2b ORFs encoded by subgroup IB strains of cucumber mosaic virus induce differential virulence on *Nicotiana* species

Zhi-You Du,1 Fei-Fei Chen,1 Qian-Sheng Liao,1,2 Hua-Rong Zhang,2 Yan-Fei Chen1 and Ji-Shuang Chen1

Correspondence
Ji-Shuang Chen
chenjs@zstu.edu.cn

1Institute of Bioengineering, Zhejiang Sci-Tech University, Hangzhou 310018, PR China
2College of Life Sciences, Zhejiang University, Hangzhou 310029, PR China

Cucumber mosaic virus (CMV)-encoded 2b protein from subgroup IA or subgroup II was shown to be a determinant of virulence in many solanaceous hosts. In this study, the virulence of 2b proteins from subgroup IB strains was analysed using four intraspecies hybrid viruses, which were generated by precise replacement of the 2b open reading frame (ORF) in subgroup IA strain Fny-CMV with the 2b ORFs of four subgroup IB strains, Cb7-CMV, PGs-CMV, Rad35-CMV and Na-CMV, generating FCb7 2b-CMV, FPGs 2b-CMV, FRad35 2b-CMV and FNa 2b-CMV, respectively. FCb7 2b-CMV was more virulent than Fny-CMV, and was similar in phenotype to its parental virus Cb7-CMV on the three *Nicotiana* species tested. FNa 2b-CMV also was virulent on these host species, equivalent to Fny-CMV or Na-CMV. However, FRad35 2b-CMV only caused mild mosaic or undetectable symptoms on all the host species tested, and was less virulent than Fny-CMV or Rad35-CMV. FPGs 2b-CMV infected all the host species systemically, and induced either mosaic or barely visible symptoms, demonstrating that the inability of PGs-CMV to infect these three *Nicotiana* species was not due to its 2b protein. The diverse virulence was shown to be mediated by the 2b proteins rather than the C-terminal overlapping parts of the 2a proteins, and was associated with the level of viral progeny RNA accumulation in systemically infected leaves, but not with the rate of long-distance viral movement in host plants. Through analysis of encapsidation of viral RNAs, there was an apparent correlation between the virulence and the high level of encapsidated RNA 2 in virions of Fny-CMV, FCb7 2b-CMV and FNa 2b-CMV.

INTRODUCTION

Cucumber mosaic virus (CMV) is a positive-sense RNA virus with a tripartite genome, designated RNAs 1, 2 and 3 in decreasing order of their sizes. CMV is the type species of the genus *Cucumovirus*, in the family *Bromoviridae*, which also includes the genera *Bromovirus*, *Alfamovirus*, *Ilavirus* and *Oleovirus*. CMV infects over 1000 plant species (Palukaitis & García-Arenal, 2003) and occurs worldwide. Numerous strains have been identified and characterized to date. There are five open reading frames (ORFs) in the CMV genome that encode five viral proteins: proteins 1a, 2a, 3a and CP (capsid protein). The proteins 1a, 2a, 3a and CP are well-studied with regard to their functions in viral replication, movement and encapsidation. The 2b protein translated from the subgenomic RNA (RNA 4A) of RNA 2 was identified relatively recently in Q-CMV-infected plants (Ding et al., 1994). Similarly, the homologous 2b protein was detected in Fny-CMV-infected hosts (Mayers et al., 2000). The 2b ORF is located at the 3’ terminus of RNA 2, and partially overlaps with the 3’ terminus of the 2a ORF (Ding et al., 1994). A 2b-like ORF is encoded by ilarviruses (Xin et al., 1998). However, species of the genera *Bromovirus* and *Alfamovirus* do not contain equivalent ORF (Ding et al., 1994).

Earlier analysis suggested that CMV strains could be divided into two subgroups, now named subgroup I and II (Owen & Palukaitis, 1988). Subgroup I strains are more heterogeneous than subgroup II strains. In general, subgroup I strains may be more virulent than subgroup II strains (Wahyuni et al., 1992; Zhang et al., 1994) or may have differences in host range from subgroup II strains (Daniels & Campbell, 1992; Wahyuni et al., 1992). Recently, phylogenetic analyses of the CP gene and 5’ non-translated region of RNA 3 showed that subgroup I can be further classified into subgroup IA and subgroup IB (Roossinck et al., 1999). Isolates of subgroups IA and II occur...
Studies to date have shown the CMV 2b protein to be a suppressor that interacts directly with Argonaute 1 protein (AGO1) and weakens its cleavage activity in RNA silencing (Zhang et al., 2006), to inhibit the activity of the RNA mobile silencing signal (Guo & Ding, 2002), and to be a determinant of long-distance movement in cucumber (Ding et al., 1995). Besides the above roles, the 2b protein has another important function as a pathogenicity determinant in solanaceous hosts. The mutant Q-CMV-Δ2b, in which 2b ORF was disrupted by inserting a termination codon in 2b ORF, caused only mild symptoms in Nicotiana glutinosa plants, and was less virulent than the subgroup II wild-type Q-CMV (Ding et al., 1995). Similarly, the mutant Fny-CMV-Δ2b, in which most (nt 2419–2713 of RNA 2) of the 2b ORF was deleted from subgroup IA wild-type Fny-CMV, produced no symptoms in tobacco plants; however, Fny-CMV caused severe symptoms, including mosaic, stunting and leaf deformation (Soards et al., 2002). An interspecies hybrid virus, CMV-qt, in which the 2b ORF of Q-CMV was replaced with that of the cucumovirus tomato aspermy virus (TAV), was more virulent than the parental viruses (Q-CMV or TAV) in seven different host species, but did not infect cucumber systemically (Ding et al., 1996). An interspecies virus, CMV-qw, in which the 2b ORF of Q-CMV was substituted by that of subgroup IA strain WAII-CMV, was found to be more virulent than wild-type Q-CMV or WAII-CMV (Shi et al., 2002). All of the above findings support the initial conclusion that the cucumoviral 2b protein is a determinant of pathogenicity and controls symptom expression (Ding et al., 1995, 1996).

In the past several years, we isolated four isolates of CMV subgroup IB in China: Na-CMV, Rad35-CMV, Cb7-CMV and PGs-CMV. The first three isolates had different symptom expression patterns in Nicotiana species, while PGs-CMV failed to systemically infect Nicotiana species by mechanical inoculation. To determine whether their biological properties of differential virulence and failure to infect Nicotiana species were associated with their 2b proteins, we herein constructed four interspecies hybrid viruses, FNa2b-CMV, FRad352b-CMV, FCb72b-CMV and FPGs2b-CMV, in which the 2b ORF of Fny-CMV was replaced with those of isolates Na-CMV, Rad35-CMV, Cb7-CMV and PGs-CMV, respectively. The biological properties of the four hybrid viruses were compared with each other and with those of their parental viruses to establish the role of 2b proteins from subgroup IB strains in virulence and host range.

**METHODS**

**Plants, viruses and plasmid constructs.** The three Nicotiana species, N. glutinosa, Nicotiana tabacum and Nicotiana benthamiana, used as host plants in this work were grown in a greenhouse at 26 °C under a 14 h photoperiod. Four parental CMV isolates were used besides wild-type Fny-CMV (Rizzo & Palukaitis, 1990): Cb7-CMV, Na-CMV, Rad35-CMV and PGs-CMV, isolated from tomato, zucchini, radish and Pinellia ternata, respectively. Pinellia ternata is a traditional Chinese medicinal plant. Four interspecies hybrid clones, pFny209Cb72b, pFny209Na2b, pFny209PGs2b and pFny209Rad352b, derivatives of pFny209, were constructed by separately replacing the 2b gene of Fny-CMV with the 2b genes of Cb7-CMV, Na-CMV, PGs-CMV and Rad35-CMV, respectively (Fig. 1). The four interspecies hybrid clones were constructed using three overlapping PCR fragments I, II and III. Fragments I and III flank Fny-CMV ORF 2b (nt 2419–2751) and contain nt 1856–2420 and 2749–3050, respectively. The two fragments, I and III, were obtained by separate PCRs using pFny209 as the template and primer pairs CF2F2398/C2R2420 and CF2F2749/C123R, respectively (Supplementary Table S1 available in JGV Online). Fragments II-Cb72b, II-Na2b, II-PGs2b and II-Rad352b represent coding region sequences of 2b ORFs in Cb7-CMV, Na-CMV, PGs-CMV and Rad35-CMV, respectively. The five fragments were obtained by separate PCRs using primer pairs Cb72b/Cb72bR, Na2b/Na2bR, PGs2b/PGs2bR and Rad352b/Rad352bR (Supplementary Table S1 available in JGV Online), and full-length cDNA clones of RNAs 2 of Cb7-CMV, Na-CMV, PGs-CMV and Rad35-CMV as PCR templates, respectively. The fragments I, II and III were mixed and used as a template for a final amplification with primer pair CF2F2398/C123R. The resultant fragments were subsequently digested with restriction endonucleases PsI and HindIII, and then cloned into pFny209 previously digested with restriction endonucleases PsI, EcoRI and HindIII.

The pFny209Δ2AΔC2I plasmid (Fig. 1), a derivative of pFny209, in which 81 codons from the C terminus of 2a ORF was rendered non-coding by substitutions at nt 2415–2417 (AAT→TGA), was constructed by an overlap-extension PCR using a pair of completely complementary mutagenic primers CF2F2398A2a81/CR2398A2a81 (Supplementary Table S1 available in JGV Online). The pFny209A2bΔII plasmid (Fig. 1), a derivative of pFny209, in which most of the 2b gene was deleted (nt 2421–2478), was constructed by an overlap-extension PCR using a pair of partially complementary primers CF2F244/C2R244 and CF2A2b (Supplementary Table S1 available in JGV Online), pFny209A2bpro plasmid (Fig. 1), a derivative of pFny209, in which 2b ORF was made non-coding by substitutions at nt 2422–2423 (CAG→TGA), and at nt 2441 and 2471 (ATG→ACG), was constructed by two overlap-extension PCRs using two pairs of partially complementary mutagenic primers, CF2F2398A2b/C2R23770 and CF2F244/C2R2466 (Supplementary Table S1 available in JGV Online). pFny209C2bΔ2bproR (Fig. 1), a derivative of pFny209C2bΔ2bR (Supplementary Table S1 available in JGV Online), in which the Cb7-CMV-encoded 2b ORF was made non-coding by substitutions at nt 2422–2423 (CAG→TGA), and at nt 2471 (ATG→ACG), was constructed by two overlap-extension PCRs using two pairs of partially complementary mutagenic primers, CF2F2398A2b/C2R23770 and CB72bpro/Cb72bproR (Supplementary Table S1 available in JGV Online). All constructed plasmids were sequenced before use.

**Plant inoculation and viral progeny RNA analysis.** These constructed plasmids were linearized by digestion with restriction endonuclease PsI and made blunt-ended with Klenow fragment (Promega) before use as templates for in vitro transcription with RiboMax in vitro transcription kit (Promega). Each transcript was combined with the in vitro transcripts of pFny109 and pFny309, and then inoculated onto Carborundum-dusted leaves of N. glutinosa seedlings to produce wild-type Fny-CMV and its derivatives (Table 1). Mock-treated plants were inoculated with distilled water. Total RNA was extracted from CMV-infected or mock-infected plants using TRIzol reagent (Invitrogen). Northern blot hybridization for viral progeny RNA analysis was performed using the protocol of Sambrook & Russell (2001). A CMV hybridization probe (probel-40) was prepared by end-labelling a DNA oligonucleotide with [γ-32P]ATP using T4 polynucleotide kinase (Takara), and used in the hybridization.
The oligonucleotide sequence (5’-ACTGACCACTTATTACGCTAA-GCTTGAGTTGCAACGCCGTC-3’) is completely complementary to a conserved sequence at the 3’ end region of all genomic RNAs in all cucumoviruses (McGarvey et al., 1995). The virions of wild-type Fny-CMV and the four intraspecies hybrids were purified from singly infected leaves of N. glutinosa plants 20 days post-inoculation (p.i.) according to a previously described protocol (Palukaitis & Zaitlin, 1984). Virion RNAs were extracted from the purified virions, and analysed by separating on a formaldehyde-containing agarose gel and by Northern blot hybridization using the probe-40 probe as described above.

Analysis of viral accumulation and movement. N. glutinosa plants were inoculated with Fny-CMV, Fny-CMV2a2b, Fny-CMV2bpro, FCb72b-CMV or FCb72b-CMV2a2bpro (Table 1) at an equal concentration of their RNA transcripts. The systemic leaves (third from top leaf) were collected at 21 days p.i., and total RNAs were extracted from equal weights of these collected leaves. The total RNAs were separated in a formaldehyde-containing agarose gel, and then blotted onto a nylon membrane (PALL). Accumulation of viral progeny RNAs of these viruses was analysed by hybridization with the probe-40 probe as described above. The hybridized membrane was exposed to a storage phosphor screen (Molecular Dynamics) for 4 h, and the storage phosphor screen was scanned by Typhoon 9200 (Amersham).

Three plant species, N. glutinosa, N. tabacum and N. benthamiana, were inoculated with Fny-CMV, FCb72b-CMV, FRad352b-CMV, FPGs2b-CMV or FNa2b-CMV (Table 1) at an equal concentration of their RNA transcripts. The systemic leaves (third from top leaf) were collected from N. glutinosa plants at 1, 3, 5, 7, 14 and 28 days p.i., from N. tabacum plants at 28 days p.i., and from N. benthamiana plants at 14 days p.i. Total RNA extraction and Northern blot hybridization were carried out as described above. Hybridization signal intensities of viral RNAs in each RNA sample were calculated after normalization of loading quantities of these RNA samples against their 28S rRNA.

cDNA clone of RNAs 2 of three CMV isolates. Full-length cDNAs of RNAs 2 of isolates Cb7-CMV, Na-CMV and Rad35-CMV were obtained by RT-PCR amplification using primers C12FBamHI and C123RPstI. The primer C12FBamHI consists of the sequence 5’-AATCAGATCTCTACGACATCAGACATCATATGTATTCTAAGAGGCTAGC-3’ with a degenerate base ‘R’ (A or G), a BamHI site (in italic), modified T7 RNA polymerase promoter (underlined), and 3’ 22 nt identical to those at 5’ end of RNAs 1 and 2 in subgroup 1 strains. The C123RPstI primer consists of the sequence 5’-AATCTCTGATTGGACAACCCGTTC-3’ with a degenerate base ‘R’ (A or G), a PstI site (in italic), and 3’ 16 nt complementary to those at 3’ end of RNAs 1, 2 and 3 in subgroup I strains. The three full-length cDNAs were digested with restriction endonucleases BamHI and PstI, and then cloned into pUC18 plasmid previously digested with the same restriction endonucleases. The resultant cDNA clones of RNAs 2 were sequenced by a DNA sequencer 3730 (Applied Biosystems). The cDNA sequences of RNAs 2 of Cb7-CMV, Na-CMV and Rad35-CMV have been submitted to GenBank under the accession nos DQ785470, DQ785471 and DQ785469, respectively. The sequence of RNA 2 of PGs-CMV has been determined previously (unpublished data, accession no. DQ399549).

**RESULTS AND DISCUSSION**

**Infectivity and stability of Fny-CMV-derived mutants**

Wild-type Fny-CMV, Fny-CMV2a2b, FRad352b-CMV, FPGs2b-CMV, FCb72b-CMV and FNa2b-CMV were generated.
by co-inoculating RNA transcripts on seedlings of *N. glutinosa* at the eight-leaf stage. cDNA clones used for preparation of the RNA transcripts are listed in Table 1. Two independent assays showed that all plants inoculated with the above viruses produced mosaic symptom on upper systemic leaves at 3 or 4 days p.i. (data not shown). The six viruses were passaged once through *N. glutinosa* at 14 days p.i. Virions were purified from equal weights of infected leaves 14 days after passage. Viral RNAs were isolated from the purified virions and amplified by RT-PCR using the primer pair C2F1856/C123R. Results obtained from RT-PCR showed that a DNA fragment of about 1.2 kb was amplified in all viral RNA samples (data not shown). All amplified DNA fragments were analysed further by sequencing. No sequence variation was observed in any RNA 2 of these intraspecies hybrid viruses, but the modified RNA 2 of Fny-CMV reverted to the RNA 2 of wild-type Fny-CMV (data not shown). All amplified DNA fragments were sequenced by co-inoculating RNA transcripts on seedlings of *N. glutinosa* at the eight-leaf stage. cDNA clones used for preparation of the RNA transcripts are listed in Table 1. Two independent assays showed that all plants inoculated with the above viruses produced mosaic symptom on upper systemic leaves at 3 or 4 days p.i. (data not shown). The six viruses were passaged once through *N. glutinosa* at 14 days p.i. Virions were purified from equal weights of infected leaves 14 days after passage. Viral RNAs were isolated from the purified virions and amplified by RT-PCR using the primer pair C2F1856/C123R. Results obtained from RT-PCR showed that a DNA fragment of about 1.2 kb was amplified in all viral RNA samples (data not shown). All amplified DNA fragments were analysed further by sequencing. No sequence variation was observed in any RNA 2 of these intraspecies hybrid viruses, but the modified RNA 2 of Fny-CMV reverted to the RNA 2 of wild-type Fny-CMV (data not shown). All amplified DNA fragments were sequenced.

**Replacement of the 2b ORF affected encapsidation of viral RNA 2**

The replacement of the 2b ORF directly resulted in changes in the nucleotide sequences of RNAs 2 and 4A, besides changes in the 2b protein and the C-terminal part of the 2a protein. To analyse whether the replacement of the 2b ORF would affect the encapsidation of each viral RNA, virions of Fny-CMV, FRad352b-CMV, FPgs2b-CMV, FCb72b-CMV and FNa2b-CMV were purified from equal weights of leaf tissues. Virion RNAs were isolated from the purified virions, separated on an agarose gel, and then stained with ethidium bromide (Fig. 2a). The intensity of each viral RNA band was measured using GeneTool software (Syngene), and the quantities of RNAs 1, 2 and 4 relative to RNA 3 in each virus were calculated (Fig. 2c). RNA 3 in each virus was regarded as an internal reference, thus the relative quantity of RNA 3 in each virus was designated as 1. Although there was some difference in the relative quantities of RNAs 1 or 4 of the five viruses, this difference did not show a correlation with the degree of virulence (Table 2). By contrast, the difference in relative quantities of RNAs 2 of the five viruses was shown to correlate with the degree of virulence. Fny-CMV, FCb72b-CMV and FNa2b-CMV all contained a very faint band with a migration faster than RNA 4, but FRad352b-CMV and FPgs2b-CMV did not (Fig. 2a). A similar result concerning this band was obtained by Northern blot hybridization analyses (Fig. 2b). Although the RNA band had the same mobility to RNA 4A shown in total RNA from Fny-CMV-infected leaves of *N. glutinosa* (data not shown), it could not be verified to be RNA 4A.

**Different virulence induced by intraspecies hybrid viruses**

The virulence of the four intraspecies hybrid viruses and four parental viruses was investigated by inoculating these viruses on three plants of *Nicotiana* species: *N. glutinosa*, *N. tabacum* and *N. benthamiana*. Symptoms on these host species are shown in Table 2. Of all the viruses, FCb72b-CMV and its parental virus Cb7-CMV were the most virulent. They showed high virulence on all the host species tested, especially on *N. glutinosa*, where FCb72b-CMV and Cb7-CMV caused top necrosis early during infection and plant death later during infection. This was more severe than the disease caused by Fny-CMV. FNa2b-CMV was also virulent on these plant species, showing similar virulence to its parental viruses Fny-CMV and Na-CMV. However, FRad352b-CMV only caused mild systemic or undetectable symptoms on these three host species, and was less virulent than its parental viruses Fny-CMV and Rad35-CMV.

<table>
<thead>
<tr>
<th>Virus construct</th>
<th>Transcription templates for</th>
<th>Derivation of 2b genes</th>
<th>Presence of functional 2b genes†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNA 1</td>
<td>RNA 2</td>
<td>RNA 3</td>
</tr>
<tr>
<td>Fny-CMV</td>
<td>pFny109*</td>
<td>pFny209*</td>
<td>pFny309*</td>
</tr>
<tr>
<td>Fny-CMVΔ2aC81</td>
<td>pFny109</td>
<td>pFny209Δ2aC81</td>
<td>pFny309</td>
</tr>
<tr>
<td>Fny-CMVΔ2b</td>
<td>pFny109</td>
<td>pFny209Δ2b</td>
<td>pFny309</td>
</tr>
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<td>pFny209Δ2bpro</td>
<td>pFny309</td>
</tr>
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<td>pFny209FCb72b-CMV</td>
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<td>FRad352b-CMV</td>
<td>pFny109</td>
<td>pFny209Rad352b-CMV</td>
<td>pFny309</td>
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<td>FPgs2b-CMV</td>
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<td>FCb72b-CMVΔ2bpro</td>
<td>pFny109</td>
<td>pFny209FCb72bΔ2bpro</td>
<td>pFny309</td>
</tr>
</tbody>
</table>

*Plasmids pFny109, pFny209 and pFny309 were previously constructed by Rizzo & Palukaitis (1990).
†Indicates the functional 2b gene was present in the corresponding virus; N indicates the functional 2b gene was absent in the corresponding virus.
FPGs2b-CMV, similar to FRad35 2b-CMV, showed little virulence on the three host species. Although both FCb72b-CMV and Cb7-CMV caused top necrosis on N. glutinosa, FCb7 2b-CMV induced this symptom to appear about 3 to 5 days later than that induced by Cb7-CMV (Table 2). A pseudorecombinant virus FIP2F3, composed of Fny-CMV RNA 1 plus RNA 3, and PGs-CMV RNA 2, caused severe mosaic and leaf deformation on N. glutinosa at later stages of infection (data not shown), which was more virulent than FPGs2b-CMV. Similarly, differences in virulence were also observed between Rad35-CMV and FRad352b-CMV (Table 2). These results suggested that the 2b protein played an important role in virulence, but it is not the only virulence determinant in these three subgroup IB strains.

Surprisingly, all the host species did not express symptoms after inoculation either with crude extracts from a PGs-CMV-infected P. ternata plant or RNA transcripts of PGs-CMV RNAs 1, 2 and 3 (Table 2). Failure to systemically infect these three Nicotiana species was confirmed by RT-PCR analyses, which did not detect RNA 3 of PGs-CMV in all the upper non-inoculated leaves of these plants, but only in all the inoculated leaves (unpublished data). Furthermore, PGs-CMV could replicate successfully in tobacco protoplasts, and its progeny RNAs had a similar accumulation level to Fny-CMV’s progeny RNAs (unpublished data). These results demonstrate that the failure of PGs-CMV to systemically infect all the Nicotiana species was most likely a consequence of these hosts blocking its long-distance movement. The absence of the Q-CMV 2b protein or replacement of its 2b ORF with that of TAV rendered this virus unable to infect cucumber systemically (Ding et al., 1995, 1996), which suggests that the 2b protein may determine the host specificity of the virus. However, systemic infection of FPGs 2b-CMV on these three Nicotiana species demonstrated that the 2b protein was not the determinant of host specificity of PGs-CMV.

Table 2. Symptoms induced by various viruses on three Nicotiana species

<table>
<thead>
<tr>
<th>Virus</th>
<th>N. glutinosa</th>
<th>N. benthamiana</th>
<th>N. tabacum</th>
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<tbody>
<tr>
<td>Fny-CMV</td>
<td>Mos/LD/St</td>
<td>LD/St</td>
<td>sMos/LD</td>
</tr>
<tr>
<td>PGs-CMV</td>
<td>-*</td>
<td>-*</td>
<td>-*</td>
</tr>
<tr>
<td>Rad35-CMV</td>
<td>Mos/St</td>
<td>LD/YS</td>
<td>sMos/LD</td>
</tr>
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<td>Cb7-CMV</td>
<td>Mos/TN/St†</td>
<td>LD/St</td>
<td>sMos/LD/St</td>
</tr>
<tr>
<td>Na-CMV</td>
<td>Mos/LD/St</td>
<td>LD/St</td>
<td>sMos/LD</td>
</tr>
<tr>
<td>FPGg2b-CMV</td>
<td>Mos</td>
<td>NoS</td>
<td>mMos</td>
</tr>
<tr>
<td>FRad352b-CMV</td>
<td>mMos</td>
<td>NoS</td>
<td>mMos</td>
</tr>
<tr>
<td>FCb72b-CMV</td>
<td>Mos/TN/St†</td>
<td>LD/St</td>
<td>sMos/LD/St</td>
</tr>
<tr>
<td>FNa2b-CMV</td>
<td>Mos/LD/St</td>
<td>LD/St</td>
<td>sMos/LD</td>
</tr>
</tbody>
</table>

*Indicates that the PGs-CMV did not systemically infect these three Nicotiana species by mechanically inoculating with either crude sap or in vitro transcription products.
†Although both FCb72b-CMV and Cb7-CMV caused top necrosis on N. glutinosa, FCb72b-CMV induced this symptom at about 3 to 5 days later than that induced by Cb7-CMV.

Fig. 2. Analysis of the relative quantities of viral RNAs in virions of wild-type Fny-CMV and the four intraspecies hybrid viruses purified from equal weights of infected leaf tissues. (a) Electrophoresis patterns of viral RNAs separated on 1.5% agarose gels in 1× TBE electrophoresis buffer. (b) Northern blot hybridization analysis of viral RNAs using the probeI-40 probe as described in the text. (c) The quantities of RNAs 1, 2 and 4 relative to RNA 3 in each virus as shown in (a). RNA 3 in each virus was regarded as an internal reference, thus the relative quantity of RNA 3 in each virus was defined as 1. The mean and standard deviation of the relative quantity were obtained from three independent experiments. The positions of RNAs 1, 2, 3 and 4 are indicated on the left (a, b). * shown in (a) and (b) indicates the position of an uncertain RNA band with a same mobility to RNA 4A.
Different virulence was mediated by the 2b proteins, rather than the C-terminal overlapping parts of the 2a proteins

As stated above, the Fny-CMVA2aC81 mutant was unstable and reverted to wild-type Fny-CMV after one passage, so we could not directly rule out the possibility of the C-terminal part of the 2a protein of Fny-CMV affecting symptom expression and accumulation of viral progeny RNAs in host plants. To address this issue, Fny-CMVΔ2b, Fny-CMVA2bpro and FCb72b-CMVΔ2bpro, all of which could not express the 2b proteins, were generated by inoculation of N. glutinosa with viral RNAs transcribed in vitro from the corresponding cDNA clones (Table 1). Fny-CMVΔ2b and Fny-CMVA2bpro caused very mild mosaic symptom in the upper systemic leaves (Fig. 3a), even by 30 days p.i., and significantly reduced virulence, when compared with wild-type Fny-CMV. FCb72b-CMVΔ2bpro also induced mild mosaic symptom early in infection similar to Fny-CMVA2b and Fny-CMVA2bpro, although the infected plants then appeared symptomless later in infection (Fig. 3a). Northern blot hybridization analysis of viral progeny RNAs in systemically infected leaves showed that although RNA 3 of Fny-CMV accumulated to a similar level to RNA 3 of Fny-CMVA2b or Fny-CMVA2bpro, the other four RNAs (RNAs 1, 2, 4 and 4A) of Fny-CMV had higher accumulation levels than those of Fny-CMVA2b or Fny-CMVA2bpro, especially RNAs 4 and 4A (Fig. 3b). The difference in viral RNA accumulation profiles between FCb72b-CMV and FCb72b-CMVΔ2bpro was similar to that between Fny-CMV and either Fny-CMVA2bpro or Fny-CMVA2b. It also could be observed that there was a similar profile of viral RNA accumulation among Fny-CMVA2b, Fny-CMVA2bpro and FCb72b-CMVΔ2bpro. Taken together, these experiments showed that the C-terminal overlapping part of the 2a protein from a subgroup IA strain or a subgroup IB strain did not affect symptom expression and accumulation of viral progeny RNAs in systemic leaves. Rather, the differential virulence was induced by the 2b proteins and not the C-terminal overlapping parts of the 2a proteins. No effects of the C-terminal overlapping part of the 2a protein of Q-CMV or V-TAV were observed on symptom expression of CMV in N. glutinosa (Ding et al., 1995, 1996).

Different virulence was associated with the level of accumulation of viral progeny RNAs, rather than the rate of long-distance viral movement

Shi et al. (2002, 2003) found that the virulence mediated by the 2b gene was not correlated with the level of viral RNA.
Fig. 4. Northern blot hybridization analysis of viral progeny RNAs of Fny-CMV and the four intraspecies hybrid viruses in systemically infected leaves of N. glutinosa (a–d), N. tabacum (e) and N. benthamiana (f). Relative concentrations of viral RNAs accumulating in N. glutinosa are shown for RNAs 1 and 2 in panel (a), for RNA 3 in panel (b), for RNA 4 in panel (c) and for RNA 4A in panel (d). Total RNAs were extracted from systemically infected leaves of N. glutinosa at 1, 3, 5, 7, 14 and 28 days p.i., of N. tabacum at 28 days p.i., and of N. benthamiana at 14 days p.i. Viral progeny RNAs were hybridized with the probe40 probe as described in the text. The inoculum corresponding to each of these five viruses applied to panels (a–f) is indicated on the upper panel. Relative quantities of the RNA samples loaded were normalized against their 28S rRNA (data not shown). Relative concentration of viral RNAs in each RNA sample was calculated after normalization of loading quantities of these RNA samples against their 28S rRNA.
Viral RNAs were detected at 3 days p.i., but only for FCb72b-RNAs were detected for any of these viruses at 1 day p.i. relative concentrations were shown in Fig. 4(a–d). No viral blot hybridization using the probeI-40 probe, and their viral movement, viral RNAs were analysed by Northern levels of viral progeny RNAs and/or rates of long-distance viral movement (Gal-on et al., 1994). To investigate whether the differential virulence of the four intraspecies hybrid viruses and wild-type Fny-CMV was associated with accumulation levels of viral progeny RNAs and/or rates of long-distance viral movement, viral RNAs were analysed by Northern blot hybridization using the probe-40 probe, and their relative concentrations were shown in Fig. 4(a–d). No viral RNAs were detected for any of these viruses at 1 day p.i. Viral RNAs were detected at 3 days p.i., but only for FCb72b-CMV and FRad35pb-CMV and not for Fny-CMV, FNa2b-CMV or FPGs2b-CMV. At 5 days p.i., viral progeny RNAs of all the viruses were detected readily. RNAs 1 and 2 of Fny-CMV, FCb72b-CMV and FNa2b-CMV accumulated to a higher level than those of FPGs2b-CMV or FRad35pb-CMV (Fig. 4a). However, RNA 3 of FRad35pb-CMV had a higher accumulation than RNA 3 of the other four viruses (Fig. 4b), and RNA 4 of FRad35pb-CMV had an intermediate accumulation among all the viruses (Fig. 4c). Furthermore, RNAs 3, 4 and 4a of FCb72b-CMV had a relatively low accumulation among all the viruses (Fig. 4b–d). At 7, 14 and 28 days p.i., the accumulation levels of most progeny RNAs of FPGs2b-CMV and FRad35pb-CMV were lower than those of Fny-CMV, FCb72b-CMV or FNa2b-CMV (Fig. 4a–d), except for the accumulation level of RNA 3 of FRad35pb-CMV, which was higher than RNA 3 of FNa2b-CMV at 7 days p.i. (Fig. 4b). To determine whether there was also a similar difference in accumulation levels of viral progeny RNAs in the other two Nicotiana species, viral progeny RNAs, extracted from systemically infected leaves of N. tabacum at 28 days p.i. and N. benthamiana at 14 days p.i., were analysed by Northern blot hybridization using the probe-40 probe. Although accumulation levels of the progeny RNAs of Fny-CMV, FCb72b-CMV and FNa2b-CMV were different to each other, all of them were higher than those of FPGs2b-CMV or FRad35pb-CMV in the two other host species (Fig. 4e, f). Taken together, the higher virulence caused by Fny-CMV, FCb72b-CMV and FNa2b-CMV was associated generally with the high levels of accumulation of their progeny RNAs, but not with their rates of long-distance movement in the systemic leaves.

CMV-encoded 2b protein was identified to be a suppressor of post-transcriptional gene silencing (PTGS) (Brigneti et al., 1998). Suppression of PTGS by 2b protein correlated with its localization in the nucleus, and it was important for virulence determination (Lucy et al., 2000). Each of the four 2b proteins tested here has an arginine-rich nuclear localization signal: 22KRQRRRR27 in the 2b proteins of Cb7-CMV and Rad35-CMV, 22KKQRRRR27 in the 2b protein of PGs-CMV, and 22KQLRRRR27 in the 2b protein of Na-CMV, presumably suggesting that the four 2b proteins could efficiently localize in the nucleus. Recently, Zhang et al. (2006) found that the Fny2b protein interfered with microRNA (miRNA) pathways by direct interaction with the AGO1 (Zhang et al., 2006), causing developmental defects in Arabidopsis. The interference in miRNA pathways posed by the Fny2b protein targeted some selectively but not all miRNAs (Lewsey et al., 2007). Although the LS2b protein from the mild strain (LS-CMV) was equally efficient as the Fny2b protein at suppressing PTGS (Lewsey et al., 2007), it and the Q2b protein from another mild strain (Q-CMV) had little effect on miRNA function and Arabidopsis development (Chapman et al., 2004; Lewsey et al., 2007), probably either due to absence of a specific symptom-inducing functional domain in them (discussed in Lewsey et al., 2007), or due to their instability and low expression levels in vivo (discussed in Zhang et al., 2006). Potyviral HC-Pro protein, as one of the first identified suppressors of PTGS, was shown to contain a symptom-inducing functional site or domain (Atreya & Pirone, 1993; Gal-on, 2000). The Fny2b protein and these four 2b proteins tested here share 70.0–93.7% identity in their amino acid sequences. The obvious difference in the sequences suggests that these four 2b proteins possess differential functional sites or domains involved in interrupting miRNA metabolism. Presumably, the 2b proteins of Cb7-CMV and Na-CMV, like the Fny2b protein, were more effective at interrupting miRNA metabolism than the 2b protein of either PGs-CMV or Rad35-CMV, by which Fny-CMV, FCb72b-CMV and FNa2b-CMV all induced higher virulence than FPGs2b-CMV or FRad35pb-CMV in these three Nicotiana species tested (Table 2). Our results showed that the high virulence of Fny-CMV, FCb72b-CMV and FNa2b-CMV was concomitant with the high accumulation level of their viral progeny RNAs in systemically infected leaves (Fig. 4), which was probably a consequence of these viruses directing hosts to produce a cellular niche advantageous for their proliferation by altering host miRNA metabolism (discussed in Zhang et al., 2006).

In summary, the different virulence resulting from the replacement of the 2b ORFs was caused by the 2b proteins, rather than the C-terminal overlapping parts of the 2a proteins. The CMV 2b protein plays an important role in symptom expression in Nicotiana species whether in a subgroup IA CMV strain or a subgroup IB CMV strain; the virulence of 2b proteins from subgroup IB strains varied, which correlated with the level of viral RNA accumulation, rather than the rate of long-distance viral movement.

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