The 2b Protein of Cucumoviruses Has a Role in Promoting the Cell-to-cell Movement of Pseudorecombinant Viruses

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Pseudorecombinant viruses (i.e., those containing a reassorted genome of closely related multipartite viruses) are often not as competitive as the parental viruses. The role of the 2b gene in hypervirulence and maintenance of a progressive infection was assessed in a pseudorecombinant virus formed between RNAs 1 plus 2 of *Cucumber mosaic virus* (CMV) and RNA 3 of *Tomato aspermy virus* (TAV). The presence of RNA 3 of TAV was found to affect the level of RNA accumulation but not the level of virulence. By contrast, the 2b genes of both TAV and a hypervirulent strain of CMV (WAIL-CMV) were found to affect the virulence of the pseudorecombinant viruses but not the levels of viral RNA accumulation. The 2b gene rather than the overlapping open reading frame encoding the C-terminal 41 amino acids of 2a protein of the corresponding virus was found to be essential for promoting infection of the pseudorecombinant viruses in planta. However, the 2b gene was not essential for replication of pseudorecombinant viruses containing CMV RNAs 1 plus 2 and TAV RNA 3. These results indicate that the 2b protein is involved in promoting the cell-to-cell movement of the pseudorecombinant viruses. These data also suggest the existence of specific interaction between the TAV 2b protein and either RNA 3 or its encoded proteins, which may be critical for promoting or maintaining infection or both.

Mixed infections of plants by two or more unrelated viruses are quite common, sometimes leading to synergistic interactions. By contrast, when two viruses are closely related in sequence, there is usually some form of competition between them, often leading to the loss of one of the viruses. Several examples of such virus exclusion have been observed for plants infected by viruses in the genus *Cucumovirus* (Sackey and Francki 1990; Crescenzi et al. 1993; Fraile et al. 1997). Sackey and Francki (1990) observed that mixed infections in several common hosts by the cucumoviruses *Cucumber mosaic virus* (CMV) and *Tomato aspermy virus* (TAV) led to loss of TAV upon passage but almost never loss of CMV. Similarly, altering the initial ratios of the two viruses in the inoculum in favor of TAV still resulted in infection by either CMV alone or both viruses but rarely by

TAV alone. This difference in selective advantage of CMV over TAV also extended to pseudorecombinant viruses (i.e., with reassorted genomes) formed between the three genomic RNAs of CMV and TAV (Sackey and Francki 1990). A pseudorecombinant virus consisting of RNAs 1 and 2 of CMV and RNA 3 of TAV (C1C2T3) was completely unable to compete with CMV in mixed infections. Similarly, the reciprocal pseudorecombinant virus T1T2C3 was unable to out-compete CMV, even when inoculated in a 19-fold excess. The inability of such viruses to maintain a long-term coexistence, either in the initially inoculated plants or upon passage, was concluded to be a consequence of differences in the rates of systemic infection by the virus combinations (Sackey and Francki 1990). If one virus is able to outpace host defense responses more efficiently than the other virus, the former virus may be able to gain a competitive advantage.

The 2b gene of different cucumoviruses has been shown to have a major effect on systemic infection by CMV as well as on the degree of virulence (Ding et al. 1995a, 1996; Ji and Ding 2001; Shi et al. 2002; Soards et al. 2002). These effects are believed to be due to the ability of the 2b protein to inhibit various defense reactions, such as by interference with the salicylic acid-mediated defense response (Ji and Ding 2001) and by suppression of the RNA silencing mechanism (Brigneti et al. 1998; Guo and Ding 2002). The approximately 11- to 13-kDa 2b protein has been localized to the nucleus (Mayers et al. 2000), and mutations affecting its nuclear localization also affected the ability of the 2b protein to suppress RNA silencing (Lucy et al. 2000). The 2b protein also may be considered a determinant of host specificity (Ding et al. 1995a; Li et al. 1999; Shi et al. 2002).

The 2b gene of TAV also has been shown to be a determinant of virulence, when expressed either from CMV in place of its own 2b gene (Ding et al. 1996) or from virus vectors such as *Tobacco mosaic virus* and *Potato virus X* (Li et al. 1999). On the other hand, wild-type TAV itself did not display the hypervirulence associated with the expression of its 2b gene from other viruses. Therefore, such hypervirulence may be associated with some incompatibility between one of the roles of the TAV 2b gene and components of the other viral genomes, including that of CMV. The failure of the CMV-TAV pseudorecombinant viruses to compete effectively with either parental virus (Sackey and Francki 1990) suggests a possible 2b protein function that can be assessed; viz., the role of the 2b protein in maintaining a progressive infection of CMV-TAV pseudorecombinant viruses. Here, we examine this role and confirm that, while the 2b gene itself is not

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essential for movement of CMV in young plants of two *Nicotiana* spp. (Ji and Ding 2001; Soards et al. 2002), the *2b* gene is essential for movement of a pseudorecombinant virus derived from CMV RNAs 1 and 2 and TAV RNA 3.

Fig. 1. Schematic representation of cucumber mosaic virus (CMV) RNA2 mutants. The various plasmids containing either the wild-type 2b open reading frame (ORF) of the Q-strain of CMV (plasmid pQCD2) or variants of the 2b ORF in Q-CMV RNA 2, as shown. The 2b ORF and overlapping C-terminus of the 2a ORF of Q-CMV were replaced with the corresponding sequences from either the WAII-strain of CMV or *Tomato aspermy virus* (TAV) in pQCD2qw and pQCD2qt, respectively. A termination codon was introduced into the sequences encoding the overlapping C-terminus of the TAV 2a protein to prevent the expression of these sequences in pQCD2qt1. Different termination codons were then introduced into pQCD2qt1 to prevent expression of the TAV 2b ORF, creating pQCD2qt2 and pQCD2qt3. The nucleotides introduced to generate termination codons are shown in bold and underlined. Arrows indicate the substituted nucleotides. The stop codon of the 2a ORF and the start and stop codon of the 2b ORF are indicated by nucleotide position numbers. All of the plasmids contained the respective Q-CMV RNA 2 cDNA flanked by cauliflower mosaic virus 35S RNA promoter [P] and terminator [T] sequences.

RESULTS

RNA 3 of TAV affects accumulation levels of CMV RNAs 1 and 2 but not virulence.

The effects of the genes encoded by TAV RNA 3 on the virulence of CMV as well as on the accumulation of viral RNAs were analyzed to explore the potential interactions during infection of the proteins encoded by RNA 3, the 3a protein, and the capsid protein (CP), either alone or in combination, with the proteins encoded by CMV RNAs 1 and 2, the 1a, 2a, and 2b proteins. Thus, the pseudorecombinant cucumovirus designated C1C2T3 was constructed. Here, C and T indicate the Q-strain of CMV and the V-strain of TAV, respectively, and 1, 2, and 3 indicate RNAs 1, 2, and 3, respectively, all derived from infectious cDNA clones (Ding et al. 1995b; Shi et al. 1997a). Initially, the differences were examined in the context of a wild-type Q-CMV RNA 2 background, before assessing the effects of chimeric RNA 2 containing the TAV *2b* gene inserted in Q-CMV RNA 2 (i.e., comparing pQCD2 with pQCD2qt [Fig. 1]).

Bioassay of C1C2C3 (i.e., Q-CMV RNAs 1 through 3) and C1C2T3 on two *Nicotiana* species, *N. clevelandii* and *N. glutinosa*, indicated that both viruses were infectious and induced either no systemic symptoms or similar mild systemic symptoms (Table 1). Analysis of the accumulation levels of these two viruses in *N. glutinosa* showed that they both accumulated, although RNAs 1 and 2 of Q-CMV accumulated to higher levels in plants infected by C1C2T3 than in plants infected by C1C2C3 (Fig. 2A, lane 5 vs. lane 2). On the other hand, the level of accumulation of TAV RNA 3 was similar or slightly lower for C1C2T3 than for T1T2T3 (i.e., V-TAV RNAs 1 through 3 [Fig. 2B, lane 5 vs. lane 3]). Nevertheless, T1T2T3 induced more severe systemic symptoms than either C1C2C3 or C1C2T3 (Table 1 and Fig. 3A). Thus, the increase in accumulation of C1 and C2 in the presence of T3 did not parallel an increase in the severity of symptoms. This is consistent with earlier observations suggesting that the symptom determinants of TAV were associated with either RNA 1 or 2 or both (Habibi and Francki 1974; Salánki et al. 1997).

The *2b* gene affects virulence but not RNA accumulation, independent of the source of the RNA 3.

The 2b open reading frame (ORF) of Q-CMV RNA 2 was replaced with that of TAV to construct pQCD2qt (Fig. 1). The plasmid pQCD2qt was combined with pQCD1 plus pQCD3 or pQCD1 plus pCass1T3 and was inoculated to plants to generate the viruses C1C2_{qt}C3 or C1C2_{qt}T3. Comparison of the symptoms induced on both *N. glutinosa* and *N. clevelandii* by the three viruses, C1C2T3, C1C2_{qt}T3, and C1C2_{qt}C3, showed that both C1C2_{qt}T3 and C1C2_{qt}C3 induced similar systemic symptoms, more severe than those induced by C1C2T3 or T1T2T3 (Table 1 and Fig. 3A). On the other hand, the chimeric cucumoviruses C1C2_{qt}T3 and C1C2_{qt}C3 showed quite different levels of accumulation of RNAs 1 plus 2 from each other (Fig. 2A, lane 6 vs. lane 7). Moreover, the virus pair C1C2C3 plus C1C2_{qt}C3 accumulated similar levels of CMV RNAs (Fig. 2A, lanes 2 and 6) as did the virus pair C1C2T3 plus C1C2_{qt}T3 (Fig. 2A, lanes 5 and 7). Similarly, the latter two viruses also accumulated similar levels of TAV RNA 3 (Fig. 2B, lanes 5 and 7). This shows that the level of accumulation of RNAs 1 plus 2 of CMV was affected by the source of RNA 3 (TAV vs. CMV) but was not affected by the source of the TAV *2b* gene (Fig. 2A, lanes 2, 5, 6, and 7). These data also suggest that the 2b protein may have determined the difference in virulence between the two viruses but was not influenced by the 3a protein or CP derived from TAV RNA 3 (Table 1).

The replacement of the 2b ORF also resulted in a change in the encoded C-terminal 41 amino acids of the 2a protein from

that of Q-CMV to that of TAV (Fig. 1). Hence, the chimeric 2a protein might have shown some effect on the virulence. Therefore, the pseudorecombinant, chimeric virus C1C2_{qt}T3 was constructed to distinguish between the role of the TAV 2b protein and the chimeric 2a protein in determining hypervirulence. C1C2_{qt}T3 was generated after infection by the plasmids pQCD1, pQCD2qt1, and pCass1T3. Plasmid pQCD2qt1 was a modification of pQCD2qt, in which a termination codon was introduced in the 2a ORF immediately upstream of the 2b ORF and that would prevent expression of the C-terminal 41 amino acids of the 2a protein (Fig. 1). Inoculation of C1C2_{qt}T3 to the above two *Nicotiana* species showed that C1C2_{qt}T3 was infectious, induced severe symptoms similar to those of C1C2_{qt}T3 on the same plant species, and accumulated to levels similar to C1C2_{qt}T3 (Table 1 and data not shown). Sequence analysis of the progeny RNA 2 revealed that the mutation introduced was maintained (data not shown). Thus, the C-terminal 41 amino acids derived from the TAV 2a ORF did not influence the hypervirulence phenotype per se. This is consistent with earlier observations that the C-terminal 41 amino acids of the 2a protein were not essential for systemic infection (Ding et al. 1995a).

The 2b gene is essential for promoting infection in planta of pseudorecombinant viruses containing CMV RNAs 1 and 2 and TAV RNA 3.

Neither the C-terminal 41 amino acids of the 2a protein nor the 2b protein of Q-CMV were required for either replication or cell-to-cell movement but did influence the long-distance movement of Q-CMV (Ding et al. 1995a; Ji and Ding 2001). Therefore, to establish the role of the 2b gene in maintaining a pseudorecombinant virus, the virus C1C2_{qt}T3 was constructed and bioassayed. C1C2_{qt}T3 was generated from the plasmids pQCD1, pQCD2qt2, and pCass1T3. Plasmid pQCD2qt2 contains termination codons introduced in both the 2a and 2b ORFs, such that neither the 2b protein nor the C-terminal 41 amino acids of the 2a protein would be expressed (Fig. 1).

Surprisingly, the virus C1C2_{qt}T3 did not appear to be infectious (Table 1; Fig. 2A, lane 9; and Fig. 2B, lane 9). Repeated inoculation of C1C2_{qt}T3 with increased concentration up to five times the standard inoculum dosage still failed to demonstrate infection on *N. clevelandii*, *N. glutinosa*, or other plant species, including *Lycopersicon esculentum* and *Physalis flori-*

dana (data not shown). Failure to infect these plant species was confirmed by Northern blot hybridization analysis, which did not detect viral RNA in the inoculated plants (Fig. 2, lane 9 and data not shown). However, when pQCD1 and pQCD3, the resultant virus, C1C2_{qt}C3, was infectious in *N. glutinosa* (Table 1 and Fig. 2A, lane 8) and in the other plant species tested (Table 1 and data not shown). Moreover, C1C2_{qt}C3 accumulated to similar levels as C1C2_{qt}C3 (Fig. 2, lane 8 vs. lane 6). This indicates that

Table 1. Symptoms induced by various viruses in two *Nicotiana* spp.^a

Viruses species ^b	<i>N. clevelandii</i>	<i>N. glutinosa</i>
C1C2C3	tMot or NS	tMot
T1T2T3	Mos	Mos
C1C2T3	tMot or NS	tMot
C1C2 _{qt} C3	Mos/LD/St	Mos/LD/St
C1C2 _{qt} T3	Mos/LD/St	Mos/LD/St
C1C2 _{qt1} T3	Mos/LD/St	Mos/LD/St
C1C2 _{qt2} C3	tMot or NS	tMot
C1C2 _{qt2} T3	NS	NS
C1C2 _{qt3} C3	tMot or NS	tMot
C1C2 _{qt3} T3	NS	NS
C1C2 _{qw} C3	Mos/LD/St	Mos/LD/St
C1C2 _{qw} T3	Mos/LD/St	Mos/LD/St

^a Systemic symptoms: tMot = transient mottle; Mos = mosaic; LD = leaf distortion; St = stunting; and NS = no symptoms.

^b Virus species derived from combinations of RNAs 1, 2, and 3 of the Q-strain of *Cucumber mosaic virus* (CMV) or the V-strain of *Tomato aspermy virus* (TAV), indicated here as C or T, respectively. Mutants of Q-CMV RNA 2 consist of various replacements of the sequences encoding the 2b gene with those derived from the corresponding gene of V-TAV or the WAII-strain of CMV, indicated by C2 followed by the subscripts qt and qw, respectively.

Fig. 2. Northern blot hybridization analysis of the accumulated viral RNAs in *Nicotiana glutinosa*. Total RNAs were extracted from *N. glutinosa* plants inoculated with the wild-type, pseudorecombinant, chimeric, or chimeric pseudorecombinant viruses indicated. Equal amounts of total RNAs were fractionated by electrophoresis, were blotted to membranes, and were hybridized with probes specific to the 3' nontranslated region of the RNAs of either the Q-strain of *Cucumber mosaic virus* or *Tomato aspermy virus*. The positions of RNAs 1, 2, 3, 4, 4A, 3B, and 5 are indicated.

pQCD2qt2 was biologically active but did not support a progressive infection with TAV RNA 3.

To confirm that the lack of infectivity of C1C2_{qt2}T3 was not due to some effect of the two substituted nucleotides in pQCD2qt2, another virus, C1C2_{qt3}T3, was constructed in which the second substituted nucleotide, A, was changed to G (Fig. 1, pQCDqt3). Inoculation of C1C2_{qt3}T3 onto both test *Nicotiana* species showed that this virus also was not infectious (Table 1; Fig. 2, lane 11; and data not shown). However, when pQCD2qt3 was combined with pQCD1 and pQCD3, the resultant virus, C1C2_{qt3}C3, infected both of the tested *Nicotiana* spp. (Table 1; Fig. 2A, lane 10; and data not shown), indicating that the plasmid pQCD2qt3 also was biologically active. Moreover, C1C2_{qt3}C3 accumulated to similar levels as C1C2_{qt}C3 (Fig. 2A, lane 10 vs. lane 6). These results indicate that the substituted nucleotide had no role in viral infection and further suggest the existence of a specific interaction between the TAV 2b protein and RNA 3 that may be critical for viral infection. However, these studies could not differentiate between effects on either the replication of TAV RNA 3 or the cell-to-cell movement of the chimeric viruses C1C2_{qt2}T3 and C1C2_{qt3}T3.

The 2b gene is not essential for replication of pseudorecombinant viruses containing CMV RNAs 1 plus 2 and TAV RNA 3.

Infectious plasmids yielding C1C2C3 or T1T2T3 after inoculation to whole plants did not yield viral RNAs after inoculation to protoplasts (B. J. Shi and R. H. Symons, *unpublished data*). Therefore, replication of C1C2_{qt2}T3, incapable of expressing the C-terminal 41 amino acids of the 2a gene and the entire 2b ORF, was assessed using a fluorescent assay in inoculated plants. The CP gene in pCass1T3 was replaced with the gene encoding the green fluorescent protein (GFP), and

replication of this modified RNA 3 was detected by confocal laser scanning microscopic visualization of the GFP. As a positive control, the CP gene in pQCD3 was also replaced with the gene encoding the GFP. Both of these modified RNA 3 plasmids, pCass1T3GFP and pQCD3GFP, containing the GFP gene in place of the CP gene were mixed with pQCD1 and pQCD2qt2 and were inoculated to *N. clevelandii* plants. Replication of the various combinations of inocula was detected by the presence of green fluorescent single-cell foci of infection (Fig. 4 and data not shown), showing similar intensities of GFP accumulation for infection by C1C2_{qt2}C3_{GFP} (Fig. 4A) and C1C2_{qt2}T3_{GFP} (Fig. 4B). Foci of infection of similar intensities also were observed when pQCD2qt2 in the inoculum was replaced with either pQCD2 or pQCD2qt1 (data not shown). Thus, the inability of C1C2_{qt2}T3 to establish infection detectable by Northern blot hybridization analysis (Fig. 2) was not due to a failure of the modified virus to support the replication of TAV RNA 3. Rather, this failure was most likely a consequence of the inability of such viruses to move from the initially infected cells to adjacent cells in the inoculated leaves.

The 2b gene of a hypervirulent CMV strain also enhances the virulence but not the RNA accumulation of the CMV-TAV pseudorecombinant virus.

An alternative approach to verifying the role of the 2b protein in hypervirulence involved replacing the whole TAV 2b ORF in pQCD2qt2 with the 2b ORF from the hypervirulent CMV strain WAII-CMV to generate pQCD2qw (Fig. 1). Coinoculation of pQCD2qw, pQCD1, and pCass1T3 yielded a virus, C1C2_{qw}T3, that infected all the plant species tested above and induced severe systemic mosaic symptoms on these species (Table 1, Fig. 3B, and data not shown). However, in contrast to observations made with C1C2qtC3 vs. C1C2qtT3, RNAs 1 and 2 generated from C1C2_{qw}T3 accumulated to levels comparable to those generated from C1C2_{qw}C3 (Fig. 2A, lane 13 vs. lane 12).

As expected, the nucleic acids extracted from the plants infected with C1C2_{qw}T3 showed hybridization to TAV RNAs 3 and 4 with the TAV-specific probe (Fig. 2B, lane 13 and data not shown). However, these same samples also showed hybridization to RNAs 3 and 4 as well as RNAs 1 and 2 with the CMV-specific probe (Fig. 2A, lane 13 and data not shown). RNAs 3 and 4 detectable by the CMV-specific probe in these samples also were larger than those of CMV RNAs 3 and 4 (Fig. 2A, lane 13 vs. lane 12) and were the same size as expected for TAV RNAs 3 and 4. Characterization of the 3' 1,200 nt of these RNAs by reverse transcription-polymerase chain reaction (RT-PCR) and sequencing using various combinations of primers showed that the recombinant RNAs consisted of TAV RNAs 3 and 4 but with the sequence of approximately 300 nt of the 3' terminal nontranslated region (NTR) derived from CMV. In addition, there were RT-PCR products obtained representing the parental sequences of TAV RNAs 3 and 4 (data not shown). Thus, there was a mixture of TAV RNAs 3 plus 4 in these plants, some containing the 3' NTR of TAV and some containing the 3' NTR of CMV. This may have had some effect on altering the accumulation profiles of the various RNAs, resulting in a large increase in TAV RNAs 3 and 4 accumulation (Fig. 2B, lane 13 vs. lane 5), with a corresponding decrease in accumulation of CMV RNAs 1 plus 2 (Fig. 2A, lanes 13 vs. lane 5). The detailed characterization of the recombinants and the factors involved in their generation will be described elsewhere.

The TAV and WAII-CMV 2b ORFs share only 53.4% identity at the nucleotide level. Therefore, using a different 2b gene, the above data again showed that the 2b gene determined the level of virulence but did not directly affect the level of

Fig. 3. *Nicotiana glutinosa* infected by *Cucumber mosaic virus* (CMV), *Tomato aspermy virus* (TAV), and various pseudorecombinant viruses containing the wild-type or chimeric mutants of the 2b gene. **A**, Top row: Plants infected by either CMV RNAs 1, 2 and 3 (C1C2C3) (left), or CMV RNAs containing a TAV 2b gene in RNA 2 replacing the Q-CMV 2b gene (C1C2_{qt}C3) (right). Bottom row: Plants infected by either TAV RNAs 1, 2, and 3 (T1T2T3) (left) or the chimeric pseudorecombinant virus C1C2_{qt}T3 (right). **B**, Plants infected by either the chimeric pseudorecombinant virus C1C2_{qw}T3 (left) or the chimeric virus C1C2_{qw}C3 (right), in which qw represents the 2b gene from the WAII-strain of CMV replacing the 2b gene of Q-CMV.

RNA accumulation. Moreover, the above results are inconsistent with any proposed role of the nucleotide sequence of the 2*b* gene itself being required to promote viral infection. Thus, we conclude that both viral infection and hypervirulence were determined only by the 2*b* protein rather than by other factors, such as either the 2*b*-gene sequence or the C-terminal 41 amino acids encoded by the TAV 2*a* gene.

DISCUSSION

Pseudorecombinant viruses involving exchange of RNA 3 can be constructed between CMV and TAV (Habili and Francki 1974; Masuta et al. 1998; Rao and Francki 1981; Perry and Francki 1992; Sackey and Francki 1990; Salánki et al. 1997). However, it is apparent that they are not as competitive as the parental viruses (Sackey and Francki 1990; Perry and Francki 1992). This lack of competitiveness may reflect differences in the ability of the various proteins encoded by the two viruses to function together optimally to facilitate movement of viral RNAs from cell to cell and through the vasculature. Alternatively, there may be a requirement for direct, coordinated interactions between these proteins and specific host components. In either case, a lack of coordinated functions could lead to differences in the accumulation profiles and the virulence of the resulting viruses. In the case of the pseudorecombinants examined here, it is apparent that there were several effects on RNA accumulation. First, in many cases, the presence of RNA 3 from TAV caused a stimulation in the levels of accumulation of the CMV RNAs 1 or 2 (Fig. 2A, lanes 5 and 6 vs. lanes 2 and 6, respectively). The exceptions to this occurred when the 2*b* protein was not expressed (Fig. 2A, lanes 9 and 11 vs. lanes 8 and 10) or when it was derived from the more virulent WAI-CMV (Fig. 2A, lane 13 vs. lane 12), although in each of these cases, further differences were seen. Second, those pseudorecombinants that showed infection in plants, with the exception of C1C2_{q_w}T3 (Fig. 2B, lane 13), showed similar levels of accumulation of TAV RNA 3 to the wild-type T1T2T3 and lower levels of accumulation of TAV RNA 4 than for the wild-type T1T2T3 (Fig. 2B, lanes 5 and 7 vs. lane 3). These differences in RNA accumulation levels had no effect on virulence vis-à-vis the corresponding virus containing CMV RNA 3 (Table 1 and Fig. 3B). On the other hand, the source of the 2*b* gene showed considerable affect on the level of virulence (Table 1 and Fig. 3). Thus, in the cases examined here, the presence of a variant 2*b* gene caused a direct effect on the virulence of the virus, irrespective of the source of the RNA 3 (Table 1 and Fig. 3), and the virulence was unrelated to any effects on accumulation of the viral RNAs (Fig. 2). These latter observations are consistent with the analysis done in a previous study on virus levels and the timecourse of viral RNA accumulation for viruses differing in virulence on several hosts; i.e., C1C2C3, C1C2_{q_t}C3, and C1C2_{q_w}C3 (Shi et al. 2002).

The CMV 2*b* gene was shown not to be essential for either cell-to-cell or long-distance movement in several *Nicotiana* spp. (Ding et al. 1995a; Ji and Ding 2001; Soards et al. 2002). Thus, it was surprising that mutations that prevented the expression of the TAV 2*b* gene from CMV RNA 2 also prevented the accumulation of viral RNAs in planta (Fig. 2) but not the replication (Fig. 4) of a chimeric pseudorecombinant virus containing TAV RNA 3 (i.e., C1C2_{q_t}T3). This indicates that the 2*b* protein, whether from CMV or TAV, is required to facilitate a progressive infection of the pseudorecombinant viruses. This would tend to suggest a further interaction is required of the 2*b* protein with RNA 3, the 3*a* protein, or the CP of TAV to promote the cell-to-cell move-

ment of C1C2_{q_t}T3 and C1C2_{q_t}T3, which is not required to facilitate the cell-to-cell movement of either C1C2_{q_t}C3 or C1C2_{q_t}C3 (Table 1 and Fig. 2). Alternatively, the 2*b* protein may be required to neutralize a host response elicited by either TAV RNA 3 or one of its encoded components that leads to a block in movement. The data presented here do not allow discrimination between these alternatives.

Differences between the ability of TAV and CMV homologous components to function in facilitating virus movement have been described before. A virus designated R1R2TR3, which consisted of RNAs 1 and 2 of the R-strain of CMV and a chimera of RNA 3, in which the 5' half derived from TAV RNA 3 and the 3' half derived from R-CMV RNA 3, was not able to infect any of four host species, although the viral RNAs were able to accumulate in protoplasts from *Nicotiana tabacum*. On the other hand, a virus with the reciprocal RNA 3 chimera R1R2RT3 was able to infect all four host plant species tested (Salánki et al. 1997). Thus, there are other examples of chimeric pseudorecombinants between CMV and TAV that do not affect virus replication but do affect the ability of the virus to move cell to cell. This example does not allow further delimitation of the possible mechanism by which some chimeric pseudorecombinants are restricted in cell-to-cell movement, since there may be other factors operating to restrict the cell-to-cell movement of R1R2TR3. However, it does support the premise that pseudorecombinant viruses and chimeric viruses are not, in general, optimized for their interactions either between the viral-encoded components, or with the host. Thus, such viruses would not be expected to compete very effectively with either of the parental viruses.

Fig. 4. Distribution of green fluorescent protein (GFP) in *Nicotiana clelandii* epidermal cells. The viruses C1C2_{q_t}C3_{GFP} and C1C2_{q_t}T3_{GFP} were inoculated to *N. clelandii* plants. At five days postinoculation, the inoculated leaves were viewed by confocal laser scanning microscopy. Fluorescence images due to expression of the GFP were obtained from epidermal cells infected with either **A**, C1C2_{q_t}C3_{GFP} or **B**, C1C2_{q_t}T3_{GFP}. Noninfected cells did not show green fluorescence (arrow).

MATERIALS AND METHODS

Infectious cDNA plasmids.

Infectious cDNA plasmids pQCD1, pQCD2, pQCD2qt, pQCD2qt1, pQCD2qt2, pQCD2qw, pQCD3, and pCass1T3 were described previously (Ding et al. 1995a, 1995b, 1996; Shi et al. 1997a, 2000). pQCD1, pQCD2, and pQCD3 contain the full-length cDNAs of RNAs 1, 2, and 3 of wild-type Q-CMV (Ding et al. 1995b), while pCass1T3 contains the full-length cDNA of RNA 3 of wild-type TAV (Shi et al. 1997a). Plasmids pQCD2qt, pQCD2qt1, pQCD2qt2, and pQCD2qw are derivatives of pQCD2, in which the 2b gene or its mutant versions come from either TAV (pQCD2qt, pQCD2qt1, and pQCD2qt2 [Ding et al. 1996]) or WAI-CMV (pQCD2qw [Shi et al. 2002]). The infectious cDNA plasmid pQCD2qt3 was also a derivative of pQCD2, constructed by adapting the same PCR-based strategy used previously for mutagenesis (Ding et al. 1996; Shi et al. 2002). Plasmids containing the GFP ORF in place of the CP ORF (pQCD3GFP and pCass1T3GFP, respectively) were prepared from pQCD3 and pCass1T3 by PCR mutagenesis and a 3-piece ligation strategy, using the *GFP* gene from plasmid pS65T-C1 (Clontech, Basingstoke, U.K.) and appropriate primers (Canto et al. 1997). All the amplified regions in the clones were fully sequenced to ensure that the inserted sequences were as expected. All the above RNA 2 plasmids and their abbreviations are given in Figure 1.

Plant materials and inoculation.

The plant species *N. clevelandii*, *N. glutinosa*, *L. esculentum*, and *P. floridana* were all grown at 25°C. Inoculation was carried out after a 24-h dark pretreatment by rubbing the infectious cDNA clones or sterile water (as the control) with sterile Carborundum onto the surface of two or three fully expanded young leaves (Shi et al. 1997a). Unless specified otherwise, all the individual inocula consisted of the three genomic cDNA clones, each at a concentration of 10 µg in 10 µl water per plant. For each test, five, four-leaf-stage plants of each species were inoculated.

Viral progeny RNA analysis.

Total RNAs were extracted from infected plants and were analyzed by Northern blot hybridization as described previously (Shi et al. 1997b). Samples were first quantified by gel electrophoresis and by staining with ethidium bromide, and then, loadings for gels processed for blotting were equalized, relative to the level of the stained rRNAs. (The viral RNAs are all positioned behind various rRNA bands.) The probes were ³²P-labeled transcript complementary to either nucleotides 2,287 to 2,386 of TAV RNA 3 or nucleotides 2,871 to 3,035 of Q-CMV RNA 2. These two riboprobes hybridize to all three genomic and the various subgenomic RNAs of either TAV or CMV, since the 3'-terminal sequences of the three genomic RNAs in each virus are highly conserved (Shi et al. 1997b).

Direct RNA sequencing was performed according to Fichot and Girard (1990). Templates used for sequencing were either the total RNAs extracted from infected plants or single RNAs purified via polyacrylamide gel electrophoresis and elution. DNA sequencing, using either PCR products or cDNA clones as templates, was carried out as described in Shi and associates (1997b).

Detection of GFP fluorescence.

Plants were inoculated with plasmids pQCD1 plus pQCD3GFP or pQCD1 plus pCass1T3GFP and were mixed with various RNA 2 variant plasmids as described above but with 2 µg of plasmid per 10 µl per plant. At three and five days

after inoculation, the inoculated leaves were examined by confocal laser scanning microscopy to visualize the GFP, as previously described (Canto et al. 1997). Digital images obtained with the confocal microscope were processed with Adobe Photoshop software (Adobe Systems, San Jose, CA, U.S.A.).

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