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Nature of Resistance of a Tobacco Cultivar to Tobacco Vein Mottling Virus

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Comparative studies were done on the reaction of a "resistant" (Tennessee 86) cultivar and a susceptible (Kentucky 14) cultivar of tobacco to tobacco vein mottling virus (TVMV). In Tennessee 86 (Tn 86), TVMV did not spread to uninoculated leaves but could be recovered from inoculated leaves as determined by infectivity assay. In tests to determine the extent of virus spread, cylindrical inclusion (CI) protein and coat protein (CP) could be detected by immunostaining strips of epidermal cells taken from inoculated Kentucky 14 (Ky 14) leaves as early as 5 days postinoculation, and by 15 days numerous epidermal cells were stained. In the epidermis of Tn 86, CI and CP were confined to a few isolated cells or groups of cells for at least 15 days postinoculation. CI was detected in Ky 14 mesophyll cells within 5 days after mechanical inoculation but was not detected in Tn 86 mesophyll cells up to 15 days postinoculation. The spread and distribution in Tn 86 of TVMV-S, an isolate of TVMV which infects Tn 86 systemically, were similar to that of TVMV in Ky 14. Electroporated protoplasts from both Ky 14 and Tn 86 supported the accumulation of TVMV, although the final amount of virus accumulated was lower in the Tn 86 protoplasts. The results suggest that resistance of Tn 86 is primarily due to restricted virus movement, although a reduction in the number of initial infection sites and in the rate of virus accumulation may also play a role.

Additional keywords: potyvirus, viral gene products.

The term "resistant," in the broad sense, may be used to describe a plant or cultivar in which the development of pathogenic symptoms is suppressed or retarded. In the case of viruses, resistance to the establishment of infection, virus replication, or virus movement can result in a "resistant" plant. An understanding of the underlying mechanisms by which plants resist viruses has become increasingly possible with the development of protoplast systems and molecular probes. Protoplasts from tomatoes with the *Tm-2* gene for resistance to tobacco mosaic virus (TMV) support the replication of TMV, suggesting that resistance is manifested at the level of cell-to-cell spread (Motoyoshi and Oshima 1975); mutations in the 30K protein gene of TMV overcome *Tm-1* resistance (Meshi *et al.* 1988b). Tomato protoplasts with the *Tm-1* gene do not support TMV replication, indicating that this resistance mechanism operates at an earlier stage (Motoyoshi and Oshima 1977); mutations in the putative replicase genes of TMV overcome *Tm-1* resistance (Meshi *et al.* 1988a). Inhibition of proteolytic processing of viral polyproteins has been implicated in the resistance of cowpea to cowpea mosaic virus (Bruening *et al.* 1987).

Little is known about resistance mechanisms against a large and economically important plant virus group, the potyviruses, and in this study we have attempted to determine at which level the observed resistance of the tobacco cultivar Tennessee 86 (Tn 86) to one of these potyviruses, tobacco vein mottling virus (TVMV), is operative. Tn 86 does not develop visible symptoms following mechanical inoculation with TVMV, while a susceptible cultivar such as Kentucky 14 (Ky 14) develops systemic symptoms 6–7 days after inoculation. This system allowed us to compare a particular TVMV isolate on two different plant phenotypes; the availability of a naturally occurring TVMV variant, TVMV-S, to which Tn 86 is susceptible, allowed us to compare these two phenotypically different viruses on the same host.

MATERIALS AND METHODS

Viruses and viral RNA. Two isolates of TVMV were compared in this study. The first was a subculture of the original Kentucky isolate (Pirone *et al.* 1973). The other was an isolate (NC 148) provided by G. V. Gooding, Jr. (North Carolina State University at Raleigh), who had determined its ability to infect the tobacco cultivar Tn 86 systemically (G. V. Gooding, Jr., unpublished). This isolate is similar, if not identical, to a naturally occurring TVMV variant isolated earlier in Kentucky (Johnson 1980). For the purpose of this study, the NC 148 isolate was designated TVMV-S. Both viruses were propagated in Ky 14 tobacco and purified by the method of Moghal and Francki (1976). RNA was prepared by sodium dodecyl sulfate (SDS) disruption of virions followed by sucrose gradient centrifugation using the method of Hellmann *et al.* (1980).

Growth and inoculation of test plants. The Burley tobacco (*Nicotiana tabacum* L.) cultivars Ky 14 and Tn 86 were compared in this study. Ky 14 develops systemic

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symptoms when inoculated with either TVMV or TVMV-S, while Tn 86 develops symptoms when inoculated with TVMV-S but not when inoculated with TVMV. Plants were grown in the greenhouse for all experiments except those used for protoplast preparation; these were grown in growth chambers at 25 ± 4° C, 6,000 lx with light:dark cycles of 14:10 hr. Unless otherwise noted, plants were mechanically inoculated with purified TVMV or TVMV-S (10 μg/ml, 10 μl per leaf), as described by Fannin and Shaw (1987). For experiments to determine infection of the epidermis, the lower leaf surface of 10-week-old plants was inoculated; for all other experiments the upper leaf surface of 8-week-old plants was inoculated. Infectivity assays were done on 5-week-old Ky 14 plants.

Protoplast preparation and electroporation. Mesophyll protoplasts were prepared from 9–10-week-old Ky 14 and Tn 86 by the method of Otsuki *et al.* (1972). Unless otherwise noted, in each electroporation experiment 1.5×10^6 freshly prepared protoplasts were electroporated with 60 μg of TVMV RNA in 1 ml of electroporation buffer as described by Luciano *et al.* (1987). Control samples were mock-inoculated.

Mesophyll cells and epidermal strips. Mesophyll cells were prepared from leaves of Ky 14 or Tn 86 plants that had been inoculated or mock-inoculated 0–15 days previously, using the same method as described for protoplasts, except that the cellulase step was omitted (Luciano *et al.* 1989). Inoculated epidermal strips removed before mesophyll cell preparation were prepared as described by Luciano *et al.* (1989).

Antisera. Antisera against TVMV coat protein (CP), TVMV cylindrical inclusion (CI) protein, and TVMV helper component (HC) were produced at the University of Kentucky, Lexington. Antisera were purified by cross-absorption against proteins extracted from uninfected tobacco leaves followed by affinity chromatography with Protein A-agarose (Goding 1976) as described by Luciano *et al.* (1989).

ELISA. The method of Clark and Adams (1977) was used to detect viral CP in leaves, mesophyll cells, and protoplasts. To study virus accumulation in whole plants, leaves were sampled in the following manner. For each cultivar there were three plants per sample, three samples per replicate, and three replicates per sampling time. For each sample, six disks, 8 mm in diameter, were collected from the leaves of three plants that had been inoculated with virus 1 to 20 days previously. Uninoculated leaves from the same plants were sampled in the same way as inoculated leaves, and samples were stored at −80° C until assayed. For each cell or protoplast sample to be tested by ELISA, 2×10^5 cells or 1×10^5 protoplasts were centrifuged in a microcentrifuge at 13,800 × *g* for 3 min, the supernatant removed, and the pellets frozen at −80° C.

Each sample was ground in 500 μl of phosphate-buffered saline, pH 7.4, containing 0.5% Tween 20, 2% polyvinylpyrrolidone, and 0.2% bovine serum albumin (PBS-Tween-PVP-BSA). The resulting extract was placed in duplicate wells of an ELISA plate previously coated with IgG (4 μg/ml). Enzyme-conjugated IgG was diluted to 1:1000 with PBS-Tween-PVP-BSA that contained filtered healthy plant sap (1:20 buffer volume) to decrease the background reaction. After thorough rinsing with PBS-Tween-PVP-BSA, and 40 min after adding the substrate solution, the absorbance at A_{405} nm was measured using a Titertek Multiskan (Flow Laboratories, McLean, VA). Control tissue from uninoculated plants was processed similarly and purified virus standards were included in each plate. Absorbance values greater than twice the average for control samples from healthy leaf tissue were considered virus positive.

Immunological detection of viral proteins. Protoplasts and mesophyll cells (1×10^4 cells per sample) and epidermal strips (4–6 strips per leaf, size range 50–120 mm^2) were assayed for TVMV CI or CP using the method of Luciano *et al.* (1989).

Immunoprecipitation analysis of [^{35}S]methionine-labeled viral proteins. Electroporation of tobacco protoplasts with viral RNA was performed as described by Luciano *et al.* (1987). In a typical experiment 1×10^6 protoplasts were cultured in 5 ml of incubation medium for 1 hr. [^{35}S]methionine (New England Nuclear, Boston, MA; normally 2 mCi) was then added, and the protoplasts or cells were allowed to incubate for varying lengths of time under conditions described by Xu *et al.* (1984). Protoplasts or cells were collected by centrifugation and lysed in a buffer containing 1% Triton X-100, 1% sodium deoxycholate, 2% SDS, 2% 2-mercaptoethanol, and 20 mM methionine. Samples were boiled for 6 min and any insoluble material was removed by centrifugation. Aliquots representing approximately 15,000 protoplasts were included in immunoprecipitation reactions performed as previously described (Hellmann *et al.* 1983). Labeled products were separated by SDS-PAGE and visualized by fluorography (Hellmann *et al.* 1980).

Immunosorbent electron microscopy. The method described by Derrick (1973), modified as described below, was used for quantitative electron microscopy. Protoplasts electroporated with viral RNA were frozen at designated times after inoculation. Samples were thawed, ground in a tissue homogenizer, and centrifuged briefly to remove debris. Trapping grids were prepared by incubating collodion-carbon-coated grids with anti-TVMV IgG followed by rinsing off the unbound IgG. Grids were floated on a drop of protoplast extract for 20 min, and then each grid was rinsed thoroughly with deionized water and stained with 1% phosphotungstic acid. Three grids were prepared for each sample and for each sampling time. Particle counts were made on 10 randomly selected 106 u^2 areas on each of 10 randomly selected grid squares of each grid.

RESULTS

Infectivity assay of Tn 86 inoculated with TVMV. To determine if Tn 86 is a symptomless host of TVMV, samples from mechanically inoculated leaves were extracted and used to inoculate Ky 14, a systemic host of TVMV. Samples from Tn 86 leaves inoculated with 10 μl of 10 μg/ml of TVMV failed to infect Ky 14 at time periods up to 3 wk after inoculation. Samples taken from Tn 86 one day after inoculation with 10 μl of 100 μg/ml of TVMV failed to infect Ky 14, whereas samples taken 7 and 14 days after

inoculation infected 10–20% of the Ky 14 test plants (data from two experiments). Samples taken from leaves of Tn 86 above the inoculated leaf at 7, 14, and 21 days after inoculation did not infect Ky 14 test plants regardless of the concentration of the initial inoculum. These results indicated that TVMV can infect Tn 86, but that systemic spread does not occur.

Extent of spread and virus content. Overall levels of virus accumulation were determined by ELISA using antiserum to TVMV. The TVMV content in both inoculated and systemically infected leaves of Ky 14 increased between 3 and 7 days after inoculation and then decreased between days 14 and 21 (Fig. 1). TVMV was not detected in either inoculated or uninoculated leaves of Tn 86 after 3, 7, 14, and 21 days (Fig. 1). Some antigen was detected in inoculated leaves at 1 hr and 1 day after inoculation, probably due to residual inoculum because the antigen was not detected 3 days after inoculation. Thus, TVMV can reach high titers in inoculated and systemically infected leaves of Ky 14 but does not accumulate to detectable levels in either inoculated or uninoculated leaves of Tn 86.

The inability of TVMV to produce detectable amounts of viral gene products in Tn 86 was confirmed by immunoprecipitation analysis of inoculated leaf tissue. Cells prepared from inoculated leaves of Ky 14 and Tn 86 tissues were incubated in the presence of [^{35}S]methionine for 24 hr, lysed, and then immunoprecipitated using antisera to TVMV CP, HC, and CI proteins. Mature-sized proteins were detected in Ky 14 samples, whereas no virus-specific proteins were detected in Tn 86 samples (data not shown).

Virus detection in epidermal strips and mesophyll cells. The extent of the spread of infection in epidermal and mesophyll cells of inoculated leaves was assayed by methods that can detect replication in individual cells. Epidermal strips and mesophyll cells were prepared from Ky 14 and Tn 86 leaves inoculated with TVMV at 0, 5, 10, and 15 days after inoculation. Ten epidermal strips were collected from two Ky 14- and two Tn 86-inoculated leaves at each sampling time and were immunostained with anti-CI or anti-CP. In a separate experiment, Tn 86 plants were inoculated with TVMV or TVMV-S; epidermal strips and mesophyll cells were then prepared from inoculated leaves at 0, 5, 10, and 15 days after inoculation, and were examined similarly.

In epidermal cells, with combinations in which systemic spread can occur (TVMV in Ky 14; TVMV-S in Tn 86), CP was detected in all 10 strips in groups of 10–100 cells (foci) 5 days after inoculation. After 10 days, the number of cells containing detectable CP had increased so that approximately 25% of the cells were stained over the entire strip. Antiserum against CI was less effective at detecting newly infected cells; only one group of cells was detected 5 days after inoculation. However, 15 days after inoculation, the number of infected cells detectable by CI staining was comparable to that detected by CP staining. In the resistant Tn 86, considerably fewer cells contained detectable antigen 5 days after inoculation. Of the 10

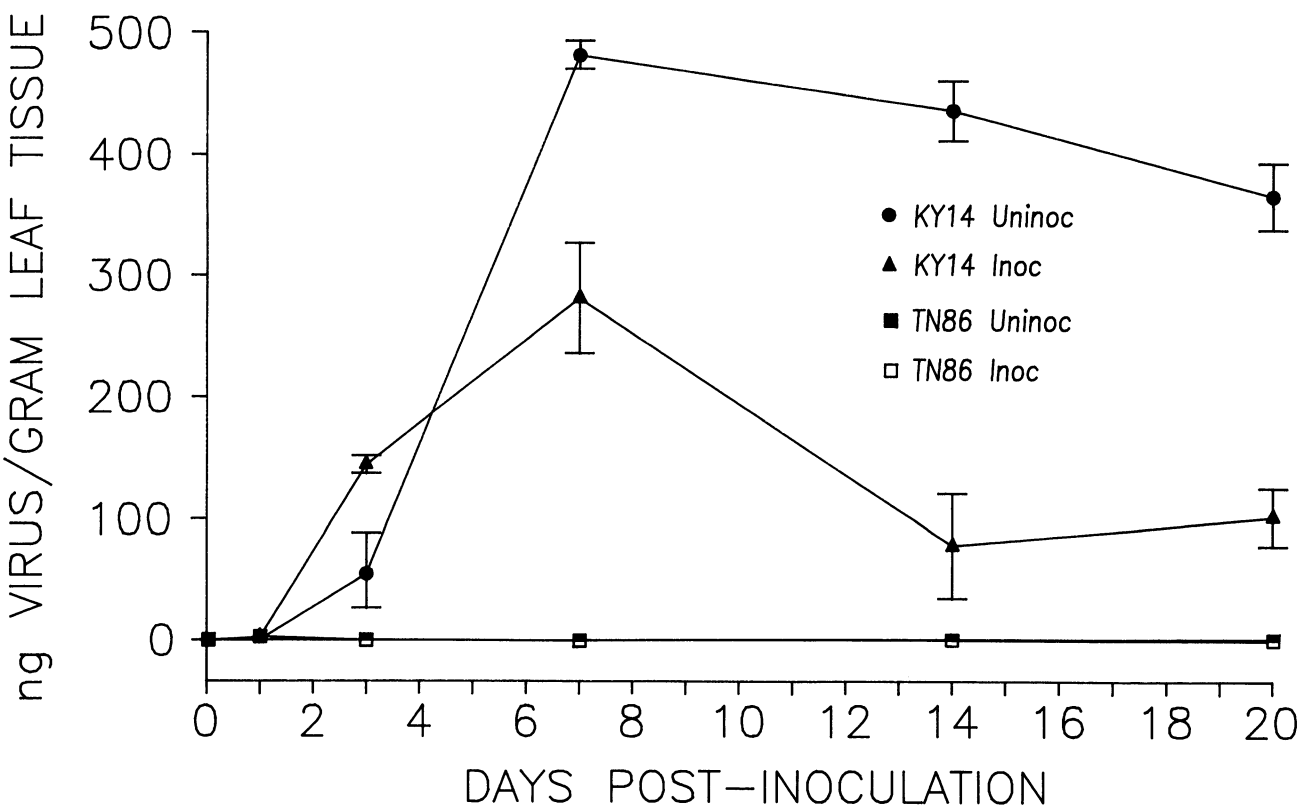

Fig. 1. Amount of viral antigen in inoculated and uninoculated leaves of Kentucky 14 (Ky 14) and Tennessee 86 (Tn 86) tobacco inoculated with tobacco vein mottling virus (TVMV) as determined by ELISA with antiserum to TVMV virions.

epidermal strips examined, CP was detected in two individual cells in each of two strips, and CI was detected in one cell of one strip. After 15 days, CP was detected in a single group of 20–100 cells in each of two strips, and CI was detected in a total of five foci, each with one to five cells, in four strips.

In mesophyll cells, with the combinations in which systemic spread occurred (TVMV in Ky 14 and TVMV-S in Tn 86), CI was detected in the mesophyll cells from inoculated leaves 5 days after inoculation. The number of TVMV-inoculated Ky 14 cells in which CI was detectable was somewhat greater than that of TVMV-S in Tn 86 (Fig. 2). No infected mesophyll cells from TVMV-inoculated Tn 86 were detected even up to 15 days after inoculation (Fig. 2). Similar results were obtained when Ky 14 and Tn 86 plants were inoculated with TVMV RNA instead of purified virus.

The amount of virus per infected cell in TVMV-inoculated Ky 14 and TVMV-S-inoculated Tn 86 reached an apparent peak 5 days after inoculation and decreased between days 5 and 15 (Fig. 2). This apparent decrease might have resulted from an underestimation of the number of cells infected at 5 days due to a lack of accumulation of CI to detectable levels in all infected cells; alternatively, the decrease may reflect the general decline in concentration of TVMV seen in inoculated Ky 14 (Fig. 1).

Virus accumulation in protoplasts. The inability to detect TVMV in Tn 86 mesophyll cells suggests that resistance may be due to restricted cell-to-cell movement or to an inability of Tn 86 mesophyll cells to support TVMV replication. The ability of TVMV-inoculated Tn 86 protoplasts to support virus replication, as measured by ELISA using antisera to CP and CI, was compared with TVMV-inoculated Ky 14 protoplasts and TVMV-S-inoculated Tn 86 protoplasts.

Ky 14 protoplasts were electroporated with TVMV RNA, and Tn 86 protoplasts were electroporated with either TVMV RNA or TVMV-S RNA. Protoplasts were sampled at 0, 24, 48, and 72 hr following electroporation. At each sample time, 10^5 protoplasts were tested by ELISA for CP accumulation and 10^5 were tested for CI accumulation by immunostaining with anti-CI. CP and CI were detected in both TVMV-inoculated Ky 14 protoplasts and TVMV-S-inoculated Tn 86 protoplasts 24 hr after electroporation, whereas CP and CI were not detected in TVMV-inoculated Tn 86 protoplasts before 48 hr (Figs. 3 and 4). Approximately twice as many TVMV-inoculated Ky 14 protoplasts and TVMV-S-inoculated Tn 86 protoplasts were infected compared with TVMV-inoculated Tn 86 protoplasts (Fig. 3).

To express CP levels on a "per-infected-cell" basis, two identical samples were prepared. CP accumulation was determined by ELISA in one sample, and the number of infected cells was determined in the other sample by staining with CI antiserum (with protoplasts, similar results were obtained with either CP or CI antiserum). In TVMV-inoculated Ky 14 and TVMV-S-inoculated Tn 86 protoplasts, CP was first detected 24 hr after electroporation, and the amount of coat protein per protoplast increased at each sample time (Fig. 4). In TVMV-

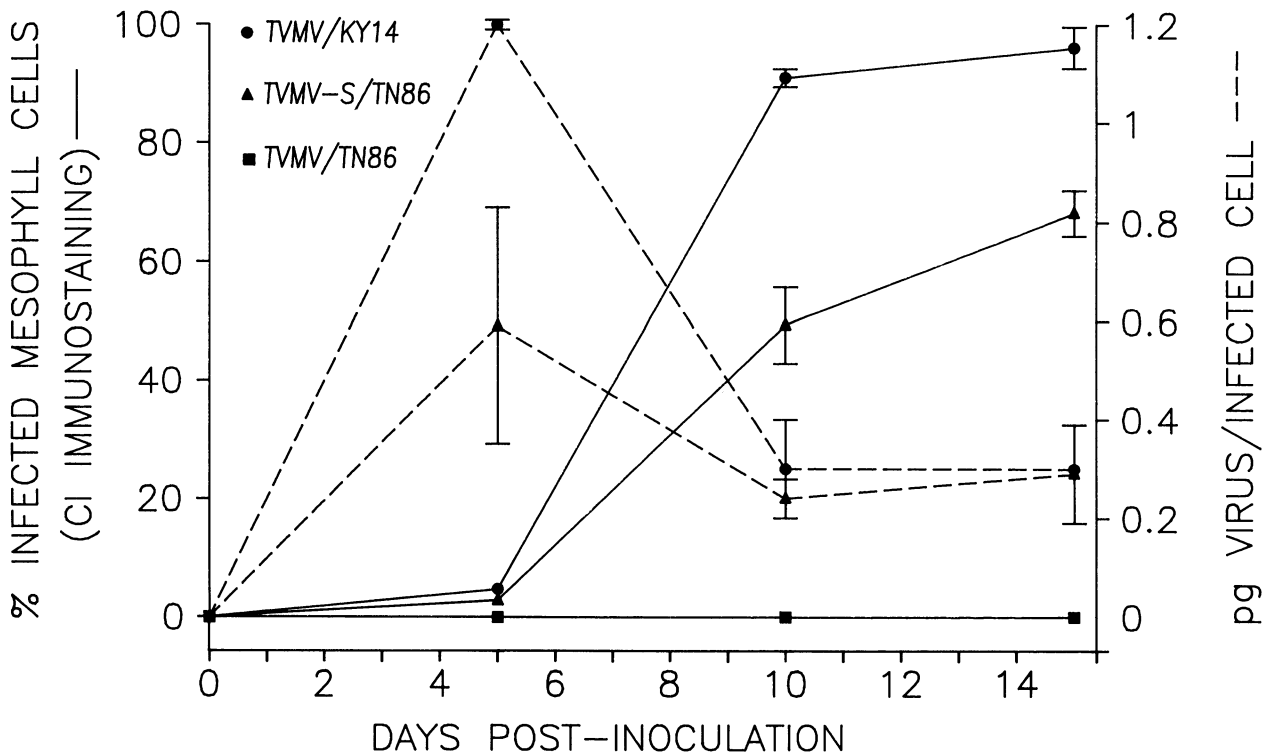

Fig. 2. Percentage of infected mesophyll cells from leaves of Kentucky 14 (Ky 14) or Tennessee 86 (Tn 86) inoculated with tobacco vein mottling virus (TVMV) or TVMV-S as determined by immunostaining with antiserum to cytoplasmic inclusion (CI) protein, and amount of virus per infected cell as determined by ELISA using antiserum to TVMV.

inoculated Tn 86 protoplasts, CP was not detected until 48 hr after electroporation, and there was no increase in CP levels per cell between 48 and 72 hr. The amount of CP per protoplast in TVMV-inoculated Tn 86 protoplasts was approximately half that of TVMV-inoculated Ky 14 protoplasts and TVMV-S-inoculated Tn 86 protoplasts (Fig. 4).

Viral protein synthesis was also monitored in Tn 86 protoplasts by incorporation of [^{35}S]methionine (Fig. 5). Twenty-four hours after electroporation, [^{35}S]methionine was added to the protoplast samples, and aliquots were removed after 15 min, 30 min, 1 hr, and 20 hr of additional incubation. Samples were lysed and then immunoprecipitated with antisera to HC or CI. After 15 min of labeling, similar patterns of labeled HC and CI proteins were obtained from cells electroporated with either RNA. Each antiserum precipitated essentially a single labeled species the size of the corresponding mature protein isolated from tissues infected with TVMV. The amount of radioactivity present in HC protein from the two samples increased at similar rates during the 20-hr labeling period (Fig. 5). Although no attempt was made to quantitate the relative amounts of labeled protein in each sample, it appears that the amount of HC at a given time point did not differ by more than a factor of two for the two TVMV isolates.

Attempts to quantitate virus replication directly by measuring the increase in viral RNA, using the method of Luciano *et al.* (1987), or modifications thereof, were unsuccessful. Direct evidence for virus replication was thus obtained by immunosorbent electron microscopy. Protoplasts of Ky 14 and Tn 86 were electroporated with TVMV RNA, and samples were taken 0, 24, 48, and 72 hr after inoculation. The time course of virus accumulation (Fig. 6) was similar to that determined for CP by ELISA (Fig. 4). Virions were detected after 24 hr in Ky 14 and after 48 hr in Tn 86. The number of virions per field after 72 hr was about three times greater in Ky 14 than in Tn 86.

DISCUSSION

Several lines of evidence suggest that TVMV is restricted to individual epidermal cells or to small foci of epidermal cells in Tn 86. The infectivity of extracts from inoculated Tn 86 leaves, following a suitable incubation period, shows that at least limited infection of Tn 86 does occur. The inability to detect TVMV in inoculated Tn 86 leaves by

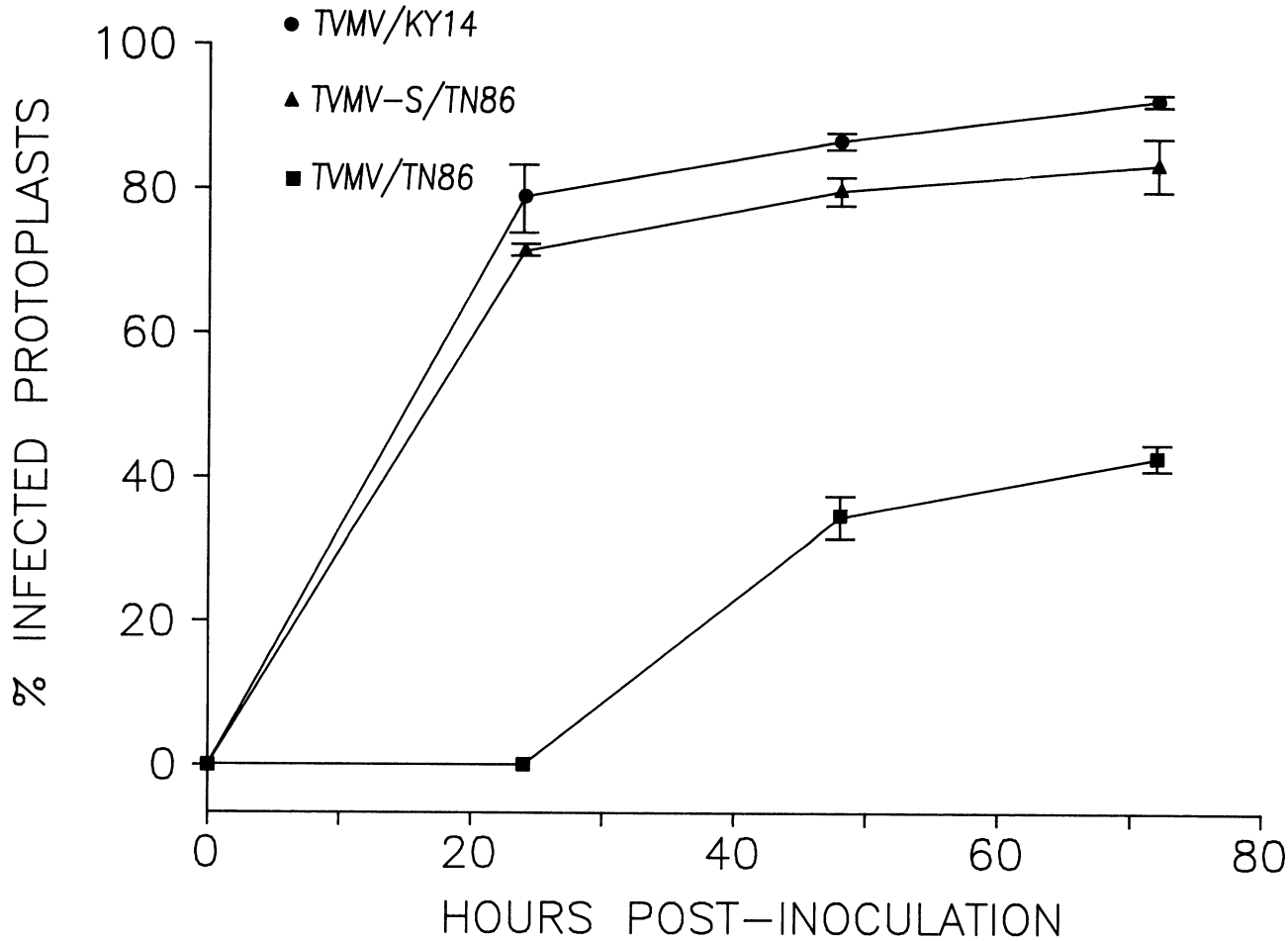

Fig. 3. Time course of detection of infected protoplasts following inoculation of Kentucky 14 (Ky 14) or Tennessee 86 (Tn 86) with RNA of tobacco vein mottling virus (TVMV) or TVMV-S. Antiserum to cytoplasmic inclusion (CI) protein was used to detect infected cells.

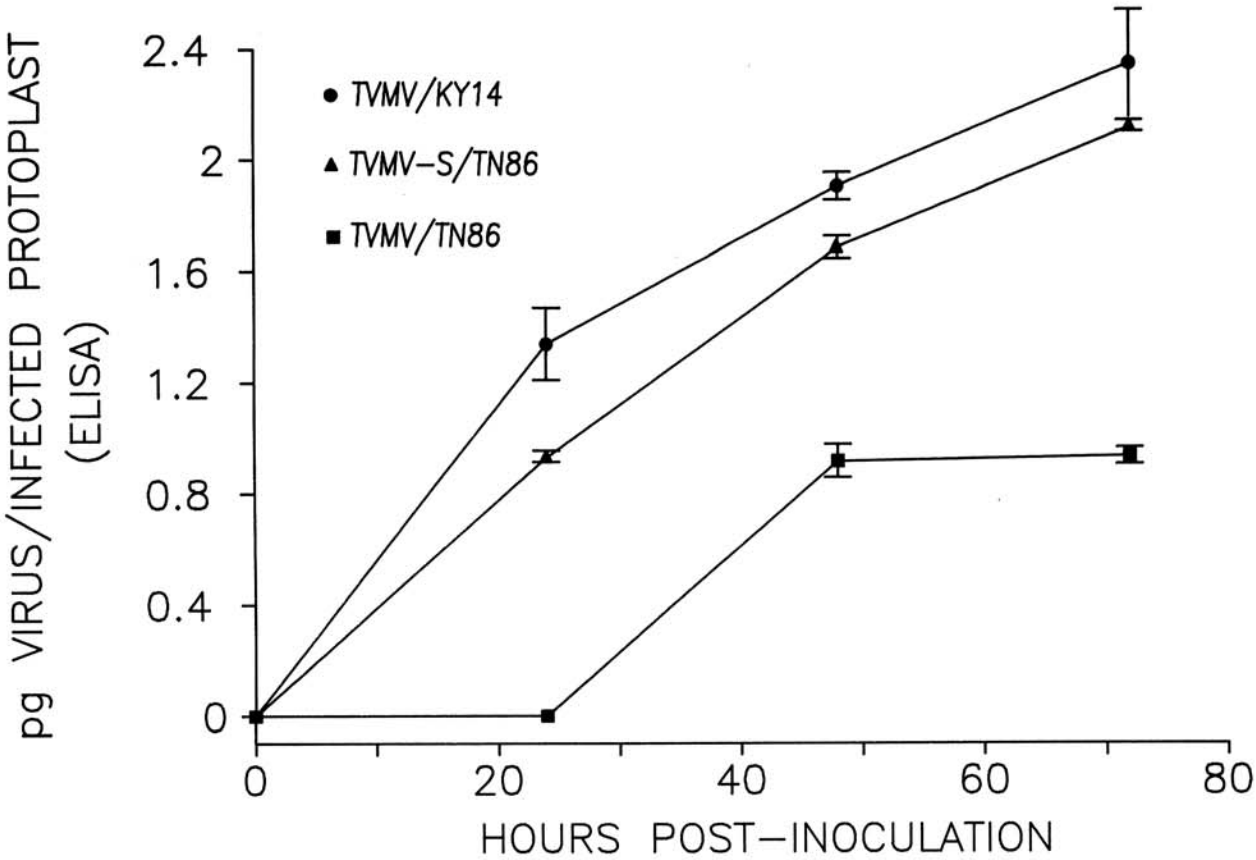

Fig. 4. Amount of virus per infected protoplast of Kentucky 14 (Ky 14) or Tennessee 86 (Tn 86) inoculated with RNA of tobacco vein mottling virus (TVMV) or TVMV-S. Quantitation was by ELISA using antiserum to TVMV coat protein.

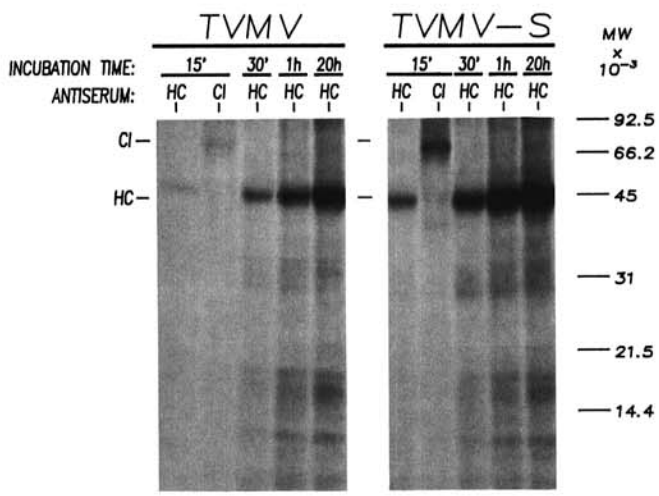

Fig. 5. Time course of detection of the viral gene products helper component (HC) and cytoplasmic inclusion (CI) by immunoprecipitation from extracts of [^{35}S]methionine-labeled Tennessee 86 (Tn 86) protoplasts inoculated with RNA of tobacco vein mottling virus (TVMV) or TVMV-S.

either ELISA (Figs. 1 and 2) or immunoprecipitation analysis is also compatible with the production of viral gene products in a very limited number of cells. The immunostaining of epidermal strips shows that the distribution of cells infected with TVMV is much more limited in Tn 86 than in Ky 14. However, groups of adjacent infected cells were occasionally found in epidermal strips of Tn 86. It is possible that these may represent adjacent cells which became infected as a result of inoculation; however, it seems more likely, on a probability basis, that these are the result of limited spread from initially infected cells.

TVMV was not detected in mesophyll cells of inoculated Tn 86 leaves while numerous mesophyll cells of the susceptible Ky 14 were infected. Because Tn86 mesophyll protoplasts inoculated with TVMV are capable of supporting virus replication, as demonstrated by the accumulation of both viral gene products (Figs. 3 and 4) and virions (Fig. 6), it is evident that mesophyll cells can be infected with TVMV. The appearance of virions and viral gene products in Tn 86 protoplasts is delayed, however, and the final concentrations are less than in Ky 14 (Figs. 4 and 6). Thus, it appears that some form of "resistance" is operative at the cellular level. The finding that a similar time course of appearance of the TVMV gene products CI and HC occurs in Ky 14 and Tn 86 (Fig. 5) suggests that processing of these proteins is unimpaired. Although no antisera are available to the adjacent 34K and 42K cistrons, the findings with CI and HC suggest that these proteins are also efficiently processed. Thus, a situation analogous to the resistance of the Arlington strain of

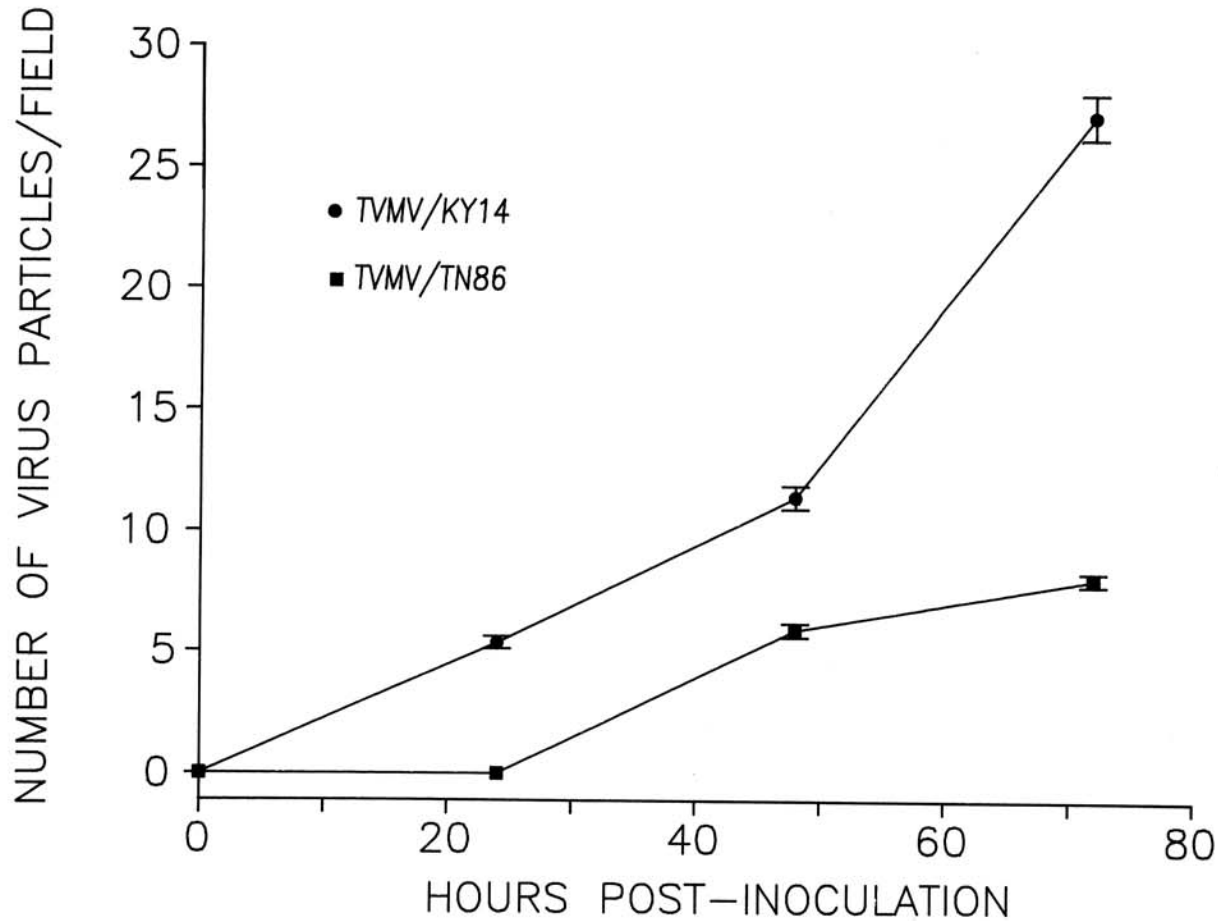

Fig. 6. Time course of virus accumulation in Kentucky 14 (Ky 14) or Tennessee 86 (Tn 86) protoplasts inoculated with tobacco vein mottling virus (TVMV) RNA as determined by quantitative immunospecific electron microscopy.

cowpeas to cowpea mosaic virus, in which the action of a protease inhibitor has been implicated (Ponz *et al.* 1988), appears not to be operating in the resistance of Tn 86 to TVMV.

The fact that Tn 86 protoplasts can support the replication of TVMV suggests that the nature of resistance may be analogous to TMV resistance in tomatoes possessing the *Tm-2* gene. Here, cell-to-cell spread is blocked (Nishiguchi and Motoyoshi 1987). In the case of *Tm-2* resistance, however, the protoplasts support TMV replication at a level comparable to that in protoplasts of a susceptible cultivar (Motoyoshi and Oshima 1975, 1977), while in Tn 86 TVMV synthesis is delayed and reduced relative to the susceptible Ky 14. The delay and reduction (Figs. 3 and 6) may play a role in resistance, but it is unlikely that it is sufficient per se to account for the high level of resistance of Tn 86. Although it can be argued that the amount of replication in protoplasts does not reflect the situation in intact mesophyll cells, the combined data from the leaf immunostaining and the protoplast inoculation studies indicate that the lack of cell-to-cell spread of TVMV is a major factor in the resistance of Tn 86.

It is evident that the spread of virus from cell-to-cell must involve an interaction between the virus (or its gene products) and the host, and there are two hypotheses to explain the difference in the response of Tn 86 to TVMV and TVMV-S. The first is that TVMV-S possesses a gene which is able to express a "movement protein" such as the TMV 30K protein (Leonard and Zaitlin 1982; Ohno *et al.* 1983; Deom *et al.* 1987) and that this protein is somehow different from the similarly hypothetical "movement protein" of TVMV, thus allowing only TVMV-S to move from cell-to-cell in Tn 86. The second hypothesis postulates a host response (or lack thereof) to infection. In this model, infection of Tn 86 by TVMV would result in the triggering of "induced immunity" that would stop the movement of the virus from the initially infected cells. TVMV-S, on the other hand, would not induce this response and, hence, would spread unimpeded. The observation that there appears to be an occasional, limited spread of TVMV in the epidermis of Tn 86 could be explained, with the host response model, on the basis that a slight delay in response might allow very limited spread. Whatever the mechanism(s) involved, the fact that infectious transcripts of TVMV have been produced (Domier *et al.* 1989) should make possible studies by which the "resistance breaking" gene(s) of TVMV-S can be identified.

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