Differential Invasion by Tobamoviruses of *Nicotiana megalosiphon* Following the Hypersensitive Response

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ABSTRACT

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The tobamoviruses tobacco mild green mosaic virus (TMGMV) and tobacco mosaic virus (TMV) differ in their behavior in *Nicotiana megalosiphon.* Both viruses induce necrotic lesion formation in the inoculated leaves, but only TMV moves from the initially infected (necrotic) areas and is able to spread systemically, overcoming the hypersensitive response. The appearance and growth of necrotic local lesions induced by TMV or TMGMV in *N. megalosiphon* were shown to follow similar kinetics; however, virus accumulation in the local lesions was about five times greater for TMV than for TMGMV. In accordance with this, TMV replicated to much higher levels than TMGMV did in *N. megalosiphon* protoplasts. In *N. tabacum* 'Xanthi-nc,' a hypersensitive host for both viruses that was used as a control, no difference was observed in the kinetics of local lesion development nor in virus accumulation, either in local lesions or in protoplasts, for TMV or TMGMV. Thus, the ability of TMV, but not of TMGMV, to overcome the hypersensitive response of *N. megalosiphon* does not appear to be related to differences in the elicitation of the hypersensitive response but rather to the differential effect of a second resistance factor over the multiplication of TMGMV but not of TMV.

The hypersensitive response (HR) of plants to virus infections is an active defense reaction that leads to the necrosis of the initially infected tissues and usually prevents further virus spread within the infected plant (5). The HR develops only for certain plant-virus combinations, and it is determined by both the plant and the virus genomes according to a gene-for-gene interaction (5,8). In plants of the genus *Nicotiana,* at least two genes for hypersensitivity to tobacco mosaic virus (TMV) and other tobamoviruses have been identified. The N gene, originally from *N. glutinosa* L. and incorporated now into several cultivars of *N. tabacum* L., such as Samsun NN or Xanthi-nc, is a single dominant gene that determines a HR to almost all tobamoviruses able to infect *Nicotiana* species (10). Although the viral component that triggers the expression of the N gene has not yet been identified, it has been shown that the N-mediated HR can be broken with a mutation in the TMV 126-kDa replicase protein (15). The second gene, N′, an intermediately dominant single gene, was found in *N. sylvestris* Speg & Comes and some cultivars of tobacco (26). The N′-mediated HR is induced by some but not all tobamoviruses and TMV strains (26). The viral coat protein has been shown to be responsible for inducing N′-mediated HR (13,17,20). Other *Nicotiana* species are also known to respond with a HR to infection with tobamoviruses (11), although the plant genes determining this reaction have not been identified.

Although various metabolic processes have been associated with the HR (9,12), the mechanisms that restrict virus spread from the necrotic lesions are still obscure in spite of considerable research efforts. To elucidate such mechanisms, systems in which the virus overcomes the HR-mediated resistance are of great interest. Here we report on one such system. While both tobacco mild green mosaic tobamovirus (TMGMV) and TMV induce a HR in inoculated leaves of *N. megalosiphon* Heurck & Muell., only the latter is not confined to the initially infected (necrotic) areas and is able to systemically colonize *N. megalosiphon.* We also show that this difference between the viruses may be caused by a more efficient replication of TMV in *N. megalosiphon* cells.

MATERIALS AND METHODS

Viruses and plants. U1-TMV and U5-TMGMV (previously called U5-TMV) are laboratory cultures that have been described (22). Two other TMV and TMGMV isolates (H23 and H6, respectively) from field-infected *N. glauca* Graham were the gift of A. Gibbs (Australian National University, Canberra, Australia). *N. tabacum* L. 'Xanthi-nc' and a laboratory accession line of *N. megalosiphon* were used as host plants.

Plant experiments. Plants with three to four expanded leaves were mechanically inoculated with 10 μl of a suspension (5 μg/ml) of TMV or TMGMV virions in 10 mM sodium-phosphate buffer, pH 7.2; in mixed infections, 10 μl of a suspension (5 μg/ml) of each virus was used. Inoculated plants were kept in a greenhouse at 22–25 C with a 16-h photoperiod. At different times postinoculation (p.i.), symptoms were recorded, and virus multiplication was analyzed on three types of samples: 1) local lesions from inoculated leaves, 2) an interlesion area from inoculated leaves, and 3) upper noninoculated leaves. Twenty local lesions (sample type 1) or the equivalent fresh weight of leaf tissues for sample types 2 and 3 were analyzed for each infected plant. Results were obtained from three independent experiments; three replicate plants per treatment and per experiment were analyzed.

Protoplast experiments. Protoplasts were isolated from fully expanded mature leaves of *N. megalosiphon* and *N. tabacum* 'Xanthi-nc' as described for *N. tabacum* (18). For inoculation, to the pellet of 1×10^6 protoplasts, 5 μg of virions in 25 μl of ice-cold water was added. After incubation for 2 min on ice, 1 ml of 40% (w/v) polyethylene glycol (MW 6,000) in 9% mannitol was added (19). The suspension was gently mixed. After incuba-

tion for 30 min at room temperature, the protoplasts were washed and incubated in Murashige and Skoog media containing 9% mannitol at 23–24 C under continuous illumination. Virus multiplication was analyzed in samples consisting of 1×10^6 protoplasts between 4 and 40 h p.i. Results were obtained from three independent experiments with two or three replicates in each.

Analysis of virus multiplication. Virus multiplication was analyzed by infectivity assay, enzyme-linked immunosorbent assay (ELISA), and dot blot hybridization. Virus infectivity in samples from infected plants was assessed by the mean number of necrotic local lesions induced in randomized half leaves of Xanthi-nc tobacco plants. Accumulation of virus antigen in infected plants and protoplasts was quantitated by double antibody sandwich ELISA (3). For the analysis, 10^6 protoplasts per sample, extracted by freeze-thawing in phosphate-buffered saline (pH 7.2) and 0.05% Tween 20, or plant tissues homogenized in the same buffer were used. Polyclonal antisera to TMV and TMGMV were gifts from M. T. Serra (Consejo Superior de Investigaciones Científicas, Madrid, Spain) and J. A. Dodds (University of California, Riverside), respectively. Accumulation of viral RNA in infected plants and protoplasts was quantitated by dot blot hybridization of nucleic acid extracts from infected tissues (16) or from protoplasts (7). ^{32}P-labeled cRNA probes were transcribed from pBS clones complementary to nucleotides 4903 to 5665 of TMV (the gift of D. Baulcombe, Norwich, England) or to nucleotides 4885 to 5534 of TMGMV. The percentage of infected cells in inoculated leaves was determined by immunofluorescence staining of protoplasts with anti-TMV serum as described by Van Lent et al (25).

RESULTS

Accumulation of TMV and TMGMV in *N. megalosiphon*. In leaves of *N. megalosiphon* inoculated with either TMV or TMGMV, local necrotic lesions were apparent 2 days p.i. and expanded gradually to form larger lesions after 6–7 days. In the plants inoculated with TMV, systemic symptoms were observed about 7 days p.i. and were characterized by stunting, mosaic, and sometimes necrosis in systemically infected leaves (Fig. 1). Development of systemic necrosis was dependent on environmental conditions and concentration of the virus. No systemic symptoms developed in TMGMV-infected *N. megalosiphon* by 30 days p.i.

Virus accumulation in necrotic local lesions and interlesion areas of inoculated leaves was analyzed 6 days p.i. and in noninoculated leaves 10 days p.i. by an infectivity assay and by quantifying viral antigen and viral RNA. The results (Table 1) showed that TMGMV was localized in the necrotic local lesions and that the location was independent of the concentrations (up to 50 μg/ml) of TMGMV in the inoculum (not shown). Conversely, TMV spread efficiently out of the necrotic lesions in the inoculated leaves and was able to systemically infect *N. megalosiphon*. Isolation and immunofluorescence screening of protoplasts from first and second systemically infected leaves 12 days p.i. showed that 66% of parenchyma cells were infected by TMV. When *N. megalosiphon* plants were coinoculated with TMV and TMGMV, the situation was not changed: both viruses induced necrotic local lesions, spread of TMV was not suppressed by TMGMV, and TMV did not complement systemic infection of TMGMV. The described virus-host interactions were the same for isolates U1 and H23 of TMV and U5 and H6 of TMGMV (not shown). As expected, in Xanthi-nc tobacco plants both TMV and TMGMV induced HRs in which the development of necrotic local lesions limited virus spread (data not shown).

The growth of necrotic local lesions induced by TMV or TMGMV in leaves of *N. megalosiphon* followed similar kinetics, which also was the case for lesions induced in Xanthi-nc tobacco leaves (Fig. 2). Nevertheless, virus accumulation in local lesions of *N. megalosiphon* assessed as viral antigen by ELISA was about five times higher for TMV than for TMGMV. Differences in virus accumulation were not detected in local lesions induced in Xanthi-nc tobacco plants (Fig. 2).

Replication of TMV and TMGMV in protoplasts. Protoplasts prepared from *N. megalosiphon* plants were inoculated with TMV and TMGMV, and viral replication was assessed by the amounts of viral antigen and viral RNA present at 4, 16, 24, and 40 h p.i. The data (Fig. 3) show that TMV replicates to much higher levels (more than five times) than TMGMV does in *N. megalosiphon* protoplasts and that this is caused by an initially faster replication rate of TMV and to the fact that RNA and coat protein accumulations plateau at lower levels in TMGMV. In protoplasts of Xanthi-nc, the kinetics of RNA and coat protein accumulation were the same for both viruses.

DISCUSSION

TMV and TMGMV, often reported as different strains of TMV (22), are two different tobamoviruses (24) that share a number of natural hosts in the family Solanaceae. Both infect *N. glauca* and *N. tabacum,* although they accumulate to different levels in these two hosts. TMV multiplies more efficiently than TMGMV in *N. tabacum,* while TMGMV accumulates to higher levels in *N. glauca* (1,23). TMV and TMGMV show about 60% homology in the nucleotide sequence of their genomic RNAs (24) and are easily differentiated by the symptoms induced in tobacco, a systemic host for both TMV and TMGMV, and in *N. sylvestris,* a systemic host for TMV and a local lesion host for TMGMV

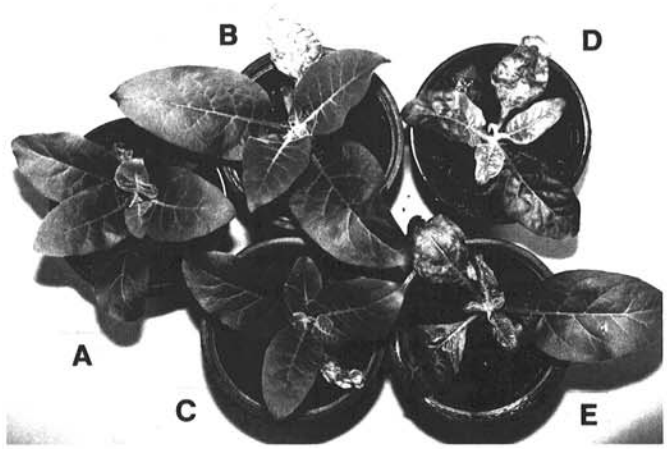

Fig. 1. Symptoms induced by tobacco mosaic virus (TMV) and tobacco mild green mosaic virus (TMGMV) in *Nicotiana megalosiphon* plants 21 days postinoculation. Plants were inoculated with 10 μl of a suspension (5 μg/ml) of U5-TMGMV (B), H6-TMGMV (C), U1-TMV (D), or H23-TMV (E) or mock inoculated with 10 μl of buffer (A).

TABLE 1. Accumulation of tobacco mosaic virus (TMV) and tobacco mild green mosaic virus (TMGMV) in *Nicotiana megalosiphon*

Virus isolate Plant part analyzed[a]	Infectivity[b]	Virus antigen[c] (μg/sample)	Viral RNA (μg/sample)
U1-TMV			
Local lesions	148 ± 22	46.66 ± 5.03	ND[d]
Interlesion area	172 ± 16	98.88 ± 9.47	1.28 ± 0.16
Noninoculated leaves	154 ± 17	109.44 ± 9.17	4.83 ± 0.23
U5-TMGMV			
Local lesions	29 ± 8	11.33 ± 1.15	ND
Interlesion area	0	0	0
Noninoculated leaves	0	0	0

[a]Samples consisted of 20 excised local lesions from inoculated leaves 6 days postinoculation, 0.1 g of interlesion area excised from inoculated leaves 6 days postinoculation, or 0.1 g of tissue from noninoculated leaves excised 10 days postinoculation.
[b]Data are mean ± standard errors for number of local lesions on five *N. tabacum* 'Xanthi-nc' half leaves.
[c]Data are mean ± standard errors from three independent experiments with three replicate plants in each.
[d]Not determined.

(22). We found that *N. megalosiphon* is also a differential host for these two tobamoviruses: both TMV and TMGMV induced a hypersensitive response, shown by the appearance of necrotic local lesions 2 days p.i. at 25 C, but only TMV induced systemic symptoms. These pathogenic reactions may be characteristic of these two tobamoviruses, because they were the same for two different isolates of each virus with widely different provenances: U5-TMGMV and H6-TMGMV, both from *N. glauca,* were isolated from California in 1950 and from New South Wales in 1971, respectively. U1-TMV came from tobacco in Wisconsin in the early 1940s, and H23-TMV was isolated from a *N. glauca* plant collected in New South Wales in 1898. When the presence of TMGMV or TMV was analyzed in different parts of the inoculated plants by local lesion assay on Xanthi-nc tobacco leaves or by quantification of viral antigen or viral RNA, it was shown that TMGMV was confined to the necrotic lesions, while TMV was found in interlesion areas of the inoculated leaves and was able to efficiently spread systemically into the upper leaves. Thus, TMV is able to overcome the hypersensitive defense reaction of *N. megalosiphon.*

The HR is an active defense reaction that occurs in certain plant-virus combinations according to a gene-for-gene model of genome interaction (5,8). Several single, dominant genes determining HR to tobamoviruses have been described in the genera *Nicotiana, Lycopersicon,* and *Capsicum* (2,8). We do not know the genetics of the HR induced in *N. megalosiphon* by TMV and to TMGMV, but other accessions of this species have been reported as systemic, nonnecrotic hosts for TMV (11). The resistance conferred by these reported HR genes is not detected in protoplasts but is associated with the limitation or blocking of the spread of the virus; for the N gene, the ability to block the modification of plasmodesmata induced by TMV infection has been shown (6). The resistance conferred by each of the reported HR genes may be overcome by different species and strains of tobamoviruses that generally do not elicit the HR gene. In a few cases, though, as we report here for TMV and *N. megalosiphon,* a virus may elicit a HR, but instead of being confined to the necrotic lesions, it is able to spread systemically in the plant (4,14,21). Systemic spread of the virus may or may not induce necrosis in systemically infected leaves, depending on poorly understood conditions. For mutants of TMV (4) or tomato mosaic virus (14) able to spread out of the elicited necrotic lesions, necrosis starts later after inoculation and/or lesions grow more slowly than for the corresponding wild types that do not overcome the HR. The overcoming of the HR may be related to its delayed development. In our case, the time p.i. at which local lesions appear in *N. megalosiphon* in response to TMV and TMGMV is the same, and kinetics of lesion growth are also the same. This is also the case for Xanthi-nc tobacco plants, a host in which neither virus is able to overcome the HR. An important difference, though, is that virus accumulation in the lesions is higher for TMV than for TMGMV in *N. megalosiphon* but not in Xanthi-nc; this agrees with the fact that TMV replicates faster and to higher levels than TMGMV in protoplasts of *N. megalosiphon.* Since the kinetics of replication of both viruses are the same in protoplasts of Xanthi-nc tobacco, a second resistance factor in *N. megalosiphon* seems to result in a less effective replication of TMGMV, and this second host component could modulate the expression of the HR. Although it cannot be ruled out that the mechanism restricting cell-to-cell spread in *N. megalosiphon* would act differentially on TMV and TMGMV, the data reported here seem to indicate that the ability of TMV to overcome the HR could be related to this second resistance factor. Thus, the ability of TMV to overcome the HR of *N. megalosiphon* could be related to its higher replication capacity in this host as compared with TMGMV and not to differences in the elicitation, or to interference with development, of the HR defense reaction. This also agrees with the fact that in mixed inoculations, TMV is unable to complement the systemic spread of TMGMV in *N. megalosiphon.*

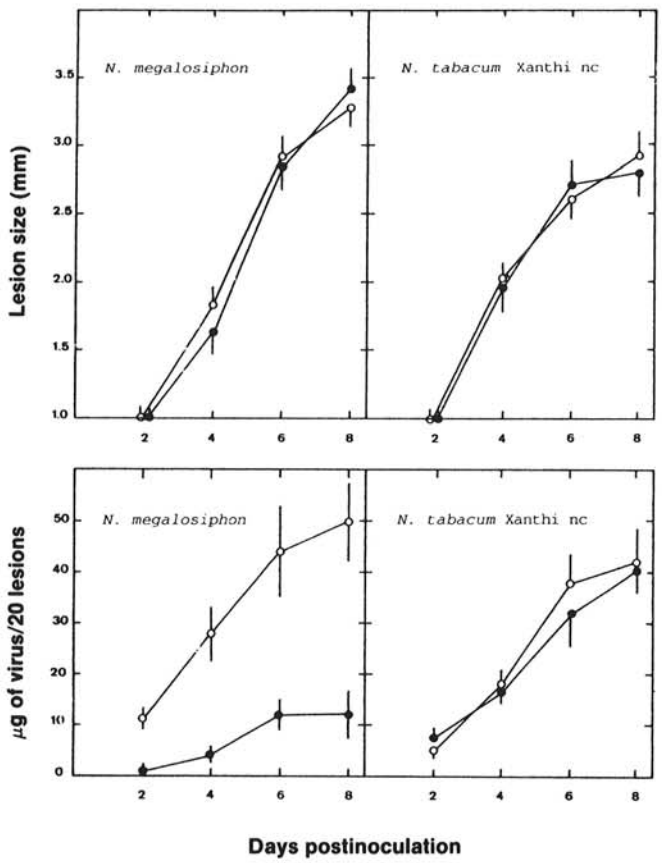

Fig. 2. Time course for lesion growth and intralesion virus accumulation in leaves of *Nicotiana megalosiphon* and *N. tabacum* 'Xanthi-nc' inoculated with tobacco mosaic virus isolate U1-TMV (○) or tobacco mild green mosaic virus isolate U5-TMGMV (●). Local lesion size data are mean and standard deviations of 40 lesions. Virus antigen content was determined by enzyme-linked immunosorbent assay in 20 lesions from each of three plants. Data are mean and standard errors from three independent experiments with three replicate plants in each.

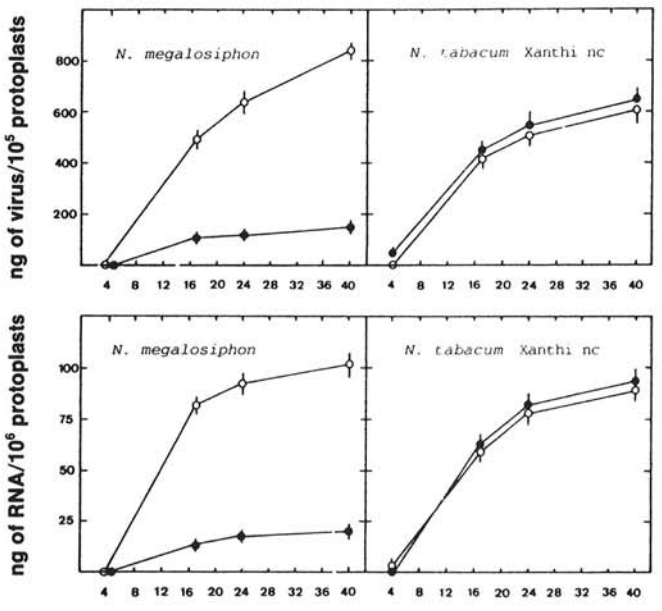

Fig. 3. Time course of virus antigen and virus RNA accumulation in protoplasts of *Nicotiana megalosiphon* and *N. tabacum* 'Xanthi-nc' inoculated with tobacco mosaic virus isolate U1-TMV (○) and tobacco mild green mosaic virus isolate U5-TMGMV (●). Data are means and standard errors from three independent experiments with two or three replicates in each.

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