normalized to the *N. benthamiana* 18S ribosomal RNA and expressed relative to the control mean. The asterisk indicates a significant difference between treatments ($P < 0.05$, Student's *t* test). **C**, Percentages of viable protoplasts prepared from control *N. benthamiana* leaves and leaves treated with 1 mM ASM. Each protoplast preparation was either uninoculated, mock-inoculated with water, or inoculated with the PIAMV-GFP RNA. Viable protoplasts were visualized and counted using fluorescein diacetate staining at 24 hpi. Bars represent means $\pm$ standard error ($n = 6$).

inhibit the cell-to-cell movement of TuMV-GFP. However, caution is needed in the interpretation of these results, since the ASM treatment also inhibited viral replication in the initially infected cells. In this and other studies, leaves were treated uniformly with ASM, and thus, we would expect that viral replication would be inhibited in neighboring cells as well as the initially infected cells. Therefore, our results for TuMV-GFP might be due to the ASM-mediated inhibition of replication in the neighboring cells rather than a specific effect of ASM on cell-to-cell movement. Regardless of this possibility, ASM treatment did not reduce fluorescent spot size in leaves inoculated with PIAMV-GFP and PVX-GFP, even though it restricted viral propagation in the initially infected cells. It is possible that different strategies of cell-to-cell movement employed by potexviruses and potyviruses can affect the outcome of ASM-mediated inhibition in their initially infected cells and their neighboring cells. Future studies should include an experiment that would reveal whether the restrictive effect of ASM on the replication (represented by the number of spots) and the cell-to-cell movement (represented by the size of spots) are dependent or not.

We also took advantage of an *Arabidopsis*-PIAMV pathosystem that has been used to identify host factors that affect the infection of plants by viruses (Yamaji et al. 2012; Hashimoto et al. 2016). NPR1 is the positive and central regulator of PR gene expression and SAR downstream of SA and ASM. We demonstrated that NPR1 was needed for the ASM-mediated

inhibition of PIAMV infection in inoculated leaves. This finding differs from previous studies showing that PA-induced antiviral responses are independent of NPR1 (Carr et al. 2010). In studies with *A. thaliana*, NPR1 was reported to be dispensable for both the SA-induced inhibition of turnip vein clearing virus and resistance to turnip crinkle virus, which is mediated by the dominant resistance gene *HRT* (Kachroo et al. 2000; Wong et al. 2002). On the other hand, in *N. benthamiana* plants transformed with the *N* gene, the *N*-mediated resistance to TMV requires NPR1, even though the *N* gene encodes a nucleotide binding site leucine-rich repeat protein similar to *HRT* (Liu et al. 2002). These findings, combined with the results of this study, highlight differences in the requirement of NPR1 for antiviral responses among plant-virus pathosystems.

An unanswered question that needs to be addressed is what kinds of genes and pathways downstream of NPR1 are involved in the restriction of virus infection. Although proteins encoded by PR genes, whose expression is induced downstream of NPR1, are candidates, molecular evidence for their antiviral roles is scarce (Ali et al. 2018). One of the more possible mechanisms is RNA silencing, which is an essential antiviral defense response. However, ASM treatment reduced the number of PIAMV-GFP fluorescent spots in the inoculated leaves of the RNA silencing-deficient *dcl2/dcl3/dcl4* triple mutant, indicating that RNA silencing is not involved in the ASM-mediated suppression of PIAMV-GFP infection (Fig. 8). This result is consistent with a previous study showing that DCL2,

Fig. 8. Effects of acibenzolar-S-methyl (ASM) treatment on green fluorescent protein-expressing plantago asiatica mosaic virus (PIAMV-GFP) infection in wild type and mutant lines of *Arabidopsis thaliana*. **A**, Representative photographs taken under UV light showing spots of PIAMV-GFP infection in inoculated leaves of plants treated with 1 mM ASM and control plants at 2 days postinoculation (dpi). Bars = 5 mm. Left, Col-0 (the wild type); middle, the *dcl2/3/4* mutant; right, the *npr1* mutant. **B**, Numbers of fluorescent spots on whole inoculated leaves at 2 dpi ($n = 8$). The results are representative of three independent experiments with similar results. The box plots represent the median, interquartile range, maximum, and minimum numbers. Small circles in the plot of *npr1* mutant represent outliers. The asterisks indicate significant differences from the control leaves inoculated with the normal concentration ($P < 0.05$, Student's *t* test).

DCL3, and DCL4 are dispensable for the SA-induced inhibition of TMV and cucumber mosaic virus infection (Lewsey and Carr 2009).

Another candidate mechanism for the ASM-mediated suppression of virus infection is programmed cell death. However, we showed that, in *N. benthamiana* protoplasts, ASM treatment decreased viral accumulation but did not increase the rate of cell death, indicating that the ASM-mediated inhibition of viral replication cannot be explained by cell death (Fig. 7). In accordance with this result, several previous reports have shown that some resistance genes against plant viruses inhibit their replication without cell death. For example, cell death did not occur in protoplasts prepared from *N. tabacum* cv. Samsun NN, which exhibits the *N*-dependent hypersensitive response upon TMV infection (Otsuki et al. 1972). Further, *Rx*-mediated resistance completely suppressed PVX infection without cell death in potato protoplasts (Bendahmane et al. 1999). Recently, the jacalin-type lectin JAX1 was shown to inhibit the replication of potexviruses by targeting the viral replication complex (Yoshida et al. 2019). It is possible that ASM treatment induces expression of an unidentified antiviral resistance gene that does not activate programmed cell death, leading to the rapid inhibition of viral replication.

An alternative but not mutually exclusive mechanism involves inhibition of viral replication as a side effect of the antifungal and antibacterial functions of the SA signaling pathway. For example, the protein secretory pathway, which is required for resistance against bacterial pathogens, is induced downstream of NPR1 (Wang et al. 2005). Massive induction of the NPR1-dependent secretory pathway upon ASM treatment may perturb the regular organization of intracellular membranes, in which plant viruses establish their replication complexes (Grangeon et al. 2012).

In this study, we have developed a system that can be used to track the progression of viral infection through three distinct steps. This system can be used with a variety of viruses and plant hosts under a variety of treatment conditions, to identify the steps affected by particular treatments in each pathosystem. Furthermore, the use of mutants such as the *Arabidopsis dcl2/dcl3/dcl4* and *npr1* mutants allows us to identify host factors that affect the progression of viral infection through each stage. This study will stimulate future research to resolve the unknown mechanisms of PA-mediated restriction of plant virus infection. This approach could also be used to identify more effective PAs, possibly leading to more efficient approaches to the control of viral diseases.

MATERIALS AND METHODS

Plant materials and PA and SA treatment.

N. benthamiana plants were grown in a growth chamber under a 16-h light and 8-h dark cycle at 25°C. *Arabidopsis thaliana* was grown in a growth chamber under a 12-h light and 12-h dark cycle at 22°C. Seeds of the *npr1-1* (CS3726) and *dcl2/dcl3/dcl4* (CS16391) *A. thaliana* mutants were provided by the Arabidopsis Biological Resource Center (Columbus, OH, U.S.A.). Four- to 5-week-old *N. benthamiana* plants and 3-week old *A. thaliana* plants were treated with ASM or water by foliar spraying and were incubated under the same conditions. ASM (50% [wt/wt] active ingredient; Syngenta, Tokyo) and sodium salicylate (Sigma-Aldrich, St. Louis) were dissolved in water containing 0.004% (vol/vol) Silwet L-77 (Nippon Unicar, Kawasaki, Japan) to a final concentration of 1 mM, unless otherwise stated. Water containing Silwet L-77 was used as a control. The ASM, SA, and water were applied to one leaf of each *N. benthamiana* plant.

Viruses, purification, and inoculation.

PIAMV-GFP (Minato et al. 2014) and PVX-GFP (Cruz et al. 1996) express their GFP as a fusion protein with the coat protein, separated by the foot-and-mouth disease virus 2A peptide. TuMV-GFP (Takeshita et al. 2012) expresses its GFP as a part of a polyprotein, in which the GFP cistron is inserted into the junction between the Nib and coat protein cistrons.

In order to produce purified virions for the mechanical inoculation of ASM- or SA-treated leaves, we first propagated the viruses in *N. benthamiana*. For PIAMV-GFP and PVX-GFP, virus infection was launched by infiltration with an agro-bacterium culture (optical density at 600 nm = 1.0) carrying a binary vector containing viral DNA (Minato et al. 2014). For TuMV-GFP, infection was initiated by mechanical inoculation with a plasmid containing the viral cDNA (Takeshita et al. 2012). Systemically infected leaves were collected 5 to 7 dpi and were stored at -80°C, until use. Virus particles of PIAMV-GFP and TuMV-GFP were prepared, from these infected leaves, using a polyethylene glycol (PEG) precipitation procedure, essentially as described previously (AbouHaidar et al. 1998; Garcia-Ruiz et al. 2010). For the preparation of PIAMV-GFP virions, a subsequent centrifugation step with a 20% sucrose cushion was also performed. For PVX-GFP, three leaf disks 5 mm in diameter were excised at 3 to 5 dpi using a cork borer, were ground with 1 ml of 0.2 M sodium phosphate buffer, and were directly used for inoculation. Each virus-enriched pellet of PIAMV-GFP and TuMV-GFP was resuspended in RNase-free water (Funakoshi, Tokyo) containing recombinant RNase inhibitor (TaKaRa Bio, Otsu, Japan).

Three days after spraying with ASM or water, treated leaves (one per plant) were mechanically inoculated with virus particles (PIAMV, 350 ng per leaf; TuMV, 660 ng per leaf) or a virus solution, in the case of PVX (10 µl per leaf), using Carborundum as an abrasive.

Fluorescence microscopy, image analysis, and trypan blue staining.

Viral-expressed green fluorescence images were acquired at 2 or 3 dpi, using a BIOREVO BZ-9000 microscope (Keyence, Osaka, Japan) with GFP filter sets, and the data was processed using the inbuilt data analysis software. Fluorescence images of inoculated leaves or whole plants infected with PIAMV-GFP or PVX-GFP were acquired using a handheld UVGL-58 UV lamp (Funakoshi) and a DS126231 camera (Canon, Tokyo), with an exposure time of 15 s. The numbers of fluorescent spots in each image was counted manually, and the size of fluorescent spots was measured using ImageJ software (National Institutes of Health, Bethesda, MD, U.S.A.). Fluorescence images of inoculated leaves or upper leaves infected with TuMV-GFP were acquired using a LAS-3000 imager (Fujifilm, Tokyo), with an exposure time of 15 s. Trypan blue staining was performed by boiling the inoculated leaves for 3 min in ethanol-lactophenol (2:1) containing 0.02% (wt/vol) trypan blue. The stained leaves were decolorized in a chloral hydrate solution (1 kg of chloral hydrate in 400 ml water) for at least 1 day.

RNA extraction and RT-qPCR.

A total of about 0.1 g of leaf tissues from two different leaves were homogenized in liquid nitrogen and total RNA was isolated, using ISOGEN reagent (Wako, Osaka, Japan) according to manufacturer instructions. Each RNA sample was treated with RQ1 RNase-free DNase (Promega, Madison, WI, U.S.A.), and about 1 µg of the DNase-treated total RNA was reverse transcribed, using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.), following manufacturer protocols. For monitoring PIAMV RNA accumulation and *PR-1* expression, 2 µl of synthesized cDNA was used

for RT-qPCR, using the Thermal cycler dice real time system II MRX [TP960] (TaKaRa Bio) with GoTaq qPCR master mix (Promega). 18S rRNA was used as an internal control.

The primer sets used for RT-qPCR were: PIAMV-3877F (5'-CCTCATTCTCCCTGCTGAAG-3') and PIAMV-4010R (5'-CTTGAGGGGGTCTTTGATGA-3') for PIAMV genomic RNA (Tanaka et al. 2019); NtPR1a-238F (5'-CATGGTCAATACGGCGAAAACCTAG-3') and NtPR1a-322R (5'-TCTCATCGACCACATCTCAACG-3') for *PR-1*; Nb18S-193F (5'-ATACGTGCAACAAACCCCGAC-3') and Nb18S-280R (5'-TGAATCATCGCAGCAACGG-3') for the *18S rRNA* (Hashimoto et al. 2012); and Nbhsr203J-F-SC57 (5'-CGCAATTCGAATCCATCCAGGCTT-3') and Nbhsr203J-R-SC58 (5'-GCAATTTAAGCTCCTCAACCGCCT-3') for *Nbhsr203J* (Wei et al. 2013).

Protoplast preparation and infection.

For inoculation of protoplasts with PIAMV-GFP, we first constructed T7-Li1-GFP, from which the infectious Li1-GFP genomic RNA can be transcribed in vitro, based on the binary vector pLi1-GFP (Minato et al. 2014), for agroinoculation with the PIAMV Li1 isolate carrying the sGFP gene. PCR was carried out using pLi1-GFP as a template, with the primer T7-Li1-1F (5'-GTAATACGACTCACTATAGAAAACAAACC TACACAAACCA-3') that has the T7 promoter sequence at its 5' terminus and a poly(T) primer with the downstream *Clal* restriction enzyme site (5'-ATCGATTV-3'), and the amplification product was cloned into the plasmid pBluescript SK+. The T7-Li1-GFP virus construct was digested at the *Clal* site just downstream of the poly(A) sequence. The viral PIAMV-GFP RNAs were transcribed from the linearized plasmids with the MEGascript T7 transcription kit (Thermo Fisher Scientific), according to manufacturer instructions. The m⁷GpppG cap analog (New England Biolabs, Ipswich, MA, U.S.A.) was included in the reaction. After the transcription, the quality and integrity of the RNA were confirmed using polyacrylamide gel electrophoresis.

Leaves of about 40-day-old *N. benthamiana* plants were used for protoplast preparation. The abaxial epidermis of each expanded leaf was peeled off and was floated in enzyme solution consisting of 0.5 M mannitol, 1.8 mM MES, 20 mM KCl, 10 mM CaCl₂, 0.05% macerozyme R-10 (Kinki Yakult Co., Nishinomiya, Japan), and 1% cellulase "Onozuka" R-10 (Kinki Yakult Co.) for 3 h at 26°C. The density of the isolated protoplasts was adjusted to 5 × 10⁵ to 10 × 10⁵ protoplasts per milliliter. The protoplast solution (1 ml) was inoculated with 1 µg of capped in vitro transcribed virus RNA in PEG-calcium transfection solution (40% [wt/vol] PEG 1540 [Polysciences, Inc., Warrington, PA, U.S.A.] and 3 mM CaCl₂). After inoculation the protoplasts were suspended in incubation buffer (0.5 M mannitol, 1× Aoki salts (0.2 mM KH₂PO₄, 1 mM KNO₃, 1 mM MgSO₄, 10 mM CaCl₂, 1 µM KI, 0.1 µM CuSO₄, pH 6.5) (Kroner and Ahlquist 1992) and were incubated for 24 h at 25°C in the dark. For analyses of the percentages of protoplast viability and infection rates of PIAMV-GFP, approximately 1,000 protoplasts were observed, using fluorescence microscopy. Extraction of total RNA from protoplasts was performed as described previously (Kroner and Ahlquist 1992). Total RNA (200 ng) extracted from the protoplasts was used for RT-qPCR.

ACKNOWLEDGMENTS

We are grateful to Y. Nakajima at Syngenta Japan for providing ASM, T. Natsuaki at Utsunomiya University for providing TuMV-GFP, and D. C. Baulcombe at the University of Cambridge for PVX-GFP. We also thank to the *Arabidopsis* Biological Resource Center for providing the *dcl*- and *npr1*-deficient lines of *A. thaliana*.

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