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Identification of an Elicitor Active Site within the Three-Dimensional Structure of the Tobacco Mosaic Tobamovirus Coat Protein

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The coat protein (CP) of tobacco mosaic tobamovirus (TMV) elicits the hypersensitive response (HR) conferred by the *N'* gene from *Nicotiana sylvestris*. This study presents evidence demonstrating a critical role for a specific CP structural site in eliciting this HR. Based on the known structure of the TMV CP, specific substitutions were created within the CP of the elicitor strain P20L to identify structural areas essential for host recognition. Of 32 substitutions made, 14 conferred either a temperature-sensitive (loss of the HR at 29°C) or a knockout (loss of the HR at 25°C) HR phenotype in *N. sylvestris*. These essential residues were noncontiguous in position; however, within the three-dimensional CP structure, all resided primarily along the right face of the molecule's helical bundle. Substitutions that did not affect the HR phenotype either were located outside of this area or were conservative in change. In addition, placing two temperature-sensitive substitutions within the same CP resulted in lowering temperature sensitivity from 29 to 27°C. This additive effect suggests that residues essential for HR elicitation contribute independently to host recognition. This feature is characteristic of recognition surfaces. The presence of a specific elicitor active site within the three-dimensional structure of the TMV CP is consistent with binding of a host-encoded receptor and demonstrates the importance of CP structure in HR specificity.

INTRODUCTION

Plants respond actively to pathogen infection via a process termed the hypersensitive response (HR). The HR embodies a cascade of biochemical events that results in a localized cell death reaction that confines the pathogen and prevents disease (Bol et al., 1990; Stintzi et al., 1993). Gene-for-gene complementarity between a specific host resistance gene (*R* gene) and a specific pathogen avirulence (*avr*) gene generally governs the HR (Flor, 1971). Current models for this gene-for-gene interaction predict that *avr* gene products either directly or indirectly produce pathogen elicitors that are recognized by specific receptors encoded by plant *R* genes (Lamb et al., 1989; Gabriel and Rolfe, 1990; Keen, 1992). Thus, specificity for the HR does not involve the defense mechanisms that confine the pathogen but rather the plants' ability to recognize an individual pathogen elicitor.

Recently, several *R* genes have been cloned and characterized at the molecular level (Staskawicz et al., 1995). Sequence comparisons show *R* genes that direct HRs against bacterial, fungal, or viral pathogens encode products with related structural features. In particular, a region of leucine-rich repeats (LRRs) has been identified in genes from Arabidopsis (*RPS2*), tobacco (*N*), tomato (*Cf-9*), and flax (L^6) (Bent et al., 1994; Jones et al., 1994; Mindrinos et al., 1994; Whitham et al., 1994; Ellis et al., 1995). In other systems, LRRs have been implicated in protein–protein interactions and ligand binding, and at least half are associated with signal transduction pathways (Kobe and Deisenhofer, 1995). These findings support the occurrence of a molecular recognition event between the product of the plant *R* gene and the product of the pathogen *avr* gene (elicitor) that culminates in a cascade of plant defense responses that confer disease resistance.

Pathogen elicitors have been identified in several systems, but in contrast to the common motifs observed among *R* genes, a variety of molecules, including proteins, polysaccharides, and low molecular weight compounds, has been shown to act as specific elicitors (Keen and Dawson, 1992). This elicitor diversity suggests flexibility within the mechanisms involved in host recognition. Understanding these mechanisms will require a comprehensive knowledge of the structure of the host and pathogen determinants involved. Because the three-dimensional structure of the tobacco mosaic tobamovirus (TMV) coat protein (CP) has been determined (Bloomer et al., 1978; Namba et al., 1989), as shown in Figure 1, and its role

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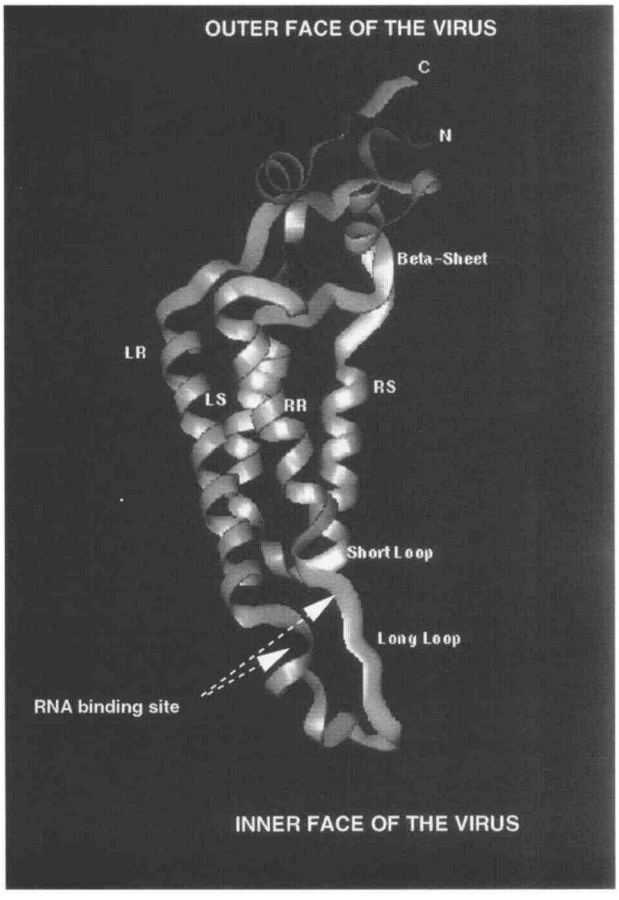

Figure 1. Ribbon Representation of the α Carbon Tracing of the CP of the U1 Strain of TMV.

At the core of the protein is a right-handed α-helical bundle of four α helices, the LS, LR, RS, and RR. The long loop connects the LR and RR helices, and a short inner loop connects the LS and RS helices. The RNA binding site is formed by residues from both loops and a part of the LR helix. The N and C termini are on the outer surface of the virus.

as the elicitor of the *N'* gene in *Nicotiana sylvestris* has been identified (Culver et al., 1991), this system provides a unique opportunity to investigate the structure–function relationship behind host–pathogen recognition.

Several studies have demonstrated conclusively that the TMV CP acts as the elicitor of the *N'* gene HR in tobacco (Saito et al., 1989; Culver and Dawson, 1991; Pfitzner and Pfitzner, 1992). In addition, specific amino acid substitutions within the CP of the U1 strain of TMV, normally a nonelicitor, result in *N'* gene host recognition and HR induction (Knorr and Dawson, 1988; Culver and Dawson, 1989a). Structure–function analyses of these HR-eliciting substitutions have demonstrated that they reside within and would predictably disrupt interface regions between adjacent CP subunits (Culver et al., 1994). In vitro analysis of these proteins has also revealed a correlation between the level of interference in CP quaternary structure and the strength of the elicited HR. Thus, the greater a substitution's ability to destabilize CP aggregation, the more rapidly the CP elicited the HR. It was hypothesized that aggregate disruption exposes a structural site normally buried within CP quaternary structure, and the exposure of this site facilitates elicitation of the HR.

Induction of the *N'* gene HR is not simply associated with an altered CP quaternary structure. The importance of tertiary structure in HR elicitation has been demonstrated by results showing that internal deletions within the CP open reading frame (ORF) interfere with its ability to elicit an *N'* gene–mediated HR (Saito et al., 1989). In addition, amino acid substitutions that were predicted to disrupt overall CP tertiary structure also affected HR induction by the elicitor CP (Culver et al., 1994). In each of these cases, the quaternary structure of the CP was disrupted. Thus, the CP must be able to fold properly, at least in part, into its three-dimensional form for host recognition to occur. This evidence suggests that a specific elicitor active site resides within the structure of the CP. To demonstrate the existence of such a site would provide additional support for the elicitor–receptor model for HR induction and would permit additional insight into the mechanisms operating during plant–pathogen recognition.

In this study, we used the known three-dimensional structure of the TMV CP to target specific amino acids for substitution. Each substitution was designed to modify various structural features of the CP molecule. This approach was utilized to identify CP structural regions essential for the elicitation of the *N'* gene HR. Data from this study indicate that the *N'* gene elicitor site is composed of a noncontiguous stretch of amino acids primarily covering the right face of the molecules' helical bundle. Substitutions in other CP structural regions either were not involved in HR elicitation or had previously been identified as leading to HR elicitation (Culver et al., 1994). The significance of this elicitor site is discussed in relation to CP function and to the structure of other known protein–protein recognition sites.

RESULTS

Mapping CP Elicitor Active Regions

Mapping experiments were performed by adding specific amino acid substitutions to the existing strong elicitor CP P20L, a TMV U1 strain containing a proline-to-leucine substitution at CP position 20 (Culver and Dawson, 1989a). Table 1 provides a complete list of substitutions. P20L was selected as the base CP for these experiments because it elicits a pronounced HR in *N. sylvestris* 2 to 3 days postinoculation. P20L also invades *N. tabacum* systemically and produces virions at levels comparable with the wild-type virus, indicating that the

P20L substitution does not greatly affect the three-dimensional structure of CP. In addition, P20L elicits necrotic lesions with a similar phenotype at temperatures between 20 and 35°C, demonstrating that the molecular requirements for HR elicitation are maintained over a broad temperature range.

We wanted to test whether substitutions of P20L CP residues that are important to elicitor activity would affect temperature stability. To test this possibility, all P20L substitutions were screened for their ability to elicit the HR at both 25 and 29°C in growth chambers under controlled temperature and light conditions. These experiments revealed that P20L substitutions displayed three different HR-eliciting phenotypes: temperature-insensitive substitutions, which had no effect on the HR, regardless of the temperature; temperature-sensitive substitutions, which inhibited HR elicitation at 29°C but not at 25°C, as shown in Figure 2A; and knockouts, substitutions that inhibited HR elicitation at both 25 and 29°C, as shown in Figure 2B. The mutants also expressed the CP at levels similar to the P20L virus at both 25 and 29°C, as shown in the protein immunoblot assay in Figure 3. In addition, none of these mutants induced necrosis on non-*N'* gene hosts, indicating that the observed responses were specific.

Table 1. Elicitation of the *N'* Gene HR by TMV P20L CP Substitutions and Deletion Mutants

Residue No.[a]	Substitution	HR Type[b]	Position in CP[c]
2	Y to S	KO	N terminus
7	P to S	TI	N terminus
10	F to Y	TI	N terminus
15	S to A	TI	N terminus
17	W to R	KO	β Sheet
32	G to Y	TI	LS helix
34	Q to R	TS	Short loop
38	Q to A	TS	RS helix
48	F to S	KO	RS helix
50	E to K	TS	RS helix
53	K to I	TS	β Sheet
59	T to N	TI	β Sheet
64	D to G	TI	β Sheet
66	D to A	TI	β Sheet
70	Y to F	TI	β Sheet
71	R to L	KO	β Sheet
72	Y to F	TS	β Sheet
77	D to N	TS	RR helix
78	P to G	TS	RR helix
81	T to R	TS	RR helix
84	L to C	TS	RR helix
88	D to I	TS	RR helix
89	T to A	TI	Long loop
90	R to D	TI	Long loop
91	N to A	TI	Long loop
92	R to D	TI	Long loop
95	E to Q	TI	Long loop
102	P to L	TI	Long loop
106	E to Q	TI	Long loop
109	D to A	TI	Long loop
116	D to N	TI	LR helix
133	I to L	TI	LR helix
ΔN(1 to 14)	NA[d]	KO	N terminus
ΔC(148 to 158)	NA	KO	C terminus

[a] Numbers represent residue positions within the linear TMV CP sequence. All mutations were created within the strong elicitor CP P20L.
[b] Phenotypes displayed by P20L substitutions and deletion mutants in *N. sylvestris*: KO, knockout phenotype designates no HR elicitation at 25° or 29°C; TS, temperature-sensitive phenotype designates HR elicitation at 25°C but not at 29°C; and TI, temperature-insensitive phenotype designates HR elicitation at both 25 and 29°C.
[c] Residue secondary structure within the CP three-dimensional structure (Bloomer et al., 1978; Namba et al., 1989).
[d] NA, not applicable.

Substitutions within the Long Loop Do Not Alter Elicitor Activity

A key functional feature of the TMV CP is its ability to bind viral RNA and form virus particles. Many of the interactions involved in this process are located along the long inner loop, residues 89 through 109, connecting the right radial (RR) and left radial (LR) α helices (Figure 1). In the absence of RNA, this loop region is flexible and does not have a defined fold (Jardetzky et al., 1978). A total of eight substitutions, T89A, R90D, N91A, R92D, E95Q, P102L, E106Q, and D109A, were created in this region of the CP of elicitor P20L, as shown in Figure 4A (see also Table 1). These substitutions span the entire loop and are both radical and conservative in change. None of these substitutions affected the ability of P20L to elicit the *N'* gene HR. This indicates that amino acids substituted within the structural region defined by the long loop, including the RNA binding residues R90 and R92 that are conserved among all tobamoviruses, are not essential for HR elicitation.

Substitutions in the Conserved Short Loop Interfere with Host Recognition

A short hairpin loop connects the left slewed (LS) and right slewed (RS) α helices (Figure 1). The residues in this loop, 32 through 38, are involved in maintaining the orientation of these helices, with residues 36, 37, and 38 being conserved among all tobamoviruses. Two substitutions were made in this region, Q34R and Q38A (Table 1 and Figure 4A). Both mutants containing these substitutions elicited a temperature-sensitive HR phenotype. These substitutions predictably alter charge and side-chain packing along the short loop, possibly affecting its hairpin structure. However, both Q34 and Q38 reside on the surface of the loop; thus, the substitutions made at these positions might not dramatically affect the bend of the loop at temperatures below 29°C. This could explain the

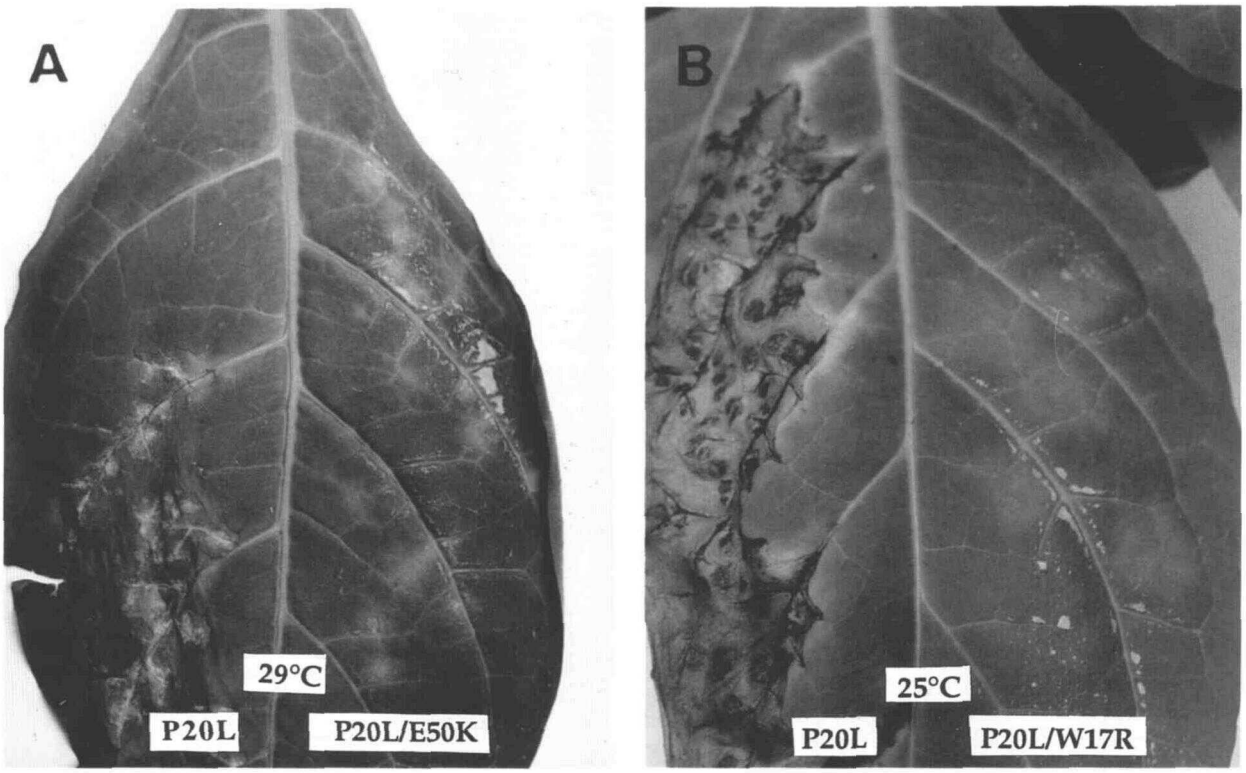

Figure 2. HR Effects of P20L-Derived Mutants in *N. sylvestris*.

(A) Left half of a leaf inoculated with TMV P20L at 29°C; right half of a leaf inoculated with the temperature-sensitive mutant P20L plus E50K.
(B) Left half of a leaf inoculated with TMV P20L at 25°C; right half of a leaf inoculated with the knockout mutant P20L plus W17R.
Photographs were taken 10 days postinoculation. Local lesions appeared within 3 days of inoculation on leaf halves inoculated with TMV P20L.

temperature-sensitive HR phenotype of mutants Q34R and Q38A. However, it is also possible that these residues interact with the putative *N'* gene product to trigger the HR.

HR Effects Are Localized in Different Regions of the Helical Bundle

Four anti-parallel α helices, LR, LS, RR, and RS, are the major structural feature of the tobamovirus CP (Figure 1). The LS and LR helical regions, composed of residues 20 to 31 and 110 to 134, respectively, were not targeted for extensive substitutions, because numerous substitutions within this region were previously shown to confer the U1 TMV strain with the ability to elicit the *N'* gene HR (Figure 4B; Culver et al., 1994). However, to determine whether double substitutions in this region affect the lesion phenotype, three substitutions, G32Y, D116N, and I133L, located in the two left-side α helices, were constructed in the P20L CP. Each of these three substitutions was previously shown to confer independently an HR-eliciting

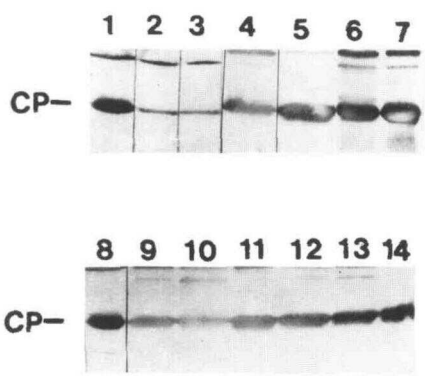

Figure 3. Protein Immunoblot of SDS-PAGE–Separated Proteins from P20L and Representative Mutant-Infected Plant Tissue at 25 or 29°C.

Lanes 1 and 8 contain P20L; lane 2, ΔN at 25°C; lane 3, ΔC at 25°C; lane 4, F48S at 25°C; lane 5, N91A at 29°C; lane 6, E50K at 25°C; lane 7, E50K at 29°C; lane 9, K53I at 25°C; lane 10, K53I at 29°C; lane 11, D77N at 25°C; lane 12, D77N at 29°C; lane 13, D88I at 25°C; and lane 14, D88I at 29°C. Bands indicate the presence of CP.

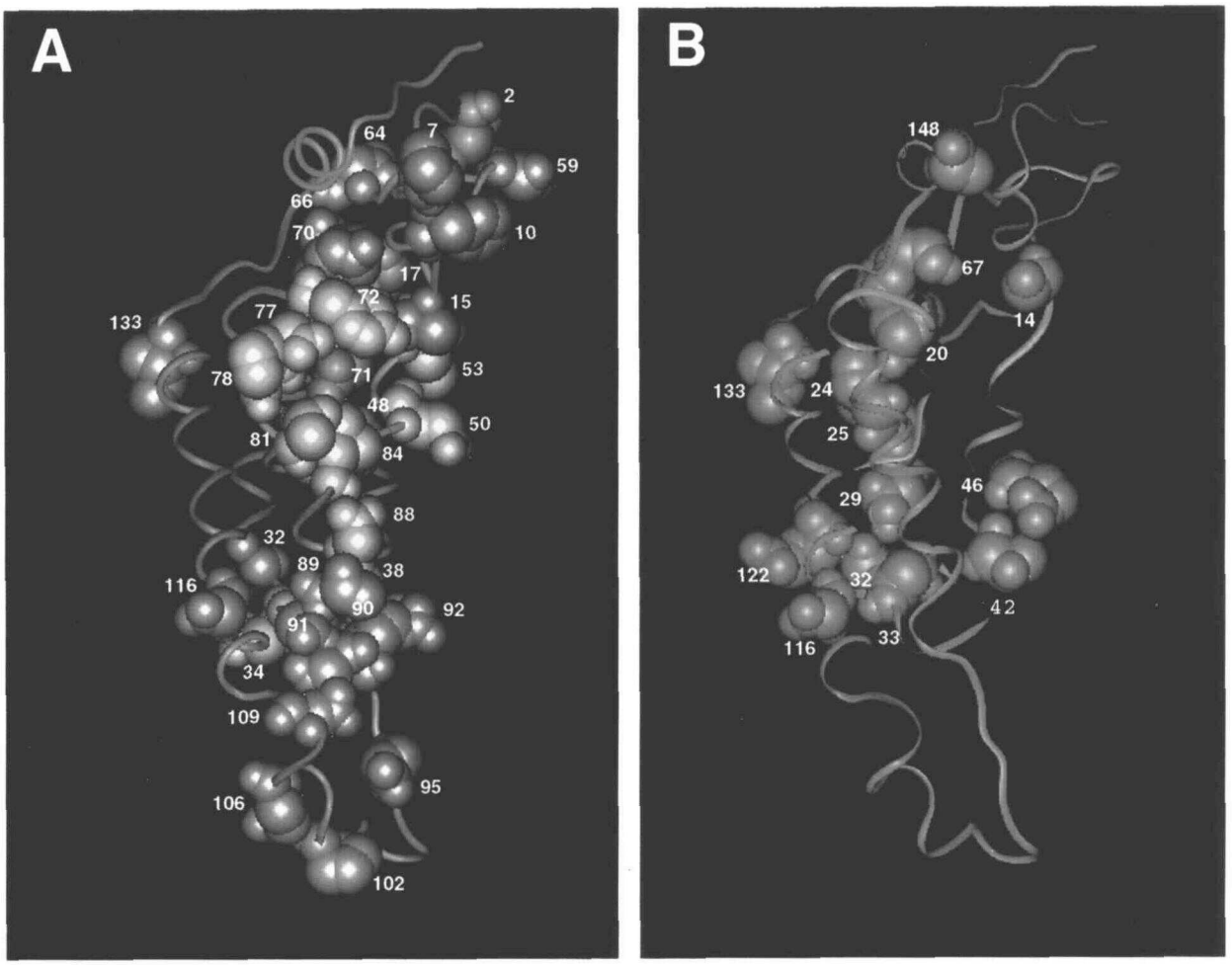

Figure 4. Spatial Locations of Substituted Residues in the TMV CP.

Residues are numbered.

(A) Substitutions made in the P20L background in this study.

(B) Locations of HR-eliciting substituted residues identified in a previous study (Culver et al., 1994).

Red, knockout substitutions; yellow, temperature-sensitive substitutions; white-gray, temperature-insensitive substitutions; green, HR-eliciting substitutions.

phenotype in the U1 CP (Culver et al., 1994). P20L-derived mutants containing these substitutions retained the ability to elicit a normal HR phenotype at both temperatures (Table 1).

The RS and RR α helices are composed of residues 38 to 51 and 74 to 88, respectively. Three P20L substitutions, Q38A, F48S, and E50K, were made along the RS α helix (Table 1 and Figure 4A). Q38 also participates in the structure of the short loop, and the temperature-sensitive phenotype elicited by the Q38A mutant was discussed previously. F48 is a buried residue that is an integral part of the hydrophobic core stabilizing the four-helix bundle. Substitution F48S would disrupt the hydrophobic nature and side-chain packing of this internal region and disrupt the ability of the four helices to associate properly with each other. A P20L mutant containing the F48S substitution was previously shown to have lost the ability to elicit the HR, that is, a "knockout" mutation (Culver et al., 1994). E50 is a surface residue that makes no significant intramolecular interactions. Besides altering side-chain length and charge substitutions, E50K should not greatly disrupt the overall CP structure. The temperature-sensitive HR phenotype elicited by mutant E50K suggests that the local structural modifications conferred by the mutation may affect a region that interacts directly with the *N'* gene product.

Five P20L substitutions, D77N, P78G, T81R, L84C, and D88I, were created within the RR α helix (Figure 4A). All of the substitutions affected surface residues, and P20L mutant derivatives containing each substitution elicited a temperature-sensitive phenotype (Table 1). When changing D77 to its amide

N, only the charge should be altered. This substitution results in the disruption of an intrasubunit salt bridge between D77 and R71. P78 resides within the RR α helix and causes an abnormal helix bend. Also, P78 as well as L84 are part of an exposed patch of nonpolar residues that reside along the right face of the helical bundle. Substitution P78G is predicted to alter the bend of the RR α helices and, along with substitution L84C, would decrease the hydrophobic nature of the nonpolar patch. T81R would alter side-chain volume and charge along the face of the helix. D88 is conserved among all tobamoviruses, and a substitution to an I residue should alter the charged nature of this region. Taken together, these data demonstrate that all P20L substitutions made within the RS and RR α helices are capable of affecting the HR, suggesting that the structure of this region is important for eliciting the *N'* gene HR.

Specific Residues within the β-Sheet Region Alter Host Recognition

In the CP molecule, a short four-stranded β-sheet structure transversely ties together the four α helices in a pairwise fashion (Figure 1). Aromatic amino acids at the distal ends of the β sheet form part of a hydrophobic cluster extending across the width of the molecule. This β-sheet region provides rigidity within the subunit, while allowing flexibility across the subunit-to-subunit interface (Bloomer et al., 1978). Eight substitutions, W17R, K53I, T59N, D64G, D66A, Y70F, R71L, and Y72F, have been made within this region, and they cover all four β-sheet strands (Table 1 and Figure 4A). One substitution, R71L, was previously shown to "knock out" the HR (Culver et al., 1994). R71 makes an intrasubunit salt bridge with residue D77, which has already been shown to confer a temperature-sensitive HR phenotype when mutated to an N residue. Substitution R71L would not only result in the loss of this salt bridge but would alter the charge and hydrophobicity. The W17R substitution was also found to knock out the HR. W17 is part of the hydrophobic core of the protein; thus, a substitution of a W residue to an R at this position would radically interfere with hydrophobic packing and disrupt overall CP structure. Mutants containing substitutions K53I and Y72F elicited temperature-sensitive HR phenotypes. K53 and Y72 are both surface residues, and the substitutions made at these positions should not affect the tertiary structure of the protein. This is especially true for Y72F because only the exposed hydroxyl group has been lost. Thus, the localized structure conferred by K53 and Y72 may interact directly with the product of the *N'* gene.

Four substitutions in the β sheet, T59N, D64G, D66A, and Y70F, had no effect on the P20L CP's ability to elicit the HR (Table 1 and Figure 4A). T59, D64, and D66 are all surface residues located within the β-sheet structure. The inability of these substitutions to affect the HR indicates that the surface structures of these regions are not important in HR elicitation. Y70 is a buried residue and part of the hydrophobic core of the protein. A substitution of Y70F would not affect the hydrophobic interactions in this region, and this conservative change also failed to affect host response.

Structurally Important Residues within the N and C Termini Affect HR Elicitation

The CP N and C termini are within 6 Å of each other and would reside on the outer surface of the TMV virion (Figure 1). The 15 N-terminal residues contain a short α helix formed by residues 7 through 13. Four substitutions, Y2S, P7S, F10Y, and S15A, were made within the N terminus. Two were within the α helix, and two were in the flanking regions (Table 1 and Figure 4A). P7, F10, and S15 are all surface residues, and the substitutions made at these positions produced no effect on the ability of P20L's CP to elicit the HR. Thus, the surface structure modifications conferred by these substitutions do not alter host response. However, substitution Y2S knocked out the HR-eliciting ability of CP P20L. Y2 is conserved among all tobamoviruses and is an important component of the molecule's hydrophobic core; consequently, the radical substitution of Y2S should destabilize a significant portion of the protein's three-dimensional structure.

We also deleted portions of the N and C termini to determine the effects on elicitation (Table 1). Residues 1 to 14 were deleted from the N terminus of CP mutant ΔN, and residues 148 to 158 were removed from the C terminus of the CP mutant ΔC. Both deletions in the P20L strain resulted in a knockout HR phenotype. Both the ΔN and ΔC deletions removed the conserved residues Y2, L150, and W153 that form a significant part of the CP hydrophobic core. Loss of these residues should dramatically affect the side-chain packing of this structurally important region and thus the structure of the protein. Lower levels of CP expression by these mutants, as determined from protein immunoblots (Figure 3), may have also contributed to the observed knockout phenotype. However, the combined data indicate that residues critical for maintaining the overall CP structure are essential for elicitation of the *N'* gene HR.

Effects of Combining Temperature-Sensitive Substitutions

The P20L substitutions led to a temperature-sensitive HR phenotype cluster in a region along the right side of the helical bundle (Figure 4A) and potentially could function in concert to elicit the *N'* gene HR. To investigate this possibility, temperature-sensitive substitutions were combined in the P20L CP, and the mutants were tested for their ability to elicit the HR. Mutants with substitutions E50K, Y72F, and D88I elicited the HR normally at 27°C, as shown in Table 2. However, when in combination, Y72F plus E50K and Y72F plus D88I lost the ability to elicit the HR at 27°C. Thus, the combination of two 29°C temperature-sensitive substitutions had an additive effect by

Table 2. Additive Effect of Temperature-Sensitive Substitutions

Substitutions[a]	HR Elicitation at 25°C	27°C	29°C
P20L plus E50K	+[b]	+	−[b]
P20L plus Y72F	+	+	−
P20L plus D88I	+	+	−
P20L plus Y72F plus E50K	+	−	−
P20L plus Y72F plus D88I	+	−	−

[a] P20L CP substitutions exhibiting temperature-sensitive HR phenotypes were tested for HR elicitation both individually and in combination with each other at 25, 27, and 29°C.
[b] (+), HR appeared within 3 days postinoculation; (−), no HR.

reducing the temperature-sensitive phenotype to 27°C. This suggests that these residues contribute independently to elicitor activity and function in concert in host recognition.

DISCUSSION

Specific protein–protein interactions are critical events in many biological processes, including host–pathogen interactions. In this study, we present evidence for the existence of a three-dimensional site within the TMV CP whose structural integrity must be maintained for host recognition and HR elicitation to occur. Of the P20L CP residues targeted for substitution, four exhibited a total loss in elicitor function, whereas 10 displayed a temperature-sensitive loss and 18 failed to affect the HR. P20L substitutions that affected HR elicitation were located mainly along the right face of the molecule, including residues in the inner loop, RR helix, RS helix, β-sheet region, and N terminus. Based on predicted structural effects, P20L substitutions that yielded a temperature-sensitive or a knockout HR phenotype can be placed into one of two categories. The first category includes substitutions that affect structural elements essential for maintaining the three-dimensional fold of the CP. These include substitutions at residues Q34 and Q38 within the conserved short hairpin loop and substitutions at residues Y2, W17, and F48 within the hydrophobic core of the protein. The N- and C-terminal mutations also removed internal hydrophobic residues that could dramatically affect overall CP structure and would therefore fit into this first category. The remainder of the P20L substitutions that affected the HR involved surface residues that would not be expected to disrupt the overall CP tertiary structure. However, these substitutions would affect localized secondary and/or tertiary CP structure primarily along the right face of the helical bundle. Thus, residues E50, K53, R71, Y72, D77, P78, T81, L84, and D88 contribute to structural elements that are important for host recognition, and they may play a direct role in the ability of the CP to elicit the *N'* gene HR.

P20L substitutions that did not affect HR elicitation were located primarily within the long inner loop, the left side of the β-sheet region, LS and LR α helices, and surface residues along the N terminus. One substitution that did not interfere with HR elicitation, Y70F, was located on the right side of the helical bundle. However, Y70 is completely buried within the core of the protein, and a conservative substitution of Y to F should not affect the CP structure. In addition, 10 of the substitutions within P20L that interfered with induction of the HR, Q34R, Q38A, F48S, E50K, K53I, R71L, Y72F, D77N, L84C, and D88I, were introduced into the parental U1 CP, which does not contain the P20L elicitor mutation. None of these substitutions in the U1 CP was able to elicit the HR (data not shown), indicating that these substitutions affect only the HR phenotype of an elicitor CP.

Fourteen additional substitutions from a previous study (Culver et al., 1994) can also be added to the CP model (Figure 4B). All of these substitutions, including the P20L base substitution used in this study, elicited the *N'* gene HR when introduced into the normally noneliciting TMV U1 strain (Culver et al., 1994). It was proposed that these substitutions resulted in HR elicitation due to their ability to interfere with the normal U1 CP quaternary structure, thereby exposing a buried receptor binding site. Thus, specific recognition of the amino acids altered by these substitutions does not seem to be required for the elicitation of the *N'* gene. Mapping HR-eliciting substitutions and temperature-insensitive P20L substitutions together clearly indicates that the active site for HR elicitation resides along the right face of the helical bundle (Figure 4B). Three exceptions are the knockout substitution made at Y2, a conserved member of the hydrophobic core, and the temperature-sensitive substitutions made at Q34 and Q38 that are involved in maintaining the orientation of the LS and RS helices. Thus, mutations made at these three residues may indirectly affect the elicitor site by disrupting overall CP structure. This suggests that the overall three-dimensional fold of the CP serves as a structural platform for presentation of the elicitor active site.

The area outlined by P20L substitutions that affected the HR potentially involves more than 25 residues with a total surface area of ~600 Å. This site is ~50% nonpolar, 20% charged, and 30% polar. This finding is consistent with the makeup of known recognition surfaces (Janin and Chothia, 1990). In addition, combinations of two 29°C temperature-sensitive substitutions, Y72F plus E50K and Y72F plus D88I, were found to lower the HR phenotype to below 27°C. This additive effect suggests that residues in this structural region act in concert to facilitate host recognition. Taken together, these data demonstrate that the outlined structural elements along the right face of the CP helical bundle confer specificity for *N'* gene recognition and possibly delineate a receptor binding site.

Precisely how the CP presents the elicitor site for host recognition remains to be determined. Many of the P20L substitutions, when examined by electron microscopy, failed to

produce any large CP aggregates, disks, or protohelices, regardless of their ability to elicit the HR (data not shown). This is consistent with previous findings (Culver et al., 1994) and demonstrates that the monomer or a small oligomer of CP is active in eliciting the HR. In addition, the P20L substitutions that displayed a knockout HR phenotype also disrupted the virus by affecting the ability to form virions and move systemically within the plant (data not shown). This result indicates that structural alterations of the magnitude required to avoid HR elicitation also affect other functions of the CP. Thus, the *N'* gene appears to target a site that is important for CP function.

Interestingly, the TMV U1 CP is normally not an elicitor of the *N'* gene, even though it carries the elicitor active site described in this study. Thus, TMV U1 has apparently evolved to counter *N'* gene host recognition without altering the structure of the elicitor site or the CP function. Previously, specific amino acid substitutions within U1 that disrupted the CP quaternary structure, such as the P20L mutation, have been shown to alter HR activity from nonelicitor to elicitor (Figure 4B; Culver et al., 1994). Presumably, these alterations lead to a form of CP in which the elicitor site is accessible for host recognition. The elicitor site, as mapped in this study, would normally be buried within the predominate disk/protohelical aggregate formed by the wild-type U1 CP. Therefore, the U1 strain of TMV has apparently evolved a quaternary structure that masks the CP elicitor site and hence prevents host recognition.

Although the *N'* gene from *N. sylvestris* has not been isolated, several phenotypically similar *R* genes have been cloned and characterized (Bent et al., 1994; Jones et al., 1994; Mindrinos et al., 1994; Whitham et al., 1994; Ellis et al., 1995). Sequence analysis of these *R* genes has revealed that they encode similar features, including a region of LRRs that typically play a role in ligand binding (Kobe and Deisenhofer, 1995). The crystal structure of LRRs reveals individual leucine repeats folded into β–α hairpin units that can present a varied array of residues for ligand binding (Kobe and Deisenhofer, 1995). This structure makes the LRRs a highly flexible binding platform capable of meeting the requirements for a number of specific interactions. Staskawicz et al. (1995) have suggested that the LRRs encoded by *R* genes specifically recognize and bind the elicitors encoded by the pathogen. The ability of specific TMV CP substitutions to interfere with HR elicitation and the mapping of these substitutions to a common site within the molecule's three-dimensional fold are consistent with a hypothesis whereby the CP binds directly to an *R* gene–encoded receptor. This work provides additional support for the elicitor–receptor model in HR induction and demonstrates the critical role that elicitor structure plays in HR specificity.

METHODS

Virus Constructs and HR Assay

Full-length infectious cDNA clones of the U1 strain of tobacco mosaic tobamovirus (TMV), joined to the phage SP6 promoter pBGC150 or to the T7 promoter pSNC004, were used as the parental constructs for all described mutations (Dawson et al., 1986; Kumagai et al., 1993; Turpen et al., 1995). TMV nucleotide numbering is from Goelet et al. (1982). A pUC119-based subclone, pDL3P20L, containing the coat protein (CP) open reading frame (ORF), including a substitution of P to L at residue 20 and the 3' untranslated region of TMV (nucleotides 5081 to 6406), was used for mutagenesis (Culver and Dawson, 1989a).

Site-directed mutagenesis was accomplished in vitro using the modified M13 method described by Geisselsoder et al. (1987). A uracil-containing single-stranded pDL3P20L template was obtained by coinfection with phage M13KO7 in *Escherichia coli* CJ236 (Vieira and Messing, 1987). Second-strand synthesis was performed using synthetic oligomers, 18 nucleotides in length, that were designed to alter a specific CP amino acid codon. Plasmid DNA from bacterial colonies transformed with second-strand products was sequenced to confirm the presence of the desired mutations. In addition, the entire CP ORF was sequenced to ensure that no other mutations had occurred during mutagenesis. Once sequenced, NcoI (nucleotide 5460) to BsiWI (nucleotide 6245) fragments containing the entire CP ORF were ligated into similarly cut TMV full-length constructs, pTMV304. CP ORFs from full-length constructs were also sequenced to confirm the presence of the desired mutation. Each CP substitution is identified by the single-letter code for the wild-type residue in front of the residue number, and this is followed by the code of the substituting amino acid.

Deletion of the N terminus was accomplished by polymerase chain reaction (PCR) amplification of a CP fragment (nucleotides 5760 to 6245), using the pDL3P20L vector as the template (Mullis et al., 1986). The 5' end PCR primer also encoded a BglII site and an AUG start codon, whereas the 3' end PCR primer carried sequences complementary to TMV nucleotides 6236 to 6253, covering a unique SpII restriction site. The amplified 477-bp fragment, carrying a deletion covering residues 1 to 14 from the N terminus, was cut with BglII and SpII and ligated into a similarly cut pDL3 vector, with a BglII site at the start codon (Culver and Dawson, 1989b). Plasmid DNA from bacterial colonies transformed with the above ligation product was sequenced to confirm the deletion. In addition, the CP ORF was sequenced to ensure that no other mutations had occurred during PCR amplification. Deletion of the C terminus was accomplished by site-directed mutagenesis, as described above. An 18-nucleotide synthetic oligomer was designed to replace residue codon S148 with a stop codon, prematurely terminating CP by 11 amino acids. Placement of both N and C deletions into full-length pSNC004 was as described in the previous section.

To determine effects on the hypersensitive response (HR), infectious transcriptions from full-length cDNA constructs were produced in vitro, as described by Kumagai et al. (1993) and Turpen et al. (1995). Transcription products were mechanically inoculated directly onto the leaves of *Nicotiana sylvestris* (*N'* gene) or *N. tabacum* cv Xanthi (systemic host). Inoculated plants were maintained in growth chambers at 25, 27, or 29°C under light (10,000 lux, 12-hr photoperiod). The appearance of symptoms was monitored for a period of 2 weeks.

Protein Extraction, SDS-PAGE, and Protein Immunoblotting

Infected leaf samples displaying necrosis or chlorosis were pulverized in liquid nitrogen, and the powdered tissue was thawed in Laemmli buffer (125 mM Tris, pH 6.8, 10% [w/v] DTT, 20% [w/v] SDS, 0.01% [w/v] bromophenol blue) at 95°C for 4 min. SDS-PAGE was conducted, as described by Laemmli (1970), using acrylamide concentrations of 4.8

and 15% for stacking and separation gels, respectively. Separated proteins were electrophoretically transferred to nitrocellulose paper and probed with rabbit anti-CP antiserum (Lehto et al., 1990), followed by alkaline phosphatase–conjugated goat anti–rabbit antibody (Sigma). CP was visualized by the addition of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (1 to 2 μg per blot), as described by Knecht and Dimond (1984). All blockings and incubations were done in Tris-buffered saline (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 10% [w/v] nonfat dry milk) for 2 hr at 37°C.

Computer Modeling of the TMV CP Structure

The three-dimensional structure of the TMV CP, determined at 2.9 Å resolution in the intact virus (Namba et al., 1989), was used to model structures of mutant CPs with the molecular graphics programs MacImdad (Molecular Applications Group, Stanford, CA) or Quanta 4.0 (Molecular Simulation Inc., Burlington, MA).

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