Induction of a Hypersensitive Response by Chimeric Helicase Sequences of Tobamoviruses U1 and Ob in *N*-Carrying Tobacco

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Recently, the helicase domain of the *Tobacco mosaic virus* (TMV)-U1 replicase proteins (designated MOREHEL:U1) was identified as the elicitor of the *N* gene-mediated hypersensitive response (HR) in tobacco. In this study, we used agroinfiltration to express the equivalent MOREHEL domain of the non-HR-inducing tobamovirus strain TMV-Ob. It appeared that this MOREHEL:Ob sequence did not elicit a HR in *N* gene-carrying tobacco. Both MOREHEL sequences were divided into eight subdomains, and chimeras of MOREHEL sequences from U1 and Ob were constructed. Expression of these chimeric MOREHEL sequences revealed that, in the TMV-U1 MOREHEL sequence, at least four domains involved in full HR induction were present. The presence of at least three of these four domains seems a minimal requirement for HR induction. Two additional domains may play a minor role in HR induction. To study the elicitor function of the chimeras during the TMV life cycle, chimeric MOREHEL domains were introduced into full-length TMV cDNA clones. These constructs, however, were unable to establish an infection in *Nicotiana benthamiana* or *Nicotiana tabacum* plants.

Additional keywords: avirulence, gene for gene, protein–protein interaction, signal transduction.

The gene-for-gene resistance mechanism postulated by Flor (1971) states that, in plants, (semi)-dominant resistance gene (*R*) products confer resistance to pathogen-derived avirulence gene (*Avr*) products. Resistance occurs only when both the *R* and *Avr* products are present in a plant–pathogen interaction. In many interactions, this resistance is accompanied by a hypersensitive response (HR), which manifests itself as necrotic lesions at the infection sites. The pathogen is confined to the vicinity of these lesions and, therefore, systemic infection is prevented. In addition, the plant develops a broad-spectrum resistance against many pathogens, a phenomenon known as systemic acquired resistance (SAR) (Sticher et al. 1997). The SAR phenotype is accompanied by induction of several pathogenesis-related (*PR*) genes (Linthorst 1991). Although many genes that are involved in the establishment of HR and SAR gene expression have been identified, a detailed knowl-

edge of the signal transduction routes is unavailable at this time (Blumwald et al. 1998; Delaney 2000; Feys and Parker 2000; Martin 1999). Moreover, the recognition mechanism of the *Avr* product by the *R* product remains elusive for most plant–pathogen interactions. So far, protein–protein interactions between *R* and *Avr* products have been identified only for three plant–pathogen systems (Jia et al. 2000; Leister and Katagiri 2000; Scofield et al. 1996; Tang et al. 1996).

In our laboratory, the *Tobacco mosaic virus* (TMV)-*N* gene interaction is being studied as a model for gene-for-gene resistance. The *N* gene was cloned several years ago and was shown to contain an amino terminal domain that resembles the cytoplasmic domains of Toll and Interleukin-1 receptors (IL-1R) and, hence, is indicated as the TIR domain (Whitham et al. 1994). This domain is followed by a putative nucleotide binding site and a leucine-rich repeat (LRR) domain. These domains are common in other *R* proteins. LRR domains are involved in protein–protein interactions and have been shown to determine resistance specificity of several *R* proteins (Ellis et al. 2000; Kobe and Deisenhofer 1995). It therefore has been postulated that LRRs interact directly or indirectly with the pathogen avirulence gene product, resulting in *R* protein-activated initiation of a signaling cascade that results in HR and resistance (Martin 1999; Van der Biezen and Jones 1998). On the basis of the presence of the TIR domain, the *N*-mediated signaling cascade, leading to HR and resistance, may resemble a Toll- or IL-1R-like signal transduction route.

The infection of *N* gene-containing plants with TMV-U1 or other tobamoviruses results in the induction of a HR and SAR. The tobamovirus genome consists of an approximately 6.4-kb, single-stranded RNA molecule of positive polarity. Tobamoviruses encode two replicase proteins from overlapping open reading frames: the 126K protein and the 183K read-through protein. The 126K replicase protein contains domains with methyltransferase and putative helicase activities, whereas the 183K protein contains an additional polymerase domain. In addition, TMV encodes a movement protein involved in cell-to-cell movement of the virus and a coat protein (CP) involved in the encapsidation of the viral RNA into virions. One tobamovirus strain (TMV-Ob) that infects *N* gene-carrying plants systemically and does not elicit *N* gene-mediated resistance has been described (Padgett and Beachy 1993). Early experiments showed that amino acid alterations in the TMV-Ob replicase proteins restored elicitation of the defense response, indicating a role for the TMV replicase proteins in HR

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Fig. 1. A, Schematic representation of the *Tobacco mosaic virus* (TMV)-U1 genome and the location of the helicase domain of the replicase proteins (MOREHEL). Open reading frames are indicated by the 126K and 183K replicase proteins, movement protein (MP), and coat protein (CP). Black circle indicates the cap structure that is present at the 5' terminus of the TMV genome. Asterisk denotes the amber termination codon. MOREHEL domain resides in both replicase proteins and comprises nucleotide 2,109–3,419. Because the schematic representation of TMV-Ob is similar to that of TMV-U1, it is not shown. **B,** Schematic representation of the TMV-U1 and TMV-Ob MOREHEL cDNA sequences. Restriction sites that were used as fusion sites for the construction of the MOREHEL chimeras are indicated above the cDNAs. Sites that are unique to the MOREHEL:U1 sequence were introduced at the same position in the corresponding MOREHEL:Ob sequence, and vice versa. **C,** Alignment of MOREHEL:U1 and MOREHEL:Ob protein sequences. Identical and similar amino acid residues are indicated with black and gray boxes, respectively. Pileup was performed with the DNA Manager software program, and GeneDoc (Pittsburgh Supercomputing Center, Pittsburgh, PA, U.S.A.) was used to create this figure. Domains that were exchanged in the construction of the MOREHEL chimeras are indicated above the amino acid sequence. A–H: U1 domains; a–h: Ob domains.

induction (Padgett and Beachy 1993; Padgett et al. 1997). Previously, we and others showed that transient expression of the 126K–183K helicase domain of TMV-U1 (designated MOREHEL:U1) induced a HR in *N* gene-carrying plants (Abbink et al. 1998; Erickson et al. 1999). This necrotic re-

Fig. 2. Western blot analysis of agroinfiltrated *Nicotiana benthamiana* leaves. NN transgenic (N) and nn wild-type (n) *N. benthamiana* leaves were agroinfiltrated with cyan fluorescent protein (CFP) fusion constructs of the helicase domain of the replicase proteins (MOREHEL):U1 (U1) and MOREHEL:Ob (Ob). An unfused MOREHEL:U1 construct (–) was infiltrated as a negative control. At 2 days postinfiltration, equal amounts of leaf tissue were ground in phosphate buffered saline, applied to standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted (Towbin et al. 1979). Blots were immunostained with an antibody against GFP, according to the manufacturer’s protocol (Clontech, Palo Alto, CA, U.S.A.). As a positive control for the immunostaining procedure, purified recombinant GFP (Clontech) also was applied to the SDS-PAGE (+). Arrows indicate the CFP fusion products and a protein breakdown product that is recognized by the GFP antibody only after expression of the CFP fusion proteins and is therefore indicated as a CFP breakdown product.

sponse was accompanied by the induction of PR genes (Abbink et al. 1998). From these experiments, it became obvious that HR induction is independent of virus replication. In addition, the protein, rather than its mRNA, triggers this response. The putative helicase activity was not required for HR induction (Erickson et al. 1999).

In the present study, we observed that transient expression of the MOREHEL sequence of TMV-Ob (MOREHEL:Ob) by agroinfiltration technique did not trigger a HR. The MOREHEL amino acid sequences of the tobamoviruses U1 and Ob are 64% identical and 80% similar (Fig. 1). To identify residues or sequence motifs in MOREHEL:U1 that play a role in the induction of the HR, we engineered a number of chimeras consisting of U1 and Ob MOREHEL sequences. These chimeras were expressed transiently in *Nicotiana tabacum* cv. Samsun either carrying (NN) or not carrying (nn) the *N* gene. Some of the chimeras were used to replace the MOREHEL sequence in the TMV-U1 and TMV-Ob infectious cDNA clones. Transcripts expressing these chimeric viruses were inoculated onto *Nicotiana benthamiana* plants to determine whether the chimeric sequences could support virus replication and to analyze the HR-inducing properties.

RESULTS

Agroinfiltration-mediated expression of U1 and OB MOREHEL chimeras.

In previous studies, the MOREHEL:U1 domain was shown to trigger an *N* gene-mediated HR (Abbink et al. 1998; Erickson et al. 1999). So far, TMV-Ob is the only tobamovirus strain that does not elicit the *N*-mediated HR and SAR (Padgett and Beachy 1993). Therefore, the MOREHEL domain of TMV-Ob was analyzed for induction of HR upon agroinfiltration. The MOREHEL:Ob sequence is 64% identical and 80% similar to the MOREHEL:U1 elicitor at the amino acid level (Fig. 1C). To rule out that the MOREHEL:Ob domain is unstable upon expression in tobacco plants, the expression levels of MOREHEL:Ob were analyzed by Western blot. For technical reasons, we used a fusion construct of cyan fluorescent protein (CFP) and MOREHEL:Ob for expression in *N. benthamiana* plants. This showed that the MOREHEL:Ob fusion protein could be detected 2 days after agroinfiltration, and its expression level

		HR induction							
		NN plants				nn plants			
1	A B C D E F G H	+++				-			
2	a b c d e f g h	-				-			
3	A B C d e f g h	-				-			
4	a b c D E F G H	-				-			

Fig. 3. Schematic representation of the chimeric helicase domain of the replicase proteins (MOREHEL) and their elicitor activities. Shaded domains indicate the MOREHEL:U1 origin, whereas open domains indicate the MOREHEL:Ob origin. Names of the chimeric proteins are indicated. Infiltrated leaves were observed for a period of at least 2 weeks. Hypersensitive response was normally induced 2 days postinfiltration. The MOREHEL:U1 elicitor activity was set at +++. –, leaves that did not induce necrosis or leaf yellowing.

seemed higher than that of a similar MOREHEL:U1 fusion protein (Fig. 2). The CFP-MOREHEL:U1 fusion product was still capable of triggering the typical *N*-dependent HR in tobacco plants (data not shown). The CFP-fused and nonfused MOREHEL:Ob domains, however, did not trigger necrosis in either NN or nn tobacco plants (Fig. 3). Apparently, the MOREHEL:Ob domain contains one or more amino acid substitutions that interfere with its recognition in NN plants. In order to identify these substitutions, we constructed various sets of MOREHEL:U1–Ob chimeras. For this purpose, we divided the MOREHEL sequences into eight equivalent domains, A through H for MOREHEL:U1 and a through h for MOREHEL:Ob (Fig. 1B). The first set of chimeras was created with the *Sst*II site as a fusion site (Fig. 3, chimeras 3 and 4). The *Sst*II site is present in both MOREHEL sequences (nucleotide position 547 in the MOREHEL cDNA sequences) and determines the border between the C/c and D/d domains. Expressing these chimeras in tobacco plants by agroinfiltration showed that neither of the two triggered a HR in Samsun plants, independent of the presence of the *N* gene (Fig. 3).

The results with chimeras 3 and 4 indicated that the ABC or DEFGH sequences of MOREHEL:U1, when separate, were insufficient to trigger a HR. In chimeras 5 and 6 (Fig. 4), the ABC sequence of MOREHEL:U1 was extended with the U1 sequences DEF (ABCDEFgh) or GH (ABCdefGH), respectively, with the use of a *Hpa*I site common to the U1 and Ob sequence (nucleotide position 1,126). Both chimeras induced the U1-specific HR in NN plants but not in nn plants (Fig. 4). This observation suggests that sequences in domains DEF and GH

play a role in HR induction. As the DEFGH sequence in chimera 4 was not sufficient to induce a HR, we surmise that elements in sequences DEF and GH act in conjunction with one or more elements in sequence ABC for HR induction.

A characteristic difference between DEF and GH and the corresponding Ob sequences is the presence of cysteine residues at positions 300 and 420 in the Ob def and gh sequences, respectively, which are absent in the U1 sequences. Possibly, C300 and C420 form a disulfide bridge, masking a HR-inducing domain in MOREHEL:Ob. Disruption of this putative disulfide bridge in chimeras 5 and 6 could enable them to induce a HR. To investigate this possibility, point mutations were introduced into chimeras 5 and 6, yielding chimeras 7 and 8, respectively (Fig. 4). In chimera 7 (ABCDEGgh-R300C), the arginine at position 300 in sequence E was mutated into cysteine, whereas in chimera 8 (ABCdefGH-K420C), the lysine at position 420 in sequence H was mutated into cysteine. The ability to form a putative disulfide bridge between C300 and C420 in chimeras 7 and 8, however, did not interfere with their ability to induce the HR (Fig. 4). We analyzed whether disruption of the putative disulfide bridge in the defgh sequence of chimera 3 would trigger a HR. In chimera 9 (ABCdefgh-C300R), the cysteine at position 300 was mutated to arginine, whereas in chimera 10 (ABCdefgh-C420K), the cysteine at position 420 was mutated into lysine (Fig. 4). Chimeras 9 and 10 did not induce a HR in NN plants (Fig. 4), which further supports the conclusion that putative disulfide bridge formation between C300 and C420 is not responsible for the inability of Ob to induce a HR.

Fig. 4. Schematic representation of the chimeric helicase domain of the replicase proteins (MOREHEL) and their elicitor activities. Shaded domains indicate the MOREHEL:U1 origin, whereas open domains indicate the MOREHEL:Ob origin. Names of the chimeric proteins are indicated. Infiltrated leaves were observed for a period of at least 2 weeks. Hypersensitive response was normally induced 2 days postinfiltration. MOREHEL:U1 elicitor activity was set at +++. -, leaves that did not induce necrosis or leaf yellowing. Amino acid alterations R300C, K420C, C300R, and C420K are indicated.

Chimeras 11 to 14 (Fig. 5) were engineered to further analyze the role of sequences D, E, and F in HR induction. To permit the construction of these chimeras, *Hind*III and *Bgl*II sites present in Ob were introduced in the U1 sequence. Chimera 5 was shown to induce the HR (Fig. 4). Replacing the F sequence by f in this chimera to form chimera 11 did not affect HR induction. Also, replacing the D sequence by d to form chimera 13 did not affect HR induction. When, in addition to sequence F, sequence E was replaced by the corresponding Ob sequence, as was done in chimera 12, HR induction was largely lost (Fig. 5). Chimera 12 did not induce the massive necrosis obtained with full-length MOREHEL:U1, but some minor necrosis was detectable at the bottom side of the infiltrated leaf area. The difference in HR-inducing activity of chimeras 13 and 14 clearly points to an important role of sequence E in HR induction.

The results (Fig. 4) indicate that either sequence DEF or GH could induce a HR in concert with sequence ABC. The contribution to HR induction of elements in sequence GH was analyzed with the constructs shown in Figure 6. The GH sequence of the HR-inducing chimera 6 differs by 24 amino acid substitutions from the gh sequence in chimera 3 that does not induce a HR. Sequences of three to five amino acids in the gh sequence were mutated into the corresponding GH sequences in constructs gh1 to gh11. When five, three, or eight amino acids of the g sequence were replaced by their G counterparts in constructs gh1, gh4, and gh10, respectively, no induction of a HR was obtained. Also, when these mutations were combined with a mutation of five amino acids at the C terminus of the h sequence (constructs gh3 and gh7), no HR was induced. Unexpectedly, the effect of other mutations in the h sequence was more complex. The mutation of four amino acids (L-KLV

into M-SII) in construct gh5 resulted in full HR induction. In the context of construct gh8, however, this mutation induced only a low level of HR, indicating that either the N- or C-terminal mutations in construct gh8 attenuated the HR induction. Moreover, the mutation of five amino acids in the h sequence, as occurred in construct gh6, induced a low level of HR that was increased in the context of construct gh9. This points to an enhancement of the effect of the gh6 mutation by either the N- or C-terminal mutations in construct gh9. The combination of all 14 amino acid substitutions that were made in the h sequence in construct gh11 resulted in a HR comparable to that of construct gh5, which contained only four mutations in this sequence. Together, the data indicate that the three-dimensional folding of the C terminus of the MOREHEL sequence is probably more important for HR induction than the primary structure.

We then focused on the role of the MOREHEL:U1 ABC domains in HR induction. The *Cla*I and *Nsi*I sites in the MOREHEL:U1 cDNA sequence were introduced into the MOREHEL:Ob cDNA and used to exchange Ob and U1 MOREHEL domains. The resulting chimeras (15 to 17) induced N gene-dependent necrosis (Fig. 7). Chimera 15, however, gave a very weak necrotic response, which was visible only at the bottom surface of the infiltrated leaf tissue. Inclusion of U1 domain A in chimera 4 resulted in chimera 17 and was sufficient to restore elicitor function to the MOREHEL:U1 level. This restoration also was achieved when the U1 domains DEF or GH were introduced into chimera 3 to create chimeras 5 and 6 (Fig. 4). These observations led to the construction of chimeras 18 and 19 (Fig. 7), which were expected to elicit a HR with a minimum of MOREHEL:U1 sequences. Expression of these chimeras in NN tobacco plants, however, did not induce necrosis, not even at the bottom surface of the leaf (Fig. 7). The addition

		HR induction									
		NN plants	nn plants								
5	<table><tr><td>A</td><td>B</td><td>C</td><td>D</td><td>E</td><td>F</td><td>g</td><td>h</td></tr></table>	A	B	C	D	E	F	g	h	+++	-
A	B	C	D	E	F	g	h				
11	<table><tr><td>A</td><td>B</td><td>C</td><td>D</td><td>E</td><td>f</td><td>g</td><td>h</td></tr></table>	A	B	C	D	E	f	g	h	+++	-
A	B	C	D	E	f	g	h				
12	<table><tr><td>A</td><td>B</td><td>C</td><td>D</td><td>e</td><td>f</td><td>g</td><td>h</td></tr></table>	A	B	C	D	e	f	g	h	+	-
A	B	C	D	e	f	g	h				
13	<table><tr><td>A</td><td>B</td><td>C</td><td>d</td><td>E</td><td>F</td><td>g</td><td>h</td></tr></table>	A	B	C	d	E	F	g	h	+++	-
A	B	C	d	E	F	g	h				
14	<table><tr><td>A</td><td>B</td><td>C</td><td>d</td><td>e</td><td>F</td><td>g</td><td>h</td></tr></table>	A	B	C	d	e	F	g	h	-	-
A	B	C	d	e	F	g	h				

Fig. 5. Schematic representation of the chimeric helicase domain of the replicase proteins (MOREHEL) and their elicitor activities. Shaded domains indicate the MOREHEL:U1 origin, whereas open domains indicate the MOREHEL:Ob origin. Names of the chimeric proteins are indicated. Infiltrated leaves were observed for a period of at least 2 weeks. Hypersensitive response (HR) normally was induced at 2 days postinfiltration. The MOREHEL:U1 elicitor activity was set at +++. -, leaves that did not induce necrosis or leaf yellowing. +, HR was induced only in cells of the bottom surface of the infiltrated leaf tissue. In all experiments, MOREHEL:U1 was expressed as a positive control.

of MOREHEL:U1 domain C to chimera 19 resulted in chimera 20. This last chimera did not elicit a HR in NN plants, indicating that the presence of domains A, C, and H is not sufficient for HR induction. Apparently, domain B also is involved in the HR elicited by chimera 6 (Fig. 4). Therefore, at least four MOREHEL:U1 domains, A, B, E, or H, are involved in full HR induction. Domains C and D may play minor roles in HR induction.

A last point of concern is the temperature sensitivity of *N*-mediated resistance (Samuel 1931). At temperatures higher than 28°C, TMV-U1 infection of NN tobacco plants does not result in HR or SAR. It has been suggested that the early step of elicitor recognition is to determine temperature sensitivity (Padgett et al. 1997). Therefore, we used an agroinfiltration system to express MOREHEL:Ob, MOREHEL:U1, and their chimeric proteins, at a temperature of 15°C. The HR-inducing capacity of the proteins at this temperature was identical to those observed at the higher temperature of 23 to 25°C (data not shown). This indicates that the agroinfiltration system is functional at 15°C and temperature sensitivity is not a likely reason for the lack of HR induction by MOREHEL:Ob and some chimeras.

Chimeric MOREHEL domains in infectious cDNA clones of TMV:U1 and TMV:Ob.

In order to test the elicitor activity of chimeric MOREHEL domains during a TMV infection, we constructed two chi-

meric full-length cDNAs. In an infectious TMV-U1-derived cDNA, the MOREHEL:U1 domains D, E, and F were exchanged for MOREHEL:Ob domains d, e, and f (Fig. 8A). In a TMV-Ob-derived cDNA, the MOREHEL:Ob d, e, and f domains were exchanged for the MOREHEL:U1 domains D, E, and F (Fig. 8A). RNA transcripts from the chimeric full-length clones were used to inoculate *N. benthamiana* and *N. tabacum* plants. Whereas RNA transcripts from the TMV-U1 and TMV-Ob full-length clones resulted in a viral infection in these host plants, RNA transcripts from the chimeric full-length clones did not result in infection. Replication of the chimeric viruses could not be detected because no symptoms developed on the host plants and Northern blot (Fig. 8B) and reverse transcriptase-polymerase chain reaction (PCR) data (not shown) were negative. As a result of this apparent lethal phenotype of the chimeric viruses, we were unable to characterize the elicitor activity of the MOREHEL chimeras in these viruses.

DISCUSSION

The resistance gene *N* confers resistance to most tobamoviruses, resulting in HR and SAR development upon infection. It has been shown that the HR-triggering domain resides in the putative RNA helicase domain of the TMV replicase pro-

Fig. 6. Schematic representation of the chimeric helicase domain of the replicase proteins (MOREHEL) and their elicitor activities. Shaded domains indicate the MOREHEL:U1 origin, whereas open domains indicate the MOREHEL:Ob origin. Names of the chimeric proteins are indicated. gh chimeras 3–11 are identical to the U:ABCdefgh chimera, except for the amino acid changes, Ob–U1, that are indicated with arrows and underlined. Chimeras gh10 and gh11 were obtained by fusion of the G and h or g and H domains at the *ScaI* site (nucleotide 1,209). Infiltrated leaves were observed for a period of at least 2 weeks. Hypersensitive response (HR) normally was induced at 2 days postinfiltration. The MOREHEL:U1 elicitor activity was set at +++. –, leaves that did not induce necrosis or leaf yellowing. +, HR was induced only in cells of the bottom surface of the infiltrated leaf tissue. In all experiments, MOREHEL:U1 was expressed as a positive control.

teins (Abbink et al. 1998; Erickson et al. 1999; Padgett et al. 1997). This MOREHEL:U1 domain induced a HR and systemic *PR* gene induction, independent of the viral life cycle or the putative helicase activity. In this study, we analyzed the equivalent MOREHEL domain of TMV-Ob. So far, this tobamovirus strain is the only strain that does not elicit *N*-mediated resistance responses (Padgett et al. 1997). Therefore, it was not surprising that the MOREHEL:Ob domain was unable to induce a HR in NN tobacco, although in some experiments, a single plant exhibited *N*-dependent leaf yellowing after agroinfiltration. One could argue that this lack of HR induction may be the result of instability of the MOREHEL:Ob protein. A CFP fusion at the amino terminus of MOREHEL:Ob, however, could be detected in agroinfiltrated leaves by Western blot analysis with anti-GFP antibodies. Therefore, it is unlikely that protein instability causes the lack of HR. An equivalent CFP fusion of MOREHEL:U1 still induced an *N*-dependent HR, similar to the unfused MOREHEL:U1 protein. This indicates that the CFP fusion at the amino terminus of the MOREHEL protein does not interfere with induction of HR.

This observation directed us to express chimeric proteins of U1 and Ob MOREHEL domains. We divided each MOREHEL sequence into eight domains on the basis of the presence of restriction sites in the MOREHEL cDNAs. Expression of these chimeras in our agroinfiltration system could not pinpoint the HR-induction capacity to a specific site in the MOREHEL:U1 sequence. Our results demonstrate that at least four domains are involved in HR induction. MOREHEL:U1 domains A, B, E, and H were shown to be very important for induction of HR, whereas domains C and D were less important but did modulate HR. Although the results may be complicated to interpret, the involvement of domains A, B, E, and H in HR in-

duction is obvious. This suggests that a MOREHEL protein that contains domains A, B, and E; A, B, and H; or A, E, and H is able to induce an *N*-mediated HR. Domains F and G did not seem to be involved in HR induction, although we cannot rule out at this point that the Ob f and g domains and U1 F and G domains fulfill equivalent functions in recognition by the *N* gene. In principle, it is possible that the Ob f and g domains are recognized by the *N* protein and elicit a HR, but this event is prevented by the overall structure of the MOREHEL:Ob protein. Other MOREHEL:Ob domains may mask this HR-inducing domain, and the MOREHEL chimeras that disrupt this masking result in HR. From this point of view, the MOREHEL:Ob a, b, c, d, e, and h domains may be involved in shielding the HR-eliciting domain from recognition by the *N* protein.

The data obtained with the MOREHEL chimera ABCdefgh, which contains clusters of MOREHEL:U1 H amino acids in domain h, suggest that the carboxy-terminal sequences of the MOREHEL:U1 protein are important for HR induction. The results, however, were confusing. Exchange of additional MOREHEL:U1 amino acid clusters positively and negatively influenced the HR induction. In addition, mutations in the TMV-Ob replicase coding sequence restore *N*-mediated resistance (Padgett et al. 1997). These mutations reside in the MOREHEL domain, but the resulting protein sequence is even less similar to the U1 sequence. Moreover, some alterations involve the substitution of a proline residue, and prolines can greatly influence the protein secondary structure because they form a bend in linear protein structure. Therefore, the Ob mutations that restore *N*-mediated resistance may influence the overall protein structure of the replicase proteins. A large effect of many small alterations in the entire protein sequence on elicitor activity also has been observed for the elicitor of the *N'*-mediated resistance

		HR induction									
		NN plants	nn plants								
4	<table><tr><td>a</td><td>b</td><td>c</td><td>D</td><td>E</td><td>F</td><td>G</td><td>H</td></tr></table>	a	b	c	D	E	F	G	H	-	-
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15	<table><tr><td>a</td><td>b</td><td>C</td><td>D</td><td>E</td><td>F</td><td>G</td><td>H</td></tr></table>	a	b	C	D	E	F	G	H	+	-
a	b	C	D	E	F	G	H				
16	<table><tr><td>A</td><td>B</td><td>c</td><td>D</td><td>E</td><td>F</td><td>G</td><td>H</td></tr></table>	A	B	c	D	E	F	G	H	+++	-
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17	<table><tr><td>A</td><td>b</td><td>c</td><td>D</td><td>E</td><td>F</td><td>G</td><td>H</td></tr></table>	A	b	c	D	E	F	G	H	+++	-
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18	<table><tr><td>A</td><td>b</td><td>c</td><td>D</td><td>E</td><td>F</td><td>g</td><td>h</td></tr></table>	A	b	c	D	E	F	g	h	-	-
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19	<table><tr><td>A</td><td>b</td><td>c</td><td>d</td><td>e</td><td>f</td><td>G</td><td>H</td></tr></table>	A	b	c	d	e	f	G	H	-	-
A	b	c	d	e	f	G	H				
20	<table><tr><td>A</td><td>b</td><td>C</td><td>d</td><td>e</td><td>f</td><td>G</td><td>H</td></tr></table>	A	b	C	d	e	f	G	H	-	-
A	b	C	d	e	f	G	H				

Fig. 7. Schematic representation of chimeric the helicase domain of the replicase proteins (MOREHEL) and their elicitor activities. Shaded domains indicate the MOREHEL:U1 origin, whereas open domains indicate the MOREHEL:Ob origin. Names of the chimeric proteins are indicated. Infiltrated leaves were observed for a period of at least 2 weeks. Hypersensitive response (HR) normally was induced at 2 days postinfiltration. The MOREHEL:U1 elicitor activity was set at +++. -, leaves that did not induce necrosis or leaf yellowing. +, HR was induced only in cells of the bottom surface of the infiltrated leaf tissue. In all experiments, MOREHEL:U1 was expressed as a positive control.

(Culver et al. 1994; Taraporewala and Culver 1996; Taraporewala and Culver 1997). The *N'* gene confers resistance against many non-tobacco tobamoviruses. The CP has been identified as the elicitor of the *N'*-mediated resistance. Upon elucidation of the CP tertiary and quaternary structure, it appeared that the quaternary structure was important for shielding of the HR-eliciting domain in the tobacco TMV CP. Throughout the entire CP sequence, amino acid alterations have been described that disrupt the CP quaternary structure important for CP multimerization, exposing a putative *N'*-recognizing site that consequently induces HR. Elucidation of the crystal structures of TMV-U1 and TMV-Ob MOREHEL domains should be very helpful in providing further information on the interpretation of the data obtained with the chimeric MOREHEL proteins.

Assuming that the HR-inducing capacity resides in specific MOREHEL:U1 sequences and not in MOREHEL:Ob, it is interesting to note that the domains important for HR induction are dispersed over the entire primary sequence of the MOREHEL:U1 protein. This may suggest that, in the tertiary protein structure, the HR-inducing determinants are in (close) proximity and the receptor recognizing the MOREHEL:U1 protein is binding to this complex binding site. Alternatively, the MOREHEL:U1 recognizing receptor is binding the MOREHEL:U1 protein at several contact points. This also could explain why MOREHEL:U1 lacking HR-active domains E or H still induces a HR, whereas both E and H domains are required in the absence of domains B (and C). Accordingly, the receptor is still able to induce the HR when a minimal number of contact points is present. R proteins may be (in)direct receptors for binding and recognition of HR elicitors. R proteins are encoded by dominant genes and expressed in uninfected tissue. Also, many R proteins contain a LRR domain, which is involved in protein-protein interactions in other proteins (Kobe and Deisenhofer 1995).

The specificity of R proteins is, in some cases, determined by this LRR domain (Ellis et al. 2000). It is tantalizing to suggest, on the basis of our results, that the MOREHEL:U1 protein is interacting with the LRR domain of the N protein. One can imagine that the putative interaction between the MOREHEL:U1 protein and the LRR domain of N is established by contact points that are spread throughout both protein sequences. Thus, such an interaction could involve several regions in the MOREHEL:U1 domain. Recently, two direct interactions between LRR-containing R proteins and avirulence products have been described (Jia et al. 2000; Leister and Katagiri 2000).

A third possibility is that amino acid alterations in MOREHEL:U1 influence the overall protein structure and indirectly disrupt the HR-eliciting sequence. This hypothesis is supported in that full-length transcripts expressing U1 and Ob chimeric replicase proteins do not result in infection, even in the highly susceptible *N. benthamiana* plants. It cannot be excluded, however, that these chimeric viruses may owe their lethal phenotype to changes in the RNA structure rather than to changes in the replicase protein. Further studies are required to elucidate the reason for the lethality. In any case, for TMV replication, the putative helicase domains of TMV-Ob and TMV-U1 are not simply interchangeable.

MATERIALS AND METHODS

Constructs.

The MOREHEL:U1 sequence has been described before, and its location (nucleotide 2,109 to 3,419) in the TMV-U1 genome is shown in Figure 1A (Abbink et al. 1998). The

TMV-Ob genome and its MOREHEL:Ob location (nucleotide 2,106 to 3,416 of the TMV-Ob genome) is very similar and therefore not shown separately. Figure 1B shows the schematic representation of the cDNA sequences of the MOREHEL domains of TMV-U1 and TMV-Ob. The MOREHEL:Ob construct was created by cloning the *NcoI*-*PstI* fragment from a PCR reaction with primers ta055 and ta056 on a TMV-Ob template into the binary pMOG800 expression vector (Table 1) (Abbink et al. 1998). The restriction sites in the U1 and Ob

Fig. 8. Infection of chimeric viruses in *Nicotiana benthamiana* plants. **A**, Schematic representation of the chimeric infectious cDNAs. Shaded boxes indicate domains of the helicase domain of the replicase proteins (MOREHEL):U1 origin, whereas open boxes represent regions of MOREHEL:Ob. Domains DEF of MOREHEL:U1 and def of MOREHEL:Ob were exchanged in the infectious cDNAs clones of *Tobacco mosaic virus* (TMV)-U1 and TMV-Ob. Asterisk denotes the amber termination codon. **B**, Northern blot analysis of viral RNA in TMV cDNA-inoculated plants. Transcripts from infectious TMV-Ob cDNA (Ob) and chimeric transcripts containing U1- and Ob-derived helicase domains (Ob-DEF, U1-def) were used to inoculate *N. benthamiana*. RNA was extracted from inoculated (L) and systemic (S) leaves. Position of TMV genomic (gRNA) and subgenomic mRNA for the coat protein (CP sgRNA) are indicated. Blot was probed with a TMV-U1- and TMV-Ob-derived ³²P-dCTP-labeled cDNA probe.

Table 1. Oligonucleotide primers used for constructing chimeric MOREHEL (*Tobacco mosaic virus* [TMV]-replicase proteins) constructs

Primer	T ^a	Nucleotide position ^b	Sequence (5' → 3') ^c
ta025	U1	2,109–2,126 (<i>Nco</i> I)	<u>CGAGTTTCC</u> ATGGCAACGGCAGATTG
ta026	U1	3,419–3,398 (<i>Pst</i> I)	<u>GCCCTGCAGTTA</u> CTATTGTGTTCTGCATCGACC
ta055	Ob	2,106–2,144 (<i>Nco</i> I)	<u>GGGCCATGGT</u> CGCAGCTGAATCAGTTATTAGTAACAAGATGGC
ta056	Ob	3,416–3,395 (<i>Pst</i> I)	<u>CCCCTGCAGTTA</u> CTATGCACACACTATCTACC
ta059	Ob	2,344–2,378 (<i>Nsi</i> I)	ATCATGCATGGGTAGTCGTTTCAAGTTCGACGG
ta060	Ob	2,357–2,329 (<i>Nsi</i> I)	CCCCATGCATGATTTTAAAGTGTGCGC
ta061	Ob	2,188–2,232 (<i>Cl</i> aI)	CTATATCGATAGTCTCGCAGCATCTCTATCGGCC
ta065	U1	3,350–3,373 (K420C)	CATTAGAGATCTAGAGTGTCTTAGCTCGTACTTG
ta066	U1	3,373–3,350 (K420C)	CAAGTACGAGCTAAGACACTCTAGATCTCTAATG
ta067	Ob	3,349–3,384 (C420K)	GTTAGAGATTTAGAGAAAGTTAGTAACCTCTTGTTGG
ta068	Ob	3,384–3,349 (C420K)	CCAACAAGAAGTTACTAACCTTCTCTAAATCTCTAAC
ta072	U1	2,991–3,029 (R300C)	CATTATCTGAACAGGTTGTATGAGGGCTTGTCTAGAGC
ta073	U1	3,029–2,991 (R300C)	GCTCATGACAAAGCCCTCATAACACCTGTTTCAGATAATG
ta075	Ob	2,986–3,023 (C300R)	CCCATTCTTAAATCAAAGATACGATGGTGCTGTCACG
ta076	Ob	3,023–2,986 (C300R)	CGTGACAGCACCATCGTATCTTTGATTAAAGAAATGGG
ta092	Ob	3,230–3,283 (gh1/3/7/8/9)	AACACCTACCCCGGTTTCATCATGCTGGAGACAGTCTCATGTACTTGTGG
ta093	Ob	3,416–3,377 (gh2/3/7/8/9)	<u>CCCCTGCAGTTA</u> CTATTGTGTTCTGTCATCTACCTTATACACATCCAAGCAAG
ta095	Ob	3,369–3,407 (gh6/9)	AGTTCGTACTTGTGGATATGTATATGGTAGATAGTGTG
ta096	Ob	3,383–3,348 (gh6/9)	CAACAAGTACGAACCTAAGTTTCTCTAAATCTCTAAC
ta097	Ob	3,333–3,366 (gh5/8)	CCTCTGGTTAGTATAATTAGAGATTAGAGTGTG
ta098	Ob	3,347–3,308 (gh5/8)	TATACTAACCAGAGGATCCATCACCAGTATAGTACTTG
ta099	Ob	3,297–3,326 (gh4/7)	ACATGTTCTCTCAAGTACTATACAGTGGTG
ta100	Ob	3,331–3,282 (gh4/7)	CTTGAGAGAACATGTGTGCTTGTCAACCC
ta101	U1	3,045–3,071 (<i>Bgl</i> II)	ATTTAGATCTGTTTCGCACCATGGTTCGGC
ta102	U1	3,051–3,026 (<i>Bgl</i> II)	CCCCAGATCTTTTAAACCGAAGAAGTGCTC
ta103	U1	2,848–2,874 (<i>Hind</i> III)	CTTTAAGCTTATGTTACGGAGACACACAGC
ta104	U1	2,853–2,828 (<i>Hind</i> III)	<u>CCCCAAGCTTTT</u> TCGCACAATGACATCGCC

^a T indicates the MOREHEL template that was used in the polymerase chain reaction (PCR) reaction.

^b Nucleotide positions of the PCR primers in the U1 and Ob TMV genomes. Restriction sites that were introduced in the MOREHEL sequence are in parentheses. The amino acid changes introduced by primers TA092 to TA100 are located in domains g and h of the MOREHEL:Ob open reading frame. The names of the chimeric constructs in which the alterations are present are indicated in parentheses (Fig. 6).

^c Nucleotides that deviate from the wild-type sequences of the U1 and Ob MOREHEL are underlined.

MOREHEL sequences that were used as fusion sites for the construction of the chimeric cDNAs are indicated. On the basis of these restriction sites in the cDNAs, the MOREHEL sequences were divided into eight domains: A to H in the MOREHEL:U1 and, correspondingly, a to h in the MOREHEL:Ob protein (Fig. 1B). Restriction sites that are unique to either MOREHEL cDNA were introduced, by PCR, into the other MOREHEL cDNA. The oligonucleotide primers that were used in these reactions are in Table 1. Non-silent point mutations were introduced into domains E, e, g, and h, which resulted in the exchange of amino acids between the Ob and U1 MOREHEL protein sequences. In general, the cloning strategy for all chimeric constructs was similar. For instance, construction of chimera 12 was initiated by a PCR reaction on the TMV-U1 template with primers ta025 and ta104. The PCR fragment was digested by *Nco*I and *Hind*III and cloned into the MOREHEL:Ob construct after digestion by the same restriction enzymes. All PCR sequences were confirmed by T7 sequencing analysis (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, U.K.).

Eventually, all chimeric cDNAs were cloned into the binary vector pMOG-800 under control of the *Cauliflower mosaic virus* 35S promoter and potato proteinase inhibitor terminator (Abbink et al. 1998). The pMOG-800 derivatives were introduced into *Agrobacterium tumefaciens* strain LB 4404 and used for agroinfiltration studies. Cultures of the transformed *A. tumefaciens* were grown overnight to an optical density at 600 nm of approximately 1 and infiltrated into leaf tissue in the presence of 200 μ M acetosyringone. A small cut was then made through the upper epidermis of the leaf where the bacte-

rial suspension was pressed with the use of a syringe without a needle. The chimeric composition is evident from the schematic representations in the figures. Amino acid positions of the MOREHEL sequences are numbered, starting from the initiation codon of the MOREHEL domains.

Construction of chimeric TMV cDNA clones.

Full-length, infectious cDNAs of TMV-U1 and TMV-Ob were a gift from W. O. Dawson (Lake Alfred, FL, U.S.A.) and R. Beachy (St. Louis, MO, U.S.A.). The MOREHEL domains DEF and def were exchanged in these infectious cDNAs with the *Sst*II and *Hpa*I sites that are present in the full-length cDNA sequences of U1 and Ob (Fig. 1). Transcripts were made in standard in vitro transcription reactions containing T7 RNA polymerase, nucleotides, and cap analog (New England Biolabs, Beverly, MA, U.S.A.). Transcript levels and quality were analyzed on 1.5% agarose gel. Similar amounts of transcript were used to infect *N. benthamiana* plants. Leaves of these plants were ground with a mortar and pestle, and the sap was used to infect NN and nn tobacco plants. Northern blot analysis was performed as described (Abbink et al. 1998).

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