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Stunting Induced by Cucumber Mosaic Cucumovirus-Infected *Nicotiana glutinosa* Is Determined by a Single Amino Acid Residue in the Coat Protein

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R-CMV, a subgroup II strain of cucumber mosaic cucumovirus (CMV) induces a very strong stunting response in *Nicotiana glutinosa* plants, while Trk7-CMV causes green mosaic in this host. The genetic determinant of this phenotype was mapped to a 534-nucleotide region at the 3′ end of RNA3 with biologically active, full-length cDNA clones of R-CMV and Trk7-CMV and RNA3 chimeras of the two strains. Within this region, R-CMV differs from Trk7-CMV by a single amino acid at position 193 in the coat protein. Changing the codon for Lys at this position to Asn or Ser, by site-directed mutagenesis, also changed the phenotype of the viruses from green mosaic to induction of stunting. Profound differences in both the spread and the accumulation of the viruses causing stunting and green mosaic were observed, although these did not correlate with the host specificity of stunting. Since expression of R-CMV coat protein with the PVX vector did not cause stunting, the data suggest that the presence of other CMV components is necessary for the induction of this symptom.

In viral infections, plants are not passive participants that only endure the invasion of the viruses; they also respond to the infections. This response involves specific defense mechanisms, such as the hypersensitive reaction (HR) or gene silencing, by which plants attempt to avoid disease or moderate disease symptoms (for review see White and Antoniw 1991; Jones and Dangl 1996; Ratcliff et al. 1997). On the other hand, viruses have evolved to overcome these mechanisms (Brigneti et al. 1998; Kasschau and Carrington 1998). Thus, disease symptoms are the results of complex interactions between the pathogens and the hosts (Goodman et al. 1986; Matthews 1991; Takahashi and Ehara 1993).

Cucumber mosaic cucumovirus (CMV) is a prevalent plant pathogen all over the world, and has an extremely large host range of over 800 plant species (for review see Palukaitis et al. 1992). The CMV genome consists of three genomic RNAs. RNA1 and 2 contain the genes *1a* and *2a,* respectively, which are primarily responsible for virus replication. Gene *2b* is located in RNA2 and has been correlated with viral long-distance movement in *Nicotiana glutinosa* and cucumber (Ding et al. 1995b), with host specificity (Ding et al. 1996; Li et al. 1999), and with suppression of gene silencing (Brigneti et al. 1998). RNA3 contains two genes, *3a* and *3b,* the latter being translated from the subgenomic RNA4. The product of the *3a* gene is necessary for cell-to-cell movement, and gene *3b* encodes the viral coat protein (CP) (Davies and Symons 1988; Ding et al. 1995a; Kaplan et al. 1995; Gould and Symons 1978).

CMV strains induce a variety of symptoms on different hosts (Palukaitis et al. 1992; Brunt et al. 1996). Genetic determinants of characteristic symptoms have been identified in a few cases. Zhang et al. (1994) analyzed the difference in local and systemic symptom induction by subgroup I and subgroup II strains. Infection of tobacco plants with a subgroup I strain, Fny-CMV, caused severe systemic mosaic, while infection with a subgroup II strain, LS-CMV, resulted in a systemic mottle. They found this phenotypic difference to be dependent on both RNAs 1 and 2. Another pathological feature specific to subgroup II is necrotic etching on inoculated tobacco leaves. The 5′ 618 nucleotides of LS-CMV RNA3 were shown to determine this symptom (Zhang et al. 1994). In another study, by Kim and Palukaitis (1997), the roles of amino acid residues 631 and 641 of the 2a protein in necrosis induction on cowpea were demonstrated. The product of the *2b* gene has also been correlated with characteristic symptoms such as stunting of *N. glutinosa* (Ding et al. 1995b), and HR in tobacco (Li et al.1999). The latter phenotype was shown to depend on amino acid residues 21 and 28 of the 2b protein (Li et al. 1999).

Precise localization of CMV symptom determinants has demonstrated the crucial role of amino acid residue 129 in the CP of subgroup I strains. Chlorosis induction by Y-CMV and M-CMV versus green mosaic produced by Fny-CMV and O-CMV as well as veinal necrosis or necrotic lesions induced by artificial mutants were found to be determined by the nature of the amino acid residue at position 129 (Shintaku et al. 1992; Suzuki et al. 1995). Based on the predicted structure of the CP, the authors concluded that the conformation of the CP is affected by changes at amino acid position 129, which could lead to alteration in other physical or biochemical properties.

Strains belonging to subgroup II of CMV generally cause green mosaic symptoms in *Nicotiana* species. However, R-CMV induces an unusual stunting symptom on *N. glutinosa* plants. In this study, we demonstrate that the amino acid residue 193 in the CP of R-CMV is responsible for induction of stunting. We analyzed the kinetics of CMV infection as well as the accumulation of the virus in different parts of the plant

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and studied the effect of ectopic expression of CMV CP genes on symptom induction in *N. glutinosa* plants with a PVX vector system.

RESULTS

Characterization of the stunting symptom expression.

Although strains belonging to subgroup II of CMV generally only cause mild green mosaic symptoms in *Nicotiana* species, R-CMV-infected *N. glutinosa* plants display a severe stunting and leaf distortion phenotype (Fig. 1). On the other hand, Trk7-CMV, another subgroup II strain, causes a typical green mosaic (Fig. 1). Despite the substantial symptom difference between the two strains on *N. glutinosa,* we found no difference between them when the following hosts were infected: *N. tabacum, N. benthamiana, Capsicum annuum, Cucumis sativus,* and *Lycopersicon esculentum.* Cell sizes in *N. glutinosa* leaves displaying the stunting symptom were found to be normal in toluidine-blue-stained, 1-μm sections, suggesting that interference with cell division rather than cell elongation is involved in the development of stunting (data not shown).

The characteristic phenotype of R-CMV was stably maintained during five passages in *N. glutinosa.* When coinfection of *N. glutinosa* plants was performed in such away that two opposite leaves were inoculated with either R-CMV or Trk7-CMV, the whole plant developed the typical stunting symptoms corresponding to R-CMV. We did not ascertain whether Trk7-CMV replicated in the systemic leaves of these plants. On the other hand, cross protection was observed when systemic leaves of Trk7-CMV-infected plants were reinoculated with R-CMV 10 days post inoculation (dpi) (data not shown).

Delimitation of induction of stunting to sequences containing the *3b* gene.

Infectious transcripts synthesized from full-length cDNA clones of R-CMV and Trk7-CMV were used to map the determinant of the stunting symptom in the R-CMV genome. Transcripts of R-CMV RNA1 and RNA2 were mixed with those of Trk7-CMV RNA3 (R12T3). The other reassortant constructed contained a combination of transcripts derived from cDNAs of Trk7-CMV RNA1 and 2 (T1 and T2) and of R-CMV RNA3 (T12R3). *N. glutinosa* plants infected with R12T3 developed mild green mosaic symptoms indistinguishable from those caused by the wild-type Trk7 virions. Conversely, infection with T12R3 resulted in a stunting characteristic of R-CMV infection. Thus, the genetic determinant of the stunting symptom expression on *N. glutinosa* is exclusively associated with RNA3 in the viral genome. This was in accordance with previous results obtained with chimeric viruses between R-CMV and P-TAV (Salánki et al. 1997).

In order to distinguish between the 5′ and the 3′ halves containing *3a* and the *3b* genes, respectively, for symptom induction two constructs were made by exchanging these fragments between the RNA3 cDNA clones of R-CMV (R3) and Trk7-CMV (T3). Infection with transcripts of the resulting chimeras named TR-ApaI and RT-ApaI in combination with those from T1 and T2 showed that the 3′ half of the RNA3 molecule containing the *3b* gene has a distinctive role in the determination of the stunting phenotype (Fig. 2).

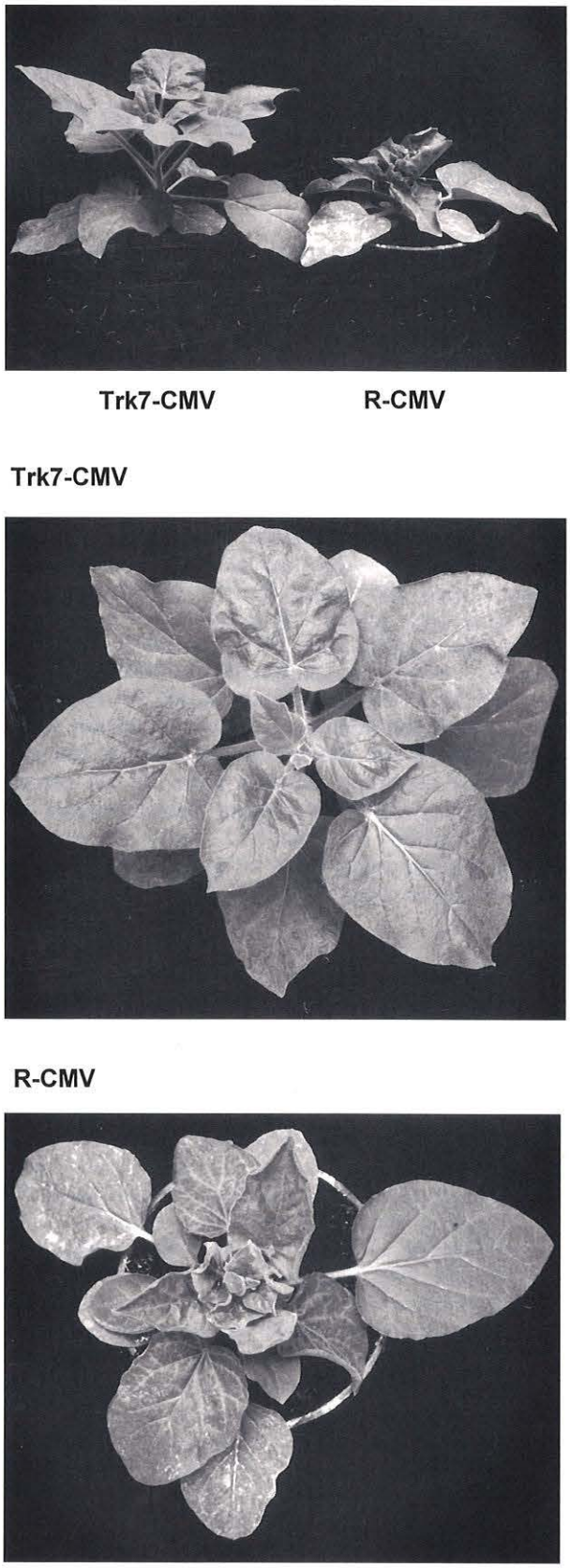

Fig. 1. Symptoms induced on *Nicotiana glutinosa* plants by Trk7-CMV and R-CMV.

Localization of the viral sequences responsible for the stunting symptom.

A series of chimeras with common restriction enzyme sites of R-CMV and Trk7-CMV were constructed to delimit the viral sequences that induce the severe host response on *N. glutinosa* (Fig. 2). Transcripts of the chimeras were mixed with those of T1 and T2 prior to inoculation. We presumed that the determinants were expressed at the amino acid rather than at the RNA level; therefore, chimeras were constructed according to the differences in the amino acid composition of the CPs. As shown in Figure 2, there were six amino acid differences between the respective CPs. Chimera RTR-Hind, which contained the N-terminal, most proximal two of the six differing amino acid residues from Trk7-CMV, caused stunting symptoms on the infected *N.*

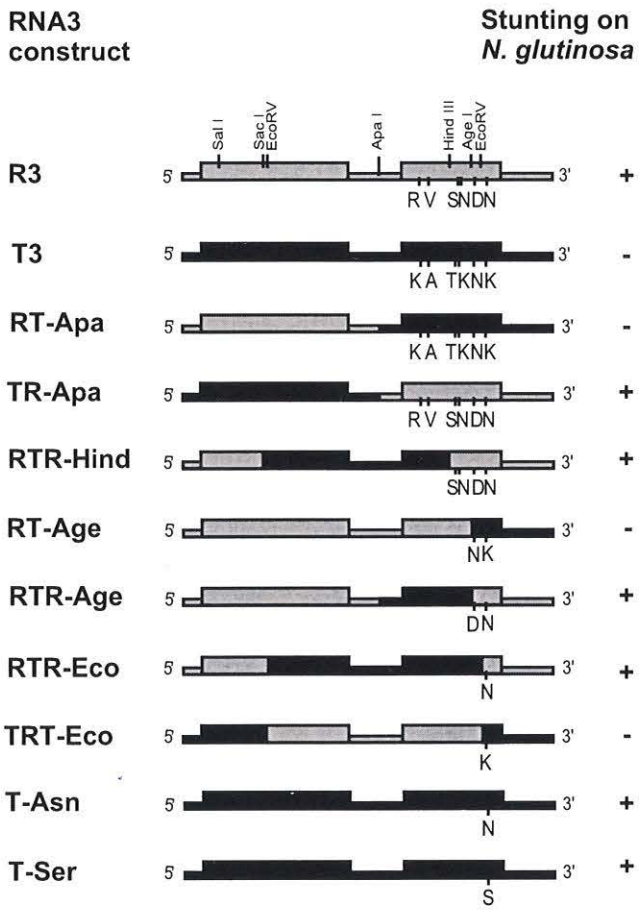

Fig. 2. Schematic diagram of RNA3 of R-CMV (R3), Trk7-CMV (T3), various chimeric R/Trk7 constructs, and mutant clones (T-Asn; T-Ser), as well as their phenotypes with respect to stunting induction. Gray and black indicate R-CMV and Trk7-CMV origin of the genomic fragments, respectively. Movement protein (*3a*) and coat protein (CP) (*3b*) genes are left and right rectangles, respectively. RNA transcripts of each construct were coinoculated with cDNA-derived transcripts of Trk7-CMV RNAs 1 and 2. Restriction enzyme sites used to construct chimeras are indicated. Amino acid residues of the CPs in which the strains differ are shown below the genes of R3 and T3. For the other constructs, only residues of interest are indicated. Construct T-Asn was created by site-directed mutagenesis of the cDNA clone T3 to replace an A by a T at nucleotide 1810. For T-Ser, the A residues at positions 1808 and 1809 were replaced by T and C. These mutations led to amino acid substitutions of Lys (K) for Asn (N) and Ser (S) in T-Asn and T-Ser, respectively.

glutinosa plants. This suggested that involvement of the sequences upstream of the *Hin*dIII site in this phenotype could be ruled out. Infection obtained with chimeras RT-AgeI and RTR-AgeI clearly showed that the symptom determinant was located in the region downstream of the *Age*I site (Fig. 2). The CP sequences of the stunting-inducing R-CMV strain and the green mosaic-inducing Trk7-CMV strain differed by only two amino acid residues in this region. R-CMV contained an aspartic acid (D) residue at position 169 and an asparagine (N) residue at position 193, while Trk7-CMV contained asparagine (N) (169) and lysine (K) (193) at the respective sites. To establish whether one or both residues were involved in symptom induction, two additional chimeras were constructed. Infection with the chimera RTR-Eco triggered severe stunting of the *N. glutinosa* plants, while plants inoculated with TRT-Eco plants displayed green mosaic symptoms characteristic of Trk7-CMV (Fig. 2). At the RNA level, there were nine nucleotide differences between the two clones, one of which resulted in a change of amino acid residue 193. To confirm that asparagine 193 or the RNA sequence specifying this amino acid is the determinant of the stunting symptom, we generated a mutant form of T3. In this mutant, named T-Asn, the lysine residue at position 193 was replaced with an asparagine (Fig. 2). Thus, the entire sequence of this clone was identical to that of T3, except for nucleotide 1810. Since the nucleotide triplet introduced in this mutant to encode asparagine was different from that found in R3, it enabled us to differentiate whether symptom expression was mediated at the RNA or at the protein level. Infection of *N. glutinosa* plants with transcripts of T-Asn induced a stunting symptom indistinguishable from those produced by R-CMV infection (not shown). Therefore, we concluded that asparagine 193 in the CP is the exclusive determinant of the severe stunting symptom on this plant species.

Another mutant, T-Ser, was also generated from T3. In this clone, the lysine at position 193 was substituted by serine (Fig. 2). Infection with transcripts of this clone resulted in stunting of *N. glutinosa* plants, although to a slightly lesser extent than did T-Asn (not shown). Thus, the nature of the amino acid at position 193 plays a crucial role in the induction of symptoms in this host.

Analysis of the kinetics of infection and the yield of the viruses.

The kinetics of infection of R-CMV and Trk7-CMV in *N. glutinosa* were compared. Systemic symptoms appeared at the same time in both cases, and usually the first leaf above the inoculated leaves remained symptomless. Therefore, no apparent difference could be observed in the long-distance movement of the strains.

To determine whether there was any difference in cell-to-cell movement between the viruses, we hybridized press blots of the inoculated leaves collected at 1-day intervals with virus-specific probes. The leaves were inoculated with virus of similar biological activity as determined by counting the lesions induced on *Chenopodium amaranticolor* and *C. quinoa* (not shown). As seen in Figure 3, by 2 dpi, detectable areas of infection were substantially larger in the case of TR-Apa, a chimera causing stunting, than in the case of RT-Apa, a chimera causing green mosaic.

The appearance of the viruses in the petioles of the inoculated leaves also was investigated. Six petioles of inoculated leaves were extracted at 1-day intervals and analyzed for the presence of the virus CP by Western blotting (immunoblotting). The presence of the viruses was first detected at 4 dpi in both cases and they could be detected in all of the samples by 5 dpi (Fig. 4). Therefore, no significant difference was found in the appearance of the chimeras in the phloem. On the other hand, TR-Apa accumulated within the petioles to higher levels in most of the samples than did RT-Apa (Fig. 4).

The levels of the viral RNA and the CP in the systemic leaves were also analyzed. The amounts of both viral RNA (Fig. 5A) and CP (Fig. 5B) were significantly higher in the TR-Apa-infected plants than in the RT-Apa-infected ones.

As mentioned above, induction of stunting was found to be specific to *N. glutinosa.* To ascertain whether the observed difference between the chimeras in the rate of spread and virus accumulation was specific to *N. glutinosa,* we did the same experiments on *N. tabacum* cv. Xanthi. Both of the chimeras TR-Apa and RT-Apa caused green mosaic symptoms in this host. Surprisingly, exactly the same differences in the kinetics and accumulation of the chimeras were observed (not shown). Hence, the stunting response by *N. glutinosa* against infection with TR-Apa may not simply be attributed to the enhanced spread within the inoculated leaves or the higher accumulation level in the petioles and in the systemic leaves.

Expression of the CMV CP in the plants with a PVX vector.

As reported above, the differences in the kinetics of infection or the accumulation did not exclusively account for the distinct symptom induction of the chimeras. Nonetheless, because of the host specificity, we speculated that an interaction between the CP and a host factor was perhaps involved in the stunting induction process. Therefore, we introduced the CPs of the two strains of CMV, either inducing or not inducing stunting, to *N. glutinosa* plants with a PVX vector system. Expression of the CMV CPs in the systemic leaves of these plants was confirmed by immunoblot analysis (Fig. 6B). As seen in Figure 6A, *N. glutinosa* plants infected with transcripts of PVX-RCP showed no stunting phenotype, and no difference between PVX-RCP and PVX-TCP could be observed in the induction of symptoms. This result suggested that, apart from the CP, the presence of other CMV components is also necessary for the induction of stunting.

DISCUSSION

The genetic determinant in the R-CMV genome for the induction of an unusually strong symptom response of *N. glutinosa* was localized to amino acid residue asparagine 193 in the CP. Comparison of the amino acid compositions of CMV subgroup II isolates reveals that only R-CMV and Kin-CMV contain asparagine in this position, while it is substituted by a

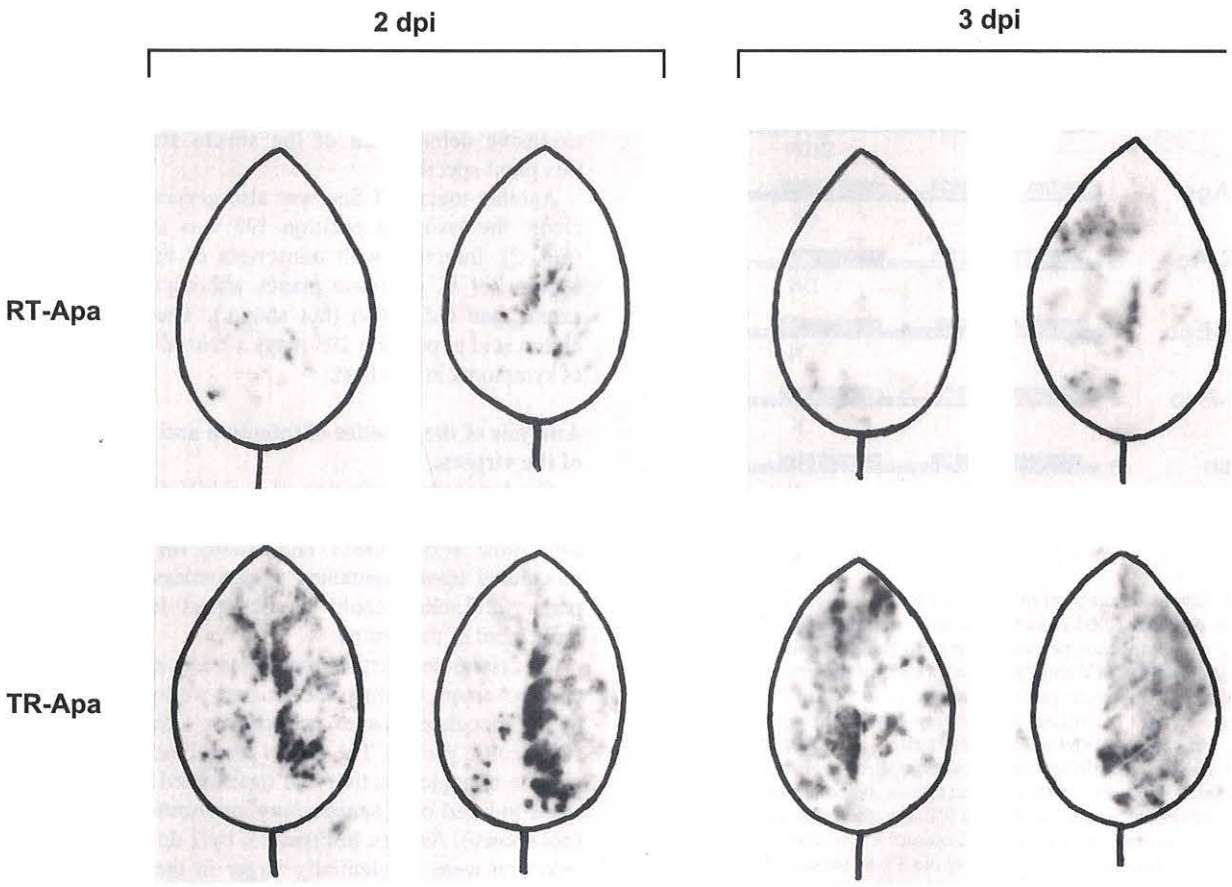

Fig. 3. Spread of cucumber mosaic cucumovirus (CMV) infections in *Nicotiana glutinosa* leaves. Press blots of the leaves inoculated with either RT-Apa or TR-Apa (displayed in Figure 2) were hybridized with radiolabeled, virus-specific probes 2 and 3 days post inoculation (dpi).

lysine in all of the other strains (Fig. 7). Based on the predicted structures of the cucumovirus CPs (Wikoff et al. 1997), it is probable that this residue is located in a region flanked by two β sheet structures (Fig. 7). An amino acid change at this location has two consequences: first, exchanging the lysine residue for asparagine at this site probably does not affect the overall structure of the CP and hence virion stability. Second, asparagine 193 of R-CMV CP may be located on the surface and thus could directly interact with other molecules. By comparison, substitutions at amino acid position 129 in the CPs of subgroup I strains were shown to have indirect effects. Numerous mutations introduced at this site revealed that the local secondary structure surrounding residue 129 rather than a particular amino acid per se induced differences in symptom development and virion stability (Shintaku et al. 1992; Suzuki et al. 1995). This was further confirmed by the demonstration that leucine 129 determined chlorosis and necrosis together with regions outside the surrounding 53 amino acids (Suzuki et al. 1995). In our system, both of the mutant constructs T-Asn and T-Ser induced stunting. This fact shows that the nature of amino acid residue 193 exclusively determines symptom severity when R-CMV and Trk7-CMV are compared. However, this is obviously not the cause of the difference between Trk7-CMV-induced and Q-CMV-induced symptoms. In contrast to Trk7-CMV, Q-CMV induces stunting in *N. glutinosa,* and this response was shown to depend on the *2b* gene (Ding et al. 1995b). Both Trk7-CMV and Q-CMV contain a lysine at position 193 in the CPs (Fig. 7). Thus, similar host responses can be triggered by different viral determinants.

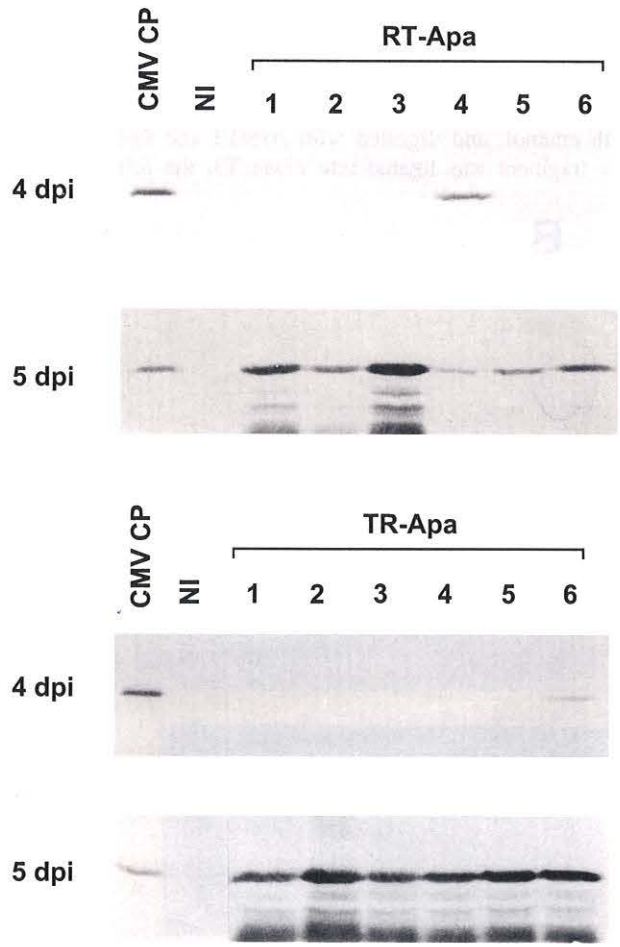

Fig. 4. Appearance of cucumber mosaic cucumovirus (CMV) in the petioles of inoculated leaves. Six petioles of leaves inoculated with either RT-Apa or TR-Apa (displayed in Figure 2) were extracted 4 and 5 days following inoculation. CMV was detected by immunoblot following polyacrylamide gel electrophoresis of total protein extracts. CMV CP: 80 ng of purified CMV CP. NI: protein extract from non-inoculated leaves.

A number of viral and plant genes are thought to be involved in symptom development (Goodman et al. 1986; Matthews 1991), and this fact is also suggested by the common characteristics of symptoms triggered by a wide range of host-virus combinations. The host-specific nature of stunting induction in *N. glutinosa* by R-CMV offers the possibility of mapping the corresponding gene(s) in the plant genome with the help of genetic crosses between *N. glutinosa* and *N. tabacum*. Analyses of plant genomes have been performed to find the host counterparts of CMV symptom determinants. In one study, severe chlorotic spot symptom formation was found to be induced by a combination of the *3b* gene of Y-CMV and two recessive host genes that were closely mapped to the *yb* genes of *N. tabacum* cv. Ky57 (Takahashi and Ehara 1993). In another study, a cowpea line was described in which the CMV localization response was mapped to a single, dominant host gene (Nasu et al. 1996).

Our results suggest that a complex interaction may occur between the CMV CP and other CMV as well as host components, since the CP alone did not induce stunting of *N. glutinosa* plants when introduced with the PVX vector system. Stunting induction by R-CMV is therefore different from that driven by the P6 protein (gene VI) of cauliflower mosaic caulimovirus (CaMV) or by the BC1 protein of tomato mottle geminivirus (Cecchini et al. 1997; Duan et al. 1997). In those cases, numerous transgenic plant lines expressing the respective proteins exhibited stunting phenotypes characteristic of virus infection. Based on the role of the CMV CP in the cell-to-cell movement of the virus (Boccard and Baulcombe 1993; Canto et al. 1997), it is possible that the R-CMV CP is able to deliver the virus into the meristemic cells of *N. glutinosa,* which constitute a separate plasmodesmatal domain, which in turn interferes with organogenesis.

Differences between chimeras TR-Apa and RT-Apa in the kinetics of local infection and virus accumulation were observed in both *N. glutinosa* and *N. tabacum* cv. Xanthi. Nevertheless, the possible involvement of these differences in the induction of stunting of *N. glutinosa* cannot be ruled out. Similar to our observations in *N. tabacum* cv. Xanthi, there was no correlation between symptom severity and the accumulation of the viruses in the cases of infections by a caulimovirus (Anderson et al. 1991) and other CMV strains (Zhang et al. 1994). In contrast, extreme disease severity induced by I17F-CMV in solanaceous hosts was correlated with high virus accumulation (Carrere et al. 1999). Similarly, the symptom character was dependent on the expression level of the CaMV P6 protein in transgenic lines (Cecchini et al. 1997). The consistently higher amount of TR-Apa in the petioles of the inoculated *N. glutinosa* leaves and in the systemic leaves may reflect a function of the CMV CP in virus accumulation or possibly virion stability. Mutations in the CP of Kin-CMV have been shown to reduce CMV RNA accumulation (Boccard and Baulcombe 1993), and such a role for the CP

has been suggested in the cases of alfalfa mosaic alfamovirus and brome mosaic bromovirus (Marsh et al. 1991; reviewed by Bol 1999; Jaspars 1999).

This is the first report on detailed localization of the genetic determinant of a severe stunting symptom caused by a CMV strain. Such symptoms have been extensively characterized in geminiviruses and caulimoviruses (Stratford and Covey 1989; Rochester et al 1990; Anderson et al. 1991; Stanley and Latham 1992; Zijlstra et al. 1996; Cecchini et al. 1997; Duan et al. 1997). Understanding the interactions of viral and host components should facilitate the development of strategies to avoid economic losses caused by severe disease symptoms.

MATERIALS AND METHODS

Viruses and characterization of their pathology.

R-CMV (Jaquemond and Lot 1981) and Trk7-CMV (Salánki et al. 1994) have previously been described. Test plants were inoculated at the two-leaf (*Nicotiana glutinosa*, *N. tabacum* cv. Xanthi, *Capsicum annuum* and *Lycopersicon esculentum*) or the four-leaf (*N. benthamiana* Domin.) stage. Inoculated plants were maintained in environmentally controlled growth chambers at 20 to 25°C with a 14-h light/10-h dark cycle. The inocula were either purified virus at 20 μg/ml or RNA transcripts from cDNA clones. Chimeric viruses were propagated on tobacco and the virions purified according to Lot et al. (1972) prior to testing for symptom response and accumulation in *N. glutinosa*.

Infectious transcripts and generation of chimeric and mutant constructs.

Infectious transcripts from full-length cDNA clones of R-CMV and Trk7-CMV have been described previously (Salánki et al. 1997; Szilassy et al. 1999). Chimeric constructs were created by standard procedures (Sambrook et al. 1989) with the common restriction enzyme sites indicated in Figure 2 as well as the unique *Pst*I and *Bam*HI sites downstream of the 3′ end of clones R3 and T3, respectively. Polymerase chain reaction (PCR)-based, site-directed mutagenesis (Higuchi et al. 1988) was applied for the generation of mutant constructs T-Asn and T-Ser. Mutagenic oligonucleotides complementary to the viral sequence were used to change the A residue at position 1810 to T in the case of T-Asn (5′ GGAATTC GTTGATGCTCGACGTCGACATGAAGTACAATCTCGTC CTTCTCTAGATTATCG 3′) and the AA residues at positions 1808 to 1809 to TC in the case of T-Ser (5′ GGAATTCGTTG ATGCTCGACGTCGACATGAAGTACAATCTCGTCCTTC TCTAGGAATCG 3′). Oligonucleotide Trk4 (5′ CGTTTAGT TGTTTACC 3′) corresponding to a region of T3 from nucleotide 1168 to 1183 was used in the PCR. Thirty-five cycles of amplification were performed with T3 as the template, with the following parameters: denaturation at 94°C for 20 s, annealing at 45°C for 30 s, and synthesis at 72°C for 1 min. PCRs were performed in 50-μl volumes with the buffers supplied by the manufacturer (Promega, Madison, WI). The PCR products were extracted with phenol-chloroform, precipitated with ethanol, and digested with *Hin*dIII and *Eco*RI. Before this fragment was ligated into clone T3, the following sub-

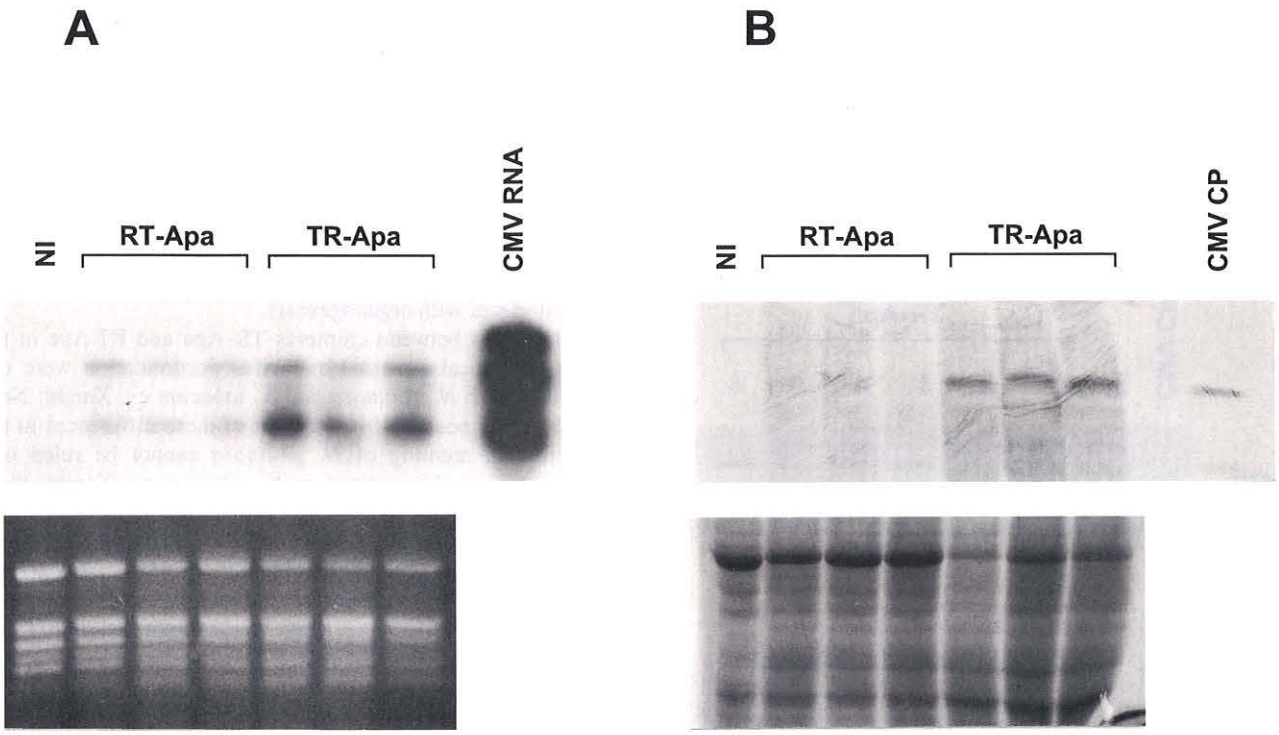

Fig. 5. Accumulation of the cucumber mosaic cucumovirus (CMV) RNAs and coat protein (CP) in the systemic leaves of *Nicotiana glutinosa* plants. **A,** Northern (RNA) blot analysis of the CMV RNAs. Equivalent amount (2 μg) of total RNA extracts from the systemic leaves was hybridized with a probe specific to the 3′ end of CMV RNA3. NI: RNA from noninoculated plant. CMV RNA: 70 ng of purified CMV RNA. Photograph of the ethidium bromide-stained gel under UV light prior to blotting is shown below the autoradiograph. **B,** Immunoblot analysis of the CMV CP. Equivalent amounts of total protein extracts were separated on sodium dodecyl sulfate-polyacrylamide gel, blotted onto Hybond C (Amersham, Uppsala, Sweden) membrane, and probed with CMV antibody. NI: protein from noninfected plant. CMV CP: 80 ng of purified CMV CP. The same amounts of total protein extracts were electrophoresed in the Coomassie blue-stained gel shown below the immunoblot.

clone had to be made because of the presence of an *Eco*RI site upstream of the 5′ end of the clone. Therefore, the *Apa*I-*Pst*I fragment representing the 3′ half of the clone T3 from nucleotides 1127 to 2209 was ligated into *Apa*I/*Pst*I-digested pBluescript KS+ (Stratagene, La Jolla, CA), producing the clone pAP. The *Hin*dIII-*Eco*RI fragment of pAP was replaced by the respective fragment of the above mutant PCR products, yielding clones pAP-Asn and pAP-Ser. The entire PCR-amplified fragments were sequenced and the presence of the expected mutations around nucleotide 1810 was verified in both cases. Sequencing was carried out according to Sambrook et al. (1989) with a T7 Sequenase version 2.0 sequencing kit (Amersham, Uppsala, Sweden). Mutated pAP-Asn and pAP-Ser clones were chosen, digested with *Apa*I and *Pst*I, and subsequently ligated into *Apa*I/*Pst*I-digested T3, producing clones T-Asn and T-Ser, respectively.

Analysis of the kinetics of infection.

Tobacco plants were coinoculated with transcripts synthesized from cDNA clones T1 and T2, together with transcripts from either RT-Apa or TR-Apa. Virions were purified from the plants in both cases and these virus preparations were used

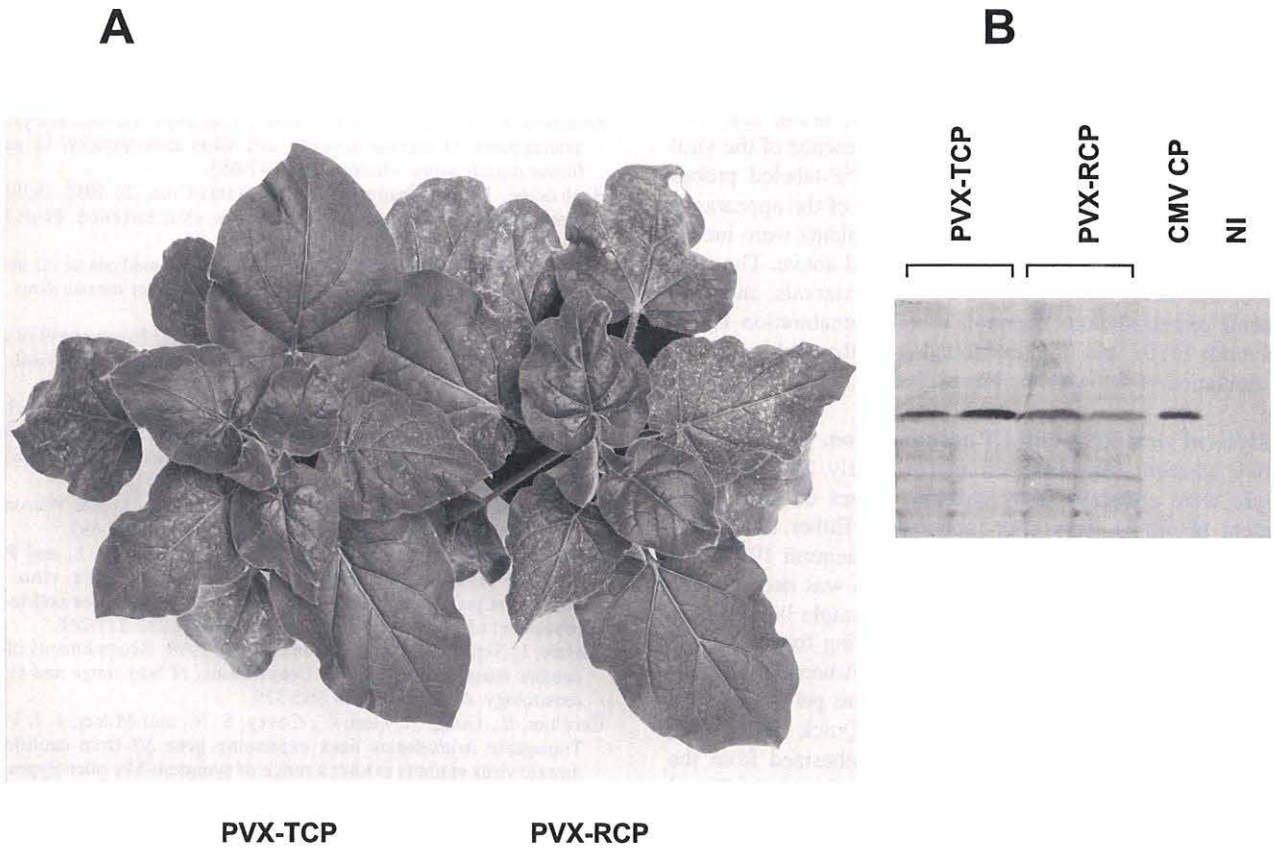

Fig. 6. Analysis of expression of the cucumber mosaic cucumovirus (CMV) coat protein (CP) by the PVX vector in *Nicotiana glutinosa*. **A,** Symptoms induced by the recombinant PVX constructs expressing the CMV CP. **B,** Immunoblot analysis of the CMV CP in the recombinant PVX-infected plants. CMV CP: 80 ng of purified CMV CP. NI: protein from noninfected plants.

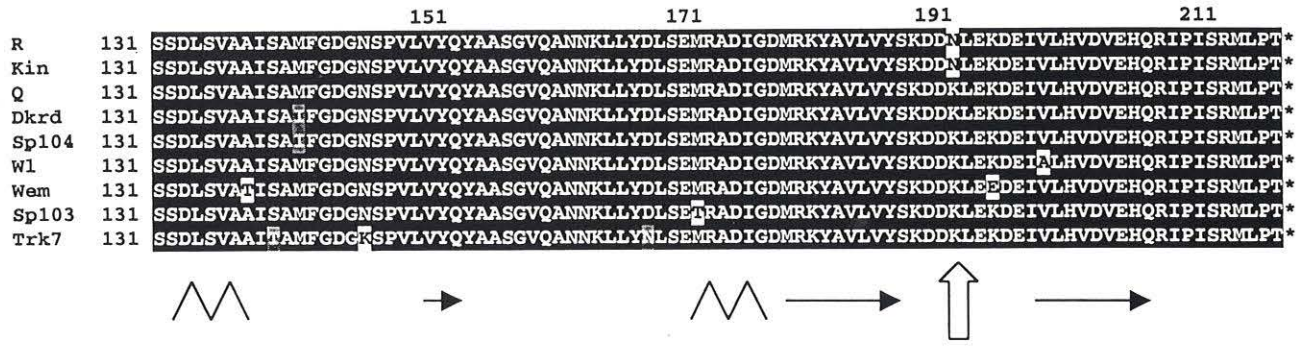

Fig. 7. Alignment of the predicted amino acid sequences of the coat proteins (CPs) of subgroup II cucumber mosaic cucumovirus (CMV) strains from amino acid position 131. The amino acid residue 193 is shown below with open arrow. Locations of the secondary structural elements are indicated under the sequences (Wikoff et al. 1997). Large solid arrow: β sheet; large "*N*": α helix.

as inocula in the analysis of the kinetics of infection. Virions at concentrations of 50 μg/ml were applied, and the infectivities of the inocula were equalized by monitoring lesion induction on *C. amaranticolor* and *C. quinoa*. Two opposite, fully expanded leaves of *N. glutinosa* or *N. tabacum* cv. Xanthi plants were inoculated at the four-leaf stage with either construct. The inoculated leaves were removed at 1-day intervals, and the lower side abraded with cellite, using a brush. After being rinsed in distilled water, the leaves were placed onto Hybond N membrane (Amersham) with the abraded surface downwards. The membrane was floated on W5 solution (125 mM $CaCl_2 \cdot 2H_2O$; 154 mM NaCl; 5.5 mM glucose; 10.7 mM KCl) containing 0.7% cellulase and 0.25% macerozyme, and incubated overnight at room temperature. The next day, the membrane together with the leaves was removed and roller pressed between Whatman papers. The membrane was subsequently rinsed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) to remove leaf remains, dried, and UV-crosslinked (Amersham crosslinker). The presence of the viral RNA was detected with random-primed, ^{32}P-labeled probes synthesized from clone T3. For the analysis of the appearance of the viruses in the petioles, *N. glutinosa* plants were inoculated with RT-Apa and TR-Apa as described above. The petioles of the leaves were removed at 1-day intervals, and total proteins extracted with Laemmli protein denaturation buffer (Laemmli 1970), and immunoblotted as in the analysis of CP accumulation in the systemic leaves (below).

Analysis of viral RNA and CP accumulation.

Two adjacent leaf disks of approximately 200 mg fresh weight were collected from systemic leaves of three independent plants 21 days after inoculation. Either total RNA (White and Kaper 1989) or total protein (Laemmli 1970) was extracted from the disks. Total RNA (2 μg) was denatured in formaldehyde and formamide-containing sample buffer, electrophoresed on a 1.2% agarose gel containing formaldehyde, and blotted onto Hybond N membrane (Amersham) (Sambrook et al. 1989). Northern blot analysis was performed with random-primed, ^{32}P-labeled probes (T7 Quick Prime Kit; Pharmacia Biotech, Uppsala, Sweden) synthesized from the *Nru*I-*Bam*HI fragment of clone T3 corresponding to the 3′ end of the clone. Approximately 2 μg of total protein was electrophoresed on a 12.5% polyacrylamide gel (Laemmli 1970), electroblotted onto Hybond C membrane (Amersham), and probed first with rabbit antibody against CMV and then with anti-rabbit alkaline phosphatase-conjugate (Boehringer, Vienna) according to Sambrook et al. (1989).

Expression of CMV CP with a PVX vector.

A 1,040-nucleotide fragment corresponding to the entire RNA4 was PCR amplified from either clone T3 or clone R3 with oligonucleotide Trk4 described above and the oligonucleotide (5′ CAGGATCCTGGTCTCCTTATGGAG 3′) that is complementary to the 3′ 16 nucleotides of both T3 and R3. Thirty-five cycles were performed with the following parameters: denaturation at 94°C for 20 s, annealing at 50°C for 30 s, and synthesis at 72°C for 1.5 min. The PCR products were ligated into *Eco*RV-linearized cDNA of the PVX vector (Baulcombe et al. 1995), producing constructs PVX-TCP and PVX-RCP carrying the *3b* genes of Trk7-CMV and R-CMV, respectively. These clones were linearized with *Spe*I prior to synthesis of transcripts with T7 RNA polymerase (Promega). *N. glutinosa* plants were inoculated with the transcripts at the two-leaf stage. Expression of the CMV CPs in the systemic leaves was confirmed by immunoblotting 21 days after inoculation as described above.

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