The Necrotic Pathotype of the *Cucumber mosaic virus* (CMV) Ns Strain Is Solely Determined by Amino Acid 461 of the 1a Protein

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The unique Ns isolate of *Cucumber mosaic virus* (CMV) induces necrotic lesions on several *Nicotiana* spp. in contrast to other strains that cause systemic mosaic on these plants. By using biologically active RNA transcripts from cDNAs of Ns-CMV and a reference subgroup I strain Rs-CMV, we confined the genetic determinant solely responsible for necrosis induction to amino acid 461 of the 1a protein translated from genomic RNA1. An Arg to Cys change at this position (R461C) rendered Rs-CMV necrotic, whereas the reciprocal C461R mutation reverted the necrotic phenotype of Ns-CMV. Necrotic (Ns-CMV, R461C) and non-necrotic (Rs-CMV and C461R) viruses accumulated to similar levels in *Nicotiana clevelandii* protoplasts. Deletion of the residue at position 461 abolished replicase activity of the Ns-CMV 1a protein. The R461C mutation also was introduced into the 1a protein of Trk7-CMV, a subgroup II isolate. Symptoms induced by the Trk7/R461C mutant were identical to those caused by wild-type Trk7-CMV, even when the mutant Trk7 RNA1 was co-inoculated with RNA2 and 3 of the necrotic Ns strain.

Additional keywords: hypersensitive response.

Symptom appearance during the course of viral infection is a result of a complex interaction between the virus and the host plant. However, host plants have evolved effective strategies to minimize the damaging effect of the infection. One such protection mechanism is the hypersensitive response (HR), elicited by a broad spectrum of pathogens, including viruses (Goldbach et al. 2003). An HR is characterized by the confinement of the infection through programmed cell death, which prevents further spread of the pathogen (Heath 2000). One of the characteristic symptoms associated with the HR is the appearance of necrotic local lesions, visible signs of mass cell necrosis. HR is considered to be a result of a receptor–ligand interaction between a host resistance gene product and a corresponding pathogen-encoded avirulence factor (Hutcheson 1998). Indeed, the gene-for-gene concept is supported by experimental evidence; moreover, many avirulence factors and several resistance genes have been identified (Hulbert et al. 2001; Hutcheson 1998).

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The small icosahedral *Cucumber mosaic virus* (CMV) is the type member of the genus *Cucumovirus* in the family *Bromoviridae*. CMV has an unusually broad host range, encompassing over 1,000 species (Palukaitis et al. 1992). Based on serological and sequence data, CMV strains have been categorized into three subgroups, IA, IB, and II (Roossinck et al. 1999). The tripartite CMV genome consists of single-stranded positive sense RNAs. RNA1 and RNA2 code for the 1a and 2a proteins, respectively, which are the core elements of the replicase complex (Hayes and Buck 1990). Two functional domains can be distinguished in the 1a protein: the N-terminal region shows sequence homology to methyltransferases while the C-terminal region contains motifs characteristic of viral and cellular helicases (Gorbalenya et al. 1988; Haseloff et al. 1984). The conserved GDD motif, characteristic of RNA polymerases, is present in the 2a protein, the other viral component of the replicase complex (Habibi and Symons 1989). RNA3 codes for two proteins, 3a and the coat protein (CP); the latter is translated from a subgenomic RNA (RNA4). Both RNA3 gene products are essential for the short-distance movement of CMV (Canto et al. 1997). The smallest CMV protein, 2b, is translated from the subgenomic RNA4A of RNA2 with a partially overlapping open reading frame to the 3' end of the 2a gene. The 2b protein is a host range determinant (Ding et al. 1995) and a suppressor of post-transcriptional gene silencing (Brigneti et al. 1998). Nonlethal mutations in any of the five genes of CMV can alter symptomatology, host range, or both (Kim and Palukaitis 1997; Li et al. 2001; Roossinck and Palukaitis 1990; Shi et al. 2002; Szilassy et al. 1999a). CP and 2a also have been identified as possible HR avirulence factors in tobacco and cowpea, respectively (Kim and Palukaitis 1997; Takahashi et al. 2000).

Although most CMV strains systemically infect *Nicotiana* spp., there are two known exceptions that induce necrotic lesions. First, CMV(Y/GM2), a derivative of the yellow CMV strain (Y-CMV), triggers an HR on nine *Nicotiana* spp. (Takahashi et al. 2000). The other necrotic strain is N-CMV, which was isolated and described as a chance mutant by Fulton (1953). In addition to *N. benthamiana* and *N. clevelandii*, which are susceptible both locally and systemically, N-CMV induces only necrotic local lesions on a wide range of *N. tabacum* cultivars as well as on other solanaceous species (e.g., *Atropa*, *Capsicum*, *Datura*, *Nicotiana*, and *Physalis* spp.) (Fulton 1953; Salamon et al. 1998). *N. glutinosa* previously has been thought to be resistant to the virus; however, the stock culture of N-CMV occasionally induced necrotic local lesions on these plants. From the lesions, a derivative of the N strain, Ns-CMV, was isolated. Apart from regularly inducing necrotic local lesions and very rarely systemic mosaic (especially after longer incubation) on *N. glutinosa*.

nosa, the pathological properties of the derivative strain are identical to those of N-CMV (Salamon et al. 1998). Based on double-gel immunodiffusion tests and on the nucleotide sequence of the RNA3, N-CMV was classified as a subgroup I isolate (Salamon et al. 1998).

Although it has been shown that the unusual symptoms of the N-CMV can be linked to genomic RNA1 (Lakshman and Gonsalves 1985), the exact localization and nature of the genetic determinant responsible for lesion induction remained unknown.

In order to map the necrotic determinant, we used Rs-CMV as a reference strain. Rs-CMV was isolated from *Raphanus sativus* and belongs to subgroup I, causing green systemic mosaic on all *Nicotiana* hosts (P. Salamon, unpublished data). By using Ns-CMV/Rs-CMV reassortant, chimeric, and point mutant viruses, one amino acid in the viral 1a protein was identified as the necrotic determinant of Ns-CMV. In the present work, we also have examined whether this single amino acid change is able to induce necrosis in a different genetic context.

RESULTS

Symptomatology and test of the full-length cDNA clones.

Although both Ns-CMV and Rs-CMV belong to subgroup I, the symptoms of infection were strikingly different. Whereas the Rs strain caused the typical systemic mosaic characteristic of CMV infection on all tested *Nicotiana*

plants (*N. clevelandii*, *N. glutinosa*, and *N. tabacum* cv. Xanthi-nc) with no local symptoms (Table 1; Fig. 1, left panel), the Ns strain induced necrotic local lesions 4 days postinoculation (dpi) on these species (Table 1; Fig. 1, right panel). The Ns-CMV infection became systemic only in *N. clevelandii* plants, where the virus caused progressive necrosis of systemically infected leaves, leading to the collapse of the plant 21 dpi (Table 1). There was also a difference in the time required for the appearance of the systemic symptoms between Ns- and Rs-CMV. The first signs of systemic necrosis were observed 5 to 6 dpi on *N. clevelandii* infected by Ns-CMV, whereas Rs-CMV induced systemic mosaic only 7 to 9 dpi on all *Nicotiana* spp.

Full-length cDNA clones were synthesized from both Ns and Rs viral genomes for further investigations. In order to verify the sequence integrity of the cDNA clones of the two strains, test plants were co-inoculated with capped in vitro RNA transcripts of the pN1, pN2, and pN3, and pR1, pR2, and pR3 clones, respectively. Both genomic clone sets were infectious and the induced symptoms were identical to the original viruses (i.e., Ns-CMV and Rs-CMV) (Table 1). Nucleotide sequence of the genomic clones have been determined and submitted to the EMBL database under accession numbers AJ580953, AJ511989, AJ511990, AJ511988, AJ517801, and AJ517802. Analysis of the sequence data has confirmed the earlier serology-based classification of the strains to subgroup I.

Table 1. Symptoms of the wild-type, reassortant, chimeric, and point mutant viruses 10 days postinoculation

Genotype	Infection phenotype ^a		
	<i>Nicotiana clevelandii</i>	<i>N. glutinosa</i>	<i>N. tabacum</i> cv. Xanthi-nc
Wild type viruses			
R1/R2/R3	1	2	3
N1/N2/N3	5	6	7
Trk1/Trk2/Trk3	9	4	4
Ns-CMV/Rs-CMV reassortants ^b			
R1/R2/N3	1	2	3
R1/N2/R3	1	2	3
R1/N2/N3	1	2	3
N1/N2/R3	5	6	7
N1/R2/N3	5	6	7
N1/R2/R3	5	6	7
RNA1 chimeras			
NR/R2/R3	5	7	7
RN/R2/R3	1	2	3
N.RNR/R2/R3	5	7	7
R.NRN/R2/R3	1	2	3
RNR-Bsm/R2/R3	5	7	7
NRN-Bsm/R2/R3	1	2	3
RNR-Csp/R2/R3	5	7	7
NRN-Csp/R2/R3	1	2	3
NRN-Csp/N2/N3	1	2	3
1a protein point mutants			
rL456F/R2/R3	1	NA	NA
rR461C/R2/R3	5	7	7
rV462Q/R2/R3	1	NA	NA
rV467A/R2/R3	1	NA	NA
rH469R/R2/R3	1	NA	NA
nC461R/N2/N3	1	2	3
nΔ461/N2/N3	0	0	0
tR461C/Trk2/Trk3	9	4	4
Intersubgroup reassortants			
N1/Trk2/Trk3	8	7	7
rR461C/Trk2/Trk3	8	7	7
tR461C/R2/R3	1	2	2
tR461C/N2/N3	1	2	2

^a Infection phenotype codes; slash (/) separates local and systemic symptoms, and rarely or slowly appearing symptoms are in parentheses: 0 = asymptomatic; 1 = none/leaf deformation, green mosaic; 2 = none/green mosaic; 3 = none/green mosaic, vein clearing; 4 = none/mild green mosaic; 5 = necrotic lesions/necrotic lesions (apical necrosis); 6 = necrotic local lesions/none (green mosaic); 7 = necrotic local lesions/none; 8 = necrotic lesions/necrotic lesions, apical necrosis; 9 = chlorotic spots/leaf deformation; NA = not assayed.

^b CMV = Cucumber mosaic virus.

Confinement of the necrosis-inducing sequence to a 175-nucleotide section of RNA1.

In order to identify which genomic RNA molecule or molecules were responsible for necrosis induction, all six possible reassortant viruses were prepared by combining the transcripts of the Rs-CMV and Ns-CMV cDNA clones. *Nicotiana* test plants were co-inoculated with the reassorted RNA1 transcripts. Symptoms caused by viruses carrying the RNA1 molecule of the Rs strain were similar to those induced by

Ns-CMV (Table 1). In contrast, reassortants bearing RNA1 of the Ns strain induced necrotic local lesions at 4 dpi that were indistinguishable from those caused by Ns-CMV (Table 1). These results confirmed that necrosis induction is determined solely by the RNA1 molecule in the reassorted genome (Lakshman and Gonsalves 1985). We did not find significant influence of the origin of RNA2 and RNA3 molecules either on the appearance of symptoms or on the kinetics of the infection.

Fig. 1. Local symptoms induced by Rs-Cucumber mosaic virus (CMV) and Ns-CMV 10 days postinoculation on *Nicotiana clelandii* (top), *N. glutinosa* (middle), and *N. tabacum* cv. Xanthi-nc (bottom). Black arrowheads indicate necrotic local lesions. On Rs-CMV-infected *N. clelandii*, white arrowheads indicate systemic symptoms; inoculated leaves are marked with asterisks. Inoculations were performed with purified virions at 10 µg/ml.

By using common restriction endonuclease sites in the pN1 and pR1 clones, a series of reciprocal cDNA1 chimeras was created to further confine the sequence responsible for the necrotic pathology (Fig. 2A). Transcripts from the chimeric

cDNA1 molecules were combined with transcripts from the pR2 and pR3 clones prior to the inoculation of different *Nicotiana* plants. The symptoms induced by the chimeric viruses are summarized in Table 1. pNR and pRN chimeras localized

A

B

Strain name			EMBL accession number
CMV-Rs	450	SKWFAALTRPLRVFFSSVVHALFPTLR	AJ580953
CMV-Ns	450	F.....CQ.....A.R.....	AJ511988
CMV-Trk7	450	W.....SMS...F.....T...KT.....	AJ007933
TAV-V	449	R.F...S....M...L.AC.KT.....	NC_003837
PSV-J	445	.Q.WK.M.K.I.T.VRL.A.T.....	D11126

Fig. 2. A, Chimeric *Cucumber mosaic virus* (CMV) cDNA1 molecules. Black and white bars indicate the Ns-CMV and Rs-CMV origin of the 1a coding segments, respectively. Restriction endonuclease sites common to pN1 and pR1 were used for the construction of the chimeras. In vitro transcripts of the chimeric cDNA1s were co-inoculated with transcripts from the pR2 and pR3 clones. **B,** Alignment of translated cucumovirus 1a protein sequences corresponding to the Csp45I to EcoRV section of CMV cDNA1. Two subgroup I CMV (Rs and Ns), a subgroup II CMV (Trk7), as well as sequences from *Tomato aspermy virus* (V-TAV) and *Peanut stunt virus* (3-PSV) were used for the alignment.

the necrotic lesion inducing genetic determinant to the first 1,616 nucleotides (nt) of the RNA1 molecule. Dissection of the 5' half of the cDNA1 by pN.RNR and pR.NRN constructs excluded the influence of the *BsmI* to *BsmI* (nucleotides 692 to 1,427) section on symptom determination. pRNR-*BsmI* and pNRN-*BsmI* chimeras demonstrated that sequences upstream of the first *BsmI* site (nucleotides 1 to 692) also have no effect on lesion induction. The close proximity of a *Csp45I* site (nucleotide 1,442) to the second *BsmI* site allowed further confinement of the necrotic determinant to the 175-nt section between

the *Csp45I* and *EcoRV* restriction enzyme recognition sites. The origin of these 175 nt apparently determines the infection phenotype: even if the RNA transcript of the pNRN-*Csp* was co-inoculated with transcripts from the pN2 and pN3 clones, the emerging symptoms were characteristic of Rs-CMV (Table 1).

The crucial role of the amino acid 461 in the 1a protein.

To our knowledge, there had been only one report describing viral genomic RNA as an elicitor of the HR (Szittyá and Burgyán 2001); therefore, we assumed that the necrotic deter-

Fig. 3. Local symptoms of **A**, rR461C/R2/R3 and **B**, nC461R/N2/N3 mutant viruses on *Nicotiana* plants. Arrowheads indicate necrotic local lesions. Photos were taken 10 days postinoculation.

minant is expressed at the amino acid level. Comparison of Rs and Ns 1a protein sequences revealed five amino acid differences in the 175-nt cDNA region (Fig. 2B). The occurrence of cysteine at position 461 is unique because, in the genus *Cucumovirus* (including *Tomato aspermy virus* and *Peanut stunt virus*), all the known 1a proteins contain arginine at this position. In order to identify which amino acid or acids are responsible for the altered infection phenotype of the Ns strain, five pR1-based cDNA1 constructs were designed bearing point mutations in the 1a protein sequence. These mutants were designated as prL456F, prR461C, prV462Q, prV467A, and prH469R, referring to the pR1 origin of the molecule and the sequence change in the encoded 1a protein. *N. clelandii* plants were co-inoculated with transcripts from pR2 and pR3 and the mutant pR1 clones. In four cases, the sequence alteration of 1a protein had no effect on the symptoms (Table 1). In contrast, the rR461C mutant induced necrotic lesions 4 dpi on all tested *Nicotiana* spp. (Table 1). The infection phenotype and the time required for the symptom induction were similar to the Ns-CMV (Fig. 3A). Based on these results, we concluded that amino acid 461 of the 1a protein is a principal determinant of host response in the Ns-CMV/Rs-CMV experimental system. The fact that the predicted secondary structures of the transcribed pR1 and prR461C clones are identical in the *Csp45I* to *EcoRV* section (data not shown) supports our preliminary hypothesis that the symptom determinant was expressed at the amino acid level. In order to analyze whether the presence of cysteine at position 461 is solely responsible for lesion induction, pnC461R, a counterpart clone of prR461C, was created based on the pN1 clone. The test plants that were co-infected with transcripts of pnC461R, pN2, and pN3 showed green systemic mosaic (Table 1) but no local symptoms (Fig. 3B). In general, the symptoms induced by the nC461R mutant were characteristic of Rs-CMV, which confirmed that amino acid position 461 of the 1a protein fundamentally determined the host response given to the infection.

To further analyze the role of amino acid 461 in symptom determination, a deletion mutant of the Ns 1a protein, lacking position 461 (n Δ 461) was constructed. The deletion resulted in a defective virus; no signs of infection were observed on any

of the tested *Nicotiana* plants (Table 1). The absence of infectivity proved to be the result of the impaired replication of n Δ 461 (discussed below).

We also performed experiments to test whether the change of the canonical arginine in position 461 to cysteine leads to necrotic lesion induction in a virus with different genetic background. The R461C mutation was introduced into the 1a protein of Trk7, a subgroup II CMV strain (Szilassy et al. 1999b), resulting in the clone ptR461C. Test plants co-inoculated with transcripts of ptR461C, pTrk2, and pTrk3 displayed symptoms identical to the wild-type Trk7-CMV: mild systemic mosaic, but no lesions were observed (Table 1). In order to test whether there are subgroup-specific factors located on the RNA2 and 3 molecules contributing to the R461C mutation-induced necrotic response, four intersubgroup reassortants were created. Symptoms of the N1/Trk2/Trk3 and the rR461C/Trk2/Trk3 reassortants indicated that lesion induction was not dependent on the presence of subgroup I RNA2 and 3 (Table 1). Similarly, viruses carrying the mutated Trk7 RNA1 and the RNA2 and 3 molecules of the subgroup I Ns or Rs strains (tR461C/R2/R3 and tR461C/N2/N3) did not induce necrosis on any test plant (Table 1), which shows that it is not the lack of subgroup I-specific factors on RNA2 and 3 that renders the tR461C/Trk2/Trk3 virus unable to induce lesions.

Accumulation in protoplasts.

To determine whether the arginine-cysteine substitution in position 461 of the 1a protein had any influence on the accumulation level of the virus in single cells, protoplasts from fully expanded leaves of *N. clelandii* were isolated and inoculated with RNAs of the unmodified Ns and Rs strains as well as with RNAs extracted from the rR461C/R2/R3 and nC461R/N2/N3 mutant viruses. The radioprobe was synthesized from the 3' end of the pN3 clone; therefore, the RNA3 and 4 gave a stronger signal than the other two genomic RNAs. At 24 h after transfection, both the wild-type and mutant viral RNAs accumulated at approximately the same level (Fig. 4A).

Protoplasts transfected by transcripts of pn Δ 461, pN2, and pN3, did not show detectable virus replication (Fig. 4B).

Fig. 4. Accumulation of *Cucurbit mosaic virus* (CMV) RNAs in *Nicotiana clelandii* protoplasts 24 h after transfection. Total nucleic acid extracts from 2.5×10^5 protoplasts were probed against the radioprobe synthesized from the *Hind*III to *Bam*HI fragment (nucleotides 1,563 to 2,216) of the pN3 clone. RNA3 and 4 show a stronger signal, although the probe also reacts with RNA1 and 2. **A**, Protoplasts transfected with 2 μ g of purified virus RNA. Lane 5, mock inoculated; lane 6, 25 ng of purified Rs-CMV RNA. **B**, Protoplasts transfected with 2 μ g of in vitro RNA transcripts. Lane 1, 25 ng of purified Rs-CMV RNA; lane 2, mock inoculated; lane 3, transfection control.

These data indicate that, although the arginine-cysteine change in position 461 did not significantly alter the accumulation level of the virus in protoplasts, deletion of amino acid 461 completely abolishes the functionality of 1a protein.

DISCUSSION

In our experiments, amino acid 461 of the 1a protein has been shown to play a central role in determining different aspects of the infection. Apart from the obvious influence on symptom manifestation on several *Nicotiana* spp., the absence of this amino acid ceased viral replication. Such single amino acid changes in viral proteins have been reported to be able to act as necrotic or avirulence determinants in various other viruses. There are examples where nonstructural viral proteins induce necrosis or HR (Jenner et al. 2003; Kim and Palukaitis 1997; Scholtof et al. 1935); however, in many cases it involves the CP (Berzalherranz et al. 1995; Cruz and Baulcombe 1993; Suzuki et al. 1995; Takahashi et al. 2000).

Involvement of CMV RNA1 in symptom determination is rather unusual, but not limited to Ns-CMV. Fny-CMV and Sny-CMV both infect zucchini squash cultivars with different kinetics and symptom severity, both of which have been associated with RNA1 (Gal-On et al. 1994; Roossinck and Palukaitis 1990). In accordance with our observations, the phenotypic difference of infection was not due to differential replication of Fny-CMV and Sny-CMV (Gal-On et al. 1994).

For interpretation of the function of amino acid 461 in triggering the host necrotic response, the gene-for-gene concept provides a good basis. The avirulence factor of the Ns-CMV is clearly the 1a protein: by using reassortant viruses, we were able to localize the necrotic determinant solely to RNA1. According to the gene-for-gene concept, we assume that the introduction of a cysteine at position 461 alters the conformation of the 1a protein or the accessibility or electrostatic pattern of a surface determinant in such a way that 1a protein becomes a ligand of a putative cellular receptor responsible for triggering the molecular cascade resulting in tissue necrosis. Conformational and functional information on the CMV 1a protein mainly derives from data gained by the detailed analysis of the closely related *Brome mosaic virus* (BMV). Despite low (55%) similarity at the amino acid level, CMV and BMV 1a proteins share identical structural features (O'Reilly et al. 1998). Secondary structure prediction placed amino acid 461 in the middle of an α -helix with >80% confidence. Three amino acid insertions in this α -helix rendered the BMV 1a protein nonfunctional by disrupting the structure of the globular C-terminal helicase domain, which also can be found in the CMV 1a protein (O'Reilly et al. 1995). This reports suggests that a mutation in the α -helix involving amino acid 461 may have a global (or even deleterious) effect on protein folding. We presume that the 1a protein completely lost its functionality in our experiments due to the Δ 461 deletion; however, the R461C mutation had less impact on the conformation or charge distribution. Nevertheless, this amino acid change might have made the protein capable of triggering a necrotic response. The presence of the cysteine instead of the canonical arginine in position 461 also raises the possibility of alternative disulphide bond formation. Such disulphide bonds can fundamentally change the structure of the 1a protein.

As mentioned above, in addition to direct conformational change of the 1a protein, surface exposure of otherwise hidden domains also can lead to necrosis induction. Like all known RNA viruses, CMV replicates in close association with intracellular membranes (Cillo et al. 2002). In the case of the BMV, this interaction is predominantly mediated by amino acids 367

to 480 of the 1a protein, which corresponds to the 397- to 496-amino acid (aa) section of the CMV 1a protein (den Boon et al. 2001). Interestingly, this region of the 1a protein largely overlaps with the particularly important structural α -helix described by O'Reilly and associates (1998), which implies a function in both conformation stabilization and membrane association. Based on these considerations, mutations in the 397- to 496-aa region of the CMV 1a protein also may lead to necrosis induction by weakening its membrane affinity and in this way exposing normally blocked surfaces of the molecule to the interacting host factor or factors.

Although our results showed the crucial importance of position 461 in the 1a protein in host response determination, experiments with viruses carrying the R461C-mutant Trk7 1a protein may shed light on other factors required for the R461C mutation-triggered necrosis induction. These additional factors are likely subgroup I-specific intramolecular interactions, which are not present in the mutated subgroup II 1a protein. The lack of such interactions could explain why the R461C mutation is ineffective in the case of the Trk7 strain.

MATERIALS AND METHODS

Viruses and characterization of their pathology.

Rs-CMV, a subgroup I strain, was isolated from *R. sativus* and kindly provided by Pál Salamon (P. Salamon, unpublished data). Host range and pathology of the Ns-CMV, a derivative of Fulton's necrotic strain (Fulton 1953), has been characterized previously (Salamon et al. 1998). Test plants were kept in environmentally controlled growth chambers at cycle of 14 h of light (23°C) and 10 h of dark (18°C). The plants were mechanically inoculated at the fourth-leaf stage (*N. glutinosa* and *N. tabacum* cv. Xanthi-nc) or at 3 weeks old (*N. clevelandii*) with in vitro RNA transcripts from cDNA clones. Symptoms were observed at 10 dpi. For the demonstration of the symptoms of the Ns and Rs strains (Fig. 1), virions at a concentration of 10 μ g/ml were used as inoculum. Viruses were propagated on *N. benthamiana* and purified (Lot et al. 1972) prior to inoculation. In each case, the indicated data represent the result of at least three independent experiments.

Generation and sequencing of the cDNA clones of viral genomes.

First-strand cDNAs were synthesized using M-MuLV reverse transcriptase (MBI Fermentas, Vilnius, Lithuania), applying the protocol provided by the manufacturer. Oligonucleotide (5'g~~cg~~g~~at~~c~~tc~~g~~gt~~c~~tc~~tt3') complementary to the 3' conserved sequence of the viral RNAs, containing a flanking *Bam*HI recognition site (underlined), was used to initiate the synthesis. For subsequent polymerase chain reaction (PCR) amplification of cDNA1 and cDNA2, the same 3' primer and a 5' primer (5'g~~gg~~g~~gg~~c~~cc~~t~~a~~a~~t~~a~~c~~g~~a~~c~~t~~a~~c~~t~~a~~g~~t~~t~~t~~a~~t~~t~~a~~c~~a~~g~~a~~g~~c~~g3'), containing a flanking *Ap*aI recognition site (underlined) and a T7 RNA polymerase promoter sequence (italics), were used. For the amplification of the cDNA3, the 5' oligonucleotide (5'g~~gc~~t~~gc~~a~~g~~t~~a~~c~~a~~c~~t~~a~~g~~t~~a~~g~~t~~a~~t~~c~~t~~t~~a~~c~~c~~a~~c~~3') contained a *Pst*I recognition site. After the PCR, the product was digested with *Ap*aI/*Bam*HI or *Pst*I/*Bam*HI, and ligated into similarly treated pBluescriptII KS+ (Stratagene, La Jolla, CA, U.S.A.) vector, resulting in the pN1, pN2, and pN3, and pR1, pR2, and pR3 clones, respectively. After assuring the infectivity of RNA 1-2-3 sets synthesized in vitro from the appropriate cDNAs, nucleotide sequences of the cDNA clones were determined using automated fluorescent dideoxy-sequencing. cDNA clones of the Trk7-CMV (pTrk1, pTrk2, and pTrk3) have been described previously (Salánki et al. 1997; Szilassy et al. 1999b).

Table 2. Oligonucleotide primers used for site-directed mutagenesis of cDNAs^a

Mutant	Forward oligonucleotide primer	Reverse oligonucleotide primer	Endonuclease recognition site
prL456F	<u>tccggcgcgctgcgtg</u>	<u>tccggcgcgagtAAAcgcg</u>	<i>Xma</i> III
prR461C	<u>tccggcgcgctgTGTgtg</u>	<u>tccggcgcgagtcaacgcg</u>	<i>Xma</i> III
prV462Q	<u>tccggcgcgctgcgtCAGtttttc</u>	<u>tccggcgcgagtcaacgcg</u>	<i>Xma</i> III
prV467A	<u>gtcTGAgttcacgcactatcc</u>	<u>ggcTGCagaggagaaaaacacac</u>	<i>Pst</i> I
prH469R	<u>cCGAgtctattcccgactttg</u>	<u>gggagcTCGaacaacactggag</u>	<i>Sac</i> I
pnC461R	<u>ggcttaAGGcagttttctctag</u>	<u>agCTtaaggacagagtaaatgc</u>	<i>Afl</i> III
pnΔ461	<u>tacggcgcgctg.cagtttttc</u>	<u>tccggcgcgagttaaatgctgc</u>	<i>Xma</i> III
prR461C	<u>tccggcgcgttcTGTgttttc</u>	<u>tccggcgcgagacatagacgc</u>	<i>Xma</i> III

^a Oligonucleotides are written in 5' to 3' direction. Embedded endonuclease recognition sites are underlined. Mutagenized codons are capitalized.

Infectious transcripts and generation of chimeric and mutant RNA1 constructs.

Infectious transcripts from full-length cDNA clones were synthesized according to the previously described protocol (Szilassy et al. 1999b).

The chimeric RNAs were constructed by using the common restriction enzyme recognition sites indicated in Figure 2A. The cloning steps were carried out according to standard laboratory procedures (Sambrook et al. 1989). Prior to PCR-based site-directed mutagenesis of the pN1, pR1, and pTrk1 clones, the *Xma*III site in the multicloning region was removed by *Xma*III linearization, T4 DNA polymerase digestion, and recircularization. Letters n, r, and t have been assigned to the name of the mutant clones based on pN1Δ*Xma*, pR1Δ*Xma*, and pTrk1Δ*Xma* templates, respectively. Oligonucleotide primers intended to insert point mutations were designed with the SilMut software package (available online from IUBio Software Archive). All oligonucleotides contained a restriction enzyme site to facilitate the circularization of the PCR product (Table 2). Templates were amplified with *Pfu* polymerase to minimize the chance of nonintentionally introduced mutations. PCR products were circularized after cutting with the endonucleases whose recognition sites have been included in the primers as silent mutations. Nucleotide sequences of the mutant cDNA1 clones were verified with automated dideoxy-sequencing. The M-fold web site (Zuker 2003) was used for the prediction of secondary RNA structures. For each calculation, the offered default parameters were accepted.

Preparation and transfection of protoplasts.

Protoplasts were isolated from fully expanded leaves of *N. clevelandii* plants and purified in K3 medium containing 0.4 M sucrose as described by Nagy and Maliga (1976). Protoplast transfection with 2 μg of purified viral RNA or in vitro RNA transcripts was carried out as described by Kroner and Ahlquist (1992). Briefly, 100 μl of a protoplast suspension of 2.5×10^6 protoplasts/ml was treated for 5 s with 200 μl of 40% polyethylene glycol (molecular weight approximately 1500) and 3 mM CaCl₂ containing 2 μg of RNA. After dilution and washing, protoplasts were incubated in 1 ml of K3 and 0.4 M glucose for 24 h in a growth chamber at a cycle of 14 h of light (23°C) and 10 h of dark (18°C). Nucleotide sequences of the purified viral RNAs were verified by reverse-transcriptase PCR followed by automated fluorescent dideoxy-sequencing prior to transfection. Protoplasts were centrifuged at 65 × g for 5 min. Supernatant was discarded, and the pellet was processed using the extraction solution and protocol described by White and Kaper (1989). Approximately 5 μg of total nucleic was denatured with formaldehyde and formamide, electrophoresed in formaldehyde-containing agarose gel, and blotted onto nylon membrane (Sambrook et al. 1989). After crosslinking, the membrane was hybridized with ³²P-labeled, random-primed probe synthesized from the *Hind*III-BamHI fragment (nucleotides 1,563 to 2,216) of the pN3 clone. Radioactivity was visualized using a storage phosphor screen and matching scanner (Molecular Dynamics,

Sunnyvale, CA, U.S.A.). The protoplast analyses were carried out in triplicates.

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