JOURNAL OF VIROLOGY, Nov. 1993, p. 6432–6438
0022-538X/93/116432-07$02.00/0

Vol. 67, No. 11

Two Amino Acid Substitutions in the Tomato Mosaic Virus 30-Kilodalton Movement Protein Confer the Ability To Overcome the *Tm-2*^{2} Resistance Gene in the Tomato

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The *Tm-2*^{2} resistance gene is used in most commercial tomato cultivars for protection against infection with tobacco mosaic virus and its close relative tomato mosaic virus (ToMV). To study the mechanism of this resistance gene, cDNA clones encompassing the complete genome of a ToMV strain (ToMV-2^2) that was able to break the *Tm-2*^{2} resistance were generated. Chimeric full-length viral cDNA clones were constructed under the control of the cauliflower mosaic virus 35S RNA promoter, combining parts of the wild-type virus and ToMV-2^2. Using these clones in cDNA infection experiments, we showed that the 30-kDa movement protein of ToMV-2^2 is responsible for overcoming the *Tm-2*^{2} resistance gene in the tomato. DNA sequence analysis revealed four amino acid exchanges between the 30-kDa proteins from wild-type ToMV and ToMV-2^2, Lys-130 to Glu, Gly-184 to Glu, Ser-238 to Arg, and Lys-244 to Glu. To clarify the involvement of the altered amino acid residues in the resistance-breaking properties of the ToMV-2^2 movement protein, different combinations of these amino acid exchanges were introduced in the genome of wild-type ToMV. Only one mutant strain which contained two amino acid substitutions, Arg-238 and Glu-244, was able to multiply in *Tm-2*^{2} tomato plants. Both amino acid exchanges are found within the carboxy-terminal region of the movement protein, which displays a high variability among different tobamoviruses and has been shown to be dispensable for virus transport in tobacco plants. These observations suggest that the resistance conferred by the *Tm-2*^{2} gene against ToMV depends on specific recognition events in this host-pathogen interaction rather than interfering with fundamental functions of the 30-kDa protein.

Three resistance genes, *Tm-1*, *Tm-2*, and *Tm-2*^{2}, have been identified in crossings between wild and cultivated species of tomato, all of which are used to prevent systemic mosaic symptoms and losses in fruit yield and quality caused by tobacco mosaic virus (TMV) and tomato mosaic virus (ToMV) (6, 15). *Tm-1* interferes with viral replication and is expressed in protoplasts (12, 25). In contrast, resistance conferred by the *Tm-2* or the allelic *Tm-2*^{2} gene is effective only in whole plants or in leaf discs (3, 12). In addition, fluorescent antibody staining of leaf epidermis of tomato plants with the *Tm-2* or *Tm-2*^{2} gene after infection with TMV indicated that virus spread was restricted in these tomato cultivars (13). These results therefore suggested that the *Tm-2* and *Tm-2*^{2} resistance genes operate at the level of cell-to-cell movement. On the other hand, it has been observed that the *Tm-2* and *Tm-2*^{2} resistance is often accompanied by a hypersensitive response, notably in heterozygous tomato plants or when infected plants are kept at elevated temperatures (6, 15, 16). Thus, the mechanism of the resistance conferred by the *Tm-2* locus comprising the *Tm-2* and *Tm-2*^{2} resistance genes is still unclear.

Tm-1 and *Tm-2* are of only limited practical use because they can be overcome by naturally occurring ToMV strains (20). For *Tm-2*^{2}, however, there have been only few reports on ToMV isolates which are able to overcome this resistance gene (6, 20). Furthermore, in contrast to *Tm-1* and *Tm-2*, the breaking of *Tm-2*^{2} results in severe stunting and distortion of the affected plants, which can be easily detected in a tomato population at an early time point. Therefore, infected plants can be removed before high virus titers which would endanger the whole population can accumulate. These specific features associated with *Tm-2*^{2}-overcoming ToMV strains may be the reason why this resistance gene is still highly effective despite decades of use in glasshouse tomato production. Because of this commercial importance of the *Tm-2*^{2} gene, it seemed highly desirable to characterize the plant-pathogen interactions associated with this resistance gene at the molecular level.

One possible approach to understand the resistance mechanism of a particular resistance gene is to characterize resistance-breaking strains of the pathogen. Subsequent comparison with avirulent strains may then give clues to what viral components interact with putative host resistance factors and how the viral and host components interact. For *Tm-1* and *Tm-2*, the mutations responsible for the ability of ToMV strains to overcome the particular resistance gene have been identified (10, 11). In both cases, two amino acid substitutions in the open reading frame for either the 130/180-kDa proteins or the 30-kDa movement protein were necessary to confer the resistance-breaking phenotype. Although differences in the sequence of *Tm-2*^{2}-breaking virus strains in comparison with wild-type ToMV have been observed (1, 23a), the mutations responsible for overcoming the *Tm-2*^{2} resistance gene have not been identified so far. Here we show by using infectious cDNA clones of recombinant viruses that two amino acid exchanges in the carboxy-terminal part of the movement protein are sufficient for ToMV to escape the *Tm-2*^{2} resistance reaction.

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MATERIALS AND METHODS

Plant material and virus strains. Seeds were obtained from the Tobacco Institute, N.C., from K.-W. Mundry, University of Stuttgart (*Nicotiana tabacum* cv. Samsun and Samsun NN), and from the Deutsche Sammlung für Mikroorganismen, branch plant viruses, Braunschweig, Germany (*Chenopodium quinoa*). Nearly isogenic lines of *Lycopersicon esculentum* cv. Craigella, GCR 26 (+/+, without any resistance gene) and GCR 267 ($Tm\text{-}2^2/Tm\text{-}2^2$), were supplied by the Institute for Horticultural Research, Littlehampton, England. ToMV and ToMV-2^2 were gifts from P. Grimbley, Institute for Horticultural Research.

Plasmids and antisera. Plasmid pSLN contains a full-length cDNA of ToMV (TMV-L [9]) fused to the cauliflower mosaic virus 35S RNA promoter and the polyadenylation signal of the nopaline synthase gene. This plasmid has been previously shown to give rise to high yields of ToMV after mechanical inoculation of *C. quinoa* plants (26). Antibodies directed against the ToMV coat protein were obtained from the American Type Culture Collection (ATCC PVAS-135B).

cDNA cloning of ToMV-2^2. Virus was purified from systemically infected GCR 267 plants as described previously (7). Genomic RNA was isolated and transcribed into cDNA as described previously (9). For first-strand synthesis, primer A (complementary to the ToMV genomic RNA from positions 6384 to 6369), primer 6 (complementary to the ToMV genomic RNA from positions 4418 to 4403), and primer 2 (complementary to the ToMV genomic RNA from positions 1875 to 1860) were used. The second strand was primed with primer 5 (identical to the ToMV genomic RNA from positions 4358 to 4373), primer 1 (identical to the ToMV genomic RNA from positions 1818 to 1834), and primer B (identical to the ToMV genomic RNA from positions 1 to 15), giving rise to cDNAs A.5, 6.1, and 2.B, respectively. The cDNAs were digested with *Kpn*I (A.5), *Pst*I and *Kpn*I (6.1), or *Pst*I (2.B) and ligated into pUC18, which was cut with *Sma*I and *Kpn*I, *Pst*I and *Kpn*I, or *Sma*I and *Pst*I, respectively. The resulting plasmids were designated pToMV-2^2A.5, pToMV-$2^2$6.1, and pToMV-$2^2$2.B.

Construction of recombinant infectious cDNA clones for ToMV. Recombinant DNA technology was performed as described previously (22). For the construction of pSL30.2^2, an 851-bp *Cla*I-*Bst*EII fragment from pToMV-2^2A.5 (nucleotides 4948 to 5799 of the ToMV genomic RNA) containing the 30-kDa open reading frame was isolated and ligated to an 8.8-kb *Cla*I-*Bst*EII fragment, which was generated from pSLN by partial digestion with *Cla*I and subsequent cleavage with *Bst*EII. Plasmids pSL30N3 and pSL30N5 were generated by exchanging the 3,537-bp *Nhe*I-*Nco*I fragment (nucleotides 1925 to 5462 of the ToMV genomic RNA) between pSLN and pSL30.2^2. pSL30N3/5 and pSL30N3/3 were constructed by oligonucleotide-directed mutagenesis from pSLN, using the Transformer site-directed mutagenesis kit as recommended by the manufacturer (Clontech, Palo Alto, Calif.). For pSL30N3/5 the synthetic oligonucleotide 5′-AAGGCCTAAACCAAA AAGGTTTGATGAA-3′ (positions 5601 to 5628 in the ToMV genomic RNA) was used, and for pSL30N3/3 the synthetic oligonucleotide 5′-AAGGCCTAAACCAAAAAGTTTTGA TGAAGTTGAAGAAGAGTTTGA-3′ (positions 5601 to 5645 in the ToMV genomic RNA) was used.

Generation of recombinant ToMV Strains by cDNA infection. *C. quinoa* plants at the four-leaf stage were dusted with carborundum, and the primary leaves were mechanically inoculated with 10 μg of supercoiled plasmid DNA of the corresponding recombinant infectious cDNA clones. After 7 days, the inoculated leaves were homogenized with an equal volume of 100 mM Tris buffer (pH 7), and the extracts were analyzed for virus content by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (8) and by Western blotting (immunoblotting) (17) with an antiserum directed against the ToMV coat protein and the enhanced chemiluminescence detection system (Amersham). All recombinant infectious cDNA clones used in this study gave similar yields of viral progeny (>0.1 μg of virus per mg of leaf material).

Infectivity assays. *L. esculentum* cv. Craigella GCR 267 ($Tm\text{-}2^2/Tm\text{-}2^2$) plants at the four-leaf stage were dusted with carborundum, and one primary leaf each was mechanically inoculated with *C. quinoa* leaf extracts containing 1 μg of the corresponding recombinant ToMV strain or with 1 μg of purified virus. After 2 weeks, the infected tomato plants were monitored for systemic infection by visual inspection, and the youngest, i.e., noninoculated, leaf of each plant was analyzed for virus content by SDS-PAGE (8) and by Western blotting (17) with an antiserum directed against the ToMV coat protein.

PCR. The polymerase chain reaction (PCR) was performed on DNA templates, using a kit from Perkin Elmer, Norwalk, Conn., according to the manufacturer's recommendations. For the amplification of viral RNA sequences, first-strand cDNA was synthesized with reverse transcriptase followed by PCR (22). The 30-kDa open reading frame was amplified from recombinant ToMV strains by using primers 30-3′ (complementary to the ToMV genomic RNA from positions 5861 to 5841) and TMV-Co (identical to the ToMV genomic RNA from positions 4800 to 4819). For direct sequence analysis, the PCR products were purified on a 1% agarose gel and sequenced (23) by using primers 30-3′, TMV-Co, 30seq (identical to the ToMV genomic RNA from positions 5257 to 5273), 30-N (identical to the ToMV genomic RNA from positions 5424 to 5473), and 30N5seq (complementary to the ToMV genomic RNA from positions 5327 to 5310).

Primer extension analysis of the ToMV 5′ end. ToMV was purified from systemically infected tissue as described previously (7). For the isolation of genomic RNA, the virus suspension was adjusted to 1% SDS and extracted with phenol-chloroform, and nucleic acids were precipitated with ethanol. Primer extension analysis was performed as described previously (19). For the extension reaction, 0.1 μg of viral RNA was annealed with 3.2 pmol of end-labeled primer rep 1 (5′-GTGTATGCCATTGTAGTT-3′; complementary to positions 64 to 82 in the ToMV sequence) at 70°C in a volume of 10 μl. The enzyme reaction was performed for 40 min at 42°C in the presence of 1 mM each dATP, dCTP, dGTP, and dTTP and 1.5 U of RNasin in 50 mM Tris-HCl (pH 8.3)–5 mM $MgCl_2$–140 mM KCl–20 mM dithiothreitol with 37.5 U of avian myeloblastosis virus reverse transcriptase (New England Biolabs, Beverly, Mass.). Extension products were analyzed on a 6% sequencing gel (19).

Immunodetection of the ToMV coat protein. Soluble proteins were extracted from leaves of infected plants with an equal volume of 100 mM Tris buffer (pH 7.0) by grinding the plant tissue with a glass rod in an Eppendorf tube. The extracts were clarified by centrifugation for 5 min at 4°C in a microcentrifuge and directly used for electrophoresis on SDS–15% polyacrylamide gels (8). Protein blotting and incubation with antisera were done as described previously (17). For detection of the ToMV coat protein-antibody complex, a second incubation with protein A coupled to horseradish peroxidase (Bio-Rad, München, Germany) was performed. Enzyme activity was localized on the nitrocellulose filter with the enhanced chemiluminescence detection system from Amersham Buchler, Braunschweig, Germany.

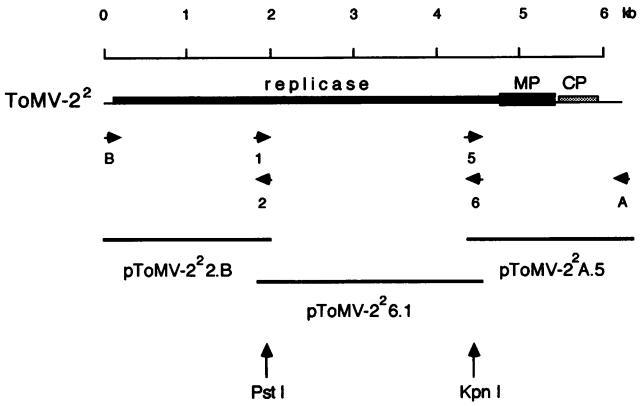

FIG. 1. cDNA cloning strategy for ToMV-2^2. The genomic organization of the ToMV RNA is represented schematically. Open reading frames are boxed (replicase, 126/183-kDa protein; MP, 30-kDa movement protein; CP, coat protein). The positions of the primers used (arrowheads) and the sizes of the corresponding cDNAs (black bars) are indicated.

TABLE 1. Alterations in the nucleotide sequence of the 30-kDa protein open reading frame of ToMV-2^2 in comparison with wild-type ToMV

Nucleotide position	ToMV		ToMV-2^2	
	Codon	Amino acid	Codon	Amino acid[a]
5082	GUA	Val	GUG	Val
5091	AUA	Ile	AUC	Ile
5115	GUC	Val	GUU	Val
5220	CUG	Leu	CUA	Leu
5244	GCU	Ala	GCA	Ala
5293	AAG	Lys	GAG	**Glu**
5456	GGA	Gly	GAA	**Glu**
5475	UCG	Ser	UCA	Ser
5619	AGU	Ser	AGG	**Arg**
5635	AAA	Lys	GAA	**Glu**
5676	UCG	Ser	UCA	Ser
5753	GUA	Val	GUG	Val

[a] Amino acids that are different from those in ToMV are boldfaced.

RESULTS

Cloning of ToMV-2^2. So far, *Tm-2^2*-breaking ToMV strains have been isolated from two locations soon after tomato cultivars containing this resistance gene were introduced. One isolate was identified in The Netherlands (20), and the other isolate was found in Sussex, England (6). Leaf extracts from tomato plants homozygous for the *Tm-2^2* gene, which were systemically infected with a Sussex strain, were obtained from the Institute for Horticultural Research, Littlehampton, England. The tomato leaf extract was inoculated on the local lesion host *N. tabacum* cv. Samsun NN, and virus was purified from a single lesion. For further characterization, this ToMV strain, designated ToMV-2^2, was amplified on a tomato cultivar homozygous for the *Tm-2^2* gene (GCR 267). Viral RNA was isolated and reverse transcribed. Using specific primers, three overlapping cDNAs (pToMV-$2^2$2.B [nucleotides 1 to 1875], pToMV-$2^2$6.1 [nucleotides 1818 to 4418], and pToMV-2^2A.5 [nucleotides 4358 to 6384]) spanning the whole genome of ToMV-2^2 were generated and inserted in pUC18 (Fig. 1). All three cDNA clones contain unique restriction enzyme sites at their ends, thus allowing the reassembly of a full-length cDNA of ToMV-2^2 (Fig. 1).

Sequence analysis of the 30-kDa movement protein of ToMV-2^2. *Tm-2* and *Tm-2^2* are allelic genes that interfere with the transport of wild-type ToMV in infected plants (13). Sequence analysis and recombination between the genes encoding the 30-kDa movement proteins of a wild-type and a *Tm-2*-breaking strain of ToMV demonstrated that two amino acid differences between the 30-kDa proteins encoded by these viruses were sufficient to overcome the resistance (11). Therefore, we speculated that mutations necessary for breaking the *Tm-2^2* resistance might also be localized in the movement protein gene of ToMV. To test this hypothesis, we first sequenced the region from nucleotides 4835 to 5894 of ToMV-2^2, which contains the complete open reading frame for the movement protein and part of the reading frame coding for the coat protein. As summarized in Table 1, 12 base substitutions were found in the sequenced region. Of these, only four substitutions (at positions 5293, 5456, 5619, and 5635) result in amino acid changes within the 30-kDa protein of ToMV-2^2.

Mapping of the mutations that confer the ability to overcome the *Tm-2^2* resistance. Although the substitutions that we found in the amino acid sequence of the ToMV-2^2 30-kDa protein suggested that this altered protein may be involved in the resistance-breaking phenotype, the possibility remained that changes responsible for breaking the *Tm-2^2* resistance are located in the unsequenced region of the ToMV-2^2 genome. In addition, all sequenced ToMV strains that have been experimentally analyzed for overcoming resistance genes in tomato plants (10, 11) as well as the sequenced wild-type ToMV (14) are isolates from Japan. Therefore, differences found between the nucleotide sequences of an English ToMV strain, as described by us, and the nucleotide sequences reported previously might reflect only variations in local ToMV strains. To clarify this point, we generated recombinant viruses containing different parts of the ToMV-2^2 movement protein and examined the ability of the recombinant viruses to overcome the *Tm-2^2* resistance. As the canonical construct, we used plasmid pSLN, which contains a full-length cDNA of ToMV (TMV-L [9]) under the control of the cauliflower mosaic virus 35S RNA promoter (26). This plasmid has been previously shown to give rise to high yields of ToMV after mechanical inoculation of *C. quinoa* plants (26). Likewise, all recombinant viruses used in this study were recovered after cDNA infection with similar yields from *C. quinoa* (>0.1 μg of virus per mg of leaf material), indicating that the introduced mutations did not interfere with basic functions of the respective variant 30-kDa movement proteins.

The first question we asked was whether the gene for the 30-kDa protein of ToMV-2^2 is sufficient to confer the ability to overcome the *Tm-2^2* resistance to wild-type ToMV. To answer this question, we exchanged the region from nucleotides 4950 to 5800 in pSLN, comprising all amino acid substitutions in the 30-kDa movement protein of ToMV-2^2, with the respective region of the ToMV-2^2 cDNA, giving rise to pSL30.2^2 (Fig. 2A). Virus ToMV-30.2^2 was obtained by inoculating *C. quinoa* plants with different concentrations of pSL30.2^2. Subsequently, primary leaves from 3-week-old plants of the tomato cultivar GCR 267 (*Tm-2^2/Tm-2^2*) were inoculated with *Chenopodium* leaf extracts containing 1 μg of wild-type ToMV, 1 μg of ToMV-2^2, or 1 μg of recombinant virus ToMV-30.2^2. After 2 weeks, the infected tomato plants were monitored for systemic infection by visual inspection and by immunodetection of the viral coat protein in the youngest, i.e., noninoculated, leaves. As shown in Fig. 2B, virus coat protein could be detected in the upper leaves of GCR 267 plants inoculated with virus strain

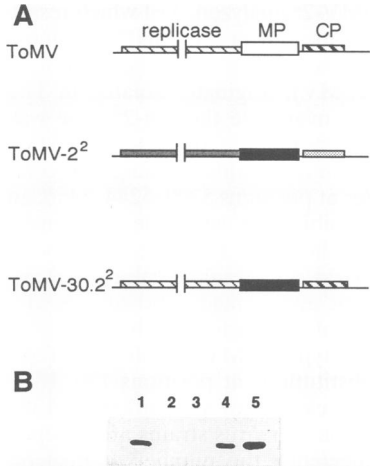

FIG. 2. Mapping of the gene of ToMV-2^2 which is responsible for breaking the *Tm-*2^2 resistance gene. (A) Schematic representation of recombinant viruses used for infection experiments. Open reading frames are boxed (replicase, 126/183-kDa protein; MP, 30-kDa movement protein; CP, coat protein). (B) Immunodetection of the ToMV coat protein in the youngest (not inoculated) leaf of *L. esculentum* cv. Craigella GCR 267 (*Tm-*2^2/*Tm-*2^2) plants 2 weeks after inoculation with different recombinant virus strains. Soluble proteins were extracted from infected leaves. Equal amounts of protein were separated on an SDS–12% polyacrylamide gel and analyzed for the ToMV coat protein by Western blotting. Lanes: 1, 0.1 μg of ToMV; 2 through 5, leaf extracts from tomato plants inoculated with buffer, 1 μg of ToMV, 1 μg of ToMV-2^2, and 1 μg of ToMV-30.2^2, respectively.

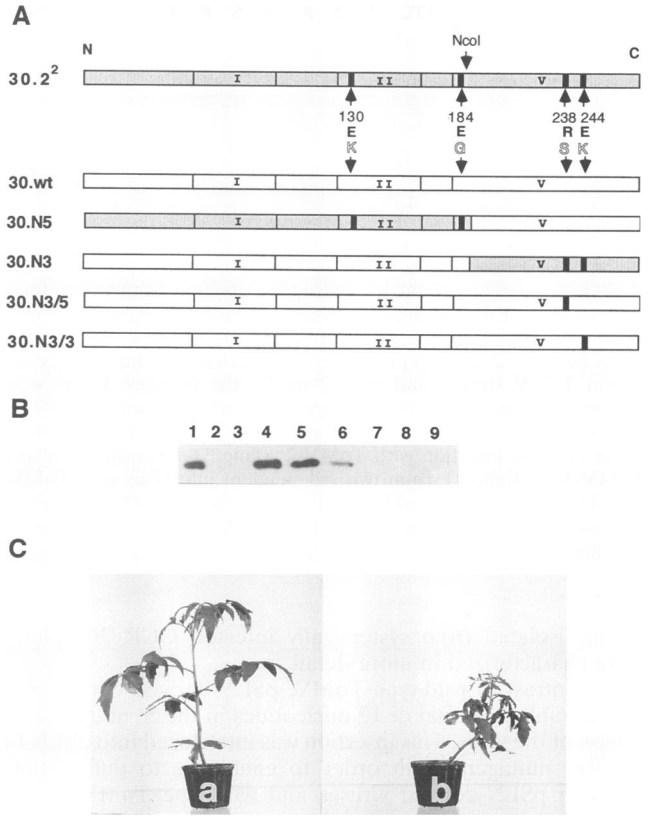

FIG. 3. Analysis of the *Tm-*2^2 resistance-breaking determinants in the ToMV-2^2 30-kDa protein. (A) Schematic representation of the structures of the 30-kDa proteins of different ToMV mutants analyzed for the ability to infect GCR 267 (*Tm-*2^2/*Tm-*2^2) tomato plants. The 30-kDa open reading frame is shown as a box (grey, ToMV-2^2 sequences; white, ToMV sequences). Protein domains defined by Saito et al. (21) are indicated (I and II, conserved regions; V, variable regions A, B, and C). The positions of the amino acid exchanges in ToMV-2^2 compared with wild-type ToMV are indicated by black bars and identified below (ToMV-2^2 in bold face letters, ToMV in outlined letters). (B) Immunodetection of the ToMV coat protein in the youngest (not inoculated) leaf of *L. esculentum* cv. Craigella GCR 267 (*Tm-*2^2/*Tm-*2^2) plants 2 weeks after inoculation with different recombinant virus strains. Soluble proteins were extracted from infected leaves. Equal amounts of protein were separated on an SDS–15% polyacrylamide gel and analyzed for the ToMV coat protein by Western blotting. Lanes: 1, 0.1 μg of ToMV; 2 through 9, leaf extracts from tomato plants inoculated with buffer, 1 μg of ToMV, 1 μg of ToMV-2^2, 1 μg of ToMV-30.2^2, 1 μg of ToMV-30.N3, 1 μg of ToMV-30.N5, 1 μg of ToMV-30.N3/5, and 1 μg of ToMV-30.N3/3, respectively. (C) Phenotype of *L. esculentum* cv. Craigella GCR 267 (*Tm-*2^2/*Tm-*2^2) plants 4 weeks after inoculation with 1 μg of ToMV-30.N5 (a) and 1 μg of ToMV-30.N3 (b).

ToMV-2^2 or ToMV-30.2^2 but not in plants infected with wild-type ToMV. In addition, plants giving rise to the multiplication of virus ToMV-30.2^2 exhibited a typical phenotype (leaf distortion, stunting, systemic necrosis), which is the consequence of resistance breaking by ToMV-2^2 (data not shown). These results clearly indicate that all information necessary for overcoming the *Tm-*2^2 resistance gene is located in the 30-kDa movement protein of ToMV-2^2.

For *Tm-2*-breaking virus strains, it has been shown that not all amino acid exchanges found in the 30-kDa gene are equally important for the resistance-breaking phenotype (11). We therefore analyzed whether all four amino acid replacements in the ToMV-2^2 30-kDa protein are necessary for the ability of ToMV-2^2 to grow on GCR 267 tomatoes. Using a unique *Nco*I restriction enzyme site at position 5463, the ToMV-2^2 30-kDa gene in pSL30.2^2 was split and the 5′ and 3′ parts of this reading frame were exchanged with the respective wild-type sequences. This procedure generated plasmid pSL30N5, which contains the amino acid substitutions at positions 130 and 184, and plasmid pSL30N3, which contains the mutations at positions 238 and 244 (Fig. 3A). Both plasmids gave rise to viral progeny on *C. quinoa*, designated ToMV-30N5 and ToMV-30N3, which were tested for the ability to overcome the *Tm-*2^2 resistance. From these, only virus ToMV-30N3 was able to multiply on GCR 267 tomatoes, causing severe symptoms and a high virus titer in the upper leaves (Fig. 3). To further test whether both or only one of the replacements is necessary for the resistance-breaking property of the ToMV-2^2 movement protein, we replaced either amino acid 238 or amino acid 244 in the 30-kDa protein of pSL30N3 with the appropriate wild-type amino acid by in vitro mutagenesis (Fig. 3A). Both mutant plasmids, pSL30N3/5 and pSL30N3/3, gave high yields of virus on *C. quinoa* after cDNA infection. Neither of the resulting virus strains was, however, able to multiply on tomato cultivars containing the *Tm-*2^2 resistance gene (Fig. 3B).

Analysis of the recombinant resistance-breaking ToMV strains. Although the results obtained with our virus mutants seemed to be very clear-cut, there are at least two sources of error which had to be excluded. On the one hand, the results could be caused by a contamination of the recombinant viruses with ToMV-2^2. On the other hand, it is well known that reversion of point mutations can occur in these kinds of experiments because of the high mutation rates of RNA viruses (10). To rule out these possibilities, recombinant virus

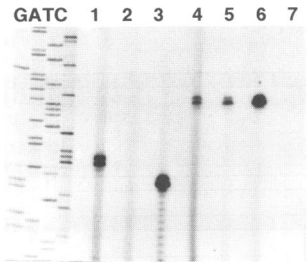

FIG. 4. Analysis of the viral progeny deriving from infection of tomato plants with the *Tm-2²* resistance gene with different recombinant virus strains by primer extension. *L. esculentum* cv. Craigella GCR 267 (*Tm-2²*/*Tm-2²*) plants were inoculated with different recombinant ToMV strains, and after 2 weeks, the youngest leaves were harvested. Primer extension reactions were performed with viral RNAs obtained from authentic ToMV (lane 1), from infection with ToMV (lane 2), from infection with ToMV-2^2 (lane 3), from infection with ToMV-30.2^2 (lane 4), from two independent infections with ToMV-30.N3 (lanes 5 and 6), and from an infection with ToMV-30.N5 (lane 7). Standard sequencing reactions (lanes G, A, T, and C) were used as size markers.

strains isolated from systemically infected GCR 267 plants were characterized in more detail.

In contrast to wild-type ToMV, pSLN-derived viruses contain a stable insertion of 12 nucleotides in the 5′ untranslated region of the virus. This insertion was introduced into pSLN by in vitro mutagenesis in order to enable us to differentiate between pSLN-derived viruses and wild-type ToMV; it has been shown not to interfere with the life cycle of ToMV (26). Accordingly, this insertion should be contained in all of the recombinant resistance-breaking ToMV strains. Genomic RNA was isolated from virus strains ToMV-30.2^2 and ToMV-30N3 after propagation on GCR 267 plants, and the 5′ ends were analyzed by primer extension experiments. As shown in Fig. 4, both virus strains still contained the 12-nucleotide insertion, thus proving that they were not contaminated with ToMV-2^2. In addition, this experiment revealed that ToMV-2^2 is four nucleotides shorter at the 5′ end of the genomic RNA than wild-type ToMV, thus displaying the same genome length at the 5′-end as does TMV (5).

To verify that no reversion of the mutations in the amino-terminal part of the 30-kDa movement protein of ToMV-30N3 had occurred, the open reading frames of the 30-kDa proteins from two independent isolates of virus ToMV-30N3 were amplified by reverse transcription and subsequent PCR. Direct sequence analysis of the PCR products revealed that no mutations apart from the original amino acid substitutions at positions 238 and 244 had accumulated in these virus mutants on GCR 267 tomatoes (data not shown). Taken together, these results confirm that both amino acid substitutions, Ser-238 to Arg and Lys-244 to Glu, in the 30-kDa movement protein of ToMV-2^2 are necessary and sufficient for overcoming the *Tm-2²* resistance in tomato.

DISCUSSION

To understand the mechanism of the *Tm-2²* resistance gene in the tomato, we have cloned the genome of a resistance-breaking strain of ToMV (ToMV-2^2) and determined the nucleotide sequence of the 30-kDa movement protein gene and part of the coat protein gene of this virus. In comparison with the published sequence of a wild-type ToMV strain from Japan, we find 12 nucleotide exchanges in the region of the genome of ToMV-2^2 analyzed, 4 of which result in amino acid substitutions all located in the 30-kDa protein. Recently, the nucleotide sequence of the same region of a different ToMV strain (LIIA-ToMV), originally isolated in The Netherlands, that was able to overcome the *Tm-2²* gene was reported (1). The 30-kDa open reading frames of ToMV-2^2 and LIIA-ToMV have seven nucleotide exchanges in common but differ from each other at positions 5220, 5244, 5456, and 5676, where LIIA-ToMV exhibits the wild-type sequence. Furthermore, LIIA-ToMV displays base substitutions at positions 4909 and 5698, where ToMV-2^2 is identical to ToMV. As a consequence, different amino acid substitutions are found in the 30-kDa proteins of both resistance-breaking virus strains compared with wild-type ToMV. While our ToMV isolate has amino acid substitutions at positions 130, 184, 238, and 244, LIIA-ToMV has exchanges at positions 2, 130, 238, and 244, indicating that the two virus strains are independent isolates.

Quite unexpectedly, the number of nucleotide exchanges observed between both *Tm-2²* resistance-breaking virus isolates (ToMV-2^2 from England and LIIA-ToMV from The Netherlands) in the 30-kDa protein genes in comparison with wild-type ToMV (from Japan) proved to be rather high. In addition, ToMV-2^2 is four nucleotides shorter at the 5′ end of the genomic RNA than the sequenced wild-type ToMV (Fig. 4), thus displaying the same genome length at the 5′ end as does TMV (5, 5a). It is, however, unlikely that these differences are due only to the natural variation among ToMV strains from different geographic origins, because we found no variation in the genome lengths at the 5′ ends and only minor nucleotide exchanges in the 30-kDa gene sequences of other European ToMV isolates compared with the Japanese ToMV strains (27). Furthermore, ToMV strains breaking the *Tm-1* and *Tm-2* resistance genes have been described as displaying only a few nucleotide exchanges compared with wild-type ToMV (10, 11). Therefore, it seems likely that ToMV-2^2 is more distantly related to *Tm-1*- and *Tm-2*-breaking ToMV strains and that ToMV-2^2 on the one hand and *Tm-1*- and *Tm-2*-breaking ToMV strains on the other hand have developed independently from different wild-type virus strains.

To determine which determinants of ToMV-2^2 are responsible for breaking the *Tm-2²* resistance gene, we analyzed recombinants between ToMV-2^2 cDNAs and the infectious cDNA construct pSLN, containing the 35S RNA promoter fused to the wild-type ToMV sequence. These experiments showed for the first time that two amino acid substitutions in close proximity at positions 238 (Ser→Arg) and 244 (Lys→Glu) of the 30-kDa movement protein are both necessary and sufficient for a systemic infection of tomato plants with the *Tm-2²* resistance gene by ToMV. Both sequence alterations have a strong effect on the local charge of the 30-kDa protein, because at position 238 an additional positive charge is introduced (Arg) and at position 244 a positive charge (Lys) is replaced by a negative one (Glu).

Concerning the mechanism of *Tm-2²* resistance, two possibilities can be imagined. One is that a putative resistance factor is an altered form of a host factor that is normally required for cell-to-cell movement of ToMV. Because of the alteration, the molecular interaction between this host factor and the wild-type 30-kDa protein is not possible, resulting in defective virus movement. A functional interaction is restored by a complementary mutation in the 30-kDa protein of resistance-breaking ToMV strains. The other possibility is that the resistance factor is unrelated to the function of the movement protein but leads to the recognition of a specific domain of the wild-type 30-kDa protein, which results in the induction of a (hypersensitive?) defense reaction. The structure and function of the movement

proteins of tobamoviruses have been studied in detail. Comparison of the amino acid sequences led to the definition of protein domains (21). Domain I (amino acids 55 to 98) and domain II (amino acids 126 to 167) are highly conserved in their sequences, whereas domains A, B, and C (amino acids 182 to the carboxy terminus) are conserved only in charge. Deletion analysis of the 30-kDa protein revealed that half of domain B and the complete domain C (amino acids 234 to the carboxy terminus) can be removed without loss of function (4), although this part of the 30-kDa protein can be phosphorylated in vivo (25). Because amino acid substitutions in domains I and II have been found in *Tm-2*-breaking ToMV strains, it has been suggested that resistance conferred by the *Tm-2* locus might be closely related to the function of the 30-kDa protein (11). Therefore, it is a very surprising result of our experiments that the two essential amino acid substitutions for overcoming the $Tm\text{-}2^2$ resistance map to the region of the 30-kDa movement protein (amino acids 234 to the carboxy terminus), which has been found for TMV to be dispensable for virus transport in tobacco plants (4). Both TMV and ToMV, however, are able to systemically infect tobacco and susceptible tomato plants. One explanation for this finding may be that there are differences in virus-host interactions in tobacco and in tomato plants and that this domain of the 30-kDa protein is important only for viral movement in tomato plants. This view is supported by the fact that there is little homology between the 30-kDa movement proteins from TMV and ToMV in the carboxy-terminal parts. A different explanation could be that at least resistance conferred by the $Tm\text{-}2^2$ resistance gene does not interfere with the function of the 30-kDa protein but rather leads to the induction of a hypersensitive defense reaction.

This hypothesis does not rule out, however, that other parts of the ToMV genome and in particular the two other amino acid substitutions in the 5′ region of the 30-kDa protein may support the virus in infecting $Tm\text{-}2^2$ tomato plants. One indication for this may be that differences between various recombinant viruses were observed in infectivity and in the velocity and severity of symptom formation. ToMV-2^2 infection of GCR 267 tomatoes results in leaf curling after 4 to 6 days postinfection (p.i.), which is followed by severe stunting and by systemic necrosis after 2 to 3 weeks p.i. In contrast, infection with ToMV-30.2^2, which contains the ToMV-2^2 30-kDa protein in a wild-type ToMV background, does not lead to visible disease symptoms until 6 to 9 days p.i. In addition, symptom formation is less severe, and systemic necrosis is observed much later than with ToMV-2^2, although the same infection rates are found for the two viruses. ToMV-30N3, which contains only the two carboxy-terminal amino acid exchanges of the ToMV-2^2 30-kDa protein in a wild-type ToMV background, shows even more delayed symptom development (9 days p.i.). The damage to the plant which is caused by ToMV-30N3 is, however, more severe than the damage caused by ToMV-2^2 because of an earlier onset of a systemic necrosis (Fig. 3C). On the other hand, the infection rate of ToMV-30N3 is reduced to 40% of the rate observed for ToMV-2^2 and ToMV-30.2^2. This effect is found only in the context of the $Tm\text{-}2^2$ resistance gene. On wild-type tomato plants or on other hosts like *C. quinoa*, all virus mutants perform equally well in systemic spread and symptom formation. One explanation for this phenomenon may be that the additional amino acid substitutions in the amino-terminal part of the 30-kDa protein are necessary to compensate for possible changes in the protein structure caused by the substitutions at positions 238 and 244. For example, the 30-kDa protein may need two domains for optimal virus spread on tomato plants which contain the $Tm\text{-}2^2$ resistance gene. One region, the carboxy-terminal part, of the 30-kDa protein may be recognized by a host factor as an avirulence gene and may lead to the induction of a defense reaction, possibly a hypersensitive response. Therefore, it may be necessary for ToMV-2^2 to change the structure of this domain to evade the recognition event. The second region of the 30-kDa protein (at the amino terminus around amino acid 130) may be important to repress the formation of severe symptoms and in that way allow a more efficient invasion of tomato plants with the $Tm\text{-}2^2$ resistance gene.

Although our experiments have shed some light on the interaction between ToMV and the $Tm\text{-}2^2$ resistance gene, further experiments are needed to elucidate the mechanism of this defense reaction. One important approach to study the interaction between the 30-kDa protein and putative host factors is the generation of transgenic plants which express the 30-kDa movement proteins of different ToMV strains. This technique has been already applied successfully for investigations concerning the mechanism of the N′ resistance gene in tobacco (2, 18). In particular, expression of 30-kDa protein mutants in transgenic tomato plants with the $Tm\text{-}2^2$ resistance gene may help to address questions regarding the function of different domains of this protein in overcoming the $Tm\text{-}2^2$ resistance.

ACKNOWLEDGMENTS

We thank T. Meshi and Y. Okada for plasmid pLFW3, P. Grimbley for different strains of *L. esculentum* cv. Craigella and of ToMV, R. G. Herrmann for support during the course of these investigations, and K.-W. Mundry for his generous gift of tobacco seeds and for many stimulating discussions. Finally, we thank U. M. Pfitzner for many fruitful discussions and for critical reading of the manuscript.

This work was supported by grant Fa138/3-7;9 from the Deutsche Forschungsgemeinschaft to A. J. P. Pfitzner.

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