Susceptibility and Resistance of *Arabidopsis thaliana* to Turnip Crinkle Virus

Anne E. Simon[1,2], Xiao Hua Li[3], Jodi E. Lew[1], Rebecca Stange[1], Chunxia Zhang[2], Mary Polacco[1], and Clifford D. Carpenter[1]

[1]Department of Biochemistry and Molecular Biology, [2]Program in Molecular and Cellular Biology, [3]Department of Plant Pathology, University of Massachusetts, Amherst 01003 U.S.A.

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The *Arabidopsis thaliana* ecotype Dijon (Di-0) is resistant to turnip crinkle virus (TCV). It exhibited either no symptoms 3 wk after inoculation or mild symptoms such as stunting, curled bolts, and seliques and early desiccation. Twenty-two other ecotypes of *A. thaliana* developed a lethal systemic necrosis within 3 wk of inoculation with TCV. Five days after inoculation, TCV genomic RNA accumulated in Di-0 plants, grown under different environmental conditions, at less than 1% of the level found in the susceptible ecotype Col-0. To monitor the accumulation of viral RNA in both inoculated and uninoculated leaves of Col-0 and Di-0, virus-specific probes were hybridized directly to the RNA within whole plants. Results using this procedure revealed that in Col-0 the virus had spread throughout the vascular tissue by 7 days postinoculation. In Di-0, signal was detected mainly in the inoculated leaf and in the opposite leaf of the same age. Di-0 protoplasts, however, were found to support virus replication. These results suggest that restricted virus spread, and not a block in virus replication, is responsible for the resistance of Di-0 to TCV.

Additional keywords: disease resistance, virus movement, virus replication, whole-plant hybridization.

Recently, several reports have described the utility of *Arabidopsis thaliana* (L.) Heyhn. for studies of the interactions between plants and fungi (Koch and Slusarenko 1990), bacteria (Simpson and Johnson 1990; Dong *et al.* 1991; Whalen *et al.* 1991), and nematodes (Sijmons *et al.* 1991). With few exceptions (Melcher 1989; Li and Simon 1990), little is known about the interactions between *A. thaliana* and viruses. We are developing a system for the study of plant/virus interactions using turnip crinkle virus (TCV) and *A. thaliana.*

TCV is a carmovirus and is one of the smallest and simplest of the plant RNA viruses. Its single, positive-strand genome of 4,054 nucleotides contains only five open reading frames, one of which requires the readthrough of an amber codon (Carrington *et al.* 1989; Hacker *et al.* 1992). TCV is easily transmissible by mechanical inoculation of either virions, isolated viral RNA, or *in vitro* synthesized transcripts (Heaton *et al.* 1989) and is one of the best characterized viruses at the structural level (Carrington *et al.* 1987; Wei *et al.* 1990). TCV is associated with small subviral RNAs including satellite (sat-) RNAs and defective interfering RNAs, some of which intensify the symptoms of the helper virus by an as yet undetermined mechanism (Simon and Howell 1986; Simon *et al.* 1988; Li *et al.* 1989). The TCV isolate used in this study, TCV-M, is associated with two avirulent sat-RNAs (D and F) and one virulent sat-RNA (C).

TCV-M was previously reported to be a pathogen of *A. thaliana* (Li and Simon 1990). Six ecotypes of *A. thaliana* were equally susceptible to the virus. Infected plants died within 3 wk of inoculation if the virulent sat-RNA C was present in the inoculum. Infection of *A. thaliana* ecotypes with TCV-M which did not contain any virulent subviral RNAs resulted in stunted, mildly chlorotic plants (Li and Simon 1990).

We have found that the ecotype Dijon (Di-0) is resistant to infection by TCV-M. Di-0 accumulates less than 1% of the viral RNA levels found in susceptible ecotypes by 5 days postinoculation. We also describe a new technique that has allowed us to investigate the patterns of virus accumulation in susceptible and resistant plants. The technique involves hybridizing probes directly to plants and may be useful not only in plant virology but also for the detection of other cellular RNAs.

Address correspondence to A. E. Simon: Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, MA 01003 U.S.A.

Current address of Xiao Hua Li: Department of Biology, Texas A&M, College Station, TX 77843 U.S.A.

Permanent address of M. Polacco: Department of Biochemistry, University of Missouri, Columbia, MO 65211 USA.

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MATERIALS AND METHODS

Plant materials and inoculations. *A. thaliana* ecotypes were obtained from the following sources: S. Somerville (Michigan State University), Bur-0, Di-0, Chi-1, Co-1, Col-glabrous, Etr, Rid, Eastland, La-0, Montcalin, Bla-4, Nd-0, Pr-0, Sch-1, Msu12, Msu14, Msu15, Msu16, Msu24, Msu25, and Msu30; F. Ausubel (Harvard Medical School), Col-0, Kas-1. Di-0 was originally obtained from A. Krans (AIS Acc. 44[300]869[82900], *Arabidopsis* stock center), and was originally isolated from the Botanical Garden of Dijon, France. All seeds were planted in Pro-Mix soil (Premier Brands Inc., New Rochelle, NY). and grown in a growth chamber under a 14-hr light cycle at 20° C and

200 μE s^{-1} m^{-2} consisting of mixed fluorescent and incandescent light (unless otherwise stated). Plants with four fully expanded leaves (approximately 2 wk postgermination) were dusted with Celite and then inoculated by rubbing each leaf with one stroke of a glass rod dipped either in RNA inoculation buffer alone (mock; Li *et al.* 1989), or in RNA inoculation buffer containing 0.1 mg/ml of total plant RNA extracted from turnip infected with TCV-M genomic RNA and the nonvirulent sat-RNA D (TCV-m + D) or TCV-M which contains TCV genomic RNA, the avirulent sat-RNAs D and F, and the virulent sat-RNA C (Li and Simon 1990).

RNA isolation and blot hybridization. Total leaf RNA was extracted using phenol and a LiCl precipitation procedure as previously described (Simon and Howell 1986) except that only a single LiCl precipitation step was used. DNA-RNA blot hybridization was as previously described (Li *et al.* 1989). Probes were a full-length cDNA of TCV-M genomic RNA (C. D. Carpenter, C. Song, and A. E. Simon, unpublished), or pea ribosomal DNA (Jorgensen *et al.* 1987), labeled with [α-^{32}P]dCTP using random primers (Sen and Murai 1991). Filters were exposed to X-ray film for various lengths of time to ensure linearity of film grain saturation. Autoradiograms were scanned with a two-dimensional gel analysis system (Microscan 1000, Technology Resources, Nashville, TN) and the data were integrated using software provided by the manufacturer.

***In situ* hybridization to RNA in whole plants.** At the four fully expanded leaf stage, one of the two oldest leaves of Col-0 was inoculated as described above. At various times following inoculation, roots were excised and shoots were either frozen at $-80°$ C until needed or treated immediately as follows. Shoots were placed in a 60-mm petri dish, immersed in 95% ethanol and subjected to mild agitation overnight at 25° C. The ethanol was then poured off and 10 ml of a freshly prepared solution of 0.1 mM NaN_3, 0.1% sodium dodecyl sulfate (SDS), 0.5 mg/ml of pronase was added, and the plants were subjected to mild agitation overnight at 37° C. The solution was poured off and replaced with 10 ml of 0.15 N HCl. After incubation at 25° C for 20 min, plants were rinsed twice in 2$\times$ SSC (1$\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then treated as described below or dried at room temperature and stored until needed. Plants were incubated in 5 ml of prehybridization solution (2$\times$ SSC, 5$\times$ Denhardt's solution, 0.2% SDS, 1.0 mg/ml denatured salmon sperm DNA, 50% formamide) overnight at 37° C with mild agitation. Probe DNA (1.0×10^6 dpm) was added for each plant, and hybridization was allowed to proceed overnight at 37° C. After hybridization, plants were washed twice in 0.1$\times$ SSC, 0.1% SDS at 55° C for 20 min and then transferred to acetate sheets and allowed to dry at 25° C. Plants were then covered with plastic wrap and exposed to Kodak XAR5 film overnight.

Preparation and infection of *A. thaliana* protoplasts. Protoplasts were prepared from axenic callus cultures of Di-0 and Col-0 according to Guzman and Ecker (1988) except that both cellulysin and macerase were purchased from Calbiochem (La Jolla, CA) and digestion was stopped after 3 hr. For infection, 8×10^7 protoplasts were added to 500 μg of total plant RNA extracted from turnip infected with TCV-M, mixed gently, and then brought to a final concentration of 3 mM $CaCl_2$, 40% polyethylene glycol in 2.7 ml. After a 15-sec incubation, the mixture was immediately diluted in 10 vol of 0.6 M mannitol, 1 mM $CaCl_2$ and kept on ice for 15 min. Protoplasts were pelleted at 900 $\times$ *g*, washed, and pelleted three times in 0.6 M mannitol, 1 mM $CaCl_2$, and then resuspended in 15 ml of Murashige-Skoog salts supplemented with (per liter unless noted) 1 mg of thiamine-HCl, 0.5 mg of pyridoxine-HCl, 0.5 mg of nicotinic acid, 100 mg of *myo*-inositol, 0.2 mg of 2,4-D, 0.1 M sucrose, 0.4 M mannitol, 60 μg/ml nystatin, 2 mg/ml carbenicillin, and 3 mM MES pH 5.8. The protoplast solution was divided into five equal portions and incubated at 25° C. After times ranging from 4 to 40 hr, protoplasts were pelleted in a microcentrifuge at 1,000 rpm and frozen at $-80°$ C. Equal volumes of phenol/chloroform (1:1) and RNA extraction buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 100 mM NaCl, 1% SDS) were added, and the aqueous layer was separated and reextracted in phenol/chloroform. After the addition of 1/10 volume of 1.5 M sodium citrate, the RNA was precipitated in ethanol.

RESULTS

***A. thaliana* ecotype Di-0 is resistant to TCV-M.** We screened 23 different *A. thaliana* ecotypes for possible resistance to TCV-M (for a listing of the ecotypes tested, see Materials and Methods). One ecotype, Di-0, was highly resistant to virus infection. Nearly all Di-0 plants remained symptomless 3 wk postinoculation by which time all other *A. thaliana* ecotypes infected with TCV-M were dead (Fig. 1A). Six to eight days after inoculation, leaves of the susceptible ecotype Col-0 had ceased expanding, displayed clear downward curling at the margins and were slightly less green than mock-inoculated plants (Fig. 1B). By 14 days postinoculation, infected Col-0 plants did not exhibit any additional growth and older leaves were completely chlorotic (Fig. 1C). By 3 wk postinoculation, all infected Col-0 plants were completely necrotic (Fig. 1D). Di-0 plants inoculated with TCV-M displayed several different reactions to the virus. At 6–8 days postinoculation, most Di-0 plants were symptomless but on about 25% of the plants, a few small necrotic lesions were seen on the inoculated leaves (Fig. 1B). These small necrotic lesions were never detected on the inoculated leaves of TCV-M-infected Col-0 plants. By 14 days postinoculation, the vast majority of Di-0 plants remained symptomless (Fig. 1C), whereas a few plants displayed early signs of symptoms such as bolt curling. About 80% of Di-0 plants observed at 23 days postinoculation remained symptomless. Plants displaying symptoms had different combinations of the following: stunted leaves and bolts, curled bolts, curled seliques, and early desiccation (Fig. 1D).

Levels of virus accumulating in Di-0 and Col-0 ecotypes. We previously reported that all cultivars of TCV-M-infected *Brassica rapa* L. spp. *chinensis* plants tested accumulated high levels of TCV-M genomic RNA, yet some varieties remained symptomless (tolerant to viral RNA accumulation) while others exhibited the severe symptoms normally associated with the virus (Li and Simon 1990).

To determine whether Di-0 plants were resistant (i.e., little or no virus accumulation) or tolerant (normal virus accumulation) to TCV-M, viral genomic RNA and associated sat-RNA levels were monitored in Col-0 and Di-0 plants between 1 and 11 days after inoculation with TCV-M. Total RNA was extracted from approximately 10 plants pooled for each time point. RNA-DNA blot hybridization analysis using a full-length TCV-M cDNA clone as a probe revealed low but detectable levels of TCV-M genomic RNA present at 3 days postinoculation in Col-0 plants that were absent in inoculated Di-0 plants (Fig. 2). Accumulation of viral genomic RNA in Col-0 plants increased dramatically by 5 days postinoculation and reached maximum levels by 7 days postinoculation. In contrast, no hybridization to the TCV-M genomic RNA probe was discernible in lanes containing Di-0 RNA until 11 days postinoculation. This result indicates that resistance of Di-0 to TCV-M is probably due to a lack of early accumulation of the virus, as

Fig. 1. Resistant and susceptible interactions between *Arabidopsis thaliana* and turnip crinkle virus (TCV-M). **A,** Col-0 plants (left) and Di-0 plants (right) 21 days after inoculation with TCV-M. **B,** Col-0 plant (left) and Di-0 plant (right) 8 days after inoculation with TCV-M. Di-0 plant has small necrotic lesions on the inoculated leaves. **C,** Same as B, 14 days after virus inoculation. **D,** Same as B, 23 days after virus inoculation. One Col-0 and two Di-0 plants are shown; the Di-0 plant on the left exhibits typical mild symptoms (curled seliques and early desiccation).

opposed to an inability to express symptoms.

Effect of environmental parameters on resistance and susceptibility to TCV-M. Because the duration of light period can affect plant susceptibility to virus infection and virus multiplication (Matthews 1991), we evaluated the influence of day length of the TCV/*A. thaliana* interaction. Col-0 and Di-0 plants were grown from seed in 200 μE s^{-1} m^{-2} for light periods (per 24 hr) of 8, 14, 16, 18, or 24 hr. Plants with four fully expanded leaves were inoculated with TCV-M. At 5 days after inoculation (when viral RNA accumulation was still increasing in Col-0 plants grown under 200 μE s^{-1} m^{-2}, 14-hr days; see Fig. 2), RNA was extracted from individual plants, and the relative accumulation of TCV-M genomic RNA was determined by RNA-DNA blot analysis. Visual examination of symptoms expressed by additional infected plants was conducted at 9 days postinoculation when symptoms were clearly discernible.

The results (Fig. 3A) indicated that TCV-inoculated Di-0 plants subjected to longer day lengths were consistently less symptomatic. Whereas 90–95% of inoculated Di-0 plants grown under 16–24 hr of light were symptomless at 9 days postinoculation, only 60–70% of Di-0 plants were symptomless when grown under shorter day length conditions. Viral RNA levels in individual Di-0 plants assayed

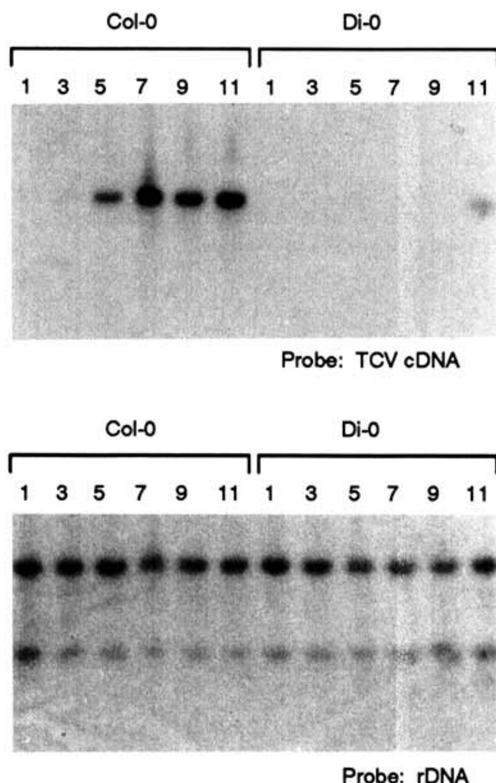

Fig. 2. Time course for the accumulation of turnip crinkle virus (TCV-M) genomic RNA in *Arabidopsis thaliana* ecotypes Col-0 and Di-0. Total RNA (4–5 μg/lane) was extracted from Col-0 or Di-0 leaves at various times following virus inoculation, subjected to electrophoresis on 1% agarose gels, and transferred to nylon membranes. The gel blot was probed with a full-length cDNA of TCV-M genomic RNA (top). To demonstrate that equal amounts of RNA were present in all lanes, the probe was stripped off and the blot reprobed with pea rDNA (bottom). Numbers at the top of each lane refer to the number of days after inoculation of plants with TCV-M when the RNA was extracted.

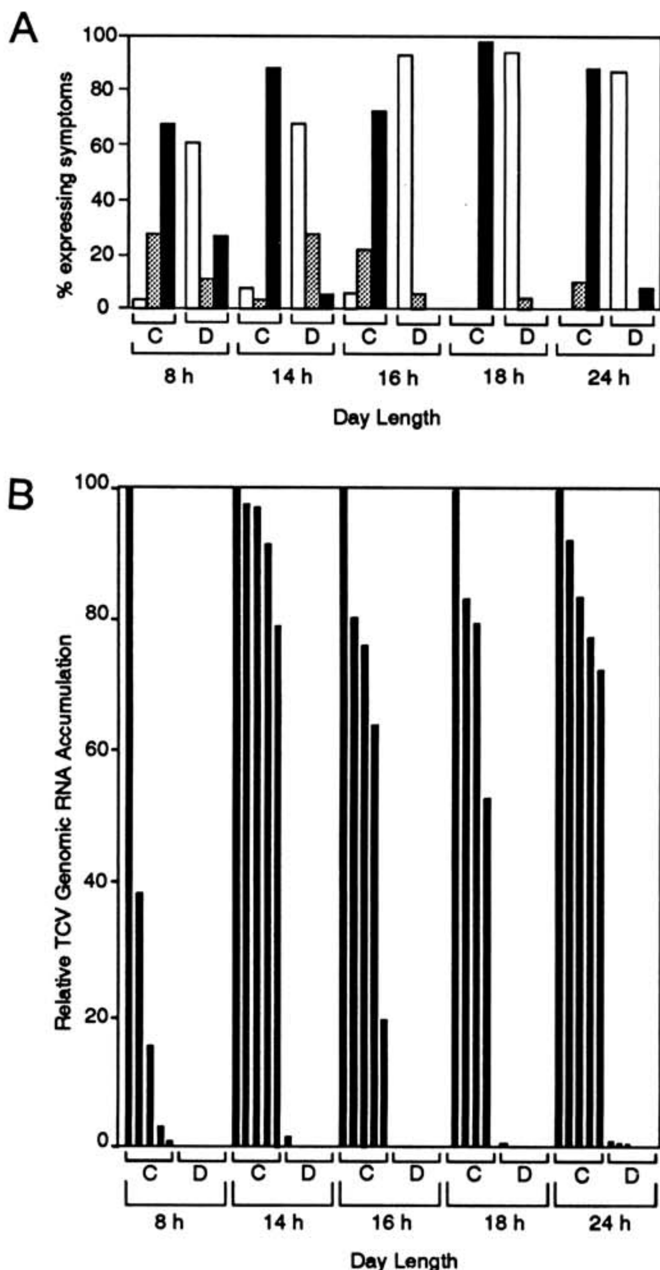

Fig. 3. Effect of day length on symptoms and accumulation of turnip crinkle virus (TCV-M) genomic RNA. Plants were grown from seed in 200 μE s^{-1} m^{-2} for the day lengths indicated. **A,** Col-0 (C) or Di-0 (D) plants were inoculated with TCV-M and symptoms observed at the peak of symptom onset, 9 days later. Open bars, no detectable symptoms; lightly shaded bars, mild symptoms consisting of slight leaf curling, slight discoloration, bolt curling; black bars, severe symptoms consisting of extreme leaf curling and shriveling, discoloration to brown/purple/yellow, failure to bolt. Data are compiled from five separate experiments containing 10 replicate Col-0 or Di-0 plants per treatment. **B,** Northern analysis of TCV-M genomic RNA accumulating in five individual plants 5 days postinoculation. Total RNA isolated from each plant was subjected to electrophoresis in 1% denaturing agarose gels, transferred to nylon membranes and probed with cDNA to TCV genomic RNA. Blots were then stripped and rehybridized with rDNA. Autoradiograms were scanned with a two-dimensional scanning densitometer and TCV genomic RNA levels normalized to the level of ribosomal RNA. The data for each set of five Col-0 or Di-0 plants are expressed as a percentage of the level of highest viral RNA accumulation for each day length condition. Only four Col-0 plants were assayed at 18 hr day length. This experiment was repeated with very similar results (data not shown).

5 days after inoculation varied only slightly under all growth conditions, with barely detectable viral RNA found in only a few plants (Fig. 3B). These results suggest that Di-0 plants are less resistant to virus infection if the plants are grown under light conditions that produce slow growth. However, the level of viral RNA accumulating in Di-0 plants grown under all conditions was not appreciably different 5 days after inoculation, suggesting that the increase in disease symptoms in slow-growing Di-0 plants was not due to more rapid virus accumulation early in the infection process.

In an opposite manner, Col-0 plants grown in long light duration showed stronger symptoms than those grown in conditions of shorter light duration. Nine days after inoculation with TCV-M, Col-0 plants growing in 8 hr of light showed a range of visible symptoms with several plants in each experiment not developing any symptoms by 21 days postinoculation. Analysis of viral RNA levels in infected Col-0 plants confirmed this result; several plants growing in 8 hr of light per day contained only a few percent of the viral RNA accumulating in other plants grown under the same conditions. In contrast, nearly all Col-0 plants growing in 16–24 hr of light per day developed severe symptoms by 9 days after inoculation. The few plants without visible symptoms when scored at 9 days eventually developed full necrosis resulting in the death of all plants. These results indicate that conditions that favor rapid symptom development in Col-0 plants (rapid plant growth) also cause Di-0 plants to exhibit more consistent resistance to the virus.

Movement of TCV-M in Col-0 and Di-0 plants. A technique for *in situ* detection of viral DNA in whole plants (Melcher *et al.* 1981) was recently used to monitor movement of CaMV in intact turnip plants (Leisner *et al.* 1992). We have modified this technique to detect the accumulation of RNA viruses in whole *A. thaliana* plants. TCV-M was inoculated onto Col-0 and Di-0, and at various times postinoculation, plants were treated in succession with 95% ethanol, a solution containing SDS and pronase, and then HCl to denature the RNA (see Materials and Methods). Plants were then prehybridized overnight in a standard "blot" solution and then hybridized to probe consisting of ^{32}P-labeled cDNA of full-length TCV-M genomic RNA. After hybridization, plants were washed, dried onto acetate sheets, and then exposed directly to X-ray film.

In initial experiments, all four leaves of Col-0 or Di-0 were inoculated with TCV-M and plants were hybridized with the TCV-M cDNA probe 8 days later (Fig. 4). At this stage of infection, Col-0 plants showed highest levels of TCV-M genomic RNA in the vascular tissue of inoculated leaves particularly in the petioles and midribs. In contrast, no viral genomic RNA was detectable in the petioles of Di-0 plants. It should be noted that this technique can give false signals at the margins of leaves and at the point of leaf attachment in the rosette (see plant labeled "mock"). However, hybridization to the vascular regions of infected Col-0 and lack (or very low levels) of such hybridization in Di-0 was very reproducible and, we believe, accurately reflects the location of virus in these plants.

To follow the movement of virus over time, one leaf from the oldest pair of leaves (at the four expanded-leaf stage) was inoculated with TCV-M and the virus allowed to multiply for 3, 5, 7, or 8 days. Either a Col-0 or Di-0 plant was also mock-treated as a hybridization control. Results indicate that TCV-M was discernible only in the inoculated leaf of both Col-0 and Di-0 as late as 5 days after inoculation (Fig. 5). Between 5 and 7 days postinoculation, the virus was able to spread to the opposite leaf of the same age in both the susceptible and resistant ecotypes. However, at 7–8 days postinoculation, only virus infecting Col-0 plants was found in large quantities in the vascular system of younger leaves. These findings suggest that resistance to the virus exhibited by Di-0 is not due to an inability of TCV-M to initially replicate in cells or undergo short-distance movement. This result supports the observation that TCV-M genomic RNA is detectable in low levels in total RNA populations extracted from individual or pooled Di-0 plants (see Figs. 2 and 3). However, accumulation of viral genomic RNA in vascular tissue and younger leaves is more restricted in Di-0 plants than in susceptible Col-0 plants.

Replication of TCV genomic RNA in isolated protoplasts of Col-0 and Di-0. Results from the whole plant hybridizations suggest that TCV-M is able to replicate in Di-0 cells. To confirm this result, protoplasts were isolated from fresh Di-0 and Col-0 callus cultures and infected with the same virus preparation used in the whole plant infections (Fig. 6). Although it is difficult to quantitatively compare results obtained using two different ecotypes, because differences in viral RNA uptake into protoplasts cannot be controlled, TCV genomic RNA seems to replicate at similar rates in both Col-0 and Di-0 protoplasts. These results confirm the findings obtained with the whole plant hybridization technique, that resistance to TCV-M exhibited by Di-0 is probably due to a block in systemic infection and not replication in the inoculated and opposite leaves.

DISCUSSION

Although many natural plant genes conferring resistance to specific viruses have been identified and their general modes of action determined (i.e., block in viral replication or movement), the large genome sizes and long life cycles of most plants have severely impeded attempts to isolate specific resistance genes. We have therefore initiated a series of experiments using *A. thaliana* and the simple RNA virus, turnip crinkle, as part of a long-term effort to demonstrate

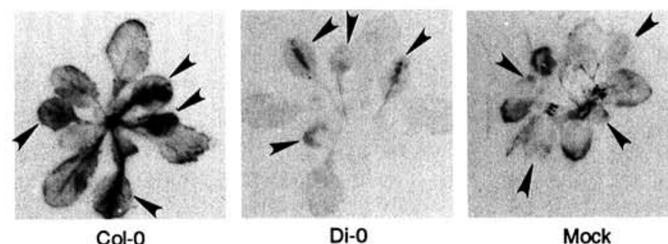

Fig. 4. Localization of turnip crinkle virus (TCV-M) genomic RNA within whole plants. Four fully expanded leaves of Col-0 or Di-0 plants were each inoculated with TCV-M or treated with buffer. Eight days later, plants were hybridized with labeled cDNA to TCV-M genomic RNA as described in Materials and Methods. Arrowheads denote inoculated leaves.

how the interaction between host and pathogen results in compatibility or incompatibility. In this paper, we characterize both resistant and susceptible ecotypes of *A. thaliana* and determine that resistance is not expressed at the cellular level but rather at the level of the whole plant.

Symptom expression and viral RNA accumulation in susceptible and resistant *A. thaliana* ecotypes. Although symptoms are not visible on infected Col-0 plants until 6 days after inoculation with TCV-M, TCV sat-RNAs are detectable in ethidium bromide-stained gels within 2 days following inoculation (not shown) and viral genomic RNA reaches near maximal levels by 5 days postinoculation. These results indicate that simple multiplication of the viral genomic or sat-RNAs is insufficient to elicit symptoms. This is not surprising because TCV-M multiplies to high levels in several cultivars of *Brassica chinensis* L. without eliciting symptoms (Li and Simon 1990).

Di-0 plants inoculated with TCV-M were either completely symptomless 3 wk after inoculation, or exhibited slight symptoms such as bolt and selique curling and stunting. At 6 days after inoculation, about 25% of Di-0 plants exhibited necrotic lesions on the inoculated leaves. This

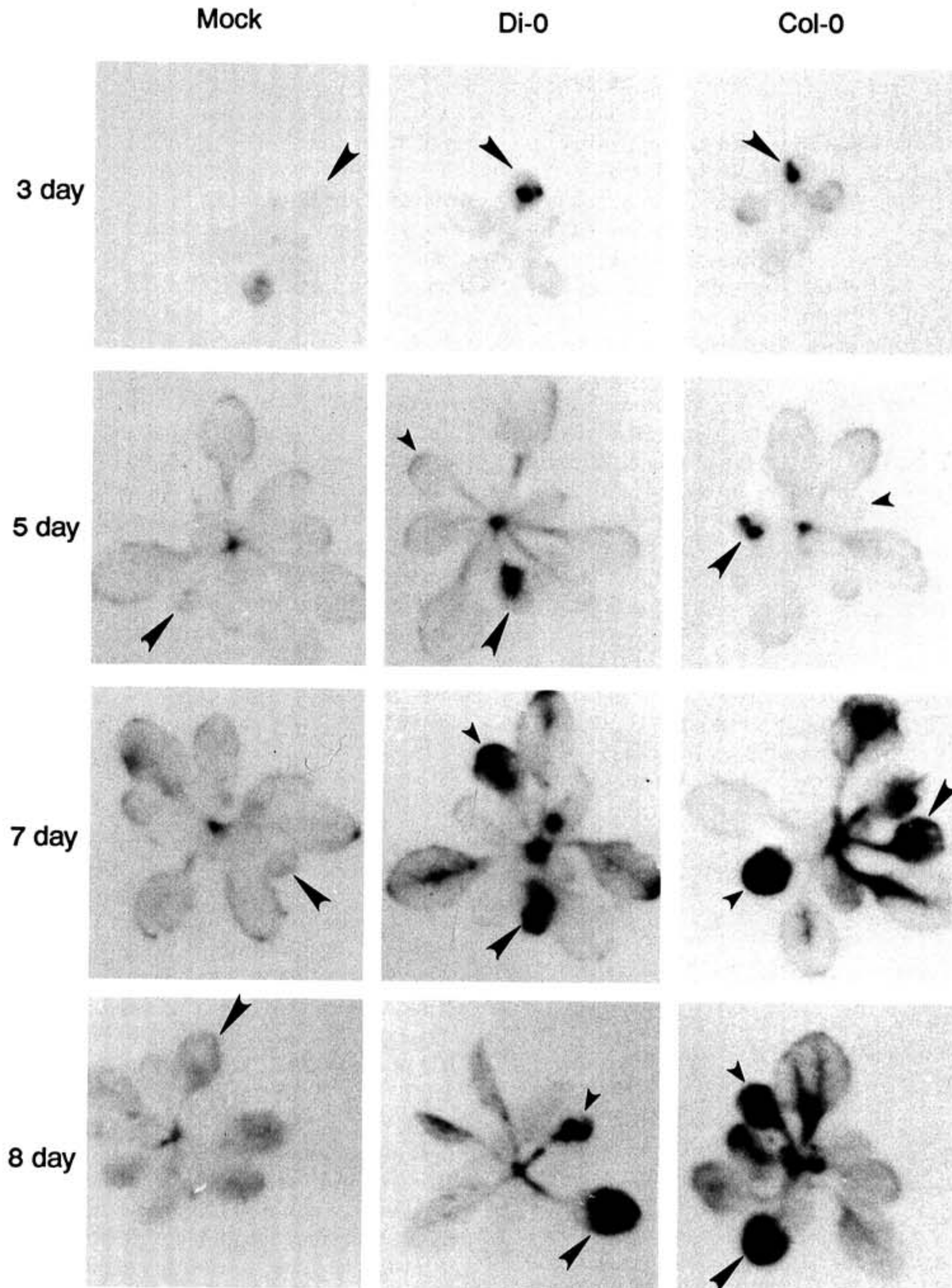

Fig. 5. Time course for localization of TCV-M genomic RNA within whole plants. Col-0 and Di-0 plants are shown 3, 5, 7 and 8 days after inoculation of a single leaf (long arrowhead). Short arrowheads denote opposite leaf of the same age as the inoculated leaf. After autoradiography, microscopic examination of the 8 day Di-0 plant indicated that the signal over the petioles corresponded with the location of un-excised root tissue.

reaction is similar to the hypersensitive response associated with some incompatible interactions between plants and viruses (Matthews 1991). There was no reliable correlation, however, between plants that had lesions, and plants that later displayed no symptoms to the virus. Hypersensitive responses of plants to pathogens have been implicated in subsequent immunity to further infection by the same or different pathogen, a process called systemic acquired resistance (reviewed by Fraser 1985). The acquisition of systemic acquired resistance is correlated with the induction of a variety of pathogenesis-related proteins in the inoculated and systemic leaves (Ward *et al.* 1991; Uknes *et al.* 1992). Recent work by others has indicated that the combination of TCV-M and Di-0 results in excellent systemic acquired resistance that is correlated with the accumulation of pathogenesis-related proteins (E. Ward and S. Uknes, personal communication).

Day length affects both resistance and susceptibility to TCV-M. When Di-0 plants were grown under a 16–24 (continuous)-hr light cycle, 90–95% of the plants did not exhibit any symptoms 9 days after inoculation, indicating that environmental conditions can influence the degree of resistance exhibited by Di-0. We also examined whether individual Di-0 plants grown under conditions of limited light duration exhibited symptoms due to the accumulation of virus at the same time and at levels similar to the susceptible ecotype. Assays of viral genomic RNA at 5 days postinoculation in individual Di-0 and Col-0 plants revealed that, in general, <1% of the level of virus accumulating in Col-0 plants was detected in Di-0 plants under all light duration conditions. This result suggests that either significant accumulation of TCV-M occurs in some slow-growing Di-0 plants between 5 and 9 days postinoculation, or that the level of virus required to elicit symptoms is quite low.

Resistance of *A. thaliana* ecotype Di-0 to TCV-M is at the level of the whole plant. Resistance of plants to viral pathogens can either be at the level of virus replication in initially inoculated cells (Watanabe *et al.* 1987), or at the level of systemic virus infection (Motoyoshi and Oshima 1975; Motoyoshi and Oshima 1977; Meshi *et al.* 1989). Results of the whole plant hybridizations and protoplast replication studies suggest that resistance to TCV-M by *Arabidopsis* ecotype Di-0 involves inhibition of systemic TCV-M infection and not a block in initial virus replication. When susceptible Col-0 plants were inoculated with virus on a single leaf, TCV-M accumulated throughout that leaf and then spread to and accumulated throughout the opposite leaf. The virus then spread to the younger leaves of the plant, with high levels of viral RNA concentrated in the vascular system of younger leaves. In Di-0 plants, TCV-M genomic RNA accumulated throughout the inoculated leaf and then spread to the opposite leaf within 7 days of inoculation. However, TCV-M RNA was not detected in high levels in the vascular tissue or in younger leaves of Di-0 plants 8 days after inoculation.

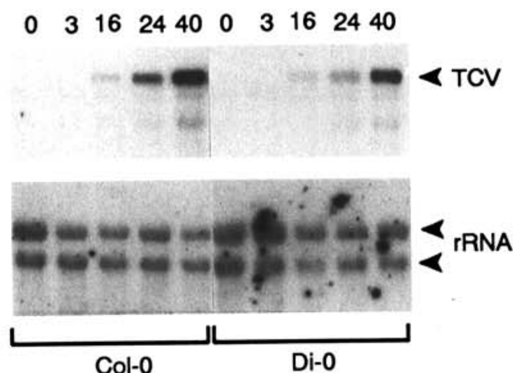

Fig. 6. Northern analysis of the accumulation of TCV-M genomic RNA in Col-0 and Di-0 protoplasts. Col-0 and Di-0 protoplasts were prepared from fresh callus cultures, divided into 5 equal portions and inoculated with TCV-M. Numbers refer to the times after inoculation when RNA was extracted. The same blot was probed with an oligonucleotide complementary to position 269-278 of the TCV-M genomic RNA (top) or cDNA to pea rDNA (bottom).

One possible explanation for these results is that virus replication and short-distance movement does occur in Di-0, whereas long-distance movement is inhibited. Two different processes are thought to result in systemic virus movement through plants: short-distance spread through plasmodesmata which link cells together by forming continuous symplastic connections (Robards and Lucas 1990; Citovsky and Zambryski 1991), and long-distance spread through the plant vascular system, a process that is not well understood (Maule 1991). At present, it is not known if long-distance movement of TCV in hosts is via phloem sieve tube cells, the case for most viruses (Matthews 1991), or through the xylem, the tissue proposed for the transport of beetle-transmitted viruses such as TCV (Gergerich and Scott 1988). Because a percentage of Di-0 plants do show mild symptoms and are therefore susceptible to TCV-M infection, virus could occasionally spread cell-to-cell in sufficient quantities to elicit symptoms before the plant matures. This would explain the observation that slow-growing Di-0 plants are more susceptible to the virus than faster-growing plants.

A second possible explanation for the resistance of Di-0 to TCV-M comes from the observation that the timing and level of TCV-M genomic RNA accumulation in the inoculated leaf and its opposite were not appreciably different for the susceptible and resistant ecotypes under conditions when a single leaf was inoculated. Rather, the ability of the virus to spread rapidly to younger leaves seemed to be more restricted in Di-0 plants than in Col-0 plants. This was in contrast to the large difference in the levels of viral RNA that accumulated in Col-0 versus Di-0 plants when all four fully expanded leaves were inoculated, as assayed by northern blots 5 days after inoculation (Fig. 3B), or by whole plant hybridizations 8 days after inoculation (Fig. 4). These results could be explained by the induction in virus-infected Di-0 plants of a systemic inhibitor that affected either virus replication or movement (reviewed in Fraser 1990). If induction of a systemic inhibitor was more rapid or expansive following widespread virus inoculation, this could account for the differences observed in the levels of virus that accumulate in plants inoculated on four leaves versus a single leaf.

Curiously, our results from single-leaf inoculations suggest that rapid virus movement occurs between one leaf and the opposite leaf of the same age, possibly preceding

movement of the virus through the vascular system to younger leaves. Southern bean mosaic virus also moves rapidly from the inoculated leaf to its opposite member in Black Valentine bean, unlike the movement of tobacco ring spot virus in the same host (Schneider 1965). This pattern of movement of TCV-M in *A. thaliana* and southern bean mosaic virus in bean differs from classic studies using tobacco mosaic virus and tomato plants (Samuel 1934). In these experiments, tobacco mosaic virus moved from the inoculated leaf down to the roots and then up to the youngest leaves before eventually accumulating in older leaves throughout the plant. Further studies will elucidate the transport route of TCV-M and determine, more clearly, the mechanism of resistance expressed by Di-0 plants.

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LITERATURE CITED

Carrington, J. C., Heaton, L. A., Zuidema, D., Hillman, B., and Morris, T. J. 1989. The genome structure of turnip crinkle virus. Virology 170:219-226.

Carrington, J. C., Morris, T. J., Stockley, P. G., and Harrison, S. C. 1987. Structure and assembly of turnip crinkle virus. IV. Analysis of the coat protein gene and implications of the subunit primary structure. J. Mol. Biol. 194:265-276.

Citovsky, V., and Zambryski, P. 1991. How do plant virus nucleic acids move through intercellular connections? BioEssays 13:373-379.

Dong, X., Mindrinos, M., Davis, K. R., and Ausubel, F. M. 1991. Induction of *Arabidopsis* defense genes by virulent and avirulent *Pseudomonas syringae* strains and by a cloned avirulence gene. Plant Cell 3:61-72.

Fraser, R. S. S. 1985. Mechanisms of Resistance to Plant Diseases. Advances in Agricultural Biotechnology. Martinus Nijhoff, Dr W. Junk, Dordrecht.

Fraser, R. S. S. 1990. Genetics of resistance to viruses. Annu. Rev. Phytopathol. 28:179-200.

Gergerich, R. C., and Scott, H. A. 1988. Evidence that virus translocation and virus infection of non-wounded cells are associated with transmissibility by leaf-feeding beetles. J. Gen. Virol. 69:2935-2938.

Guzman, P., and Ecker, J. R. 1988. Development of large DNA methods for plants: Molecular cloning of large segments of *Arabidopsis* and carrot DNA into yeast. Nucleic Acids Res. 16:11091-11105.

Hacker, D. L., Petty, I. T. D., Wei, N., and Morris, T. J. 1992. Turnip crinkle virus genes required for RNA replication and virus movement. Virology 186:1-8.

Heaton, L. A., Carrington, J. C., and Morris, T. J. 1989. Turnip crinkle virus infection from RNA synthesized *in vitro*. Virology 170:214-218.

Jorgensen, R. A., Cuellar, R. E., Thompson, W. F., and Kavanagh, T. A. 1987. Structure and variation in ribosomal RNA genes of pea. Plant Mol. Biol. 8:3-12.

Koch, E., and Slusarenko, A. 1990. *Arabidopsis* is susceptible to infection by a downy mildew fungus. Plant Cell 2:437-445.

Leisner, S. M., Turgeon, R., and Howell, S. H. 1992. Long distance movement of cauliflower mosaic virus in infected turnip plants. Mol. Plant-Microbe Interact. 5:41-47.

Li, X. H., and Simon, A. E. 1990. Symptom intensification on cruciferous hosts by the virulent sat-RNA of turnip crinkle virus. Phytopathology 80:238-242.

Li, X. H., Heaton, L. A., Morris, T. J., and Simon, A. E. 1989. Defective interfering RNAs of turnip crinkle virus intensify viral symptoms and are generated de novo. Proc. Natl. Acad. Sci. USA 86:9173-9177.

Matthews, R. E. F. 1991. Plant Virology. 3rd ed. Harcourt Brace Jovanovich, San Diego.

Maule, A. J. 1991. Virus movement in infected plants. Crit. Rev. Plant Sci. 9:457-473.

Melcher, U. 1989. Symptoms of cauliflower mosaic virus infection in *Arabidopsis thaliana* and turnip. Bot. Gaz. 150:139-147.

Melcher, U., Gardner, C. O., Jr., and Essenberg, R. C. 1981. Clones of cauliflower mosaic virus identified by molecular hybridization in turnip leaves. Plant Mol. Biol. 1:63-74.

Meshi, T., Motoyoshi, F., Maeda, T., Yoshiwoka, S., Watanabe, H., and Okada, Y. 1989. Mutations in the tobacco mosaic virus 30-kD protein gene overcome Tm-2 resistance in tomato. Plant Cell 1:515-522.

Motoyoshi, F., and Oshima, N. 1975. Infection with tobacco mosaic virus of leaf mesophyll protoplasts from susceptible and resistant lines of tomato. J. Gen. Virol. 29:81-91.

Motoyoshi, F., and Oshima, N. 1977. Expression of genetically controlled resistance to tobacco mosaic virus infection in isolated tomato leaf mesophyll protoplasts. J. Gen. Virol. 34:499-506.

Robards, A. W., and Lucas, W. J. 1990. Plasmodesmata. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41:369-419.

Samuel, G. 1934. The movement of tobacco mosaic virus within the plant. Ann. Appl. Biol. 21:90-111.

Schneider, I. R. 1965. Introduction, translocation and distribution of viruses in plants. Adv. Virus Res. 11:163-221.

Sen, P., and Murai, N. 1991. Oligolabeling DNA probes to high specific activity with Sequenase. Plant Mol. Biol. Rep. 9:127-130.

Simons, P. C., Grundler, F. M. W., von Mende, N., Burrows, P. R., and Wyss, U. 1991. *Arabidopsis thaliana* as a new model host for plant-parasitic nematodes. Plant J. 1:245-254.

Simon, A. E., Engel, H., Johnson, R., and Howell, S. H. 1988. Identification of determinants affecting virulence, RNA processing and infectivity in the virulent satellite of turnip crinkle virus. EMBO J. 7:2645-2651.

Simon, A. E., and Howell, S. H. 1986. The virulent satellite RNA of turnip crinkle virus has a major domain homologous to the 3′-end of the helper virus genome. EMBO J. 5:3423-3428.

Simpson, R. B., and Johnson, L. J. 1990. *Arabidopsis thaliana* as a host for *Xanthomonas campestris* pv. *campestris*. Mol. Plant-Microbe Interact. 3:233-237.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. 1992. Acquired resistance in *Arabidopsis*. Plant Cell 4:645-656.

Watanabe, Y., Kishibayashi, N., Motoyoshi, F., and Okada, Y. 1987. Characterization of Tm-1 gene action on replication of common isolates and a resistance-breaking isolate of TMV. Virology 161:527-532.

Wei, N., Heaton, L. A., Morris, T. J., and Harrison, S. C. 1990. Structure and assembly of turnip crinkle virus. VI. Identification of coat protein binding sites on the RNA. J. Mol. Biol. 214:85-95.

Whalen, M. C., Innes, R. W., Bent, A. F., and Staskawicz, B. J. 1991. Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. Plant Cell 3:49-59.

Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., Ahl-Goy, P., Metraux, J. P., and Ryals, J. A. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell 3:1085-1094.