A cucumber mosaic virus mutant lacking the 2b counter-defence protein gene provides protection against wild-type strains

Heiko Ziebell,1 Tina Payne,2 James O. Berry,3 John A. Walsh2 and John P. Carr1

Correspondence
John P. Carr
jpc1005@hermes.cam.ac.uk

1Department of Plant Sciences, University of Cambridge, Cambridge CB2 3EA, UK
2Warwick HRI, Wellesbourne, Warwick CV35 9EF, UK
3Department of Biological Sciences, State University of New York at Buffalo, NY 14260, USA

Several plant virus mutants, in which genes encoding silencing suppressor proteins have been deleted, are known to induce systemic or localized RNA silencing against themselves and other RNA molecules containing homologous sequences. Thus, it is thought that many cases of cross-protection, in which infection with a mild or asymptomatic virus mutant protects plants against challenge infection with closely related virulent viruses, can be explained by RNA silencing. We found that a cucumber mosaic virus (CMV) mutant of the subgroup IA strain Fny (Fny-CMVΔ2b), which cannot express the 2b silencing suppressor protein, cross-protects tobacco (Nicotiana tabacum) and Nicotiana benthamiana plants against disease induction by wild-type Fny-CMV. However, protection is most effective only if inoculation with Fny-CMVΔ2b and challenge inoculation with wild-type CMV occurs on the same leaf. Unexpectedly, Fny-CMVΔ2b also protected plants against infection with TC-CMV, a subgroup II strain that is not closely related to Fny-CMV. Additionally, in situ hybridization revealed that Fny-CMVΔ2b and Fny-CMV can co-exist in the same tissues but these tissues contain zones of Fny-CMVΔ2b-infected host cells from which Fny-i-CMV appears to be excluded. Taken together, it appears unlikely that cross-protection by Fny-CMVΔ2b occurs by induction of systemic RNA silencing against itself and homologous RNA sequences in wild-type CMV. It is more likely that protection occurs through either induction of very highly localized RNA silencing, or by competition between strains for host cells or resources.

INTRODUCTION

Cucumber mosaic virus (CMV) is the type species of the genus Cucumovirus of the family Bromoviridae (Van Regenmortel et al., 2000). Based on serological relationships and sequence criteria, the species is divided into three distinct subgroups: IA, IB and II (Roossinck et al., 1999). CMV has the largest host range of any known plant virus and is transmitted in a non-persistent manner by aphids belonging to over 80 species. Taken together, these factors probably contribute to the worldwide distribution of the virus and its economic importance (Palukaitis et al., 1992; Palukaitis & García-Arenal, 2003). The CMV genome consists of three positive-sense RNA molecules. These are RNAs 1, 2 and 3, which also function as mRNAs for the synthesis of the 1a and 2a replicase proteins, and the 3a movement protein, respectively. During replication, subgenomic mRNAs encoding additional proteins are synthesized. The subgenomic RNA 4, derived from RNA 3, is the mRNA for CMV coat protein (CP) and RNA 4A, which is derived from RNA 2, is the mRNA for the multifunctional 2b protein (Ding et al., 1994).

Direct control of CMV or prevention of its transmission by aphids by using insecticides is difficult to achieve. One promising approach for controlling CMV is the use of pathogen-derived transgenes in genetically modified plants (Palukaitis & Zaitlin, 1997; Gaba et al., 2004). Pathogen-derived resistance to CMV and other viruses works either through the triggering of RNA silencing against the transgene-encoded RNA, or through disruption of one or more stages of viral infection by constitutive expression of wild-type or mutant viral proteins by the host plant (Hellwald & Palukaitis, 1995; Beachy, 1997; Winterrmantel & Zaitlin, 2000; Lindbo & Dougherty, 2005). Another means of providing protection through interference with the life cycle of a virus is cross-protection. Cross-protection is a phenomenon in which infection with a mild virus strain protects a plant against infection by closely related, more severe strains of the same virus. Cross-protection was
described as early as the 1920s by McKinney (1929) who showed that tobacco plants that had previously been inoculated with a tobacco mosaic virus (TMV) strain causing mild green mosaic symptoms were resistant to a subsequent challenge with a TMV strain that caused yellow mosaic symptoms. Cross-protection has been deployed commercially against a variety of viruses, including TMV and tomato mosaic virus (Rast, 1967a, b), papaya ringspot virus (Yeh & Gonsalves, 1984) and citrus tristeza virus (Costa & Muller, 1980), as well as CMV (Rodriguez Alvarado et al., 2001).

The mechanism, or mechanisms, behind cross-protection has remained obscure but a number of explanations have been proposed. Currently, the leading hypothesis used to explain cross-protection is that the protective strain induces RNA silencing against its own RNA and homologous sequences, such as those occurring in closely related strains of the same virus (Ratcliff et al., 1999; Hull, 2002; Gal-On & Shibolet, 2006). Thus, it is hypothesized that the protective strain is acting as an elicitor of a natural antiviral response, RNA silencing, which underlies other natural resistance phenomena, such as recovery and ‘green island’ formation, as well as many instances of pathogen-derived resistance in transgenic plants (Ratcliff et al., 1997; Moore et al., 2001; Voinnet, 2001; Goldbach et al., 2003). Other ideas that have been used to explain cross-protection include competition between protective and challenge virus strains for host cells, intracellular replication sites, host translational apparatus and/or other host factors, or inhibitory interactions between the proteins or nucleic acids of the competing viral strains (Hull & Plaskitt, 1970; Palukaitis & Zaitlin, 1984; Sequeira, 1984; Hull, 2002). Mechanisms such as these may explain ‘exclusion’, in which closely related strains of the same virus infect adjacent cells but do not produce mixed infections within the same host cell (Dietrich & Maiss, 2003; Hull & Plaskitt, 1970).

Many, if not most, viruses have adapted to host resistance mediated by RNA silencing by acquiring silencing suppressor proteins that enable them to evade or blunt the effect of this defence mechanism (Voinnet et al., 1999). Viral suppressor proteins target different points of the machinery regulating induction, amplification and maintenance of RNA silencing (Palukaitis & MacFarlane, 2006), for example by interacting with the small interfering (si) RNAs that confer specificity on RNA silencing (Chapman et al., 2004; Lakatos et al., 2004).

CMV encodes a well studied suppressor of RNA silencing, the 2b protein (Brigneti et al., 1998; Guo & Ding, 2002; Lewsey et al., 2007; Mlotshwa et al. 2002; Zhang et al., 2006). The 2b protein can also act as a symptom determinant; it induces disease symptoms by interfering with microRNA-mediated gene regulation (Lewsey et al., 2007; Zhang et al., 2006). However, the severity of the symptoms induced depends upon the CMV strain and the effects on the host plant of environmental and physiological factors (Handford & Carr, 2007; Lewsey et al., 2007; Zhang et al., 2006).

Genetic engineering of viruses may provide a means of designing and generating mild, potentially cross-protective virus strains (discussed by Gal-On & Shibolet, 2006). We speculated that if RNA silencing is the mechanism behind cross-protection, then a mutant virus lacking the ability to express a silencing suppressor would be a particularly potent cross-protecting agent. This is because the mutant might act as a trigger of silencing against its own RNA and homologous viral RNA sequences, but would lack the means to inhibit or evade the establishment of RNA silencing. In the present study, we investigated the ability of the CMV mutant CMVΔ2b, which is unable to express the 2b silencing suppressor protein, to cross-protect plants against infection with wild-type CMV strains.

**METHODS**

**Plants and viruses.** Tobacco (Nicotiana tabacum cv. xanthi-nc and xanthi) and *Nicotiana benthamiana* seeds were germinated in compost and grown in a glasshouse, with supplementary lighting when required. Infectious viral RNA from the subgroup IA CMV strain Fny-CMV (Roossinck & Palukaitis, 1990) and its deletion mutant Fny-CMVΔ2b was reconstituted by mixing *in vitro* transcription products of full-length cDNA clones encoding RNA 1 (pFny109), RNA 3 (pFny309), and wild-type RNA 2 (pFny209) or a mutant RNA 2 lacking the 2b open reading frame (ORE) (pFny209/M3), as described previously (Rizzo & Palukaitis, 1990; Ryabov et al., 2001; Soards et al., 2002). Equal volumes of transcripts were combined and gently rubbed with a frosted microscope slide onto tobacco leaves at the three-to-five leaf stage. To prepare inoculum for subsequent experiments, virions of CMV were extracted from the infected plant tissue according to the method of Roossinck & White (1998). A naturally occurring UK isolate of CMV, TC-CMV (N.J. Spence & A. Baker, unpublished data), was maintained in plants of zucchini squash (courgette) (*Cucurbita pepo* cv. ‘Goldrush’) and propagated for virion purification in tobacco plants. RNA 2 of TC-CMV has been sequenced and has the GenBank accession number EF40931. Experiments with TMV were carried out using xanthi, rather than xanthi-nc, which is resistant to this virus. The naturally occurring TMV mutant YSI/1 was used since it induces clearly observable yellow mosaic symptoms (Banerjee et al., 1995).

**Inoculation of plants and RNA extraction.** For cross-protection experiments, tobacco and *N. benthamiana* plants at the three-to-four leaf stage were inoculated with Fny-CMVΔ2b virions suspended in water at a concentration of 100 μg ml⁻¹. After a period of 9–18 days, the plants were challenge inoculated with Fny-CMV or TC-CMV at either 1 or 10 μg ml⁻¹. Samples for nucleic acid extraction were harvested from inoculated leaves as well as non-inoculated leaves immediately above the inoculated leaves, or from the uppermost non-inoculated leaves at various times between 17 and 25 days after the challenge inoculation. Nucleic acid was extracted using TRizol reagent (Invitrogen), DNA was degraded using RNAse-free DNase (Promega), and the RNA further purified using a Qiagen RNeasy Mini kit, according to the various manufacturers’ instructions.

**Detection of Fny-CMV and Fny-CMVΔ2b.** RT-PCR was used to detect, and distinguish between, the RNAs 2 of wild-type Fny-CMV and Fny-CMVΔ2b occurring in plant RNA samples. The primers were designed to amplify a region of RNA 2 sequence (nt 2367–3031) flanking the 2b ORF of wild-type Fny-CMV as well as the
corresponding region in the RNA 2 of Fny-CMVΔ2b containing a deletion in the 2b ORF (Ryabov et al., 2001) (Fig. 1a). Reverse transcription was carried out using the reverse primer (5'-CCACAAAAGTGGGGGGACCCG-3') followed by PCR using the reverse and forward primers (5'-AGTACAGATTCAGGTTGAGCGTG-3'). PCR reaction conditions were as follows: 94 °C 5 min, 94 °C 30 s, 65 °C 30 s, 72 °C 1 min 30 s for 30 cycles, final extension at 72 °C for 7 min. PCR products were analysed on 1 % (w/v) agarose gels. In some experiments, the presence of CMV (Fny-CMV or Fny-CMVΔ2b) was detected in leaf tissue by using a rapid immunodiagnostic test kit (Pocket Diagnostics).

Detection of viral RNAs in inoculated tissue by in situ hybridization. Tobacco leaves were simultaneously inoculated with Fny-CMV and Fny-CMVΔ2b on opposite surfaces of the leaf or on adjacent areas on the same surface. At 3–4 weeks after inoculation these co-inoculation zones were excised from leaves and prepared for in situ hybridization by using a protocol adapted from previously published methods (Long & Berry, 1996; Patel et al., 2004; Ruzin, 1999; Wang et al., 1993). Tissue samples were fixed by vacuum infiltration in 1 % (v/v) glutaraldehyde and incubated overnight at 4 °C, then dehydrated through an ethanol/tert-butyl alcohol series and embedded in Paraplast Plus (Monoject Scientific). In some cases, paraformaldehyde was used for fixation (Jackson, 1992). A microtome was used to prepare 5 μm transverse sections through embedded leaf tissues that were stained using ‘Superfrost Ultra plus’ slides (VWR) and dried overnight at 42 °C. Slides were deparaffinized by two 15 min incubation steps in xylene. Sections were rehydrated through an ethanol series consisting of 5 min sequential incubations in absolute ethanol (twice), 95, 80, 70, 50 and 30 % (v/v) ethanol. Slides were washed twice for 2 min with sterile, RNase-free water. After exposure to 0.2 M HCl for 20 min at room temperature and incubation in 2 x saline sodium citrate (SSC) at 70 °C for 30 min, sections were further incubated with 1 μg proteinase K ml⁻¹ for 30 min at 37 °C and then blocked with 2 mg glycine ml⁻¹ in PBS. Sections were fixed in 10 % (v/v) formalin, washed twice for 5 min in PBS and incubated for 10 min in 0.1 M triethanolamine buffer (pH 8.0) containing 0.5 % (v/v) acetic anhydride. The slides were washed twice for 5 min in PBS and dehydrated through an ethanol series (5 min for each sequential step): 30, 50, 70, 80, 95 % (v/v) and absolute ethanol (two incubations). Finally, the slides were dried and immediately used for pre-hybridization. Slides were incubated for 10 min in 2 x SSC before pre-hybridization and hybridization at 50 °C, followed by washing in various dilutions of SSC as described previously (Long & Berry, 1996; Patel et al., 2004; Wang et al., 1993).

Three biotin-labelled riboprobes were synthesized for use in hybridization. The first riboprobe was complementary to the 3' conserved region of all of the Fny-CMV RNAs. This riboprobe, which has been described previously (Carr et al., 1994; Gal-On et al., 1994), can act as a ‘general’ probe capable of detecting the presence of both wild-type Fny-CMV RNAs and the mutant CMV RNA 2 component of CMVΔ2b. The second riboprobe was designed to detect specifically to a region of sequence within the 2b ORF to detect the presence of wild-type RNA 2 but not the mutant RNA 2 of CMVΔ2b, which contains a deletion in this region (Ryabov et al., 2001; Soards et al., 2002). This probe was synthesized by in vitro transcription of a DNA template generated by PCR from wild-type Fny-CMV RNA 2 cDNA sequence using the primers CMV2BF (5'-GAACGAGGTCACAAAAGTCC-3') and CMV2.R_T7 (5'-TAATACGACTCAGTGTCACAAAAGTCC-3'). The use of primer CMV2.R_T7 introduces a T7 RNA polymerase promoter sequence into the transcription template. The third riboprobe was used as a negative control and was complementary to an approximately 300 bp long green fluorescent protein (gfp) sequence. Primers GFP-F3 (5'-CGTGCTGAAGTCAGATTGT-3') and GFP-R3_T7 (5'-TAATACGACTCAGTGTCACAAAAGTCC-3') were used to

Fig. 1. Detection of mutant and wild-type CMV by RT-PCR. (a) Schematic map of Fny-CMV RNA 2 with ORFs for the 2a and 2b proteins indicated by boxes. The forward (5'-AGTACAGAGTTCAGGTTGAGCGTG-3') and reverse (5'-CCACAAAAGTGGGGGGACCCG-3') primers were designed to amplify the sequences between nt 2367 and 3031 (target region indicated with arrow heads) of RNA 2 of Fny-CMV. This includes the ORF for the 2b protein (nt 2419–2749), which is deleted between nt 2419 and 2713 of RNA 2 of Fny-CMVΔ2b (indicated by the dotted line). Consequently, the RT-PCR product generated using Fny-CMVΔ2b RNA as a template is 370 bp, whereas the product from wild-type RNA 2 is 664 bp (b). (c) A selection of RT-PCR results for a cross-protection experiment is shown. Plants were either mock-inoculated or inoculated with 100 μg ml⁻¹ Fny-CMVΔ2b, prior to challenge with either 1 or 10 μg ml⁻¹ Fny-CMV. Numbers indicate individual plants and L and U indicate lower inoculated leaf and upper, non-inoculated leaf, respectively. X represents an empty lane.
amplify a fragment from pF:GFP/CP (Canto et al., 1997) introducing the T7 promoter sequence for in vitro transcription of antisense RNA. The riboprobes were labelled with biotin by including biotin-16-uridine-5'-triphosphate in the transcription reactions, according to the instructions of the manufacturer (Roche Applied Sciences). Biotin-labelled riboprobe binding to tissue sections was detected by using streptavidin-alkaline phosphatase conjugate (NeutrAvidin; Pierce Biotechnology) and the Sigma Fast 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium alkaline phosphate substrate. Developed slides were imaged by using a Nikon ECLIPSE 50i microscope and images were recorded by using a digital camera control unit (Nikon).

**RESULTS**

**Fny-CMVΔ2b protects against a challenge infection with wild-type Fny-CMV**

In tobacco, Fny-CMVΔ2b infections are symptomless, whereas the wild-type subgroup IA strain Fny-CMV induces strong symptoms such as leaf distortion and severe stunting of whole plants (Figs 2 and 3 and Soards et al., 2002). Plants that had previously been inoculated with Fny-CMVΔ2b remained symptomless even when they were later challenged with the wild-type virus on the same leaf. In contrast, plants that had not been pre-inoculated

<table>
<thead>
<tr>
<th>Primary inocula</th>
<th>Fny-CMVΔ2b</th>
<th>Mock</th>
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<tr>
<td>Challenge inocula</td>
<td>Fny-CMV</td>
<td>TC-CMV</td>
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<td>Fny-CMV</td>
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<td>Fny-CMVΔ2b</td>
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<td>TC-CMV</td>
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Fig. 2. Fny-CMVΔ2b protects tobacco against disease induction by wild-type CMV strains. Tobacco plants that were pre-inoculated with a primary inoculum of Fny-CMVΔ2b were protected not only from challenge with the wild-type Fny-CMV (CMV subgroup IA) but also from challenge with TC-CMV (a member of CMV subgroup II). In contrast, mock-inoculated plants subsequently challenged with either of the wild-type viruses showed significant stunting of growth compared with the mock-inoculated control plant. Plants photographed at 19 days post-challenge.

Fig. 3. Fny-CMVΔ2b cross-protects against its wild-type parental strain but not against an unrelated virus. Strong symptoms including severe leaf distortion were caused on upper, non-inoculated leaves of tobacco (cv. xanthi) by Fny-CMV (photographed at 27 days post-inoculation). Upper leaves from plants inoculated with Fny-CMVΔ2b, or with Fny-CMVΔ2b followed by wild-type Fny-CMV, did not become distorted and were similar in appearance to leaves from mock-inoculated plants. As expected, pre-inoculation with Fny-CMVΔ2b did not protect against challenge by an unrelated virus, TMV strain YSI/1. Bar, 5 cm. See colour version as supplementary Fig. S3 in JGV Online.

with Fny-CMVΔ2b showed typical Fny-CMV-induced symptoms of stunting and distortion of the leaves (Figs 2 and 3). Pre-inoculation with Fny-CMVΔ2b protected plants against Fny-CMV challenge inocula at concentrations of, at least, up to 10 μg ml⁻¹ (Supplementary Fig. S1 available in JGV Online). Experiments in which plants were infected with Fny-CMVΔ2b and challenged with Fny-CMV were carried out independently six times comprising more than 108 plants in total, and out of 60 Fny-CMVΔ2b-infected plants challenged with Fny-CMV none exhibited
stunting or any other disease symptoms characteristic of infection by the wild-type virus (Table 1).

Classical cross-protection works most effectively between closely related strains of the same virus. To exclude the possibility that Fny-CMVΔ2b triggers a general plant defence mechanism against all viruses, plants were challenged with an unrelated virus. TMV strain YSI/1 is a yellowing strain of TMV that induces strong, easily observable symptoms on tobacco (Banerjee et al., 1995). As expected, Fny-CMVΔ2b did not protect plants against challenge infection with TMV YSI/1 (Fig. 3). To explore the broadness of protection provided by Fny-CMVΔ2b, we tested whether it could protect against disease induction by TC-CMV, a CMV subgroup II strain that has an RNA 2 sequence with 70% similarity to the subgroup IA strain Fny-CMV (unpublished data). Surprisingly, in three independent experiments (24 plants) Fny-CMVΔ2b was able to protect against challenge with TC-CMV (Fig. 2 and data not shown).

To detect the presence of the protective mutant and challenge wild-type viruses in infected plants, RT-PCR was used to analyse total RNA extracted from upper non-inoculated leaves (Fig. 1b–c). In all plants from four separate experiments that had been inoculated with Fny-CMVΔ2b and subsequently challenged with wild-type Fny-CMV on the same leaf (total of 22 plants), no accumulation of wild-type viral RNA was detected by RT-PCR in the inoculated leaves, whereas over 75% of these leaves contained detectable levels of Fny-CMVΔ2b RNA at 21 days post-challenge (Fig. 1c and data not shown). From the analysis of viral RNA occurring in the upper, non-inoculated leaves, over 63% of plants showed evidence of systemic spread of Fny-CMVΔ2b (Table 1). Interestingly, four plants (7.4%) in one of the experiments contained low but detectable amounts of challenge strain RNA (Table 1 and examples in Fig. 1c). Nevertheless, these plants, like all others challenged with the wild-type virus, were symptom free and contained detectable levels of RNA 2 of the Fny-CMVΔ2b mutant. Overall, the results indicate that in the majority of cases inoculation of tobacco plants with Fny-CMVΔ2b protects them against the disease symptoms induced by Fny-CMV and against detectable levels of infection with the challenging virus (Table 1). However, it appears that inoculation with Fny-CMVΔ2b cannot completely prevent some host cells becoming infected with the challenge virus and, in a small proportion of challenged plants, giving rise to a low level, asymptomatic infection with the challenge virus.

### Cross-protection by Fny-CMVΔ2b also works against wild-type Fny-CMV in N. benthamiana plants

Due in part to defects in its RNA silencing system and the properties of its plasmodesmata the plant *N. benthamiana* is exceptionally susceptible to a wide range of wild-type viruses as well as mutants that spread poorly in other hosts (Christie & Crawford, 1978; Howard et al., 2004; Murphy et al., 2004; Yang et al., 2004). We were curious to see if this highly susceptible host plant is protected by Fny-CMVΔ2b against challenge with Fny-CMV. Plants were inoculated with Fny-CMVΔ2b at a concentration of 100 µg ml⁻¹ and challenged after a period of 11–15 days on the same leaf with wild-type Fny-CMV at concentration of 10 µg ml⁻¹. Symptoms were observed over a period of 4 weeks following the challenge inoculation. In three independent experiments, 12 plants that had previously been inoculated with Fny-CMVΔ2b were protected against the symptoms of Fny-CMV infection. In contrast, unprotected plants challenged with Fny-CMV showed symptoms of vein clearing, yellowing, leaf distortion and stunting of whole plants after 15 days. The results demonstrate that the protection induced by Fny-CMVΔ2b against Fny-CMV also works in hosts other than tobacco (Fig. 4).

### The relative locations on the plant of primary and challenge inoculations affect the degree of cross-protection

In all experiments up to this point, both the protective and challenge inoculation were carried out using the same leaf. Although this was a useful method for establishing whether or not Fny-CMVΔ2b could protect plants from Fny-CMV and TC-CMV, it is not a realistic model for the potential practical application of Fny-CMVΔ2b-mediated cross-protection. Therefore, experiments were conducted in which Fny-CMVΔ2b-inoculated plants were challenged on a different leaf. Tobacco plants were inoculated with Fny-CMVΔ2b and 6 days later tested with an immunodiagnostic test kit to ensure that the inoculation had been successful. Confirmed Fny-CMVΔ2b-infected plants were then inoculated with Fny-CMV on a randomly chosen upper leaf 8 days following the primary inoculation. Symptom development was monitored for 8 weeks following the first inoculation, after which RNA was extracted from the upper leaf.

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**Table 1.** Symptom expression and viral RNA accumulation in plants inoculated sequentially with Fny-CMVΔ2b and Fny-CMV

<table>
<thead>
<tr>
<th>Primary inoculation</th>
<th>Challenge inoculation</th>
<th>Disease symptoms*</th>
<th>Wild-type RNA 2†</th>
<th>Fny-CMVΔ2b (Δ2b) RNA 2†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fny-CMVΔ2b</td>
<td>Fny-CMV</td>
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<td>4/54</td>
<td>34/54</td>
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<td>Fny-CMV</td>
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<td>14/19</td>
<td>0/19</td>
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</tr>
<tr>
<td>Mock</td>
<td>Mock</td>
<td>0/13</td>
<td>0/11</td>
<td>0/11</td>
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</tbody>
</table>

*Stunting of plant growth and deformation and/or mosaic symptoms in non-inoculated leaves.
†The presence of mutant and wild-type RNA 2 sequences was assessed by RT-PCR (for strategy and examples see Fig. 1) in upper, non-inoculated leaves of a subset of plants used in the study of disease symptom development.
non-inoculated leaves of the plants (Fig. 5). Fny-CMVΔ2b-infected *N. benthamiana* plants were challenge inoculated at 15 days post-inoculation, monitored for symptom development for 6 weeks, at which point samples were taken for RT-PCR analysis (data not shown). In both tobacco and *N. benthamiana* plants, a total of 16 plants inoculated with Fny-CMVΔ2b and subsequently challenged on the same leaf with Fny-CMV did not develop any local or systemic symptoms at all, consistent with previous results. However, almost 50% of plants challenged with Fny-CMV on a different leaf (total of 17 plants) were not protected from the induction of visible systemic disease symptoms. RT-PCR analysis showed that the upper leaves of plants displaying symptoms contained wild-type Fny-CMV RNA, whereas equivalent samples from symptomless plants contained Fny-CMVΔ2b RNA (Fig. 5 and data not shown).

**In situ** hybridization of doubly infected tobacco leaves

Because cross-protection by Fny-CMVΔ2b did not appear to be caused by induction of systemic RNA silencing it was possible that the mutant was inhibiting infection by the challenging virus by excluding it. That is, by competition for host cells. We predicted that if this idea was correct, co-infected tissue would contain islands of cells infected only by the mutant. It was possible to investigate the distribution of the two viruses in doubly infected tobacco leaf tissue by taking sequential, serial sections through the same piece of inoculated leaf tissue and incubating them with biotin-labelled riboprobes specific to either the 2b sequence of Fny-CMV or the 3'-terminal sequence shared by the RNAs 2 of Fny-CMV and Fny-CMVΔ2b (Fig. 6). In sections of tissue where infection of cells by both viruses had occurred, sectors of tissue were labelled by the 3'-end-specific riboprobe, but not by the probe specific for the 2b gene sequence, while nearby regions of tissue were labelled by both riboprobes (Fig. 6 and Supplementary Fig. S2 for additional controls and examples). This indicated that cells infected with Fny-CMVΔ2b had not become doubly infected with the wild-type virus and the data suggest that cells infected with the mutant resist entry by the wild-type virus. Since RNA sequences belonging to both the wild-type and mutant versions of Fny-CMV were readily detectable in adjacent cells, the data further support the idea that infection with Fny-CMVΔ2b does not induce strong systemic silencing of homologous RNA sequences.

**DISCUSSION**

A genetically engineered mutant of Fny-CMV, lacking the ability to express the 2b silencing suppressor protein,
induced cross-protection against wild-type Fny-CMV in two species of *Nicotiana*. Fny-CMVΔ2b also protected against a less closely related strain, TC-CMV. One of the leading theories put forward to explain cross-protection is that the protective virus strain triggers RNA silencing directed against homologous sequences occurring in the genome of the challenged strain (Kurihara & Watanabe, 2003; Ratcliff *et al.*, 1999; Valkonen *et al.*, 2002). On the face of it, the cross-protection afforded by Fny-CMVΔ2b appears to be consistent with this hypothesis. This is because a virus lacking an RNA silencing suppressor should be a particularly effective cross-protecting strain by virtue of inducing RNA silencing against itself and viral strains possessing homologous sequences.

Previous work has shown that viral mutants compromised in the expression of a silencing suppressor can induce protection against homologous viral sequences. Mutant tombusviruses unable to express the gene for the P19 silencing suppressor protein can infect plants, but the plants subsequently recover from infection due to the degradation of viral RNA mediated by virus-specific siRNAs (Silhavy *et al.*, 2002). Recovered plants show silencing-mediated resistance to viral constructs with similarity to the inducing virus. Thus, plants initially infected with Cym19stop, a mutant of cymbidium ringspot virus (CymRSV), were resistant to infection by potato virus X (PVX)-derived vectors carrying CymRSV sequences (Szittya *et al.*, 2002).

Some of our findings appear to be inconsistent with a model for Fny-CMVΔ2b-mediated cross-protection based on RNA silencing. For example, the ability of the mutant, which is derived from a subgroup IA strain, Fny-CMVΔ2b,
Our results indicate that it is unlikely that infection with Fny-CMV sequence was derived (Carr et al., 1994; Hellwald & Palukaitis, 1995; Wintermantel & Zaitlin, 2000; Zaitlin et al., 1994). Early in infection of tobacco, wild-type CMV and TC-CMV share only 70% sequence similarity overall. Thomas et al. (2001) determined that 23 nt of identity or near identity between sequences was the minimum needed in principle to generate identity-based silencing, but alignment of the two RNA 2 sequences indicates that they have few regions in common with identical sequence exceeding 20 nt (data not shown). In contrast, most conventional examples of cross-protection only work when the strains are more closely related than this (Hull, 2002). Similarly, where plants have been genetically engineered to resist CMV with a virus-derived transgene and the protection results from a combination of RNA-mediated and protein-mediated mechanisms, resistance is only effective against CMV strains belonging to the same subgroup as the virus strain from which the transgene sequence was derived (Carr et al., 1994; Hellwald & Palukaitis, 1995; Wintermantel & Zaitlin, 2000; Zaitlin et al., 1994).

Our results indicate that it is unlikely that infection with Fny-CMVΔ2b results in the generation of a strong systemic silencing signal directed against CMV-specific RNA sequences. It was found that the location of the challenge inoculation site relative to the site inoculated with the protective strain had a clear effect on the degree of protection. Thus, when tobacco and N. benthamiana plants were challenged with Fny-CMV on a leaf different from that inoculated with Fny-CMVΔ2b, almost 50% of plants displayed symptoms typical of the challenging virus, although the progression of disease in these plants was slowed down by up to a week (Fig. 5 and data not shown).

Since Fny-CMVΔ2b appeared unlikely to be providing protection based on its similarity to sequences in the challenge viruses or by inducing a strong systemic silencing signal, this suggested that one of two potential mechanisms might explain how this form of cross-protection may operate. Firstly, Fny-CMVΔ2b might induce highly localized, non-systemic RNA silencing against homologous sequences in the challenging virus. Highly localized RNA silencing can be induced by viral mutants lacking the gene for a silencing suppressor protein; for example, a P38 (a CP) deletion mutant of tobacco crinkle virus (Ryabov et al., 2004). Highly localized RNA silencing, without detectable accumulation of siRNAs, can also occur in certain lines of transgenic plants overexpressing the gfp reporter gene (Kalantidis et al., 2006). Secondly, it is possible that the presence of the protecting virus may exclude the challenge strain from cells that it has infected by occupying sites within the host cell or titrating out host factors needed by the challenging virus strain.

Mutual exclusion of closely related strains of the same virus has been demonstrated previously. For example, it was found that two genetically modified versions of the same plum pox virus (PPV) strain, one expressing GFP and the other a red fluorescent protein, very infrequently infected the same host cells (Dietrich & Maiss, 2003). In contrast, PPV expressing either of the fluorescent proteins was able to co-infect cells with PVX expressing GFP or the red fluorescent protein (Dietrich & Maiss, 2003). Hull & Plaskitt (1970), using electron microscopy to identify strain-specific ultrastructural features in infected cells, demonstrated a similar exclusion effect in tissues infected with closely related strains of alfalfa mosaic virus. Similarly, using in situ hybridization, Takeshita et al. (2004) found that two strains of CMV did not mix in cells of co-infected cowpea plants. Interestingly, these CMV strains belonged to different CMV subgroups (Takeshita et al., 2004), possibly making it less likely that this exclusion effect resulted from RNA silencing.

Early in infection of tobacco, wild-type CMV and CMVΔ2b move preferentially into different cell types with the mutant moving more rapidly into and through the mesophyll cell layer, which is the predominant cell type in leaves (Soards et al., 2002). To investigate the possibility that Fny-CMVΔ2b excludes wild-type CMV from the cells it infects, we investigated the relative distribution of wild-type Fny-CMV and Fny-CMVΔ2b in doubly infected tissue by using in situ hybridization. Our prediction that we should observe zones of cells infected with Fny-CMVΔ2b immediately adjacent to cells harbouring wild-type viral sequences was substantiated. The fact that we could observe this pattern of viral RNA distribution in simultaneously inoculated areas of tissue is suggestive that exclusion of the unmodified virus by Fny-CMVΔ2b does not require induction of RNA silencing. However, our results cannot exclude a role for highly localized silencing and only further experiments using mutant plants compromised in the silencing machinery (for example, see Deleris et al., 2006) may provide a definitive answer.

Recently, it was described how introduction of specific mutations into the gene for the HC-Pro-silencing suppressor protein of zucchini yellow mosaic virus (ZYMV) yielded mild strains that cross-protected host plants from a severe ZYMV strain (Lin et al., 2007). Despite this, the HC-Pro of the mild ZYMV mutant was still an effective suppressor of RNA silencing (Lin et al., 2007). These results, together with our data on cross-protection afforded by Fny-CMVΔ2b, show that modification or deletion of genes encoding silencing suppressor proteins is a viable method of generating mild or non-symptom-inducing strains for testing as potential cross-protecting agents. However, it may not necessarily follow that the cross-protecting properties of these mutant viruses are due to the induction of RNA silencing against homologous sequences in the challenge virus.

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