Functional analysis of the *Cucumber mosaic virus* 2b protein: pathogenicity and nuclear localization

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The 2b protein encoded by *Cucumber mosaic virus* (CMV) has been shown to be a silencing suppressor and pathogenicity determinant in solanaceous hosts, but a movement determinant in cucumber. In addition, synergistic interactions between CMV and *Zucchini yellow mosaic virus* (ZYMV) have been described in several cucurbit species. Here, it was shown that deletion of the 2b gene from CMV prevented extensive systemic movement of the virus in zucchini squash, which could not be complemented by co-infection with ZYMV. Thus, ZYMV expressing a silencing suppressor with a different target could not complement the CMV 2b-specific movement function. Expression of the 2b protein from an attenuated ZYMV vector resulted in a synergistic response, largely restoring infection symptoms of wild-type ZYMV in several cucurbit species. Deletion or alteration of either of two nuclear localization signals (NLSs) did not affect nuclear localization in two assays, but did affect pathogenicity in several cucurbit species, whilst deletion of both NLSs led to loss of nuclear localization. The 2b protein interacted with an *Arabidopsis thaliana* karyopherin \(\alpha\) protein (AtKAP\(\alpha\)) in the yeast two-hybrid system, as did each of the two single NLS-deletion mutants. However, 2b protein containing a deletion of both NLSs was unable to interact with AtKAP\(\alpha\). These data suggest that the 2b protein localizes to the nucleus by using the karyopherin \(\alpha\)-mediated system, but demonstrate that nuclear localization was insufficient for enhancement of the 2b-mediated pathogenic response in cucurbit hosts. Thus, the sequences corresponding to the two NLSs must have another role leading to pathogenicity enhancement.

INTRODUCTION

Synergistic interactions between plant viruses can lead to increased disease in crops that are susceptible to the various virus combinations (Anjos et al., 1992; Bennett, 1952; Hunter et al., 2002; Karyeija et al., 2000; Palukaitis & Kaplan, 1997; Pio-Ribeiro et al., 1978; Poolpol & Inouye, 1986; Rochow & Ross, 1955; Sano & Kojima, 1989; Wang et al., 2002). In addition, interviral synergy can lead to resistance breakage (Choi et al., 2002; Murphy & Kyle, 1995; Wang et al., 2004) or limited spread of another virus (Sáenz et al., 2002). In some cases, these synergistic interactions are mediated by proteins that have been shown to be suppressors of RNA silencing (Brigneti et al., 1998; Pruss et al., 1997; Qiu et al., 2002; Sáenz et al., 2001; Seth et al., 2004). Expression of such proteins from heterologous viral expression vectors results in increased disease and/or virus accumulation in some host species, but not in others (Brigneti et al., 1998; Li et al., 1999; Pruss et al., 1997; Qiu et al., 2002), as has been observed for interviral synergy (Fukumoto et al., 2003; Garces-Orejuela & Pound, 1957; González-Jara et al., 2004).

Mixed infections involving *Cucumber mosaic virus* (CMV) and a number of other viruses have been found to result in viral synergy in some plant species (Fukumoto et al., 2003; Palukaitis & Kaplan, 1997; Pio-Ribeiro et al., 1978; Poolpol & Inouye, 1986; Sano & Kojima, 1989; Wang et al., 2002), whilst CMV interferes with resistance to some potyviruses (Murphy & Kyle, 1995; Sáenz et al., 2002) and vice versa (Choi et al., 2002; Wang et al., 2004). The 2b protein encoded by CMV enhanced pathogenicity in *Nicotiana benthamiana* when expressed from a *Potato virus X* (PVX) vector (Brigneti et al., 1998; Lucy et al., 2000), but did not facilitate the systemic infection of *Plum pox potyvirus* (PPV) when expressed from PPV in the same host (Sáenz et al.,...
2002). Thus, the 2b protein may not be the sole CMV determinant for facilitation of interviral synergy involving CMV.

The absence of the CMV 2b protein was shown not to affect virus replication in protoplasts (Ding et al., 1996; Soards et al., 2002), but did affect the extent and pattern of CMV movement in tobacco (Soards et al., 2002) and cucumber (Ding et al., 1995a). Insertion of an early translational termination sequence or complete deletion of the 2b gene had the same effect on the accumulation of CMV in protoplasts and whole plants (Ding et al., 1996), as well as on the ability of a chimeric CMV–umbravirus to promote cell-to-cell movement of the phloem-limited Potato leafroll virus (Ryabov et al., 2001). The 2b protein was shown to be localized primarily in the nucleus (Lucy et al., 2000; Mayers et al., 2000). A nuclear localization signal (NLS) of CMV Q strain was identified and localization to the nucleus was determined to be necessary, but not sufficient, for silencing suppression in N. benthamiana, whereas neither the ability to suppress silencing nor nuclear localization alone appeared to be sufficient for enhancement of the pathogenicity of PVX (Lucy et al., 2000).

The effect of the 2b protein on synergy between CMV and potyviruses has not been examined. Moreover, interactions between CMV and potyviruses in cucurbit hosts showed several characteristics that are different from interviral interactions in N. benthamiana or Nicotiana tabacum (Choi et al., 2002; Pruss et al., 1997; Szénez et al., 2002; Wang et al., 2002, 2004). Thus, we examined the role of the CMV 2b protein compared with the 3a movement protein (MP) and capsid protein (CP) in synergy between CMV and the potyvirus Zucchini yellow mosaic virus (ZYMV) in several cucurbit species. In addition, we assessed the role of nuclear localization of the 2b protein in enhancing pathogenicity in cucurbit hosts.

**METHODS**

**Plant material and virus strains.** Squash (Cucurbita pepo L. cv. Mar'yan), cucumber (Cucumis sativus L. cvs Bet Alpha and Delila), melon (Cucumis melo L. cv. Arava) and Nicotiana glutinosa L. plants were grown in a temperature-controlled growth room at 25 °C with a photoperiod of 14 h. Biologically active cDNA clones of the wild-type, virulent AT strain and the attenuated (asymptomatic) AGII strain of ZYMV (ZYMV-AT and ZYMV-AGII, respectively) were used (Gal-On, 2000; Gal-On et al., 1992). Additionally, biologically active cDNA clones of the Fny strain of CMV (Fny-CMV) (Rizzo & Palukaitis, 1990), as well as a mutated Fny-CMV with the 2b ORF deleted from the cDNA of RNA 2 (Fny-CMVΔ2b) (Soards et al., 2002) were used.

**Inoculation procedure and virus detection.** Cotyledons of cucurbit seedlings were inoculated by bombardment with cDNA clones of ZYMV-AT, ZYMV-AGII or ZYMV-AGII carrying foreign genes, as described by Gal-On et al. (1997). Fny-CMV and Fny-CMVΔ2b RNA transcripts were synthesized individually from the appropriate cDNA templates, as described previously (Soards et al., 2002; Zhang et al., 1994). The mixed in vitro CMV RNA transcripts were bombarded with a suspension of tungsten particles in calcium nitrate buffer at pH 8.5 (Gal-On et al., 1997) to inoculate N. glutinosa leaves and squash cotyledons. In double inoculations, ZYMV-AGII cDNA and Fny-CMVΔ2b transcripts were bombarded separately onto the same cotyledons. CMV RNA accumulation was detected by RT-PCR. In addition, RT-PCR of viral progeny derived from ZYMV-AGII or ZYMV-AGII carrying the CMV 2b gene or various mutated 2b genes was performed by using a one-tube, single-step RT-PCR method, as described by Arazi et al. (2001), with the primers described below. ZYMV CP accumulation was detected by Western blot analysis, as described previously (Wang et al., 2002).

**Construction of CMV MP, CP and 2b genes for expression via the ZYMV-AGII vector.** The Fny-CMV MP, CP and 2b genes were amplified by PCR and cloned into the vector pGEM-T (Promega) using the following primers: for the MP gene, 5'-GA-TGCTGGATCCGTTCATCATTCTTACAGGATTGAGT-3' and 5'-CT-GATCTGGATCCGTTCATCATTCTTACAGGATTGAGT-3' (PstI sites underlined); for the CP gene, 5'-GATATGCTGGATCCGAT-GAACAAATCTGGATCCAGT-3' and 5'-TGCTGGATCCGAT-ACGATGCTGGATCCGATGACTCCAGA-3' (PstI sites underlined); and for the 2b gene, 5'-AACCTCGAGGCGAATGTTGAATGCTGGAAT-3' and 5'-AGCAGGATCCGATGACTCCAGGAGGAAACCTCCTCC-GGCACTTC-3' (PstI and SalI sites underlined). The MP and CP genes were cloned into the ZYMV-AGII (AGII) viral expression vector by using the PstI sites. The 2b gene was introduced into the PstI/SalI sites, as described by Arazi et al. (2001). The resultant clones were designated AGII-MP, AGII-CP and AGII-2b, respectively, and were used for standard bombardment inoculation as described above.

**Mutagenesis of the putative NLSs of the Fny-CMV 2b gene.** Mutagenesis of the 2b gene was done according to the method of Kunkel et al. (1987), using the following primers: 5'-GCT-GTGATTCGTGGAGGTACACACAAAGAGAGA-3' for 2bANLS1; 5'-GCTGATTCGTGGAGGTACACACAAAGAGAGA-3' for 2bANLS2; and 5'-TGGTCAAGACCTGAGGAATCT-3' and 5'-TATGCAAGACCTGAGGAATCT-3' (PstI sites underlined). The 2b gene was introduced into the ZYMV-AGII vector by using the PstI sites. The 2b gene was cloned into the PstI/SalI sites, as described by Arazi et al. (2001). The resultant clones were designated 2bANLS1, 2bANLS2, 2bANLS1 + 2, for construction of mutagenesis of the plasmid containing 2bANLS1, used for the mutagenesis of 2bANLS2. The PGE-2b clone served as a template for single-stranded DNA generation and mutagenesis with the appropriate primers. The resultant clones were designated 2bPGEANLS1 (deletion of the first putative NLS1), 2bPGEANLS2 (replacement of the second putative NLS1 residues with six alanine residues), 2bPGEANLS2 (replacement of the second putative NLS2 residues with six alanine residues) and 2bPGEANLS2 (replacement of the second putative NLS2 residues with six alanine residues) and 2bPGEANLS2 (replacement of the second putative NLS2 residues with six alanine residues) and 2bPGEANLS2 (replacement of the second putative NLS2 residues with six alanine residues) and 2bPGEANLS2 (replacement of the second putative NLS2 residues with six alanine residues) and 2bPGEANLS2 (replacement of the second putative NLS2 residues with six alanine residues). The modified 2b genes were inserted into the ZYMV-AGII vector by using the PstI and SalI sites, as for the cloning of the native 2b gene described above (Arazi et al., 2001). The resultant clones, designated AGII-2bANLS1, AGII-2bANLS2, AGII-2bANLS1, AGII-2bANLS2, AGII-2bANLS1, AGII-2bANLS2, AGII-2bANLS1, AGII-2bANLS2, AGII-2bANLS1, AGII-2bANLS2, were used for standard bombardment inoculations as described above.

**Verification of gene stability in progeny viruses.** RT-PCR of the recombinant viruses harbouring the 2b gene or its mutants was performed with primers flanking the cloning region on the AGII genome: 5'-AGCTCCATCATAAGCAGTAACA-3' (forward primer from the Nlb gene) and 5'-TGGTGGAGACAGAGG-3' (reverse primer from the CP gene), as described by Arazí et al. (2001). To confirm the authenticity of the ZYMV-AGII vector in plants infected with ZYMV-AGII expressing 2b or its mutated variants, RT-PCR was performed on the HC-Pro sequence of ZYMV-AGII compared with ZYMV-AT, as described by Gal-On (2000), using the following primers: 5'-GTTCTGAGTCTAGAGACAAGG-3' (forward) and 5'-AGCAGTGCCTCTCGAGTTAATG-3' (reverse).
Nuclear localization assay using the yeast nuclear import system. The nuclear import assay of the 2b protein and its mutants was performed by using the pNIA vector according to Rhee et al. (2000). Native and mutant 2b genes were fused to mLexA-Gal4AD fusion in the pNIA vector by using the BamHI/PstI sites in the primers 5′-BamHI-2b (5′-CGGGATCCGTAGATAGGTTGAAATGTTGCAATG-3′; BamHI site underlined) and 3′-PstI-2b (5′-GCTCGAGGAAAGGACCTTCCGGCCATTCGTT3′; PstI site underlined), to obtain clones pNIA-2b, pNIA-2bANLS1, pNIA-2bANLS2, and pNIA-2bANLS1+2. The Agrobacterium tumefaciens genes virD2 and virE2 were used as positive and negative controls (Tzfira & Citovsky, 2001), respectively, for nuclear localization in the pNIA expression vector (Rhee et al., 2000). The yeast L40 strain was transformed and the nuclear import assay was performed according to Rhee et al. (2000).

Nuclear localization assay in onion cells. The intact 2b gene and the mutant variants 2bANLS1, 2bANLS2, 2b4ANLS2 and 2bANLS1+2 were amplified by PCR and cloned in place of the VP1 gene in the plTR2-GUS-VP1 vector (Tzfira et al., 2001), resulting in an N-terminal fusion of 2b to the β-glucuronidase (GUS) reporter protein. Nuclear localization of fusion proteins GUS–2b, GUS–2bANLS1, GUS–2bANLS2, GUS–2b4ANLS2 and GUS–2bANLS1+2 was examined by following the method of Varagona et al. (1992). The fusion constructs were bombarded (Varagona et al., 1992) onto the inner face of onion inner epidermal layers, covering approximately 30–50 cells for each bombardment, by using a Helios portable gene gun at a pressure of 150 p.s.i. Onion inner epidermal layers were peeled and placed on Petri dishes containing 6% agar and 100 µg ampicillin ml⁻¹. The bombarded onion epidermal layers were assayed histochemically with X-glucuronide for GUS expression. After blue staining was visible, tissues were stained immediately with the nuclear-specific stain 4,6-diamidino-2-phenylindole (DAPI) 16 h after bombardment, as described by Varagona et al. (1992). Photomicrographs were prepared by using differential-interference optics.

Yeast two-hybrid protein–protein interaction assay. The native 2b and the 2b mutant genes were amplified by PCR as BamHI–PstI fragments, using the primers 5′-BamHI-2b and 3′-PstI-2b and cloned into the corresponding sites of pSTT91 (TRPI+) (Sutton et al., 2001), producing fusions with the LexA gene. These clones were designated pSTT91-2b, pSTT91-2bANLS1, pSTT91-2bANLS2, pSTT91-2b4ANLS2 and pSTT91-2bANLS1+2. The gene encoding A. thaliana karyopherin α protein (AtKAP2α) was cloned into pGAD424 (LEU2+) Clontech, producing a fusion with the GAL4 activation domain, as described previously (Ballas & Citovsky, 1997). For the two-hybrid assay, the potential interactors were introduced into the Saccharomyces cerevisiae strain TAT7 (140-ura3) (Hollenberg et al., 1995) and grown for 2 days at 30°C on a leucine-, tryptophan- and histidine-deficient medium. Histidine prototrophy indicated protein–protein interaction (SenGupta et al., 1996). Qualitative determination of β-galactosidase activity was performed as described by Stachel et al. (1985). High-fidelity Pfu DNA polymerase (Stratagene) was used in all PCRs and all DNA constructs were verified by dyeoxyxynucleotide sequencing.

RESULTS

Involvement of the Fny-CMV 2b gene product in symptom expression in cucurbits

To determine whether the Fny-CMV 2b gene was involved in pathogenicity in cucurbits, two approaches were taken. In the first approach, the 2b gene was deleted from RNA 2 of Fny-CMV to generate Fny-CMVΔ2b (Fig. 1a) and infection of various hosts with in vitro-capped RNA transcripts of the Fny-CMV RNA and Fny-CMVΔ2b RNA was compared. Fny-CMVΔ2b infected N. glutinosa plants systemically, as described previously for infection of N. tabacum by Soards et al. (2002); however, Fny-CMVΔ2b accumulated poorly compared with Fny-CMV, as determined by ELISA (data not shown). Fny-CMVΔ2b elicited only very mild symptoms in N. glutinosa, compared with wild-type Fny-CMV (Fig. 1b, plants 1 and 2). Interestingly, squash plants infected with Fny-CMVΔ2b also showed only very mild mosaic symptoms on the first systemic leaf (Fig. 1c, leaf 1). However, in the upper systemic leaves, infection of Fny-CMVΔ2b produced a recovery-like phenotype (Fig. 1b, plant 4, and Fig. 1c, leaves 2–4). This was in contrast to squash plants infected with wild-type Fny-CMV, in which systemic infection produced typical, severe CMV symptoms 5–7 days post-inoculation (Fig. 1b, plant 3). The accumulation of Fny-CMVΔ2b was undetectable by either Western blot analysis or ELISA in both symptomatic and asymptomatic leaves of infected squash plants (data not shown). Moreover, sap extracted from asymptomatic upper leaves of Fny-CMVΔ2b-infected squash plants failed to infect N. glutinosa test plants. The presence of Fny-CMVΔ2b in the symptomatic leaves of infected squash plants (Fig. 1c, leaf 1) was confirmed by RT-PCR amplification of the region flanking the 2b gene on RNA 2 (Fig. 1d, lane 1).

We previously described a synergistic, enhanced accumulation of Fny-CMV by the attenuated ZYMV-AGII (Wang et al., 2002), and Choi et al. (2002) showed that attenuated ZYMV-AGII (identical to ZYMV-AGII with the exception of the introduced cloning sites) could assist the M strain of CMV in overcoming a systemic movement defect in doubly infected squash plants. Therefore, we assessed the possibility that Fny-CMVΔ2b would move systemically in squash plants in a double infection with ZYMV-AGII. However, double inoculation with Fny-CMVΔ2b and ZYMV-AGII did not assist the systemic movement of Fny-CMVΔ2b in squash plants (Fig. 1c, leaves 10–12). Very mild symptoms could be seen only on the first true leaves of the doubly infected squash plants, exactly as observed in squash plants infected by Fny-CMVΔ2b alone (Fig. 1c, leaf 1 vs leaf 9). Moreover, in the doubly infected plants (Fig. 1c, leaves 9–12), symptoms that were induced by ZYMV-AGII remained mild from the second true (systemic) leaf upwards (Fig. 1c, leaves 10–12), as for infection by ZYMV-AGII alone (Fig. 1c, leaves 6–8). Thus, while the absence of the 2b gene resulted in lower virulence of CMV in squash, it also resulted in poor systemic movement in this host, which could not be complemented by co-infection with ZYMV.

The second approach to determine whether the Fny-CMV 2b protein was involved in pathogenicity in cucurbits did not depend on movement by CMV. In this approach, the 2b gene was expressed from the viral expression vector ZYMV-AGII (Arazi et al., 2001). This approach also took advantage of the symptom attenuation by the ZYMV-AGII vector to evaluate the potential roles of other CMV genes.
in pathogenicity in cucurbits. The CMV genes for MP, CP and 2b were cloned between the Niβ- and CP-encoding sequences of the ZYMV-AGII expression vector (Fig. 2a). The inserted genes were designed to create in-frame translational fusions with the ZYMV-AGII-encoded polyprotein. Proteolysis of the nascent AGII polyprotein by the Niα protease in trans was predicted to yield expressed CMV-encoded proteins with one additional amino acid residue (serine) at the N-terminus and seven amino acid residues (VDTVMLQ) at the C-terminus. All of the modified viruses (designated AGII-MP, AGII-CP and AGII-2b) were inoculated by bombardment into cucurbit hosts at the cotyledon stage and the symptoms caused by infection with the modified ZYMV-AGII were examined in cucumber, melon and squash plants. The modified viruses AGII-MP, AGII-CP and AGII-2b were detectable by ELISA by 6–8 days post-inoculation, similar to infection by the parental virus ZYMV-AGII. Our efforts to express the full-length 1a and

**Fig. 1.** Construction, pathogenicity and accumulation of CMVΔ2b. (a) Schematic illustration of the 2b gene deletion in RNA 2 of Fny-CMV. Orientations and locations of the primers for RT-PCR verification of 2b deletion in the progeny virus are indicated by arrows with numbers. (b) Symptoms on *N. glutinosa* (1, 2) and squash (3, 4) plants inoculated with infectious transcripts of wild-type Fny-CMV (1, 3) or CMV without the 2b gene (CMVΔ2b) (2, 4). Photographs of squash and *N. glutinosa* were taken 10 and 18 days post-inoculation, respectively. (c) Symptoms on squash infected systemically by Fny-CMVΔ2b (CMVΔ2b) (1–4), ZYMV-AGII (5–8) and ZYMV-AGII together with CMVΔ2b (AGII+CMVΔ2b) (9–12) 3 weeks post-inoculation. In each sequence, leaves are numbered from the lowest systemically infected leaf, above the inoculated cotyledons. (d) Confirmation of the presence of CMV with a 2b deletion by RT-PCR using total RNA extracted from squash plants infected with Fny-CMVΔ2b (lane 1) and wild-type Fny-CMV-infected plants (lane 2), 3 weeks post-inoculation. Lane 3 is a control PCR product, using the plasmid pFny209Δ2b with the 2b gene deleted as the template. Lane M, molecular mass marker (bp).
Expression of Fny-CMV MP or CP caused only very mild symptoms, as caused by the parental ZYMV-AGII in infected melon plants (Fig. 2c). However, expression of Fny-CMV 2b from ZYMV-AGII exacerbated disease symptom development in melon, cucumber and squash plants (Fig. 2b, c, e and f). In particular, in the CMV-resistant cucumber cv. Delila, infection by AGII-MP and AGII-CP caused no symptoms (Fig. 2b), as was observed in double infection of this cucumber cultivar by ZYMV-AG plus Fny-CMV (Wang et al., 2004). Interestingly, the symptoms induced by infection with AGII-2b (Fig. 2b) largely resembled those symptoms that were caused by infection with the wild-type strain ZYMV-AT in cucumber cultivars Delila and Bet Alpha (Wang et al., 2004), as well as in squash (Fig. 2e). Moreover, expression of CMV CP and 2b genes via ZYMV-AGII did not affect ZYMV-AGII CP accumulation (Fig. 2d). However, a slight reduction in ZYMV-AGII CP accumulation was detected reproducibly in CMV-tolerant cucumber cv. Delila (Fig. 2d) and melon (data not shown) infected by AGII-MP.

Pathogenicity of the CMV 2b protein and the effect of the putative NLSs

We examined whether the pathogenicity associated with the Fny-CMV 2b protein in cucurbits required an NLS. A putative NLS 22KKQRRR27 (NLS1) (Fig. 3) was deleted from the Fny-CMV 2b protein to produce 2bANLS1 and the mutant 2bANLS1 was expressed from ZYMV-AGII (AGII-2bANLS1). The expression of 2bANLS1 from AGII-2bANLS1 was not able to enhance disease symptoms, compared with the parental ZYMV-AGII in infected squash (Fig. 2e), melon or cucumber (data not shown). In addition, the ZYMV-AGII CP accumulated to a similar level in squash plants that were infected with AGII, AGII-2b or AGII-2bANLS1 (Fig. 2g). The stability of the sequences encoding 2b and 2bANLS1 within the AGII expression vector was confirmed by sequencing the RT-PCR products that were amplified from viral RNA extracted from squash plants infected by AGII-2b and AGII-2bANLS1 (Fig. 2h).

To reduce the likelihood that the results obtained were due to disruption of the conformational structure of the 2b protein by deleting the putative NLS1, the putative NLS1 was substituted with six alanine residues (2bANLS1). In the CMV-susceptible cucumber cv. Bet Alpha, expression of 2bANLS1 from ZYMV-AGII induced symptoms similar to those seen in infection with AGII-2bANLS1 (Fig. 4a). In this experiment, as well as in others that are not shown, the native and modified 2b genes were stable within the ZYMV-AGII expression vector, as determined by RT-PCR of the progeny viruses after several passages (Fig. 4b). Moreover, the AGII-2b, AGII-2bANLS1 and AGII-2bANLS1 constructs did not affect accumulation of the ZYMV-AGII vector, as determined by Western blot analysis of CP accumulation (Fig. 4c). In summary, none of the 2b mutants with modified NLS1 produced symptoms as severe as those induced by the wild-type 2b gene expressed from ZYMV-AGII (Figs 2 and 4).

To ensure the authenticity of the ZYMV-AGII expression vector, the entire HC-Pro coding sequence was amplified by RT-PCR from progeny of the modified viruses (Fig. 4d) and digested with Eco47III (Fig. 4e). The presence of the Eco47III restriction site in the HC-Pro gene confirmed the identity of the attenuated virus ZYMV-AGII and distinguished it from wild-type ZYMV-AT (Gal-On, 2000). This eliminated the possibility that ZYMV-AGII had reverted to the wild-type ZYMV-AT. Thus, the severe symptoms induced by AGII-2b were due to expression of the CMV 2b gene, i.e. the 2b protein was the pathogenicity determinant.

A second putative NLS motif (NLS2), 33RRER36 (Fig. 3), was predicted in the Fny-CMV 2b protein by using the PredictNLS server (http://cubic.bioc.columbia.edu/services/predictNLS/). This sequence was completely conserved in all subgroup I CMV strains and varied by only one amino acid (E35 to A35) in subgroup II CMV strains (Fig. 3). There were only five amino acid residues between the putative NLS1 and NLS2 sequences. To examine the role of the putative NLS2 in 2b-mediated pathogenicity, the NLS2 was either deleted (2bANLS2) or substituted by four alanine residues (2b4ANLS2). Infection of cucurbit hosts with ZYMV-AGII expressing either 2bANLS2 or 2b4ANLS2 reduced or eliminated the pathogenicity caused by the parental AGII-2b in squash and cucumber (data not shown).

Nuclear import of the CMV 2b protein and its NLS mutants

Nuclear localization of the 2b protein and its mutants was examined by two different methods (Fig. 5). In the first method, histochemical analysis was performed on onion epidermal cells following particle bombardment with a construct allowing transient expression of genes fused to the GUS reporter gene (Varagona et al., 1992). Specifically, the intact 2b gene and its NLS-modified mutants were fused to sequences encoding the C-terminus of the GUS reporter gene in the expression vector pRTL2-GUS. Following bombardment, GUS expressed from the control construct diffused through the cytoplasm and there was no apparent accumulation of the GUS gene product in the nucleus (Fig. 5a). By contrast, expression of GUS fused to the 2b gene product was localized in the nucleus (Fig. 5a), as was the positive control fusion between the nuclear protein VIP1 (Tzfira et al., 2001) and the GUS reporter gene. Interestingly, the GUS protein fused to 2bANLS1 (GUS–2bANLS1) was localized in the onion nucleus as effectively as the GUS–2b fusion protein (Fig. 5a). These results indicated that deletion of NLS1 did not affect nuclear localization of the 2b protein. Moreover, GUS gene product fused to 2bANLS2 (GUS–2bANLS2) or to 2b4ANLS2 (GUS–4ANLS2) was also localized in the nucleus (Fig. 5a).
(a) VPg P1 P3 CI NIa CMV genes

(b) Healthy ZYMV-AGII AGII-CP AGII-2b AGII-MP

(c) ZYMV-AGII ZYMV-AGII-2b

(d) M He AGII AGII-CP AGII-MP AGII-2b

(e) Healthy ZYMV-AGII ZYMV-AT AGII-2b AGII-2b\(\Delta\)

(f) ZYMV-AGII ZYMV-AGII-2b

(g) M He AG 2b 2b\(\Delta\)

(h) M He AG 2b 2b\(\Delta\) p2b RT-PCR
These results suggested that either NLS1 or NLS2 is sufficient to localize the 2b protein in the nucleus.

To provide further support for this conclusion, both the NLS1- and NLS2-encoding sequences were deleted and the resultant 2b mutant gene (2b\(^{DNLS1+2}\)) was fused to the GUS gene (GUS–2b\(^{DNLS1+2}\)) for the nuclear localization assay. Histochemical analysis of GUS production following bombardment with the GUS–2b\(^{DNLS1+2}\) construct demonstrated that the GUS–2b\(^{DNLS1+2}\) fusion protein was unable to localize in the nucleus of onion cells (Fig. 5a).

A second method was used to confirm that the native 2b
protein localized to the nucleus, by fusion of the wild-type 2b protein and its mutants to the mLexA–Gal4AD fusion in the pNIA system (Rhee et al., 2000). The mLexA–Gal4AD chimaeric protein is unable to localize to the nucleus, as the NLS in mLexA is disabled. Due to this disability, the transcriptional activator Gal4AD cannot activate the mLexA operon in yeast cells, resulting in arrest of yeast-cell growth on histidine-deficient medium (Rhee et al., 2000). Only when the test protein fused to the mLexA–Gal4AD chimaera possesses a functional NLS will mLexA be able to target the mLexA operator into yeast nuclei and allow yeast-cell growth on media without histidine (Rhee et al., 2000; Tzfira et al., 2000). Yeast cells were transformed with pNIA expressing a fused wild-type 2b grew as well as those transformed with fusions containing the A. tumefaciens VirD2 (Fig. 5b), corroborating that 2b is a nuclear protein. Removal of either NLS domain (NLS1 or NLS2) did not affect 2b nuclear localization, as 2bΔNLS1- or 2bΔNLS2-transformed yeast cells were able to grow on selective medium (Fig. 5b). Moreover, substitution of NLS1 or NLS2 with alanine residues (2b6AlaNLS1 or 2b4AlaNLS2, respectively) did not affect nuclear transport (Fig. 5b and data not shown). However, removal of both NLS domains (2bΔNLS1 + 2) completely abolished 2b protein-mediated nuclear transport, as did expression of a fusion to A. tumefaciens VirE2 (Fig. 5b).

**Interaction of NLS1 and NLS2 with nuclear localization protein AtKAPα**

It is known that protein localization to the nucleus requires the co-operation of a karyopherin-like molecule for the docking process with the nuclear membrane pore (Suntharalingam & Wente, 2003) and that plant-pathogen proteins use the host nuclear import machinery for their nuclear import (e.g. Ballas & Citovsky, 1997; Tzfira et al., 2002). Therefore, we examined whether a plant RNA virus-encoded nuclear protein, such as the 2b protein of CMV, used a similar mechanism.

We first tested whether the native 2b protein interacted with AtKAPα (Ballas & Citovsky, 1997) in the yeast two-hybrid system (SenGupta et al., 1996). Our results showed that the native 2b protein bound to AtKAPα, as determined by an enzymic assay for the expression of β-galactosidase (Fig. 6). The 2b/AtKAPα interaction activity was scored as 100%. Deletion of both NLS domains (2bΔNLS1 + 2) disrupted the interaction between 2b and AtKAPα completely, as no β-galactosidase activity was observed (Fig. 6). On the other hand, deletion or alanine substitution of either NLS1 or NLS2 did not seem to affect the interaction between 2b and AtKAPα (Fig. 6).

**DISCUSSION**

To determine which genes of Fny-CMV were involved in disease symptom elicitation, we used the attenuated...
Fig. 5. Nuclear localization of 2b and its NLS mutants by (a) transient expression in onion cells and (b) yeast nuclear import assay. (a) Onion epidermal cells were stained with X-glucuronide for GUS expression (lower row of panels) or with DAPI for nuclear staining (upper row of panels) 48 h after transfection with the various constructs fused to the 3’ end of the GUS reporter gene: GUS construct alone (GUS), VIP1 gene fused to GUS as a positive control, Fny-CMV 2b gene fused to GUS (2b), 2b gene with a deleted NLS1 fused to GUS (2bΔNLS1), 2b gene with a deleted NLS2 fused to GUS (2bΔNLS2), 2b gene with its NLS2 substituted with four alanines fused to GUS (2b4AlaNLS1) and 2b gene with both the NLS1 and NLS2 deleted fused to GUS (2bΔNLS1+2). (b) Yeast import assay. Each of the above constructs except for 2b4AlaNLS1 was cloned into the pNIA vector. In addition, 2b6AlaNLS1 (with NLS1 substituted with six alanines) was also tested, as were the A. tumefaciens nuclear transport protein VirD2 (positive control) and the non-nuclear transport protein VirE2 (negative control). Following transformation into yeast cells (L40), each transformant was grown on selective minimal medium deficient for both tryptophan and histidine and, for the control, on non-selective minimal medium deficient for only tryptophan.
ZYMV-AGII vector system (Arazi et al., 2001; Gal-On, 2000). By this means, we provided additional evidence that the 2b protein elicits severe disease symptoms in cucurbits (melon, cucumber and squash), as demonstrated previously in solanaceous plants (Ding et al., 1995a, b), with a delay and milder symptoms. The CP and MP of Fny-CMV do not in themselves elicit disease symptoms in cucurbits.

It was demonstrated previously that deletion of the 2b gene from RNA 2 of Fny-CMV caused slower viral movement, lower virus accumulation and amelioration of disease symptoms in tobacco, but did not affect virus replication (Soards et al., 2002). In this study in squash, Fny-CMVΔ2b exhibited movement limited from the inoculated cotyledons to only the first true leaf. Similarly, either deletion of the 2b gene from RNA 2 of the Q strain of CMV, a member of CMV subgroup II, or introduction of a translation termination mutation in the 2b gene (2bΔNLS1, 2bAlaNLS1, 2bΔNLS2 and 2bΔNLS1+2) were subcloned into the plasmid pSTT91, whereas the gene for AtKAPα was cloned in the pGAD424 expression vector. Protein–protein interaction was performed in yeast strain L40 for AtKAPα was cloned in the pGAD424 expression vector. Protein–protein interaction was performed in yeast strain L40 and β-galactosidase activity was assessed. The histogram presents activity relative to that of 2b (100%) as measured by β-galactosidase activity from five independent experiments. Bars represent SD of the mean.

**Fig. 6.** Interaction of CMV 2b protein with the AtKAPα in a two-hybrid system. The wild-type 2b gene (2b) and its NLS mutants (2bΔNLS1, 2bAlaNLS1, 2bΔNLS2 and 2bΔNLS1+2) were subcloned into the plasmid pSTT91, whereas the gene for AtKAPα was cloned in the pGAD424 expression vector. Protein–protein interaction was performed in yeast strain L40 and β-galactosidase activity was assessed. The histogram presents activity relative to that of 2b (100%) as measured by β-galactosidase activity from five independent experiments. Bars represent SD of the mean.

ZYMV-AGII is an asymptomatic, attenuated ZYMV strain derived from the severe strain ZYMV-AT, in which the sequence of the FRNK motif was altered to FINK (Gal-On, 2000). Restoration of disease symptoms by expression of Fny-CMV 2b protein via ZYMV-AGII suggests that the 2b protein may have substituted for the disabled HC-Pro in interactions with host factors, eliciting disease symptoms. Perhaps the 2b protein substituted for the disabled HC-Pro in interactions with host microRNAs, as potyviral-related disease symptoms could, in part, be accounted for by interference with targeting and/or cleavage functions of host microRNAs by HC-Pro (Kasschau et al., 2003). Thus, examination of interactions between ZYMV-AGII HC-Pro or Fny-CMV 2b and cucurbit microRNAs may provide specific insights into disease symptom restoration by the 2b protein.

We have demonstrated that the 2b protein of a subgroup IA strain of CMV localized to the host nucleus, has two NLSs and that either of the NLSs is sufficient to localize the 2b protein into the nucleus (Figs 5 and 7). Interestingly, both of the NLS sequences were found to be associated with symptom elicitation in cucurbits (Figs 2, 4 and 7). Moreover, we have provided evidence that both NLSs are capable of binding to AtKAPα, an A. thaliana karyopherin that is known to be an essential nuclear membrane protein (Figs 6 and 7).

We used two different methods to follow viral protein nuclear localization: importation of GUS fused to a viral protein in onion epidermal cells and a yeast one-hybrid nuclear import assay (Fig. 5). In both plant and yeast systems, the 2b protein showed nuclear localization. Surprisingly, deletion or replacement by six alanines of the homologous NLS (22KKQRR27) from a CMV subgroup IA 2b protein did not prevent translocation of the density between veins and the companion cell of solanaceous and cucurbit plants (van Bel, 1993). Thus, it is conceivable that the 2b protein is more important for CMV long-distance movement in squash than in tobacco. It is also possible that the CMV 2b protein is less effective in suppression of host responses in squash than in *Nicotiana* species.

In contrast to symptomatic infection by ZYMV-AGII-2b of the CMV-tolerant cucumber cv. Delila, we have shown previously that mixed infection of the same host with ZYMV-AG and Fny-CMV was symptomless (Wang et al., 2004). This discrepancy in disease symptom elicitation between ZYMV-AGII-2b infection and a mixed infection by ZYMV-AG + Fny-CMV may be due to differences in the relative levels of expression of the 2b genes in each cell in two distinct systems, as well as an additive suppressor function to host RNA silencing. In addition, in mixed infection, the proportion of the infected cells containing both HC-Pro and 2b might be much lower than with the viral vector, where every infected cell contained both the HC-Pro and 2b proteins.
shown to contain a second NLS (33RRER36), modification of the ZYMV-AGII vector. The Fny-CMV 2b protein was impaired by modification of the NLS when expressed via protein into the host nucleus. However, the function of the CMV, a subgroup II CMV strain (Lucy NLS, as demonstrated in the 2b protein of the Q strain of two NLSs, which do not resemble a classical monopartite nuclear localization can be implemented by either of the sufficient for induction of pathogenicity. Thus, Fny-CMV 2b protein to localize to the nucleus is not pathogenicity. These results indicate that the ability of the 2b protein and its mutants by the yeast one-hybrid nuclear import assay is marked as (+) for localization and (−) for non-localization to the nucleus. Binding of the 2b protein and its mutants to AtKAPα is marked as positive (+) or negative (−). NT, Not tested.

Fig. 7. Summary of the effects of 2b mutations on pathogenicity, nuclear localization and interaction with AtKAPα. A schematic diagram of the location of the 2b gene within CMV RNA 2 is shown at the top. The putative NLS1 and NLS2 sites within the 2b mutations are indicated by black bars. Replacement of NLS1 or NLS2 by alanine or deletion of each NLS within the 2b gene are marked by striped and white bars, respectively. The various characteristics examined for each construct are presented to the right of the diagram. Symptom severity in the inoculated cucurbit plants is indicated as symptomatic (+) or symptomless (−). Nuclear localization of the GUS fusions is indicated as (+) for nuclear localization and (−) for diffusion in the cytoplasm. Nuclear localization of the 2b protein and its mutants by the yeast one-hybrid nuclear import assay is marked as (+) for localization and (−) for non-localization to the nucleus. Binding of the 2b protein and its mutants to AtKAPα is marked as positive (+) or negative (−). NT, Not tested.

It has been shown that plant viral proteins, as with other nuclear proteins, need to ‘dock’ on the outside of the nuclear pore complex in order to enter the nucleus (Görlich & Mattaj, 1996). This has been shown previously only for structural proteins of DNA viruses, such as Cauliflower mosaic virus (Leclerc et al., 1999) and Tomato yellow leaf curl virus (Kunik et al., 1998) and a negative-strand RNA virus, Sonchus yellow net virus (Goodin et al., 2001). Docking may occur via karyopherin z proteins, analogous to the importin z proteins of animals (Görlich & Mattaj, 1996), as was observed for the A. tumefaciens VirD2 protein, but not the VirE2 protein (Ballas & Citovsky, 1997). In this study, we showed an interaction between an A. thaliana karyopherin and a non-structural protein of an RNA virus that possesses two NLSs. This indicates that either of the two NLSs of the Fny-CMV 2b protein is capable of interaction with the importin-like docking and nuclear transport system. Each NLS of the viral protein bound separately to the karyopherin, although deletion of both prevented binding. Therefore, modification of either NLS of Fny-CMV 2b protein did not prevent 2b nuclear transport through the nuclear pore complex, but did affect intra-nuclear activity, preventing symptom elicitation. Whether this occurs by preventing 2b interactions with microRNAs needs to be established.

ACKNOWLEDGEMENTS

This research was supported in part by Research Grant Award no. US-2541095R from BARD, the United States–Israel Binational Agricultural Research and Development Fund. P. P. received support via a Grant-in-Aid from the Scottish Executive Environment and Rural Affairs Department. This is contribution no. 502/04 series from the Agricultural Research Organization, the Volcani Center, Bet Dagan, Israel.

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