The roles of the *Cucumber mosaic virus* 2b protein in promoting movement and inducing or sustaining symptom induction in *Arabidopsis* and *Nicotiana* plants

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ABSTRACT

The cucumber mosaic virus (CMV) 2b protein is a counter-defense factor and symptom determinant. Conserved domains in the 2b protein sequence were mutated in the *2b* gene of strain Fny-CMV. The effects of these mutations were assessed by infection of *Nicotiana tabacum*, *N. benthamiana* and *Arabidopsis thaliana* (ecotype Col-0) with mutant viruses and by expression of mutant *2b* transgenes in *A. thaliana*. We confirmed that two nuclear localization signals were required for symptom induction and found that the N-terminal domain was essential for symptom induction. The C-terminal domain and two serine residues within a putative phosphorylation domain modulated symptom severity. Further infection studies were conducted using Fny-CMVΔ2b, a mutant which cannot express the 2b protein and that induces no symptoms in *N. tabacum*, *N. benthamiana* or *A. thaliana* ecotype Col-0. Surprisingly, in plants of *A. thaliana* ecotype C24, Fny-CMVΔ2b induced severe symptoms, similar to those induced by the wild-type virus. However, C24 plants infected with the mutant virus recovered from disease whilst those infected with the wild-type virus did not. Whereas expression of Fny *2b*-transgenes induced symptom-like phenotypes in Col-0, this was rarely seen in the C24 background. Expression of *2b*-transgenes from either Fny-CMV or from LS-CMV (a mild strain) in Col-0 plants enhanced systemic movement of Fny-CMVΔ2b and permitted symptom induction by Fny-CMVΔ2b. Taken together, the results indicate that while the 2b protein is an important symptom determinant in certain hosts, it can also synergize symptom induction by other CMV-encoded factors.

INTRODUCTION

Cucumber mosaic virus (CMV) is the type *Cucumovirus* species and CMV strains are further classified into one of three subgroups (IA, IB, or II) (Palukaitis and García-Arenal 2003; Roossinck et al. 1999). Cucumoviruses possess tripartite, positive sense RNA genomes encoding five proteins (Habili and Francki 1974; Palukaitis and García-Arenal 2003; Wikoff et al. 1997). One of these proteins is the multifunctional 2b protein (c. 12 kDa) encoded by the second open reading frame (ORF) of RNA 2 and synthesized by the translation of a sub-genomic mRNA, RNA 4A (Ding et al. 1994).

The 2b protein influences local and systemic viral movement and inhibits host defense mechanisms based on salicylic acid (SA)-induced resistance and RNA silencing (Béclin et al. 1998; Brigneti et al. 1998; Ding et al. 1995; Guo et al. 2005; Ji and Ding 2001; Li et al. 1999; Mourrain et al. 2000; Shi et al. 2003; Soards et al. 2002). The severity of the symptoms induced by subgroup IA, IB and II CMV strains and by tomato aspermy virus, another cucumovirus, is determined in large part by the properties of the 2b proteins of these viruses (Du et al. 2007; Shi et al. 2002, 2003). Thus, a mutant of the subgroup II CMV strain Q that cannot express the 2b protein (Q-CMVΔ2b) was unable to move systemically in cucumber and displayed decreased symptom induction in *Nicotiana glutinosa* and tobacco (*N. tabacum*) (Ding et al. 1996; Ji and Ding 2001). A *2b* deletion mutant of the subgroup IA strain Fny (Fny-CMVΔ2b) moves systemically in tobacco and *N. benthamiana* but does not induce symptoms (Soards et al. 2002; Ziebell et al. 2007).

Constitutive expression of *2b* genes from various subgroup II and subgroup IA strains of CMV in transgenic *Arabidopsis thaliana* (ecotype Col-0) and *Nicotiana* spp. (Du et al. 2007; Siddiqui et al., 2008) provided evidence that the severity of symptoms induced by these strains was related to the ability of their respective 2b proteins to disrupt the regulation of host gene expression by micro(mi)RNAs (Chapman et al. 2004; Lewsey et al. 2007; Zhang et al. 2006). Thus, transgenic plants expressing Fny-CMV 2b protein displayed strong symptom-like phenotypes (distortion of leaves, general stunting and disturbance of root architecture: Lewsey et al. 2007), whereas transgenic plants expressing Q- or LS-CMV 2b proteins are similar in appearance to non-transgenic plants (Chapman et al. 2004; Lewsey et al. 2007; Zhang et al. 2006). Using the subgroup IA strain Fny-CMV we have investigated the importance of specific domains within the 2b protein for symptom induction and the requirement for the 2b protein in symptom induction by the virus in a number of hosts.

RESULTS

Mutagenesis of specific domains in the 2b protein affects CMV-induced symptoms

The *2b* genes and protein sequences from different CMV strains share several highly conserved features. For example, there is an overlap between the 5' region of the 2b open reading frame (ORF) and the 3' region of the 2a replicase protein gene. Other conserved features include: a conserved bipartite, arginine-rich nuclear localization sequence (NLS); a putative phosphorylation sequence, and a C-terminal sequence of approximately 17 amino acids (Lucy et al. 2000; Mayers et al. 2000; Wang et al. 2004) (Fig. 1A). To investigate the roles of these conserved regions and domains in protein function, we carried out site-directed mutagenesis of the 2b ORF in an infectious cDNA clone of Fny-CMV RNA 2 (pFny209: Rizzo and Palukaitis 1990) (Supp. Table 1).

A number of mutant plasmids were made (Fig. 1 and Supp. Table 1). In plasmid pFny209:Δ5T the nucleotides encoding the first 17 amino acids of the Fny-CMV 2b protein sequence were deleted (Fig. 1). Plasmids pFny209:ΔNLS1, pFny209:ΔNLS2 and pFny209:ΔNLS1+2 encode, respectively, RNAs 2 in which one of the two segments of the 2b protein NLS (1 or 2) or both segments were deleted (Fig. 1). Plasmid pFny209:ΔKSPSE encodes an RNA 2 in which the sequence for the putative phosphorylation motif KSPSE in the 2b protein was entirely deleted (Fig. 1). Plasmids pFny209:S40A and pFny209:S42A encode, respectively, 2b protein sequences in which serine residues 40 and 42 have been replaced by alanine residues (Fig. 1). These two serine residues are the most probable phosphorylation sites within

the putative phosphorylation domain. Plasmid pFny209:Δ3T encodes a truncated RNA 2 lacking the 3' terminal RNA sequence of the 2b ORF, corresponding to the C-terminal 16 amino acids of the 2b protein (see Fig. 1 and Supp. Table 1). For all newly made constructs, successful mutation of pFny209 was confirmed by DNA sequencing (data not shown).

Wild-type (WT) and mutant RNAs 2 were generated by *in vitro* transcription and combined as appropriate with *in vitro*-synthesized RNAs 1 and 3 using the plasmids pFny109 and pFny309 as templates (Rizzo and Palukaitis 1990; Supp. Table 2). Infectious transcripts of the mutant Fny-CMVΔ2b, in which most of the 2b ORF is deleted, were also reconstituted (Ryabov et al. 2001; Soards et al. 2002; Supplementary Supp. Table 2). These were mechanically inoculated onto leaves of tobacco (cv. Xanthi-nc), *N. benthamiana* and *A. thaliana* plants and the development of symptoms was observed (Table 1; Fig. 2; Supp. Figs. 1 and 2). In all host/virus combinations, systemic accumulation of the virus was confirmed by RT-PCR (Fig. 3 and data not shown), using PCR primers flanking the *2b* coding region (Ziebell et al. 2007). Conservation of the mutations within the viral progeny was confirmed by DNA sequencing of RT-PCR products (data not shown).

Infections of tobacco with Fny-CMVΔ5T, Fny-CMVΔNLS1, Fny-CMVΔNLS2, Fny-CMVΔNLS1+2 or Fny-CMVΔKSPSE were asymptomatic (Table 1; Fig. 2A; Supp. Fig. 1). As previously described, infection of tobacco by Fny-CMVΔ2b was also asymptomatic (Table 1; Fig. 2A; Supp. Fig. 1; Soards et al., 2002). Infection with Fny-CMVS40A or Fny-CMVS42A caused mild symptoms in tobacco. These included leaf distortion, mild vein clearing and very slight stunting (Table 1;

Fig. 2A; Supp. Fig. 1). Tobacco plants infected with Fny-CMVΔ3T exhibited severe symptoms, which included mosaic and leaf distortion and a more marked yellowing than those induced by Fny-CMV (Table 1; Fig. 2A; Supp. Fig. 1).

Similarly, infection of *N. benthamiana* plants with Fny-CMVΔ5T, Fny-CMVΔNLS2, Fny-CMVΔNLS1+2 or Fny-CMVΔKSPSE did not induce any obvious symptoms (Table 1; Fig. 2B). However, symptoms induced by Fny-CMVΔ3T were more severe than those induced by Fny-CMV in *N. benthamiana*, in that plants infected with Fny-CMVΔ3T exhibited more extensive necrosis than those infected with Fny-CMV (Table 1; Fig. 2B; Supp. Fig. 2). Infection with Fny-CMVΔNLS1 caused mild stunting, whilst Fny-CMVS40A caused slight leaf distortion and slight stunting (Table 1; Fig. 2B; Supp. Fig. 2). Infection with Fny-CMVS42A caused moderate stunting and leaf distortion (Table 1; Fig. 2B; Supp. Fig. 2).

In *A. thaliana* ecotype Col-0 plants, infection with Fny-CMVΔ2b, Fny-CMVΔ5T, Fny-CMVΔNLS1, Fny-CMVΔNLS2, Fny-CMVΔNLS1+2 or Fny-CMVΔKSPSE did not induce symptoms (Table 1; Supp. Fig. 2C). Fny-CMVS40A or Fny-CMVS42A infections caused mild stunting, whilst Fny-CMVΔ3T infection caused severe symptoms similar to those induced by WT Fny-CMV infection (Table 1; Supp. Fig. 2C). These reactions were similar to those of tobacco and *N. benthamiana* (Table 1; Fig. 2; Supp. Figs. 1 and 2).

Constitutive expression of the Fny-CMV 2b ORF in *A. thaliana* induced a symptom-like phenotype (Lewsey et al. 2007). Expression was achieved using the binary expression vector pBI121 Fny 2b, which contains a cauliflower mosaic virus

(CaMV) 35S promoter to drive constitutive expression of the *2b* gene (Lewsey et al. 2007). The Δ5T and Δ3T truncations (Fig. 1) of the 2b protein were recreated in this vector by introducing stop codons at amino acids 2 and 9 (Δ5T) or amino acid 95 (Δ3T) (Supp. Fig. 3). In transgenic *A. thaliana* (Col-0) plants the 35S:Fny2bΔ5T construct induced no obvious changes in phenotype, whilst 35S:Fny2bΔ3T induced a symptom-like phenotype similar to that induced by constitutive expression of a wild-type Fny-CMV *2b* transgene (Supp. Fig. 4; Lewsey et al. 2007). These modified plant phenotypes are consistent with the symptoms induced by infection of non-transgenic plants with the corresponding mutant viruses. This indicates that the differences in symptoms induced by Fny-CMVΔ5T and Fny-CMVΔ3T, compared with those induced by wild-type Fny-CMV, are due solely to the properties of the mutant 2b proteins and not to altered interactions between the 2b protein and other CMV gene products.

A. thaliana ecotypes exhibit different responses to a CMV mutant lacking the _2b_ gene

Fny-CMVΔ2b infection is symptomless in plants of two cultivars of *N. tabacum* (Soards et al. 2002; Ziebell et al. 2007), as well as in *N. benthamiana* (Ziebell et al. 2007) and *A. thaliana* (ecotype Col-0) (Lewsey et al. 2007). We also found that infection with this mutant virus was symptomless in *N. clevelandii* and *N. rustica* plants (data not shown). However, Wang and colleagues (2004) previously observed that *Cucurbita pepo* L. cv. Ma'yan plants infected with Fny-CMVΔ2b exhibited mild symptoms early in infection followed by recovery. We have observed similar results in *C. pepo* cv. Goldrush (data not shown) and the Warwick accession of *Chenopodium quinoa* (Supp. Fig. 5). *N. occidentalis* plants exhibited symptoms of

systemic mosaic and leaf curling when infected with Fny-CMV or Fny-CMVΔ2b, but recovery was not assessed (data not shown). These observations suggest that in certain hosts the 2b protein cannot be the sole determinant required for full pathogenicity. Consistent with this idea, we found that *A. thaliana* plants belonging to the ecotypes Wassilewskija, Landsberg erecta, Nössen and RLD (Fig. 4) exhibited stunting, but no other symptoms, in response to infection with Fny-CMVΔ2b and that this mutant evoked easily recognizable symptoms in plants of the C24 ecotype (Fig. 5A). These symptoms included necrosis and disturbance of normal leaf development (Fig. 5A). This contrasted with infection of *A. thaliana* ecotype Col-0 by Fny-CMVΔ2b, which was asymptomatic (Table 1; Supp. Fig. 1).

When infected with either wild-type Fny-CMV or the mutant Fny-CMVΔ2b, C24 plants exhibited symptoms that included extensive necrosis, which was not observed on plants of the other ecotypes following infection with either of these viruses (Fig. 5A and data not shown). Observation over time indicated that symptom induction on C24 plants by Fny-CMVΔ2b was delayed relative to Fny-CMV and that the symptoms induced by the mutant were slightly milder (Fig. 5A). *A. thaliana* ecotype C24 plants infected with Fny-CMV eventually died, whilst those infected with Fny-CMVΔ2b eventually exhibited recovery from disease in the form of apparently symptom-free new growth (Fig. 5A and B). Fny-CMVΔ2b RNA was detected by RT-PCR in the emerging leaves that exhibited recovery from disease (Fig. 5C). This indicates that the plants had not undergone a true recovery, where the initially infecting virus is usually undetectable in recovered tissue.

Symptom determination by the 2b protein is independent of its ability to promote viral movement

We explored further the roles of the 2b protein in enhancement of systemic movement and the induction of symptoms when expressed in 2b-transgenic plants. Transgenic *A. thaliana* (Col-0) plants expressing the Fny-CMV 2b protein (35S:Fny2b: Lewsey et al. 2007) were infected with Fny-CMV or Fny-CMVΔ2b. Virus accumulation and the induction of symptoms in systemically infected leaves were examined at various times following inoculation. The systemic movement of wild-type and mutant CMV was monitored by taking samples of protein from non-inoculated leaves at 6, 12 and 21 days post-inoculation (dpi). Virus accumulation was detected by immunoblot analysis of leaf proteins using an antiserum specific for the CMV coat protein.

In non-transgenic plants of *A. thaliana* ecotype Col-0, Fny-CMVΔ2b spread to non-inoculated leaves less rapidly than wild-type Fny-CMV (Fig. 6A), consistent with the pattern of Fny-CMVΔ2b systemic movement in tobacco (Soards et al. 2003). In transgenic plants constitutively expressing the Fny 2b protein, Fny-CMVΔ2b movement to non-inoculated tissues occurred as rapidly as movement of wild-type Fny-CMV (Fig.4A). However, the amount of mutant virus that accumulated in the non-inoculated leaves was still less than that achieved by the wild-type virus (Fig. 6A).

Experiments were conducted to further delineate the abilities of 2b proteins from CMV strains from different subgroups to promote movement and induce symptoms. Transgenic *A. thaliana* ecotype Col-0 harboring the *2b* coding region of a

subgroup II CMV strain (LS-CMV) under the control of a CaMV 35S promoter (35S:LS2b: Lewsey et al. 2007) were utilized. The 35S:LS2b transgene induces only very slight changes in plant phenotype and infection of non-transgenic *A. thaliana* ecotype Col-0 with LS-CMV is symptomless (Lewsey et al. 2007). Non-transgenic *A. thaliana* ecotype Col-0 plants, 35S:LS2b and 35S:Fny2b plants were inoculated with Fny-CMVΔ2b. Samples of protein were extracted from non-inoculated leaf tissue at 5, 7, 9 and 11 dpi and analyzed for CMV coat protein accumulation by immunoblotting (Fig. 6B). It was found that the 35S:LS2b transgene complemented accumulation of Fny-CMVΔ2b in systemically infected tissues as effectively as 35S:Fny2b (Fig. 6B).

During the experiments investigating systemic movement of Fny-CMV and Fny-CMVΔ2b in *2b*-transgenic plants, virus-induced symptom development was monitored. *A. thaliana* ecotype Col-0 plants harboring 35S:Fny2b or 35S:LS2b transgenes were infected with Fny-CMV, Fny-CMVΔ2b or were mock-inoculated. Fny-CMVΔ2b infection was confirmed by RT-PCR for Fny-CMVΔ2b RNA (Fig. 7). Plants confirmed to be infected with Fny-CMVΔ2b were assessed visually for symptoms one month post-inoculation and photographed (Fig. 8). It should be noted that non-infected plants of these 35S:Fny2b lines exhibit a mild symptom-like phenotypic change in response to the transgene and that non-infected plants of the 35S:LS2b lines utilized exhibit very mild developmentally perturbed phenotype changes (Lewsey et al. 2007). Infection of all *2b*-transgenic plants with wild-type Fny-CMV resulted in very severe disease symptoms. These were more severe than those induced by infection of non-transgenic *A. thaliana* ecotype Col-0 (Fig. 8). This is most likely to be because development of these plants is affected by both the 2b

protein expressed from the transgene and the 2b protein expressed by the virus. Fny-CMVΔ2b did not induce symptoms in non-transgenic *A. thaliana* ecotype Col-0 plants (Fig. 5, 8; Supp. Fig. 1C). Contrastingly, plants expressing either the 35S:Fny2b or the 35S:LS2b transgene infected with Fny-CMVΔ2b exhibited obvious stunting compared to mock-inoculated controls (Fig. 8). This demonstrates that the 2b protein of either LS- or Fny-CMV was able to complement symptom induction by Fny-CMVΔ2b.

We examined the possibility that complementation of systemic movement of Fny-CMVΔ2b and the induction of symptoms in 2b-transgenic plants was a result of recombination between the transgenic *2b* mRNA and the truncated RNA 2 of the mutant virus, resulting in the generation of reconstituted wild-type or chimeric virus. We carried out RT-PCR reactions on RNA from systemically infected leaves using a primer combination that can be used to distinguish between wild-type and mutant RNA 2 molecules (Ziebell et al. 2007). Full length CMV RNA 2 was not detected in any RT-PCR reactions (Fig. 6 and data not shown), indicating that recombination had not occurred between the transgene-encoded mRNAs and RNA 2 of Fny-CMVΔ2b. Thus, the effects on systemic movement (Fig. 6) and on symptom development (Fig. 8) are caused by 2b protein supplied *in trans*, as RT-PCR data indicated that 2b transgene-derived mRNAs had not recombined with the virus (Fig. 7).

DISCUSSION

The results of this study show that the CMV 2b protein influences disease induction in two ways. Firstly, and consistent with earlier studies, the 2b protein can act directly as an inducer of symptoms (Lewsey et al. 2007; Lucy et al. 2000; Wang et al., 2004; Zhang et al. 2006). Secondly, it can synergize and sustain symptom induction by other viral gene products. This supporting role in symptom induction appears to be independent of the ability of a 2b protein to induce symptoms autonomously.

The roles in symptom induction of specific domains within the 2b protein

The 2b protein of the subgroup IA strain of CMV, Fny-CMV, is a strong inducer of symptoms in several hosts. Amino acid sequence domains within the 2b protein were found to affect its ability to induce symptoms in plants from three host species: tobacco, *N. benthamiana* and *A. thaliana* ecotype Col-0. In all three hosts, deletion of the entire putative phosphorylation sequence (in the mutant Fny-CMVΔKSPSE) completely abolished symptom induction. Point mutations in Fny-CMVS40A and Fny-CMVS42A, whereby the potential for phosphorylation of the 2b protein was abolished by exchange of serine for alanine residues greatly decreased but did not completely abolish CMV-induced symptom induction in these three hosts. Deletion of the corresponding five residues from the 2b protein of Q-CMV (subgroup II) also abolished symptom induction (Ding et al. 1995). These results are consistent with our suggestion that these serine residues are likely to be phosphorylatable and indicate that the phosphorylation state of the 2b protein modulates its symptom-inducing activity. Sequence analysis by Lucy and colleagues (2000) indicated that

these five residues may contain motifs for phosphorylation that occur in substrates for casein kinase II (CKII) and cyclin-dependent kinase 2 (CDK2). They proposed that the combination of NLSs with CDKII and CDC2 phosphorylation sites constitutes a CcN motif, which has been shown to be involved in regulation of nuclear import for many proteins (Lucy et al. 2000). They also noted that several aligned 2b sequences possessed potential nuclear export signals, suggesting that the 2b protein may shuttle in and out of the nucleus (Lucy et al. 2000).

Previous studies have demonstrated that functional NLS(s) are required for 2b protein to act as a symptom determinant when expressed from vectors derived from potato virus X and zucchini yellow mosaic virus (Lucy et al. 2000; Wang et al. 2004). Our data also demonstrate that both NLS domains are required for 2b protein to operate as an effective symptom determinant of CMV. However, this is the first demonstration that they are required for symptom determination during an authentic CMV infection, rather than when wild-type and mutant 2b proteins are expressed from heterologous viruses. Deletion of the N-terminal region of the 2b protein (in mutant CMVΔ5T) also rendered the virus unable to induce symptoms, indicating that this region is required for symptom induction. It should be noted, though, that such a large deletion (seventeen amino acids) may have altered 2b protein function by affecting protein structure. Interestingly, deletion of the sixteen C-terminal amino acids (in CMVΔ3T) did not attenuate symptom induction. Rather, it seemed to alter the precise nature of symptoms in tobacco and slightly increase severity in *N. benthamiana*. A previous study found that the region had transcriptional activation activity in yeast (Ham et al. 1999). Our results suggest that in certain hosts, this region of the 2b protein may down-regulate symptom severity.

Symptom production and systemic movement by the Fny-CMVΔ2b mutant virus in non-transgenic and *2b*-transgenic *A. thaliana* plants

In this study and in previous work, we found that the Fny-CMVΔ2b mutant can spread systemically in *A. thaliana* ecotype Col-0 but induces no symptoms (Lewsey et al. 2007), which was consistent with work with the same mutant in tobacco and *N. benthamiana* (Table 1; Supp. Fig. 1). However, Wang and colleagues (2004) noted that Fny-CMVΔ2b induces transient symptoms in squash. This finding was confirmed by our data and it was noted that Fny-CMVΔ2b has similar effects on *C. quinoa* and *N. occidentalis* and, surprisingly, on plants of the C24 ecotype of *A. thaliana*.

The induction of symptoms in C24 plants by Fny-CMVΔ2b was investigated in greater detail. It was observed that Fny-CMVΔ2b induced symptoms of leaf distortion, chlorosis and necrosis. However, new growth in these plants did not show clear symptoms, although these young tissues were systemically infected with Fny-CMVΔ2b (Fig. 5). This suggests that these host plants had adapted in some manner and had recovered from virus-induced disease, although this was not a true recovery from virus infection. In contrast, *A. thaliana* ecotype C24 plants did not recover from disease caused by infection with wild-type Fny-CMV. The symptoms induced by the wild-type virus were stronger than those induced by Fny-CMVΔ2b, or by wild-type Fny-CMV infection of plants of the Col-0 ecotype, and eventually resulted in death of the plants.

The 2b protein enhances systemic movement of CMV in several hosts and preliminary work using Fny-CMVΔ2b had indicated that it was required for efficient long-distance movement in *A. thaliana* (Soards 2003). To further examine the functionality of the 2b protein expressed in 2b-transgenic plants we inoculated them with Fny-CMVΔ2b and monitored virus accumulation in directly inoculated and non-inoculated leaves at various times. In non-transformed *A. thaliana* plants Fny-CMVΔ2b accumulated and spread less rapidly than wild-type Fny-CMV and induced no disease symptoms, similar to previous results seen in Fny-CMVΔ2b-infected tobacco (Soards et al. 2003). However, in transgenic plants expressing the 2b protein of the Fny-CMV strain, Fny-CMVΔ2b accumulation and spread occurred as rapidly as for wild-type virus, although the amount of mutant virus that accumulated in non-inoculated leaves was still less than that achieved by the wild-type CMV (Fig. 6A). A previous report indicated that CMV infection ameliorated 2b-induced phenotype changes in 2b-transgenic tobacco (Praveen et al. 2008). Contrastingly, we found that infection of Fny 2b-transgenic *A. thaliana* ecotype Col-0 plants with Fny-CMV resulted in extremely severe disease symptoms and that even infection with Fny-CMVΔ2b exacerbated the stunting exhibited by the *2b*-trangenic plants. Transgenic expression of the LS 2b protein complemented movement of Fny-CMVΔ2b as effectively as Fny 2b protein. More surprisingly, Fny-CMVΔ2b induced disease symptoms in these plants. Since LS-CMV infection is essentially asymptomatic in *A. thaliana* ecotype Col-0 plants and transgenic expression of LS 2b protein causes only very mild changes in plant phenotype (Lewsey et al. 2007), the symptoms must have been caused by one or more gene products expressed by Fny-CMVΔ2b.

The 2b protein can induce symptoms but also facilitates symptom induction by other CMV gene products

The 2b protein of a severe CMV strain can induce symptoms autonomously of other CMV gene products (Lewsey et al. 2007; Lucy et al. 2000; Wang et al. 2004; Zhang et al. 2006) and the characteristics of 2b-induced symptoms depend upon specific domains within the 2b protein sequence (data from this study; Lucy et al. 2000; Ham et al. 1999). However, whereas expression of Fny 2b in transgenic Col-0 plants induces strong disease-like phenotypes (this study; Lewsey et al. 2007; Zhang et al. 2006), this was not seen in the majority of lines created in the C24 background. Thus, the host background also conditions symptom induction by 2b proteins. Furthermore, a 2b protein, whether it originates from a severe or a mild strain, can also facilitate and sustain symptom induction by other viral gene products even when it induces no symptoms or symptom-like phenotypes of its own. Thus, the 2b protein from LS-CMV, a strain that does not induce strong symptoms in *A. thaliana*, does not induce a strong symptom-like phenotype when expressed in transgenic plants. However, expression of 2b in transgenic plants converted a symptomless infection by Fny-CMVΔ2b into a severe infection.

Taken together, our results indicate that although the 2b protein may be the predominant determinant of symptom induction, it is not the only CMV protein involved in symptom determination. This is consistent with previous studies that have mapped determinants of CMV symptoms to all three genomic RNA segments and to all five known CMV protein genes (Palukaitis and García-Arenal 2003). For example, work by Zhang et al. (1994) demonstrated that in tobacco symptoms were determined by both RNAs 1 and 2 of Fny-CMV. However, the results obtained by

infecting *A. thaliana* ecotype C24 plants and transgenic ecotype Col-0 plants expressing the 2b protein of LS-CMV with Fny-CMVΔ2b indicate that successful symptom induction and/or sustained induction of symptoms by other CMV gene products requires the 2b protein.

Interestingly, previous work has suggested that CMV may exert effects on RNA silencing and symptom induction that are not dependent solely upon the 2b protein itself. It was shown that in transgenic *N. benthamiana*, infection by Fny-CMVΔ2b could to some extent relieve the silencing of an amplicon derived from potato leafroll virus (Taliansky et al. 2004). The subgroup II CMV strain Q-CMV, and 2b deletion mutants derived from it (Q-CMVΔ2b), do not induce strong disease symptoms in *A. thaliana* plants belonging to the Col-0 ecotype. Remarkably, Diaz-Pendon and colleagues (2007) found that both wild-type Q-CMV and Q-CMVΔ2b induced strong disease symptoms in mutant *A. thaliana* plants lacking functional genes for dicer-like (DCL) enzymes: specifically in double *dcl 2/4* and triple *dcl 2/3/4* mutants. These DCLs are largely responsible for RNA silencing-mediated resistance to viruses (Deleris et al. 2006). Diaz-Pendon et al. (2007) argued that these results indicate that 2b is dispensable for symptom induction. However, our results demonstrate that the ability of 2b to induce symptoms in CMV-infected plants, or symptom-like phenotypes in transgenic plants, is both host plant specific and virus strain specific. Furthermore, even in plants where a given 2b protein has no symptom-inducing activity it can still support or potentiate the symptom induction by other CMV gene products and it appears to be needed to prevent recovery of the plant. Our work demonstrates that the 2b protein does not operate in isolation; rather, it is a key component of a complex, precision attack on plant development mounted by the virus.

MATERIALS AND METHODS

Site-directed mutagenesis

Targeted mutations of the *2b* coding region were introduced into plasmids pFny209 (Rizzo and Palukaitis, 1990) and pBI121 Fny *2b* (Lewsey et al., 2007) by site-directed mutagenesis using the Quick-Change II XL kit (Stratagene; http://www.stratagene.com/) according to the manufacturer's instructions. The sequences of primers used are detailed in Supp. Table 1.

Plant growth and virus inoculation

A. thaliana seeds were planted on a 4:1 compost/sand mixture and maintained at 21°C with an 8 h photoperiod. *Nicotiana* spp., *C. quinoa* (Warwick HRI accession) and *C. pepo* seeds were planted on compost and maintained at 25 °C either in a greenhouse (with supplementary lighting) or in a custom built growth chamber with an 8 h photoperiod and a light intensity of 200 $\mu mol.m^{-2}.s^{-1}$ (Conviron; www.conviron.com).

Infectious RNAs for Fny-CMV and mutants thereof were regenerated from infectious cDNA clones by *in vitro* transcription as described previously (Soards et al., 2002), using the combinations of plasmids described in Supplementary Supp. Table 2. *A. thaliana* plants were inoculated with infectious transcripts at the 4 to 6 leaf stage by applying solutions of transcripts to Carborundum-dusted leaves using a cotton bud. Plants of all other species were inoculated at 2 to 5 weeks post-germination by applying transcripts to Carborundum-dusted leaves using a roughened glass microscope slide. Infection was confirmed in transcript-inoculated plants by

reverse transcription coupled to the polymerase chain reaction (RT-PCR). To inoculate plants with infectious sap leaves from plants confirmed to be infected were ground in 0.1M potassium phosphate buffer (pH 7) and the clarified homogenate was applied to Carborundum-dusted leaves of plants with a gloved finger. Virus purification was conducted by the method of Ng and Perry (2004) and inoculated to *A. thaliana* plants as per infectious transcripts, using a suspension of 100μg.ml^{-1} viral particles. Infected plants were photographed with a Nikon Coolpix digital camera (http://www.nikondigital.com/main.html).

Experiments assessing symptom induction of Fny-CMV mutants in tobacco were conducted twice using infectious transcript inocula, with plants maintained in a greenhouse, and once using infectious sap, with plants maintained in a growth chamber. These experiments were conducted twice in *N. benthamiana* using infectious transcript inocula, with plants grown in a greenhouse. In *A. thaliana* the experiment was conducted once, with each virus inoculated onto 4-6 plants. Results were consistent between plants of the same species inoculated with the same virus.

RT-PCR and DNA sequencing

RNA extraction and RT-PCR to detect Fny-CMV or mutants thereof was performed using primers flanking the *2b* coding region, according to Ziebell et al. (2007). PCR products were purified for sequencing by extraction from agarose gels using the Qiaquick gel extraction kit (Qiagen; http://www1.qiagen.com/). Sequencing of purified RT-PCR products from wild-type and mutant CMV-infected plants and of mutant plasmids was performed by Geneservice Ltd. (http://www.geneservice.co.uk/).

Arabidopsis transformation

Agrobacterium tumefaciens-mediated transformation was performed by floral dipping (Clough and Bent, 1998) and selection of transformants was conducted according to Lewsey et al (2007).

Immunoblotting

Immunoblotting for CMV coat protein was conducted according to Naylor et al. (1998) except that bound primary antibody was detected using an anti-rabbit horseradish peroxidase conjugate as the secondary antibody and visualized using Western Lightning enhanced luminol chemiluminescence reagent (PerkinElmer; http://las.perkinelmer.com) according to manufacturer's instructions.

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LITERATURE CITED

Béclin, C., Berthomé, R., Palauqui, J-C, Tepfer, M. and Vaucheret, H. 1998. Infection of tobacco or *Arabidopsis* plants by CMV counteracts systemic post-transcriptional silencing of nonviral (*trans*)genes. *Virology*, **252,** 313-317.

Brigneti, G., Voinnet, O., Li, W-X., Ji, L-H., Ding, S-W. and Baulcombe, D. C. 1998. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *The EMBO Journal*, **17,** 6739-6746.

Chapman, E. J., Prokhnevsky, A. I., Gopinath, K., Dolja, V. V. and Carrington, J. C. 2004. Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. *Genes and Development*, **18,** 1179-1186.

Clough, S. J. and Bent, A. 1998. Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal*, **16,** 735-743.

Deleris, A., Gallego-Bartolome, J., Bao, J., Kasschau, K. D., Carrington, J.C. and Voinnet, O. 2006. Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science*, **313**, 68-71.

Diaz-Pendon, J.A., Li, F., Li, W-X. and Ding, S-W. 2007. Suppression of Antiviral Silencing by Cucumber Mosaic Virus 2b Protein in *Arabidopsis* Is Associated with Drastically Reduced Accumulation of Three Classes of Viral Small Interfering RNAs. *The Plant Cell*, **19,** 2053-2063.

Ding, S-W., Anderson, B. J., Haase, H. R. and Symons, R. H. 1994. New overlapping gene encoded by the cucumber mosaic virus genome. *Virology*, **198,** 593-601.

Ding, S-W., Li, W-X. and Symons, R. H. 1995. A novel naturally occurring hybrid gene encoded by a plant RNA virus facilitates long distance virus movement. *The EMBO Journal*, **14,** 5762-5772.

Du, Z-Y., Chen, F-F., Liao, Q-S., Zhang, H-R., Chen, Y-F. and Chen, J-S. 2007. 2b ORFs encoded by subgroup IB strains of cucumber mosaic virus induce differential virulence on *Nicotiana* species. *Journal of General Virology*, **88,** 2596-2604.

Guo, H-S., Xie, Q., Fei, J-F. and Chua, N-H. 2005. MicroRNA directs mRNA cleavage of the transcription factor *NAC1* to down regulate auxin signals for arabidopsis lateral root development. *The Plant Cell*, **17,** 1376-1386.

Habili, N. and Francki, R. I. B. 1974. Comparative studies on tomato aspermy and cucumber mosaic viruses. I. Physical and chemical properties. *Virology*, **57,** 392-401.

Ham, B-K., Lee, T-H., You, J-S., Nam Y-W., Kim, J-K. and Paek, K-H. 1999. Isolation of a putative tobacco host factor interacting with cucumber mosaic virus-encoded 2b protein by yeast two-hybrid screening. *Molecules and Cells*, **9,** 548-555.

Ji, L-H. and Ding, S-H. 2001. The suppressor of transgene RNA silencing encoded by *Cucumber mosaic virus* interferes with salicylic acid-mediated virus resistance. *Molecular Plant-Microbe Interactions*, **14,** 715-724.

Lewsey, M., Robertson, F.C., Canto, T, Palukaitis, P., and Carr, J.P. 2007. Selective targeting of miRNA-regulated plant development by a viral counter-silencing protein. *Plant Journal,* **50,** 240-252.

Li, H. W., Lucy, A. P., Guo, H. S., Li, W-X., Wong, S. M. and Ding, S-W. 1999. Strong host resistance targeted against a viral suppressor of the plant gene silencing defence mechanism. *The EMBO Journal*, **18,** 2683-2691.

Lucy, A. P., Guo, H-S., Li, W-X. and Ding, S-W. 2000. Suppression of post-transcriptional gene silencing by a plant viral protein localized in the nucleus. *The EMBO Journal*, **19,** 1672-1680.

Mayers, C. N., Palukaitis, P. and Carr, J. P. 2000. Subcellular distribution analysis of the cucumber mosaic virus 2b protein. *Journal of General Virology*, **81,** 219-226.

Mourrain, P., Béclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J-B., Jouette, D., Lacombe, A-M., Nikic, S., Picault, N., Rémoué, K., Sanial, M., Vo, T-A. and Vaucheret, H. 2000. *Arabidopsis SGS2* and *SGS3* genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell*, **101,** 533-542.

Ng, J. C. K. and Perry, K. L. 2004. Transmission of plant viruses by aphid vectors. *Molecular Plant Pathology*, **5,** 505-511.

Palukaitis, P. and García-Arenal, F. 2003. Cucumoviruses. *Advances in Virus Research*, **62,** 242-323.

Praveen, S., Mangrauthia, S.K., Singh, P. and Mishra, A.K. 2008. Behaviour of RNAi suppreesor 2b of *Cucumber mosaic virus in planta* in presence and absence of virus. *Virus genes*, **37**, 96-102.

Rizzo, T. M. and Palukaitis, P. 1990. Construction of full-length cDNA clones of cucumber mosaic virus RNAs 1, 2 and 3: Generation of infectious RNA transcripts. *Molecular and General Genetics*, **222,** 249-256.

Roossinck, M. J., Zhang, L., and Hellewald, K. H. 1999. Rearrangements in the 5' nontranslated region and phylogenetic analyses of cucumber mosaic virus RNA 3 indicate radial evolution of three subgroups. *Journal of Virology*, **73,** 6752-6758.

Ryabov, E. V., Roberts, I. M., Palukaitis, P. and Taliansky, M. 2001. Umbravirus gene expression helps *Potato leafroll virus* to invade mesophyll tissues and to be transmitted mechanically between plants. *Virology*, **286,** 363-372.

Shi, B-J., Palukaitis, P. and Symons, R. H. 2002. Differential virulence by strains of *Cucumber mosaic virus* is mediated by the 2b gene. *Molecular Plant-Microbe Interactions*, **15,** 947-955.

Shi, B-J., Miller, J., Symons, R. H. and Palukaitis, P. 2003. The 2b protein of cucumoviruses has a role in promoting the cell-to-cell movement of pseudorecombinant viruses. *Molecular Plant-Microbe Interactions*, **16,** 261-267.

Siddiqui, S.A., Sarmiento,C., Truve, E., Lehto, H. and Lehto, K. 2008. Phenotypes and functional effects caused by various viral RNA silencing suppressors in transgenic *Nicotiana benthamiana* and *N. tabacum*. *Molecular Plant-Microbe Interactions*, **21,** 178-187.

Soards, A. J. 2003. *The* Cucumber mosaic virus *2b protein: Influences on the plant-virus interaction*. Ph.D thesis, Cambridge University, UK.

Soards, A. J., Murphy, A. M., Palukaitis, P. and Carr, J. P. 2002. Virulence and differential local and systemic spread of *Cucumber mosaic virus* in tobacco are affected by the CMV 2b protein. *Molecular Plant-Microbe Interactions*, **15,** 647-653.

Taliansky, M., Kim, S. H., Mayo, M. A., Kalinina, N. O., Fraser, G., McGeachy, K.D. and Barker, H. 2004. Escape of a plant virus from amplicon-mediated RNA silencing is associated with biotic or abiotic stress. *The Plant Journal*, **39,** 194-205.

Wang, Y., Tzfira, T., Gaba, V., Citovsky, V., Palukaitis, P. and Gal-On, A. 2004. Functional analysis of the *Cucumber mosaic virus* 2b protein: pathogenicity and nuclear localization. *Journal of General Virology*, **85,** 3135-3147.

Wikoff. W. R., Tsai, C. J., Wang, G., Baker, T. S. and Johnson, J. E. 1997. The structure of cucumber mosaic virus: Cryoelectron microscopy, X-ray crystallography and sequence analysis. *Virology*, **232,** 91-97.

Ziebell, H., Payne, T., Berry, J.O., Walsh, J.A. and Carr, J.P. 2007. *Journal of General Virology*, **88,** 2862-2861.

Zhang, L., Hanada, K. and Palukaitis, P. 1994. Mapping local and systemic symptom determinants of cucumber mosaic cucumovirus in tobacco. *Journal of General Virology*, **75,** 3185-3191.

Zhang, X., Yuan, Y.R., Pei, Y., Lin, S.-S., Tuschl, T., Patel, D.J., and Chua, N.-H. 2006. Cucumber mosaic virus-encoded 2b suppressor inhibits *Arabidopsis* Argonaute1 cleavage activity to counter plant defense. *Genes and Development*, **20,** 3255-3268.

TABLES

Table 1 Symptoms induced by Fny-CMV variants in *A. thaliana* ecotype Col-0, *N. benthamiana* and tobacco. N/O indicates no obvious symptoms.

	Symptoms induced in host species		
Fny-CMV variant	***A. thaliana* ecotype Col-0**	***N. benthamiana***	**Tobacco**
Wild type Fny-CMV	Stunting; leaf distortion	Stunting; leaf distortion	Stunting; leaf distortion; systemic mosaic
Fny-CMVΔ2b	N/O	N/O	N/O
Fny-CMVΔ5T	N/O	N/O	N/O
Fny-CMVΔNLS1	N/O	Mild stunting	N/O
Fny-CMVΔNLS2	N/O	N/O	N/O
Fny-CMVΔNLS1+2	N/O	N/O	N/O
Fny-CMVS40A	Mild stunting	Mild stunting	Mild stunting; mild leaf distortion; mild systemic mosaic
Fny-CMVS42A	Mild stunting	Mild stunting; mild leaf distortion	Mild stunting; mild leaf distortion; mild systemic mosaic
Fny-CMVΔKSPSE	N/O	N/O	N/O
Fny-CMVΔ3T	Stunting; leaf distortion	Stunting; leaf distortion; necrosis	Leaf distortion; systemic mosaic; chlorosis

FIGURE LEGENDS

Figure 1. Schematic map describing locations of mutations, indicated by amino acid residue numbers, created in the 110 amino acid 2b protein of Fny-CMV. Mutations were generated by site-directed mutagenesis using primers described in Supp. Table 1.

Figure 2. Symptoms induced by Fny-CMV variants in tobacco plants (A; scale bars 5cm), *N. benthamiana* plants (B; scale bars 5cm) and *A. thaliana* ecotype Col-0 plants (C; scale bars 3cm). Symptoms in tobacco and *N. benthamiana* were photographed approximately 5 weeks post-inoculation (wpi), whilst those of *A. thaliana* were taken approximately 3 wpi.

Figure 3. Confirmation of systemic infection by Fny-CMV variants in *A. thaliana* ecotype Col-0 (A) and *N. benthamiana* (B). Non-inoculated tissue was tested for the presence of viral RNA by RT-PCR. The identity of the virus is indicated above each lane. The expected product size from Fny-CMVΔ2b was 370bp and the expected product from WT Fny-CMV is 664 bp (Ziebell et al., 2007). "M" denotes lanes loaded with molecular weight marker and "-ve" denotes lanes containing the results of RT-PCR reactions conducted using RNA from mock-inoculated plants.

Figure 4. Symptoms induced by Fny-CMVΔ2b and Fny-CMV in *A. thaliana* ecotypes Wassilewskija (Ws), Landsberg erecta (Ler), Nössen (Nö) and RLD. Plants were inoculated with purified virions. Infection was confirmed by RT-PCR using primers flanking the *2b* coding region and symptoms observed at 19 dpi. Scale bars indicate 3 cm.

Figure 5. Symptoms induced by Fny-CMVΔ2b and Fny-CMV in *A. thaliana* ecotype C24. Plants were inoculated with purified virions. Infection was confirmed by RT-PCR using primers flanking the *2b* coding region and symptoms observed at intervals up to 20 dpi (A; Scale bar indicates 3 cm). Control plants were photographed at 21 dpi (A; scale bars indicate 3cm). Recovery from disease was photographed in Fny-CMVΔ2b inoculated plants at 30 dpi (B; scale bar indicates 3 cm). Newly emerged tissue exhibiting recovery from disease was tested for the presence of Fny-CMVΔ2b RNA by RT-PCR (C). Lanes R1 and R2 contain RT-PCR products from recovered tissue of two independent plants; Lane I contains an RT-PCR product from directly inoculated tissue; Lane M contains a molecular weight marker, with 300, 400 and 600 bp bands indicated. The lane denoted "-ve" contains the results of an RT-PCR reaction conducted using RNA from a mock-inoculated plant.

Figure 6. Accumulation of Fny-CMV or Fny-CMVΔ2b in non-inoculated leaves of non-transgenic *A. thaliana* ecotype Col-0 and plants harboring the 35S:Fny2b transgene or 35S:LS2b transgene. Plants were inoculated on three lower leaves with purified virions and non-inoculated leaves were collected for analysis. The accumulation of Fny-CMV and Fny-CMVΔ2b in two independent lines (2.11C and 2.30F) harboring the 35S:Fny2b transgene was assessed at 6, 12 and 21 dpi (A). Infecting viruses are denoted F (Fny-CMV), Δ (Fny-CMVΔ2b) and U (uninfected control). M denotes marker lanes loaded with 1 μg of purified CMV. X indicates non-loaded lanes. Accumulation of Fny-CMVΔ2b in one 35S:Fny2b and one 35S:LS2b line was assessed at 5, 7, 9 and 11 dpi (B). Lanes loaded with protein from non-transgenic *A. thaliana* plants are labeled NT. In both experiments proteins were

extracted, separated by SDS-PAGE and equal loading assessed by staining with Ponceau S. CMV accumulation was analyzed by immunoblotting. Each test lane represents a separate plant. Individual plants were analyzed at one time point only.

Figure 7. Confirmation of Fny-CMVΔ2b infection of plants harboring the 35S:Fny2b and 35S:LS2b transgenes. Plants of two independent transgenic lines harboring the 35S:Fny2b transgene (lines 2.11C and 2.30F), two independent transgenic lines harboring the 35S:LS2b transgene (lines 4.31A and 5.7D) and non-transgenic *A. thaliana* ecotype Col-0 plants were inoculated with Fny-CMVΔ2b. Successful infection was confirmed by RT-PCR for Fny-CMVΔ2b RNA. Lanes are labeled with the 2b-derived transgene the plant harbored, the identity of the independent line and a number indicating the specific plant tested. Lanes M contain molecular weight markers, with 300 and 400 bp bands labeled. The lane denoted "-ve" contains a no RNA negative control RT-PCR.

Figure 8. Symptoms of infection by Fny-CMV and Fny-CMVΔ2b of plants harboring the 35S:Fny2b and 35S:LS2b transgenes. Plants of two independent transgenic lines harboring the 35S:Fny2b transgene (lines 2.11C and 2.30F), two independent transgenic lines harboring the 35S:LS2b transgene (lines 4.31A and 5.7D) and non-transgenic *A. thaliana* ecotype Col-0 plants were infected with Fny-CMV, Fny-CMVΔ2b or mock-inoculated. After one month, typical examples of infected plants (confirmed by RT-PCR; Fig. 6) were photographed. The stunting of plants harboring the 35S:Fny2b or 35S:LS2b transgene induced by Fny-CMVΔ2b infection can be observed, as can the asymptomatic infection of non-transgenic *A. thaliana* by Fny-CMVΔ2b (row labeled NT). Scale bars indicate 3 cm.

1

2

SUPPLEMENTARY TABLE

Supplementary Table 1 Sequences of primers used in site-directed mutagenesis of the Fny-CMV *2b* coding region and the resulting mutations. Nucleotide co-ordinates refer to the Fny-CMV RNA 2 sequence available from Genbank (accession NC_002035).

Mutation	**Direction**	**Primer sequences**	**Mutation(s) made**
Δ5T	Forward	CAA ACA GCG AAA GAA TTA TGG TGG AGG CGA AG	Nucleotides 2419 – 2469 deleted
	Reverse	CTT CGC CTC CAC CAT AAT TCT TTC GCT GTT TG	
ΔNLS1	Forward	CGT ATG GAG GCG TCT CAC AAA CAG AAT CG	Nucleotides 2482 – 2499 deleted
	Reverse	CGA TTC TGT TTG TGA GAC GCC TCC ACC ATA CG	
ΔNLS2	Forward	GTC TCA CAA ACA GAA TGG TCA CAA AAG TCC CAG C	Nucleotides 2515 – 2526 deleted
	Reverse	GCT GGG ACT TTT GTG ACC ATT CTG TTT GTG AGA C	
ΔNLS1+2	Forward	ΔNLS1 and ΔNLS2 Forward primers, sequentially	Nucleotides 2482 – 2499 and 2515 – 2526 deleted
	Reverse	ΔNLS1 and ΔNLS2 Reverse primers, sequentially	
S40A	Forward	GAA CGA GGT CAC AAA GCT CCC AGC GAG AGA GCG	Nucleotides 2536 – 2537 changed from AG to GC
	Reverse	CGC TCT CTC GCT GGG AGC TTT GTG ACC TCG TTC	
S42A	Forward	GGT CAC AAA AGT CCC GCC GAG AGA GCG CGT TCA	Nucleotides 2542 – 2543 changed from AG to GC
	Reverse	TGA ACG CGC TCT CTC GGC GGG ACT TTT GTG ACC	
ΔKSPSE	Forward	CGG GAA CGA GGT CAC AGA GCG CGT TCA AAT	Nucleotides 2533 – 2547 deleted
	Reverse	GAT TTG AAC GCG CTC TGT GAC CTC GTT CCC G	
Δ3T	Forward	GAA GAC CAT GAT TTT TGA AAC CTC CCC TTC GGC	Nucleotides 2701 – 2748
	Reverse	GCG GAA GGG GAG GTT TCA AAA ATC ATG GTC TTC	

Supplementary Supp. Table 2 Combinations of plasmids from which transcripts were utilized to produce the Fny-CMV variants used during this study. Variants of Fny-CMV were regenerated as previously described by Rizzo and Palukaitis (1990), but using transcripts from the combinations of plasmids described here.

Fny-CMV variant	**Plasmid combination required**
Wild-type Fny-CMV	pFny109; pFny209; pFny309
Fny-CMVΔ5T	pFny109; pFny209:Δ5T; pFny309
Fny-CMVΔNLS1	pFny109; pFny209:ΔNLS1; pFny309
Fny-CMVΔNLS2	pFny109; pFny209:ΔNLS2; pFny309
Fny-CMVΔNLS1+2	pFny109; pFny209:ΔNLS1+2; pFny309
Fny-CMVS40A	pFny109; pFny209:S40A; pFny309
Fny-CMVS42A	pFny109; pFny209:S42A; pFny309
Fny-CMVΔKSPSE	pFny109; pFny209:ΔKSPSE; pFny309
Fny-CMVΔ3T	pFny109; pFny209:Δ3T; pFny309
Fny-CMVΔ2b	pFny109; pFny209/M3; pFny309

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Symptoms induced by Fny-CMV variants in non-inoculated leaves of tobacco (scale bar 5 cm). Tobacco plants were inoculated with infectious sap and symptoms were photographed approximately 5 weeks post-inoculation (wpi).

Supplementary Figure 2. Symptoms induced in *N. benthamiana* by Fny-CMV variants, approximately 5 wpi. Photographs are from a replicate experiment, where plants were inoculated later (age approximately 5 weeks post germination) than those used in the experiment shown in Supp. Fig. 1. Scale bars indicate 5 cm.

Supplementary Figure 3. Protein sequences of mutagenized Fny 2b constructs. In 2b Δ5T the codons encoding amino acids 2 and 9 were mutated to stop codons (*****); translation of this construct is expected to start at the third start codon (**M**). In the 2b Δ3T construct the codon corresponding to amino acid 95 has been mutated to a stop codon (*****) and translation of this sequence is expected to stop at this amino acid.

Supplementary Figure 4. Phenotypic changes induced in transgenic *A. thaliana* ecotype Col-0 plants by 35S:Fny2b (B), 35S:Fny2b Δ3T (C) and 35S:Fny2b Δ3T constructs (D), compared to non-transgenic control (A). Scale bars indicate 3 cm.

Supplementary Figure 5. Symptoms induced by Fny-CMV and Fny-CMVΔ2b in *Chenopodium quinoa* (panel A). Panel B shows a close up of lesions induced by Fny-

CMVΔ2b in directly-inoculated leaves. Plants were photographed 5 weeks post-inoculation. Scale bar indicates 10 cm in panel A and 2 cm in panel B.