Cross-protection is the phenomenon in which infection of a plant with a mild virus strain protects it from disease if it is subsequently challenge-inoculated with a severe strain of the same virus (Hull, 2002; Ziebell, 2008). Despite many years of study since its discovery by McKinney (1929), cross-protection is still poorly understood mechanistically (Ziebell, 2008). In certain instances, successful cross-protection between viral strains involves the coat protein, presumably where the presence of the coat protein of the protective strain inhibits the uncoating of challenge-strain virions (Culver, 1996; de Zoeten & Fulton, 1975; Koo et al., 2004; Lu et al., 1998; Sherwood, 1987; Sherwood & Fulton, 1982). However, not all instances of cross-protection can be explained this way (Gerber & Sarkar, 1989; Zaitlin, 1976). Coat protein-mediated effects cannot explain cross-protection between viroid strains or protection against virus infection provided by most satellite RNAs, with the exception of turnip crinkle virus–satellite RNA interactions (Gallitelli et al., 1991; Manfre & Simon, 2008; Montasser et al., 1991; Nibllett et al., 1978; Sayama et al., 1993, 2001; Tien & Wu, 1991).

RNA silencing is a potential cross-protection mechanism (Gal-On & Shibolet, 2006; Ratcliff et al., 1997, 1999). We explored this concept by using a mutant of the Fny strain of cucumber mosaic virus (Fny-CMV) lacking the 2b silencing-suppressor protein gene (Fny-CMVΔ2b), reasoning that this deletion mutant might induce silencing against RNA sequences shared with the wild-type virus (Ziebell et al., 2007). Although CMVΔ2b protected to some extent against subsequent infection with Fny-CMV and another CMV strain, there was no indication that this mutant induced a strong, systemic silencing signal (Ziebell et al., 2007). In situ hybridization showed that, although both strains occurred in doubly infected leaves, they did not co-infect the same cells (Ziebell et al., 2007). Such spatial separation (exclusion) has been described for mixed infections between several virus strains (Dietrich et al., 2007; Hull & Plaskitt, 1970; Takahashi et al., 2007; Takeshita et al., 2004). However, it is not known whether exclusion results from highly localized RNA silencing or from competition between virus strains for host cells or cellular resources.

To test the idea that localized RNA silencing induced by the protective strain may underlie cross-protection between Fny-CMVΔ2b and Fny-CMV, we carried out experiments in Arabidopsis thaliana plants (ecotype Col-0) harbouring mutations in genes for dicer-like (DCL) endoribonucleases (Deleris et al., 2006). A. thaliana has four DCL enzymes that use small RNAs to direct
homology-dependent cleavage of host or viral RNA targets and that are required for specific types of RNA silencing (Deleris et al., 2006). DCL4 is the primary antiviral DCL enzyme, but DCL2 can substitute for it to some extent in dcl4 mutants (Deleris et al., 2006). In addition, DCL3 may also play a minor role in antiviral silencing, with DCL1 having an indirect effect through negative regulation of DCL4 and DCL3 gene expression (Qu et al., 2008). Cross-protection experiments were carried out using dcl4-2 (n=23 plants), dcl2-1 (n=16), dcl3-1 (n=12), dcl2-1/4-2 double mutant (n=16) and dcl2-1/3-1/4-2 triple mutant (n=20) plants. These mutant lines have been described by Deleris et al. (2006).

Wild-type and mutant A. thaliana (ecotype Col-0) seeds were stratified in darkness at 4 °C on moist compost for 5 days before transfer to a growth room (Conviron) for germination of seeds and plant growth at 21 °C with a photoperiod of 8 h light (200 μE m⁻² s⁻¹) and 16 h darkness. Using a glass rod (1 cm in diameter, with a roughened tip), plants at the three- to five-true leaf stage were inoculated mechanically on three leaves with either Fny-CMVΔ2b or Fny-CMV (both at a concentration of 200 μg ml⁻¹) or were mock-inoculated. Eight to 12 days following primary inoculation, plants were challenged with a secondary inoculum of either Fny-CMV or Fny-CMVΔ2b, applied to the same leaves or, where this was not possible, to an immediately adjacent leaf.

Plants were photographed 4 weeks after challenge inoculation and samples were collected to authenticate virus infection in one of two ways. In some cases, a previously described RT-PCR assay (Ziebell et al., 2007) was used to detect and distinguish between the RNAs 2 of Fny-CMVΔ2b and Fny-CMV, using systemically infected leaf tissue for RNA extraction. When it was not necessary to distinguish between the two virus strains, an ELISA was used. For ELISA, plant tissue was freeze-dried, its dry mass was determined and it was processed further by using a Bioreba CMV ELISA Complete kit to detect viral coat protein accumulation, following the manufacturer’s instructions. A₄0₅ was determined 30 min after adding p-nitrophenylphosphate substrate with a Titertek Multiscan PLUS MKIII. Using purified Fny-CMV, a calibration curve was drawn in OpenOffice (Sun Microsystems) to quantify virus levels.

As observed previously, infection with Fny-CMVΔ2b did not induce symptoms in wild-type plants of ecotype Col-0 (Fig. 1, row 1, column 2), whereas Fny-CMV caused severe stunting, crinkling and deformation of the youngest plant tissue, resulting in a compact appearance (Fig. 1, row 1, column 4) (Lewsey et al., 2009; Ziebell et al., 2007). Fny-CMVΔ2b was able to provide some cross-protection.

**Fig. 1.** Symptoms in cross-protection experiments in wild-type and dcl mutant A. thaliana plants. Plants (of wild-type Col-0 or the mutant or double mutant lines dcl2-1, dcl4-2 and dcl2-1/4-2) were inoculated (primary inoculum) with Fny-CMVΔ2b, Fny-CMV or were mock-inoculated, or left untreated (–). Ten days later, plants were challenge-inoculated (secondary inoculum) as indicated. Four weeks after challenge inoculation, plants were photographed and non-inoculated leaf samples were collected for further analysis. Bar, 8 cm.
against Fny-CMV in wild-type *A. thaliana* plants. Over 77% of Fny-CMVΔ2b-infected plants were protected against development of disease symptoms following challenge with Fny-CMV. The experiment was carried out four times using 40 plants in total. A typical protected plant is shown in Fig. 1 (row 1, column 3).

The responses of *dcl*-1 mutant and *dcl*-3 mutant plants to the two CMV variants and to challenge with Fny-CMV following a previous inoculation with CMVΔ2b were indistinguishable from those of wild-type *A. thaliana* plants (Fig. 1; Supplementary Fig. S1, available in JGV Online). In *dcl*-1 mutant plants, Fny-CMVΔ2b infection was symptomless (Fig. 1, row 2, column 2), but Fny-CMV infection provoked stunting of plants and crinkling of leaves (Fig. 1, row 2, column 4). Pre-inoculation with Fny-CMVΔ2b prevented symptom induction in *dcl*-1 mutant and *dcl*-3 mutant plants challenged with wild-type Fny-CMV (Fig. 1, row 2, column 3; Supplementary Fig. S1). The results showed that cross-protection works as well in *dcl*2 or *dcl*3 mutant plants as in wild-type *A. thaliana* plants. Interestingly, Fny-CMVΔ2b infection elicited stunting and leaf crinkling in *dcl*-4 mutant plants (Fig. 1, row 3, column 2). Fny-CMV infection also stunted the growth of *dcl*-4 plants and induced distortion of younger leaves (Fig. 1, row 3, column 4), but these symptoms were different from those induced by Fny-CMVΔ2b and were similar to those induced by CMV in wild-type *A. thaliana* (Fig. 1, row 1, column 4). The majority of *dcl*-4 plants inoculated with Fny-CMVΔ2b and subsequently challenged with Fny-CMV exhibited symptoms characteristic of infection with Fny-CMVΔ2b (Fig. 1, row 3, column 3). Thus, a form of ‘cross-protection’ occurred between Fny-CMVΔ2b and Fny-CMV in plants unable to express DCL4 activity.

Wild-type Fny-CMV and its deletion mutant Fny-CMVΔ2b both caused extremely severe symptoms in *dcl*-2/1/4-2 double mutant plants (Fig. 1, row 4, column 2; Supplementary Fig. S2, available in JGV Online). In this double-mutant background, infection with both viruses induced development of small tissue ‘domes’ in symptomically infected tissues (Fig. 1, row 4, column 2; Supplementary Fig. S2). Also, older leaves showed enhanced senescence compared with wild-type plants, resulting in early death (visible in some plants in Supplementary Fig. S2). The presence of longer, better-developed leaves in plants infected solely with Fny-CMV is due to the fact that these plants were inoculated 10 days later than those infected with Fny-CMVΔ2b (Fig. 1, columns 2 and 4, respectively). The reactions of triple mutant *dcl*-2/3/4 mutant plants to these infections were indistinguishable from those of the *dcl*-2/4 double mutant plants (Supplementary Fig. S1) and so were not investigated further. The effect of Fny-CMVΔ2b on *dcl*-1/4-2 double mutant plants is highly reminiscent of the effect of Q-CMVΔ2b on this plant line that was observed by Diaz-Pendon *et al.* (2007). However, a significant difference between the two studies is that, whilst Fny-CMV induces strong symptoms in Col-0, Q-CMV (the parent strain for Q-CMVΔ2b) induces only very mild symptoms in Col-0 plants with intact DCL2 and 4 genes (Diaz-Pendon *et al.*, 2007). Taken together, our data and those of Diaz-Pendon *et al.* (2007) indicate that the induction of symptoms by CMV is not determined exclusively by the 2b protein and is affected by the interplay between multiple viral gene products.

Based on visual symptoms, it could not be distinguished whether Fny-CMVΔ2b protected against infection with Fny-CMV in plants inoculated sequentially with the two viruses (Fig. 1, row 4, column 3). Therefore, plants were analysed by using RT-PCR to discriminate between wild-type and mutant CMV (Ziebell *et al.*, 2007). Between six and 11 plants (out of a total of 12–40 plants per mutant or wild-type line) that had been inoculated sequentially with Fny-CMVΔ2b followed by Fny-CMV (Fig. 1, column 3) were chosen randomly and used for this analysis (Table 1). In wild-type plants inoculated with Fny-CMVΔ2b and challenged with Fny-CMV, 73% of plants were infected solely with Fny-CMVΔ2b (Table 1). Almost one-third of these sequentially inoculated plants became infected with both viruses and showed signs of infection (Table 1). However, these results are consistent with our observations

Table 1. Prevalence of single and mixed infections in wild-type and *dcl* mutant *A. thaliana* Col-0 plants

<table>
<thead>
<tr>
<th>Plant line</th>
<th>No. of plants tested</th>
<th>No. (%) of plants infected with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CMVΔ2b</td>
</tr>
<tr>
<td>Wild-type</td>
<td>11</td>
<td>8 (73)</td>
</tr>
<tr>
<td><em>dcl</em>-2</td>
<td>9</td>
<td>5 (56)</td>
</tr>
<tr>
<td><em>dcl</em>-3</td>
<td>6</td>
<td>5 (83)</td>
</tr>
<tr>
<td><em>dcl</em>-4</td>
<td>7</td>
<td>6 (86)</td>
</tr>
<tr>
<td><em>dcl</em>-2/1/4-2</td>
<td>10</td>
<td>9 (90)</td>
</tr>
</tbody>
</table>
in tobacco when primary and challenge inocula were applied to different leaves (Ziebell et al., 2007).

The pattern of virus infection seen in dcl2-1 mutant plants inoculated with Fny-CMVΔ2b followed by Fny-CMV was similar to that seen in wild-type plants (Table 1). However, in both dcl3-1 and dcl4-2 mutant plants, the proportion of plants infected either with Fny-CMV or with both viruses was reduced relative to what was seen in wild-type plants, whereas the proportion of plants infected with Fny-CMVΔ2b alone was increased (Table 1). In striking contrast to the situation in wild-type and single dcl mutant plants, the majority of dcl2-1/dcl4-2 mutant plants that had been inoculated with Fny-CMVΔ2b followed by Fny-CMV became infected almost solely by Fny-CMVΔ2b, and none were doubly infected (Table 1). However, if plants were inoculated first with Fny-CMV and subsequently with Fny-CMVΔ2b (Fig. 1, column 5), RT-PCR analysis showed that all of these plants were infected with Fny-CMV and none were infected with Fny-CMVΔ2b (data not shown). Thus, once established, an Fny-CMV infection could not be displaced by Fny-CMVΔ2b, even in the dcl2-1/dcl4-2 double mutant background in which Fny-CMVΔ2b accumulation and spread are unaffected by RNA silencing.

Thus, in wild-type plants, DCL4 and, in a subsidiary role, DCL2 inhibit the accumulation and spread of CMV, whilst the 2b protein can to some extent counteract these effects. This is in line with previous findings (Diaz-Pendon & Ding, 2008). However, in dcl2/4 plants where there is no DCL2 or DCL4 activity and no significant antiviral silencing can occur, the deletion mutant Fny-CMVΔ2b appears to spread faster than Fny-CMV. We suggest that this is because, in dcl2/4 double mutant plants, Fny-CMVΔ2b is not subjected to silencing-mediated inhibition of its accumulation or spread. In this host background, the inability of CMVΔ2b to express a silencing suppressor is no handicap. In addition, because Fny-CMVΔ2b RNA 2 is shorter than the RNA 2 of Fny-CMV, the mutant may be able to replicate faster. Consistent with this idea, ELISA showed that Fny-CMVΔ2b accumulated to higher levels in dcl2-1/dcl4-2 double mutant plants than in wild-type plants (Fig. 2). Interestingly, accumulation of Fny-CMV in dcl2-1/dcl4-2 plants was not significantly different from the levels seen in wild-type plants (Fig. 2), which contrasts with the findings of Diaz-Pendon et al. (2007), who found that Q-CMV accumulated to higher levels in plants of the dcl2-1/dcl4-2 background.

In conclusion, it appears that DCLs 2, 3 and 4 are not required for cross-protection between Fny-CMVΔ2b and Fny-CMV. Our results are more consistent with an exclusion model where related viral strains ‘race’ each other to reach vital host-cell sites and resources and, once established there, cannot be displaced by the competing strain. Exclusion of either wild-type or mutant Fny-CMV by the other does not appear to require elicitation of a defence response in the plant. It is known that, in a systemically infected host plant, a virus can give rise to genetically diverse subpopulations of sequence variants that do not co-infect the same cells or tissues (García-Arenal et al., 2001; Iridi et al., 2006). We may speculate that the cross-protection that we see between Fny-CMVΔ2b and Fny-CMV may reflect a mechanism that has evolved to limit the extent of interference between different CMV sequence variants. This may benefit the variants by preventing internecine struggle over host resources, whilst preservation of RNA sequence diversity within a viral population may, among other things, provide an additional means of evading resistance mediated by RNA silencing.

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**References**


