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# Coat protein gene sequence of tobacco mosaic virus encodes a host response determinant

**(recombinant virus/*in vitro* transcription/*N'* gene/necrotic local lesion)**

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**ABSTRACT** **The common strain and tomato strain of tobacco mosaic virus (TMV) are known to be closely related to each other. However, plants with the *N'* gene, such as *Nicotiana sylvestris* and *Nicotiana tabacum* L. cv. Bright Yellow, respond differently to infections by these viruses. In the *N'* plants, TMV-OM (common strain) spreads systemically with mosaic symptoms, whereas TMV-L (tomato strain) induces the necrotic response of plants, causing local lesions. To reveal the viral factor of TMV-L inducing the necrotic response, we have constructed several recombinant viruses between the two strains, in which TMV-L RNA was partly replaced by TMV-OM RNA. The recombinant viruses having the coat protein gene sequence of TMV-OM in place of TMV-L produced no necrotic local lesions but spread systemically with mosaic symptoms in the *N'* plants. On the other hand, the recombinant viruses having TMV-OM-derived sequences other than the coat protein gene sequence, and in which the coat protein gene sequence of TMV-L still remained, produced necrotic local lesions. These observations indicate that the viral factor of TMV-L responsible for the necrotic response of the *N'* plants is coded in the coat protein gene sequence.**

Tobacco mosaic virus (TMV) is the most characterized viral pathogen of plants (1). The genome of TMV is a positive-sense, single-stranded RNA encoding at least four proteins (2, 3): 130-kDa and 180-kDa proteins thought to be components of the replicase (4–7), 30-kDa protein thought to be involved in cell-to-cell movement (8, 9), and the coat protein. Symptoms induced by TMV infection on some plants are known to be controlled by genes called *N* and *N'* (10). In plants carrying the *N* gene, naturally found in *Nicotiana glutinosa* (11), all strains of TMV so far tested are confined to small areas around their points of entry, causing necrotic local lesions (12). Another gene, *N'* from *Nicotiana sylvestris*, confers localization to some strains of TMV with necrotic lesions (13). Tobacco varieties with recessive genes *n* and *n'* permit systemic infection; TMV usually spreads throughout the plants, causing mosaic symptoms. The necrotic response of plants with either *N* or *N'* gene has been thought to involve a similar mechanism (14) and has been assumed to be the result of sequential reactions activated by a viral factor produced in the course of multiplication. However, there is no conclusive evidence identifying the viral factor and whether this factor is common between the *N* and *N'* gene systems.

The common (tobacco) and tomato strains of TMV are shown to be closely related from the aspects of serology (15) and nucleotide sequence homology (3). However, the two strains can be discriminated on the basis of their biological properties, and so the tomato strain is also recognized as a distinct virus (16). One criterion distinguishing the two strains is the response of plants with the *N'* gene to infection: the tomato strain causes necrotic local lesions, whereas the common strain spreads systemically with mosaic symptoms.

We have established an *in vitro* system to transcribe infectious TMV RNAs from cloned cDNA copies of a Japanese tomato strain, TMV-L (17). Here, to reveal the viral factor of TMV-L responsible for the necrotic response of the *N'* plants and to find a clue to resolve the *N* and *N'* gene mechanism, we constructed several cDNA derivatives in which parts of the TMV-L sequences were replaced by the corresponding sequences of a Japanese common strain, TMV-OM. These were transcribed into infectious RNAs and analyzed for the responses of the *N'* plants to their infections.

## MATERIALS AND METHODS

**Plasmids.** pLFW3 (17) carries a full-length cDNA copy of TMV-L RNA just downstream of the $P_M$ promoter (18). Infectious RNA can be transcribed *in vitro* from linearized pLFW3 with *Escherichia coli* RNA polymerase (17). pL-1-13 carries an ≈1.6-kilobase-pair (kb) cDNA derived from the 3' end of TMV-L RNA (19). pOMT-G30 (unpublished) and pOM5H2 (19) have ≈3.8-kb and 2.0-kb cDNAs derived from the 3' end of TMV-OM RNA (Fig. 1). pOMT-G30 was constructed as described (3).

*Construction of pOL-BC and pOL-CN.* All procedures were performed essentially according to standard methods (20). pOL-BC and pOL-CN were constructed by replacing the *Bam*HI/*Nco* I fragment [residues 3336–5463 of the genomic sequence of TMV-L (3)] and the *Nco* I/*Nsi* I fragment (residues 5463–6188) of pLFW3 with the corresponding fragments of pOMT-G30 and pOM5H2, respectively (Fig. 1). Constructions were confirmed by restriction mapping.

*Construction of pOL-CD.* To recombine the cDNAs of TMV-L and TMV-OM RNAs at the junction between the 30-kDa and coat protein genes, the *Dra* I site of TMV-OM cDNA was used (Fig. 2). However, since the *Dra* I site does not exist in the corresponding site of TMV-L cDNA, the DNA fragment carrying the coat protein gene of TMV-L, with the sequence generated by the *Dra* I cut at one end, was prepared according to Ahlquist *et al.* (18) and Ishikawa *et al.* (7). Briefly, the 0.27-kb filled-in *Mbo* II/*Sau*3AI fragment (residues 5665–5937) of pL-1-13 was cloned into the *Hin*cII/*Bam*HI sites of M13mp18 replicative form DNA. The single-stranded genomic DNA of this phage, having cDNA sequence of TMV-L RNA, was hybridized with a synthetic oligonucleotide [d(AAATATGTCTTACTCAATC), corresponding to residues 5699–5717] (Fig. 2) and converted to a double-stranded form as described (7). After the DNA was heat-denatured and hybridized with a reverse-sequencing primer (Pharmacia), the second strand was synthesized to generate the *Dra* I cutting end at one end with *E. coli* Klenow

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Abbreviation: TMV, tobacco mosaic virus.

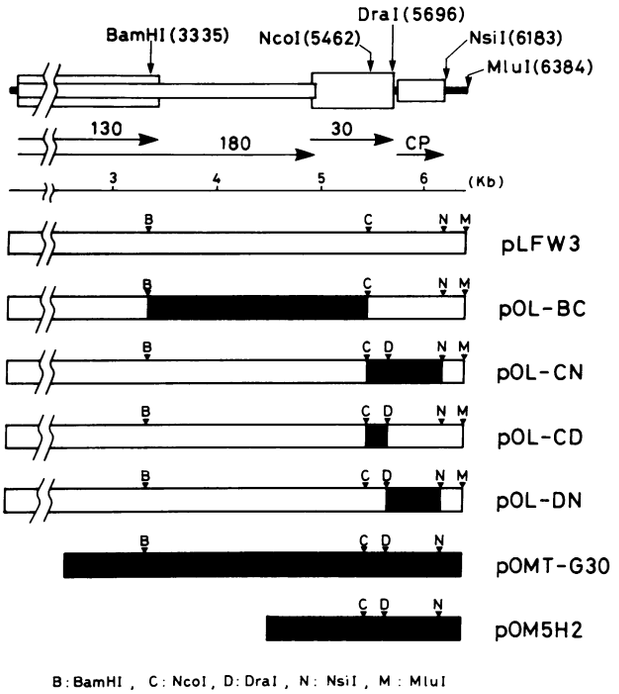


FIG. 1. Schematic representation of the genomic organization of TMV and recombinant cDNA clones. The genes of the 130-kDa, 180-kDa, and 30-kDa proteins and the coat protein (CP) are illustrated by open boxes and arrows over the scale, and the noncoding regions are illustrated by thick lines. Restriction enzyme sites used for the construction of plasmids are shown at the top with the numbers of residues (the first bases of their recognition sequences) from the 5′ end of the TMV-L genomic sequence in parentheses. The *Dra* I site does not exist in the TMV-L genomic sequence and thus not in pLFW3 or pOL-BC. Below the scale, the cloned region in each plasmid is shown by a thick bar. Open and closed areas are derived from TMV-L and TMV-OM cDNAs, respectively.

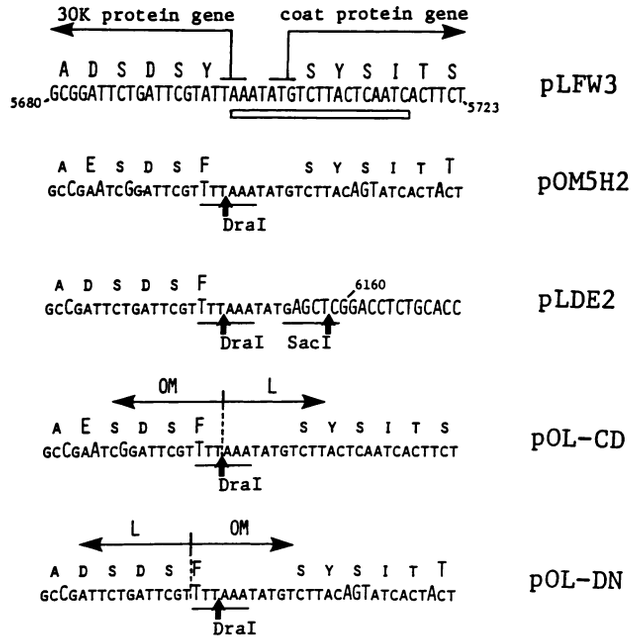


FIG. 2. Sequences surrounding the junctions between the genes of the 30-kDa and coat proteins. The nucleotide and deduced amino acid sequences of pLFW3, pOM5H2, pLDE2, pOL-CD, and pOL-DN are aligned. Large and small letters in the sequences of pOM5H2, pLDE2, pOL-CD, and pOL-DN indicate nucleotides and amino acids different from and identical to those of pLFW3, respectively. The open bar under the sequence of pLFW3 indicates the primer used to construct pOL-CD. The derivation of the sequences in pOL-CD and pOL-DN is shown by two-headed arrows. The recognition sequences of *Dra* I and *Sac* I and their cutting sites are indicated under the nucleotide sequences. Nucleotide residue numbers of the genomic RNA of TMV-L are indicated in pLFW3 and pLDE2.

fragment as described (21). After the DNA was digested with *Bst*EII (residue 5799), the resultant 0.11-kb fragment with *Bst*EII cohesive and *Dra* I blunt ends was ligated with the 0.25-kb *Nco* I/*Dra* I fragment of pOM5H2 and the 9.7-kb *Bst*EII/*Nco* I fragment of pLFW3 to create pOL-CD (Figs. 1 and 2). Generation of the *Dra* I site was confirmed by the *Dra* I digestion.

*Construction of pOL-DN.* The DNA fragment having the 30-kDa protein gene of TMV-L with the *Dra* I cutting sequence at one end was prepared from pLDE2 (unpublished). pLDE2, a pLFW3 derivative, is lacking in most of the coat protein gene (residues 5706–6159) and a *Sac* I linker is inserted instead. pLDE2 also has two base substitutions at residues 5682 and 5696, which are converted to the same bases as those of the TMV-OM sequence by the use of synthetic nucleotides (Fig. 2). The latter substitution generates the *Dra* I site at the junction between the coat and 30-kDa protein genes, although this causes a change of the last amino acid residue of the 30-kDa protein. pOL-DN was constructed by ligating the 0.23-kb *Nco* I/*Dra* I fragment (residues 5463–5698) of pLDE2, the 9.1-kb *Mlu* I/*Nco* I fragment of pLFW3, and the 0.68-kb *Dra* I/*Mlu* I fragment of pOL-CN.

**Host Response Analysis.** *In vitro* transcription of capped RNA, reconstitution, and inoculation of *in vitro* transcripts were carried out essentially as described (17). *Nicotiana tabacum* L. cvs. Xanthi nc with the *N* gene and Samsun with the *n* and *n′* genes were used as local lesion and systemic hosts, respectively. The responses to TMV infection of plants carrying the *N′* gene were analyzed using *N. sylvestris* and a Japanese tobacco cultivar, *N. tabacum* L. cv. Bright Yellow. The latter has been thought to carry the *N′* gene based on the observation of the same responses to infections of TMV strains as those of *N. sylvestris* (22). Accumulation of infectious virus in the upper uninoculated leaves was tested by local lesion assay of leaf homogenates as described (7).

## RESULTS

It has been observed that infections of TMV-OM (common strain) and TMV-L (tomato strain) cause different responses of plants carrying the *N′* gene (23); TMV-OM spreads systemically, causing mosaic symptoms, whereas TMV-L produces necrotic local lesions on the inoculated leaves (Table 1). It is assumed that TMV-L encodes a determinant responsible for the induction of the necrotic response of the *N′* plants. When the sequence of TMV-L encoding the determinant is replaced by the corresponding one of TMV-OM, the resultant recombinant virus should spread systemically without causing necrosis in the *N′* plants. To assign the relevant portion of the TMV-L genomic RNA, we first constructed two TMV-L-derived recombinant cDNA clones, partially having the TMV-OM-derived sequence, by using the following common restriction sites: the *Bam*HI site (residue 3335 of the L genomic sequence) near the end of the 130-kDa protein gene, the *Nco* I site (residue 5462) in the 30-kDa protein gene, and the *Nsi* I site (residue 6183) immediately downstream of the coat protein gene (Fig. 1). Resulting plasmids, pOL-BC and pOL-CN (Fig. 1), were different from pLFW3, the parent cDNA clone of TMV-L, in that they carried the sequences derived from TMV-OM between the *Bam*HI/*Nco* I sites and the *Nco* I/*Nsi* I sites, respectively.

*In vitro* transcripts derived from pLFW3, pOL-BC, and -CN (hereafter referred to as W3, BC, and CN transcripts,

Table 1. Symptoms of plants with different genetic backgrounds to infections of TMV and *in vitro* transcripts

| Inoculum | Plant* | | | |
|---|---|---|---|---|
| | *N. tabacum* Xanthi nc (*N*) | *N. tabacum* Samsun (*nn'*) | *N. tabacum* Bright Yellow (*N'*) | *N. sylvestris* (*N'*) |
| TMV-L | L | M | L | L |
| W3 transcript | L | M | L | L |
| BC transcript | L | ND | L | L |
| CN transcript | L | M | M | M |
| CD transcript | L | M | L | L |
| DN transcript | L | M | M | M |
| TMV-OM | L | M | M | M |

W3, BC, CN, CD, and DN transcripts were produced *in vitro* from pLFW3, pOL-BC, pOL-CN, pOL-CD, and pOL-DN, respectively. Each inoculum contained 0.1 μg of purified virus (TMV-L or TMV-OM) or virions reconstituted from transcripts and TMV coat protein, derived from 5 μg of linearized template plasmid, per ml of 10 mM sodium phosphate (pH 7.0). Fifty microliters and 50–100 μl of inocula were used for a half leaf of Xanthi nc tobacco and a whole leaf of the other plants, respectively. L, necrotic local lesions were observed in the inoculated leaves 2–4 days after inoculation, but mosaic symptoms did not appear in the upper uninoculated leaves. No spreading of viruses into the upper leaves of the *N'* plants was confirmed by local lesion assay of leaf homogenate using Xanthi nc tobacco. M, systemic mosaic symptoms were observed in upper uninoculated leaves 1–2 weeks after inoculation. The systemic spreading of progeny viruses into the upper leaves of the *N'* plants was confirmed as above. ND, systemic symptoms were not detected even 3 weeks after inoculation.
*Genes given in parentheses.

respectively) were prepared and their biological activities were assayed according to the described methods (17). The CN transcript produced local lesions on the inoculated leaves of a local lesion host, *N. tabacum* L. cv. Xanthi nc with the *N* gene (Fig. 3*A*) and spread systemically with typical mosaic symptoms when inoculated on a systemic host, *N. tabacum* L. cv. Samsun without either *N* or *N'* gene as the parental W3 transcript and TMV-L (Table 1). The lesions produced by infection of the BC transcript on Xanthi nc tobacco were small (Fig. 3*B*). Moreover, when the BC transcript was inoculated to Samsun tobacco, no mosaic symptoms appeared in the upper uninoculated leaves even 3 weeks after inoculation (Table 1). This may be explained by reduced replicability due to the chimeric 130-kDa/180-kDa proteins and/or low efficient cell-to-cell movement due to the chimeric 30-kDa protein.

We used two kinds of plants with the *N'* gene on different genetic backgrounds: *N. sylvestris* and *N. tabacum* L. cv. Bright Yellow. The BC transcript produced necrotic local lesions on the inoculated leaves of both kinds of plants, although they were smaller than those of the W3 transcript and TMV-L. The CN transcript, however, produced no local lesions. One to 2 weeks after inoculation, mosaic symptoms appeared in the upper uninoculated leaves and the virus spread systemically as was the case with TMV-OM (Fig. 3 *E* and *F*). Thus, the sequence of TMV-L between the *Nco* I and *Nsi* I sites, but not that between the *Bam*HI and *Nco* I sites, encodes the determinant responsible for the necrotic response of the *N'* plants to infection.

The *Nco* I/*Nsi* I sequence contains a part of the 30-kDa protein gene and the whole coat protein gene. For further assignment of the region responsible for the *N'* host response, the sequence between the *Nco* I and *Nsi* I sites of TMV-OM cDNA was divided into two parts at the junction between the 30-kDa protein and the coat protein genes using *Dra* I to create pOL-CD and pOL-DN (Figs. 1 and 2). pOL-CD carried the 30-kDa protein gene sequence downstream of the *Nco* I site of TMV-OM cDNA, whereas pOL-DN carried the sequence encoding the C-terminal amino acid of the 30-kDa protein and the coat protein gene of TMV-OM.

CD and DN transcripts produced local lesions indistinguishable from those of the W3 transcript on Xanthi nc tobacco and spread systemically with typical mosaic symptoms in Samsun tobacco (Table 1). When inoculated on the *N'* plants, the CD transcript produced necrotic local lesions on the inoculated leaves (Fig. 3*D*), whereas the DN transcript caused systemic mosaic symptoms in the upper uninoculated leaves without local lesions on the inoculated leaves (Fig.

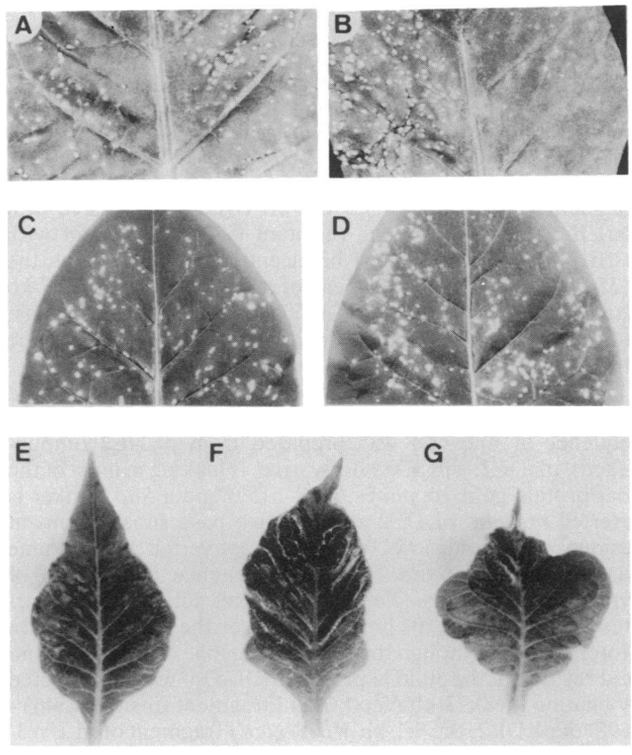


FIG. 3. Responses of *N. tabacum* L. cvs. Xanthi nc and Bright Yellow to infections of TMV and recombinant transcripts. Xanthi nc leaves were inoculated with TMV-L (left half of *A*), the CN transcript (right half of *A* and left half of *B*), and the BC transcript (right half of *B*), and necrotic local lesions were photographed 4 days after inoculation. Bright Yellow leaves were inoculated with the W3 transcript (*C*) and the CD transcript (*D*), with photography 5 days after inoculation. (*E–G*) Upper leaves of Bright Yellow showing mosaic symptoms 2 weeks after being infected with TMV-OM (*E*), the CN transcript (*F*), and the DN transcript (*G*).

3G). These results, summarized in Table 1, show that the determinant of TMV-L causing the necrotic response of the *N'* plants is coded in the coat protein gene sequence.

## DISCUSSION

The necrotic response of *N. sylvestris* to infection of some TMV strains has long been used to screen TMV mutants (24). Amino acid changes have been detected in the coat proteins of most chemically induced mutants becoming lesion-forming strains by mutagenesis (25, 26). However, from these reports, it could not be concluded that only the altered coat protein induced the necrotic response because it was not known whether there were other mutations in the other genes of the mutants. Another report suggests that the determinant for the necrotic response might be coded about one-quarter length of the genomic RNA from the 3' end (27), where the C terminus of the 180-kDa protein and the 30-kDa and coat proteins were coded. Separate evidence has suggested the involvement of the coat protein in the *N'* gene system: a strong correlation between lesion size and the temperature sensitivity of the coat protein subunits (28).

Here, we have revealed that the determinant (viral factor) of TMV-L that induces the necrotic response of the *N'* plants is coded in the coat protein gene sequence. This finding is supported by our recent observation that a TMV-L-derived coatless mutant, synthesized *in vitro* from the coatless cDNA construct (pLDCS29 in ref. 29), produced no local lesions on either *N. sylvestris* or Bright Yellow tobacco leaves even 10 days after inoculation.

It has been thought that the necrotic response of plants with either *N* or *N'* gene involves a similar mechanism (14). We have shown that the above described coatless mutant of TMV-L produces necrotic local lesions on Xanthi nc tobacco leaves carrying the *N* gene (29). This means that the viral factor responsible for the activation of the necrotic response of plants with the *N* gene is not coded in the coat protein gene sequence. Together with the results in this report, it is clear that different viral factors are involved in the activation of the two gene systems.

The most probable candidate for the determinant inducing the necrotic response of the *N'* plants is the coat protein. This protein synthesized in the infected cells would react with a host component and activate the subsequent reactions, resulting in the formation of necrotic local lesions on the *N'* plants. If so, the host component possibly could be identified by its presumed affinity for the TMV-L coat protein but not for the TMV-OM coat protein. We do not know whether the putative host component is the *N'* gene product itself.

Amino acid sequences of the TMV-OM and TMV-L coat proteins are similar; both are composed of 158 residues, and only 27 differences are found between the two (19). It will be interesting to know which substitution is crucial for the activation of the *N'* gene system. We constructed another recombinant cDNA by replacing the *Acc* I/*Nsi* I fragment of pOL-CN with the corresponding one (residues 6046–6188) of pLFW3. The resultant virus with the chimeric coat protein (N-terminal 113 residues from OM and C-terminal 45 residues from L) produced necrotic lesions on the inoculated leaves of the *N'* plants (not shown), suggesting that the crucial site is located in the C-terminal one-third of the L coat protein. However, we must interpret this result carefully, since we have no idea how many host genes have potential to be activated in the same way as the *N* and *N'* genes. The chimeric coat protein is different from the OM and L proteins, and therefore the necrotic response may not be necessarily the result of activation of the *N'* gene system. Identification and purification of the host factor would clarify the remaining ambiguities.

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