***Tm-2²* Resistance in Tomato Requires Recognition of the Carboxy Terminus of the Movement Protein of Tomato Mosaic Virus**

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The *Tm-2²* resistance gene is used in most commercial tomato cultivars for protection against infection with tomato mosaic virus (ToMV). It has been suggested that *Tm-2²* resistance interferes with viral cell-to-cell movement in plants; ToMV strain ToMV-2² requires two amino acid (aa) exchanges in the carboxy-terminal region of the viral 30-kDa movement protein (at positions 238 and 244) to overcome *Tm-2²* resistance. For further analysis of this region of the 30-kDa protein, two stop codons were introduced into ToMV movement proteins at aa positions 235 and 237, leading to deletion of the terminal 30 aa. The mutant virus strains were able to infect wild-type tomato plants systemically, suggesting the carboxy-terminal portion of the ToMV 30-kDa protein is dispensable for virus transport in tomato. Even more important, the deletion mutants overcame the *Tm-2²* resistance gene. These data indicate the carboxy-terminal domain of the ToMV movement protein serves as a recognition target in the context of the *Tm-2²* resistance gene. Furthermore, expression of the 30-kDa movement protein from wild-type ToMV, but not from ToMV-2², in transgenic tomato plants with the *Tm-2²* resistance gene led to elicitation of a necrotic reaction in tomato seedlings, showing that the 30-kDa protein on its own is able to induce the plant's defense reaction.

Additional keywords: avirulence gene, hypersensitive reaction, transgenic plants.

Resistance against viral infection in plants, in principle, can be divided into two classes. Because viruses depend on their hosts in every step of their propagation, resistance can be achieved by altered host factors that lead to inhibition of specific processes in the viral life cycle. On the other hand, inactivation of the pathogen can be accomplished by an interaction, according to the gene-for-gene model (Flor 1971). In this case, avirulence genes of the pathogen are recognized by host plants carrying specific resistance genes. As a result, a localized necrotic response is elicited in the plants at sites of primary infection (hypersensitive reaction [HR]). A classic ex-

ample of this type of resistance against viral infection in plants is the resistance of tobacco against tobacco mosaic virus (TMV). In this case, elicitation of the plant's defense reaction is mediated by two resistance genes, the *N* gene, which has been isolated recently (Whitham et al. 1994), and the *N'* gene (Melchers et al. 1966). Studies on the molecular interaction between the *N'* resistance gene and TMV revealed some features of general importance for understanding HR elicitation. Although wild-type TMV is able to systemically infect tobacco plants carrying the *N'* gene, tomato mosaic virus (ToMV) and a variety of naturally occurring or artificially created mutants of TMV induce the HR after inoculation of plants carrying the *N'* gene. By generating recombinant viruses between wild-type TMV and HR-inducing virus strains, Culver and Dawson (1989) and Saito et al. (1987) showed that in the case of *N'* resistance the viral coat protein (CP) gene is responsible for the necrotic response. Further experiments revealed that the CP gene behaves as expected for a typical avirulence gene, according to the gene-for-gene model (Flor 1971). On one hand, deletion mutants of the viral CP showed that a functional CP has to be present to induce the HR (Saito et al. 1989). On the other hand, the avirulent CP on its own is sufficient to elicit a necrotic reaction in transgenic plants with the *N'* resistance gene (Culver and Dawson 1991; Pfitzner and Pfitzner 1992). In addition, the combined data from all available CP mutants could be used to identify a protein domain on the surface of the CP that may interact with a plant receptor (Culver et al. 1994; Taraporewala and Culver 1997).

In tomato, the *Tm-2²* resistance gene has been used for decades to control infection of plants by ToMV (Hall 1980). The mechanism of this resistance, however, is unclear. Fluorescence antibody staining of the leaf epidermis of infected tomato plants carrying the *Tm-2²* gene indicated that virus spread is restricted in the plants (Nishigushi and Motoyoshi 1987). In addition, sequence analysis of two independent resistance-breaking ToMV isolates, ToMV LIIA and ToMV-2², revealed four amino acid (aa) exchanges in the viral 30-kDa movement protein compared to the wild-type ToMV 30-kDa protein, three of which were common to both isolates (at aa positions 130, 238, and 244, respectively; Calder and Palukaitis 1992; Weber et al. 1993). Generation of recombinant viruses demonstrated that two of these aa exchanges (at positions 238 and 244) are responsible for the resistance-breaking phenotype of ToMV-2² (Weber et al. 1993). These results suggested that the *Tm-2²* resistance gene operates at the level of viral cell-to-

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cell movement. On the other hand, Hall (1980) observed that the *Tm-2²* resistance is accompanied by a necrotic reaction, notably in heterozygous tomato plants and when infected plants are kept at elevated temperatures. Gafny et al. (1992) showed that, for TMV, deletion of aa 234 to 268 from the carboxy terminus of the 30-kDa movement protein does not interfere with virus movement in infected tobacco plants.

To further explore the mechanism of *Tm-2²* resistance in tomato, we generated ToMV mutants with movement proteins truncated at the carboxy terminus. Infection of plants with these deletion mutants revealed that the terminal 30 aa of the movement protein are not necessary for cell-to-cell spread of the virus. In addition, truncation of the movement protein enabled the viral mutants to overcome *Tm-2²* resistance. Expression of the 30-kDa movement protein from wild-type ToMV in transgenic tomato plants with the *Tm-2²* resistance gene elicited a necrotic reaction, whereas expression of the movement protein from ToMV-2² was tolerated by plants carrying the *Tm-2²* gene.

RESULTS

Effect of deletion of 30 aa at the carboxy terminus of the ToMV 30-kDa movement protein on virus spread in tomato plants lacking the *Tm-2²* resistance gene.

To generate ToMV mutants with movement proteins truncated at the carboxy terminus, in vitro mutagenesis was performed on infectious plasmid pSLN, which contains a full-length cDNA clone of ToMV under control of the CaMV 35S RNA promoter (Weber et al. 1992). Codon AAA, giving rise to lysine residues at positions 235 and 237 in the 30-kDa movement protein, was changed at both positions to produce a stop codon (TAA). The resulting plasmid was named pSL30stop (Fig. 1). The same mutations were introduced in plasmid pSL30N5 to produce pSL30N5stop (Fig. 1). Plasmid pSL30N5 contained an infectious ToMV cDNA clone with two amino-

Fig. 1. Schematic representation of the structures of 30-kDa movement proteins from wild-type and mutant tomato mosaic virus (ToMV). The amino- (N) and carboxy-terminal (C) regions and protein domains, as defined by Saito et al. (1989), are indicated as I and II, conserved regions; V, variable regions A, B, and C. 30.2² is the movement protein from ToMV-2² (shaded box), a virus strain able to break the *Tm-2²* resistance. 30.wt is the movement protein from wild-type ToMV (open box), a virus strain unable to overcome the *Tm-2²* resistance. Both movement proteins differ by four amino acid (aa) exchanges at positions 130, 184, 238, and 244, respectively, indicated by black bars and denoted. 30.N5stop and 30.wtstop carry stop codons at amino acid positions 235 and 237, yielding virus strains with movement proteins truncated by 30 aa at the carboxy terminus. Mutant 30.N5stop also contains two aa exchanges found in ToMV-2² at positions 130 and 184 of the movement protein.

terminal aa exchanges (at positions 130 and 184) of the four substitutions in the 30-kDa movement protein of ToMV-2². These two mutations were not sufficient to break host resistance (Weber et al. 1993).

The two plasmids were used to infect primary leaves of *Chenopodium quinoa* plants, using a cDNA infection procedure (Weber et al. 1992), giving rise to the virus progeny ToMV-stop and ToMV-N5stop, respectively. Virus was harvested 1 week after infection. Subsequently, 1 µg of each virus strain was used to infect the primary leaf of tomato plants (*Lycopersicon esculentum* cv. Craigella GCR 26) at the 4-leaf stage, which is susceptible to ToMV. After 2 weeks, all plants displayed typical symptoms of a systemic ToMV infection (data not shown). Proteins were extracted from the newly developed fifth leaf and subjected to immunodetection of the viral CP. As shown in Figure 2A, CP from both virus strains, ToMV-stop and ToMV-N5stop, had accumulated to high levels in the youngest leaves of infected tomato plants. These results demonstrate that the carboxy terminus of the ToMV 30-kDa movement protein is not important for the movement function of the protein, as has been reported previously for the truncated 30-kDa movement protein of TMV (Gafny et al. 1992).

Fig. 2. Immunodetection of tomato mosaic virus (ToMV) in *Lycopersicon esculentum* cv. Craigella GCR 26 (lacking the *Tm-2²* resistance gene) and cv. Craigella GCR 267 (*Tm-2²/Tm-2²*) after infection with virus strains expressing mutant 30-kDa movement proteins. Soluble proteins were extracted from the youngest, noninoculated leaf of tomato plants 2 weeks after infection. Equal amounts of protein were separated on a 15% sodium dodecyl sulfate polyacrylamide gel and analyzed for ToMV by Western blotting and immunodetection of the ToMV coat protein. **A**, Proteins were isolated from tomato plants lacking the *Tm-2²* resistance gene (*L. esculentum* cv. Craigella GCR 26) after infection with 1 µg of ToMV (lane 2), 1 µg of ToMV-stop (lane 3), or 1 µg of ToMV-N5stop (lane 4). Lane 1 contains 0.1 µg of ToMV inoculum. Both ToMV mutants carrying movement proteins truncated at position 234, like ToMV, were able to spread systemically in GCR 26 tomato plants. **B**, Proteins were isolated from tomato plants containing the *Tm-2²* resistance gene (*L. esculentum* cv. Craigella GCR 267, *Tm-2²/Tm-2²*) after infection with 1 µg of ToMV (lane 2), 1 µg of ToMV-2² (lane 3), 1 µg of ToMV-stop (lane 4), or 1 µg of ToMV-N5stop (lane 5). Lane 1 contains 0.1 µg of ToMV inoculum. Both ToMV mutants carrying movement proteins truncated at position 234, like ToMV-2², were able to overcome the *Tm-2²* resistance gene.

Effect of deletion of 30 aa at the carboxy terminus of the 30-kDa movement protein on the ability of ToMV to overcome *Tm-2*² resistance.

To analyze the effects of the mutant virus strains containing truncated movement proteins in plants carrying the *Tm-2*² resistance gene, the primary leaves of tomato plants (*L. esculentum* cv. Craigella GCR 267, *Tm-2*²/*Tm-2*²) at the 4-leaf stage were infected with 1 µg of ToMV, ToMV-2², ToMV-stop, and ToMV-N5stop, respectively. For each virus strain, 10 plants were inoculated. After 4 weeks, plants were scored for symptom formation and virus accumulation in the newly developed fifth (i.e., noninoculated) leaf by immunodetection of the viral CP. The results are shown in Figure 2B. Although the plants were resistant to infection by wild-type ToMV (Fig. 2B, lane 2), the two virus strains carrying mutant movement proteins, ToMV-stop and ToMV-N5stop, as well as ToMV-2², were able to overcome the *Tm-2*² resistance gene, accumulating to high titers in the upper leaves of infected plants (Fig. 2B, lanes 4 and 5 versus lane 3). Likewise, both mutant virus strains gave rise to symptom formation on GCR 267 tomato plants, as did ToMV-2² (Fig. 3). Interestingly, however, symptom formation with mutant virus strain ToMV-N5stop, carrying two aa exchanges in the amino-terminal portion of the ToMV 30-kDa protein that are not involved in breaking *Tm-2*² resistance, repeatedly was significantly less severe than with strain ToMV-2² or ToMV-stop (Fig. 3).

Because RNA viruses exhibit a high rate of mutation, it was essential to analyze whether the mutant movement protein genes with stop codons at aa positions 235 and 237 were maintained in recombinant virus strains ToMV-stop and ToMV-N5stop, evading *Tm-2*² resistance. To this end, virus was harvested from the upper, noninoculated leaves of GCR 267 tomato plants. Genomic ToMV RNA was extracted from the virus isolates and amplified via reverse-transcription (RT) followed by polymerase chain reaction (PCR) in the region of the 30-kDa movement protein gene, from nucleotide 4800 to 5861. DNA sequence analysis of gel-purified PCR products demonstrated that both viral mutants, ToMV-stop and ToMV-N5stop, still contained the stop codons corresponding to aa positions 235 and 237 of the 30-kDa movement protein, as introduced by in vitro mutagenesis (data not shown). In addition, virus strain ToMV-N5stop still contained the two nucleotide exchanges at positions 5293 and 5456 (data not shown).

Expression of the 30-kDa movement protein from ToMV in transgenic tomato plants with the *Tm-2*² resistance gene.

To analyze whether the terminal region of the 30-kDa protein is able to induce a defense reaction in plants, we generated transgenic tomato plants, expressing either the movement protein of wild-type ToMV or that of ToMV-2², able to break the *Tm-2*² resistance gene. If resistance against ToMV in tomato is due to induction of the HR, the ToMV movement protein on its own should be able to elicit a defense response in plants carrying the *Tm-2*² resistance gene, as shown previously for the CP of ToMV in tobacco plants containing the *N*⁹ gene (Culver and Dawson 1991; Pfitzner and Pfitzner 1992) and the *Avr9* avirulence gene product of *Cladosporium fulvum* in tomato plants containing the *Cf-9* resistance gene (Jones et al. 1994).

The open reading frames (ORFs) of the 30-kDa movement proteins from ToMV and ToMV-2² were integrated in binary

vector pBin19 under control of the CaMV 35S RNA promoter to produce plasmids pTA.30-L and pTA.30-2², respectively (Fig. 4A). Both constructs were introduced via *Agrobacterium*-mediated gene transfer in *L. esculentum* cv. Craigella GCR 26 susceptible to ToMV. A number of independent primary transformants were obtained for each construct (30-L/1-15 for pTA.30-L and 30-2²/1-7 for pTA.30-2²). To verify the transfer of the constructs, genomic DNA was isolated from the primary transformants and used for Southern blotting experiments. In most cases, the chimeric genes were correctly integrated in the tomato genome (data not shown). In addition, Western blot analysis with an antiserum directed against the ToMV movement protein showed accumulation of the 30-kDa protein in cell wall extracts from GCR 26 plants infected with ToMV and from transgenic plants 30-L/10, 30-2²/4₁, and 30-2²/6 but not from noninfected wild-type plants (Fig. 4B and data not shown).

To study the effect of the two movement proteins on tomato plants containing the *Tm-2*² resistance gene, pollen from transgenic lines 30-L/10, 30-2²/4₁, and 30-2²/6 was transferred to emasculated flowers of GCR 267 plants homozygous for the *Tm-2*² resistance gene. Tomato fruits from these crosses were harvested, and seeds were germinated on medium containing kanamycin at 100 µg/ml. As a control, seeds from self-fertilized transgenic tomato plants also were germinated on kanamycin-containing medium. Although the progeny of self-fertilized transgenic tomato plants yielded about 75% kanamycin-resistant seedlings, the progeny of the crosses of transgenic plants with GCR 267 tomato plants resulted in about 50% kanamycin-resistant seedlings, indicating a single active insertion of the respective transgene in the genomes of the parental transgenic lines. The seedlings from self-fertilized transgenic plants, as well as the progeny from the crosses of transgenic plants expressing the ToMV-2² movement protein (30-2²/4₁, 30-2²/6) with GCR 267 tomato plants carrying the *Tm-2*² resistance gene, exhibited a normal phenotype, as shown in Figure 5C. In contrast, all kanamycin-resistant seedlings resulting from the cross of transgenic line 30-L/10 containing the wild-type ToMV movement protein with GCR 267 plants began to develop necrotic areas at the hypocotyl 2 days after germination (Fig. 5A and B). These necrotic areas subsequently spread all over the plants, except the roots, resulting in the death of the seedlings 10 days after germination.

DISCUSSION

Our results strongly support the notion that *Tm-2*² resistance against ToMV operates via elicitation of the HR and that recognition of the 30-kDa movement protein as an avirulence gene product relies on sequences contained within its 30 aa residues at the carboxy terminus. These findings on the mechanism of the *Tm-2*² resistance gene raise new questions. One question, for example, is what is the relationship of allelic *Tm-2* resistance to the *Tm-2*² resistance gene? Analysis of *Tm-2* resistance-breaking virus strains revealed two aa exchanges that are responsible for overcoming the resistance gene also located in the movement protein gene (Meshi et al. 1989; H. Weber and A. J. P. Pfitzner, *unpublished data*). These aa exchanges are contained in the amino-terminal portion of the movement protein (at aa position 52 or 68 and position 133). Although Meshi et al. (1989) strongly favor the

hypothesis of direct interference with the *Tm-2* resistance gene product by virus movement in plants, it seems highly unlikely, considering our findings on the *Tm-2²* gene that two allelic genes should result in two completely different resistance mechanisms. Indeed, by generating transgenic plants expressing the movement protein of ToMV in tomato cultivars with the *Tm-2* resistance gene, we recently obtained preliminary evidence indicating that *Tm-2* resistance also involves a necrotic reaction, although in a somewhat milder form (M. Silber and A. J. P. Pfitzner, *unpublished data*). These data support the assumption that the *Tm-2* resistance gene, as well, operates via elicitation of the HR. Analysis of other movement protein mutants is needed, however, to clarify the resistance mechanism of the *Tm-2* gene.

Another question concerns the function of the carboxy-terminal portion of the ToMV movement protein. Although the terminal 30 aa of the movement protein seem to be dispensable for cell-to-cell movement of ToMV in tomato plants,

Fig. 3. Phenotype of leaves from *Lycopersicon esculentum* cv. Craigella GCR 267 (*Tm-2/Tm-2*) after infection with tomato mosaic virus (ToMV) strains expressing mutant 30-kDa movement proteins. The fifth leaf from the top of each plant is shown 4 weeks after infection with **A**, 1 µg of ToMV-stop, **B**, 1 µg of ToMV-N5stop, **C**, 1 µg of ToMV-2², and **D**, 1 µg of ToMV. Both ToMV mutants carrying movement proteins truncated at position 234, like ToMV-2², caused symptom formation in plants carrying the *Tm-2* resistance gene.

Fig. 4. Expression of the 30-kDa movement proteins from tomato mosaic virus (ToMV) and ToMV-2² in *Lycopersicon esculentum* cv. Craigella GCR 26 (lacking the *Tm-2* resistance gene). **A**, The open reading frames of the 30-kDa movement proteins from ToMV and ToMV-2² (hatched boxes) were inserted between the cauliflower mosaic virus 35S RNA promoter and the nopaline synthase gene polyadenylation site in binary vector pBin19, producing plasmids pTA.30-L and pTA.30-2², respectively. **B**, The gene constructs were integrated via *Agrobacterium* into *L. esculentum* cv. Craigella GCR 26. Equal amounts of cell wall proteins (20 µg each) were separated on a 15% sodium dodecyl sulfate polyacrylamide gel and analyzed by Western blotting and immunodetection of the 30-kDa movement protein. Proteins were isolated from wild-type plants (lane 2) and transformants with integrated pTA.30-L (30-L/10-12; lanes 3 through 5) or pTA.30-2² (30-2²/4₁, 30-2²/4-6; lanes 6 through 9). Lane 1 contains 0.5 ng of purified 30-kDa protein over-expressed from *Escherichia coli*. Plants 30-L/10 (lane 3), 30-2²/4₁ (lane 6), and 30-2²/6 (lane 9) express the 30-kDa movement protein from ToMV or ToMV-2².

Fig. 5. Phenotype of seedlings of *Lycopersicon esculentum* (*Tm-2*^{-/-}) expressing the 30-kDa movement proteins from tomato mosaic virus (ToMV) or ToMV-2². Pollen from transgenic plants (*L. esculentum* cv. Craigella GCR 26) expressing the movement protein of ToMV or ToMV-2² was transferred to emasculated flowers of *L. esculentum* cv. Craigella GCR 267 (*Tm-2*²/*Tm-2*³). The progeny were selected for kanamycin resistance. **A and B**, Hybrids (*Tm-2*^{-/-}) expressing the movement protein of ToMV develop necrotic regions at the hypocotyl 2 days after germination. **C**, Expression of the movement protein of ToMV-2² had no effect on the development of the seedlings.

this region of the ToMV movement protein was maintained in the gene during evolution. Furthermore, the *Tm-2²* resistance gene, which obviously requires recognition of a domain within the carboxy-terminal region of the 30-kDa protein, seems to be an effective resistance gene; only a few ToMV strains able to overcome *Tm-2²* resistance have been isolated (Hall 1980). Taken together, these data indicate that the carboxy-terminal portion must be an important region in the ToMV movement protein that is not easily subject to mutations. One hint pointing to a possible function of this region may come from a recent report on recombinant TMV strains containing the movement protein gene from odontoglossum ringspot virus (ORSV), a tobamovirus infecting orchids (Fenczik et al. 1995). The recombinant virus strains were unable to establish a systemic infection in tobacco plants unless 11 aa from the carboxy terminus of the ORSV movement protein were removed. The ORSV movement protein deletion mutants, however, were far less effective than wild-type TMV in long-distance movement in tobacco plants, suggesting the carboxy-terminal region of the movement protein may be involved in interactions with specific factors of the host for phloem-dependent accumulation.

Although in this paper we do not describe differences in systemic infection of tomato plants, the ToMV movement protein deletion mutants, ToMV-stop and ToMV-N5stop, may have an effect on long-distance movement. In all experiments reported, we used young tomato plants at the 4-leaf stage for virus inoculation, because young plants at this stage display the strongest disease symptoms when the *Tm-2²* resistance gene is overcome. Coincidentally, we observed that infection of older tobacco and tomato plants with ToMV-stop resulted in delayed symptom development and strongly reduced virus accumulation in the youngest parts of the plants, especially in the flowers, compared to wild-type ToMV (A. J. P. Pfitzner, unpublished data). This finding may point to hampered long-distance transport of ToMV movement protein deletion mutants in older tomato plants, similar to the effect found for the ORSV movement protein deletion mutants in tobacco (Fenczik et al. 1995). Thus, efficient long-distance transport appears to depend on a functional 30-kDa protein, including its native carboxy terminus, at least in older plants. Given this hypothesis, virus spread in tomato populations would rely strictly on the carboxy-terminal aa residues of the 30-kDa movement protein, because a major source of ToMV in tomato populations is infected seed material (Broadbent 1976). To test this hypothesis, a detailed analysis of the local accumulation of different ToMV mutants in tomato plants at various developmental stages is needed.

MATERIALS AND METHODS

Plant material and virus strains.

Seeds were obtained from the Tobacco Institute, NC; K.-W. Mundry, University of Stuttgart, Germany (*Nicotiana tabacum* cv. Samsun nn); and the Deutsche Sammlung für Mikroorganismen, Branch Plant Viruses, Braunschweig, Germany (*C. quinoa*). Nearly isogenic lines of *L. esculentum* cv. Craigella GCR 26 (+/+, without any resistance gene) and GCR 267 (*Tm-2²/Tm-2²*) were supplied by the Institute for Horticultural Research, Littlehampton, England. ToMV and ToMV-2² were gifts from P. Grimbley, Institute for Horticultural Research.

Plasmid construction.

For infection of plants with ToMV strains expressing truncated 30-kDa movement proteins, plasmids pSL30stop and pSL30N5stop were generated by in vitro mutagenesis, using plasmids pSLN and pSL30N5 (Weber et al. 1992, 1993) as templates. Mutant DNA synthesis was primed with oligonucleotide 5'-GAAGGCCTTAACCATAAAGT-3', corresponding to nucleotides 5600 to 5619 in the ToMV genomic RNA. The oligonucleotide contains two nucleotide exchanges (in bold) compared to the ToMV wild-type sequence, thus, introducing two stop codons at aa positions 235 and 237 into the ORF of the movement protein gene. In vitro mutagenesis was performed, using the Transformer site-directed mutagenesis kit (Clontech Laboratories, Palo Alto, CA). The mutant plasmids were verified by DNA sequence analysis.

To construct plant expression vectors, the coding sequences of the movement protein genes from ToMV and ToMV-2² were isolated as *Bst*NI fragments (corresponding to nucleotides 4833 to 5892 in the ToMV genomic RNA) from cDNA clones of ToMV and ToMV-2², respectively. The fragments were blunted and cloned into the *Sma*I site of pUC18. To generate plasmids pTA.30-L and pTA.30-2², the movement protein genes from ToMV and ToMV-2² were isolated as *Bam*HI-*Sac*I fragments and inserted between the cauliflower mosaic virus 35S RNA promoter and the polyadenylation signal of the nopaline synthase gene in the binary vector pBin19 35S[GUS] (Pfitzner and Pfitzner 1992) by replacing the *gus* gene.

Plant infection and detection of virus.

Inoculation of *C. quinoa* with infectious cDNA and infection of *L. esculentum* with virus were as described previously (Weber et al. 1993). For the analysis of virus content, soluble proteins were extracted from the upper (i.e., noninoculated) leaves of plants and subjected to immunodetection with an antiserum directed against the ToMV CP (Weber et al. 1993).

Transgenic plants.

The genes coding for the movement proteins of ToMV and ToMV-2² were introduced into tomato plants (*L. esculentum* cv. Craigella GCR 26) by *A. tumefaciens*-mediated gene transfer, as described previously (Chang and Pfitzner 1994). To introduce the transgenes into *L. esculentum* cv. Craigella GCR 267, pollen from transgenic GCR 26 plants with integrated movement protein genes from ToMV or ToMV-2² were transferred to emasculated flowers of GCR 267 plants homozygous for the *Tm-2²* resistance gene. The resulting seeds were germinated in the dark on medium containing MS salts at 4.6 g/liter (Murashige and Skoog 1962), 0.4% agar, and kanamycin at 100 µg/ml, pH 5.7. After the appearance of primary roots, seeds were transferred to standard light conditions (25°C; 16 h of light at 10 to 20 µE m⁻² s⁻¹; 8 h of dark).

Isolation of viral RNA and RT-PCR analyses.

For the analysis of viral RNA sequences, genomic ToMV RNA was isolated from systemically infected plant tissue and amplified by PCR after first-strand cDNA synthesis with reverse-transcriptase. Briefly, infected tissue was homogenized in buffer (50 mM Tris/HCl, pH 7.6, 20 mM Na₂SO₃), centrifuged at 10,000 × *g*, and the supernatant was heated at 55°C for 10 min. After a second centrifugation,

100 µl of the supernatant was diluted with the same volume of water, mixed with 20 µl of 10% sodium dodecyl sulfate (SDS), and extracted twice with phenol/chloroform. Viral RNA from the extract was precipitated with sodium acetate and ethanol. First-strand cDNA synthesis and PCR were performed according to standard protocols (Sambrook et al. 1989), using oligonucleotides TMV-Co (identical to the ToMV genomic RNA from positions 4800 to 4819) and 30-3' (complementary to the ToMV genomic RNA from positions 5841 to 5861). PCR products were purified from 1% agarose gel and sequenced, using oligonucleotide coatB (complementary to the genomic ToMV RNA from positions 5690 to 5715) as a primer.

Immunodetection of the ToMV 30-kDa movement protein.

For detection of the ToMV 30-kDa movement protein in virus-infected and transgenic plants, cell wall protein extracts were prepared according to Deom et al. (1990). Protein extracts were dialyzed against TE (10 mM Tris/HCl, pH 6.8, 1 mM EDTA) and stored at -70°C. Samples of 20 µg of protein each were used for electrophoresis on 15% SDS polyacrylamide gels. Protein blotting and immunodetection with a rabbit anti-serum produced against the purified ToMV 30-kDa protein overexpressed in *Escherichia coli* were performed, as described previously (Weber et al. 1993).

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