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Two concomitant base substitutions in the putative replicase genes of tobacco mosaic virus confer the ability to overcome the effects of a tomato resistance gene, *Tm-1*

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A resistance-breaking strain of tobacco mosaic virus (TMV), Lta1, is able to multiply in tomatoes with the *Tm-1* gene, unlike its parent strain, L. Comparison of the genomic sequences of L and Lta1 revealed two base substitutions resulting in amino acid changes in the 130 and 180 kd proteins: Gln-979 → Glu and His-984 → Tyr. To clarify their involvement in the resistance-breaking property of Lta1, the two substitions were introduced into L by an *in vitro* transcription system to generate a mutant strain, T1. T1 multiplied in *Tm-1/Tm-1* tomatoes with symptoms as did Lta1. Two additional mutant strains were constructed, each of which had one base substitution which caused a His-984 → Tyr change (T2) or a Gln-979 → Glu change (T3). T3 multiplied in tomato plants and protoplasts with the *Tm-1* gene, indicating that the single base substitution is sufficient to overcome the resistance. T2 also multiplied, but its multiplication was greatly decreased. Although no sequence changes were detected in any progeny viruses recovered from plants without the *Tm-1* gene, progeny viruses recovered from T2- or T3- inoculated *Tm-1/Tm-1* tomatoes contained in most cases viruses with additional second base substitutions. They caused amino acid changes near the mutagenized residues, suggesting that the ability of T3 to overcome the resistance is not the same as that of Lta1. Sequencing of the genomic RNAs of other independently isolated resistance-breaking strains revealed the same two base substitutions found in the Lta1 RNA. These observations suggest that the two concomitant base substitutions, and possibly also the resulting amino acid changes, guarantee successful replication of these TMV strains in tomatoes containing the *Tm-1* gene. A strong correlation was found between the ability to overcome the resistance and a decrease in local net charge, suggesting the involvement of an electrostatic interaction between the viral 130 and 180 kd proteins and a putative host resistance factor.

Key words: nucleotide sequence/*Tm-1* resistance/tobacco mosaic virus/viral multiplication

Introduction

Three tomato resistance genes, *Tm-1*, *Tm-2* and *Tm-2*^{2}, have been identified in crossings between wild and cultivated species of tomatoes, and used in practice to prevent systemic mosaic symptoms and the losses in fruit yield and quality caused by tobacco mosaic virus (TMV) (Pelham, 1966, 1972; Fraser, 1985). In tomato plants with these genes, multiplication of TMV is inhibited. In the case of the *Tm-1* gene, the inhibition of multiplication is more effective in homozygotes (*Tm-1/Tm-1*) than in heterozygotes (*Tm-1*/+) (Fraser and Loughlin, 1980; Fraser *et al.*, 1980; Motoyoshi, 1982; Watanabe *et al.*, 1987b). Unlike the resistance conferred by the *Tm-2* and *Tm-2*^{2} genes (Motoyoshi and Oshima, 1975, 1977), *Tm-1* resistance is also expressed in protoplasts isolated from both plants and suspension-cultured cells even in the presence of actinomycin D (Motoyoshi and Oshima, 1977; Motoyoshi, 1982; Watanabe *et al.*, 1987b). Since inoculation with viral RNA does not prevent the inhibition, the inhibition must operate after uncoating and before assembly of progeny during multiplication (Motoyoshi and Oshima, 1979). However, little is known at present about what viral and host components are involved in the *Tm-1* resistance.

The genomic structure of TMV has been well characterized, as a single-stranded, positive-sense RNA of ~6400 nt (Van Regenmortel and Fraenkel-Conrat, 1986). The genomic RNA encodes at least three non-structural proteins (130, 180 and 30 kd proteins) and the coat protein (Goelet *et al.*, 1982; Ohno *et al.*, 1984). The 130 and 180 kd proteins are N-coterminal and overlapping; the latter is synthesized by read-through of the amber termination codon of the 130 kd protein gene (Pelham, 1978). Involvement of the 130 and 180 kd proteins in replication (Ishikawa *et al.*, 1986 and unpublished observation) and of the 30 kd protein in cell-to-cell movement (Deom *et al.*, 1987; Meshi *et al.*, 1987) has been demonstrated.

Characterization of resistance-breaking (virulent) strains and subsequent comparison with avirulent strains are one possible approach to understanding the resistance mechanisms, i.e. what viral component(s) interact(s) with the putative host resistance factor and, next, how the viral and host components interact. As for the resistance conferred by the *Tm-1* gene, only the amino acid composition of the coat protein has been compared between virulent and avirulent strains, but no strong correlation with the virulence was observed (Dawson *et al.*, 1979). TMV Lta1 was recently isolated from a *Tm-1*-gene-bearing tomato plant (Watanabe *et al.*, 1987b), that had been inoculated with a Japanese tomato strain, L (Ohno *et al.*, 1984). Unlike L, Lta1 causes mosaic symptoms and spreads systemically in the *Tm-1/Tm-1* tomatoes. Thus these two strains of TMV are closely related and suitable for extensive comparisons.

An *in vitro* transcription system has been established that allows the *in vitro* regeneration of infectious TMV RNAs from the cloned full-length cDNA copies (Dawson *et al.*, 1986; Meshi *et al.*, 1986). As a result, manipulation of TMV RNA (via manipulation of cloned DNAs) has become possible (Ishikawa *et al.*, 1986; Takamatsu *et al.*, 1987). Here

we describe the differences found between the genomic sequences of L and Lta1 and, from the analyses of strains constructed by the *in vitro* transcription system, the base substitutions responsible for the ability of Lta1 to overcome *Tm-1* resistance.

Results

Nucleotide sequence of the genomic RNA of Lta1

Since Lta1 arose spontaneously from L, a few base substitutions were expected between their genomic RNAs. The nucleotide sequence of the genomic RNA was determined directly using the dideoxy method with reverse transcriptase and end-labeled primers, except for the 3′ portion, where a chemical method was used. To clarify the base changes, the samples of L and Lta1 were always loaded on parallel lanes of the sequencing gels, and the two sequencing gel patterns were compared. With this approach it was possible to confirm the published sequence of L that had been deduced from cloned cDNA sequences (Ohno *et al.*, 1984).

As summarized in Figure 1, five clear differences were found between L and Lta1; four were base substitutions in the coding sequence and one was a one-base insertion in the 5′ non-coding sequence. Among the four substitutions, two occurred close together, at residues 3006 and 3021, and caused amino acid changes in the 130 and 180 kd proteins, respectively: Gln-979 → Glu (L → Lta1) and His-984 → Tyr (Figure 2). The other two substitutions, in the 30 kd and coat protein genes, did not cause amino acid changes. The latter two substitutions have also been found in the sequences of other L-derived strains, Ls1 (a temperature-sensitive mutant as to cell-to-cell movement; Ohno *et al.*, 1983) and $L_{11}A$ (an attenuated strain; Nishiguchi *et al.*, 1985), and, therefore, they are thought to be unrelated to the ability of TMV Lta1 to overcome the resistance. The difference in length of the U cluster near the 5′ end of the genomic RNA (five residues for L RNA and six for Lta1 RNA) is also thought to be unrelated to that ability, because polymorphism in this U cluster has previously been reported in another tomato strain, *dahlemense* (Kukla *et al.*, 1979), and because we have found that the genomic RNA of another stock of L had six U residues at this position (data not shown). It is, therefore, likely that either or both of the two substitutions at residues 3006 and 3021 are responsible for overcoming the effects of the *Tm-1* gene. Several bases could not be identified by the direct RNA sequencing, however, mainly because of premature terminations of reverse transcriptase and also because of faint bands that suggest possible sequence heterogeneity.

Construction and bioassay of a strain (T1) with the two base substitutions at residues 3006 and 3021

To clarify whether, as speculated above, the two base substitutions correlate with the resistance-breaking property of Lta1, we generated a strain with the same genomic sequence as L, except for the two bases at residues 3006 and 3021, by the use of the *in vitro* transcription system and assayed its ability to overcome the *Tm-1* resistance. Plasmid pLFW3 has a full-length cDNA of the L RNA (Meshi *et al.*, 1986), and is that which we have used as the standard clone (Ishikawa *et al.*, 1986; Takamatsu *et al.*, 1987). *Escherichia coli* RNA polymerase transcribes an infectious L RNA from pLFW3. First we constructed a plasmid,

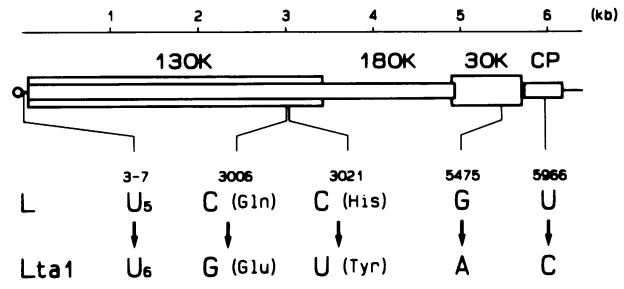

Fig. 1. The schematic representation of the genomic organization of TMV RNA and the difference in the genomic sequence between L and Lta1. The coding regions of the 130 (130K), 180 (180K) and 30 kd (30K), and coat (CP) proteins are illustrated with open boxes. Non-coding regions are illustrated with thin lines. An open circle at the left indicates a cap structure. Base changes from L to Lta1 are shown below. Residue numbers are counted from the 5′ end of the genomic RNA (Ohno *et al.*, 1984). Where base substitutions resulted in amino acids changes, the amino acids are shown in parentheses.

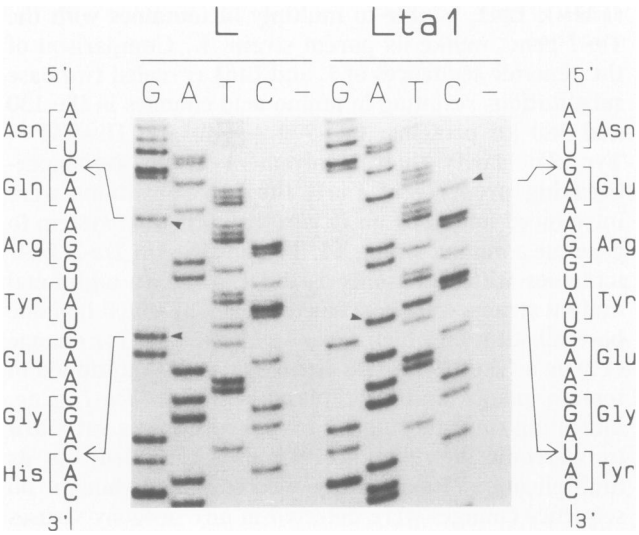

Fig. 2. Base substitutions at residues 3006 and 3021. The genomic RNAs of L and Lta1 were sequenced as described in Materials and methods. A 51-base ssDNA, derived from a *Bst*NI−*Afl*II restriction fragment, complementary to residues 3062−3112 was used as the primer. Bands corresponding to positions where the substitutions were found are marked with arrowheads. Deduced RNA sequences and amino acid sequences are shown beside the ladders. G, A, T, and C refer to reactions in the presence of the corresponding dideoxynucleotides, and − indicates a reaction in the absence of dideoxynucleotides.

pLFT1, by replacing the *Sal*I−*Bam*HI fragment (residues 2940−3335) of pLFW3 (Figure 3) with the corresponding fragment derived from a TMV Lta1 cDNA clone. *In vitro* transcripts from pLFW3 and pLFT1 were inoculated into tobacco plants (*Nicotiana tabacum* L. cv. Samsun) and the respective progeny viruses were purified from the inoculated leaves a week later. The progeny viruses derived from pLFW3 and pLFT1 were designated W3 and T1, respectively. The sequences of progeny viral RNAs around the mutagenized region were confirmed prior to further use.

The biological properties of W3 and T1 were assayed using two nearly isogenic tomato lines, GCR 26 without the *Tm-1* gene (+/+) and GCR 237 with the *Tm-1* gene (*Tm-1*/*Tm-1*). Both viruses caused typical mosaic symptoms on systemic leaves of GCR 26 about a week after inoculation, as did L and Lta1 (Table I). When inoculated into GCR

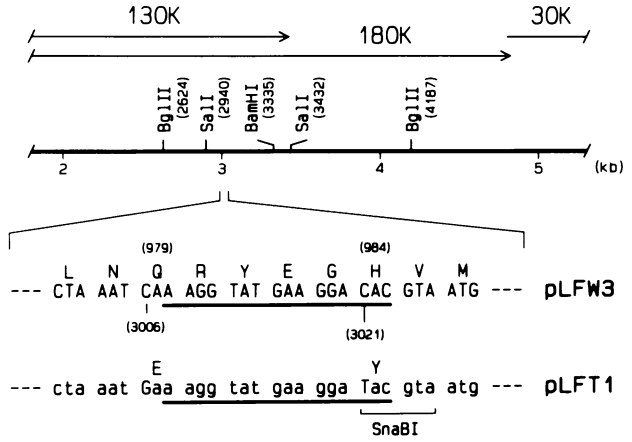

Fig. 3. The sequences surrounding the different nucleotides at residues 3006 and 3021 between pLFW3 and pLFT1. Restriction enzyme sites used for the construction of plasmids are shown along the scale. The numbers of the first bases of their recognition sequences are shown in parentheses. Below the scale, the pLFW3 and pLFT1 sequences, corresponding to residues 3000–3029 of L, are shown. In the pLFT1 sequence, nucleotides differing from pLFW3 are denoted with capitals. Above the pLFW3 sequence, deduced amino acids are shown. In the case of pLFT1, only the different amino acids are shown. Numbers of the different amino acids and nucleotides are shown in parentheses. Amino acid positions are counted from the N-terminal methionine residue. The thick bars below the nucleotide sequences correspond to the oligonucleotides synthesized for the construction of pLMT2 and pLMT3 (see Materials and methods). The *Sna*BI site shown below the pLFT1 sequence was newly generated by a base substitution.

237 leaves, T1 caused mosaic symptoms in the upper systemic leaves about a week after inoculation, as did Lta1, while W3 did not cause any symptoms 3–5 weeks after inoculation (duration of observation) as was the case with L (Table I). At 12–14 days post inoculation, similar amounts of progeny viruses were recovered from the upper systemic leaves of symptom-showing plants regardless of the genotype. The genomic RNA sequence around the mutagenized portion of each progeny virus was confirmed to be unchanged (data not shown). These results show that T1 overcomes the effects of the *Tm-1* gene and that the base substitutions resulting in the amino acid changes in the 130 and 180 kd proteins are responsible for overcoming the resistance.

Construction and bioassay of strains (T2 and T3) with one base substitution at residue 3006 or 3021

To reveal whether the concomitant substitutions are necessary or either substitution alone is sufficient to overcome the resistance, we constructed two clones, pLFT2 and pLFT3, as described in Materials and methods. These two clones have the same sequence as pLFW3 except for having single base substitutions at residue 3006 (pLFT3) or at residue 3021 (pLFT2) (Figures 3 and 4). Progeny viruses derived from pLFT2 and pLFT3, designated T2 and T3, respectively, were prepared and assayed as in the cases of T1 and W3. Both strains were purified from tobacco plants in normal recovery amounts (5–10 mg/g of tissue) and without any detectable sequence alteration around the mutagenized portion (data not shown).

Both strains caused mosaic symptoms on GCR 26 (+/+) about a week after inoculation (Table I). Their progenies were recovered with a yield similar to the cases of L and

Table I. The ability of *in vitro* generated strains to overcome *Tm-1* resistance

	Amino acid position[a]		GCR 16 (+/+)		GCR 237 (*Tm-1*/*Tm-1*)	
Strain	979	984	symptom[b]	multiplication[c]	symptom[b]	multiplication[c]
L	Gln	His	mosaic	++	none	–
Lta1	Glu	Tyr	mosaic	++	mosaic	++
W3	Gln	His	mosaic	++	none	–
T1	Glu	Tyr	mosaic	++	mosaic	++
T2	Gln	Tyr	mosaic	++	none	+
T3	Glu	His	mosaic	++	mosaic	++

[a]Amino acids at positions 979 and 984 of the 130 and 180 kd proteins of each strain.
[b]Symptoms were judged 14 days after inoculation. Systemic mosaic symptoms always appeared 6–9 days after inoculation.
[c]Multiplication was defined as accumulation of virus both in systemic leaves 12–14 days after inoculation and in protoplasts 24 h after inoculation (see Figure 6 and Materials and methods). ++, accumulation of a given virus was similar to that of L in GCR 26 or Lta1 in GCR 237; +, accumulation was detected by $\leq 10^{-1}$ of that of Lta1 in GCR 237; –, no virus was detected in most assays, but, in one assay, a few lesions were seen to be caused by back-inoculation of leaf-homogenates derived from W3-inoculated GCR 237.

Lta1 and also without detectable sequence changes. The introduced base changes in the putative replicase gene would not seem to cause any significant defectiveness in multiplication in plants without the *Tm-1* gene.

When inoculated on GCR 237 (*Tm-1*/*Tm-1*), T3 caused typical mosaic symptoms indistinguishable from those caused by Lta1 and T1 ~1 week after inoculation (Table I). Viral accumulation, estimated as 1–10 mg/g of tissue, was also similar to that for Lta1 and T1. These results show that the single base substitution at residue 3006 is sufficient to confer on TMV L the ability to overcome those effects of the *Tm-1* gene which relate to viral propagation and symptom development.

T2 did not cause any visible symptoms at least within 2 weeks after inoculation (Table I), although weak mosaic symptoms were occasionally observed ~3 weeks after inoculation or later. Various amounts of viruses accumulated in T2-inoculated GCR 237 12–14 days after inoculation, at which time no visible symptoms had developed; the amounts ranged from undetectable (<1 μg/g of tissue) to nearly 1 mg/g of tissue, although the amounts detected were always lower than those detected in T1- and T3-inoculated GCR 237. Since sequencing analyses, as described below, showed that the propagating viruses did not necessarily retain the sequence of the inoculated viruses (T2 and T3), the accumulation of T2 and T3 in GCR 237 plants could not be quantitated with certainty.

Progeny viruses of T2 and T3 were extracted 12–14 days after inoculation and their genomes were sequenced. In eight preparations (designated T2p#1–4 for T2 progeny and T3p#1–4 for T3 progeny), surprisingly, all but one T2 progeny (T2p#4) contained viruses with additional second base substitutions of various ratios. Two typical examples are shown in Figure 5. As summarized in Figure 4, base changes resulting in the same sequence as T1 were found in three preparations (T2p#1, T3p#1 and T3p#2). Interestingly, substitutions at other residues were also found in five preparations and all caused amino acid changes in the 130 and 180 kd proteins: a C → U substitution at residue

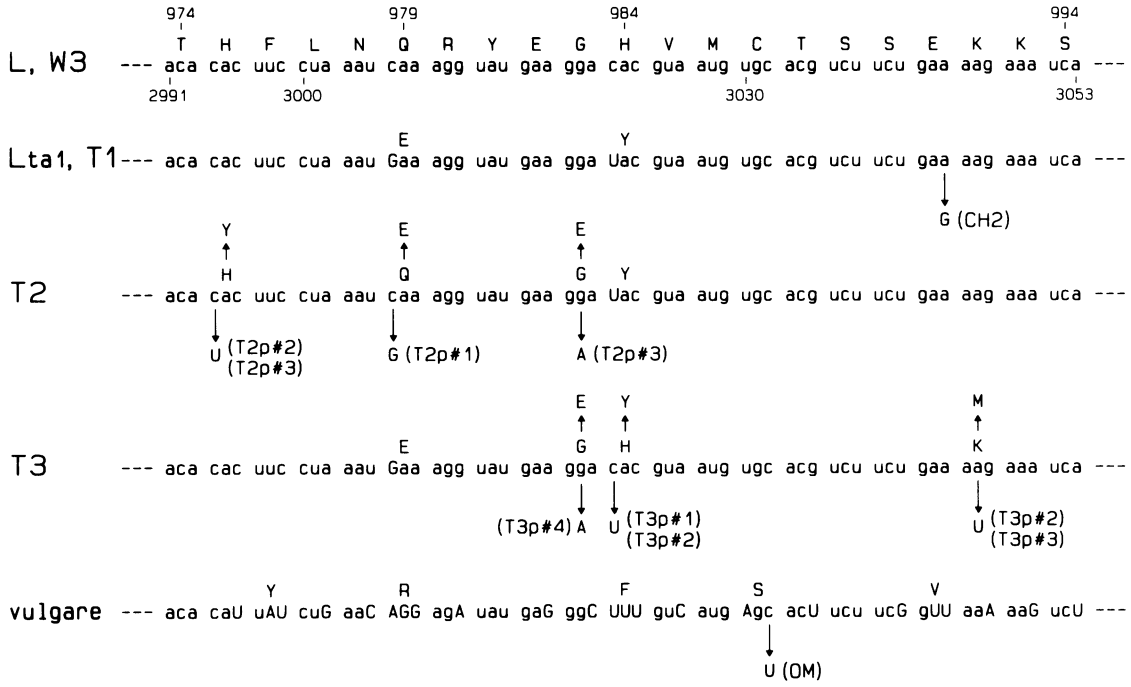

Fig. 4. The genomic sequences of TMV strains between residues 2991–3053. Nucleotides different from the corresponding residues of L RNA are shown with capitals and the others are shown with lower-case letters. Above the L sequence, deduced amino acid sequence of the 130 and 180 kd proteins is shown. As for the other strains, amino acids different from the corresponding amino acids of the L proteins and those changed by the second base substitutions in T2 and T3 progenies are shown. The second base substitutions observed are shown below the T2 and T3 sequences with names of progenies in parentheses. Silent base substitutions found in CH2 and OM are shown below the Lta1 and *vulgare* sequences. The *vulgare* sequence is from Goelet *et al.* (1982).

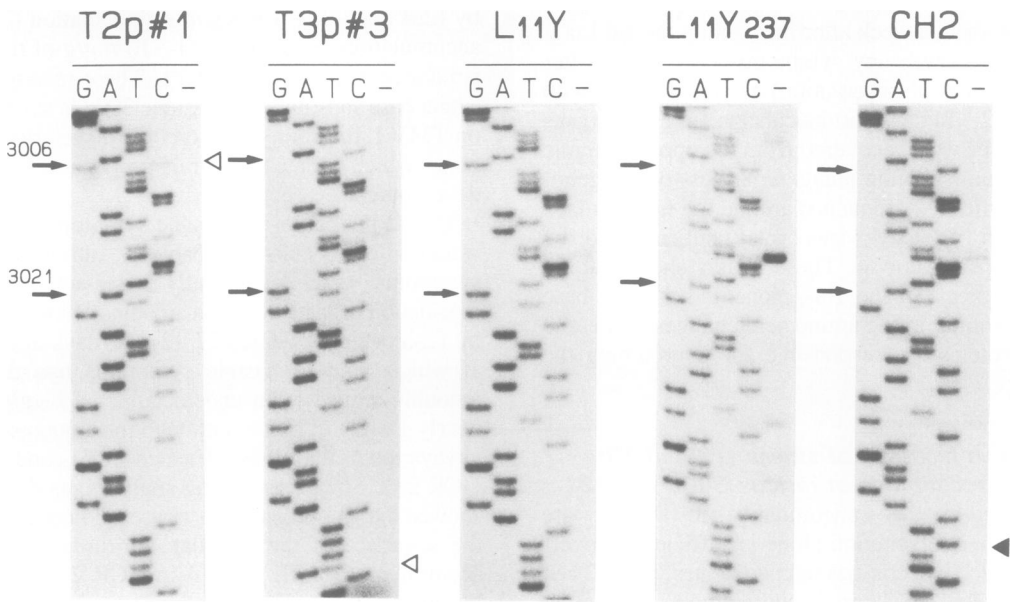

Fig. 5. Nucleotide sequences of progeny viruses (T2p#1 and T3p#3) and independently isolated resistance-breaking strains (L_{11}Y237 and CH2) with a parent strain (L_{11}Y) surrounding the responsible bases. Nucleotides at residues 3006 and 3021 are indicated with arrows. Second base substitutions found in T2p#1 and T3p#3 are marked (△). A silent base substitution found in the CH2 sequence is also marked (▲).

2994 causing a His-975 → Tyr change in T2p#2 and T2p#3, a G → A substitution at residue 3019 causing a Gly-983 → Glu change in T2p#3 and T3p#4, and an A → U substitution at residue 3046 causing a Lys-992 → Met change in T3p#2 and T3p#3 (Figure 4). Since the genomic sequences of progeny viruses determined were only ~150 nt (residues 2910–3060), it is not known whether there were any other substitutions in unsequenced regions. However, the fact that the second substitutions found were all close to the mutagenized sites suggests the specific importance of the region for overcoming the resistance (see Discussion).

Replication (accumulation) of constructed strains in protoplasts

Uncertainty regarding the multiplication of T2 and T3 still remained because of the appearance of viruses with the second base substitutions and, in addition, because of the uneven recovery of T2 progeny from the systemic leaves about 2 weeks after inoculation. However, since, regarding the sequence around the mutagenized residues, viruses with the same sequence as T2 and T3 were always detected, it was thought that T2 and T3 multiplied by themselves at least for a few days. Therefore, we investigated their multiplication at an earlier stage using a protoplast system (Watanabe *et al.*, 1987b).

Tomato protoplasts prepared from suspension-cultured cells of GCR 26 and GCR 237 were inoculated with W3, T1, T2 and T3 RNAs, and, 24 h after inoculation, viral accumulation was examined by Northern blotting. All strains multiplied in GCR 26-derived protoplasts without significant differences (Figure 6A). In GCR 237-derived protoplasts, T1 and T3 multiplied similarly (Figure 6B), confirming that T3 has the ability to overcome the resistance. Multiplication of T2 was also confirmed with longer exposure, while multiplication of W3 or L could not be detected (Figure 6B). All results were consistent with those obtained from the assays in plants.

Analysis of independently isolated resistance-breaking strains

The above results indicate that both of the closely occurring base substitutions are involved in the ability of Lta1 to propagate in tomatoes with the *Tm-1* gene. To learn whether the same substitutions are found in common among other resistance-breaking strains or whether there are many other possible equivalent substitutions for overcoming the resistance, we analysed two other resistance-breaking strains, L_{11}Y237 and CH2. The former occurred spontaneously from L_{11}Y (Umekawa and Oshima, 1971; Oshima *et al.*, 1980) and the latter was an isolate from a field-grown tomato plant with the *Tm-1* gene (Motoyoshi and Oshima, 1977). As shown in Figure 5, the genomic RNAs of both strains carried the same base substitutions as Lta1: C → G and C → U substitutions at residues 3006 and 3021, respectively. CH2 RNA had an extra silent substitution within the sequenced region (Figures 4 and 5). In contrast to that, L_{11}Y, the parent strain of L_{11}Y237, had the same sequence as TMV L in the examined sequence.

Discussion

We have determined the two base substitutions responsible for the ability of TMV Lta1 to overcome the effects of the tomato resistance gene, *Tm-1*: one is a C → G substitution at residue 3006 causing a Gln-979 → Glu amino acid change in the 130 and 180 kd proteins and the other is a C → U substitution at residue 3021 causing a His-984 → Tyr change in the same proteins. Analyses of *in vitro* generated strains, T2 with the latter substitution and T3 with the former substitution, showed that the former substitution is sufficient for propagation and symptom development in the *Tm-1/Tm-1* tomato plants. The appearance of viruses having the second substitutions in the T3-inoculated *Tm-1/Tm-1* tomatoes, however, suggested that the ability of T3 to overcome the resistance is not the same as the ability of TMV Lta1 and T1 (with both substitutions), although we could not experimentally detect the apparent difference in multiplication. On the other hand, multiplication of T2 in the *Tm-1/Tm-1* tomatoes was considerably lower than that of Lta1 but apparently higher than that of L and W3. Considering the fact that T1 can propagate in the *Tm-1/Tm-1* tomatoes without any detectable sequence alteration and the fact that both L_{11}Y237 and CH2, independently isolated resistance-breaking strains, have the same substitutions as Lta1, the two substitutions may be mutually enhancing. Viruses with the two concomitant substitutions would seem to propagate most successfully in tomatoes with the *Tm-1* gene, and a genomic RNA with the two substitutions might be at least one possible ultimate structure.

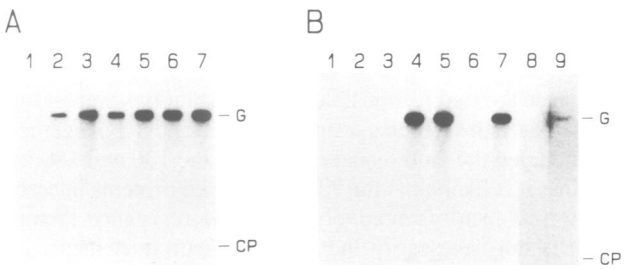

Fig. 6. Accumulation of *in vitro* generated strains in protoplasts derived from GCR 26 without the *Tm-1* gene (**A**) and derived from GCR 237 with the *Tm-1* gene (**B**). Protoplasts were mock-inoculated (**lane 1**) and inoculated with L (**lane 2**), W3 (**lanes 3** and **8**), Lta1 (**lane 4**), T1 (**lane 5**), T2 (**lanes 6** and **9**) and T3 (**lane 7**) RNAs. Total RNAs were extracted 24 h after inoculation, and electrophoresed on 1% agarose gel. The genomic RNA (G) and the coat protein subgenomic RNA (CP) were made visible by the Northern blotting. In panel B, lanes 8 and 9 are long exposed patterns of lanes 3 and 6.

All the *in vitro* constructed strains, T1, T2, T3 and W3, propagated similarly in both tobacco and susceptible (+/+) tomato plants without detectable sequence alteration. This suggests that the activity of the 130 and 180 kd proteins is affected little, if at all, by either of the two amino acid changes in Gln-979 and His-984. Furthermore, the corresponding residues of the common strain proteins are different, i.e. Arg-979 and Phe-984, respectively (Figure 4). Changeability of amino acids at positions 979 and 984, and possibly in their vicinity, would appear to be advantageous for acquiring the ability to overcome the resistance. Other possible changes might be prevented by a functional constraint of the 130 and 180 kd proteins.

Tm-1 resistance is known to be effective to various TMV strains (Pelham, 1972; Dawson *et al.*, 1979; Watanabe *et al.*, 1987b), including several distantly related strains, such as *vulgare*, U2, and a cowpea strain, among which little or very limited sequence homology has been observed (Gibbs, 1986). Assuming that a common resistance mechanism operates against TMV strains, the responsible host factor would recognize a certain conserved feature found among TMV strains. Considering the fact that the 30 kd and coat proteins are dispensable for replication (multiplication in protoplasts) (Meshi *et al.*, 1987), the viral component(s) recognized or attacked is(are) deduced to be the 130 and 180 kd proteins or a conserved structure of the genomic RNA. Since there are many base substitutions around the responsible residues even between the closely related

common (*vulgare*) and tomato (L) strains (Figure 4), this portion of the L genomic RNA would not be expected to be the site where the host factor attacks.

Because the two responsible base substitutions and all the second base substitutions found in the T2 and T3 progenies accompanied the amino acid changes in the 130 and 180 kd proteins, it is likely that the 130 and 180 kd proteins interact with a host factor, tentatively called the resistance factor, which is not necessarily the *Tm-1* gene product itself.

In support of this, an interesting feature is found concerning the direction of amino acid changes from avirulent strains to virulent strains against the *Tm-1* tomatoes. Both of the responsible amino acid changes, i.e., the Gln-979 → Glu and His-984 → Tyr changes, cause a decrease in the local net charge. In the region shown in Figure 4 (21 amino acids between Thr-974 and Ser-994), there are five positively or potentially positively charged residues (His-975, Arg-980, His-984, Lys-992 and Lys-993) and two negatively charged residues (Glu-982 and Glu-991) in the L 130 and 180 kd proteins, and in this situation the local net charge is calculated to be +3, when +1 is given to a His residue. In contrast to this, the local net charges of the resistance-breaking strains decrease to +1. As for the *in vitro* constructed strains, T2 and T3, the net charges are calculated to be +2, an intermediate value. The appearance of viruses with the second changes is thought to result from the fact that they had a higher ability to overcome the resistance than T2 or T3. Second changes found in their progenies include His-955 → Tyr, Gly-983 → Glu, Lys-992 → Met, all of which decrease the local net charge. Thus, the decrease of the local net charge correlates strongly with the ability to overcome the resistance. Supposing that His-984 is only partly protonated [the 130 and 180 kd proteins are localized in cytoplasm (Saito *et al.*, 1987) and cytoplasmic pH may be nearly neutral (Brodelius and Vogel, 1985)], the difference in the ability to multiply in the *Tm-1*/*Tm-1* tomatoes between T2 and T3 might also be explained by the difference in the local net charge. In addition, in the cases of common strains, OM and *vulgare,* whose multiplication is known to be inhibited in the *Tm-1* tomatoes (Motoyoshi and Oshima, 1977; Dawson *et al.*, 1979), the local net charges are calculated as +4, higher than those of avirulent tomato strains. It is, therefore, very likely that an electrostatic interaction between the resistance factor and the 130 and 180 kd proteins is involved.

Although it is still unclear how the resistance factor inhibits multiplication or how the 130 and 180 kd proteins of Lta1 restrict the resistance, our findings may provide some suggestions. The most straightforward explanation for the *Tm-1* resistance would seem to be that the resistance factor directly attacks a certain portion of the 130 and 180 kd proteins and inactivates them, as, for example, by degradation, modification, or non-covalent binding. When the local net charge of the 130 and 180 kd proteins is decreased, these proteins, i.e. those of the resistance-breaking strains, could evade the attack by the resistance factor. This possibility appears compatible with presently available data. However, it can not be excluded that the 130 and 180 kd proteins of Lta1 might acquire an ability, by the amino acid changes, to disable the resistance factor. Another possibility still remains; the resistance factor might be an altered form of a host factor involved in viral multiplication and the altered factor might not have access to viral components because of positively charged residues around the responsible amino acids. In any case, interaction between the resistance factor and the 130 and 180 kd proteins would be expected, and, in some particular cases, it may be possible to discover the resistance factor by using, as probes, polypeptides containing the amino acids responsible for overcoming the resistance.

Materials and methods

Viruses

TMV L is a Japanese tomato strain (Ohno *et al.*, 1984). Lta1 was isolated without mutagenesis from an L-inoculated tomato with the *Tm-1* gene (GCR 237) (Watanabe *et al.*, 1987b). TMV $L_{11}Y$ is a spontaneously occurring yellow mutant derived from an L-derived attenuated strain, TMV L_{11} (Umekawa and Oshima, 1971). TMV $L_{11}Y237$, derived from $L_{11}Y$, is a spontaneously occurring resistance-breaking strain that multiplies in tomatoes with the *Tm-1* gene (Oshima *et al.*, 1980). CH2 was isolated from a field-grown tomato as a tomato strain having the ability to overcome the resistance conferred by the *Tm-1* gene (Motoyoshi and Oshima, 1977).

RNA sequencing

Sequencing of the genomic RNA was performed by the dideoxy chain-terminating method using reverse transcriptase and 5′ end-labeled primers as described by Meshi *et al.*, (1983) with slight modifications. Primers were prepared from cDNA clones, pL-D66, pLT-C42 (Ohno *et al.*, 1984) and pL-1-13 (Takamatsu *et al.*, 1983), by restriction digestions, and end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase as described by Maxam and Gilbert, (1980). The 3′ end of the genomic RNA was sequenced by a chemical method (Peattie, 1979).

Plasmid construction

Standard recombinant DNA techniques used were essentially according to Maniatis *et al.* (1982) unless otherwise specified.

Double-stranded cDNA of Lta1 RNA was synthesized as described by Meshi *et al.* (1986). After the ds-cDNA was digested by *Bgl*II (see Figure 3), the internal 1.56 kb fragment (residues 2624−4187) was isolated from an acrylamide gel and cloned into the *Bam*HI site of pBR322 to create pLta1-28. The 0.49 kb *Sal*I fragment (residues 2940−3432) of pLta1-28 was isolated and sequenced by the method of Maxam and Gilbert (1980). The cloned sequence was confirmed to be consistent with the Lta1 genomic sequence except for one base at residue 3427, where an unexpected substitution thought to be a cloning artifact was found (see Ishikawa *et al.*, 1986).

pLFW3 is a full-length cDNA clone of TMV L, and from it an infectious transcript can be produced by the *in vitro* transcription system (Meshi *et al.*, 1986). pLFT1 was constructed by replacing the 0.4 kb *Sal*I−*Bam*HI fragment (residues 2940−3335) of pLFW3 with the corresponding fragment of pLta1-28, and in it the artificial base change at residue 3427 was not included.

pLFT2 and pLFT3 were constructed by oligonucleotide-aided mutagenesis essentially as described by Ahlquist and Janda (1984) and Ishikawa *et al.* (1986). The 0.49 kb *Sal*I fragments of pLFW3 and pLta1-28 were first subcloned into M13mp18 to generate mL1P and mL3P, respectively, whose genomes carry the sequences with the TMV-plus polarity. The *Sal*I fragment of pLta1-28 was also cloned into a pUC-derived vector to generate pLMT1. The genomic DNAs of mL1P and mL3P were annealed with synthetic oligonucleotides, dGTATCCTTCATACCTT (complementary to residues 3008−3023 of the Lta1 RNA) and dGTGTCCTTCATACCTT (complementary to residues 3008−3023 of the L RNA), respectively (see Figure 3), and the first strands were synthesized as described by Ishikawa *et al.* (1986). After the second strands were synthesized using the reverse sequence primer as described by Hong (1981) and cut with *Eco*RI at the polylinker sequence, the generated 0.1 kb fragments were purified. The respective fragments were cloned between the *Eco*RI and *Hin*dIII sites of pUC9 together with the 0.4 kb *Sna*BI−*Hin*dIII fragment pLMT1. The *Sna*BI site of pLMT1 was newly generated by a base substitution at residue 3021 from L to Lta1 (see Figure 3), and the *Hin*dIII site was derived from the polylinker sequence. pLMT2 and pLMT3 had one base substitution from the L sequence at residues 3006 and 3021, respectively. After the sequences around the mutagenized portions were confirmed (Zagursky *et al.*, 1985), the 0.4 kb *Sal*I−*Bam*HI fragments (residues 2940−3335) of pLMT2 and pLMT3 were isolated and introduced into pLFW3 in place of the corresponding fragment to create pLFT2 and pLFT3, respectively. The resulting plasmids had one-base substitutions from pLFW3: at residue 3021 for pLFT2 and at residue 3006 for pLFT3.

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 essentially as described by Ahlquist *et al.* (1984). *In vitro* transcripts were reconstituted with the coat protein and inoculated into tobacco leaves (*N.tabacum* L. cv. Samsun) (Meshi *et al.*, 1986). Progeny viruses were purified from the inoculated leaves 1 week later as described by Otsuki *et al.* (1977). For each construct except pLFW3, progeny viruses derived from two independently isolated clones were prepared and used throughout the work to confirm the results. The sequence around the mutagenized residues of the genomic RNA of each progeny was confirmed by the dideoxy method prior to further use.

Infectivity assay

Tomato plants (*Licopercicon esculentum* Mill.) used were nearly isogenic lines, GCR 26 (without the *Tm-1* gene) and GCR 237 (homozygous for the *Tm-1* gene) (Pelham, 1972). Viruses were inoculated into the primary and secondary true leaves at a concentration of 10 μg/ml. Viral propagation was estimated in two ways: by back-inoculation of homogenates of the upper systemic leaves into *N. tabacum* L. cv. Xanthi nc and, in most later experiments, by electrophoretic anlaysis of the coat protein contained in the leaf homogenates. Leaf homogenates were prepared by collecting leaflets of the upper systemic leaves of 4–7 plants (one or two leaflets per plant) and subsequently homogenizing them. After brief centrifugation to remove debris, the homogenates were boiled in the presence of SDS and β-mercaptoethanol and loaded on SDS–polyacrylamide gels. The coat protein was made visible by staining and by the Western blotting method essentially as described by Saito *et al.* (1986). For the purification of the progeny virus, 0.5–1 g leaflets were collected from 4–7 plants and homogenized.

Protoplast inoculation

Protoplasts were prepared from suspension-cultured cells of GCR 26 and GCR 237 (Watanabe *et al.*, 1987b). Inoculation of the genomic RNA (2 μg) into protoplasts (1×10^6) was performed by electroporation (Watanabe *et al.*, 1987a) with modification (Watanabe *et al.*, 1987b). Northern blotting was carried out essentially as described by Ishikawa *et al.* (1984) using a nick-translated full-length cDNA plasmid as probe.

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References

Ahlquist,P. and Janda,M. (1984) *Mol. Cell. Biol.*, **4**, 2876–2882.
Ahlquist,P., French,R., Janda,M. and Loesch-Fries,L.S. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7066–7070.
Brodelius,P. and Vogel,H.J. (1985) *J. Biol. Chem.*, **260**, 3556–3560.
Dawson,J.R.O., Rees,M.W. and Short,M.N. (1979) *Ann. Appl. Biol.*, **91**, 353–358.
Dawson,W.O., Beck,D.L., Knorr,D.A. and Grantham,G.L. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1832–1836.
Deom,C.M., Oliver,M.J. and Beachy,R.N. (1987) *Science*, **237**, 389–394.
Fraser,R.S.S. (1985) *Mechanisms of Resistance to Plant Diseases*, Martinus Nijhoff/Junk, Dordrecht.
Fraser,R.S.S. and Loughlin,S.A.R. (1980) *J. Gen. Virol.*, **48**, 87–96.
Fraser,R.S.S., Loughlin,S.A.R. and Connor,J.C. (1980) *J. Gen. Virol.*, **50**, 221–224.
Gibbs,A. (1986) In Van Regenmortel,M.H.V. and Fraenkel-Conrat,H. (eds), *The Plant Viruses 2. The Rod-shaped Plant Viruses*. Plenum, New York, pp. 167–180.
Goelet,P., Lomonossoff,G.P., Butler,P.J.G., Akam,M.E., Gait,M.J. and Karn,J. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5818–5822.
Ishikawa,M., Meshi,T., Ohno,T., Okada,Y., Sano,T., Ueda,I. and Shikata,E. (1984) *Mol. Gen. Genet.*, **196**, 421–428.
Ishikawa,M., Meshi,T., Motoyoshi,F., Takamatsu,N. and Okada,Y. (1986) *Nucleic Acids Res.*, **14**, 8291–8305.
Hong,G.F. (1981) *Biosci. Rep.*, **1**, 243–252.
Kukla,B.A., Guilley,H.A., Jonard,G.X., Richards,K.E. and Mundry,K.W. (1979) *Eur. J. Biochem.*, **98**, 61–66.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499–560.
Meshi,T., Ishikawa,M., Takamatsu,N., Ohno,T. and Okada,Y. (1983) *FEBS Lett.*, **162**, 282–285.
Meshi,T., Ishikawa,M., Motoyoshi,F., Semba,K. and Okada,Y. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 5043–5047.
Meshi,T., Watanabe,Y., Saito,T., Sugimoto,A., Maeda,T. and Okada,Y. (1987) *EMBO J.*, **6**, 2557–2563.
Motoyoshi,F. (1982) In Fujisawa,A. (ed.), *Plant Tissue Culture*. Maruzen, Tokyo, pp. 659–660.
Motoyoshi,F. and Oshima,N. (1975) *J. Gen. Virol.*, **29**, 81–91.
Motoyoshi,F. and Oshima,N. (1977) *J. Gen. Virol.*, **34**, 499–506.
Motoyoshi,F. and Oshima,N. (1979) *J. Gen. Virol.*, **44**, 801–806.
Nishiguchi,M., Kikuchi,S., Kiho,Y., Ohno,T., Meshi,T. and Okada,Y. (1985) *Nucleic Acids Res.*, **13**, 5585–5590.
Ohno,T., Takamatsu,N., Meshi,T., Okada,Y., Nishiguchi,M. and Kiho,Y. (1983) *Virology*, **131**, 255–258.
Ohno,T., Aoyagi,M., Yamanashi,Y., Saito,H., Ikawa,S., Meshi,T. and Okada,Y. (1984) *J. Biochem.*, **96**, 1915–1923.
Oshima,N., Motoyoshi,F. and Nishiguchi,M. (1980) *Ann. Phytopath. Soc. Japan* (In Japanese), **46**, 424.
Otsuki,Y., Takebe,I., Ohno,T., Fukuda,M. and Okada,Y. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 1913–1917.
Peattie,D.A. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1760–1764.
Pelham,H.R.B. (1978) *Nature*, **272**, 469–471.
Pelham,J. (1966) *Euphytica*, **15**, 258–267.
Pelham,J. (1972) *Ann. Appl. Biol.*, **71**, 219–228.
Saito,T., Watanabe,Y., Meshi,T. and Okada,Y. (1986) *Mol. Gen. Genet.*, **205**, 82–89.
Saito,T., Hosokawa,D., Meshi,T. and Okada,Y. (1987) *Virology*, **160**, 477–481.
Takamatsu,N., Ohno,T., Meshi,T. and Okada,Y. (1983) *Nucleic Acids Res.*, **11**, 3767–3778.
Takamatsu,N., Ishikawa,M., Meshi,T. and Okada,Y. (1987) *EMBO J.*, **6**, 307–311.
Van Regenmortel,M.H.V. and Fraenkel-Conrat,H. (1986) *The Plant Viruses 2. The Rod-shaped Plant Viruses*. Plenum Press, New York.
Umekawa,M. and Oshima,N. (1971) *Ann. Phytopath. Soc. Japan* (in Japanese), **37**, 364.
Watanabe,Y., Meshi,T. and Okada,Y. (1987a) *FEBS Lett.*, **219**, 65–69.
Watanabe,Y., Kishibayashi,N., Motoyoshi,F. and Okada,Y. (1987b) *Virology*, **161**, 527–532.
Zagursky,R.J., Baumeister,K., Lomax,N. and Berman,N.L. (1985) *Gene Anal. Tech.*, **2**, 89–94.

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