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Mutations in the Tobacco Mosaic Virus 30-kD Protein Gene Overcome *Tm-2* Resistance in Tomato

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A resistance-breaking strain of tobacco mosaic virus (TMV), Ltb1, is able to multiply in tomatoes with the *Tm-2* gene, unlike its parent strain, L. Nucleotide sequence analysis of Ltb1 RNA revealed two amino acid changes in the 30-kD protein: from Cys^{68} to Phe and from Glu^{133} to Lys (from L to Ltb1). Strains with these two changes generated in vitro multiplied in tomatoes with the *Tm-2* gene and induced essentially the same symptoms as those caused by Ltb1. Strains with either one of the two changes did not overcome the resistance as efficiently as Ltb1, although increased levels of multiplication were observed compared with the L strain. Results showed that both mutations are involved in the resistance-breaking property of Ltb1. Sequence analysis indicated that another resistance-breaking strain and its parent strain had two amino acid changes in the 30-kD protein: from Glu^{52} to Lys and from Glu^{133} to Lys. The fact that the amino acid changes occurred in or near the well conserved regions in the 30-kD protein suggests that the mechanism of *Tm-2* resistance may be closely related to the fundamental function of the 30-kD protein, presumably in cell-to-cell movement.

INTRODUCTION

Three genes, *Tm-1*, *Tm-2*, and its allele, $Tm\text{-}2^2$, have been recognized in tomatoes as conferring resistance to tobacco mosaic virus (TMV) (Pelham, 1966; Hall, 1980). Unlike the resistance conferred by the *Tm-1* gene (Motoyoshi and Oshima, 1977; Watanabe et al., 1987), the resistance conferred by the *Tm-2* or $Tm\text{-}2^2$ gene is expressed only in whole plants or in leaf disks but not in isolated protoplasts (Motoyoshi and Oshima, 1975, 1977; Stobbs and MacNeill, 1980). Fluorescent antibody staining of leaf epidermis of tomato plants with the *Tm-2* or $Tm\text{-}2^2$ genes indicated that, in the initial stage of infection, spread of wild-type TMV in these tomatoes was restricted (Nishiguchi and Motoyoshi, 1987; F. Motoyoshi and M. Nishiguchi, unpublished observation). In addition, *Tm-2* resistance is known to be broken by pre-inoculation with a helper virus, such as potato virus X, that belongs to another taxonomic group, and this observation can be interpreted as complementation in cell-to-cell movement (Taliansky et al., 1982; Atabekov and Dorokhov, 1984). These observations thus suggest that *Tm-2* and $Tm\text{-}2^2$ resistance operates at the level of cell-to-cell movement of the virus. *Tm-2* and $Tm\text{-}2^2$ resistance is often accompanied by a hypersensitive response, especially in heterozygous tomato plants (*Tm-2*/+ and $Tm\text{-}2^2$/+) and at higher temperatures (Pelham, 1966, 1972; Hall, 1980), but the relationship between this response and the blockage of cell-to-cell movement is unclear.

It has been evident that many plant viruses encode the function required for cell-to-cell movement (Atabekov and Dorokhov, 1984). In the case of TMV, the 30-kD protein has been shown by molecular genetic techniques to be involved in cell-to-cell movement (Deom, Oliver, and Beachy, 1987; Meshi et al., 1987). Immunocytochemical localization has shown that the 30-kD protein accumulates in plasmodesmata (Tomenius, Clapham, and Meshi, 1987). However, little is known about the molecular mechanism of cell-to-cell movement. Since *Tm-2* and $Tm\text{-}2^2$ resistance may be manifested as a restriction of cell-to-cell movement, an interaction might be expected between the 30-kD protein and the putative host resistance factor existing in the resistant tomatoes. Analyses of resistance-breaking strains will clarify this point and, in addition, provide a basis for further investigation concerning the resistance mechanisms as well as cell-to-cell movement.

TMV Ltb1 (Motoyoshi, 1984; F. Motoyoshi and M. Nishiguchi, in preparation) occurred spontaneously from a wild-type tomato strain, TMV L (Ohno et al., 1984); tomato strains of TMV are also referred to as tomato mosaic virus (Van Regenmortel and Fraenkel-Conrat, 1986). The Ltb1 mutant can propagate in tomatoes with the *Tm-2* gene, unlike the L strain. Recently, we have identified the mutations responsible for the ability to overcome *Tm-1* resist-

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ance by genetic analyses of biologically active cDNA clones derived from a resistance-breaking strain, TMV Lta1 (Meshi et al., 1988). Using this same strategy, we have now mapped the mutations that enable Ltb1 to overcome *Tm-2* resistance. As expected, the results show a strong correlation between the ability to overcome *Tm-2* resistance and alterations of the 30-kD protein.

RESULTS

Comparison of the Nucleotide Sequence Between L and Ltb1

Unlike TMV L, Ltb1 propagates in tomatoes with the *Tm-2* gene. In GCR 236, homozygous for the *Tm-2* and *nv* genes, Ltb1 induces mosaic symptoms in systemic leaves and, in addition, when inoculated into cotyledons, causes about half the plants to develop necrosis in hypocotyls (Table 1) (Motoyoshi, 1984; F. Motoyoshi and M. Nishiguchi, in preparation). Since *Tm-2* resistance may be related to the function of the 30-kD protein, we first sequenced the 3′-terminal one-fourth of the genomic RNA of Ltb1, including the 30-kD and coat protein genes and the 3′-noncoding region, and compared this sequence with the parental L sequence.

As summarized in Figure 1, five base substitutions were found in the sequenced region. Of these, two substitutions (at positions 5108 and 5302) resulted in amino acid changes in the 30-kD protein: from Cys^{68} to Phe and from Glu^{133} to Lys (from L to Ltb1; see Figure 2). The other three substitutions did not affect amino acid sequences. The substitutions in the 30-kD protein gene at position 5475 and in the coat protein gene at position 5966 were identical to those found in other L-derived strains, Ls1 (a temperature-sensitive mutant in cell-to-cell movement; Ohno et al., 1983), $L_{11}A$ (an attenuated strain; Nishiguchi et al., 1985), and Lta1 (a resistance-breaking strain against the *Tm-1* gene; Meshi et al., 1988). In the cases of Ls1 and Lta1, these substitutions have been concluded to be unrelated to their phenotypes (Meshi et al., 1987, 1988).

Mapping of the Mutations That Confer the Ability To Overcome *Tm-2* Resistance

We expected that the mutations responsible for resistance breaking would be one or both of the base substitutions causing amino acid changes in the 30-kD protein. How-

Table 1. Characteristics of Strains Generated in Vitro

	Amino Acid[a]		GCR 26 (+/+)			GCR 236 (*Tm-2*, *nv*/*Tm-2*, *nv*)		
				Symptoms[c]			Symptoms[c]	
Strain	At 68	At 133	Multiplication[b]	Total	Diseased	Multiplication[b]	Total	Diseased
L	Cys	Glu	++	29	M (29)	–	34	None
W3	Cys	Glu	++	11	M (11)	–	20	None
Ltb1	Phe	Lys	++	18	M (16)	++	47	MSt (47) Nh (19)
B1	Phe	Lys	++	28	M (28)	++	36	MSt (35) Nh (11)
B3	Phe	Glu	++	58	M (57)	+	104	MSt (54) Nh (7) Nc (76)
B4	Cys	Lys	++	45	M (43)	+ or –	100	Nc (36)
B7	Phe	Lys	++	24	M (23)	++	46	MSt (43) Nh (25)

[a] Amino acids at positions 68 and 133 of the 30-kD protein of each strain.

[b] Multiplication was assessed as accumulation of the coat protein in the inoculated cotyledons 5 to 8 days after inoculation. ++, accumulation (in GCR 26) was similar to (at least more than 10^{-1}) that of L (5 to 10 mg/g of tissue) or (in GCR 236) that of Ltb1 (usually 1 to 3 mg/g of tissue); +, accumulation was less than 10^{-1} of that of Ltb1 in GCR 236; –, accumulation was undetected.

[c] Typical symptoms caused by each strain. The number of plants inoculated is shown in the "Total" column; the number of those developing each symptom is shown in parentheses in the "Diseased" column. M, systemic mosaic symptoms; MSt, systemic mosaic and/or stunting (mosaic symptoms are unclear on severely stunted plants); Nh, necrosis in hypocotyls; Nc, local necrotic lesions on the inoculated cotyledons. In some assays, Ltb1, B1, or B7 also caused necrosis (but not local necrotic lesions) on the inoculated cotyledons of GCR 236. Note that multiplication and visible symptoms on GCR 236 seem to be affected by uncontrollable environmental factors, particularly in the cases of B3 and B4. We repeated assays throughout the year but symptom severity, including the number of plants developing Nh and/or Nc, varied from assay to assay.

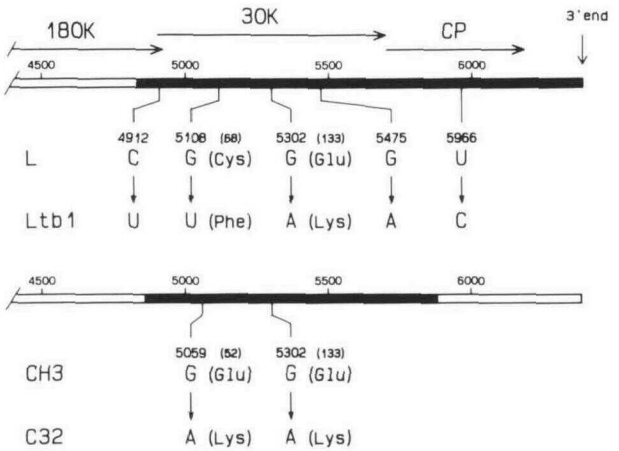

Figure 1. Summary of Nucleotide Substitutions and Resultant Amino Acid Changes Found between TMV L and Ltb1 and between CH3 and C32.

Scaled horizontal bars denote the genomic RNA (upper, L and Ltb1; lower, CH3 and C32). The regions sequenced and compared are shown in black. At the top, locations of the 180-kD protein (180k), the 30-kD protein (30k), and the coat protein (CP) genes are shown with horizontal arrows. Nucleotide positions of the genomic RNA are numbered from the 5′-terminal G residue (Ohno et al., 1984), and amino acid residues of the 30-kD protein are numbered from the N-terminal methionine residue.

ever, the possibility remained that the responsible change(s) might be located in the upstream unsequenced region. To clarify this, we generated cDNA clones containing these substitutions and used RNA transcripts generated in vitro to examine their ability to overcome *Tm-2* resistance.

pLFW3, carrying a full-length cDNA copy of TMV L RNA, has been used as the standard plasmid to produce in vitro transcripts that are biologically equivalent to TMV L RNA (Meshi et al., 1986). In this work, progeny viruses derived from the respective pLF plasmids shown in Figure 3 were first prepared from the inoculated leaves of tobacco and used as inocula for further assays with tomatoes (see Methods). All the progeny viruses were recovered with a similar yield, so the introduced mutations were not thought to cause any serious decrease in the activity of the 30-kD protein. Progeny viruses are hereafter indicated by the last two letters of the corresponding plasmid names. Accordingly, W3, derived from pLFW3, is equivalent to TMV L.

B1 was derived from pLFB1 (Figure 3), and, consequently, it differs from W3 only in three bases in the 30-kD protein gene. B1 multiplied in GCR 236 (*Tm-2*, *nv*/*Tm-2*, *nv*) as well as in GCR 26 (+/+) and induced symptoms indistinguishable from those caused by Ltb1 (Figure 4 and Table 1). The level of accumulation of B1 in the inoculated cotyledons was also similar to that of Ltb1. Therefore, these three base substitutions in the 30-kD protein gene are sufficient to explain the ability of Ltb1 to overcome *Tm-2* resistance.

We also generated three additional strains, B3, B4, and B7, which had one or both of the base substitutions causing the amino acid changes (Figure 3). All three strains multiplied to levels similar to those of L and Ltb1 in GCR 26, which lacks the *Tm-2* gene, and caused mosaic symptoms (Figure 4 and Table 1). In the *Tm-2* tomatoes, B3 multiplied, causing mosaic symptoms in the systemic leaves and necrosis in the hypocotyls of several plants, although the frequency was low relative to Ltb1 (Table 1). In addition, B3 induced necrosis (usually necrotic local lesions) on the inoculated cotyledons in most assays (Table 1). In the cases of Ltb1, B1, and B7, necrotic local lesions were not visible on the cotyledons. Accumulation of B3 was always lower than that of Ltb1 both in the cotyledons (Figure 4) and in the systemic leaves (about one-tenth the level of Ltb1 or less). Progeny viruses were recovered from the systemic leaves of B3-inoculated GCR 236 and their genomic RNAs were partially sequenced. At least in the

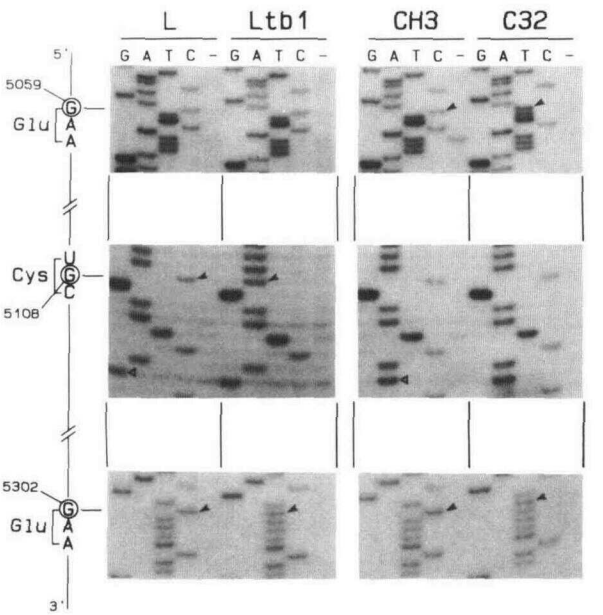

Figure 2. Nucleotide Sequencing of the Genomic RNAs of L, Ltb1, CH3, and C32.

The sequence at the left of each pair represents that of TMV L and CH3. Nucleotides whose substitutions result in amino acid changes are circled and the corresponding bands in the autoradiograms are indicated with arrowheads. Triangles point to a base difference at position 5115 between L and CH3 that does not cause an amino acid change. G, A, T, and C indicate the reaction in the presence of the corresponding dideoxynucleotide, and − indicates the reaction in the absence of dideoxynucleotides.

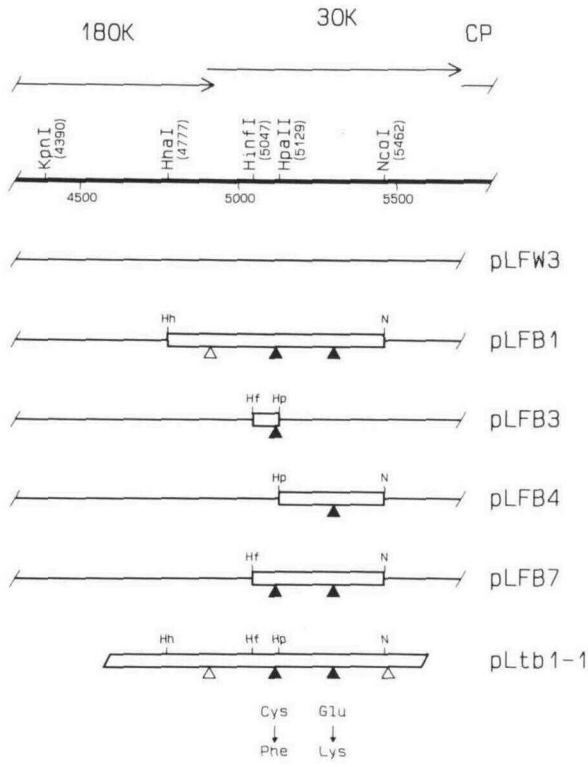

Figure 3. Structures of Constructed Plasmids.

Restriction enzyme sites used are shown on the scale. The numbers of the first bases of the recognition sequences are indicated in parentheses. TMV L- and Ltb1-derived sequences are shown with lines and boxes, respectively. The ends of the cDNA insert of pLtb1-1 have not been well characterized. Nucleotide substitutions from L to Ltb1 are shown with triangles under the Ltb1-derived sequence: △, silent substitutions; ▲, substitutions causing amino acid changes. Abbreviations used are: Hh, HhaI; Hp, HpaII; Hf, HinfI; N, NcoI.

sequenced region of 300 to 500 nucleotides, including both positions 5108 and 5302, no obvious base alterations were detected in four independent preparations (not shown). It is likely, therefore, that the accumulating virus causing symptoms in GCR 236 was B3 itself, and that B3 has an ability to multiply in the *Tm-2* tomatoes, although not as well as Ltb1.

Another mutant, B4, did not cause apparent visible systemic symptoms on the *Tm-2* tomatoes but sometimes elicited necrotic lesions on the inoculated cotyledons (Table 1). The accumulation of B4 in the inoculated cotyledons was very low (Figure 4) or undetectable (at most 0.2 mg/g of tissue). Quantities of progeny viruses sufficient for sequencing could not be recovered from the systemic leaves of B4-inoculated GCR 236 in five independent trials.

In contrast, B7, whose 30-kD protein had both of the amino acid changes present in Ltb1, multiplied in the *Tm-2* tomatoes as efficiently as Ltb1 (Figure 4); these plants developed visible symptoms indistinguishable from those caused by Ltb1 and B1 (Table 1). From these observations, we conclude that both base substitutions, presumably through the resultant amino acid changes, are involved in the ability to overcome the *Tm-2* resistance expressed in GCR 236 tomatoes.

Analysis of an Independently Isolated Resistance-Breaking Strain

To determine whether the changes in the Ltb1 30-kD protein are commonly found in other strains that overcome *Tm-2* resistance, we sequenced the 30-kD protein gene of an independently isolated resistance-breaking strain, C32. C32 occurred spontaneously from a wild-type tomato strain, CH3. C32 and Ltb1 cause similar symptoms on tomato plants.

Sequencing revealed two base substitutions in the 30-kD protein gene between CH3 and C32, both of which caused the same type of amino acid change, from Glu to

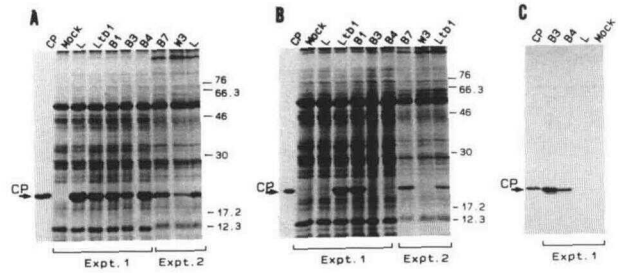

Figure 4. Accumulation of the TMV Coat Protein in Cotyledons.

Viruses and buffer (mock) were inoculated into cotyledons of GCR 26 [+/+; **(A)**] and GCR 236 [*Tm-2*, *nv*/*Tm-2*, *nv*; **(B)** and **(C)**] and harvested 7 days (Expt. 1) and 6 days (Expt. 2) after inoculation. Expts. 1 and 2 are independent assays.

(A) Proteins from 1 mg of tissue and 3 μg of the coat protein (lane CP) were loaded on an SDS-12% polyacrylamide gel. The position of the coat protein is indicated at the left of the gel. Positions of the molecular weight markers (British Drug House Ltd.) are shown at the right as molecular weight × 10^{-3}; 12.3, cytochrome c; 17.2, myoglobin; 30, carbonic anhydrase; 46, ovalbumin; 66.3, bovine serum albumin; 76, ovotransferrin.

(B) Proteins from 2 mg (Expt. 1) and 0.8 mg (Expt. 2) of tissue and 1.5 μg of the coat protein (lane CP) were loaded on a gel. Reasons for differences in the intensities of several host protein bands between Expts. 1 and 2 are not known.

(C) Protein gel blotting analysis of the coat protein. Proteins from 1.3 mg of tissue and 10 ng of the coat protein (lane CP) were loaded. The coat protein was visualized by an avidin-biotin-alkaline phosphatase complex method (ABC kit, Vector Co.).

Lys (Figures 1 and 2). The Glu^{133}-to-Lys change was the same as that found in Ltb1. Interestingly, this mutation alone was less effective in its ability to overcome *Tm-2* resistance than the other mutation (B4 versus B3, Table 1). The Glu^{52}-to-Lys change in C32 occurred close to the position of the second substitution found in the Ltb1 RNA involved in resistance breaking (at position 5108). Between L and CH3, two silent substitutions were found: C at position 5115 and G at position 5475 in the L sequence were, respectively, U and A in the CH3 sequence (Figure 2).

Although we do not know whether there are other changes in the unsequenced regions, both amino acid changes in the 30-kD protein are probably necessary (based on our results from Ltb1) for C32 to overcome *Tm-2* resistance. Evidently, there are at least two ways to confer on TMV the ability to overcome *Tm-2* resistance.

DISCUSSION

From the comparative sequence analysis between TMV L and Ltb1 and subsequent reversed-genetic analysis, we have identified two base substitutions causing amino acid changes in the 30-kD protein (Cys^{68}-to-Phe and Glu^{133}-to-Lys) that enable TMV to overcome the resistance conferred by the *Tm-2* gene. Sequence comparison between an independently isolated resistance-breaking strain, C32, and its parental strain, CH3, has also revealed two amino acid changes in the 30-kD protein: Glu^{52}-to-Lys and Glu^{133}-to-Lys. Although the Glu^{133}-to-Lys change was found in both resistance-breaking strains, the analysis of strains constructed in vitro showed that this change alone (corresponding to B4) was not sufficient to overcome the *Tm-2* resistance expressed in a tomato line, GCR 236. It is likely, therefore, that both amino acid changes are involved in the ability of C32 to overcome *Tm-2* resistance, as is also the case with Ltb1. These results, showing that *Tm-2* resistance is overcome by alterations of the 30-kD protein, imply an interaction between the 30-kD proteins of one or both of the wild-type and resistance-breaking strains and a putative host resistance factor. The facts that, (1) the 30-kD protein is a viral factor involved in cell-to-cell movement (Deom, Oliver, and Beachy, 1987; Meshi et al., 1987), (2) its expression is unnecessary for replication in protoplasts (Meshi et al., 1987), and (3) temperature-sensitive defectiveness in cell-to-cell movement can be complemented by a *trans*-acting factor of helper viruses (Atabekov and Dorokhov, 1984), can explain the previous observations that, (1) TMV L is localized at early stages of infection in single isolated epidermal cells of *Tm-2* tomatoes (F. Motoyoshi and M. Nishiguchi, unpublished observation), (2) *Tm-2* resistance is not expressed in protoplasts (Motoyoshi and Oshima, 1977; Stobbs and MacNeill, 1980), and (3) the resistance can be broken by a helper virus (Taliansky et al., 1982). As a result, three resistance genes, *Tm-1* and *Tm-2* in tomato and *N′* in tobacco, have been related to the 130-kD/180-kD protein genes (Meshi et al., 1988), the 30-kD protein gene, and the coat protein gene (Saito et al., 1987; Knorr and Dawson, 1988), respectively.

Alignment of the 30-kD protein sequences of several TMV strains reveals two relatively well-conserved regions in the middle portion of the 30-kD protein, which probably are important for the cell-to-cell movement function (Saito et al., 1988). Temperature-sensitive mutations for cell-to-cell movement have been found in one of these two well-conserved regions (Ohno et al., 1983; Zimmern and Hunter, 1983; Meshi et al., 1987). As shown in Figure 5, the amino acid changes responsible for overcoming *Tm-2* resistance have been mapped at or close to the well-conserved regions, and *Tm-2* resistance would therefore seem to correlate closely with the function of the 30-kD protein.

Concerning the mechanism of *Tm-2* resistance, two possibilities can be imagined. One is that a putative resistance factor might be an altered form of a host factor that is normally required for cell-to-cell movement of TMV. The molecular interaction between this host factor and the wild-type 30-kD protein might be perturbed, resulting in defective cell-to-cell movement. A productive interaction might subsequently be restored through mutations in the 30-kD protein. The other possibility is that the resistance factor might be unrelated to the normal transport function and might be synthesized solely to inhibit the function of the wild-type 30-kD protein. Altered 30-kD proteins of resistance-breaking strains could thus evade or repress the resistance reaction(s). The 30-kD protein has recently been detected in plasmodesmata of TMV-infected tobacco leaves (Tomenius, Clapham, and Meshi, 1987). It will be interesting to determine whether the resistance factor also localizes and functions in plasmodesmata, or whether resistance is related to the localization process per se.

It is known that the *Tm-2* locus is also involved in induction of a hypersensitive response (Pelham, 1972; Hall, 1980). At present, the hypersensitive response and the inhibition of cell-to-cell movement cannot necessarily be ascribed to a single gene because it is still unclear how many genes the *Tm-2* locus contains. Since visible necrosis should follow virus multiplication and spread in tissue, strains causing necrosis, including B3 and B4, probably overcome impediments to cell-to-cell movement at least partially, and their low levels of multiplication might result from secondary effects of necrogenesis, as is also the case with tobacco carrying the *N* gene (Moser et al., 1988). On the other hand, a direct relationship between the 30-kD protein and the induction of the hypersensitive response in the *Tm-2* tomato can also be hypothesized. Some types of 30-kD proteins (including the wild-type)

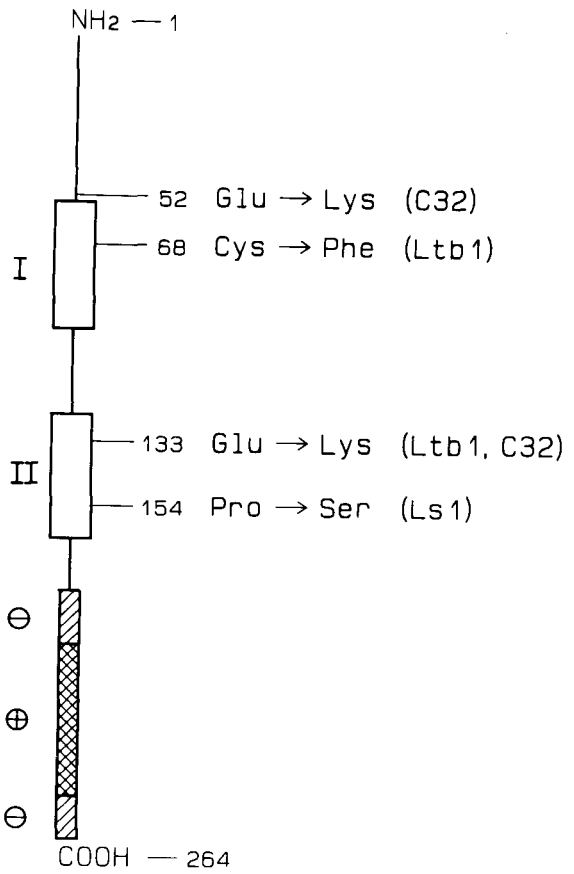

Figure 5. Schematic Representation of the Characteristics of the 30-kD Protein and Location of the Substitutions Found in L- and CH3-Derived Mutants.

The L 30-kD protein, drawn as a representative, is composed of 264 amino acids. Ls1 is a mutant that is temperature-sensitive in cell-to-cell movement; its temperature sensitivity is conferred by a single base substitution in the 30-kD protein gene (Meshi et al., 1987). Well-conserved regions (I and II) are indicated with boxes. Regions rich in positively (+) and negatively (−) charged residues are emphasized with cross-hatched and hatched boxes, respectively. Characterization is according to Saito et al. (1988).

might be recognized as triggers for a hypersensitive response elicited by the *Tm-2* and *nv* genes under certain developmental and environmental conditions. The 30-kD proteins of resistance-breaking strains could evade recognition by the host resistance factor, leading to subsequent visible necrosis, or could repress some step in the induction process. One simple explanation is that an abnormal interaction between the 30-kD protein and the host resistance factor, that normally results in defective cell-to-cell movement, might also trigger the hypersensitive response. Further investigations are required to elucidate the molecular mechanisms underlying these phenomena.

METHODS

Viruses

TMV L is a Japanese tomato strain (Ohno et al., 1984) isolated in Hokkaido. Ltb1 was isolated without mutagenesis from a TMV L-inoculated tomato with the *Tm-2* gene (GCR 236; see below) (Motoyoshi, 1984; F. Motoyoshi and M. Nishiguchi, in preparation). Briefly, TMV L was inoculated onto cotyledons of GCR 236 and a resistance-breaking strain was recovered from a leaf with weak mosaic symptoms at about 7 weeks postinoculation. After six successive single lesion transfers using *Nicotiana tabacum* cv Xanthi nc, the virus was amplified in GCR 236 and named TMV Ltb1. TMV CH3 is also a wild-type tomato strain, similar to TMV L but isolated in Chiba prefecture. C32, which has the ability to overcome *Tm-2* resistance, was derived from CH3 without mutagenesis (F. Motoyoshi and M. Nishiguchi, in preparation).

Nucleotide Sequencing

Sequencing of genomic RNA was performed by the dideoxy chain termination method using reverse transcriptase and 5′ end-labeled primers as described by Meshi et al. (1983) with slight modifications. Primers were prepared from a cDNA clone, pL-1-13 (Takamatsu et al., 1983), by restriction digestion, and end-labeled with γ-^{32}P-ATP and T4 polynucleotide kinase (Maxam and Gilbert, 1980). Two synthetic oligonucleotides, also used as primers, hybridized to nucleotides 5131 to 5157 and 5361 to 5387 of the genomic RNA. Residues not identified by this method were determined by the method of Maxam and Gilbert (1980) using cDNA clones of Ltb1. The 3′ end of the genomic RNA was sequenced by the method of Peattie (1979).

Plasmid Construction

Standard recombinant DNA techniques used were essentially according to Maniatis, Fritsch, and Sambrook (1982) unless otherwise specified.

cDNA clones of TMV Ltb1 were prepared as described by Takamatsu et al. (1983). pLtb1-1 carries an approximately 1-kb insert covering the sequences coding for the C-terminal portion of the 180-kD protein and most of the 30-kD protein (Figure 3). pLFW3 is a full-length cDNA clone of TMV L, which produces an infectious RNA after in vitro transcription (Meshi et al., 1986). pLFB1 was constructed by replacing the 0.68-kb HhaI/NcoI fragment (positions 4780 to 5462) of pLFW3 with the corresponding fragment of pLtb1-1 (Figure 3). For pLFB3, pLFB4, and pLFB7, the HhaI/NcoI fragment was further divided into three fragments with HinfI at position 5047 and HpaII at position 5129, and one or two of the three fragments generated were replaced with the corresponding fragments derived from pLtb1-1 (Figure 3).

In Vitro Transcription of Infectious RNAs and Purification of Progeny Viruses

In vitro transcription of infectious TMV RNAs from full-length cDNA clones was performed as described previously (Ahlquist et al.,

1984; Meshi et al., 1987). Viruses derived from in vitro transcripts were prepared from the inoculated leaves of tobacco (*N. tabacum* L cv Samsun) as described by Meshi et al. (1988). For each construct, progeny viruses derived from two or three independently isolated clones were prepared and used throughout the work to confirm the results. The genomic sequence of each progeny virus around the positions at which base substitutions were found between L and Ltb1 was confirmed by the dideoxy method prior to further use.

Infectivity Assay

Tomato plants (*Lycopersicon esculentum* Mill.) used were nearly isogenic lines, GCR 26 (+/+; without either the *Tm-2* or *nv* gene) and GCR 236 (*Tm-2*, *nv*/*Tm-2*, *nv*), originally supplied by the Glasshouse Crops Research Institute, Littlehampton, United Kingdom. GCR 236 carries the *nv* mutation, which is closely linked to the *Tm-2* gene and causes yellowing and stunting (Pelham, 1966). Viruses were inoculated into cotyledons at concentrations of 10 to 50 μg/ml. Viral propagation was assessed by the accumulation of the coat protein in cotyledons 3 to 8 days after inoculation, and in systemic leaves 3 to 4 weeks after inoculation, essentially as described previously (Meshi et al., 1988). The coat protein on SDS-polyacrylamide gels was visualized by Coomassie staining or by protein gel blotting essentially as described previously (Ey and Ashman, 1986; Saito et al., 1986).

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