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e-Xtra*

The Role of the *Cucumber mosaic virus* 2b Protein in Viral Movement and Symptom Induction

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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 that 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 while those infected with the wild-type virus did not. 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 the 2b protein itself is an important symptom determinant in certain hosts. However, they also suggest that the protein may somehow synergize symptom induction by other CMV-encoded factors.

Cucumber mosaic virus (CMV) is the type species of the genus *Cucumovirus*, which also includes *Peanut stunt virus* and *Tomato aspermy virus* (TAV) (Palukaitis and García-Arenal 2003). 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 (Palukaitis and García-Arenal 2003; Roossinck 2001). One of these proteins is the multifunctional 2b protein (about 12 kDa) encoded by the second open reading frame (ORF) of RNA 2 and synthesized by the translation of a subgenomic mRNA, RNA 4A (Ding et al. 1994).

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The 2b protein influences local and systemic viral movement and inhibits host defense mechanisms based on salicylic acid (SA)-induced resistance and RNA silencing (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 TAV 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) moved systemically in tobacco and *N. benthamiana* but did 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. 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 the 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 were similar in appearance to non-transgenic plants (Chapman et al. 2004; Lewsey et al. 2007; Siddiqui et al., 2008; 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 ORF and the 3′ region of the 2a replicase protein gene. Other conserved features include a 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 a biologically active cDNA clone of Fny-CMV RNA 2 (pFny209 [Rizzo and Palukaitis 1990]).

Mutant plasmids were made from which CMV RNA 2 harboring the mutations could be transcribed in vitro (Fig. 1A). RNA 2, transcribed from plasmid pFny209:Δ5T, contains a deletion of the sequence encoding the first 17 amino acids of the Fny-CMV 2b protein (Fig. 1A). RNA 2, transcribed from plasmids pFny209:ΔNLS1, pFny209:ΔNLS2, or pFny209:ΔNLS1+2, contains deletions of the sequences encoding one of the two segments of the 2b protein NLS (1 or 2) or both segments, respectively (Fig. 1A). RNA 2, transcribed from plasmid pFny209:ΔKSPSE, contains a deletion of the sequence encoding the putative phosphorylation motif KSPSE (Fig. 1A). Mutant RNA 2, transcribed from plasmids pFny209:S40A and pFny209:S42A, contain substitutions of the sequences encoding serine residues 40 and 42, respectively, changing them to alanine residues (Fig. 1A). These two serine residues are the most probable phosphorylation sites within the putative phosphorylation domain. The mutant RNA 2, transcribed from plasmid pFny209:Δ3T, contains a deletion of the sequence encoding the 16 C-terminal residues of the 2b protein, yielding a truncated 2b protein (Fig. 1A). For all newly made plasmids, successful mutation of pFny209 was confirmed by DNA sequencing (data not shown).

Wild-type (WT) and mutant RNA 2 were generated by in vitro transcription and were combined as appropriate with in vitro-synthesized RNAs 1 and 3 using the plasmids pFny109 and pFny309 as templates (Rizzo and Palukaitis 1990). Infectious transcripts of the mutant Fny-CMVΔ2b, in which most of

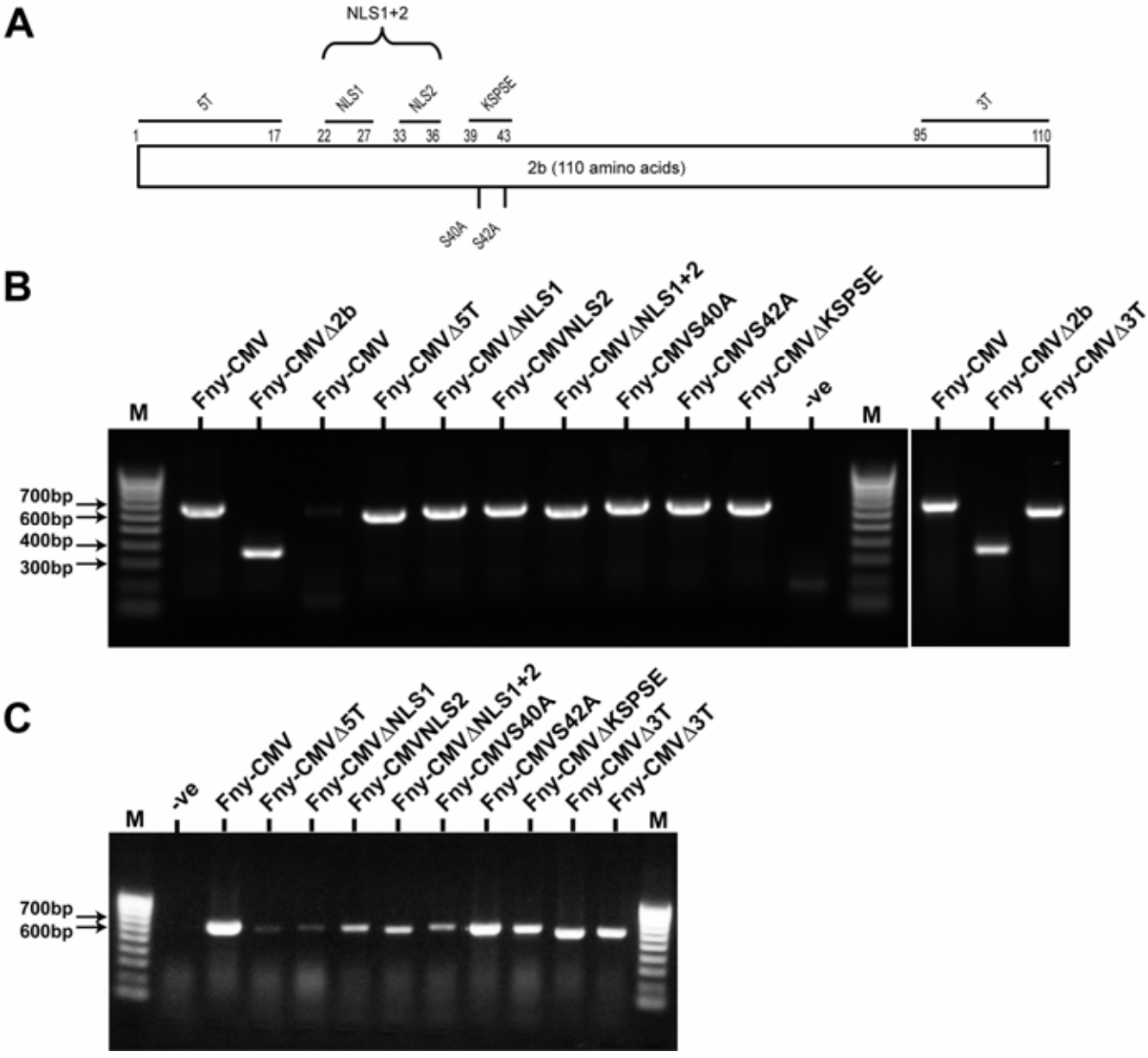

Fig. 1. A, Schematic map describing locations of mutations, indicated by amino acid residue numbers, created in the 110–amino acid 2b protein of Fny-CMV. Mutations were generated by site-directed mutagenesis using primers. **B** and **C,** Confirmation of systemic infection by Fny-CMV variants in *Arabidopsis thaliana* ecotype Col-0 (B) and *Nicotiana benthamiana* (C). Noninoculated tissue was tested for the presence of viral RNA by reverse-transcription-polymerase chain reaction (RT-PCR). The identity of the virus is indicated above each lane. The expected product size from Fny-CMVΔ2b was 370 bp, and the expected product from wild-type 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.

the 2b ORF is deleted, also were reconstituted (Ryabov et al. 2001; Soards et al. 2002). These were inoculated mechanically onto leaves of tobacco (cv. Xanthi-nc), *N. benthamiana*, and *A. thaliana* plants, and the development of symptoms was observed (Table 1; Fig. 2; Supplementary Figs. 1 and 2). In all host-virus combinations, systemic accumulation of the virus was confirmed by reverse-transcription-polymerase chain reaction (RT-PCR) (Fig. 1B and C; 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). As previously described, infection of tobacco by Fny-CMVΔ2b was also asymptomatic (Table 1; Fig. 2A; Soards et al. 2002). Infection with Fny-CMVS40A or Fny-CMVS42A caused mild symptoms in tobacco. These included mild leaf distortion, mild systemic mosaic, and mild stunting (Table 1; Fig. 2A). Tobacco plants infected with Fny-CMVΔ3T exhibited severe symptoms, including systemic mosaic, leaf distortion, and chlorosis (Table 1; Fig. 2A).

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). Infection with Fny-CMVΔNLS1 caused mild stunting, while Fny-CMVS40A caused mild leaf distortion and mild stunting (Table 1; Fig. 2B). Infection with Fny-CMVS42A caused intermediate stunting and intermediate leaf distortion (Table 1; Fig. 2B).

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; Fig. 2C). Infection by Fny-CMVS40A or Fny-CMVS42A caused mild stunting, while Fny-CMVΔ3T infection caused symptoms of stunting and leaf distortion, comparable to those induced by WT Fny-CMV infection (Table 1; Fig. 2C). These reactions were similar to those of tobacco and *N. benthamiana* (Table 1; Fig. 2).

Immunoblot analysis with an anti–Fny-CMV 2b serum was used to detect the mutant 2b proteins encoded by CMV variants in infected *N. benthamiana* plants approximately 6 weeks postinoculation (Fig. 3A). The 2b protein of WT Fny-CMV localizes to the host cell nucleus (Lucy et al. 2000; Mayers et al. 2000). Crude nuclei-enriched fractions of proteins were extracted, as described previously (Mayers et al. 2000), from infected plants. Immunoblot analysis revealed that the mutant 2b proteins encoded by Fny-CMVΔNLS1, Fny-CMVS40A, and Fny-CMVS42A accumulated to levels comparable to that of WT Fny-CMV (Fig. 3A). The 2b protein detected was in its monomeric form, which migrates with an apparent molecular mass of 17 kDa (Chen et al. 2008; Mayers et al. 2000). Fny-CMVΔ3T also produced a stable, detectable 2b protein, which migrated further in the gel than WT Fny-CMV 2b protein, indicating a lower apparent molecular mass (Fig. 3A). Severe degradation of the large subunit of RUBISCO was consistently observed in protein extracts from plants infected with this variant, as shown by Ponceau S staining (Fig. 3A), which may have affected the accuracy of loading. The protein degradation was likely due to the severe symptoms (leading to host death) that Fny-CMVΔ3T induced. The 2b protein was not detected in the nuclei-enriched fraction from plants infected with Fny-CMVΔ5T, Fny-CMVΔNLS2, Fny-CMVΔNLS1+2, or Fny-CMVΔKSPSE (Fig. 3A).

It is possible that the 2b protein was not detected in plants infected with Fny-CMVΔ5T, Fny-CMVΔNLS2, Fny-CMVΔNLS1+2, or Fny-CMVΔKSPSE because those variants exhibited lower viral accumulation, resulting in concomitantly lower 2b protein accumulation. To determine whether lowered viral accumulation might explain lowered 2b protein abundance, accumulation of CMV variants was assessed relative to WT Fny-CMV in infected *N. benthamiana* (Fig. 3B) and tobacco (Fig. 3C) plants, using immunoblotting for CMV coat protein. Coat protein was detected during infections with all CMV variants but at different abundances, indicating that relative accumulation of the variants differed. Fny-CMVS40A, Fny-CMVS42A, and Fny-CMVΔ3T coat protein accumulated to levels comparable to that of WT Fny-CMV (Fig. 3B and C), correlating with the accumulation of 2b protein in plants infected with these variants (Fig. 3A). However, it is notable that variants Fny-CMVS40A and Fny-CMVS42A induced milder symptoms than WT Fny-CMV (Table 1; Fig. 2). This demonstrates that the mutations in Fny-CMVS40A and Fny-CMVS42A diminished the ability of 2b protein to induce symptoms without altering the protein's stability and that altered viral accumulation was not responsible for the changes in symptomology. Accumulation of coat protein in Fny-CMVΔNLS1–infected plants was greatly reduced relative to that in plants infected with Fny-CMV. This was true also in plants infected with Fny-CMVΔ5T, Fny-CMVΔNLS2, Fny-CMVΔNLS1+2, or Fny-CMVΔKSPSE (Fig. 3B). Given that the 2b protein encoded by Fny-CMVΔNLS1 accumulated to levels comparable to WT Fny-CMV (Fig. 3A), this demonstrates that reduced viral accumulation does not necessarily result in decreased accumulation of 2b protein. Furthermore, as it was observed that, during infections with variants Fny-CMVS40A and Fny-CMVS42A, the viruses accumulated to levels comparable to

Table 1. Symptoms induced by Fny-CMV variants in *Arabidopsis thaliana* ecotype Col-0, *Nicotiana benthamiana*, and tobacco

	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; necrosis	Stunting; leaf distortion; systemic mosaic
Fny-CMVΔ2b	No obvious symptoms	No obvious symptoms	No obvious symptoms
Fny-CMVΔ5T	No obvious symptoms	No obvious symptoms	No obvious symptoms
Fny-CMVΔNLS1	No obvious symptoms	Mild stunting	No obvious symptoms
Fny-CMVΔNLS2	No obvious symptoms	No obvious symptoms	No obvious symptoms
Fny-CMVΔNLS1+2	No obvious symptoms	No obvious symptoms	No obvious symptoms
Fny-CMVS40A	Mild stunting	Mild leaf distortion; mild stunting	Mild stunting; mild leaf distortion; mild systemic mosaic
Fny-CMVS42A	Mild stunting	Intermediate stunting; intermediate leaf distortion	Mild stunting; mild leaf distortion; mild systemic mosaic
Fny-CMVΔKSPSE	No obvious symptoms	No obvious symptoms	No obvious symptoms
Fny-CMVΔ3T	Stunting; leaf distortion	Stunting; leaf distortion; necrosis	Leaf distortion; systemic mosaic; chlorosis

WT Fny-CMV (Fig. 3B) but that these variants have a diminished ability to induce symptoms (Table 1; Fig. 2), it can be concluded that the level of CMV accumulation does not determine symptom induction.

As several mutant 2b proteins were not detected in nuclei-enriched fractions of protein extracts, we examined the cytoplasm- and membrane-enriched supernatant fractions of the protein extracts by immunoblotting for the presence of protein cross-reacting with the anti-2b antibody. This was to identify whether all CMV variants expressed a stable 2b protein. The 2b protein can form a dimer, which migrates with an apparent molecular mass of approximately 25 kDa and is stable against denaturation by sodium dodecyl sulfate (Chen et al. 2008). A cross-reacting protein of this apparent molecular mass was detected in supernatant samples from plants infected with WT Fny-CMV, Fny-CMVΔNLS2, Fny-CMVΔNLS1+2, and Fny-CMVS42A (Fig. 3D). It is notable, though, that the 2b protein was detected as its monomeric form in the nuclei-enriched fraction from Fny-CMV–infected plants but as its putative dimeric form in the cytoplasm- and

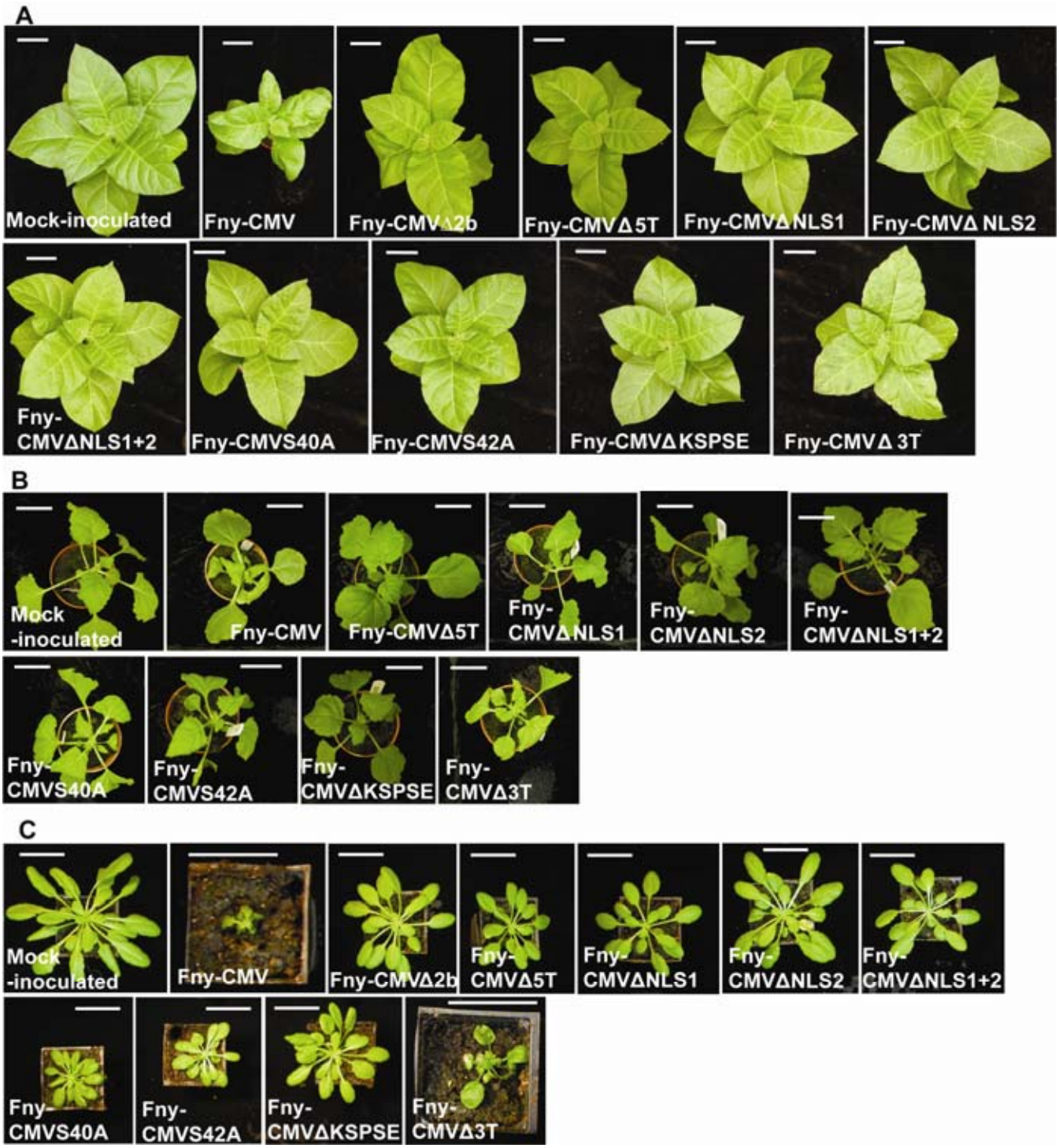

Fig. 2. Symptoms induced by **A,** Fny-CMV variants in tobacco plants (scale bars = 5 cm), **B,** *Nicotiana benthamiana* plants (scale bars = 5 cm), and **C,** *Arabidopsis thaliana* ecotype Col-0 plants (scale bars = 3 cm). Symptoms in tobacco and *N. benthamiana* were photographed approximately 5 weeks postinoculation (wpi), while those of *A. thaliana* were taken approximately 3 wpi.

membrane-enriched fraction from the same plants (Fig. 3D). These data suggest that Fny-CMVΔNLS2 and Fny-CMV ΔNLS1+2 do produce stable 2b proteins but that they may have altered subcellular localization. This suggestion is supported by previous studies expressing 2b-reporter protein fusions from heterologous viral vectors, which observed that disruption of one or more NLS of 2b altered the protein's subcellular localization (Lucy et al. 2000; Wang et al. 2004). The mutant 2b proteins produced by Fny-CMVΔKSPSE and Fny-CMVΔ5T were not detected in any experiment, and therefore, the possibility that they are unstable cannot be excluded (Fig. 3A and D; data not shown).

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 truncated forms (Fig. 1A) of the 2b protein were recreated in this vector by introducing stop codons at amino acids 2 and 9 (Δ5T) or

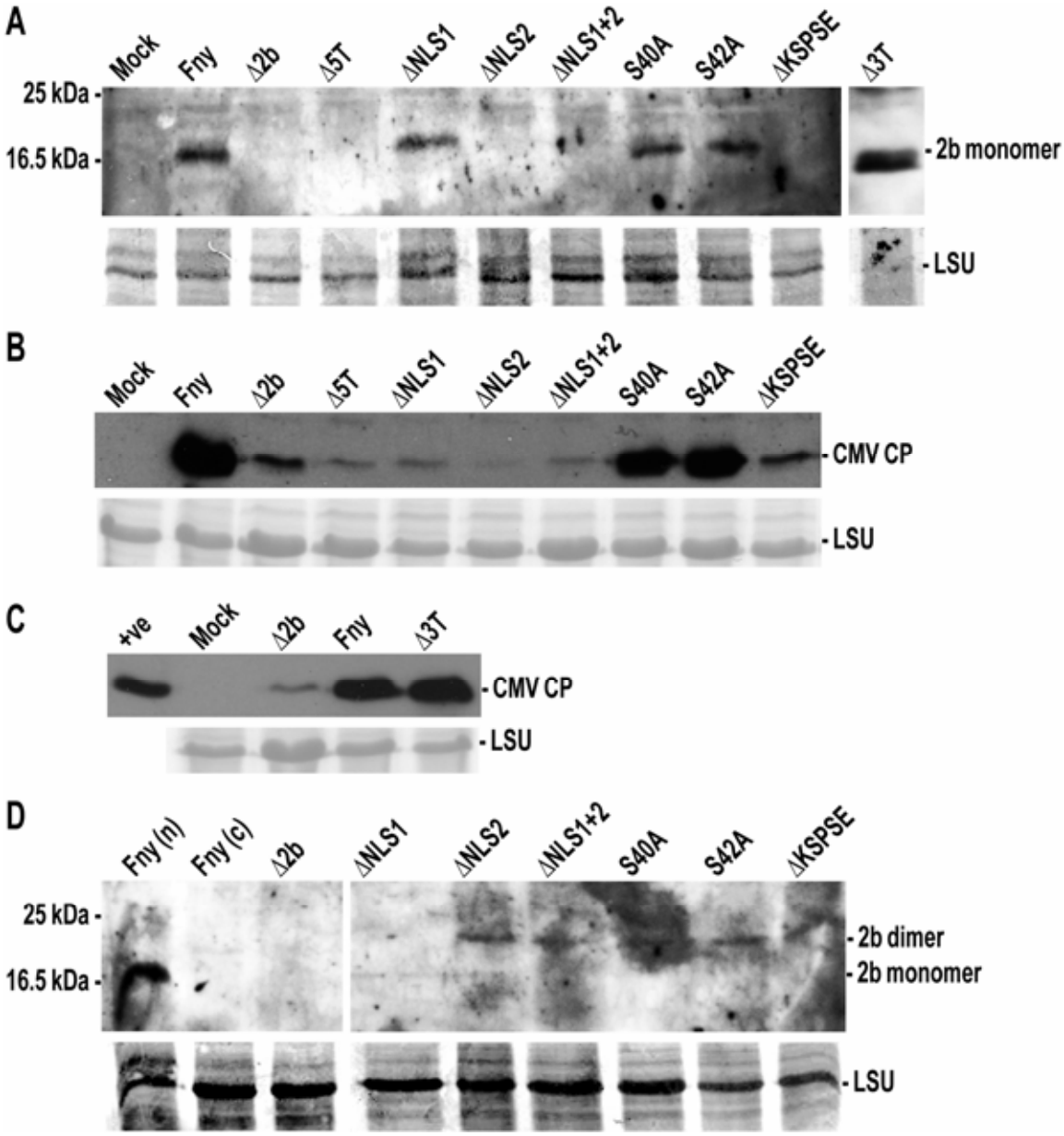

Fig. 3. Immunoblot detection of mutant 2b proteins and *Cucumber mosaic virus* (CMV) coat protein produced during infections with CMV variants. Plants were inoculated with Fny-CMV or variants encoding mutant 2b proteins. Approximately six weeks postinoculation (wpi), noninoculated upper leaves were harvested for the extraction of protein and detection of wild-type (WT) or mutant 2b proteins. **A,** Crude nuclei-enriched and **D,** cytoplasm- and membrane-enriched fractions were analyzed separately (A and D, in *Nicotiana benthamiana*). In both cases, 30 μg of total proteins per lane were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Equal loading was verified by staining of the large subunit of RUBISCO with Ponceau S (panels labeled LSU). The identity of CMV variants is indicated by labeling lanes with the mutation they harbored, with WT Fny-CMV denoted as Fny. In A, the lane labeled Mock contains a sample from a mock-inoculated control plant. Note that the 2b protein encoded by Fny-CMVΔ3T migrated with a lower apparent molecular mass than that of Fny-CMV, consistent with the fact that it harbors a deletion. In D, samples from the nuclei-enriched versus the cytoplasm- and membrane-enriched fractions of Fny-CMV 2b protein extracts are distinguished by (n) and (c), respectively. The monomeric and putative dimeric forms of 2b protein are indicated as appropriate. **B,** Accumulation of CMV coat protein (labeled CMV CP) was also assessed by SDS-PAGE and immunoblotting in independent plants at 2 wpi (in *N. benthamiana*) and **C,** 10 wpi (*N. tabacum*). Blots were loaded with equal quantities of protein, and equal loading was verified by staining of the large subunit of RUBISCO with Ponceau S (panels labeled LSU). Lanes are labeled as in A and D. +ve denotes a lane loaded with 0.5 μg of purified CMV particles as a positive control.

amino acid 95 (Δ3T) (Supplementary Fig. 3). In transgenic *A. thaliana* (Col-0) plants, the 35S:Fny2bΔ5T construct induced no obvious changes in phenotype, while 35S:Fny2bΔ3T induced a symptom-like phenotype similar to that induced by constitutive expression of a WT Fny-CMV *2b* transgene (Fig. 4; Lewsey et al. 2007). These modified plant phenotypes are consistent with the symptoms induced by infection of nontransgenic plants with the corresponding mutant viruses. This indicates that the differences in symptoms induced in *A. thaliana* (Col-0) plants 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 *Cucurbita pepo* cv. Goldrush (data not shown) and in plants of a *Chenopodium* species

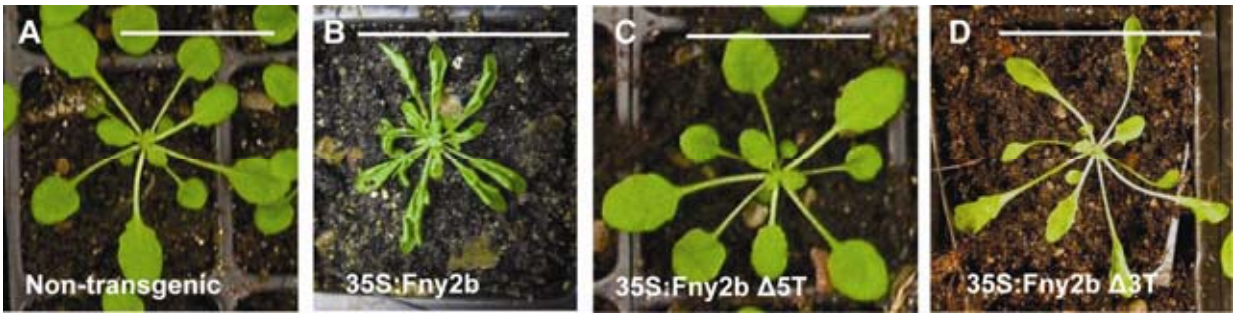

Fig. 4. Phenotypic changes induced in transgenic *Arabidopsis thaliana* ecotype Col-0 plants. **A,** Nontransgenic control. Induced by **B,** 35S:Fny2b; **C,** 35S:Fny2b Δ3T; and **D,** 35S:Fny2b Δ3T constructs. Scale bars indicate 3 cm.

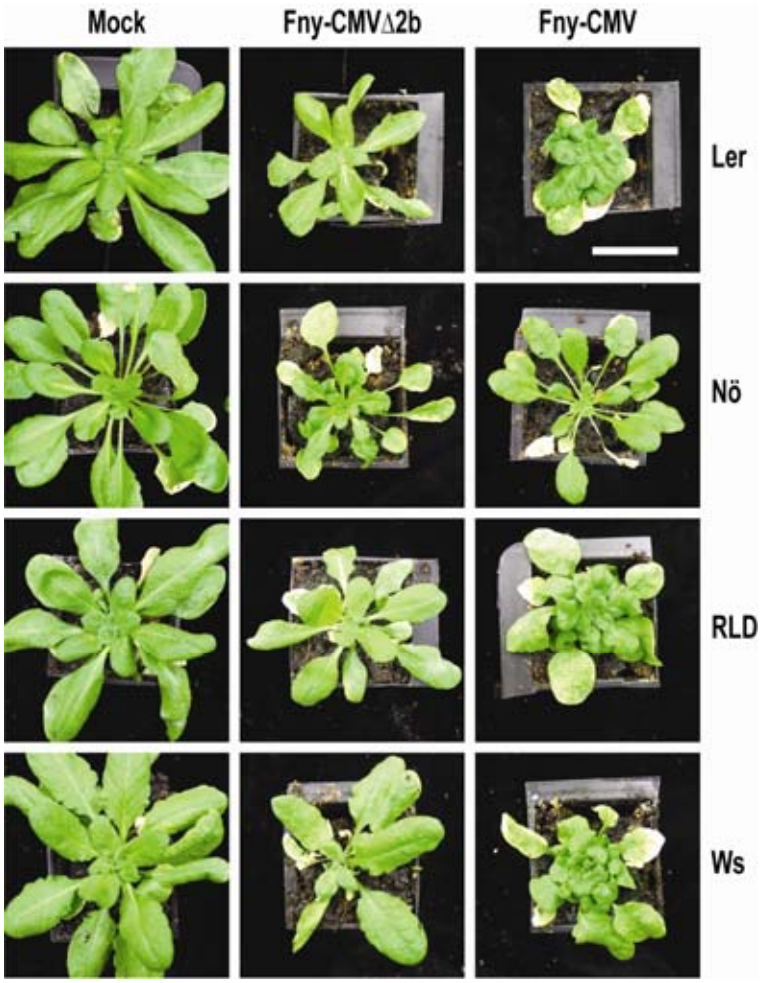

Fig. 5. Symptoms induced by Fny-CMVΔ2b and Fny-CMV in *Arabidopsis thaliana* ecotypes Landsberg erecta (Ler), Nössen (Nö), RLD, and Wassilewskija (Ws). Plants were inoculated with purified virions. Infection was confirmed by reverse-transcription-polymerase chain reaction using primers flanking the *2b* coding region, and symptoms were observed at 19 days postinoculation. Scale bar indicates 3 cm and is relevant to all panels.

(Supplementary Fig. 4). *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 may not 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. 5) 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. 6A). These symptoms included necrosis and disturbance of normal leaf development (Fig. 6A). This contrasted with infection of *A. thaliana* ecotype Col-0 by Fny-CMVΔ2b, which was asymptomatic (Table 1; Fig. 2C).

When infected with either WT 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 (Figs. 5 and 6A). Observation over time indicated that symptom induc-

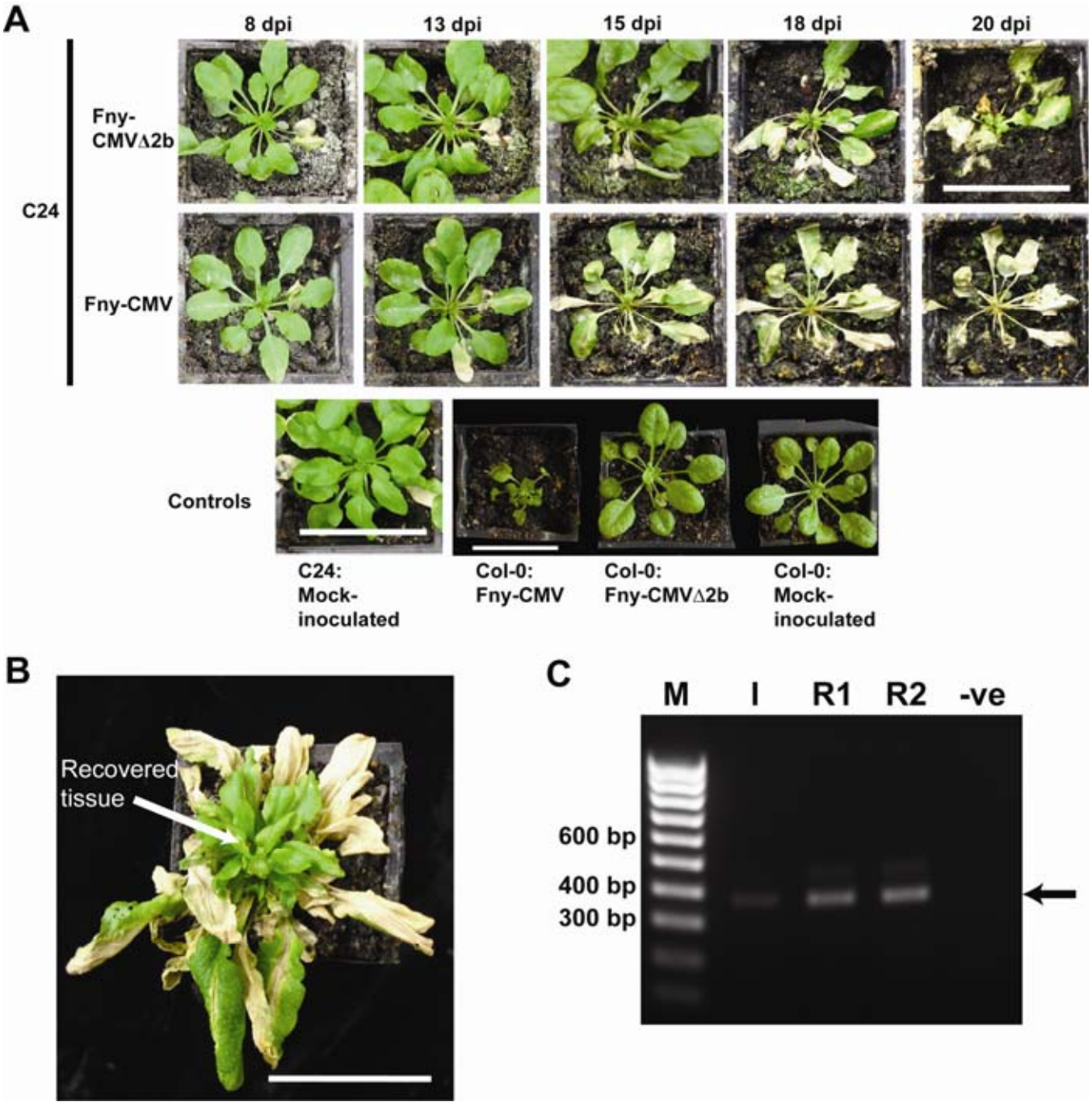

Fig. 6. Symptoms induced by Fny-CMVΔ2b and Fny-CMV in *Arabidopsis thaliana* ecotype C24. Plants were inoculated with purified virions. **A,** Infection was confirmed by reverse-transcription-polymerase chain reaction (RT-PCR) using primers flanking the *2b* coding region and symptoms observed at intervals up to 20 days postinoculation (dpi). Control plants were photographed at 21 dpi (scale bars indicate 3 cm). **B,** Recovery from disease was photographed in Fny-CMVΔ2b–inoculated plants at 30 dpi (scale bar indicates 3 cm). **C,** Newly emerged tissue exhibiting recovery from disease was tested for the presence of Fny-CMVΔ2b RNA by RT-PCR. 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 (negative) contains the results of an RT-PCR reaction conducted using RNA from a mock-inoculated plant.

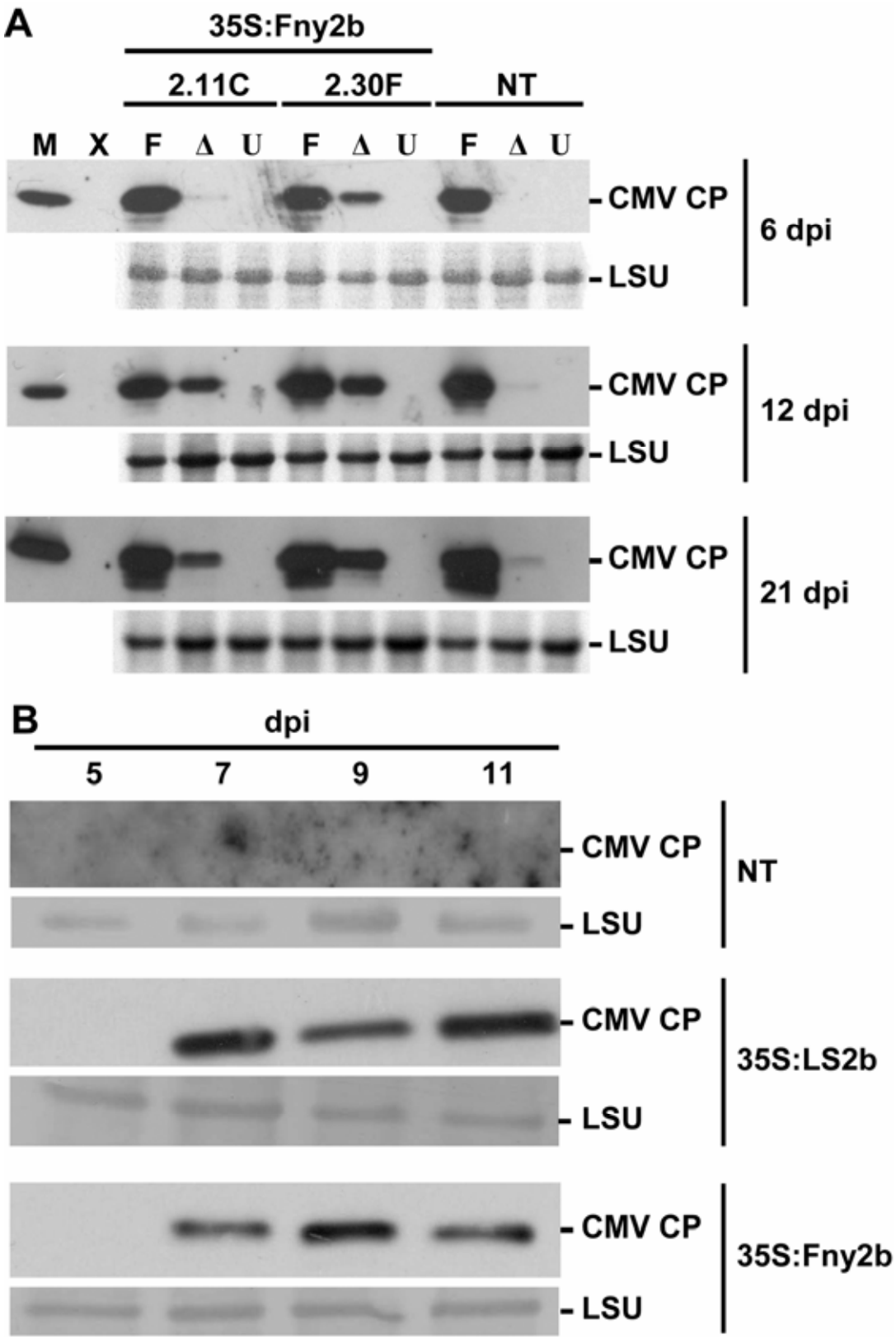

Fig. 7. Accumulation of Fny-CMV or Fny-CMVΔ2b in noninoculated leaves of nontransgenic *Arabidopsis thaliana* ecotype Col-0 and plants harboring the 35S:Fny2b transgene or 35S:LS2b transgene. Plants were inoculated on three lower leaves with purified virions, and noninoculated leaves were collected for analysis. **A,** The accumulation of coat protein 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 days postinoculation (dpi). Infecting viruses are denoted F (Fny-CMV), Δ (Fny-CMVΔ2b), and U (uninfected control). M denotes marker lanes loaded with 1 μg of purified *Cucumber mosaic virus* (CMV). X indicates nonloaded lanes. **B,** Accumulation of Fny-CMVΔ2b in one 35S:Fny2b and one 35S:LS2b line was assessed at 5, 7, 9, and 11 dpi. Lanes loaded with protein from nontransgenic *A. thaliana* plants are labeled NT. In both experiments, proteins were extracted and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All blots were loaded with equal quantities of protein, and equal loading was verified by staining of the large subunit of RUBISCO with Ponceau S (panels labeled LSU). CMV accumulation was analyzed by immunoblotting for CMV coat protein (panels labeled CMV CP). Each test lane represents a separate plant. Individual plants were analyzed at one timepoint only.

tion on C24 plants by Fny-CMVΔ2b was delayed relative to Fny-CMV and that the symptoms induced by the mutant were slightly milder (Fig. 6A). *A. thaliana* ecotype C24 plants infected with Fny-CMV eventually died, while those infected with Fny-CMVΔ2b eventually exhibited recovery from disease in the form of apparently necrosis-free new growth (Fig. 6A and B). Fny-CMVΔ2b RNA was detected by RT-PCR in the emerging leaves that exhibited recovery from disease (Fig. 6C). This indicates that the plants had not undergone a true recovery in which the initial 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

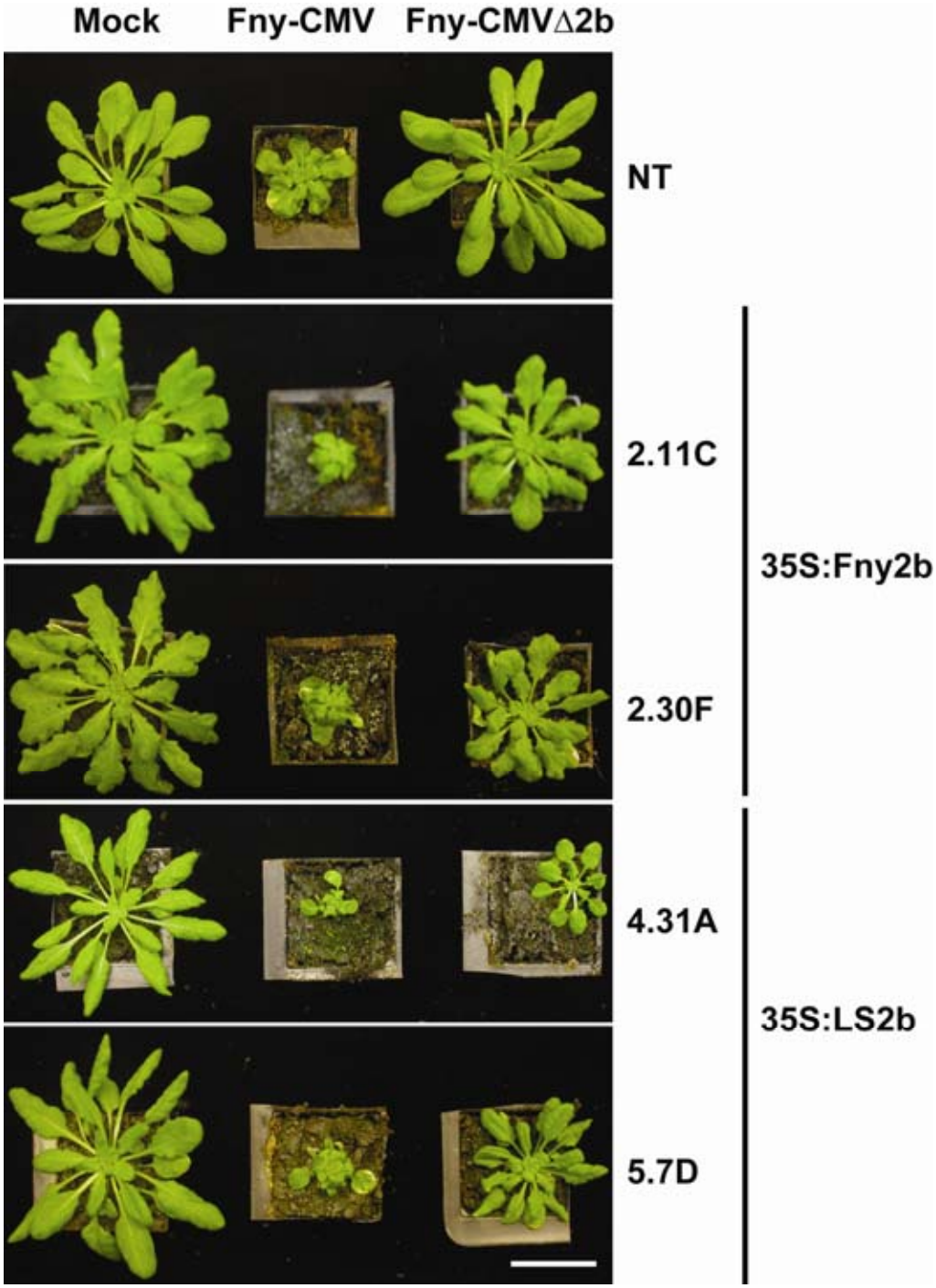

Fig. 8. Symptoms of infection by Fny-CMV and Fny-CMVΔ2b of plants harboring the 35S:Fny2b and 35S:LS2b transgenes. Plants of two independent transgenic lines harboring the 35S:Fny2b transgene (lines 2.11C and 2.30F), two independent transgenic lines harboring the 35S:LS2b transgene (lines 4.31A and 5.7D), and nontransgenic *Arabidopsis thaliana* ecotype Col-0 plants were infected with Fny-CMV, Fny-CMVΔ2b, or mock-inoculated. After 1 month, typical examples of infected plants (confirmed by reverse-transcription-polymerase chain reaction) 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 nontransgenic *A. thaliana* (row labeled NT) by Fny-CMVΔ2b. Scale bar indicates 3 cm.

(35S:Fny2b; Lewsey et al. 2007) were infected with Fny-CMV or Fny-CMVΔ2b. Relative virus accumulation and the induction of symptoms in systemically infected leaves were examined at various times following inoculation. The systemic movement of WT and mutant CMV was monitored by taking samples of protein from noninoculated leaves at 6, 12, and 21 days postinoculation (dpi). Virus accumulation was detected by immunoblot analysis of leaf proteins using antiserum specific for the CMV coat protein.

In nontransgenic plants of *A. thaliana* ecotype Col-0, Fny-CMVΔ2b accumulated less rapidly in noninoculated leaves relative to WT Fny-CMV (Fig. 7A), consistent with Fny-CMVΔ2b systemic movement in tobacco (Soards 2003). In transgenic plants constitutively expressing the Fny 2b protein, Fny-CMVΔ2b movement to noninoculated tissues occurred more rapidly and the accumulation of the virus was increased, relative to nontransgenic plants (Fig. 7A). However, the amount of mutant virus that accumulated in the noninoculated leaves was still less than that achieved by the WT virus (Fig. 7A).

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 nontransgenic *A. thaliana* ecotype Col-0 with LS-CMV is symptomless (Lewsey et al. 2007). Nontransgenic *A. thaliana* ecotype Col-0 plants and 35S:LS2b and 35S:Fny2b plants were inoculated with Fny-CMVΔ2b. Samples of protein were extracted from noninoculated leaf tissue at 5, 7, 9, and 11 dpi and were analyzed for CMV coat protein accumulation by immunoblotting (Fig. 7B). It was found that the 35S:LS2b transgene complemented accumulation of Fny-CMVΔ2b in systemically infected tissues as effectively as 35S:Fny2b (Fig. 7B).

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 (Supplementary Fig. 5). Plants confirmed to be infected with Fny-CMVΔ2b were assessed visually for symptoms 1 month postinoculation and were photographed (Fig. 8). It should be noted that noninfected plants of these 35S:Fny2b lines exhibit a mild symptom-like phenotypic change in response to the transgene and that noninfected 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 WT Fny-CMV resulted in very severe disease symptoms. These were more severe than those induced by infection of nontransgenic *A. thaliana* ecotype Col-0 (Fig. 8). Most likely, this is 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 nontransgenic *A. thaliana* ecotype Col-0 plants (Figs. 2C and 8). In contrast, plants expressing either the 35S:Fny2b or the 35S:LS2b transgene infected with Fny-CMVΔ2b exhibited obvious stunting compared with 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 WT 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 WT and mutant RNA 2 molecules (Ziebell et al. 2007). Full-length CMV RNA 2 was not detected in any RT-PCR reactions (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. 7) and on symptom development (Fig. 8) were caused by 2b protein supplied in trans, as RT-PCR data indicated that *2b* transgene–derived mRNAs had not recombined with the virus (data not shown).

DISCUSSION

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

The 2b protein of the subgroup IA strain of CMV, Fny-CMV, is a strong inducer of symptoms in several hosts. We have demonstrated that several amino-acid sequence domains (5′ terminal 17 aa, NLS1, NLS2, NLS1 + 2, serine residue 40, serine residue 42, the motif KSPSE, 3′ terminal 16 aa) within the 2b protein affect its ability to induce symptoms in a range of host species (tobacco, *N. benthamiana*, and *A. thaliana* ecotype Col-0). Our study provides the first demonstration that both NLS domains 1 and 2 are required for 2b protein to operate as an effective symptom determinant during an authentic CMV infection rather than when expressed from heterologous viral vectors (Lucy et al. 2000; Wang et al. 2004). Furthermore, we have shown that mutation of serine residue 40 or 42 reduces the ability of CMV to induce symptoms without impairing viral or 2b protein accumulation. Since the CMV variants carrying mutations of serines 40 or 42 accumulated to relative levels comparable to WT Fny-CMV but exhibited reduced symptom induction, it can be concluded that abundance of CMV did not determine symptom severity. However, low accumulation or instability of 2b protein may have been a cause of reduced symptom induction by some CMV variants. All four variants exhibiting reduced 2b protein accumulation relative to WT Fny-CMV (Fny-CMVΔ5T, Fny-CMVΔNLS2, Fny-CMVΔNLS1+2, and Fny-CMVΔKSPSE) were also impaired in their ability to induce symptoms.

In this work, we observed that deletion of the first 17 aa of Fny-CMV 2b greatly reduced stability of the protein. A recent study by Chen and colleagues (2008) analyzed the effects of several conserved residues within the 2b protein of TAV on multimer formation and double stranded (ds)RNA binding. They demonstrated that conserved residues among the first 18 aa are required for tetramer formation. In combination, these data suggest that formation of multimers may be important for stability of the 2b protein in planta.

We have demonstrated here that the mutant 2b proteins encoded by CMV variants lacking either NLS 2 or both NLS 1 and NLS 2 could be detected only in the cytoplasm- and membrane-enriched fraction of protein extractions and only in their putative dimeric form. This indicates that deletion of NLS 2 or of NLS 1 and NLS 2 simultaneously may alter subcellular localization and the dimerization properties of the protein. Conserved residues within NLS 1 and NLS 2 were also crucial for the TAV 2b protein to bind dsRNA (Chen et al. 2008). Therefore, our data may indicate that dsRNA-binding ability, nuclear localization, or both in concert may be required for symptom induction by the 2b protein.

Our study indicates that the phosphorylation state of the 2b protein may modulate its symptom-inducing activity. We observed that deletion of the entire putative phosphorylation sequence KSPSE (Fny-CMVΔKSPSE) abolished symptom induction and that point mutations of serine residues 40 and 42

(Fny-CMVS40A or Fny-CMVS42A) greatly decreased CMV-induced symptoms. This is consistent with previous studies suggesting that these serine residues are phosphorylatable (Lucy et al., 2000). It has been proposed that the five residues KSPSE constitute motifs for casein kinase II and cyclin-dependent kinase 2–dependent phosphorylation, which regulate nuclear import of the 2b protein and that, in combination with several potential nuclear-export signals, they act to shuttle the 2b protein in and out of the nucleus (Lucy et al. 2000). However, Chen and colleagues (2008) described an important role for the conserved residues 40 to 42 (serine-proline-serine) of TAV 2b in 2b protein structure and stabilization of 2b-dsRNA interactions. Based on our results and the work of Chen and colleagues (2008), we conclude that modifications in this region of the 2b protein may diminish symptom induction in three ways. i) Deletion of the sequence KSPSE may have altered the stability of the protein, thereby decreasing 2b-mediated symptom induction. ii) Deletion of this sequence as well as substitution of residues 40 and 42 may have reduced dsRNA binding affinity of the 2b protein, thereby decreasing 2b-mediated symptom induction. iii) The sequence KSPSE may be a phosphorylation sequence, disruption of which has decreased the symptom-inducing activity of the 2b protein. These hypotheses are not mutually exclusive, which highlights the multifunctional nature of domains within the 2b protein and indicates that rigorous testing is required to definitely attribute specific functions to specific domains.

Our results also suggest that in certain hosts, the C-terminal region of the 2b protein may moderate symptom severity. Deletion of the sixteen C-terminal amino acids (in Fny-CMVΔ3T) did not attenuate symptom induction or reduce 2b protein stability. Rather, it seemed to alter the precise nature of symptoms in tobacco and increase severity in *N. benthamiana*. A previous study found that this region has transcriptional activation activity in yeast (Ham et al. 1999).

A final point of note is that the coding region for the 2b protein has substantial overlap with the coding region for the 2a protein. All 2b deletion mutants, except Fny-CMVΔ3T, will have carried concomitant deletions in the 2a protein. The mutants Fny-CMVS40A and Fny-CMVS42A will have carried point mutations in the 2a protein also (lysine 825 to serine and glutamine 827 to arginine, respectively). The possibility that changes to the 2a protein may have affected the symptoms elicited by CMV variants cannot be excluded (Du et al. 2008).

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

In this study, we demonstrated that Fny-CMVΔ2b induced symptoms in plants of a *Chenopodium* species, *N. occidentalis*, and five ecotypes of *A. thaliana* (C24, Wassilewskija, Landsberg erecta, Nössen, and RLD). The virus was able to spread systemically in both *A. thaliana* ecotype Col-0 and ecotype C24 but did not induce symptoms in Col-0. Previous studies have shown that Fny-CMVΔ2b induces symptoms transiently in squash but is asymptomatic in tobacco and *N. benthamiana* (Soards 2003; Soards et al. 2002; Wang et al. 2004). Further investigation revealed that *A. thaliana* ecotype C24 plants infected with Fny-CMVΔ2b exhibited some form of recovery from symptoms, whereas those infected with WT Fny-CMV did not. This was not, however, a true recovery phenomenon, as viral RNA was detectable in the recovered tissue. A similar effect has been observed in *N. benthamiana* plants recovering from a nepovirus infection (Jovel et al. 2007).

Experiments conducted using 35S:*2b* transgenic plants (*A. thaliana* ecotype Col-0) demonstrated that both the LS 2b and Fny 2b proteins were able to complement systemic movement of Fny-CMVΔ2b. Furthermore, we found that infection of Fny *2b*-transgenic *A. thaliana* ecotype Col-0 plants with Fny-CMV resulted in extremely severe disease symptoms, in contrast to a previous report that CMV infection ameliorated 2b-induced phenotype changes in *2b*-transgenic tobacco (Praveen et al. 2008). Infection of LS *2b*-transgenic plants with Fny-CMVΔ2b also induced symptoms (Fig. 8), even though LS-CMV infection of *A. thaliana* ecotype Col-0 with LS-CMV is normally asymptomatic and transgenic expression of LS 2b protein causes only very mild changes in plant phenotype (Lewsey et al. 2007). Consequently, we propose that some other CMV gene product may play a role in inducing CMV symptoms.

Such a conclusion would be consistent with previous studies that have mapped determinants of CMV symptoms to all three genomic RNA segments and to all five known CMV genes (Palukaitis and García-Arenal 2003). For example, work by Zhang and colleagues (1994) demonstrated that symptoms in tobacco 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 suggest that successful or sustained symptom induction, or both, by other CMV gene products may require 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. Diaz-Pendon and colleagues (2007) found that both WT 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 DCL enzymes are largely responsible for RNA silencing–mediated resistance to viruses (Deleris et al. 2006). Diaz-Pendon and colleagues (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 is both host plant specific and virus strain specific, as well as suggesting that during symptom induction the 2b protein does not act in isolation.

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, La Jolla, CA, U.S.A.) according to the manufacturer's instructions. The sequences of primers used are detailed in Supplementary Table 1.

Plant growth and virus inoculation.

A. thaliana seeds were planted on a 4:1 compost/sand mixture and were maintained at 21°C with an 8-h photoperiod. *Nicotiana* spp., *Chenopodium* sp., and *Cucurbita pepo* seeds were planted on compost and were 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 μmol m^{-2} s^{-1} (Conviron, Winnipeg, Canada). The species designation of the *Chenopodium* sp. used is currently under investigation but was previously used at Warwick HRI in the belief that it was *Chenopodium quinoa*.

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 Table 2. *A. thaliana* plants were inoculated with infectious transcripts at the four- to six-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 postgermination by applying transcripts to Carborundum-dusted leaves, using a roughened glass microscope slide. Infection was confirmed in transcript-inoculated plants by RT-PCR. To inoculate plants with infectious sap, leaves from plants confirmed to be infected were ground in 0.1 M 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 Lot and colleagues (1972) and purified virions were inoculated to *A. thaliana* plants as per infectious transcripts, using a viral-particle suspension of 100 μg ml^{-1}. Infected plants were photographed with a Nikon Coolpix digital camera (Tokyo).

Experiments assessing symptom induction by 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 four to six 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 were performed using primers flanking the *2b* coding region, according to Ziebell and colleagues (2007). PCR products were purified for sequencing by extraction from agarose gels using the Qiaquick gel extraction kit (Qiagen; Chatsworth, CA, U.S.A.). Sequencing of purified RT-PCR products from WT and mutant CMV-infected plants and of mutant plasmids was performed by Geneservice Ltd. (Cambridge).

***Arabidopsis* transformation.**

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

Immunoblotting.

Protein extraction and immunoblotting for CMV coat protein was conducted according to Naylor and colleagues (1998), except that bound primary antibody was detected using an anti–rabbit horseradish peroxidase conjugate as the secondary antibody. Extraction and immunoblotting of Fny 2b protein was conducted as described by Mayers and associates (2000), using polyclonal rabbit anti-2b serum, with one modification. Following the 10,000 × *g* centrifugation step, both the pellet (the nuclei-enriched fraction) and the supernatant (the cytoplasm- and membrane-enriched fraction) were collected for analysis. Total soluble proteins were quantified using the Bio-Rad (Hercules, CA, U.S.A.) protein assay solution. After immunoblotting for both CMV coat protein and 2b protein, bound secondary antibody was visualized using Western Lightning enhanced luminol chemiluminescence reagent (Perkin-Elmer, Norwalk, CT, U.S.A.) according to the manufacturer's instructions.

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