

# Exogenous ascorbic acid derivatives and dehydroascorbic acid are effective antiviral agents against *Turnip mosaic virus* in *Brassica rapa*

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**Abstract** Plants may activate posttranscriptional gene silencing (PTGS) as an immunity system when they are infected with viruses. Viruses may in turn interfere with this system by producing RNA silencing suppressor (RSS) proteins; most RSSs bind to viral small interfering RNAs (siRNAs). We previously reported that ascorbic acid (AsA) has the ability to interfere with the binding between viral siRNAs and viral RSSs in vitro. We thus expected that AsA-treated plants would show some tolerance to virus infection because the host PTGS will be strengthened by AsA. *Brassica rapa* subsp. *rapa* was inoculated with *Turnip mosaic virus* and treated with the AsA derivatives, L-(+)-ascorbic acid 2-sulfate disodium salt dihydrate (AsA-SO<sub>4</sub>), L-(+)-ascorbyl palmitate (AsA-Pal) and dehydroascorbic acid (DHA) at 1 h postinoculation. The number of infection sites on inoculated leaves decreased by around 40 % after AsA-SO<sub>4</sub> and AsA-Pal treatments and by 80 % after DHA treatment compared with the untreated control. As evidenced by an enzyme-linked immunosorbent assay, viral accumulation was significantly reduced after regular sprays with the AsA derivatives and DHA. In a detached leaf assay, AsA clearly functioned as a viral inhibitor in cells. Additionally, we confirmed that DHA also worked in the silencing pathway because its antiviral effect was not observed in the silencing-defective double mutant *dcl2/dcl4* of *Arabidopsis thaliana*. On the basis of these results, we concluded that the AsA derivatives and DHA can

significantly reduce viral infection and accumulation and that we can develop those compounds as a practical antiviral agent in the future.

**Keywords** Ascorbic acid · Dehydroascorbic acid · Antiviral effect

## Introduction

L-Ascorbic acid (AsA) plays an important role as an antioxidant in plants and animals. In plants, AsA is very abundant in leaves (Wheeler et al. 1998) and involved in regulating the cellular redox balance to contribute to tolerance to environmental stresses (Chen and Gallie 2005; Eltayeb et al. 2006). Endogenous AsA levels increase in plants in response to viral infection and oxidative stress such as chilling (Kanda and Tsuda 2008; Sayama 2003; Tsuda et al. 2005; Xu et al. 2008). For example, tomato plants, inoculated with an attenuated *Cucumber mosaic virus* (CMV) strain that can induce cross-protection, significantly increased the AsA level (Sayama 2003; Tsuda et al. 2005), suggesting that plants increase the cellular AsA level against viruses during their defense response.

In human cells, AsA and dehydroascorbic acid (DHA) inhibited the multiplication of three animal viruses, and DHA had a much stronger antiviral effect than AsA (Furuya et al. 2008). Examining the effects of exogenous AsA on viral infection in plants, Wang et al. (2011) recently reported that AsA treatment actually alleviated the symptoms and inhibited viral accumulation in *Arabidopsis* plants infected with CMV or with *Turnip crinkle virus* (TCV). However, considering the underlying antiviral mechanism of AsA, it is difficult to interpret some of their results. For example, the effects of AsA on the virus

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appeared only after 12 days postinoculation (DPI) although the AsA treatment was given every day.

In our previous study, we screened for inhibitor chemicals that bind viral RNA silencing suppressors (RSSs) to develop an effective antiviral agent (Shimura et al. 2008) because viruses produce RSSs to counter-attack host RNA silencing, a plant immunity system to viruses. Through this screening, we eventually obtained several compounds as relatively strong inhibitors such as an oxidized form of croconic acid (NS6390 described in Shimura et al. 2008), which was found to be similar to AsA in the structure. Because AsA is an abundant antioxidant in animal and plant cells, this compound should have little toxicity to plants and no impact on the environment. Therefore, we examined the effect of AsA as an inhibitor against RSSs and confirmed its antiviral effect (Masuta et al. 2009; Sano et al. 2007).

In this study, we tested whether exogenous treatments with AsA derivatives and DHA are indeed effective against viral diseases when plants are occasionally treated with them even after viral infection.

## Materials and methods

### Plants and viruses

The turnip (*Brassica rapa* subsp. *rapa*) cultivars Wase-ohkabu and Taibyohikari were purchased from Takii Co. (Kyoto, Japan). Plants were grown at 21 °C with a 12-h photoperiod. *Turnip mosaic virus*-TuR1-expressing the yellow fluorescent protein (TuMV-TuR1-YFP) was kindly provided by Dr. T. Natsuaki (Utsunomiya University, Japan). TuMV-C2, isolated from a diseased plant in a field, was kindly provided by Mr. R. Arimoto (Takii Co., Kyoto, Japan). These viruses had been maintained, respectively, in the turnip cultivar Yukihime-kabu (Tohoku Seed Co., Utsunomiya, Japan) and Chinese cabbage (*B. rapa* subsp. *pekinensis*) cultivar Yushun (Atariya-nouen Co., Katori, Japan).

The Columbia (Col-0) ecotype of *Arabidopsis thaliana* and the double mutant dcl2/dcl4 (CS66078) were obtained from Arabidopsis Biological Resource Center. The plants were grown under the same condition as the turnip plants.

### Chemicals

An AsA derivative, L(+)-AsA 2-sulfate disodium salt dihydrate (AsA-SO<sub>4</sub>) and dehydroascorbic acid (DHA) were purchased from Wako Co. (Tokyo, Japan) and dissolved in water containing 0.2 % (v/v) Tween 20. Because endogenous AsA are present at millimolar levels in plant tissues, we used 20 mM AsA-SO<sub>4</sub> and 20 mM DHA for

the exogenous treatment of leaves for efficient incorporation of those compounds into tissues (cells). Fat-soluble ascorbyl palmitate (AsA-Pal) was supplied as a micro-emulsion formulation that included a detergent (Nippon Soda Co., Tokyo, Japan) and suspended in water to be 1 mM.

### Treatment and virus detection

The second true leaves of turnip plants were dusted with carborundum and rub-inoculated with the sap from TuMV-infected tissues. For AsA treatment, we developed a half-leaf treatment to compare the susceptibility of the AsA-treated and nontreated tissues to the virus. One hour post-inoculation (HPI), one half leaf was first treated with an AsA derivative, and the opposite half was not treated. The AsA derivative solution was applied to a half leaf with a cotton swab. The viral infection spots were counted under blue light and expressed as a percentage relative to the control considered as 100 %. To evaluate the effect of regular treatment, we first sprayed AsA-Pal and DHA on the entire plant 1 HPI, and again every 24 h for a total of six sprays. Viral accumulation levels were measured by the conventional enzyme-linked immunosorbent assay (ELISA). For ELISA, the polyclonal antibodies against TuMV were purchased from the Japan Plant Protection Association (Tokyo, Japan), and the virus was detected according to the manufacturer's instructions. In a detached leaf method, the first and second true leaves of turnip plant were excised from the basal part of the petiole 1 HPI, and the cut edge of the petiole was kept in a Petri dish containing 20 mM AsA-SO<sub>4</sub> for 18 h. After this treatment, the sample leaves were transferred to the Petri dish containing only distilled water and incubated at 21 °C with a 12-h photoperiod for 6 days.

## Results and discussion

### Development of assay system to evaluate antiviral effects of exogenous AsA derivatives

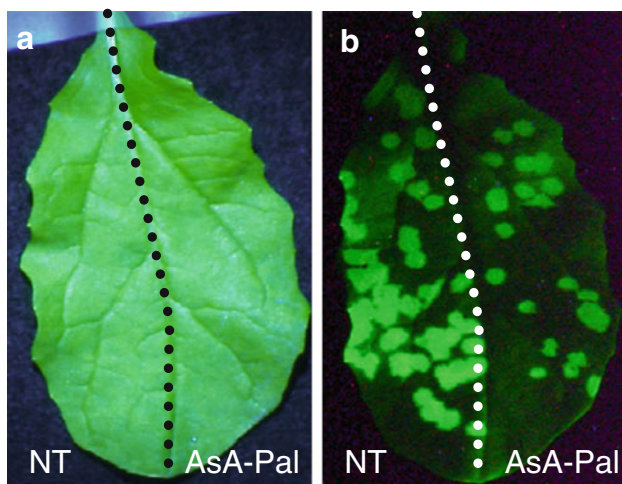
We first developed an assay system to assess the effects of AsA derivatives on viral infection. Because tissue susceptibility to viral infection should be equal between AsA-treated and nontreated leaves, the half-leaf treatment was used for the assay; we first inoculated the entire leaf with the virus, then applied AsA solutions only on one half with a cotton swab. To visualize and count the number of infection spots on inoculated leaves, we used the infectious TuMV clone (TuMV-TuR1-YFP) that produces YFP at infection sites. For the AsA compounds tested, because AsA is easily oxidized in water solution and because DHA

is known to be a labile chemical, we first selected two stable AsA derivatives: water-soluble AsA-SO<sub>4</sub> and fat-soluble AsA-Pal. As shown in Fig. 1 (e.g., the results of AsA-Pal), on the right side of the leaf where the AsA-Pal was applied, the number of infection sites significantly decreased in comparison with the nontreated side (Fig. 2a). No visible damage was observed on the leaves after treatment with AsA-SO<sub>4</sub> or AsA-Pal. We chose the half-leaf treatment as the AsA assay system for further analyses.

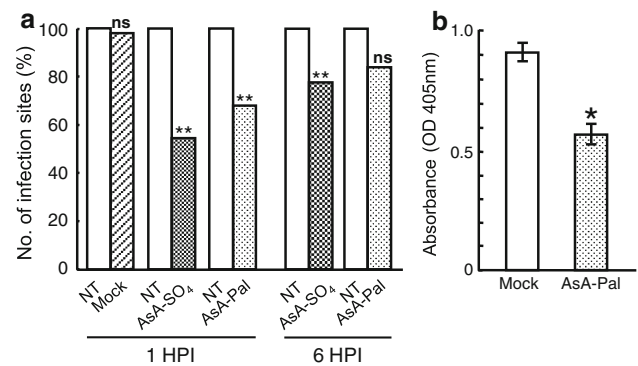
Time interval between TuMV inoculation and AsA treatment and frequency of AsA treatment

We then investigated how the time between viral inoculation and the treatment can influence the antiviral effects of AsA derivatives. As shown in Fig. 2a, leaves were treated with AsA derivatives 1 and 6 HPI, and 3 days later the fluorescent viral spots were counted. The number of spots was reduced by 45 and 32 % after treatment with AsA-SO<sub>4</sub> and AsA-Pal at 1 HPI, respectively, and both AsA-SO<sub>4</sub> and AsA-Pal had greater antiviral activity when applied at 1 HPI than at 6 HPI. These results suggested that AsA treatment would be more effective when applied closer to the time of inoculation.

To evaluate the effect of regular treatment of the AsA derivative, we first sprayed AsA-Pal on the entire plant 1 HPI and again every 24 h for a total of six sprays because we considered that AsA in leaf tissues might gradually be oxidized and degraded in the cells. Thus, the effects of the



**Fig. 1** Assay system using half-leaf treatments to observe the effect of ascorbic acid (AsA)-derivatives (e.g., AsA-Pal) on *Turnip mosaic virus* (TuMV) in *Brassica rapa* cv. Wase-ohkabu. Plants were grown for 14 days at 21 °C with a 12-h photoperiod. The second true leaf was mechanically inoculated with TuMV-TuR1-expressing the yellow fluorescent protein (TuMV-TuR1-YFP). **a** AsA-Pal was applied only to the right side of the leaf 1 h later with a cotton swab. **b** Viral infection sites are seen as YFP-fluorescent spots 4 days postinoculation. NT nontreated

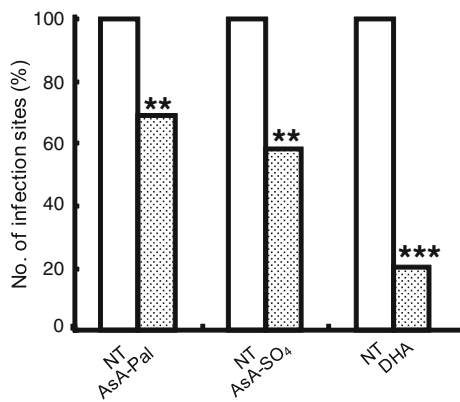


**Fig. 2** Effect of ascorbic-acid (AsA) derivatives on *Turnip mosaic virus* (TuMV) infection in turnip plants. **a** Number of infection sites on inoculated leaves of *Brassica rapa* cv. Wase-ohkabu at 3 days postinoculation (DPI) when treated with AsA derivatives at 1 h or 6 h postinoculation (HPI) with TuMV-TuR1-expressing the yellow fluorescent protein (TuMV-TuR1-YFP). Plants were grown and treated as described for Fig. 1. Mock treatment was done with water containing 0.2 % (v/v) Tween 20. Data are from at least eight replicates. Statistical significance was evaluated with Student's *t* test for paired sample; \*\* $P < 0.01$  and ns no significant difference. NT nontreated. **b** Effect of regular AsA-Pal treatment on viral accumulation in inoculated leaves of *B. rapa* cv. Wase-ohkabu. Plants were prepared as described for Fig. 1. AsA-Pal was sprayed on the entire plant 1 HPI with TuMV-TuR1-YFP, then every 24 h for a total of six sprays. The enzyme-linked immunosorbent assay was carried out at 8 DPI. Data are from six replicates. Error bars indicate standard errors. Statistical significance was evaluated with Student's *t* test for independent samples; \* $P < 0.05$

AsA derivatives would diminish with time. After the regular treatment, the levels of the virus in the inoculated leaves were assayed by ELISA. The regular treatment of AsA-Pal did not damage plants visibly, and viral level was reduced by 38 % (Fig. 2b).

#### DHA treatment efficiently inhibited TuMV infection

Because DHA was shown to be a strong antiviral agent against animal viruses such as herpes virus, influenza virus and poliovirus (Furuya et al. 2008), we then compared the antiviral effects of DHA with those of other AsA derivatives (AsA-SO<sub>4</sub> and AsA-Pal). According to the number of TuMV infection sites, 20 mM DHA significantly reduced the virus levels by 80 % in Wase-ohkabu without damage to plants (Fig. 3). To avoid the effect of differences in cultivar susceptibility to AsA, TuMV or both and to observe the antiviral effect of DHA on a naturally isolated TuMV strain, we used another host–virus combination, turnip cultivar Taibyo-hikari and TuMV-C2, which causes necrotic spots. In Fig. 4, the number of infection sites was reduced by 65 % after 20 mM DHA treatment. In addition, as shown by the ELISA, virus accumulation was reduced approximately by 40 % after the regular spray of DHA (Fig. 4c). These results therefore demonstrated that DHA



**Fig. 3** Comparison of antiviral effect of the ascorbic acid (AsA) derivatives and dehydroascorbic acid in *Brassica rapa* cv. Wase-ohkabu after inoculation with *Turnip mosaic virus*-TuR1-expressing the yellow fluorescent protein. Experiment was done as in Fig. 2. Data are from at least eight replicates. Statistical significance was evaluated with Student’s *t* test for paired samples; \*\**P* < 0.01, \*\*\**P* < 0.001 and *ns* no significant difference. *NT* nontreated

could function as a strong antiviral agent in plants as reported in animals (Furuya et al. 2008).

**AsA treatment affected viral replication and/or cell-to-cell movement**

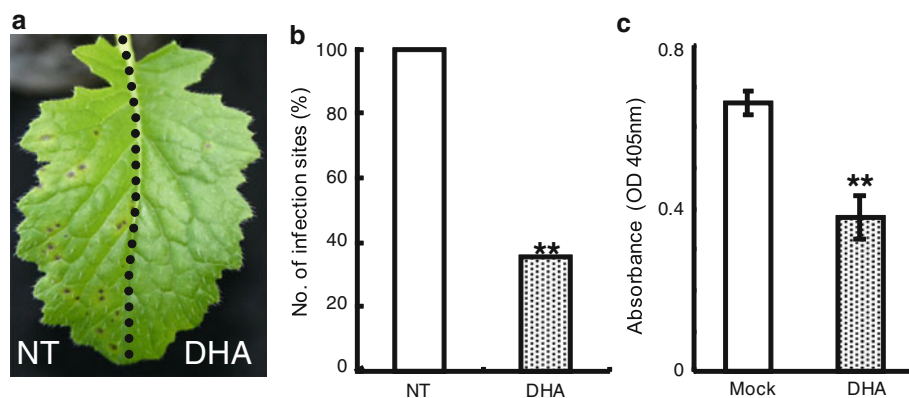
On the basis of our previous studies, the exogenous AsA derivatives appeared to act, at the least, as inhibitors of RSSs in cells after their uptake from the surface of leaves. To exclude the possibility that AsA derivatives or DHA might directly contact TuMV virions, destabilizing the virus, we performed the following two experiments. First, AsA derivatives (or DHA) were included in the TuMV

inocula: the final concentrations of AsA–Pal, AsA–SO<sub>4</sub> and DHA in the saps were 0.1, 2 and 2 mM, respectively. In this experiment, those compounds did not affect the number of infection sites on the leaves of Wase-ohkabu (Fig. 5). These results thus indicated that the AsA derivatives and DHA did not inhibit TuMV through direct interaction with TuMV virions.

Second, turnip plants were treated with an AsA derivative, AsA–SO<sub>4</sub>, using the detached leaf method in which the first and second true leaves are detached and only the cut edge of the petiole is placed in 20 mM AsA–SO<sub>4</sub>. The number of fluorescent viral spots was reduced by 70 % (Fig. 6a), and the spots on the leaves spread much slower than those on the control leaves treated only by distilled water (Fig. 6b). These results suggested that the AsA derivatives inhibited viral replication and/or the cell-to-cell movement through a resistant mechanism in tissues, possibly host RNA silencing.

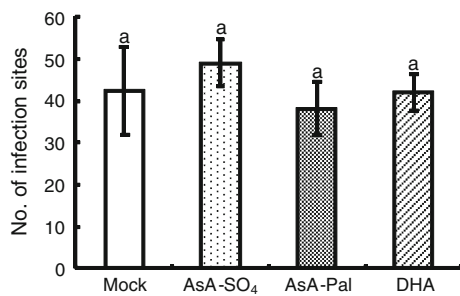
**Antiviral effect of DHA functions in the host RNA silencing mechanism**

Because we found AsA derivatives (or DHA) act as inhibitors of viral RNA silencing suppressors, to provide evidence that DHA actually works in the silencing pathway in plants, we analyzed whether DHA is effective against TuMV even in a silencing-defective plant. We here chose the *Arabidopsis* dcl2/dcl4 double mutant because DCL2 and DCL4 have been found to be the major dicers that promote TuMV degradation in the silencing pathway (Garcia-Ruiz et al. 2010). As a result, DHA was indeed not effective in the dcl2/dcl4 mutant when we observed that the viral accumulation level was reduced in the DHA-treated



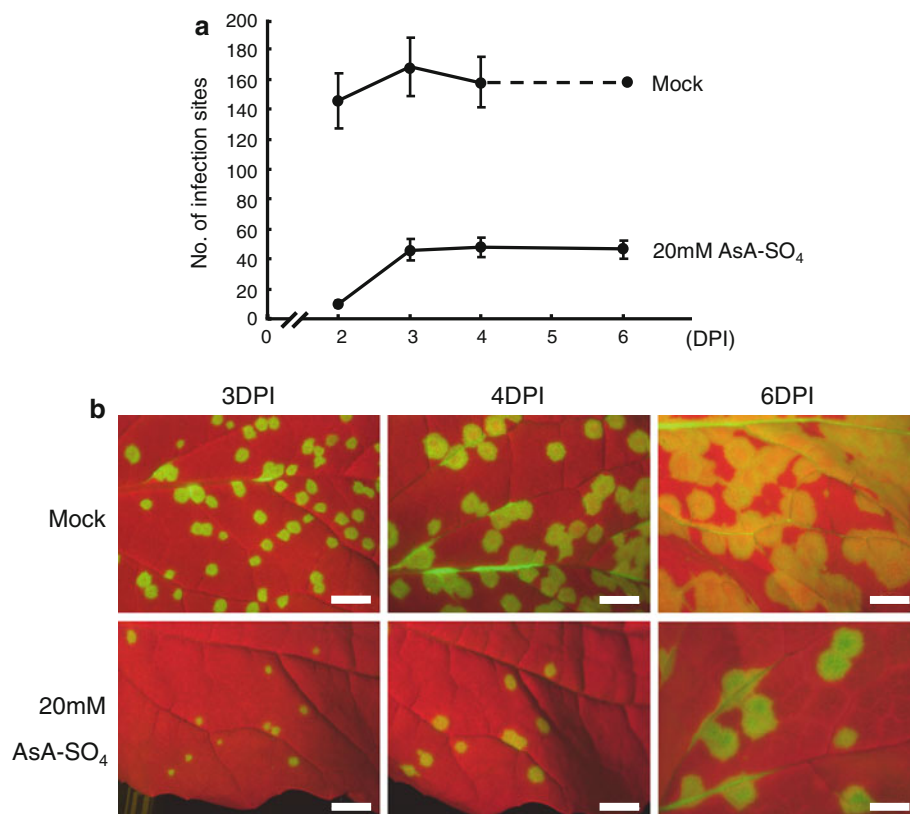
**Fig. 4** Effect of treatment with 20 mM dehydroascorbic acid (DHA) on number of virus infection sites and viral accumulation in leaves of *Brassica rapa* cv. Taiyo-hikari inoculated by *Turnip mosaic virus* (TuMV) strain C2. Plants were grown as described for Fig. 1. **a** Treated, inoculated leaf at 3 days postinoculation (DPI). **b** Number of infection sites after nontreatment or DHA treatment at 3 DPI. **c** Viral accumulation in inoculated leaves after control or regular

DHA treatment at 7 DPI as measured in an ELISA with absorbance at 405 nm. DHA was sprayed on the entire plant 1 h postinoculation with TuMV-C2, then every 24 h for a total of six sprays. Data are from at least six replicates. Error bars indicate standard errors. Statistical significance was evaluated with the paired sample *t* test (b) or the independent samples *t* test (c); \*\**P* < 0.01. *NT* nontreated



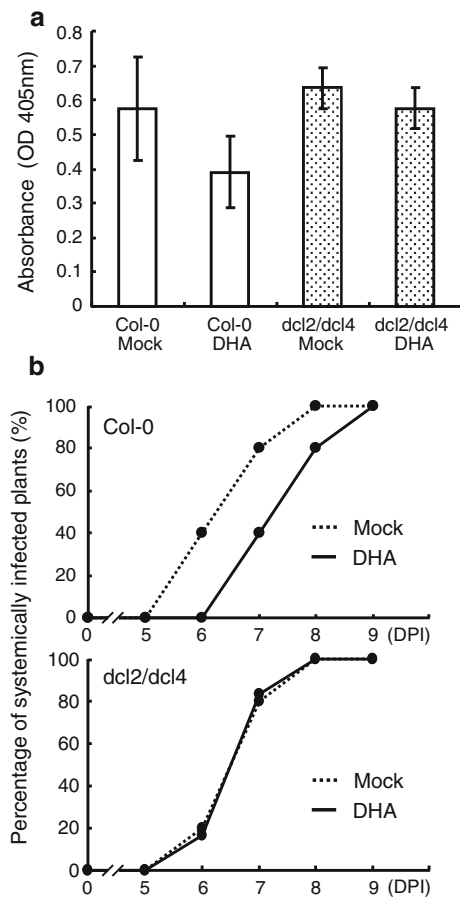
**Fig. 5** Inoculation tests of *Brassica rapa* cv. Wase-ohkabu using sap containing the ascorbic acid (AsA) derivatives or dehydroascorbic acid (DHA). The *Turnip mosaic virus*-TuR1-expressing the yellow fluorescent protein (TuMV-TuR1-YFP)-infected leaf was ground in 10 volumes of 0.1 M phosphate buffer and AsA-SO<sub>4</sub>, AsA-Pal or DHA were added to the sap for final concentrations of 2, 0.1 and 2 mM, respectively. Viral infection sites were observed as YFP fluorescent spots 4 days postinoculation. Data are from six replicates. Means followed by the same letter did not differ significantly (Tukey's test at  $P = 0.05$ ). Error bars indicate standard errors

wild type Col-0 compared to the untreated control (Fig. 7a). Unlike turnip plants as shown in Figs. 3 and 4, the DHA effect was not here statistically supported due to the difficulty in inoculating the small leaves of *Arabidopsis thaliana*, which varies greatly in susceptibility to the virus. In addition, we obtained a clear result on systemic infection; no difference in long-distance movement of TuMV-TuR1-YFP was observed between the DHA-treated *dcl2/dcl4* mutant and the mock control, while systemic movement of TuMV was delayed in the DHA-treated wild-type Col-0 (Fig. 7b). These results indicate that the DHA treatment inhibited viral accumulation in the inoculated leaves, leading to a delay in viral movement to upper leaves, but the inhibitory effect of DHA on TuMV was not fully functional in the silencing-defective *Arabidopsis* plant. We therefore consider that the antiviral effect of DHA may be associated with the RNA silencing mechanism.



**Fig. 6** Effect of an ascorbic acid (AsA) derivative, AsA-SO<sub>4</sub> on *Turnip mosaic virus* (TuMV) in detached leaf assay. First and second true leaves of *Brassica rapa* cv. Wase-ohkabu were cut out from the basal part of the petiole 1 h postinoculation, and the cut edge of the petiole was put into the Petri dish containing 20 mM AsA-SO<sub>4</sub>. After 18 h, sample leaves were transferred to a Petri dish containing water and incubated at 21 °C with a 12-h photoperiod for 6 days. **a** Change

in the number of infection sites on the leaves infected with TuMV-TuR1-expressing the yellow fluorescent protein (TuMV-TuR1-YFP). Data for the 20 mM AsA-SO<sub>4</sub> treatment at 6 days postinoculation (DPI) were judged to be equal to that at 4 DPI because infection spots had merged and could not be differentiated from each other. **b** Fluorescent stereomicrographs of second leaves at various times after inoculation with TuMV-TuR1-YFP. Scale bar is 3 mm



**Fig. 7** Effect of dehydroascorbic acid (DHA) on viral accumulation in *Arabidopsis thaliana* ecotype Col-0 and the *dcl2/dcl4* mutant. Plants were grown for 30 days at 21 °C with a 12-h photoperiod. Three rosette leaves were mechanically inoculated with *Turnip mosaic virus*-TuR1-expressing the yellow fluorescent protein (TuMV-TuR1-YFP). DHA was sprayed on the entire plant 1 h postinoculation and then every 24 h for a total of six sprays. **a** Viral accumulation levels in inoculated leaves were measured at 7 days postinoculation using an ELISA. Data are from three replicates. Error bars indicate standard errors. **b** Systemic infection of *dcl2/dcl4* mutant by TuMV-TuR1-YFP after DHA treatment. Upper, noninoculated leaves were monitored for YFP fluorescence for 10 days after viral inoculation. Five to six plants were used for each test. Similar results were obtained twice

#### Possible mechanism for AsA- and DHA-mediated antiviral activity

To explain the action of AsA and DHA, Furuya et al. (2008) previously hypothesized that either free radical formation or direct binding to the virus might be responsible for the antiviral activity of AsA and DHA against animal viruses. According to our previous results from the in vitro screening of viral inhibitors, we believe that the negatively charged AsA family, including ascorbate anion, ascorbyl radical and so on, may bind to positively charged

viral proteins, including RSSs, in cells. At this stage, we do not know whether antioxidant activity of AsA is involved. In fact, there is no report that supports an antioxidant mechanism driven by the AsA family; other factors have been suggested to be involved in the antiviral activity of AsA (Furuya et al. 2008).

On the basis of our data here and in previous studies, we believe that AsA derivatives and DHA function as inhibitors against RSSs. One interesting observation is that DHA conferred the strongest resistance against TuMV, suggesting that the hemiketal DHA structure is the best for the viral inhibition. However, we also have another explanation. In humans, DHA, but not AsA, is preferentially transported into cells via glucose transporters (Vera et al. 1995). The transported DHA is then reduced back to AsA and function as an antioxidant in cells. If the same situation occurs in plants, then the reduced form of DHA (AsA) might function as an antiviral compound in cells. Indeed, DHA is enzymatically reduced by the dehydroascorbic acid reductase in plant cells (e.g., Chen et al. 2003). The disproportionation of the ascorbyl radical anion, which is driven by the formation of the hemiketal structure of DHA, is also considered to be important for increasing intracellular concentrations of AsA (DiLabio and Wright 2000). We therefore consider that the stronger antiviral effect of DHA in relation to AsA is at least partially due to efficient transport of DHA into cells.

Our hypothesis on the mechanism underlying AsA antiviral activity is compatible with the fact that endogenous AsA levels in plants increase after virus infection (Kanda and Tsuda 2008; Sayama 2003; Tsuda et al. 2005). It is reasonable to postulate that increasing the endogenous AsA levels may be a strategy for plants to survive a virus attack. We are now investigating the expression levels of the genes involved in AsA biosynthesis, oxidation and recycling to elucidate the mechanism for regulating endogenous AsA levels against virus infection.

In this study, we demonstrated that AsA derivatives, especially DHA had strong antiviral activities. Our research on the conditions for appropriate treatments with AsA derivatives is now well under way for developing AsA derivatives as an effective antiviral agent for practical application.

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