

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

Yongzeng Wang,^{1†} Tzvi Tzfira,^{2‡} Victor Gaba,¹ Vitaly Citovsky,² Peter Palukaitis³ and Amit Gal-On¹

Correspondence

Amit Gal-On

amitg@volcani.agri.gov.il

¹Department of Virology, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel

²Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, Stony Brook, NY 11794-5215, USA

³Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK

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 α protein (AtKAP α) 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 α . These data suggest that the 2b protein localizes to the nucleus by using the karyopherin α -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.

Received 3 May 2004

Accepted 30 June 2004

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, intervirial 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; Selth *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 intervirial synergy (Fukumoto *et al.*, 2003; Garcés-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.*,

[†]Present address: Department of Plant Pathology, Iowa State University, Ames, IA 50011-1020, USA.

[‡]These authors contributed equally to this work.

2002). Thus, the 2b protein may not be the sole CMV determinant for facilitation of intervirial 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 chimaeric CMV-umbrovirus 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 intervirial interactions in *N. benthamiana* or *Nicotiana tabacum* (Choi *et al.*, 2002; Pruss *et al.*, 1997; Sáenz *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. Ma'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'-GATGCTGCAGTCAGCTTTCCAAGGTACCAGTAGG-3' and 5'-CTGACTGCAGCATCACAGTGTCAAGACCGTTAACCACCTGCGG-3' (*Pst*I sites underlined); for the CP gene, 5'-GATGCTGCAGTCAATGGACAAATCTGAATCAACCAGT-3' and 5'-TGACTGCAGCATTACAGTGTGACTGGGAGCACTCCAGA-3' (*Pst*I sites underlined); and for the 2b gene, 5'-AAACTGCAGAGCGAATTGAACGTAGGTGCAATG-3' and 5'-ACGGACGTCGACGAAAGCACCTTCCGCCATTTC-3' (*Pst*I and *Sal*I sites underlined). The MP and CP genes were cloned into the ZYMV-AGII (AGII) viral expression vector by using the *Pst*I sites. The 2b gene was introduced into the *Pst*I/*Sal*I 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 NLSs was done according to the method of Kunkel *et al.* (1987), using the following primers: 5'-GCTC-GTATGGTGGAGGCTAGCCACAAACAGAATCGA-3' for 2bΔNLS1; 5'-CGTATGGTGGAGGCTGCGGCAGCAGCAGCGACCCACAAAC-AGAATCGA-3' for 2b6AlaNLS1; 5'-TCTCACAACAGAATGGTCAAAAAGTCCCAGCAGAGAGA-3' for 2bΔNLS2; and 5'-TCTCACAACAGAATGCTGCTGCTGCTCACA AAAAGTCCCAGCAGCAGAGA-3' for 2b4AlaNLS2. 2bΔNLS1+2 was constructed by mutagenesis of the plasmid containing 2bΔNLS1, using the primer for mutagenesis of 2bΔNLS2. The pGEM-2b clone served as a template for single-stranded DNA generation and mutagenesis with the appropriate primers. The resultant clones were designated pGEM-2bΔNLS1 (deletion of the first putative NLS1), pGEM-2b6AlaNLS1 (replacement of the putative NLS1 residues with six alanine residues), pGEM-2bΔNLS2 (deletion of the second putative NLS2), pGEM-2b4AlaNLS2 (replacement of the putative NLS2 residues with four alanine residues) and pGEM-2bΔNLS1+2 (deletion of both NLS1 and -2). The modified 2b genes were inserted into the ZYMV-AGII vector by using the *Pst*I and *Sal*I sites, as for the cloning of the native 2b gene described above (Arazi *et al.*, 2001). The resultant clones, designated AGII-2bΔNLS1, AGII-2b6AlaNLS1, AGII-2bΔNLS2, AGII-2b4AlaNLS2 and AGII-2bΔNLS1+2, 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'-AGCTCCATACATAGCTGAGACA-3' (forward primer from the *NIb* gene) and 5'-TGTTGAACCAAGAGGCGAA-3' (reverse primer from the CP gene), as described by Arazi *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'-GTGTTTCGAGGTAGAGACGACG-3' (forward) and 5'-CAGCTGCTCCTCGAGTTTAATG-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 *Bam*HI/*Pst*I sites in the primers 5'-*Bam*HI-2b (5'-CGGGATCCGTGAATTGAACGTAGGT-GCAATG-3'; *Bam*HI site underlined) and 3'-*Pst*I-2b (5'-GCTGC-AGGAAAACACCTTCCGCCCATTCGTT-3'; *Pst*I site underlined), to obtain clones pNIA-2b, pNIA-2bΔNLS1, pNIA-2bAlaNLS1, pNIA-2bΔNLS2 and pNIA-2bΔNLS1+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 2bΔNLS1, 2bΔNLS2, 2b4AlaNLS2 and 2bΔNLS1+2 were amplified by PCR and cloned in place of the *VIP1* gene in the pRTL2-GUS-VIP1 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-2bΔNLS1, GUS-2bΔNLS2, GUS-2b4AlaNLS2 and GUS-2bΔNLS1+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 *Bam*HI–*Pst*I fragments, using the primers 5'-*Bam*HI-2b and 3'-*Pst*I-2b and cloned into the corresponding sites of pSTT91 (TRP1+) (Sutton *et al.*, 2001), producing fusions with the *LexA* gene. These clones were designated pSTT91-2b, pSTT91-2bΔNLS1, pSTT91-2b4AlaNLS1, pSTT91-2bΔNLS2, pSTT91-2b4AlaNLS2 and pSTT91-2bΔNLS1+2. The gene encoding *A. thaliana* karyopherin α protein (AtKAPα) 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 (L40-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 dideoxynucleotide 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-AG (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

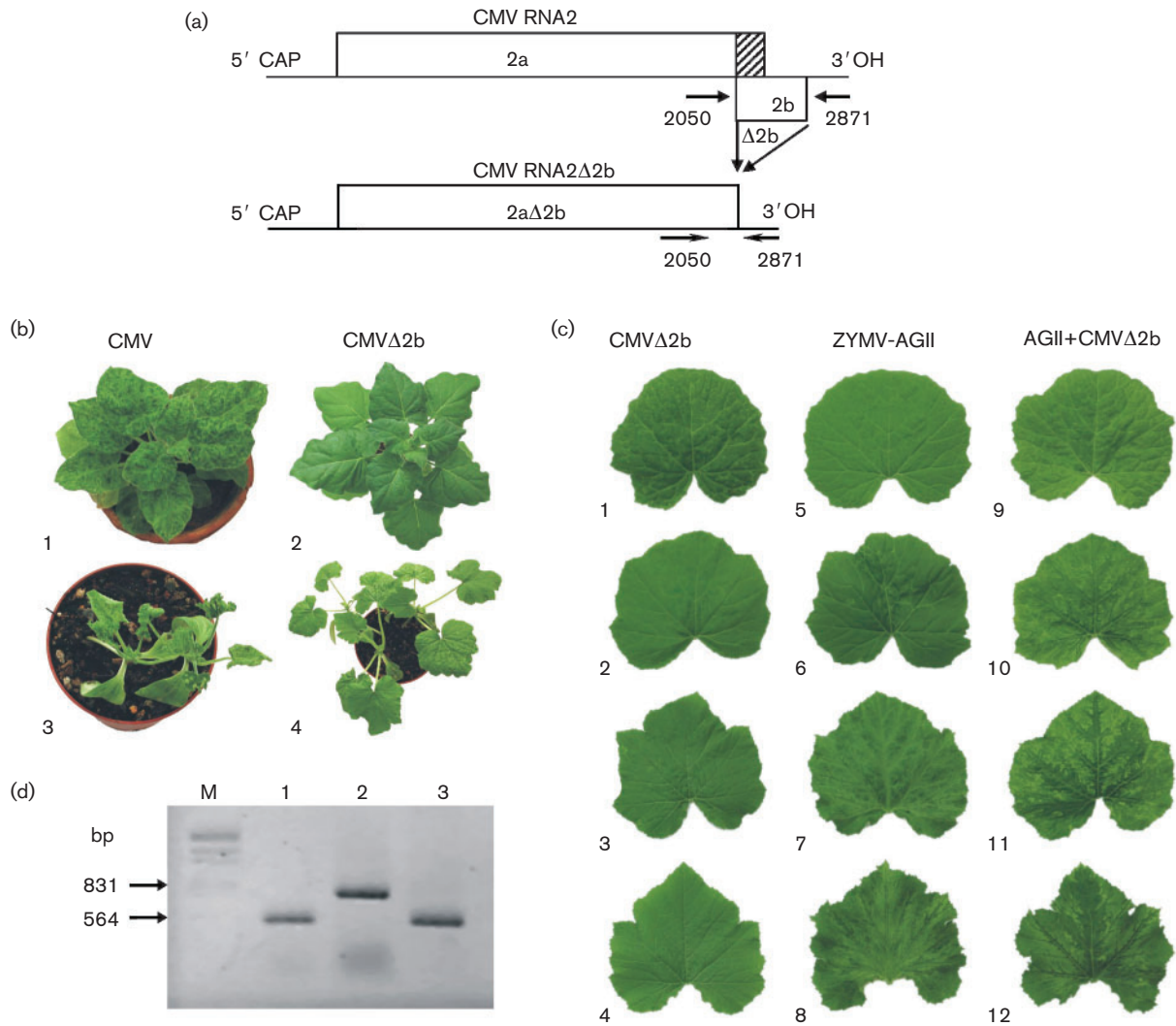


Fig. 1. Construction, pathogenicity and accumulation of CMVΔ2b. (a) Schematic illustration of the 2b gene deletion in RNA 2 of Fny-CMV. Orientations and locations of the primers for RT-PCR verification of 2b deletion in the progeny virus are indicated by arrows with numbers. (b) Symptoms on *N. glutinosa* (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).

in pathogenicity in cucurbits. The CMV genes for MP, CP and 2b were cloned between the NIb- 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 NIA 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

2a genes via the ZYMV-AGII vector were unsuccessful, probably because of a cloning-size limitation of the ZYMV-AGII vector.

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 2bΔNLS1 and the mutant 2bΔNLS1 was expressed from ZYMV-AGII (AGII-2bΔNLS1). The expression of 2bΔNLS1 from AGII-2bΔNLS1 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-2bΔNLS1 (Fig. 2g). The stability of the sequences encoding 2b and 2bΔNLS1 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-2bΔNLS1 (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 (in 2b6AlaNLS1). In the CMV-susceptible cucumber cv. Bet Alpha, expression of 2b6AlaNLS1 from ZYMV-AGII induced symptoms similar to those seen in infection with AGII-2bΔNLS1 (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-2bΔNLS1 and AGII-2b6AlaNLS1 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 (2bΔNLS2) or substituted by four alanine residues (2b4AlaNLS2). Infection of cucurbit hosts with ZYMV-AGII expressing either 2bΔNLS2 or 2b4AlaNLS2 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 2bΔNLS1 (*GUS*-2bΔNLS1) 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 2bΔNLS2 (*GUS*-2bΔNLS2) or to 2b4AlaNLS2 (*GUS*-4AlaNLS2) was also localized in the nucleus (Fig. 5a).

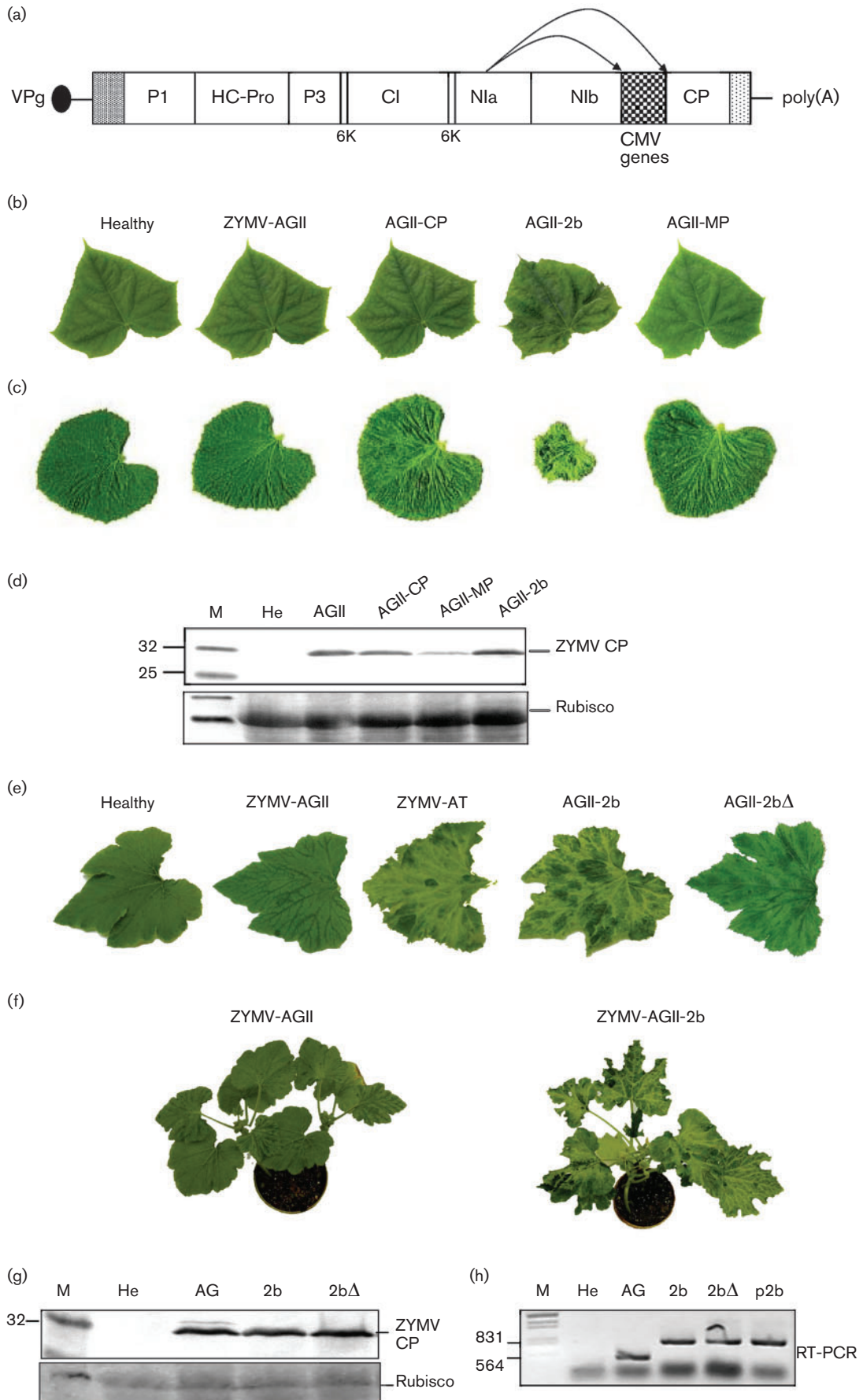


Fig. 2. Transient expression of CMV genes encoding MP, CP, 2b and 2bΔ in cucurbits via the ZYMV-AGII vector expression system and symptom appearance. (a) Schematic diagram of insertion of CMV genes into the ZYMV-AGII vector and their cleavage from the ZYMV-AGII polyprotein. (b, c) Leaves of CMV-resistant cucumber cv. Delila (b) or CMV-tolerant melon cv. Arava (c) infected with ZYMV-AGII or ZYMV-AGII recombinant viruses expressing CMV CP (AGII-CP), 2b (AGII-2b) or MP (AGII-MP). Leaves were photographed 18 days post-inoculation. (d) Western blot analysis of ZYMV CP accumulation after infection by ZYMV-AGII (AGII) and the recombinant viruses ZYMV-AGII-CP (AGII-CP), ZYMV-AGII-MP (AGII-MP) and ZYMV-AGII-2b (AGII-2b) in infected cucumber cv. Delila. Equal loading of each sample is shown by Ponceau staining of Rubisco. Lane M, molecular mass marker (kDa); lane He, proteins from a mock-inoculated (healthy) plant. (e, f) Symptoms of squash leaves cv. Ma'yan (e) and whole plants (f) infected with the attenuated ZYMV-AGII (AGII), wild-type ZYMV-AT (AT) or ZYMV-AGII chimaeric viruses expressing the Fny-CMV 2b gene (AGII-2b) or the 2b gene with a deleted NLS1 (AGII-2bΔ). (g) Western blot analysis of ZYMV CP accumulation in squash plants, mock-inoculated (He) or infected with ZYMV-AGII (AGII), ZYMV-AGII expressing the wild-type Fny-CMV 2b (2b) or a mutated 2b with a deleted NLS1 (2bΔ). Lane M, molecular mass marker. Equal loading of each lane is shown by Ponceau staining of Rubisco. (h) RT-PCR confirmation of the stability of the inserted 2b (2b) and NLS1-deleted 2b (2bΔ) sequences in progeny of the recombinant ZYMV-AGII in infected squash plants. Lane AG, ZYMV-AGII-infected control; lane He, mock-inoculated control; lane p2b, PCR product obtained by using the ZYMV-AGII-2b plasmid as a template.

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 (2bΔNLS1+2) was fused to the *GUS* gene (*GUS*-2bΔNLS1+2) for the nuclear

localization assay. Histochemical analysis of *GUS* production following bombardment with the *GUS*-2bΔNLS1+2 construct demonstrated that the *GUS*-2bΔNLS1+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

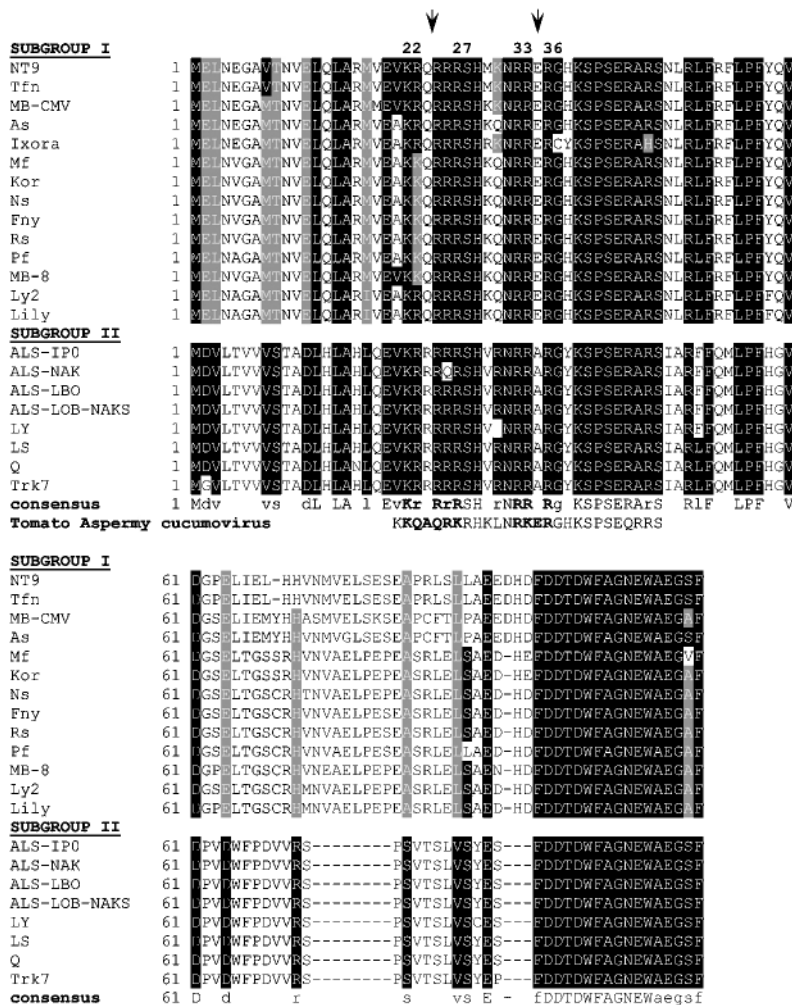


Fig. 3. Amino acid sequence alignment of 2b proteins of CMV strains subgroup I (A plus B) and II. Consensus amino acids are indicated by letters below each alignment block. Consensus amino acid residues of two putative NLSs are highlighted in bold letters. The locations of the two putative NLSs on the subgroup I 2b proteins are indicated by arrows. All sequences were retrieved from GenBank.

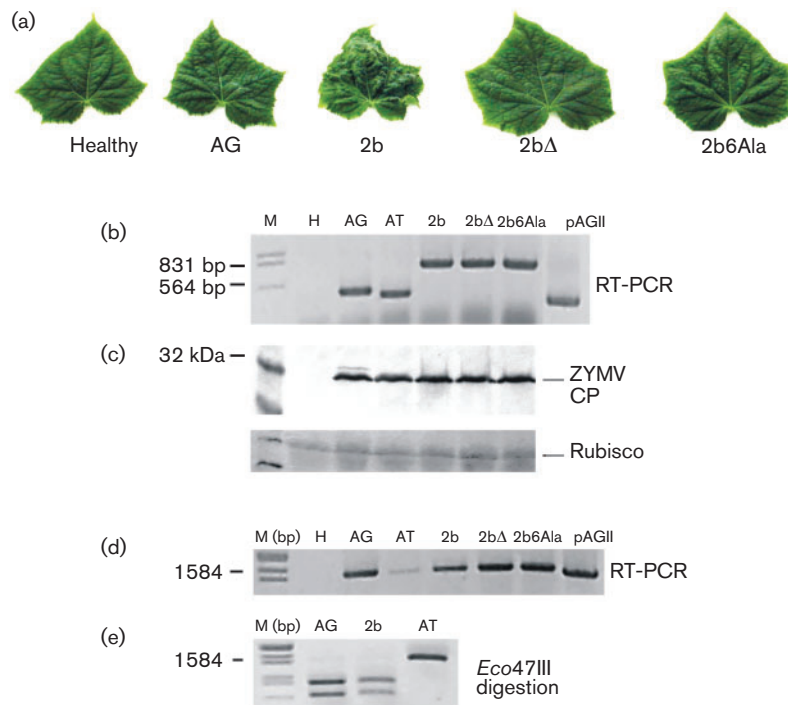


Fig. 4. Characterization of the various ZYMV-AGII-2b recombinant viruses in CMV-susceptible cucumber cv. Bet Alpha. (a) Symptom expression in cucumber leaves mock-inoculated (healthy) or inoculated with ZYMV-AGII (AG) or ZYMV-AGII chimaeric virus expressing the wild-type Fny-CMV 2b gene (2b), the 2b gene with the sequences encoding the NLS1 deleted (2b Δ) or the 2b gene with the sequences encoding NLS1 substituted with six alanines (2b6Ala). (b) RT-PCR confirmation of stability of the 2b gene and its NLS mutants in progeny viruses. Lane M, molecular mass marker (bp); lane H, mock-inoculated plants; lane pAGII, control for the PCR products using the plasmid containing ZYMV-AGII as a template. (c) Western blot analysis of ZYMV CP accumulation in cucumber cv. Bet Alpha infected by AGII-2b recombinant viruses. Equal loadings of each sample are shown by Ponceau staining of Rubisco. (d) RT-PCR amplification of ZYMV HC-Pro-encoding sequences from progeny viruses. (e) *Eco*47III digestion of the HC-Pro fragments amplified by RT-PCR in (d) from ZYMV-AGII (AG), ZYMV-AGII expressing Fny-CMV 2b (2b) and ZYMV-AT (AT).

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.*, 2001). Yeast cells were transformed with pNIA expressing 2b or 2b with modified NLSs. All the transformed cells grew well on non-selective medium (Fig. 5b). On selective medium, however, cells 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 2b Δ NLS1- or 2b Δ NLS2-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 (2b Δ NLS1 + 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 AtKAP α

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 & Went, 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 AtKAP α (Ballas & Citovsky, 1997) in the yeast two-hybrid system (SenGupta *et al.*, 1996). Our results showed that the native 2b protein bound to AtKAP α , as determined by an enzymic assay for the expression of β -galactosidase (Fig. 6). The 2b/AtKAP α interaction activity was scored as 100%. Deletion of both NLS domains (2b Δ NLS1 + 2) disrupted the interaction between 2b and AtKAP α 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 AtKAP α (Fig. 6).

DISCUSSION

To determine which genes of Fny-CMV were involved in disease symptom elicitation, we used the attenuated

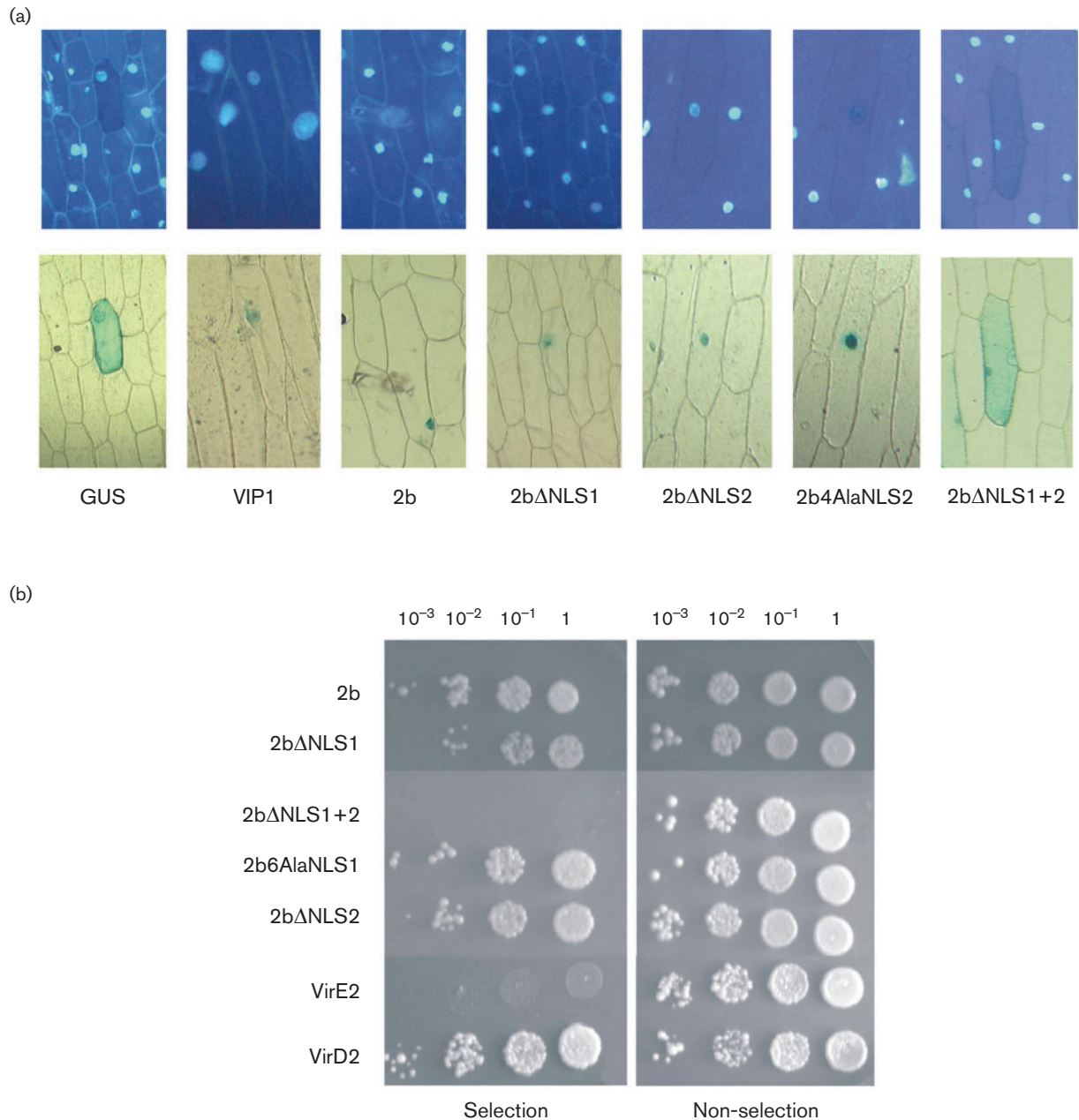


Fig. 5. Nuclear localization of 2b and its NLS mutants by (a) transient expression in onion cells and (b) yeast nuclear import assay. (a) Onion epidermal cells were stained with X-glucuronide for GUS expression (lower row of panels) or with DAPI for nuclear staining (upper row of panels) 48 h after transfection 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.

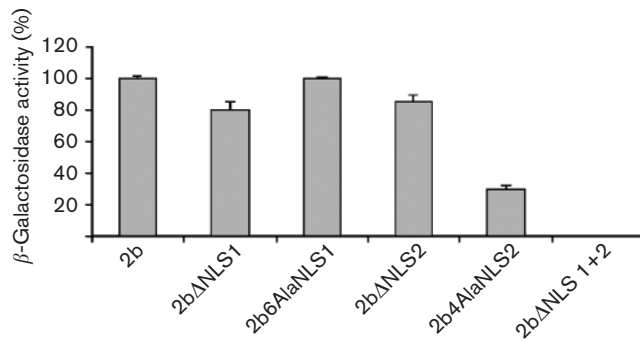


Fig. 6. Interaction of CMV 2b protein with the AtKAP α in a two-hybrid system. The wild-type 2b gene (2b) and its NLS mutants (2b Δ NLS1, 2b6AlaNLS1, 2b Δ NLS2 and 2b Δ NLS1 +2) were subcloned into the plasmid pSTT91, whereas the gene for AtKAP α was cloned in the pGAD424 expression vector. Protein–protein 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 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, 1996; Ji & Ding, 2001; Shi *et al.*, 2002; Soards *et al.*, 2002). In addition, we demonstrated that 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-CMV Δ 2b 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 terminator after the third amino acid resulted in the inability of the virus to infect cucumber plants systemically, but the virus could still infect *N. glutinosa* systemically (Ding *et al.*, 1995a, b), with a delay and milder symptoms. The 2b protein was not required for the replication of either CMV or its satellite RNA (Ding *et al.*, 1995b), but was shown to play a role in promoting cell-to-cell movement (Shi *et al.*, 2003) and long-distance movement (Ding *et al.*, 1995a; Ji & Ding, 2001; Soards *et al.*, 2002). Whether this is due to a direct effect on movement or an effect on the suppressor activity of the CMV 2b protein that affects movement indirectly cannot be determined, although differences in pathogenicity mediated by different 2b proteins did not correlate with either increased RNA accumulation or more rapid movement *per se* (Shi *et al.*, 2002). There are differences in plasmodesmata network connection, size and

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.

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 AtKAP α , 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 (²²KKQRRR²⁷) from a CMV subgroup IA 2b protein did not prevent translocation of the

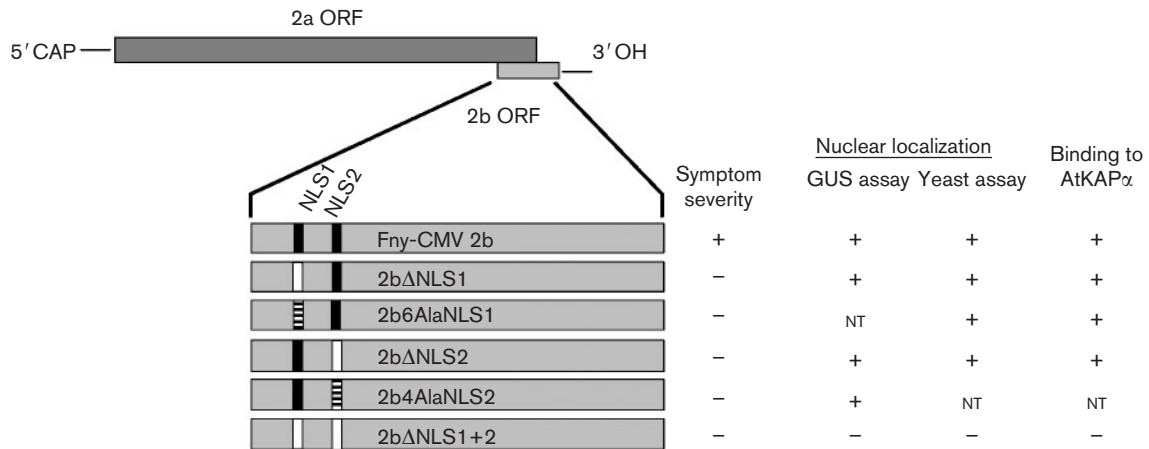


Fig. 7. Summary of the effects of *2b* mutations on pathogenicity, nuclear localization and interaction with AtKAP α . A schematic diagram of the location of the *2b* gene within CMV RNA 2 is shown at the top. The putative NLS1 and NLS2 sites within the *2b* mutants are indicated by black bars. Replacement 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 AtKAP α is marked as positive (+) or negative (-). NT, Not tested.

protein into the host nucleus. However, the function of the Fny-CMV 2b protein as a pathogenicity elicitor was impaired by modification of the NLS when expressed via the ZYMV-AGII vector. The Fny-CMV 2b protein was shown to contain a second NLS (33 RRER 36), modification of which also impaired the ability of the 2b protein to enhance pathogenicity. These results indicate that the ability of the Fny-CMV 2b protein to localize to the nucleus is not sufficient for induction of pathogenicity. Thus, Fny-CMV 2b nuclear localization can be implemented by either of the two NLSs, which do not resemble a classical monopartite NLS, as demonstrated in the 2b protein of the Q strain of CMV, a subgroup II CMV strain (Lucy *et al.*, 2000).

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 α proteins, analogous to the importin α 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 intranuclear 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.

REFERENCES

- Anjos, J. R., Jarlfors, U. & Ghabrial, S. A. (1992). Soybean mosaic potyvirus enhances the titer of two comoviruses in dually infected soybean plants. *Phytopathology* **82**, 1022–1027.
- Arazi, T., Slutsky, S. G., Shibolet, Y. M., Wang, Y., Rubinstein, M., Barak, S., Yang, J. & Gal-On, A. (2001). Engineering zucchini yellow mosaic potyvirus as a non-pathogenic vector for expression of heterologous proteins in cucurbits. *J Biotechnol* **87**, 67–82.
- Ballas, N. & Citovsky, V. (1997). Nuclear localization signal binding protein from *Arabidopsis* mediates nuclear import of *Agrobacterium* VirD2 protein. *Proc Natl Acad Sci U S A* **94**, 10723–10728.

- Bennett, C. W. (1952).** Interactions between viruses and virus strains. *Adv Virus Res* **1**, 39–67.
- Brigneti, G., Voinnet, O., Li, W.-X., Ji, L.-H., Ding, S.-W. & Baulcombe, D. C. (1998).** Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J* **17**, 6739–6746.
- Choi, S. K., Yoon, J. Y., Ryu, K. H., Choi, J. K., Palukaitis, P. & Park, W. M. (2002).** Systemic movement of a movement-deficient strain of *Cucumber mosaic virus* in zucchini squash is facilitated by a cucurbit-infecting potyvirus. *J Gen Virol* **83**, 3173–3178.
- Ding, S.-W., Li, W.-X. & Symons, R. H. (1995a).** A novel naturally occurring hybrid gene encoded by a plant RNA virus facilitates long distance virus movement. *EMBO J* **14**, 5762–5772.
- Ding, S.-W., Rathjen, J. P., Li, W.-X., Swanson, R., Healy, H. & Symons, R. H. (1995b).** Efficient infection from cDNA clones of cucumber mosaic cucumovirus RNAs in a new plasmid vector. *J Gen Virol* **76**, 459–464.
- Ding, S.-W., Shi, B.-J., Li, W.-X. & Symons, R. H. (1996).** An interspecies hybrid RNA virus is significantly more virulent than either parental virus. *Proc Natl Acad Sci U S A* **93**, 7470–7474.
- Fukumoto, F., Masuda, Y. & Hanada, K. (2003).** Pea tissue necrosis induced by *Cucumber mosaic virus* alone or together with *Watermelon mosaic virus*. *Plant Dis* **87**, 324–328.
- Gal-On, A. (2000).** A point mutation in the FRNK motif of the potyvirus helper component-protease gene alters symptom expression in cucurbits and elicits protection against the severe homologous virus. *Phytopathology* **90**, 467–473.
- Gal-On, A., Antignus, Y., Rosner, A. & Raccach, B. (1992).** A zucchini yellow mosaic virus coat protein gene mutation restores aphid transmissibility but has no effect on multiplication. *J Gen Virol* **73**, 2183–2187.
- Gal-On, A., Meiri, E., Elman, C., Gray, D. J. & Gaba, V. (1997).** Simple hand-held devices for the efficient infection of plants with viral-encoding constructs by particle bombardment. *J Virol Methods* **64**, 103–110.
- Garces-Orejuela, C. & Pound, G. S. (1957).** The multiplication of tobacco mosaic virus in the presence of cucumber mosaic virus or tobacco ringspot virus in tobacco. *Phytopathology* **47**, 232–239.
- González-Jara, P., Tenllado, F., Martínez-García, B., Atencio, F. A., Barajas, D., Vargas, M., Díaz-Ruiz, J. & Díaz-Ruiz, J. R. (2004).** Host-dependent differences during synergistic infection by Potyviruses with potato virus X. *Mol Plant Pathol* **5**, 29–35.
- Goodin, M. M., Austin, J., Tobias, R., Fujita, M., Morales, C. & Jackson, A. O. (2001).** Interactions and nuclear import of the N and P proteins of sonchus yellow net virus, a plant nucleorhabdovirus. *J Virol* **75**, 9393–9406.
- Görlich, D. & Mattaj, I. W. (1996).** Nucleocytoplasmic transport. *Science* **271**, 1513–1518.
- Hollenberg, S. M., Sternglanz, R., Cheng, P. F. & Weintraub, H. (1995).** Identification of a new family of tissue-specific basic helix-loop-helix proteins with a two-hybrid system. *Mol Cell Biol* **15**, 3813–3822.
- Hunter, P. J., Jones, J. E. & Walsh, J. A. (2002).** Involvement of *Beet western yellows virus*, *Cauliflower mosaic virus*, and *Turnip mosaic virus* in internal disorders of stored white cabbage. *Phytopathology* **92**, 816–826.
- Ji, L.-H. & Ding, S.-W. (2001).** The suppressor of transgene RNA silencing encoded by *Cucumber mosaic virus* interferes with salicylic acid-mediated virus resistance. *Mol Plant Microbe Interact* **14**, 715–724.
- Karyeija, R. F., Kreuze, J. F., Gibson, R. W. & Valkonen, J. P. T. (2000).** Synergistic interactions of a potyvirus and a phloem-limited crinivirus in sweet potato plants. *Virology* **269**, 26–36.
- Kasschau, K. D., Xie, Z., Allen, E., Llave, C., Chapman, E. J., Krizan, K. A. & Carrington, J. C. (2003).** P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function. *Dev Cell* **4**, 205–217.
- Kunik, T., Palanichelvam, K., Czosnek, H., Citovsky, V. & Gafni, Y. (1998).** Nuclear import of the capsid protein of tomato yellow leaf curl virus (TYLCV) in plant and insect cells. *Plant J* **13**, 393–399.
- Kunkel, T. A., Roberts, J. D. & Zakour, R. A. (1987).** Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol* **154**, 367–382.
- Leclerc, D., Chapdelaine, Y. & Hohn, T. (1999).** Nuclear targeting of the cauliflower mosaic virus coat protein. *J Virol* **73**, 553–560.
- Li, H.-W., Lucy, A. P., Guo, H.-S., Li, W.-X., Ji, L.-H., Wong, S.-M. & Ding, S.-W. (1999).** Strong host resistance targeted against a viral suppressor of the plant gene silencing defence mechanism. *EMBO J* **18**, 2683–2691.
- Lucy, A. P., Guo, H.-S., Li, W.-X. & Ding, S.-W. (2000).** Suppression of post-transcriptional gene silencing by a plant viral protein localized in the nucleus. *EMBO J* **19**, 1672–1680.
- Mayers, C. N., Palukaitis, P. & Carr, J. P. (2000).** Subcellular distribution analysis of the cucumber mosaic virus 2b protein. *J Gen Virol* **81**, 219–226.
- Murphy, J. F. & Kyle, M. M. (1995).** Alleviation of restricted systemic spread of pepper mottle potyvirus in *Capsicum annuum* cv. Avelar by coinfection with a cucumovirus. *Phytopathology* **85**, 561–566.
- Palukaitis, P. & Kaplan, I. B. (1997).** Synergy of virus accumulation and pathology in transgenic plants expressing viral sequences. In *Virus-Resistant Transgenic Plants: Potential Ecological Impact*, pp. 77–84. Edited by M. Tepfer & E. Balázs. Berlin: Springer.
- Pio-Ribeiro, G., Wyatt, S. D. & Kuhn, C. W. (1978).** Cowpea stunt: a disease caused by a synergistic interaction of two viruses. *Phytopathology* **68**, 1260–1265.
- Poolpol, P. & Inouye, T. (1986).** Enhancement of cucumber mosaic virus multiplication by zucchini yellow mosaic virus in doubly infected cucumber plants. *Ann Phytopathol Soc Jpn* **52**, 22–30.
- Pruss, G., Ge, X., Shi, X. M., Carrington, J. C. & Bowman Vance, V. (1997).** Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. *Plant Cell* **9**, 859–868.
- Qiu, W., Park, J.-W. & Scholthof, H. B. (2002).** Tombusvirus P19-mediated suppression of virus-induced gene silencing is controlled by genetic and dosage features that influence pathogenicity. *Mol Plant Microbe Interact* **15**, 269–280.
- Rhee, Y., Gurel, F., Gafni, Y., Dingwall, C. & Citovsky, V. (2000).** A genetic system for detection of protein nuclear import and export. *Nat Biotechnol* **18**, 433–437.
- Rizzo, T. M. & Palukaitis, P. (1990).** Construction of full-length cDNA clones of cucumber mosaic virus RNAs 1, 2 and 3: generation of infectious RNA transcripts. *Mol Gen Genet* **222**, 249–256.
- Rochow, W. F. & Ross, A. F. (1955).** Virus multiplication in plants doubly infected by potato viruses X and Y. *Virology* **1**, 10–27.
- Ryabov, E. V., Fraser, G., Mayo, M. A., Barker, H. & Taliensky, M. (2001).** Umbravirus gene expression helps *Potato leafroll virus* to invade mesophyll tissues and to be transmitted mechanically between plants. *Virology* **286**, 363–372.
- Sáenz, P., Quiot, L., Quiot, J.-B., Candresse, T. & García, J. A. (2001).** Pathogenicity determinants in the complex virus population of a *Plum pox virus* isolate. *Mol Plant Microbe Interact* **14**, 278–287.
- Sáenz, P., Salvador, B., Simón-Mateo, C., Kasschau, K. D., Carrington, J. C. & García, J. A. (2002).** Host-specific involvement

- of the HC protein in the long-distance movement of potyviruses. *J Virol* **76**, 1922–1931.
- Sano, Y. & Kojima, M. (1989).** Increase in cucumber mosaic virus concentration in Japanese radish plants co-infected with turnip mosaic virus. *Ann Phytopathol Soc Jpn* **55**, 296–302.
- Selth, L. A., Randles, J. W. & Rezaian, M. A. (2004).** Host responses to transient expression of individual genes encoded by *Tomato leaf curl virus*. *Mol Plant Microbe Interact* **17**, 27–33.
- SenGupta, D. J., Zhang, B., Kraemer, B., Pochart, P., Fields, S. & Wickens, M. (1996).** A three-hybrid system to detect RNA–protein interactions *in vivo*. *Proc Natl Acad Sci U S A* **93**, 8496–8501.
- Shi, B.-J., Palukaitis, P. & Symons, R. H. (2002).** Differential virulence by strains of *Cucumber mosaic virus* is mediated by the 2b gene. *Mol Plant Microbe Interact* **15**, 947–955.
- Shi, B.-J., Miller, J., Symons, R. H. & Palukaitis, P. (2003).** The 2b protein of cucumoviruses has a role in promoting the cell-to-cell movement of pseudorecombinant viruses. *Mol Plant Microbe Interact* **16**, 261–267.
- Soards, A. J., Murphy, A. M., Palukaitis, P. & Carr, J. P. (2002).** Virulence and differential local and systemic spread of *Cucumber mosaic virus* in tobacco are affected by the CMV 2b protein. *Mol Plant Microbe Interact* **15**, 647–653.
- Stachel, S. E., An, G., Flores, C. & Nester, E. W. (1985).** A Tn3 lacZ transposon for the random generation of beta-galactosidase gene fusions: application to the analysis of gene expression in *Agrobacterium*. *EMBO J* **4**, 891–898.
- Suntharalingam, M. & Wente, S. R. (2003).** Peering through the pore: nuclear pore complex structure, assembly, and function. *Dev Cell* **4**, 775–789.
- Sutton, A., Heller, R. C., Landry, J., Choy, J. S., Sirko, A. & Sternglanz, R. (2001).** A novel form of transcriptional silencing by Sum1-1 requires Hst1 and the origin recognition complex. *Mol Cell Biol* **21**, 3514–3522.
- Tzfira, T. & Citovsky, V. (2001).** Comparison between nuclear localization of nopaline- and octopine-specific *Agrobacterium* VirE2 proteins in plant, yeast and mammalian cells. *Mol Plant Pathol* **2**, 171–176.
- Tzfira, T., Vaidya, M. & Citovsky, V. (2001).** VIP1, an *Arabidopsis* protein that interacts with *Agrobacterium* VirE2, is involved in VirE2 nuclear import and *Agrobacterium* infectivity. *EMBO J* **20**, 3596–3607.
- Tzfira, T., Vaidya, M. & Citovsky, V. (2002).** Increasing plant susceptibility to *Agrobacterium* infection by overexpression of the *Arabidopsis* nuclear protein VIP1. *Proc Natl Acad Sci U S A* **99**, 10435–10440.
- van Bel, A. J. E. (1993).** Strategies of phloem loading. *Annu Rev Plant Physiol Plant Mol Biol* **44**, 253–281.
- Varagona, M. J., Schmidt, R. J. & Raikhel, N. V. (1992).** Nuclear localization signal(s) required for nuclear targeting of the maize regulatory protein Opaque-2. *Plant Cell* **4**, 1213–1227.
- Wang, Y., Gaba, V., Yang, J., Palukaitis, P. & Gal-On, A. (2002).** Characterization of synergy between *Cucumber mosaic virus* and potyviruses in cucurbit hosts. *Phytopathology* **92**, 51–58.
- Wang, Y., Lee, K. C., Gaba, V., Wong, S. M., Palukaitis, P. & Gal-On, A. (2004).** Breakage of resistance to *Cucumber mosaic virus* by co-infection with *Zucchini mosaic virus*: enhancement of CMV accumulation independent of symptom expression. *Arch Virol* **149**, 379–396.
- Zhang, L., Handa, K. & Palukaitis, P. (1994).** Mapping local and systemic symptom determinants of cucumber mosaic cucumovirus in tobacco. *J Gen Virol* **75**, 3185–3191.