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Genetics

A point mutation in the tobacco mosaic virus capsid protein gene induces hypersensitivity in *Nicotiana sylvestris*

(*in vitro* transcription/recombinant DNA/genetic mapping/*N'* gene)

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ABSTRACT **In *Nicotiana sylvestris*, the *N'* gene confers hypersensitive resistance to some strains of tobacco mosaic virus (TMV) but not to the common strain. TMV sequences responsible for inducing local lesion formation in this host were identified by using cDNA clones to construct genomic recombinants between the common strain genome and a local-lesion-inducing mutant. To assay for sequences conferring the mutant phenotype, *in vitro* transcripts of recombinants were inoculated onto leaves of *N. sylvestris* and observed for the formation of either local lesions or a systemic infection. Sequences from the mutant that converted the hybrid genome to the mutant phenotype were located between nucleotides 5972 and 6206. Sequence analysis of this region revealed point mutations in the mutant at nucleotides 6157 (cytosine to uracil) and 6199 (adenine to guanine). The mutation at 6157 changes the capsid protein gene to specify phenylalanine rather than serine at position 148; nucleotide 6199 occurs in the 3' nontranslated region. When each point mutation was individually substituted into the wild-type background, transcripts containing only the alteration at 6157 produced local lesions on *N. sylvestris*, whereas transcripts containing only the alteration at 6199 produced systemic mosaic symptoms. The frequency of mutation was examined by partially sequencing virion RNA from six additional independent local-lesion mutants. Five mutants had the same alteration at 6157 as the original mutant and none had the alteration at 6199. This work demonstrates that the capsid protein gene of TMV is multifunctional, both encoding the virion structural protein and mediating the outcome of infection in *N. sylvestris*.**

During interactions between plants and parasites, specific incompatibility often is manifested by rapid necrosis of infected and neighboring host cells. Occurrence of this phenomenon, termed the hypersensitive reaction (HR), frequently results in resistance of plants to diseases caused by a variety of pathogens (1–3). Because plant virus diseases are controlled most effectively by host resistance, which often is conferred by single dominant genes, HR-type resistance is useful in plant breeding programs. Unfortunately, sources for HR-type resistance are unavailable for many plant-virus systems, due in many cases to difficulty in transferring plant-virus HR genes by conventional breeding practices (3). Better characterization of host and pathogen genetic elements involved in hypersensitivity has great potential for developing new methods to control losses from plant disease.

Hypersensitivity usually results when host genes for resistance interact with complementary genes for avirulence in the pathogen. Through genetic studies, a number of pathogen-specific host resistance genes and specific avirulence genes in bacterial pathogens have been identified. For instance, avirulence genes have been isolated and characterized by Staskawicz *et al.* (4) and Gabriel *et al.* (5). Both groups isolated avirulence genes by transferring cloned DNA library fragments between different pathogen races, then testing these for incompatibility on host plants with defined race-specific resistance genes. Schoelz *et al.* (6), using engineered recombinations between two strains of cauliflower mosaic virus, showed that gene *VI* determines whether infection will be compatible or result in the HR in *Datura stramonium*.

In RNA plant virus–host systems, many host genes for hypersensitive resistance have been identified. In *Nicotiana* species, the *N* gene, originally from *Nicotiana glutinosa*, confers resistance to all strains of tobacco mosaic virus (TMV). Plants containing the *N* gene respond to TMV by forming small areas of necrosis, termed local lesions, surrounding infection sites (7). The *N'* gene from *Nicotiana sylvestris* confers resistance to some strains of TMV but not to the common strain. However, mutants of the common strain may be isolated that form local lesions on hosts containing the *N'* gene (8). Although there is little understanding of viral genes involved in eliciting this response, some workers have found differences in TMV associated with the ability to induce the HR in *N'* gene hosts (9, 10). However, exact viral sequences involved in conferring the HR phenotype have not been identified.

We have recently generated a system that allows functions of TMV to be precisely mapped. This was accomplished by cloning the entire viral genome as cDNA from which infectious transcripts may be produced *in vitro* (11), thereby extending TMV replication through an artificial DNA phase. This allows the viral genome to be manipulated as DNA, then converted into RNA for observation of biological effects. Using chimeric TMV genomes constructed by exchanging cloned cDNA segments between a local-lesion mutant and its wild-type parent, we have identified a point mutation at nucleotide 6157 that alters the capsid protein gene to specify phenylalanine rather than serine at position 148 and that is responsible for conferring the ability of TMV to elicit the HR in *N. sylvestris*.

MATERIALS AND METHODS

Virus Strains and Mutants. The parent virus for these studies, TMV204, was obtained by infecting Xanthi tobacco (*Nicotiana tabacum* Linnaeus var. Xanthi) with *in vitro* transcripts of plasmid pTMV204 as described previously (11). Purified TMV204 progeny virions were treated with nitrous acid as described by Siegel (12), diluted to 60 ng/μl in 0.1 M potassium phosphate buffer (pH 7.0), then inoculated onto leaves of *N. sylvestris* Spegazzini and Comes. Necrotic local lesions that appeared 3–5 days after inoculation were ground individually between sterilized glass microscope slides in 0.01 M potassium phosphate buffer (pH 7.0) and (1% wt/vol) Celite, then transferred to separate leaves of *N. sylvestris*.

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Abbreviations: HR, hypersensitive reaction; TMV, tobacco mosaic virus.

After several transfers, mutants producing only local lesions without systemic spread were propagated by systemic infection in tomato or Xanthi tobacco, then purified by differential centrifugation. Care was taken to ensure that mutants derived by nitrous acid treatment were separately maintained, at first on individual leaves and later on separate plants.

cDNA Synthesis and Cloning. Procedures for RNA preparation and cDNA cloning were essentially as described previously (11). Mutant double-stranded cDNA was prepared for cloning by adding synthetic *Xho* I linker sequences, then digesting with *Bam*HI and *Xho* I. To accept mutant cDNAs, plasmid pBR322-XhoI was constructed by removing overhanging nucleotides from *Hin*dIII-digested pBR322 with mung bean nuclease (Pharmacia), then adding *Xho* I linkers. Mutant cDNAs then were inserted between the *Bam*HI and *Xho* I sites of pBR322-XhoI.

Construction of Recombinant Genomes. Subclones and engineered recombinants between mutant and wild-type sequences were generated by ligating specific DNA restriction fragments excised from low melting-point agarose electrophoresis gels according to Crouse *et al.* (13). pDK4 was constructed by ligating the promoter-containing (14) 5′ *Pst* I(−856)–*Bam*HI(3333)* fragment from pTMV204 to the *Bam*HI–*Xho* I(6402) fragment from pDK3, a cDNA clone of the mutant, and inserting both together into *Pst* I–*Xho* I-digested pBR322-XhoI (map in Fig. 1). Two recombinants were generated with mutant sequences between the *Bam*HI-(3333) and *Nco* I(5460) sites (pDK15), and between the *Nco* I(5460) and the *Xho* I(6402) site at the 3′ terminus (pDK14), by exchanging *Nco* I(5460)–*Sal* I fragments between pTMV204 and pDK4 (Fig. 2). To make further recombinations at restriction sites not unique within the TMV sequence, pDK16 was constructed by inserting the 1.8-kb *Hin*dIII-(5081)–*Sau* I fragment from pDK14 into pUC19. Recombinations made with pDK16 as the recipient (Fig. 1) were confirmed by restriction map analysis. In a separate ligation reaction, genomic-length recombinants were regenerated by replacing the *Nco* I(5460)–*Sal* I fragment from pTMV204 with the same fragment from one of the intermediate subclones (Fig. 2). Intermediate pDK17 was constructed by replacing the *Sau* I(5805)–*Sal* I restriction fragment from pDK16 with the same fragment from pTMV204. Intermediate pDK18 was constructed similarly by replacing the *Hin*dIII(5081)–*Sal* I(5805) fragment from pDK16 with the corresponding wild-type fragment. Intermediates pDK21, pDK22, and pDK23 were constructed by replacing the *Asu* II(6364)–*Sal* I, *Nsi* I(6207)–*Sal* I, and *Bsm* I(5971)–*Sal* I fragments from pDK18 with the corresponding wild-type fragments (Fig. 1).

A subclone of pTMV204, p3F1, contains the *Bam*HI-(3333)–*Pst* I(6406) fragment in pUC19. To construct pDK27, the *Acc* I(6056)–*Sfa*NI(6192) fragment from pDK17 and the *Sfa*NI(6192)–*Pst* I(6406) fragment from pDK21 were ligated into p3F1 from which the *Acc* I(6056)–*Pst* I(6406) fragment had been removed. Plasmid pDK28 was constructed similarly, but using the *Acc* I(6056)–*Sfa*NI(6192) fragment from pDK21 and the *Sfa*NI–*Pst* I fragment from pDK17. The *Nco* I(5460)–*Pst* I fragments from pDK27 and pDK28 were separately ligated into pDK17, generating pDK29 and pDK30 (Fig. 1). Recombinations engineered into subclones were incorporated into pTMV204 as illustrated in Fig. 2.

Transcripts of plasmids containing recombinant viral genomes were prepared as described previously (11) and inoculated directly onto *N. sylvestris*.

Nucleotide Sequencing. Nucleotide sequences for cloned TMV cDNAs were determined by using the dideoxy chain-terminating method (15) or by the chemical method (16). Virion

*Numbers in parentheses beside restriction endonucleases indicate positions of sites with respect to the 5′-terminal nucleotide of TMV.

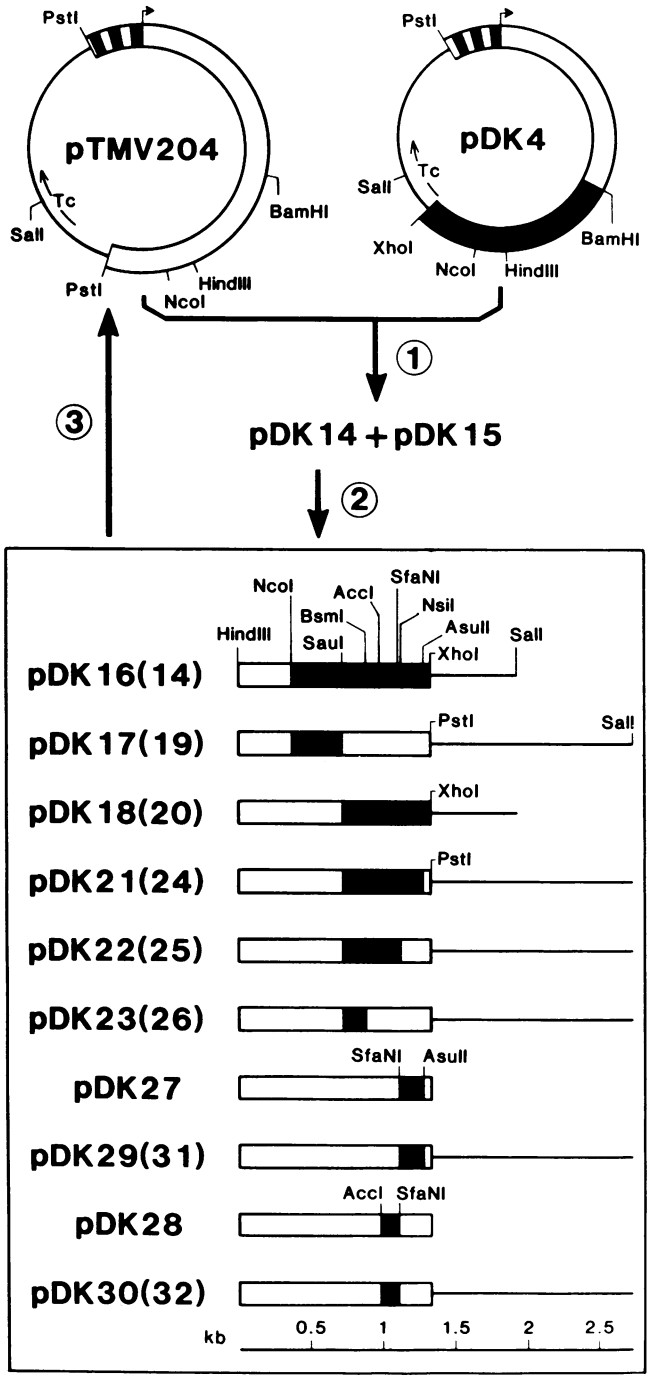

FIG. 1. Intermediate subclones generated for constructing recombinant TMV genomes used to map the mutation responsible for the local-lesion phenotype of mutant TMV204-D1. Circular maps of wild-type pTMV204 and pDK4, a local-lesion-producing recombinant, are represented above. Alternating dark and light bands represent promoter sequences, with transcription starts indicated by bent arrows. Dark areas represent sequences from a mutant cDNA clone (pDK3), open areas represent sequences from pTMV204, and single lines represent vector (pBR322) sequences (Tc, tetracycline resistance gene). Different combinations of restriction fragments from pTMV204 or pDK4 inserted into pUC19, or into p3F1 for pDK27 and pDK28, to form the intermediate subclones are represented in the box below. Step 1, exchange *Nco* I–*Sal* I fragments. Step 2, subclone pDK14 *Hin*dIII–*Sal* I fragment in pUC19. Step 3, replace *Nco* I–*Sal* I fragment from pTMV204 with *Nco* I–*Sal* I fragments from intermediate subclones. Numbers in parentheses indicate the genomic-length recombinant constructed from each subclone. Scale in kilobases (kb) is shown at the bottom.

RNA sequences were obtained essentially as described by Zimmern and Kaesberg (17). Synthetic oligonucleotide primers complementary to specific regions of the genome were annealed with template RNA in water at a primer-to-template molar ratio of 2:1 at 70°C, slowly cooled to 20°C, and then stored on ice. Individual termination reaction mixtures contained 0.2 μg of annealed primer-template, a 5:1 molar ratio of a single deoxynucleoside/dideoxynucleoside triphosphate for each reaction, and 2.0 units of avian myeloblastosis virus reverse transcriptase (Molecular Genetics Resources, Tampa, FL) in a total volume of 6.0 μl.

RESULTS

The initial objective was to obtain TMV mutants that induce the HR in *N. sylvestris* instead of systemically invading this host and causing mosaic symptoms as with the wild-type parent. This mutant phenotype has been previously described (8, 12). Since cDNA clones of these mutants ultimately would be used to construct hybrids with wild type, it was important that they be generated from a uniform population whose cDNA would have common restriction endonuclease sites. Thus, the wild-type parental TMV used in these experiments was obtained from *in vitro* transcripts of pTMV204. This source was expected to provide a near-homogeneous virus population free of contaminating strains that are normally present in naturally derived wild-type virus populations.

Seven nitrous acid mutants of TMV204 that produced local lesions on *N. sylvestris* were isolated, purified, and used in this study. These mutants generally were initially contaminated with wild-type virus that systemically infected the assay host, *N. sylvestris*. Mutants free of the wild-type virus were selected after several single-lesion transfers on the basis of their inability to spread systemically in *N. sylvestris*. Only local-lesion mutants that failed to spread systemically were further analyzed.

Mutant TMV204-D1 was chosen for detailed study to determine which genomic sequences were responsible for conferring the local-lesion phenotype. A library of cDNA clones of mutant TMV204-D1 was made. Restriction map analysis indicated that clone pDK3 contained a cDNA insert corresponding to the 3-terminal 3062 nucleotides of TMV. This cDNA of the mutant TMV204-D1 was used to determine if sequences responsible for altered phenotype mapped in the 3′ half of the TMV genome. The recombinant plasmid pDK4 (Fig. 1) contains viral 5′ sequences from wild-type pTMV204 and viral 3′ sequences from mutant pDK3. Transcripts made *in vitro* from *Xho* I-digested pDK4 produced local lesions on *N. sylvestris*, whereas transcripts of pTMV204 did not, indicating that the local-lesion phenotype of TMV204-D1 maps within the 3′ half of the genome.

To find specific sequences in the 3′ segment of TMV204-D1 responsible for the induction of local lesions in *N. sylvestris*, we constructed a series of recombinants that, in effect, progressively substituted smaller segments of mutant cDNA into the cloned wild-type genome (Figs. 1 and 2). To ensure that changes outside of the segments altered by engineered recombination were not responsible for changes in phenotype, only pTMV204 was used as the genetic background for this study, and *in vitro* transcripts of pTMV204 were inoculated into test plants as a control in each experiment. In no case did transcripts of pTMV204 produce local lesions on *N. sylvestris*. Furthermore, independent transcriptions from each of the engineered genomic-length recombinants were tested several times and in each case the phenotype was consistent.

The 3-kb segment of mutant sequence in pDK4 was split at the *Nco* I(5460) site to give pDK14 and pDK15 (Fig. 2). *In vitro* transcripts from pDK14 produced local lesions on *N. sylvestris*, whereas those from pDK15 produced systemic mosaic symptoms. Because further definition of mutant sequences would require digesting with restriction enzymes recognizing more than one site within the entire TMV cDNA, subclone pDK16 was used as an intermediate for constructing additional changes (Fig. 1). Segments of mutant sequences in pDK16 were progressively replaced by the corresponding wild-type segments from pTMV204. The resulting intermediate constructs then were directionally inserted into pTMV204 in place of the *Nco* I(5460)–*Sal* I restriction fragment (Fig. 1). In the case of pDK19 and pDK20, in which the mutant segment from pDK14 was split at the *Sau* I(5805) restriction site, pDK20 produced local lesions in *N. sylvestris*, whereas pDK19 produced only systemic infection. Three additional plasmids, pDK24, pDK25, and pDK26, were constructed by progressively replacing the mutant sequences in pDK20 with wild-type sequences. Symptoms produced after inoculation with *in vitro* transcripts of the full-length cDNAs indicated that ability to induce local lesions was retained in pDK24 within the 559-base-pair (bp) *Sau* I(5805)–*Asu* II(6364) fragment, and in pDK25, within the 402-bp *Sau* I(5805)–*Nsi* I(6207) fragment. However, pDK26 contains the 166-bp *Sau* I(5805)–*Bsm* I(5971) fragment from the mutant and produces a systemic mosaic rather than local lesions (Fig. 2). Therefore, the difference between ability to induce either local lesions or systemic infection in *N. sylvestris* resided between nucleotides 5971 and 6206.

The nucleotide sequence of the segment that alters phenotype of mutant TMV204-D1 was compared with the same region in the wild-type parent by both chemical and dideoxynucleotide sequencing methods. Two point mutations were found within the *Bsm* I(5971)–*Nsi* I(6207) fragment of pDK21 (Fig. 1). At nucleotide 6157, cytosine found in the wild type was replaced by uracil in the mutant, and at nucleotide 6199, wild-type adenine was replaced by guanine. The same changes within this region were found in TMV204-D1 by sequencing progeny virus RNA. The mutation at nucleotide 6157 occurs within the capsid protein gene (nucleotides 5712–6191) and alters the codon triplet for serine at position 148 to that for phenylalanine. Nucleotide 6199 occurs in the 3′ nontranslated region.

To determine whether both exchanges were required for conferring the mutant phenotype, each point mutation was separately engineered into the wild-type genome (Fig. 1). Sequence analysis of the carboxyl-terminal region of the capsid protein genes showed that pDK31 contains only the point mutation at nucleotide 6199 and pDK32 contains only the mutation at 6157. *In vitro* transcripts of pDK32 produced necrotic local lesions without systemic infection in *N. sylvestris*, whereas those from pDK31 produced only systemic symptoms with no necrosis. Also, the single nucleotide changes in pDK31 and pDK32 were retained by the RNA of progeny virus. Thus, a point mutation at nucleotide 6157 in pDK32 is responsible for conferring the local-lesion phenotype of TMV204-D1.

All TMV mutants with the local-lesion phenotype may not contain the same nucleotide alteration. The frequency of this mutation was examined by determining virion RNA sequences between nucleotides 6115 and 6217 for six other independently derived local-lesion mutants. In five of the mutants, cytosine was replaced by uracil at nucleotide 6157. None of the mutants contained the additional change found in TMV204-D1 at nucleotide 6199, and no other differences from the wild-type sequence were detected. Although sequence data for mutant TMV204-D7 were extended to include nucleotides 5916–6217, no alterations from wild type were detected. A total of six of seven independent mutants that were examined had the same alteration at nucleotide 6157, indicating that this mutation apparently occurred with high frequency. On the other hand, mutant TMV204-D7 did

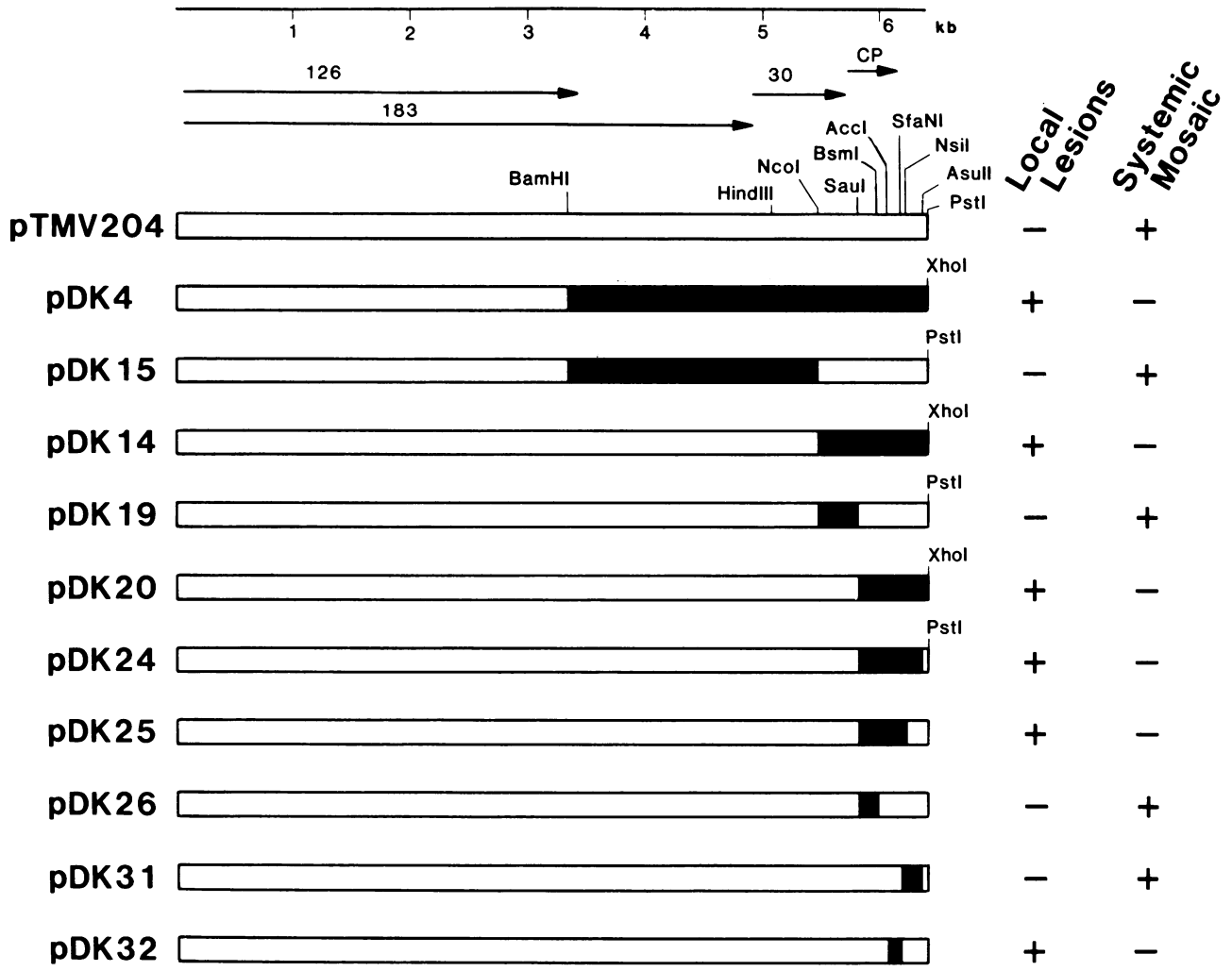

FIG. 2. Composition and symptomatology of wild-type and 10 recombinant TMV genomes. Dark portions of genomes show sequences from TMV204-D1, a mutant that induces local-lesion formation on *N. sylvestris*. Light portions of genomes represent sequences from the wild-type parental strain, TMV204. Relative positions of restriction endonuclease sites used in engineering the constructs are shown for pTMV204. The 3′ termini derived from pDK4 contain a *Xho* I restriction site, whereas all other 3′ termini contain a *Pst* I restriction site. Symptoms produced on *N. sylvestris* plants inoculated with *in vitro* transcripts of the plasmids are indicated beside each genome. The size of the TMV genome in kb is represented above. Arrows represent open reading frames, with the mass of product in kilodaltons; CP, capsid protein.

not contain the same alteration, indicating that changes in another region of the genome could also account for this phenotype.

DISCUSSION

In this study, we identified a point mutation in the coat protein gene of one TMV mutant that is responsible for inducing production of local lesions in *N. sylvestris*. This was accomplished by exchanging cloned cDNA fragments between the mutant and its wild-type parent to engineer a set of defined viral recombinants. The initial construct, pDK4, contained a recombinant TMV genome with a 3-kb cDNA fragment from mutant TMV204-D1. In subsequent constructs, sequences from the mutant cDNA were systematically replaced by corresponding sequences from the wild type. Ultimately, we constructed pDK32, containing only a single nucleotide difference from the wild-type parent virus. The cytosine-to-uracil alteration in this construct changes the capsid protein gene to encode phenylalanine in exchange for serine at position 148. Virus from pDK32 *in vitro* transcripts expressed the mutant phenotype, demonstrating that the single nucleotide substitution was sufficient to induce local lesions in *N. sylvestris*.

This study demonstrates that mutations conferring the ability to induce *N′* gene-associated HR can map within the viral capsid protein gene. This strengthens interpretations of earlier work that associated amino acid exchanges and altered physical properties in the capsid protein with this class of TMV mutants. Amino acid exchanges in the capsid proteins of numerous TMV mutants were identified in the laboratory of Fraenkel-Conrat in early efforts to decipher the genetic code (9). Later, Fraser described differences in the physical properties of capsid proteins of TMV local-lesion mutants (10). Although, several of the mutants studied had phenotypes similar to the ones we have described, the technology available did not allow a positive demonstration that capsid protein changes were related to phenotype changes. It is possible that the ability to induce HR in *N. sylvestris* may be associated with various other amino acid changes. However, because earlier data suggested the likelihood of random changes, we were surprised that six of seven independent mutants had identical changes in the capsid protein gene.

It is possible that the altered capsid protein induces the HR. However, we cannot exclude the possibility that RNA is the active molecule. The exchange in the capsid protein of TMV204-D1 occurs within a loop structure near the protein

surface that is not highly conserved within the tobamovirus group and can undergo rearrangement (18). Other mutants with this same exchange were previously reported by Funatsu and Fraenkel-Conrat (9). Analyzing one of these, van Regenmortel reported it to be distinguishable serologically from wild type (19), suggesting that the mutant capsid protein is structurally different from wild type. However, the mutation probably does not significantly impair the virion assembly function, since normal amounts of stable virions were formed. This implies that the capsid protein gene has a secondary function involved in establishing virus–host compatibility during the infection process. In a number of cellular recognition systems, conformational changes in proteins can alter receptor–target binding characteristics (reviewed in ref. 20). For instance, receptors for fibronectin and vitronectin both depend upon the same tripeptide sequence for binding, yet recognition remains mutually exclusive. Interpretation of analysis of binding with partial peptides indicates that size and conformation of targets may play a role in specific recognition by receptors. With TMV, it is conceivable that altered capsid protein from necrosis-inducing mutants is specifically recognized by cellular receptors involved in induction of the HR. It is not clear whether the mutant, but not the wild-type, capsid protein may act as an elicitor, or if the wild type, but not the mutant, suppresses the plant HR induced by virus-mediated processes.

Sequence alterations outside the capsid protein gene may also induce the *N'* gene HR. Mutant TMV204-D7 produced local lesions on *N. sylvestris* but did not contain the point mutation at nucleotide 6157, nor was a mutation detected in the sequence of approximately two-thirds of the capsid protein gene. Determining which alterations confer phenotype in this mutant would require substitution experiments similar to those used to map TMV204-D1. However, from other experiments, we now know that some viral product other than that related to the capsid protein gene is responsible for induction of hypersensitivity in plants with the *N* gene, because engineered TMV mutants with the entire capsid protein gene deleted induce the HR in Xanthi-nc tobacco (unpublished results). This situation suggests that *N'* gene recognition is triggered by viral products, such as the capsid protein, that may not normally be required for maintaining infection. In contrast, the *N* gene, which no variant of TMV is known to overcome, may recognize a factor essential for virus survival, such as replicase. The data of Kado and Knight (21, 22) suggested that a region in the gene encoding the 30-kDa protein or the carboxyl terminus of the 183-kDa protein might be involved with induction of the HR in *N. sylvestris*. With cauliflower mosaic virus, Schoelz and coworkers have shown that a segment of open reading frame *VI*, which encodes the inclusion body protein, controls the ability to produce necrotic lesions in *Datura stramonium* (6). In addition, mutants of TMV exist that are altered in ability to induce the HR in numerous hosts outside of the genus *Nicotiana*. It will be interesting to determine whether these mutations map within the capsid protein gene or are dispersed throughout the genome.

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