Susceptibility and Symptom Development in *Arabidopsis thaliana* to Tobacco mosaic virus Is Influenced by Virus Cell-to-Cell Movement

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To identify host factors that regulate susceptibility to Tobacco mosaic virus (TMV), 14 *Arabidopsis thaliana* ecotypes were screened for their ability to support TMV systemic movement. The susceptibility phenotypes observed included one ecotype that permitted rapid TMV movement accompanied by symptoms, nine ecotypes that allowed a slower intermediate rate of systemic movement without symptoms, and four ecotypes that allowed little or no systemic TMV movement. Molecular comparisons between ecotypes representing the rapid (Shahdara), intermediate (Col-1), and slow (Tsu-1) movement phenotypes revealed a positive correlation between the ability of TMV to move cell to cell and its speed of systemic movement. Additionally, protoplasts prepared from all three ecotypes supported similar levels of TMV replication, indicating that viral replication did not account for differences in systemic movement. Furthermore, induction of the pathogenesis-related genes PR-1 and PR-5 occurred only in the highly susceptible ecotype Shahdara, demonstrating that reduced local and systemic movement in Col-1 and Tsu-1 was not due to the activation of known host defense responses. Genetic analysis of F2 progeny derived from crosses made between Shahdara and Tsu-1 or Col-1 and Tsu-1 showed the faster cell-to-cell movement phenotypes of Shahdara and Col-1 segregated as single dominant genes. In addition, the Shahdara symptom phenotype segregated independently as a single recessive gene. Taken together, these findings suggest that, within *Arabidopsis* ecotypes, at least two genes modulate susceptibility to TMV.

Additional keywords: pathogenesis, protoplasts.

The ability of viruses to cause disease is determined by molecular interactions between host and virus factors. These interactions directly affect virus replication, cell-to-cell movement, systemic movement, symptom development, and the elicitation of host defense responses (Carrington and Whitham 1998; Lazarowitz 1999). Although much information exists regarding the viral components involved in these processes,

comparatively little is known about the host factors involved. Understanding how specific host components affect viral disease and resistance responses will be important in the development of new methods to control these pathogens.

In the past few years, the genetic and molecular advantages of the model plant system *Arabidopsis thaliana* have begun to be exploited for the identification of host factors that contribute to virus biology (Simon 1994; Kunkel 1996). Subsequently, a number of loci within *Arabidopsis* spp. genes have been identified that affect the replication, movement, and host responses of several positive-sense RNA viruses. For example, *RTM1* and *RTM2* are two loci that cooperatively control the systemic movement of *Tobacco etch virus* (TEV) in different *Arabidopsis* ecotypes (Mahajan et al. 1998; Whitham et al. 1999). *RTM1* was recently found to encode a jacalin-like protein implicated in pathogen defense responses, and *RTM2* encodes a multidomain protein with homology to heat shock proteins (Chisholm et al. 2000; Whitham et al. 2000). The precise role of these proteins in blocking TEV movement remains to be determined. Additional *Arabidopsis* mutants affecting the cell-to-cell movement of *Cucumber mosaic virus* (CMV), *cum1*, and both the CMV and *Turnip crinkle virus*, *cum2*, have also been identified (Yoshii et al. 1998a, 1998b). For tobamoviruses, *Arabidopsis* mutants *tom1* and *tom2* were found to support only low levels of *Tobacco mosaic virus* (TMV) Cg (crucifer strain) replication in protoplasts (Ishikawa et al. 1993; Ohshima et al. 1998), whereas mutant *vsm1* was shown to limit the systemic movement of the tobamovirus *Turnip vein-clearing virus* (Lartey et al. 1998). Together, these findings clearly demonstrate the diversity of genes present within *Arabidopsis* spp. that affect virtually all aspects of the virus infection and disease cycle.

TMV is the type member of the genus *Tobamovirus*. Both functional and structural characteristics pertaining to TMV's RNA and encoded proteins have been the focus of intensive studies that have made TMV one of the best characterized positive-sense RNA viruses (Creager et al. 1999). Unfortunately, the commonly used *Arabidopsis* ecotypes are poor hosts for TMV and display no visible symptoms when infected. Consequently, much of the work that has been done with *Arabidopsis* spp. has utilized other tobamoviruses that more readily infect *Brassica* spp. To further establish a system to study TMV–host interactions, different *Arabidopsis*

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ecotypes were screened for susceptibility to TMV. A range of phenotypes were observed including one ecotype, Shahdara, that allowed rapid TMV spread accompanied by visible symptoms, as well as several ecotypes that permitted only slow or no systemic movement. Using the ecotypes Shahdara, Col-1, and Tsu-1, which allow fast, intermediate, and slow TMV systemic movement, respectively, the molecular and genetic basis for naturally occurring TMV susceptibility in *Arabidopsis* spp. was studied.

RESULTS AND DISCUSSION

Susceptibility of *Arabidopsis* ecotypes to TMV.

Fourteen *Arabidopsis* ecotypes were screened for their ability to support systemic virus movement. The accumulation of TMV coat protein (CP) in systemic noninoculated leaf samples was used as a marker for virus movement. Samples were taken at 7, 10, 14, 18, 24, and 36 days postinoculation (dpi) and CP levels determined by comparison to known CP standards (Table 1). Based on this assay, three different systemic movement phenotypes, slow, intermediate, and rapid, were observed. The slow movement phenotype was characterized by ecotypes Sn(5)-1, Tsu-1, Cnt-1, and M3385S, which supported little or no systemic accumulation of TMV, even after 36 dpi. These ecotypes also did not exhibit any necrosis or lesion formation that would indicate the induction of a host defense response. An intermediate movement phenotype was observed in nine ecotypes, including Columbia. In these ecotypes, TMV accumulation appeared in the upper noninoculated leaves between 14 and 24 dpi. These plants also remained asymptomatic even after accumulating high levels of virus. Only one ecotype, Shahdara, displayed a rapid systemic movement phenotype, permitting detectable levels of CP accumulation in systemic tissues as early as 7 dpi. TMV-infected Shahdara plants also exhibited disease symptoms that included leaf and bolt curling, stunting, late flowering, and necrosis of mature rosette leaves (Fig. 1). Thus, among the *Arabidopsis* ecotypes tested, at least three distinct TMV susceptibility phenotypes were identified.

TMV accumulation in inoculated leaves of ecotypes Shahdara, Col-1, and Tsu-1.

Three ecotypes, Shahdara (rapid), Columbia (intermediate), and Tsu-1 (slow), were chosen for further analysis to determine the molecular and genetic basis behind the different susceptibility phenotypes. To determine whether or not different movement phenotypes resulted from reduced levels of local virus accumulation in inoculated leaves, tissue print immunoblots were performed to visualize TMV cell-to-cell movement and accumulation in inoculated leaves (Fig. 2A). Results showed that Shahdara supported detectable levels of virus accumulation and spread by 4 dpi. Conversely, Col-1 accumulated only low levels of virus by 6 dpi, and virus was not reliably detected in Tsu-1. In order to quantify virus accumulation in inoculated leaves, time course assays for the accumulation of TMV CP were performed (Fig. 2B). These studies showed that local virus accumulation in Shahdara occurred rapidly, reaching maximum levels between 7 and 10 dpi. In contrast, Col-1 accumulated virus 2 to 3 days slower than Shahdara, and significant quantities of CP were not detected in Tsu-1 until 12 to 14 dpi. Interestingly, Col-1 and Tsu-1 accumulated 30 to 40% less CP than Shahdara in the inoculated leaves. Taken together, these findings suggest that the susceptibility phenotypes in Shahdara, Col-1, and Tsu-1 result from different rates of local TMV accumulation.

Induction of pathogenesis-related genes.

To determine if innate host defenses, such as the hypersensitive response (HR), play a role in the susceptibilities of Shahdara, Col-1, and Tsu-1, all three ecotypes were tested for the induction of pathogenesis-related (PR) genes upon infection with TMV. PR-1 and PR-5 genes were used as markers for the induction of the HR and systemic acquired resistance defense responses. Each ecotype was assayed for virus induction of PR-1 and PR-5 at 3 and 7 dpi (Fig. 3). Interestingly, both PR-1 and PR-5 were induced as early as 3 dpi in the susceptible host, Shahdara, whereas no induction was observed in either Col-1 or Tsu-1. The significance of PR gene induction in the highly susceptible and symptomatic ecotype, Shahdara, is not yet clear. PR-1 and PR-5 are typically associated with

Table 1. Accumulation of *Tobacco mosaic virus* (TMV) coat protein in noninoculated systemic leaves of various *Arabidopsis thaliana* ecotypes at different days post inoculation (dpi)

Ecotype	Uninoculated rosette leaves (dpi) ^a						Cauline ^b
	7	10	14	18	24	36	36
Shahdara	+	++	+++	+++	+++	+++	+++
Rld-2	–	–	+	++	+++	+++	+++
Col-1	–	–	+	++	+++	+++	+++
Oy-	–	–	+	+	++	+++	+++
H-O-	–	–	–	+	++	+++	+++
En-T	–	–	–	+	++	+++	+++
Condara	–	–	–	+	++	+++	+++
Wei-0	–	–	–	–	++	+++	+++
Nd-1	–	–	–	–	++	+++	+++
Est	–	–	–	–	++	+++	+++
Sn(5)-1	–	–	–	–	+	+	+
Tsu-1	–	–	–	–	–	++	++
Cnt-1	–	–	–	–	–	–	LF
M3385S	–	–	–	–	–	–	LF

^a Symbols represent approximate levels of coat protein per microgram of tissue as determined by Western immunoblot; – = no coat protein detected, + = <50 ng, ++ = 50 to 200ng, and +++ = >200ng.

^b Samples taken from cauline leaves on the flower bolt. LF indicates late flowering with no cauline leaves produced.

avirulent interactions such as the HR-induced necrosis and systemic acquired resistance (Ryals et al. 1996). However, weak induction of the HR has been shown to occur in tobacco by specific TMV mutants that cause HR-like necrosis but retain their virulence (Culver et al. 1991). Consequently, the necrosis phenotype exhibited by Shahdara may also arise from the weak induction of the HR. Regardless of this possibility, it appears that active host defense responses do not play a role in slowing TMV systemic movement in either Col-1 or Tsu-1 ecotypes.

TMV replication in *Arabidopsis* protoplasts.

To determine if Shahdara, Col-1, and Tsu-1 support different levels of virus replication, TMV accumulation was monitored in protoplasts generated from each ecotype. Protoplasts were inoculated with TMV and total RNA was purified from samples taken at 4, 8, 12, 16, and 20 h postinoculation. The rate and level of both genomic and subgenomic viral RNA accumulation was determined by Northern blot hybridization analysis (Fig. 4). No consistent differences in viral RNA accumulation were detected among Shahdara, Col-1, and Tsu-1. Thus, the observed susceptibility phenotypes are not the result of alterations in TMV replication at the single cell level.

Fig. 1. Symptoms exhibited by *Arabidopsis thaliana* ecotype Shahdara when infected with *Tobacco mosaic virus* (TMV). **A**, Healthy and TMV-infected Shahdara plant. Photo taken 3 weeks post infection. **B**, Healthy and TMV-infected Shahdara plant at 6 weeks post infection.

Analysis of 30-kDa movement protein in Shahdara, Col-1, and Tsu-1.

TMV susceptibility phenotypes in Shahdara, Col-1, and Tsu-1 result from differences in cell-to-cell movement; therefore, expression of the viral 30-kDa movement protein was further ana-

Fig. 2. Accumulation of *Tobacco mosaic virus* (TMV) in inoculated leaf tissue. **A**, Tissue print immunoblot showing the development of TMV infection foci in Shahdara, Col-1, and Tsu-1. Tissue print immunoblots of leaves taken at 0, 4, and 6 days post inoculation (dpi) are shown for each ecotype. Leaves taken at day 0 were inoculated and immediately used for tissue printing. Dark spots indicate TMV infection foci, lighter spots present at day 0 represent nonspecific binding of the antiserum. **B**, Accumulation of TMV coat protein (CP) in inoculated leaves of Shahdara, Col-1, and Tsu-1 as measured by Western immunoblot analysis. Graph shows the accumulation of TMV CP in inoculated leaves of each ecotype. Data points represent the average of 4 independent experiments $\pm$ the standard deviation.

lyzed in each ecotype. Previous work by Hughes et al. (1995) demonstrated that the viral-encoded 30-kDa movement protein is proteolytically processed in ecotype Columbia. Western blots showed that the 30-kDa protein was similarly cleaved in Shahdara, Col-1, and Tsu-1, suggesting that altered rates of cell-to-cell movement do not result from differential processing of the 30-kDa protein (Fig. 5). Moreover, the ability of Shahdara to support rapid systemic and cell-to-cell movement while still maintaining the mechanisms of 30-kDa cleavage demonstrates that this form of proteolytic processing does not significantly limit virus movement.

Fig. 3. Induction of pathogenesis-related (PR) genes. Northern blot showing the accumulation of PR-1 and PR-5 transcripts in Col-1 (C) Shahdara (S) and Tsu-1 (T) at 3 and 7 days post-inoculation (dpi). Plants were either inoculated with 5 µg of purified virus or mock inoculated with an equal volume of Na₂HPO₄ buffer. A positive control was obtained by syringe infiltration of rosette leaves with avirulent *Pseudomonas syringae* (P.s.) diluted to an optical density 600 of 0.2 in 10 mM MgCl₂.

Genetic analysis of susceptibility traits.

The genetic basis for the different TMV susceptibility phenotypes was determined by performing crosses between Shahdara and Tsu-1, and between Col-1 and Tsu-1. Three-week-old F1 plants were mechanically inoculated with 5 µg of virus and monitored for TMV systemic movement via Western or dot-immunoblot analysis at 8 and 15 dpi. F1 progeny from Shahdara × Tsu-1 crosses exhibited rapid Shahdara-like levels of virus accumulation and systemic movement. Similarly, when Col-1 was crossed with Tsu-1, F1 plants allowed intermediate levels of TMV accumulation and systemic movement. Therefore, TMV systemic movement phenotypes in Shahdara and Col-1 are conferred by dominant traits. Self-pollination of the F1 generations was performed for segregation analysis. F2 progeny from Shahdara × Tsu-1 and Col-1 × Tsu-1 crosses were similarly inoculated and monitored for TMV

Fig. 5. Processing of the 30-kDa cell-to-cell movement protein in *Arabidopsis* spp. Western immunoblot showing the *Tobacco mosaic virus* (TMV) 30-kDa cell-to-cell movement protein in tissue extracts from (shown left to right) tobacco, healthy Col-1, TMV-infected Col-1, TMV-infected Shahdara, and TMV-infected Tsu-1. Infected samples were taken at 30 days post inoculation. Double arrows on right indicate the positions of the two bands present in extracts from infected *Arabidopsis* spp. plants.

Fig. 4. *Tobacco mosaic virus* (TMV) replication in Col-1, Shahdara, and Tsu-1 protoplasts. Protoplasts prepared from Col-1 (C), Shahdara (S), and Tsu-1 (T) were inoculated with 20 µg of purified TMV RNA via electroporation and cells were harvested at 4, 8, 12, 16, and 20 hours post inoculation (top). A radiolabeled cDNA probe complementary to the 3' end of the TMV genome was used to detect the genomic as well as the 30-kDa and coat protein subgenomic RNAs (indicated on right). The positions of the 3.6- and 1.8-kb ribosomal RNAs are indicated on the left. Note: the apparently higher levels of coat protein subgenomic mRNA in Shahdara samples was not observed in subsequent duplicate assays.

systemic movement. For Shahdara $\times$ Tsu-1, 269 out of 349 F2 plants allowed rapid TMV movement for a segregation ratio of 3.4:1 ($\chi^2 = 0.80$). Of 215 Col-1 $\times$ Tsu-1 F2 plants analyzed, 163 retained the intermediate movement phenotype of Col-1 for a segregation ratio of 3.1:1 ($\chi^2 = 0.08$). These observed segregation ratios indicate that a single dominant gene confers the faster movement phenotypes of Shahdara and Col-1.

Genetic analysis of Shahdara symptom phenotype.

Although all of the Shahdara $\times$ Tsu-1 F1 progeny allowed rapid TMV movement, none were found to display the severe leaf curling and necrosis observed for the Shahdara parent. However, analysis of the Shahdara $\times$ Tsu-1 F2 generation revealed several plants that did display severe Shahdara-like symptoms. Of 271 Shahdara $\times$ Tsu-1 F2 plants scored for both virus accumulation and symptoms, only 45 displayed the characteristic Shahdara symptoms, while the other plants showed either mild stunting with no obvious signs of leaf curling and necrosis or no symptoms at all. The 45 F2 plants that displayed Shahdara-like symptoms also allowed rapid TMV movement. These results indicate a segregation ratio for Shahdara-like symptoms of approximately 15:3. One interpretation of this ratio is that a single recessive gene that requires a second dominant gene necessary for rapid TMV movement controls the Shahdara symptom phenotype. Such a scenario would give an expected ratio of 13:3, rapid or slow systemic movement without severe symptoms to rapid systemic movement with leaf curling and necrosis, and is supported by a calculated χ^2 value of 0.81. Thus, it appears that at least one recessive host gene present in Shahdara regulates the development of virus symptoms.

Conclusions.

Identification of host genes that control plant susceptibility to viruses is essential for both understanding the mechanisms of virus infection and controlling these pathogens. As a means to expand the use of *Arabidopsis* spp. for the study of TMV, we have identified a series of *Arabidopsis* ecotypes that display varying susceptibilities to TMV. Molecular analysis of a representative group of these ecotypes indicates that susceptibility is predominantly controlled at the level of virus cell-to-cell movement. Additionally, a single dominant gene in ecotypes Shahdara and Col-1 appears to control the rate of TMV cell-to-cell movement. It is possible that these different ecotype genes are allelic; consequently, differences in these alleles could account for the variation in the ability of these ecotypes to support the cell-to-cell movement of TMV. However, the mapping and cloning of these genes will be required to verify this possibility. Also, of importance is the identification of one *Arabidopsis* ecotype, Shahdara, that supports the rapid accumulation and movement of TMV along with the display of distinctive disease symptoms. Genetic analysis of this ecotype's susceptibility phenotype indicates that it is controlled at two levels by at least two genes. One gene confers rapid TMV cell-to-cell movement while an additional gene appears to confer the severe symptom phenotype. These findings provide a starting point for the molecular dissection of the multiple host factors that are involved in determining *Arabidopsis* spp. susceptibility to TMV.

MATERIALS AND METHODS

Plant materials, growth conditions, and inoculations.

Single-seed propagation of the *Arabidopsis* ecotypes Shahdara, Rld-2, Col-1, Oy-1, H-O-G, En-T, Condara, Wei-0, Nd-1, Est, Sn(5)-1, Tsu-1, Cnt-1, and M3385S were obtained from the Arabidopsis Biological Resource Center (ABRC) (Ohio State University, Columbus, U.S.A.). Plants were grown and maintained in environmental growth chambers under a 12-h light cycle at 22°C. Three- to four-week-old plants were used for virus inoculations. Mature rosette leaves were mechanically inoculated with either 5 μ g of purified TMV U1 or 5 μ g of purified viral RNA diluted with 10 μ l of FES buffer (0.1 M glycine; 0.06 M potassium phosphate; 1% [wt/vol] sodium pyrophosphate; 1% [wt/vol] macaloid; and 1% [wt/vol] celite, pH 8.5) as described by Turpen et al. (1995). Plants were monitored for a period of 4 weeks for the appearance of symptoms. F1 generations of Shahdara $\times$ Col-1 and Shahdara $\times$ Tsu-1 crosses required 24 h of light to initiate flowering.

Protein extraction and immunoblotting.

Infection assays were performed by punching leaf disks (1 cm in diameter) from inoculated or systemic tissues. Leaf disks were ground in 25 μ l of Laemmli buffer and incubated at 95°C for 5 min (Laemmli 1970). Five microliters of leaf protein extract was loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, electrophoretically separated, and transferred to nitrocellulose paper. Western blot analysis was performed as previously described (Dardick and Culver 1997). Blots were scanned and CP levels were quantified against known CP standards using the program NIH Image (National Institutes of Health, Bethesda, MD, U.S.A.). Immunoblots to detect the 30-kDa protein were performed similarly; however, rabbit anti-30-kDa antiserum was used (1:5,000 dilution) (Deom and He, 1997).

Screens for TMV accumulation were done by dot-immunoblot analysis. For these screens, leaf disks (1 cm in diameter) were taken from systemic and inoculated leaves of each *Arabidopsis* ecotype or cross. Sap from disks ground in 0.01 M sodium phosphate (pH 7.4) was applied to nitrocellulose paper via a 96-well vacuum manifold (Schleicher & Schuell, Keene, NH, U.S.A.). Blots were probed with CP-specific antibody as described above (Dardick and Culver 1997).

Tissue print immunoblots were performed as follows. Inoculated leaves were cut from plants and placed onto nitrocellulose sheets. This arrangement was sandwiched between two sheets of filter paper and pressed using a rolling pin. Squashed leaves were carefully removed from the nitrocellulose, leaving an imprint of the leaf. Tissue prints were washed briefly in 1 $\times$ Tris-borate EDTA and blocked in 5% nonfat dry milk for 20 min. Prints were then probed with rabbit anti-CP antiserum for 1 h, followed by a 1-h incubation with alkaline phosphatase conjugated goat anti-rabbit antiserum. Viral infection sites were visualized by the addition of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium, as described by Knecht and Dimond (1984).

RNA extraction and Northern blot hybridization analysis.

Total RNA was extracted from 100 mg of leaf tissue or 2.5 $\times$ 10⁶ protoplasts using the RNeasy Plant Miniprep kit (Qiagen, Valencia, CA, U.S.A.). Total RNA was separated in 1.4% aga-

rose formaldehyde gels and transferred to Immobilon-Ny+ nylon membranes (Millipore, Bedford, MA, U.S.A.). ³²P-labeled cDNA probes were prepared as follows. PR-1, PR-5, and Ubq5 fragments were polymerase chain reaction amplified from *Arabidopsis* genomic DNA as described by Glazebrook et al. (1996) and cloned into the TOPO TA cloning vector (Invitrogen, San Diego, CA, U.S.A.). A 900-bp TMV cDNA fragment made by cutting a pUC119-based TMV subclone with *Nco*I and *Bsi*WI was used to generate a probe complementary to the 3' end of the TMV genome. All DNA probes were labeled using the Prime-a-Gene Labeling System (Promega, Madison, WI, U.S.A.). After hybridization, Northern blots were washed and visualized using a phosphorimager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Preparation and inoculation of protoplasts.

Protoplasts were prepared as described by Simon et al. (1992). Callus tissue was grown using surface-sterilized seeds from Shahdara, Col-1, and Tsu-1 plated onto MS media (3% sucrose; 1× Murashige-Skoog salts; 1% agar; 3 mM MES, pH 5.8; and 1× Gamborg's vitamin solution) supplemented with kinetin at 0.5 µg/ml and 2,4-D at 0.5 µg/ml. Thirty milliliters of mannitol (0.6 M) was added to 3-week-old callus cultures propagated from approximately 100 seeds and incubated for 20 min at 25°C with shaking. Callus was centrifuged at 70 × g and the supernatant discarded. To the pellet, 50 ml of protoplast isolation medium (1× Murashige-Skoog salts; 0.1 M sucrose; 3 mM MES, pH 5.8; 0.5 M mannitol; 1× Gamborg's vitamins; kinetin at 0.4 µg/ml; and 2,4-D at 0.4 µg/ml) supplemented with Cellulysin at 10 mg/ml (Calbiochem, San Diego, CA, U.S.A.) and Pectolyase at 2 mg/ml (Sigma-Aldrich, St. Louis, U.S.A.) was added. Enzymatic digestion was allowed to proceed for 3 h with shaking at 25°C. Protoplasts were filtered through Nylon mesh, 53 µm-pore-size (Small Parts Inc., Miami Lakes, FL, U.S.A.), centrifuged at 70 × g and washed three times with cold 0.6 M mannitol. The protoplast concentration and viability were determined by staining with fluorescein diacetate (FDA) and visualized on a hemacytometer under a fluorescence microscope (Leica Microsystems Inc., Deerfield, IL, U.S.A.).

Protoplasts were inoculated as described by Ishikawa et al. (1993). Briefly, 1.15×10^7 protoplasts were resuspended in 600 µl of electroporation buffer (5 mM MES; 70 mM KCl; and 0.5 M mannitol, pH 5.8). Purified TMV RNA (20 µg) was added and the protoplast suspension was electroporated once (47 µF, 300 V). Five milliliters of protoplast culture medium (1× Murashige-Skoog salts; 0.1 M sucrose; 3 mM MES; 0.4 M mannitol; 1× Gamborg's vitamin solution; kinetin at 0.4 µg/ml; and 2,4-D at 0.4 µg/ml, pH 5.8) was added and protoplasts were incubated at 25°C in the dark. Samples were taken at 4, 8, 12, 16, and 20 h postinoculation. Cells were centrifuged and stored at -80°C.

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