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Analysis of a Tobacco Mosaic Virus Strain Capable of Overcoming *N* Gene-Mediated Resistance

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The genome of Ob, a tobamovirus that overcomes the *N* gene-mediated hypersensitive response (HR), was cloned as a cDNA, and its nucleotide sequence was determined. The genomic organization of Ob is similar to that of other tobamoviruses, consisting of 6506 nucleotides and containing at least four open reading frames. These open reading frames encode a 126-kD polypeptide with a 183-kD readthrough product, a 30.6-kD movement protein, and an 18-kD coat protein. A bacteriophage T7 promoter sequence was fused to the full-length cDNA clone to obtain infectious RNA transcripts. These transcripts, when inoculated onto tobacco plants, induced disease symptoms indistinguishable from plants inoculated with Ob viral RNA. To determine which viral factor is responsible for the resistance-breaking character of Ob, a recombinant virus was constructed in which the movement protein gene of tobacco mosaic virus was replaced with that of Ob. Cultivar Xanthi NN tobacco plants infected with this virus responded with an HR, indicating that the Ob movement protein alone does not act to overcome the *N* gene-mediated response. Following mutagenesis of the infectious Ob cDNA clone with hydroxylamine, populations of transcripts from the mutagenized DNA were inoculated onto Xanthi NN tobacco, and a variant that induced the HR was identified. The mutant was analyzed and found to contain a single nucleotide change in the 126-kD gene. Recreating the mutation in the Ob cDNA clone by site-directed mutagenesis resulted in a virus that caused symptoms identical to the chemically induced mutant.

INTRODUCTION

Tobacco (*Nicotiana tabacum*) plants respond to infection by tobacco mosaic virus (TMV) and other tobamoviruses in a variety of ways. Some host–virus combinations result in a systemic disease characterized by mosaic symptoms that vary in severity depending on the virus strain. Other combinations are characterized by a hypersensitive response (HR), in which the development of necrosis limits the virus to the initial infection sites and prevents systemic spread. The local necrotic response is determined by both the plant and viral genomes; although many of the metabolic processes associated with the production of necrotic lesions are not pathogen specific, the induction of the HR is highly specific (Fritig et al., 1987). Virus strains that overcome some of the known resistance genes have been isolated and characterized by nucleotide sequence analysis (Saito et al., 1987; Knorr and Dawson, 1988; Meshi et al., 1988, 1989; Calder and Palukaitis, 1992).

The *N* gene, originally identified in *N. glutinosa* (Holmes, 1938), is a single locus, dominant gene and a durable source of resistance against tobamoviruses. Whereas the genetic history of the *N* locus has been described (Dunigan et al., 1987), little is known about the product of the *N* gene or the viral elicitor responsible for inducing the HR. A tobamovirus uniquely capable of overcoming the *N* gene–mediated HR has been isolated and designated tomato mosaic virus-Ob (ToMV-Ob) (Tobias et al., 1982; Csillery et al., 1983) but was not further characterized. Here, we report the cloning and nucleotide sequence of a ToMV-Ob cDNA clone from which infectious transcripts can be generated using bacteriophage T7 RNA polymerase. To better understand the capacity of Ob to overcome the *N* gene–mediated HR, we conducted a gene replacement experiment in which the Ob movement protein (MP) was expressed in the context of a movement-deficient infectious clone of the U1 (common) strain of TMV (TMV-U1), which elicits the *N* gene–mediated HR. We also present an analysis of a chemically induced mutant of the cloned Ob virus that induces local lesions on a tobacco cultivar carrying the *N* gene.

RESULTS

Molecular Characterization of Ob

The tobamovirus Ob has the capacity to overcome the *N* resistance gene against tobamoviruses and to produce a

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systemic infection in tobacco plants containing the *N* gene (Tobias et al., 1982; Csillery et al., 1983). Because of this novel characteristic and our interest in the nature of the *N* gene, full-length cloned cDNAs of Ob were constructed as described in the Methods section. In vitro transcripts from 13 of the 15 full-length clones tested were infectious on the indicator plant, *Chenopodium amaranticolor.* Transcripts from 10 of these clones gave systemic symptoms on *N* gene–containing tobacco plants (*N. tabacum* cv Xanthi NN) that were indistinguishable from those caused by inoculation with RNA isolated from purified Ob virions.

The sequence of a cloned Ob cDNA from which infectious transcripts were derived was determined and compared to the sequences of several other tobamoviruses, including TMV-U1 (Goelet et al., 1982), ToMV (Ohno et al., 1984), tobacco mild green mosaic virus (Solis and Garcia-Arenal, 1990), pepper mild mottle virus (Alonso et al., 1991), and cucumber green mottle mosaic virus (Ugaki et al., 1991). The Ob tobamovirus consists of 6506 nucleotides compared with 6395 for TMV-U1. As shown in Figure 1, computer-assisted analysis of the Ob sequence reveals that it has a genomic structure similar to that of other tobamoviruses, with characteristic 5′ and 3′ untranslated regions and four open reading frames (ORFs) in the (+) strand. The first ORF starts at nucleotide 69, extends to 3416, and encodes a predicted 126,012-D polypeptide. An amber termination codon is found at position 3414; translational readthrough into the second ORF would result in a polypeptide of 183,072 D that terminates at nucleotide 4919. The third ORF, which has no overlap with the 183-kD cistron, begins at position 4927 and extends to 5751, and is predicted to encode a 30,619-D MP. The ORF encoding the 17,981-D coat protein (CP) begins three nucleotides downstream of the stop codon of the MP gene and extends to nucleotide 6239. Analysis of the predicted RNA secondary structure of Ob indicates that the origin of virion assembly lies in the sequence of the MP gene. The 5′ untranslated leader of Ob, in contrast to TMV, ToMV, and pepper mild mottle virus, contains a single guanidine ribonucleotide. As in 3′ untranslated regions of other tobamoviruses (van Belkum et al., 1985), the last 267 nucleotides of Ob appear to be highly structured (data not shown).

Comparisons of the deduced amino acid sequences of Ob with those of other tobamoviruses are shown in Table 1. Ob was initially identified as a strain of ToMV based upon symptomatological and serological analyses of the virus (Tobias et al., 1982; Csillery et al., 1983). However, from the data in Table 1, it is evident that Ob is sufficiently distinct to be regarded as a separate tobamovirus.

Constructing a TMV/Ob Hybrid Virus

After completing the DNA sequence analysis and the biological characterization of Ob, experiments were initiated to identify the determinants that enable this tobamovirus to systemically infect tobacco cultivars that contain the *N* gene. Because other workers (Takamatsu et al., 1987; Dawson et al., 1988; Culver

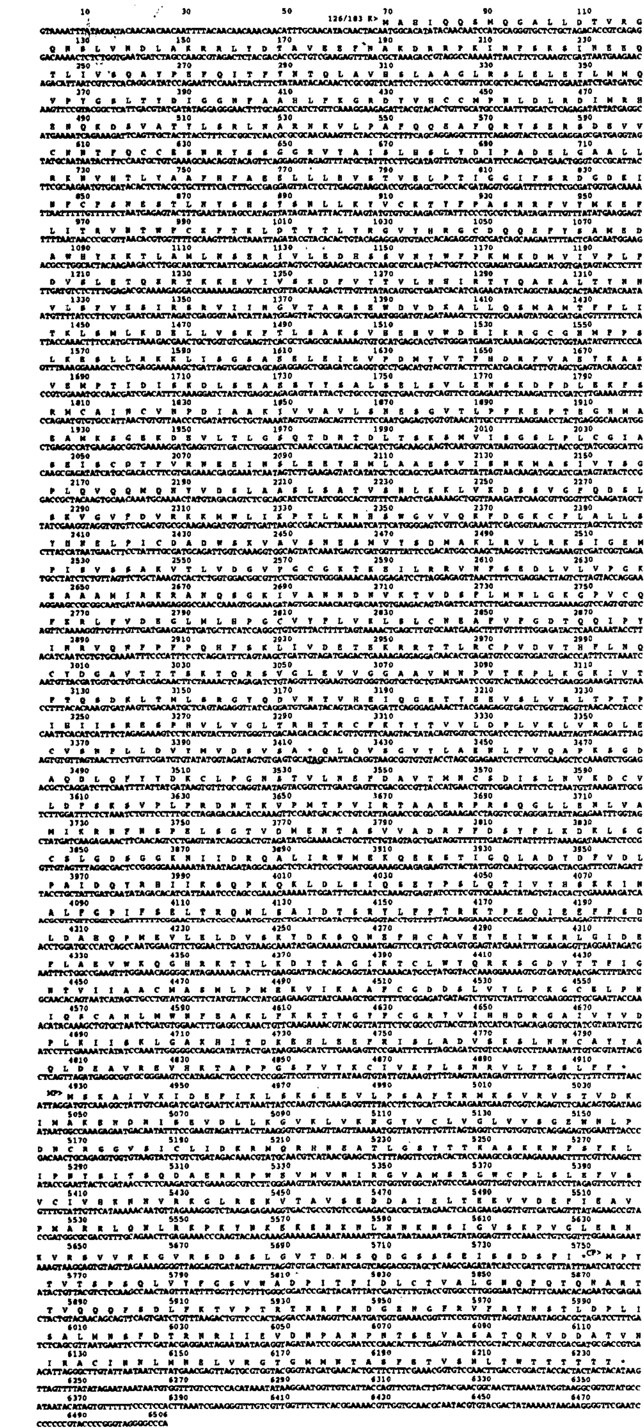

Figure 1. Nucleotide Sequence of the Ob Genome Contained in the Plasmid pOb.

The deduced amino acid sequences of the ORFs are displayed in single-letter code above the DNA sequence. Asterisks indicate the termination codons and the amber readthrough codon linking the 126- and 183-kD ORFs. The nucleotide sequence of Ob has been submitted to GenBank as accession number L11665.

Table 1. Percent Similarity of Ob-Encoded Proteins to Proteins of Other Tobamoviruses

Ob	TMV	ToMV	TMGMV	PMMV-S	CGMMV-SH
126/183 kD Replicase	67/69	67/70	61/63	65/67	38/43
Movement protein	54	56	59	60	28
Coat protein	59	55	69	68	33

TMV, tobacco mosaic virus (Goelet et al., 1982); ToMV, tomato mosaic virus (Ohno et al., 1984); TMGMV, tobacco mild green mosaic virus (Solis and Garcia-Arenal, 1990); PMMV-S, pepper mild mottle virus, Spanish isolate (Alonso et al., 1991); CGMMV-SH, cucumber green mottle mosaic virus, strain SH (Ugaki et al., 1991).

and Dawson, 1989) have shown that the CP of TMV is not responsible for induction of the *N* gene-mediated HR, it seems likely that the CP of Ob is not responsible for overcoming the *N* gene. To address the possibility that the MP of Ob is responsible for overcoming the *N* gene, we employed a virus recombination strategy. The MP of an infectious clone of TMV-U1 was replaced with the MP from Ob, as diagrammed in Figure 2. In the hybrid construct pT/O-M, the ATG initiation codon for the MP is spaced 13 nucleotides downstream of the termination codon for the 183-kD protein as compared to eight nucleotides in the parental Ob virus (Figure 1).

RNA transcripts generated in vitro from the pT/O-M construct were inoculated onto tobacco cultivar Xanthi NN and transgenic plant line 2005 (Deom et al., 1991). The 2005 line expresses the TMV-U1 MP gene under the control of the 35S promoter of cauliflower mosaic virus in Xanthi NN. This line was selected as a control because it complements the movement of a mutant of TMV that lacks a functional MP gene (Holt and Beachy, 1991). Approximately equal numbers of necrotic lesions were observed on the Xanthi NN and 2005 plants (data not shown), indicating that the Ob MP facilitates the local spread of the hybrid virus. No systemic disease symptoms were observed on plant line 2005 or Xanthi NN by 14 days after infection by T/O-M, demonstrating that the MP gene of Ob was not uniquely responsible for the capacity of Ob to cause systemic infection of Xanthi NN tobacco plants. No molecular analysis of progeny virus was performed.

The CP subgenomic promoter sequence and the sequences for nucleating virus assembly are in the MP gene sequences of both TMV (Goelet et al., 1982) and Ob (see above). Because of concerns that these Ob sequences might not be compatible with the TMV nucleotide sequences or encoded proteins in T/O-M, the TMV origin of assembly and subgenomic promoter were included in the hybrid construct. This led to a tandem duplication of Ob and TMV sequences and the possibility of sequence rearrangement or recombination. However, because roughly equal numbers of lesions were produced on Xanthi NN and 2005 plants infected with transcripts from pT/O-M, it is unlikely that rearrangement occurred. If rearrangement was a prerequisite for replication and spread, one might expect to see many fewer lesions on the NN line compared to the transgenic line 2005.

Mutagenesis of Ob and Characterization of a Mutant That Causes Local Lesions

To identify the nucleic acid or protein sequence of Ob that is involved in avoidance of local lesion formation in Xanthi NN tobacco, hydroxylamine mutagenesis was conducted with the infectious Ob cDNA clone. Mutagenized plasmid DNA was subsequently used as template for in vitro transcription reactions, and the resulting RNA was inoculated onto Xanthi NN plants. A local lesion appeared on one of these leaves, and the virus contained in the lesion was then analyzed. The lesion-inducing variant was first biologically isolated by repeated passage through the local lesion hosts Xanthi NN and *C. amaranticolor.* The necrotic local lesions caused on Xanthi NN tobacco by the variant, designated ObNL-1, are shown in Figure 3. These lesions were observed at 3 to 4 days after inoculation on Xanthi NN plants, whereas necrosis resulting from infection with TMV begins at 2 days after inoculation. As shown in Figure 3, the morphology of lesions induced by ObNL-1 differs from those caused by TMV in that the ObNL-1 lesions are somewhat smaller and have less pigmentation at their periphery than TMV-induced lesions. No virus could be recovered from the upper leaves of Xanthi NN plants infected with ObNL-1, confirming that the mutant was indeed localized to the inoculated leaf. No necrosis was observed when Xanthi nn plants, which do not carry the *N* gene, were infected with the virus.

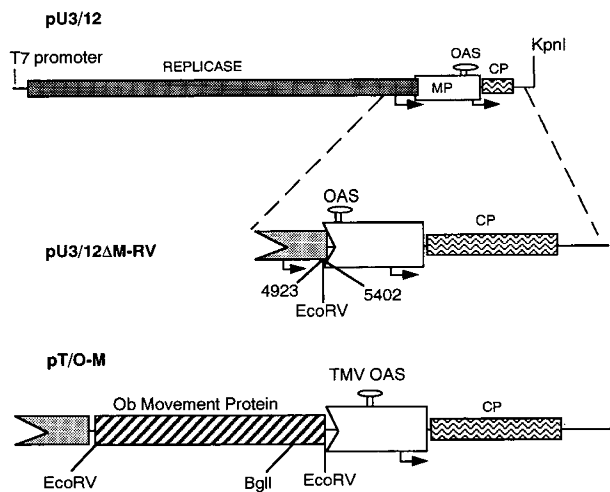

Figure 2. Construction of the TMV and Ob Hybrid pT/O-M.

Diagrammed are the infectious TMV clone pU3/12 and its derivative pU3/12ΔM-RV from which most of the MP had been deleted. The MP gene coding sequence of pOb was ligated to pU3/12ΔM-RV as described in Methods. pT/O-M is pictured at the bottom. OAS, origin of assembly.

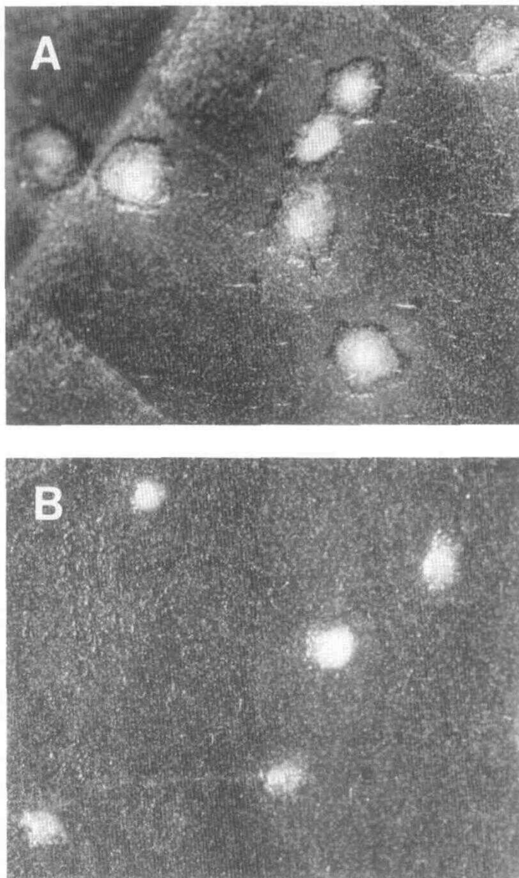

Figure 3. Local Lesions Produced on Leaves of Tobacco Cultivar Xanthi NN by TMV and ObNL-1.

(A) Symptoms on the inoculated leaf 4 days after infection with TMV-U1 virions.

(B) Symptoms on the inoculated leaf 4 days after infection with ObNL-1 virions.

To identify the nucleotides in ObNL-1 that were different from the parental Ob clone, a cloned cDNA of ObNL-1 was produced. A SphI-XmaI restriction fragment (nucleotides 445 to 6491), which represents all but the first 444 nucleotides and the last 10 nucleotides of ObNL-1, was substituted for the same fragment in the parental Ob clone, pOb, to create the clone pObNL-1. Transcripts from pObNL-1 induced symptoms on Xanthi NN plants that were indistinguishable from those caused by the virus ObNL-1.

A series of Ob-ObNL-1 hybrid viruses was constructed to map the determinant in ObNL-1 that caused the virus to induce necrotic local lesions. The cloned cDNAs of Ob and ObNL-1 were first arranged in tandem in a cloning vector, as diagrammed in Figure 4. Shared unique restriction sites were then used to delete specific sequences to obtain the hybrid viruses that are shown in Figure 5. This strategy simplified the generation of multiple hybrid viruses from the two starting clones. The hybrids that were first restricted and then ligated at the SphI sites, pO/L-SphI and pL/O-SphI, are essentially reconstructions of the parental clones pOb and pObNL-1, and they were included in this experiment to confirm the integrity of the tandem repeat clones. The other five sets of reciprocal hybrids shown in Figure 5 were used to identify the region of ObNL-1 that carries the mutation responsible for inducing necrotic local lesions on Xanthi NN tobacco. Transcripts derived from each of the hybrid virus constructs were inoculated onto Xanthi NN plants, and the plants were observed for local lesion formation. The results of these experiments (Figure 5) indicate that the 826-nucleotide segment of ObNL-1 between

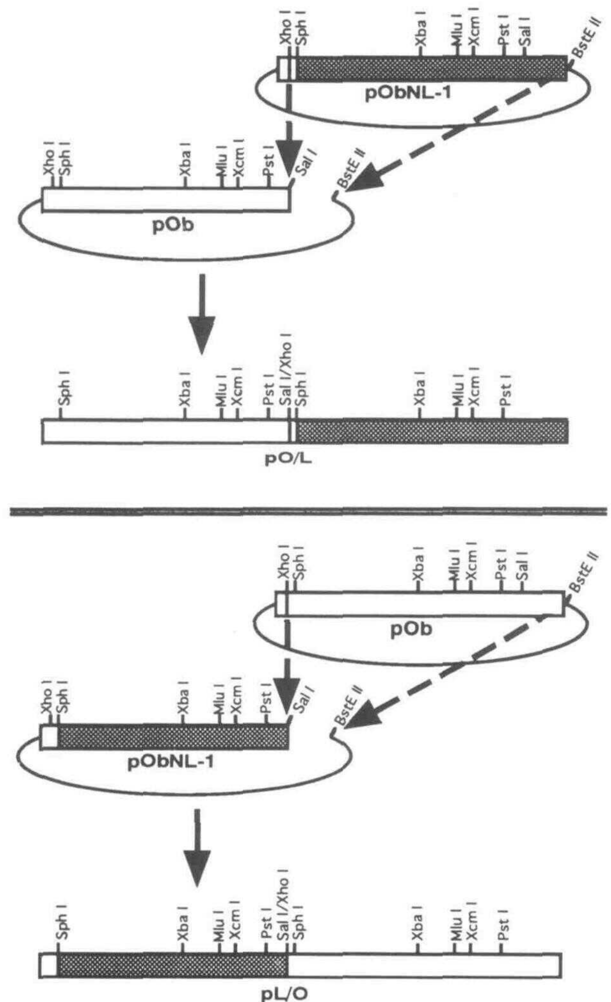

Figure 4. Schematic Representation of the Strategy Used to Construct Clones with Sequences of pOb and pObNL-1 in Direct Tandem Repeat.

After aligning the cloned virus cDNAs in tandem to obtain pO/L and pL/O, the restriction sites indicated were used to eliminate intervening sequences, thereby creating the hybrid viruses described in Figure 5.

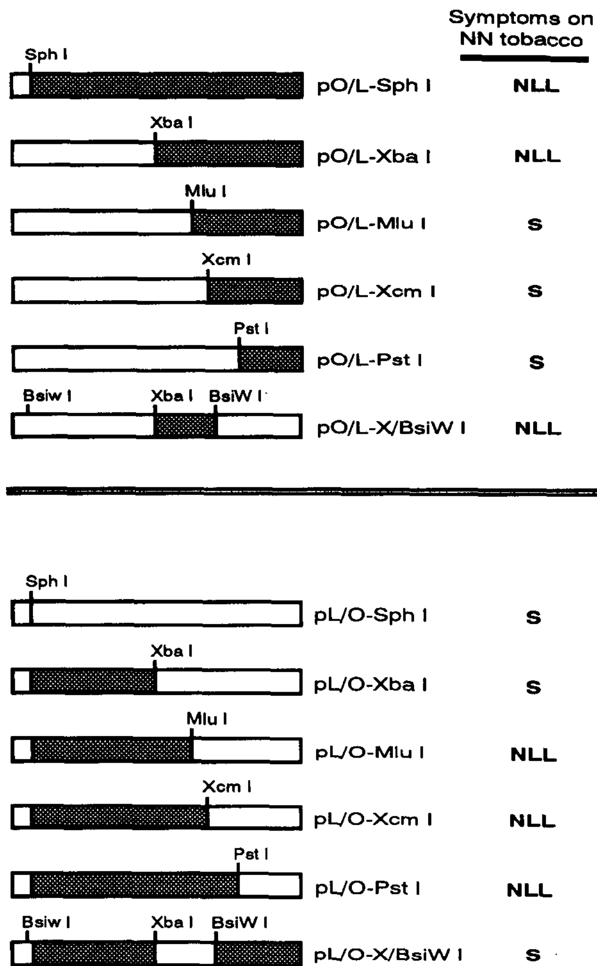

Figure 5. Hybrid Viruses Comprised of Sequences Derived from pOb and pObNL-1.

Sites used to construct the splices are as follows: SphI (nucleotide 445), XbaI (nucleotide 3255), MluI (nucleotide 4081), XcmI (nucleotide 4406), PstI (nucleotide 5166), and BsiWI (nucleotide 367, 4618). Unshaded regions in the diagrams represent sequences from pOb. Shaded regions represent pObNL-1 sequences. The symptoms induced on tobacco cultivar Xanthi NN plants by transcripts from these constructs are described to the right of each representation. S, systemic infection on Xanthi NN; NLL, necrotic local lesions on Xanthi NN.

the XbaI and MluI sites is responsible for eliciting necrotic local lesions. DNA sequence analysis of this segment revealed a single nucleotide alteration of C to T at position 3334. The C-to-T transition detected at this site is consistent with the type of mutation expected from hydroxylamine treatment (Busby et al., 1982). This mutation resulted in a predicted amino acid change from proline to leucine at amino acid 1089 in the 126-kD ORF.

Site-Directed Mutagenesis of pOb

The cloned cDNA pOb was engineered by oligonucleotide-directed mutagenesis to recreate the mutation at position 3334 that is found in pObNL-1. Transcripts derived from this clone, called pObNL-1.1, elicited local lesions when inoculated onto Xanthi NN tobacco plants. The lesions that were formed were identical in appearance to local lesions induced by ObNL-1. This result confirmed that the single nucleotide change in ObNL-1 was responsible for induction of the local lesion (HR) response in Xanthi NN tobacco. Progeny virus from several lesions was collected and inoculated to Xanthi nn tobacco plants, and virus was isolated from inoculated leaves at 7 days after infection. Direct RNA sequence analysis from nucleotides 3120 to 3535 confirmed that the sequence of the progeny virus was identical in this region to that of the in vitro mutant pObNL-1.1.

DISCUSSION

We have constructed full-length cDNA clones of the tobamovirus Ob. In vitro transcripts derived from some of these clones induced systemic symptoms on cultivars of *N. tabacum* carrying the *N* gene (i.e., cv Xanthi NN) that were similar to symptoms caused by Ob. By using a hybrid virus construct, we showed that the MP of Ob can complement the cell-to-cell movement of a movement-deficient TMV, but cannot confer the ability to overcome the *N* gene. Chemical mutagenesis of a full-length Ob clone produced a mutation in the 126-kD protein gene that caused a complete loss of resistance-breaking ability in the mutant virus, resulting in necrotic local lesion formation and localization of the mutant virus to the inoculated leaf of Xanthi NN plants. These experiments provide a unique approach to identify the gene in Ob and perhaps the gene in other tobamoviruses that is involved in the induction of the *N* gene–mediated HR.

Based on the gene-for-gene model of disease resistance described by Flor (1971), specific interactions between a host resistance factor and a pathogen-encoded gene product are considered crucial for recognition and subsequent induction of the HR. Because of the limited size of most viral genomes, it is unlikely that a viral gene will have as its sole function the determination of virulence or avirulence. Therefore, it may be predicted that a virus will acquire virulence by a change in its resistance-eliciting function, provided that it maintains its pathogenic function but no longer participates in the recognition event with the host resistance factor (Fraser, 1990).

There are several known cases in which changes in a viral gene determine whether the virus exhibits virulence or avirulence against a host resistance gene. For example, amino acid sequences in the CP of TMV determine whether *N. sylvestris*, which carries the *N′* gene, responds to infection with a local lesion or systemic infection (Saito et al., 1987; Knorr and Dawson, 1988). Mutations in the 126-kD protein of a ToMV strain

overcame the resistance conferred by the *Tm-1* gene in tomato (Meshi et al., 1988). Mutations in strains of ToMV that overcome *Tm-2* and *Tm-2*^{2} resistance genes map to the MP gene (Meshi et al., 1989; Calder and Palukaitis, 1992).

With the exception of Ob, all of the tobamoviruses described in the literature induce localized necrosis on *Nicotiana* species that carry the dominant *N* gene. Necrosis is prevented at temperatures greater than 30°C. It is of interest to note that while Ob does not induce necrosis at temperatures above 25°C, we have occasionally observed necrotic symptoms on inoculated leaves of Ob-infected plants maintained at somewhat lower temperatures (data not shown). This is in keeping with an earlier report (Csillery et al., 1983) that Ob can induce necrotic lesions on *N* gene–containing tobacco varieties at 18 to 20°C. However, virus recovered from the necrotic tissues that developed at lower temperatures did not induce necrosis when reinoculated onto plants held above 25°C, therefore eliminating the likelihood that necrosis was due to a change in the virus. Thus, induction of local necrosis at lower temperatures in no way interfered with the studies presented in this paper.

Although it is currently unknown which tobamovirus gene(s) induces the *N* gene–mediated HR, it is known that the CP is not responsible for its induction (for review, see Culver et al., 1991). It has also been shown that protoplasts isolated from leaves of *N* gene–containing tobacco plants fully support TMV infection without an HR (Otsuki et al., 1972), whereas infected callus (Beachy and Murakishi, 1971) and leaf tissues (Holmes, 1938) of varieties of *N* gene tobacco plants respond with an HR. Thus, multicellular infection or the presence of cell walls appears to be necessary for the induction of the *N* gene–mediated HR. Because the MP plays a central role in cell-to-cell movement of TMV and other tobamoviruses and is found in cell wall fractions (Deom et al., 1990), it might well be implicated as the inducer of the HR. However, the simple presence of the 30-kD MP evidently does not elicit the HR, because transgenic Xanthi NN tobacco plants that accumulate the TMV MP do not exhibit necrosis in the absence of infection (Deom et al., 1991). In contrast, expression of an elicitor CP either in transgenic *N. sylvestris* (Culver and Dawson, 1991) or *N. tabacum* cv Samsun EN calli (Pfitzner and Pfitzner, 1992), both of which carry the *N'* gene, can bring about a necrotic response in the absence of TMV infection.

Infection of Xanthi NN tobacco plants with a hybrid virus in which the TMV MP gene was replaced with the MP gene of Ob resulted in necrotic local lesions. This demonstrates that the Ob MP does not, in and of itself, confer the ability to overcome the *N* gene. Therefore, it is likely that the MP is not responsible for triggering the *N* gene.

To address the question of induction of the *N* gene–mediated HR more directly, we used chemical mutagenesis to generate a mutant of Ob, ObNL-1, that induces the formation of necrotic local lesions on Xanthi NN tobacco plants. It has been postulated that the ability to overcome resistance is a positive function of the resistance-breaking strain (van Loon, 1987). Following this model, nonbreaking strains should arise rather easily through mutation, leading to loss of that function as long as the mutation does not impair necessary virus functions. After cloning the local lesion mutant ObNL-1, the mutation that caused induction of the *N* gene response was mapped to the 126-kD ORF. Sequence analysis of the region of interest revealed a C-to-T transition at nucleotide 3334 of the infectious cDNA, leading to a predicted proline-to-leucine change at amino acid residue 1089. This change is 80 nucleotides upstream of the amber termination codon of the gene encoding the 126-kD protein. The introduction of this alteration into the parental Ob cDNA clone by site-directed mutagenesis demonstrated that a single nucleotide change is sufficient to bring about conversion of Ob from systemic infection to local lesion induction in Xanthi NN tobacco. A comparison of the 126-kD ORF sequences from amino acids 1073 to 1105 of Ob with the corresponding regions of the replicase (126-kD protein) in other tobamoviruses is shown in Figure 6. It is important to note that the proline in wild-type Ob at amino acid position 1089 is also present in most of the other tobamoviruses listed. On the basis of this single example, it is difficult to explain how the proline-to-leucine change could induce the HR.

Molecular analyses of several resistance-breaking tobamovirus mutants have resulted in the identification of changes in viral proteins that alter the charge of the protein, implying a disruption of an electrostatic interaction(s) between the viral protein and a host resistance factor. Strains of ToMV that break the *Tm-1* gene–mediated resistance in tomato differ from the wild-type ToMV in two amino acid residues of the replicase. These changes alter the net local charge of the protein (Meshi et al., 1988). Similarly, changes in ToMV strains that overcome *Tm-2* and *Tm-2*^{2} resistance genes map to residues in the MP gene that alter its charge (Meshi et al., 1989; Calder and Palukaitis, 1992). In the case of ObNL-1, however, the proline-to-leucine change does not alter charge, but may alter the

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Ob      (1073-1105)  LTRHTRCFKYYTVVLDPLVKLVRDLECVSNFLL
ObNL-1  (1073-1105)  LTRHTRCFKYYTVVLDLLVKLVRDLECVSNFLL
TMGMV   (1069-1101)  LTRHTKSFKYYTVVLDPLVQIISDLSSLSSFLL
TMV     (1074-1106)  LSRHTCSLKYYTVVMDPLVSIIRDLEKLSSYLL
ToMV    (1074-1106)  LSRHTKSLKYYTVVMDPLVSIIRDLERVSSYLL
PMMV-S  (1075-1107)  LSRHTRSIKYYTVVlDAVVSVLRDLECVSSYLL
CGMMV   (1101-1133)  LTRHTKAMVYYTVVFDAVTSIIADVEKVDQSIL
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Figure 6. Comparison of Partial Amino Acid Sequences of the 126-kD Proteins of Tobamoviruses.

The sequences shown correspond to the region surrounding the mutation in ObNL-1 that is responsible for the loss in its ability to overcome the *N* gene. Numbers within parentheses indicate the amino acid residues displayed in the alignment. The shaded bar indicates the proline of pOb that is changed to leucine in pObNL-1 and the in vitro mutant pObNL-1.1. Sources of amino acid sequences are as given in Table 1.

structural conformation of the protein. Computer-assisted analysis predicts that two separate α-helices in the wild-type protein would be replaced by a single, long α-helix in the mutant (data not shown).

The apparent importance of structural features of proteins in virus–host recognition with respect to the TMV CP and *N′* gene interaction has been described by Culver et al. (1991). In this example, alterations in a defined region of the CP that is involved in interactions between adjacent subunits lead to induction of the HR. In the case of ObNL-1, one could predict a scenario in which the induction of the *N* gene–mediated HR might result from a process associated with the viral replicase, for example, the rate of replication or expression of viral mRNAs. Continued mutagenesis of pOb will likely lead to the isolation of additional mutants that will result in a more complete understanding of how the *N* gene–mediated HR is induced during the course of tobamovirus infection.

METHODS

Virus Isolation and cDNA Synthesis

The Ob tobamovirus (Tobias et al., 1982; Csillery et al., 1983) was serially passaged twice on leaves of the local lesion indicator host *Chenopodium amaranticolor* (Tobias et al., 1982) before inoculation to *Nicotiana tabacum* cv Xanthi NN plants. Systemically infected leaves were harvested and the virus was purified by the method of Tobias et al. (1982), with the exception that the extraction buffer was 0.5 M sodium phosphate, pH 7, containing 0.1% β-mercaptoethanol, and an additional PEG precipitation step was performed in place of differential centrifugation.

Viral RNA was prepared by conventional SDS-phenol extraction of purified virus (Bruening et al., 1976). First strand cDNAs were produced with avian myeloblastosis virus reverse transcriptase as described by Holt et al. (1990). The second strand of DNA, unless otherwise stated, was synthesized using T7 DNA polymerase and a modified Sequenase sequencing kit (U.S. Biochemical Corp.) protocol. Briefly, the products of the reverse transcriptase reaction were extracted once with phenol/chloroform, precipitated with ethanol and 2.5 M NH_4OAc, and electrophoresed on a 1.5% low-melting-point alkaline agarose gel (Maniatis et al., 1982). Single-stranded cDNA was prepared for the second strand synthesis reaction by first equilibrating the gel overnight in 40 mM Tris-HCl, pH 7.5, with ethidium bromide (0.5 μg/mL), excising the desired band, and melting the agarose at 65°C. The melted agarose containing the single-stranded cDNA was brought to 40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, and 50 mM NaCl. A 10-fold molar excess of second strand primer was added, and the mixture was heated to 65°C for 2 min and annealed by slow cooling to 30°C. One microliter of 100 mM DTT, 0.8 μL of 10 mM of each deoxyribonucleotide triphosphate, and 2 μL of a 1:8 dilution of T7 DNA polymerase were added per 10 μL of annealing reaction. The reaction was allowed to proceed at 37°C for 30 min. Five volumes of Tris-EDTA buffer, pH 7.5, was added, followed by two extractions with buffer saturated phenol, one extraction with chloroform, and, finally, the nucleic acids were precipitated with ethanol and NH_4OAc. After resuspension of the nucleic acid in Tris-EDTA buffer, the DNA was ready for restriction enzyme digestion and cloning.

Initial cDNA clones of Ob were obtained by polymerase chain reaction (PCR) using primers specific for tobacco mosaic virus strain U1 (TMV-U1). A primer complementary to the 3′ end of TMV-U1 (nucleotides 6372 to 6395; Goelet et al., 1982) was used for first strand cDNA synthesis. PCR was performed on this template with the first strand primer and a primer originally designed to clone the 30-kD gene of TMV-U1 (nucleotides 4854 to 4874). The PCR product was cloned into the SmaI site of pUC19 and was subsequently sequenced with a Sequenase 2.0 sequencing kit. This sequence was used to derive a partial sequence of Ob from which more precise primers were designed.

Determining the Sequence of the 3′ End of Ob

To determine the sequence of the 3′ end of Ob, RNA was polyadenylated (Smith et al., 1988) and first strand cDNA was primed with an oligod(T) primer. Second strand synthesis was performed with a forward Ob primer (nucleotides 4891 to 4913), which was designed from the sequence derived from the PCR clones described above. The double-stranded cDNA was digested with EcoRI, and the fragment containing the 3′ terminus of viral RNA was cloned into SmaI-EcoRI–digested pUC19 and sequenced. From this information, we constructed a primer (Ob 1ST) that is complementary to the 3′ terminal 21 nucleotides of Ob and contains a 3′ flanking BstEII site.

Determining the Sequence of the 5′ End of Ob

Clones bearing sequences near the 5′ terminus were obtained by first strand cDNA synthesis using a reverse Ob primer representing nucleotides 5197 to 5215, followed by second strand replacement synthesis (Gubler and Hoffman, 1983). Double-stranded cDNAs were made blunt and cloned into the SmaI site of pUC19, and the ends of several resultant cloned cDNAs were sequenced. A primer representing nucleotides 266 to 288 was then used to directly sequence the 5′ terminus of viral RNA using the reverse transcriptase method of Fichot and Girard (1990) with several minor modifications. The labeling mix contained 1 μM dCTP, dGTP, and dTTP, 0.1 M Hepes, pH 8.0, and 10 μCi ^{35}S-dATP (1000 Ci/mmol), and the elongation mixes contained 2.5 mM of each deoxynucleotide triphosphate (dNTP), 1 mM Hepes, pH 8.0, 80 mM KCl, 6 mM $MgCl_2$, and 0.5 mM ddATP, ddTTP, or ddGTP or 0.25 mM ddCTP. Terminal deoxynucleotidyl transferase was used (DeBorde et al., 1986) to extend the reaction products that had not been terminated by a dideoxynucleotide triphosphate. The sequence thus obtained was used to design a sense strand primer (T7Ob-1) that matched nucleotides 1 to 21 and included a T7 RNA polymerase promoter such that subsequent transcription of the cloned cDNA with T7 RNA polymerase begins at the first nucleotide of the Ob genome.

Construction of a Full-Length cDNA of Ob

A full-length cloned cDNA of Ob was constructed by a two part strategy. First, a cDNA clone comprising nucleotides 1 to 5215 of Ob was generated using a primer from sequences within the movement protein (MP) and primer T7Ob-1. The double-stranded cDNA was digested by SmaI, which cuts upstream of the T7 promoter, and PstI, which cuts the DNA at nucleotide 5166. The double-stranded cDNA was ligated with pUC19 between the HindIII site, made blunt with the Klenow fragment of DNA polymerase I, and the PstI site. Second, a PCR product made with

the primer Ob 1ST and a second primer from within the MP sequence was digested at the unique PstI site. This fragment was inserted into the cDNA clone representing the 5′ portion of the RNA after digestion with PstI and SmaI. After confirming the nucleotide sequences at both ends of the cDNA insert, a set of multiple full-length Ob clones was constructed by splicing new cDNAs into the full-length clone using the XhoI (nucleotide 336) and XmaI (nucleotide 6491) sites of the original full-length clone. Resulting full-length clones were linearized with BstEII, transcribed with T7 RNA polymerase, and inoculated onto leaves of Xanthi NN tobacco and *C. amaranticolor.*

Sequence Analysis of an Infectious Ob Clone

Both strands of a representative infectious clone, plasmid pOb, were sequenced with the use of dITP where necessary to resolve compressions. End point deletion mutants were generated using the exonuclease III deletion method (S. Henikoff. Exonuclease III–generated deletions for DNA sequence analysis. Promega Notes 8, 1987). For the set of 5′ terminal deletions, plasmid was prepared by digestion with XhoI (nucleotide 336) and protection of this site with Klenow polymerase and α-phosphorothioate dNTPs (Putney et al., 1981), followed by digestion with SphI (nucleotide 445) and Klenow polymerase. Deletion reactions were performed and the resulting deletion series, as well as the ends of the full-length clone, were sequenced with an Ob sense strand primer (nucleotides 293 to 310). The sequence of the 340 nucleotides at the 5′ end was obtained using the pUC universal reverse primer. Deletions from the 3′ terminus were made by digesting the clone with BstEII and protecting the ends with α-phosphorothioate dNTPs before digestion with SmaI (nucleotide 6491) and allowing the exonuclease III reaction to proceed from the SmaI site toward the 5′ end. The resulting set of deletion clones was sequenced using a pUC universal forward primer. Sequence data were analyzed using DNASTAR computer programs (Madison WI).

Construction of a TMV/Ob Hybrid cDNA

The plasmid pOb was used as template for 10 cycles of PCR that employed primers flanking the Ob MP gene (nucleotides 4923 to 4942 and 5721 to 5751). The primers included EcoRV sites that were designed to be located outside of the MP sequence. The reverse primer used in this experiment also changed the A residue at position 5730 to C, producing a silent mutation that created a BglII site and destroyed the EcoRV site within the MP gene coding region. The PCR product was treated with EcoRV and ligated into EcoRV-digested ΔM-RV (Figure 2), a cloned cDNA of TMV-U1 from which a portion of the MP gene had been deleted with insertion of an EcoRV restriction site (Nejidat et al., 1991). Clones were screened for the proper orientation of the insert, and the resulting hybrid virus construct was linearized with KpnI and transcribed as given above.

Generation and Analysis of a Local Lesion Mutant of Ob

pOb was subjected to hydroxylamine mutagenesis as described by Busby et al. (1982). *Escherichia coli* transformed with 1 μg of the hydroxylamine-treated plasmid was grown overnight, and a plasmid miniprep was performed the following day. Transcript from 1 μg of this DNA was prepared as given above and inoculated onto Xanthi NN leaves. Virus from a necrotic lesion that appeared at 3 days after inoculation was passaged twice in Xanthi NN, twice in *C. amaranticolor,* and twice again in Xanthi NN before increasing the virus in Xanthi nn, a systemic host. Virus preparation and cDNA synthesis of this mutant, ObNL-1, were performed as described above for the wild-type Ob. The cDNA from ObNL-1 was digested with SphI and XmaI and inserted into these unique sites in pOb, replacing nucleotides 445 to 6491 of the wild-type clone with the homologous sequence of ObNL-1.

Hybrids of pOb and pObNL-1 were constructed after first placing sequences from the two genomic clones in tandem to create pO/L and pL/O, as shown in Figure 4. The compatible cohesive termini generated by XhoI (nucleotide 336) and SalI (nucleotide 6102) served to create the junction between the two cloned cDNAs. Following digestion with enzymes that cut only once in each viral genome, the fragment containing the vector sequences was self-ligated. Resultant clones, pO/L-SphI, pL/O-SphI, pO/L-XbaI, pL/O-XbaI, pO/L-MluI, pL/O-MluI, pO/L-XcmI, and pL/O-XcmI contained hybrid, unit-length, viral genomes, each with the site of ligation located at the restriction enzyme digestion site denoted in the name of the clone (Figure 5). pO/L-X/BsiWI and pL/O-X/BsiWI were derived from BsiWI fragments (nucleotides 367 to 4618) of pO/L-XbaI and pL/O-XbaI inserted into BsiWI-digested pOb and pObNL-1, respectively.

In Vitro Mutagenesis of pOb

pObNL-1.1 is a point mutant of pOb that was generated using directed mutagenesis essentially as described by Higuchi et al. (1988). Two primers, one in each orientation corresponding to Ob nucleotides 3319 to 3349, were designed such that they contained a single base substitution at position 3334, which changed the C/G base pair at position 3334 to T/A. Two initial PCR reactions were performed in which each reaction contained one of the mismatched primers paired with a distal primer. O/TF3.2, a forward primer annealing at nucleotides 3219 to 3238, was paired with the reverse mismatched primer, O/NL-1R. The forward mismatched primer, O/NL-1F, was paired with Ob4.15R, a reverse primer that anneals to nucleotides 4136 to 4154. After 15 cycles of PCR amplification and removal of the primers, the two PCR products were mixed and subjected to 15 further rounds of PCR with the two distal primers. The product was digested with XbaI and MluI and cloned into pOb between the XbaI and MluI sites. DNA sequence analysis confirmed that the mutation had been incorporated into the wild-type sequence. These clones of pObNL-1.1 were linearized with BstEII, transcribed, and inoculated onto Xanthi NN as described above.

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REFERENCES

Alonso, E., Garcia-Luque, I., de la Cruz, A., Wicke, B., Avila-Rincon, M.J., Serra, M.T., Castresana, C., and Diaz-Ruiz, J.R. (1991).

Nucleotide sequence of the genomic RNA of pepper mild mottle virus, a resistance-breaking tobamovirus in pepper. J. Gen. Virol. 72, 2875–2884.

Beachy, R.N., and Murakishi, H.H. (1971). Local lesion formation in tobacco tissue culture. Phytopathology **61**, 877–878.

Bruening, G., Beachy, R.N., Scalla, R., and Zaitlin, M. (1976). *In vitro* and *in vivo* translation of the ribonucleic acids of a cowpea strain of tobacco mosaic virus. Virology **71**, 498–517.

Busby, S., Irani, M., and de Crombrugghe, B. (1982). Isolation of mutant promoters in the *Escherichia coli* galactose operon using local mutagenesis on cloned DNA fragments. J. Mol. Biol. **154**, 197–209.

Calder, V.L., and Palukaitis, P. (1992). Nucleotide sequence analysis of the movement genes of resistance breaking strains of tomato mosaic virus. J. Gen. Virol. **73**, 165–168.

Csillery, G., Tobias, I., and Rusko, J. (1983). A new pepper strain of tomato mosaic virus. Acta Phytopathol. Acad. Sci. Hung. **18**, 195–200.

Culver, J.N., and Dawson, W.O. (1989). Tobacco mosaic virus coat protein: An elicitor of the hypersensitive reaction but not required for the development of mosaic symptoms in *Nicotiana sylvestris.* Virology **173**, 755–758.

Culver, J.N., and Dawson, W.O. (1991). Tobacco mosaic virus elicitor coat protein genes produce a hypersensitive phenotype in transgenic *Nicotiana sylvestris* plants. Mol. Plant-Microbe Interact. **4**, 458–463.

Culver, J.N., Lindbeck, A.G.C., and Dawson, W.O. (1991). Virus-host interactions: Induction of chlorotic and necrotic responses in plants by tobamoviruses. Annu. Rev. Phytopathol. **29**, 193–217.

Dawson, W.O., Bubrick, P., and Grantham, G.L. (1988). Modifications of the tobacco mosaic virus coat protein gene affecting replication, movement, and symptomatology. Phytopathology **78**, 783–789.

DeBorde, D.C., Naeve, C.W., Herlocher, M.L., and Maassab, H.F. (1986). Resolution of a common RNA sequencing ambiguity by terminal deoxynucleotidyl transferase. Anal. Biochem. **157**, 275–282.

Deom, C.M., Schubert, K.R., Wolf, S., Holt, C.A., Lucas, W.J., and Beachy, R.N. (1990). Molecular characterization and biological function of the movement protein of tobacco mosaic virus in transgenic plants. Proc. Natl. Acad. Sci. USA **87**, 3284–3288.

Deom, C.M., Wolf, S., Holt, C.A., Lucas, W.J., and Beachy, R.N. (1991). Altered function of the tobacco mosaic virus movement protein in a hypersensitive host. Virology **180**, 251–256.

Dunigan, D.D., Golemboski, D.B., and Zaitlin, M. (1987). Analysis of the *N* gene of *Nicotiana.* In Plant Resistance to Viruses, D. Evered and S. Harnett, eds (Chichester, UK: Wiley), pp. 120–135.

Fichot, O., and Girard, M. (1990). An improved method for sequencing of RNA templates. Nucl. Acids Res. **18**, 6162.

Flor, H.H. (1971). Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. **9**, 275-296.

Fraser, R.S.S. (1990). The genetics of resistance to plant viruses. Annu. Rev. Phytopathol. **28**, 179–200.

Fritig, B., Kauffmann, S., Dumas, B., Geoffroy, P., Kopp, M., and Legrand, M. (1987). Mechanism of the hypersensitivity reaction of plants. In Plant Resistance to Viruses, D. Evered and S. Harnett, eds (Chichester, UK: Wiley), pp. 92–108.

Goelet, P., Lomonossoff, G.P., Butler, P.J.G., Akam, M.E., Gait, M.J., and Karn, J. (1982). Nucleotide sequence of tobacco mosaic virus RNA. Proc. Natl. Acad. Sci. USA **79**, 5818–5822.

Gubler, U., and Hoffman, B.J. (1983). A simple and very efficient method for generating cDNA libraries. Gene **25**, 263–269.

Higuchi, R., Krummel, B., and Saiki, R.K. (1988). A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: Study of protein and DNA interactions. Nucl. Acids Res. **16**, 7351–7367.

Holmes, F.O. (1938). Inheritance of resistance to tobacco-mosaic disease in tobacco. Phytopathology **28**, 553–561.

Holt, C.A., and Beachy, R.N. (1991). *In vivo* complementation of infectious transcripts from mutant tobacco mosaic virus cDNAs in transgenic plants. Virology **181**, 109–117.

Holt, C.A., Hodgson, R.A.J., Coker, F.A., Beachy, R.N., and Nelson, R.S. (1990). Characterization of the masked strain of tobacco mosaic virus: Identification of the region responsible for symptom attenuation by analysis of an infectious cDNA clone. Mol. Plant-Microbe Interact. **3**, 417–423.

Knorr, D.A., and Dawson, W.O. (1988). A point mutation in the tobacco mosaic virus capsid protein gene induces hypersensitivity in *Nicotiana sylvestris.* Proc. Natl. Acad. Sci. USA **85**, 170–174.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Meshi, T., Motoyoshi, F., Adachi, A., Watanabe, Y., Takamatsu, N., and Okada, Y. (1988). 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.* EMBO J. **7**, 1575–1581.

Meshi, T., Motoyoshi, F., Maeda, T., Yoshiwoka, S., Watanabe, H., and Okada, Y. (1989). Mutations in the tobacco mosaic virus 30-kD protein gene overcome *Tm-2* resistance in tomato. Plant Cell **1**, 515–522.

Nejidat, A., Cellier, F., Holt, C.A., Gafny, R., Eggenberger, A.L., and Beachy, R.N. (1991). Transfer of the movement protein gene between two tobamoviruses: Influence on local lesion development. Virology **180**, 318–326.

Ohno, T., Aoyagi, M., Yamanishi, Y., Saito, H., Ikawa, S., Meshi, T., and Okada, Y. (1984). Nucleotide sequence of the tobacco mosaic virus (tomato strain) genome and comparison with the common strain genome. J. Biochem. **96**, 1915–1923.

Otsuki, Y., Shimomura, T., and Takebe, I. (1972). Tobacco mosaic virus multiplication and expression of the N gene in necrotic responding tobacco varieties. Virology **50**, 45–50.

Pfitzner, U.M., and Pfitzner, A.J.P. (1992). Expression of a viral avirulence gene in transgenic plants is sufficient to induce the hypersensitive defense reaction. Mol. Plant-Microbe Interact. **5**, 318–321.

Putney, S.D., Benkovic, S.J., and Schimmel, P.R. (1981). A DNA fragment with an α-phosphorothioate nucleotide at one end is asymmetrically blocked from digestion by exonuclease III and can be replicated *in vivo.* Proc. Natl. Acad. Sci. USA **78**, 7350–7354.

Saito, T., Meshi, T., Takamatsu, N., and Okada, Y. (1987). Coat protein gene sequence of tobacco mosaic virus encodes a host response determinant. Proc. Natl. Acad. Sci. USA **84**, 6074–6077.

Smith, O.P., Harris, K.F., Toler, R.W., and Summers, M.D. (1988). Molecular cloning of potato leaf roll virus complementary DNA. Phytopathology **78**, 1060–1066.

Solis, I., and Garcia-Arenal, F. (1990). The complete nucleotide sequence of the genomic RNA of the tobamovirus tobacco mild green mosaic virus. Virology **177,** 553–558.

Takamatsu, N., Ishikawa, M., Meshi, T., and Okada, Y. (1987). Expression of bacterial chloramphenicol acetyltransferase gene in tobacco plants mediated by TMV-RNA. EMBO J. **6,** 307–311.

Tobias, I., Rast, A.Th.B., and Maat, D.Z. (1982). Tobamoviruses of pepper, eggplant, and tobacco: Comparative host reactions and serological relationships. Neth. J. Plant Pathol. **88,** 257–268.

Ugaki, M., Tomiyama, M., Kakutani, T., Hidaka, S., Kiguchi, T., Nagata, R., Sato, T., Motoyoshi, F., and Nishiguchi, M. (1991). The complete nucleotide sequence of cucumber green mottle mosaic virus (SH strain) genomic RNA. J. Gen. Virol. **72,** 1487–1495.

van Belkum, A., Abrahams, J.P., Pleij, C.W.A., and Bosch, L. (1985). Five pseudoknots are present at the 204 nucleotides long 3′ noncoding region of tobacco mosaic virus RNA. Nucl. Acids Res. **13,** 7673–7686.

van Loon, L.C. (1987). Disease induction by plant viruses. Adv. Virus Res. **33,** 205–255.