Cucumber mosaic virus 2b protein compensates for restricted systemic spread of *Potato virus Y* in doubly infected tobacco

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Tobacco plants (*Nicotiana tabacum* cv. Xanthi-nc) inoculated with a necrotic strain of *Potato virus Y* (PVY, T01 isolate) developed necrotic symptoms in some systemically infected leaves, but not in younger leaves. However, PVY expressed distinct symptoms not only in the older leaves, but also in the younger leaves, of plants that had been doubly inoculated with PVY and with *Cucumber mosaic virus* (CMV, strain Pepo). A tissue blot immunoassay of tissues from various positions of the stem detected PVY weakly in each stem, but not in the shoot apex, of singly infected plants, whereas PVY was detected at high levels in almost all sections of doubly infected plants. CMV was also detected at high levels in sections of singly and doubly infected plants. Immunohistochemistry of stem tissues showed that in singly infected plants, PVY was confined to external phloem cells and was not detected in internal phloem cells. However, in doubly infected plants, PVY was distributed uniformly throughout whole tissues, including the external phloem, xylem parenchyma and internal phloem cells. In plants that were doubly infected with PVY and PepoΔ2b, a modified CMV that cannot translate the 2b protein, the spread of PVY was restricted as in singly infected plants. These results suggested that the plant host has a counterdefence mechanism that restricts systemic spread of PVY T01, and that the 2b protein of CMV strain Pepo negates this restriction.

INTRODUCTION

A plant virus must evade the defence system of a potential plant host before it can infect the plant systemically. Extensive research on cell-to-cell movement of viruses within their hosts has been published, but much less is known about the process and function of systemic spread (Lucas & Gilbertson, 1994; Carrington *et al.*, 1996; Gilbertson & Lucas, 1996; Séron & Haenni, 1996; Nelson & van Bel, 1998; Lazarowitz & Beachy, 1999).

Almost all eukaryotic organisms possess a sequence-specific RNA-degradation system, referred to as RNA silencing (Voinnet, 2001; Waterhouse *et al.*, 2001). Observations that plant viruses encode proteins to suppress RNA silencing provide the most compelling evidence that RNA silencing functions as an inducible, host RNA-surveillance system (Marathe *et al.*, 2000; Li & Ding, 2001; Voinnet, 2001; Waterhouse *et al.*, 2001). The coat protein (CP) is often, but not invariably, essential for systemic spread of plant viruses (Gilbertson & Lucas, 1996; Séron & Haenni, 1996; Nelson & van Bel, 1998; Kobori *et al.*, 2002). The helper component proteinase (HC-Pro) of potyviruses, the 2b protein of cucumoviruses, the p19 protein of tombusviruses and the P1 protein of sobemoviruses are also involved in systemic spread (Cronin *et al.*, 1995; Ding *et al.*, 1995; Scholthof *et al.*, 1995; Bonneau *et al.*, 1998; Kasschau & Carrington, 2001; Soards *et al.*, 2002). These proteins, which have been characterized as important pathogenicity determinants, have been identified as suppressors of RNA silencing (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Voinnet *et al.*, 1999).

Mixed virus infections can result in the complementation of a movement defect in heterologous viruses in non-host plants (Malyshenko *et al.*, 1989; Taliansky & García-Arenal, 1995; Hacker & Fowler, 2000; Takeshita & Takanami, 2000; Choi *et al.*, 2002). Several studies have shown that different viral proteins expressed from transgenes (Giesman-Cookmeyer *et al.*, 1995; Kaplan *et al.*, 1995; Cooper *et al.*, 1996), heterologous sequences cloned into defective genomes (De Jong & Ahlquist, 1992; Ryabov *et al.*, 1999; Spitsin *et al.*, 1999) or cotransfected plasmids (Agranovsky *et al.*, 1998; Fedorkin *et al.*, 2001) can functionally replace non-homologous proteins from other viruses.

In this report, we have shown that *Potato virus Y* (PVY, T01 isolate) accumulated in inoculated leaves (the oldest pair of leaves along the stem) of tobacco plants and spread systemically up the stem to sequential pairs of leaves but, atypically, did not move to young, developing tissues. However, when tobacco plants were doubly infected with PVY and *Cucumber mosaic virus* (CMV, strain Pepo), PVY moved to and accumulated in young, developing tissues and the resulting infection resulted in systemic symptoms distinct from those caused by each virus alone. Systemic spread of PVY in tobacco appeared to be regulated in phloem tissues.

METHODS

Viruses, plants and detection. Tobacco plants (*Nicotiana tabacum* cv. Xanthi-nc) and *Nicotiana benthamiana* plants at the five-leaf stage were used for inoculation. The Pepo strain of CMV (subgroup IA) was originally obtained from *Cucurbita pepo* in Japan (Osaki *et al.*, 1973; Saiga *et al.*, 1998); alternatively, a modified CMV propagated in tobacco was purified as described by Takanami (1981). Plants were inoculated mechanically with either purified CMV strain Pepo or the modified CMV, diluted to a final concentration of 50 μg ml^{-1} in 100 mM phosphate buffer, pH 7·0 (PB). PVY isolate T01 is a necrotic strain provided by Japan Tobacco (Yokohama, Japan). This strain was classified as T-PVY in Japan, but it has been reclassified as N-PVY (Hataya *et al.*, 1994). PVY for inoculation was extracted from infected tobacco leaves by grinding in 100 mM PB (1 g in 10 ml PB). In the mixed-inoculation experiments, the two inocula were mixed just before inoculation.

Inoculated plants were grown in a growth chamber (NK systems) at 24 °C with a 14 h light/10 h dark cycle. Virus accumulation was assessed at different times post-inoculation (p.i.) by double-antibody sandwich (DAS)-ELISA (Clark & Adams, 1977). Four 6 mm diameter discs per leaf were collected from six plants for each sample. Samples were ground in 400 μl PBS containing 0·05 % Tween 20 (PBST), and 100 μl samples were tested. Coating antibodies were diluted 1 : 1000 in sodium carbonate buffer (pH 9·6) and alkaline phosphatase-conjugated IgG secondary antibody was diluted 1 : 1000 in PBST. A_{405} was measured with a SPECTRAmax 250 (Molecular Devices) ELISA plate reader. Experiments were done in triplicate. Tissue immunoblot analysis was carried out independently on two plants as described by Andrianifahanana *et al.* (1997). Cuts were made across the axes of petioles or stems. The cut surface of the tissue was then pressed directly on to nitrocellulose membranes (Bio-Rad) that had been treated with 0·2 M $CaCl_2$ prior to blotting. PVY or CMV CP was detected in tissue blots by using specific antibodies, diluted 1 : 2000, as the primary antibody, and alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma), diluted 1 : 4000, as the secondary antibody. Alkaline phosphatase was detected by using a BCIP/NBT liquid substrate system (Sigma). Experiments were done in triplicate.

Immunostaining of infected tissues. Sampled pieces were immersed immediately in fixative (50 % ethanol, 5 % acetic acid, 3·7 % formalin) and left overnight at 4 °C. After dehydration and infiltration in a graded series of ethanol solutions (50, 70, 90 and 100 %), each for 30 min, samples were embedded in paraffin (Paraplast-plus; Sigma). Sections (12 μm thick) of stem tissue were cut with a rotary microtome (Yamato Kohki) and placed on glass slides (Matsunami Glass). Sections were dewaxed in xylene and washed in 100 % ethanol. After hydration in a graded series of ethanol solutions (70, 50 and 30 %) and distilled water for 10 min each, sections were incubated in PBST and 1 % BSA for 1 h and then incubated with PVY CP-specific antibody, diluted 1 : 200 in PBST/BSA, for 2 h at 37 °C. After washing in PBST, sections were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma), diluted 1 : 200 in PBST/BSA, for 2 h at 37 °C and then washed three times for 10 min in PBST. After washing, samples were stained by using the BCIP/NBT liquid substrate system. Stained sections were then washed in distilled water and observed with a BX-50 microscope (Olympus).

Infectious cDNA plasmids. Plasmids pCP1TP1, pCP2TP1 and pCP3TP2, containing full-length cDNA copies of CMV strain Pepo genomic RNAs 1, 2 and 3, respectively (Saitoh *et al.*, 1999), were used to generate CMV inocula. To prepare a modified CMV containing RNA 2 that was unable to translate the 2b protein (PepoΔ2b), nucleotide changes were introduced into pCP2TP1. As the 2b protein of CMV strain Pepo contains three methionines, at amino acid positions 1, 8 and 18, we generated two translational termination codons at the beginning of the 2b gene, one after the first AUG codon and another after the third, by site-directed mutagenesis (Kunkel, 1985). A pair of complementary mutagenic primers (5′-GCGAAAGAAATATGGAATAGAACGTAGGTGCAAT-GAC-3′ and its complement) was used. An additional translational termination codon was then introduced into this plasmid by using a pair of complementary mutagenic primers (5′-GCTGGCTCAC-ATGTAGGAGGCGAAGAAGC-3′ and its complement). Neither mutated sequence induced C-terminal amino acid changes in the 2a protein, which overlaps the 2b protein. In the PCR, 12 cycles of amplification followed incubation at 95 °C for 30 s under the following conditions, using 0·05 U *Pfu* Turbo DNA polymerase μl^{-1} (Stratagene): denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min and extension at 68 °C for 12 min. After treatment with *Dpn*I (Stratagene), the products were transformed into *Escherichia coli* DH5α. The nucleotide sequences for the cDNA clone (pCP2Δ2bM3stp) constructed were determined by using a Beckman Coulter DNA sequencer, model CEQ 8000. *In vitro* transcripts of PepoΔ2b by T7 RNA polymerase (TaKaRa Bio) were generated by combining pCP2Δ2bM3stp with infectious clones of pCP1TP1 and pCP3TP2.

RESULTS

Symptom expression and accumulation of PVY in singly and doubly infected tobacco

The PVY isolate used in this study, T01, did not fully infect *N. tabacum* systemically, although it easily infected *N. benthamiana* systemically (Fig. 1a, b). In *N. tabacum* cv. Xanthi-nc plants inoculated with PVY, symptoms started to appear at 5 days p.i. Initial chlorotic spots were followed by necrotic spots, which developed gradually, within 15 days p.i., into veinal necrosis and leaf collapse in the inoculated leaves and the fully developed (older), systemically infected leaves above the inoculated leaves. The inoculated and older leaves of *N. tabacum* plants that were doubly inoculated with PVY and CMV showed the same symptoms as in PVY singly inoculated plants. However, the appearance of viral symptoms in intermediate-aged (middle), systemically infected leaves and younger leaves differed drastically in the singly and doubly inoculated plants. In singly inoculated plants, mild chlorotic spots were induced in the middle leaves and no symptoms were observed in the younger leaves (Fig. 1b). In doubly inoculated plants, the middle leaves developed severe necrosis and leaf collapse and the younger leaves had severe stunting

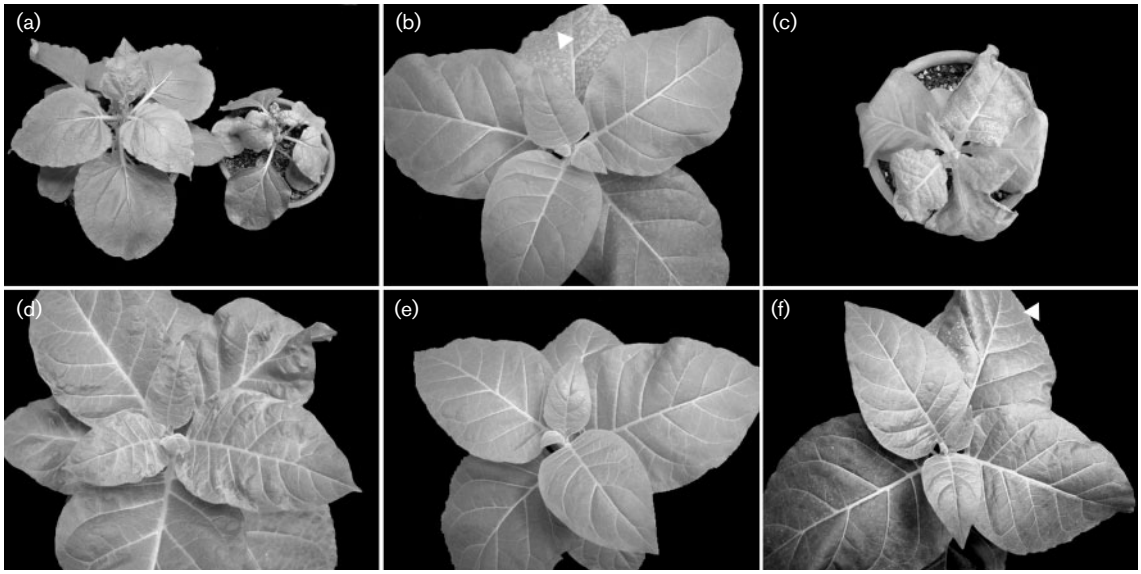

Fig. 1. Symptoms of plants infected singly with PVY, or doubly with PVY and CMV or a modified CMV (PepoΔ2b) at 15 days p.i. (a) *N. benthamiana* infected with PVY (right), showing leaf stunting and yellow mosaic, or with PB (left); (b–f) *N. tabacum* cv. Xanthi-nc infected with PVY (b), showing chlorotic spots in an older leaf (arrowhead), but not in younger leaves; PVY plus CMV (c), showing severe stunting and severe mosaic symptoms in younger leaves; CMV (d), showing distinct mosaic symptoms in younger leaves; PepoΔ2b (e), showing very mild mosaic symptoms; or PVY plus PepoΔ2b (f), showing chlorotic spots in an older leaf (arrowhead) and very mild mosaic symptoms in younger leaves.

and severe mosaic, with chlorotic lesions in dark-green islands, by 15 days p.i. (Fig. 1c). *N. tabacum* plants inoculated with CMV only had distinct mosaic symptoms in middle and younger leaves (Fig. 1d).

Virus accumulation in singly and doubly infected *N. tabacum* plants was measured by ELISA. PVY accumulation correlated with symptom expression. PVY levels were significantly greater in middle and younger leaves of doubly infected plants than in those of singly infected plants (Fig. 2a, +5U and +7U). This was particularly true in +7 leaves (seventh upper leaves above the inoculated leaves) at 13 days p.i., where PVY was detected at high levels in doubly infected plants, but was not detected in singly infected plants (Fig. 2a). However, amounts of virus were similar in the inoculated leaves and older leaves (Fig. 2a, I and +3). There were no significant differences in CMV levels between the doubly and singly infected plants (Fig. 2b).

Systemic spread of PVY in singly and doubly infected *N. tabacum*

To determine when PVY was transported from the inoculated leaf to the stem in singly and doubly infected *N. tabacum* plants, inoculated leaves and their petioles were detached from the stem at 48, 54 or 60 h p.i. Virus accumulation in +2 leaves was then analysed by ELISA at 14 days p.i. (Table 1). PVY was detected in the upper leaves of less than half of the singly infected plants at 48 h p.i. and in all plants at 54 h p.i. (Table 1). Contrary to our expectations, PVY was not detected in the upper leaves of doubly infected plants at 48 h p.i., but was detected in half of the plants at 54 h p.i. (Table 1). CMV was detected at 48 h p.i. in the upper leaves of all singly and doubly infected plants (Table 1).

Virus accumulation in the plants was further analysed by tissue immunoblot analysis to visualize the distribution of viral antigen in cross-sections of stems or petioles (Fig. 3a). PVY was detected at high levels in almost all sections, including apical tissues, in doubly infected plants (Fig. 3b). PVY was detected in each stem section, but the reaction was much weaker in tissues from middle or young stems and petioles, and PVY was not detected in the shoot apex (Fig. 3b, position K) of singly infected plants. CMV, however, was detected in almost all sections of both singly and doubly infected plants (Fig. 3d and data not shown).

To clarify whether PVY was localized in particular tissues in the singly infected plants, cross-sections of stems from plants at 7 days p.i. were further analysed by tissue immunoblot analysis. In singly infected plants, PVY was confined mostly to the external phloem, other than a few tissues of internal phloem below the inoculated leaves. However, in doubly infected plants, PVY was detected in both external and internal rings of phloem tissue (Fig. 3c, position A). PVY was not detected in the internal phloem in older sections of the stem in singly infected plants, but was distinctly detected in both external and internal rings

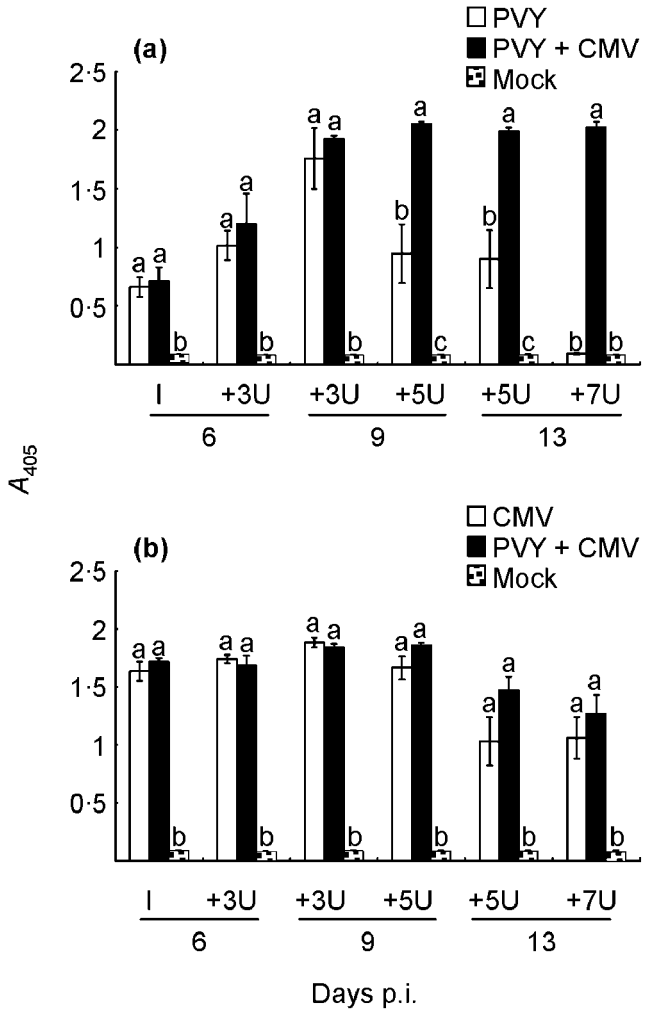

Fig. 2. Accumulation of PVY (a) or CMV (b) in inoculated leaves and systemically infected leaves after inoculation with either PVY or CMV, or with PVY plus CMV. Virus accumulation was determined by ELISA. Each histogram represents mean ELISA values (A_{405}) obtained from six individual plants. Different letters represent a significant difference of the means at $P=0{\cdot}05$, according to Bonferroni's multiple-range test. Error bars represent SD. I, Inoculated leaves at 6 days p.i.; U, upper infected leaves at 6 days p.i. (+3 old leaves above inoculated), 9 days p.i. (+3 old and +5 middle leaves above inoculated) and 13 days p.i. (+5 middle and +7 younger leaves above inoculated).

in doubly infected plants (Fig. 3c, position E). CMV was detected in external and internal phloem tissues in singly and doubly infected plants (Fig. 3d).

Immunohistochemistry revealed details of the distribution of PVY in the stem tissues (Fig. 3a, position E) in singly and doubly infected plants. PVY antigen was confined to external phloem cells and was not detected in internal phloem cells in singly infected plants (Fig. 4b). However, it was distributed uniformly in whole tissues, including the external phloem, xylem parenchyma and internal phloem cells in doubly infected plants (Fig. 4c). Therefore, CMV assisted the accumulation of PVY in stem tissues, which could be associated with enhanced spread of PVY into younger tissues.

Table 1. Transport of PVY and CMV from inoculated leaves into stems

Inoculated leaves and their petioles were detached from the stem at 48, 54 and 60 h p.i. Individual plants were used at each time point. Values are no. plants positive by ELISA for PVY or CMV in systemically infected leaves per total no. inoculated plants at 14 days p.i. Results are the sum of data from two independent experiments.

Virus detected	Inoculum	Time of sampling (h p.i.)		
		48	54	60
PVY	PVY	3/8	8/8	6/6
PVY	PVY + CMV	0/8	4/8	6/6
CMV	CMV	8/8	8/8	6/6
CMV	PVY + CMV	8/8	8/8	6/6

In the previous experiments, plants were examined for systemic spread of PVY after simultaneous inoculation with PVY and CMV. To examine whether the restricted systemic spread of PVY was affected by CMV when one virus was inoculated before the other, *N. tabacum* plants inoculated with CMV either 5 days before or 5 days after PVY inoculation were assayed by tissue blotting at 14 days after PVY inoculation. After one virus was inoculated, the other virus was inoculated on to the +2 leaves above the first-inoculated leaves. The younger leaves of plants inoculated with CMV and then with PVY developed severe mosaic symptoms and became distorted (data not shown). Plants inoculated with PVY and then with CMV also developed severe mosaic symptoms and distortion in younger leaves (data not shown). Therefore, the order of infection did not affect the assistance of PVY systemic spread by CMV in doubly infected plants.

CMV 2b protein assists PVY systemic spread in doubly infected plants

The CMV 2b protein is a known suppressor of RNA silencing (Brigneti *et al.*, 1998). To examine its possible contribution to the successful systemic spread of PVY, we inoculated *N. tabacum* plants simultaneously with PVY and PepoΔ2b, a modified CMV in which the 2b protein has been rendered untranslatable (Fig. 5a). Systemic spread of PVY was then traced by tissue immunoblot of stem sections and symptoms were observed. Plants inoculated with PepoΔ2b had very mild mosaic symptoms (Fig. 1e). Plants inoculated with PVY and PepoΔ2b developed mild chlorotic spots in older leaves without any PVY-induced symptoms in the younger leaves, as in the case of plants inoculated with PVY only (Fig. 1f). PVY was detected in the

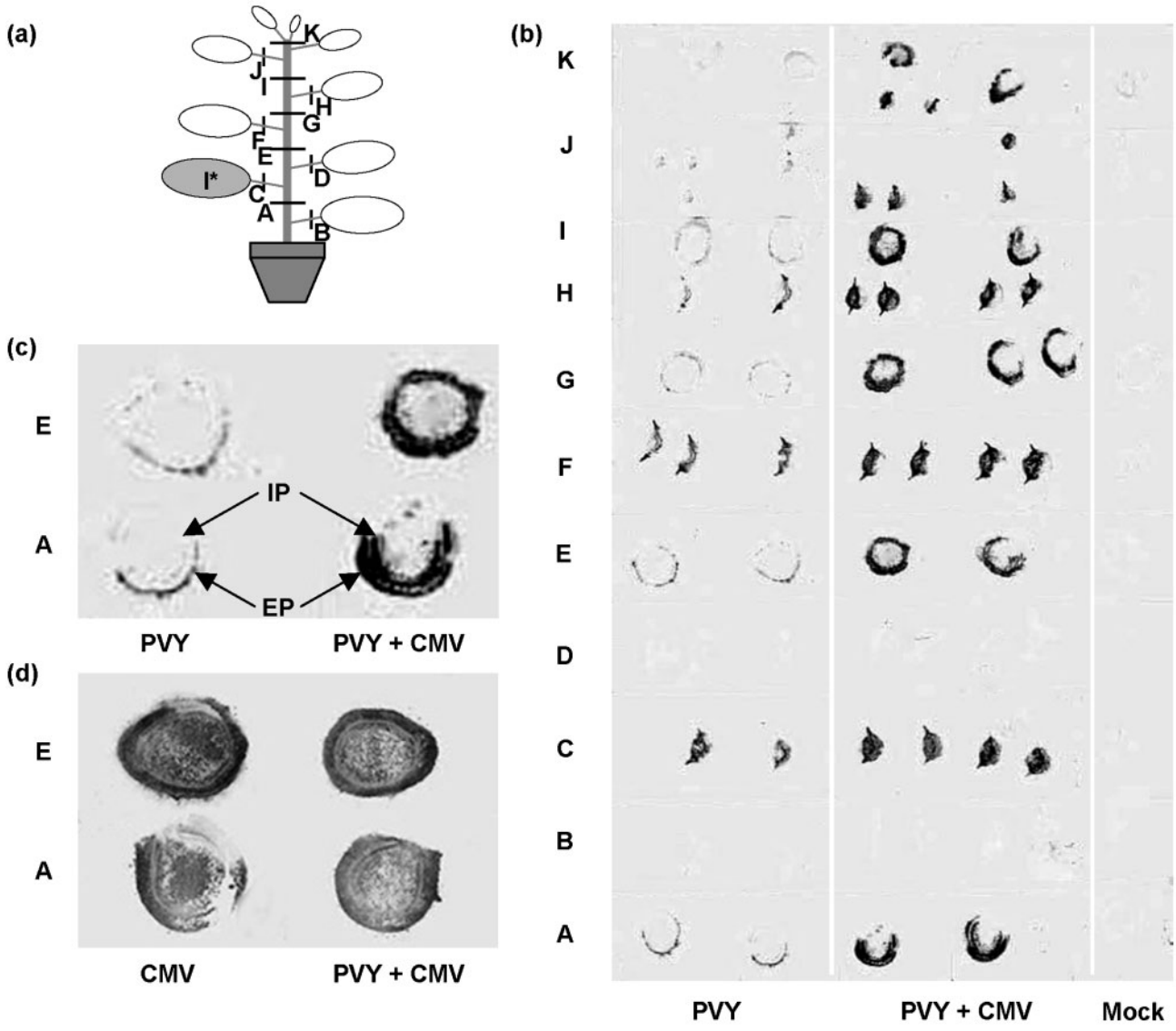

Fig. 3. Tissue immunoblot analysis of stem and petiole sections of *N. tabacum* plants. (a) Positions of tested sections of internodes and petioles. (b) Tissue prints for detection of PVY at 7 days p.i. Locations of the sections are shown by lines labelled (A) to (K). I*, Inoculated leaf. (c) Localization of PVY in cross-sections of stem tissues of plants infected singly or doubly with CMV. Magnified from (b) at positions (A) or (E). (d) Localization of CMV in cross-sections [positions (A) or (E)] of stem tissues of plants infected singly or doubly with PVY at 7 days p.i. Blots from doubly infected *N. tabacum* in (c) and in (d) were from the same plant. EP, External phloem; IP, internal phloem.

external phloem only of systemically infected stem tissues, but PepoΔ2b was detected in external and internal phloem tissues (Fig. 5a). Similar results were obtained for plants inoculated with PepoΔ2b and then with PVY (data not shown).

Virus accumulation in singly and doubly infected *N. tabacum* plants was measured by ELISA. PepoΔ2b accumulation was consistently higher in doubly infected plants than in singly infected plants; however, differences were not significant (Fig. 5c). In contrast, PVY levels in plants doubly infected with PepoΔ2b were significantly lower than in plants doubly infected with PVY and CMV or in singly infected plants (Fig. 5b). These results suggested that the CMV 2b protein is necessary to assist systemic spread of PVY.

DISCUSSION

The PVY T01 isolate used in this study did not fully infect plants of *N. tabacum* cv. Xanthi-nc systemically and was found only in the older and middle leaves. As the virus readily infected *N. benthamiana* systemically, the ability of

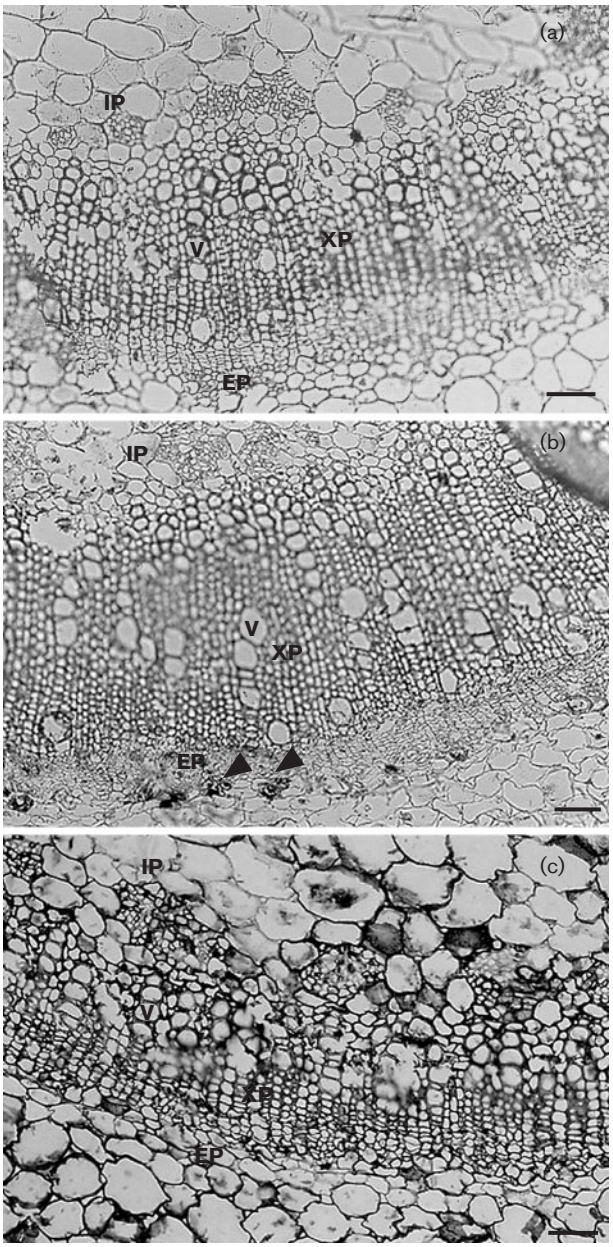

Fig. 4. Immunohistochemical localization of PVY in phloem-associated cells [stem position (E) in Fig. 3a] of *N. tabacum* at 7 days p.i. (a) Mock-inoculated plants. (b) Plants singly infected with PVY. The anti-PVY signal was present in the external phloem only (arrowheads). (c) Plants doubly infected with PVY and with CMV. The anti-PVY signal is present in the external phloem, internal phloem and xylem parenchyma. Dark spots correspond to PVY CP-specific signals. EP, External phloem; IP, internal phloem; XP, xylem parenchyma; V, vessel. Bars, 100 μm.

this PVY isolate for systemic spread appeared to be defective in *N. tabacum* cv. Xanthi-nc. We demonstrated that this restricted spread could be alleviated by infection of PVY T01 together with CMV. However, PVY was transported into the phloem faster in singly infected plants than in doubly infected plants (Table 1). This indicated that systemic spread of PVY was restricted after it entered the phloem tissue of singly infected plants. We found that the amounts of PVY in the inoculated and older leaves were similar in singly infected plants and in plants doubly infected with PVY and CMV (Fig. 2a, I and +3U), but the amounts of PVY in the middle leaves were significantly lower in singly infected plants than in doubly infected plants (Fig. 2a, +5U) and PVY was not detected at all in the younger leaves (Fig. 2a, +7U) in singly infected plants. The anti-PVY signal in tissue prints of stem tissues was always less intense for singly infected plants than for doubly infected plants and no signal was detected in apical tissues in singly infected plants (Fig. 3b). In addition, the distribution of PVY was partially restricted in phloem tissues in singly infected plants (Figs 3c and 4b). Therefore, we considered that the spread of PVY was impeded in singly infected plants as a result of restricted accumulation and transport in phloem tissues. Another possibility is that lower levels of PVY in upper leaves might be due to inhibition of replication or of cell-to-cell movement following unloading from the phloem. However, this is unlikely as PVY levels in the older leaves were similar in singly and doubly infected plants (Fig. 2a, +3U).

Members of the plant family Solanaceae have two phloem tissues – external and internal. The structural differences of these have functional consequences for photoassimilate transport: metabolites descend in the external phloem and ascend in the internal phloem (Turgeon, 1989). Previous studies have indicated that *Pepper mottle virus* (PepMoV) in *Capsicum annuum* and *Tobacco mosaic virus* (TMV) in *N. benthamiana* accumulate first in the external phloem in inoculated leaves and stem tissues below the inoculated leaves, and then accumulate in the internal phloem tissues in systemically infected stem sections or leaves (Andrianifahanana *et al.*, 1997; Cheng *et al.*, 2000). Our results indicate that PVY was transported readily from the inoculated leaves to the external phloem in stem tissues, but had no or little ability to gain access to the internal phloem tissues in singly infected plants. In plants doubly infected with PVY and CMV, invasion of stem tissues by PVY was not restricted to the external phloem and the virus gained access to the xylem parenchyma and internal phloem tissues. Although the nature of the connecting cells between the external and internal phloem is not fully understood, these cells may have an important role in regulating movement of plant viruses. Guerini & Murphy (1999) observed a similar restriction of PepMoV in the external phloem of resistant *C. annuum* cv. Avelar plants and resistance was overcome by dual infection with CMV. In *Tetragonia expansa*, which possesses temperature-sensitive systemic resistance against CMV, systemic transport of virus is considered to be inhibited in the tissue between the external and internal phloem (Kobori *et al.*, 2002, 2003). We also consider that the internal phloem is the pathway for rapid transport of PVY up the stem to the

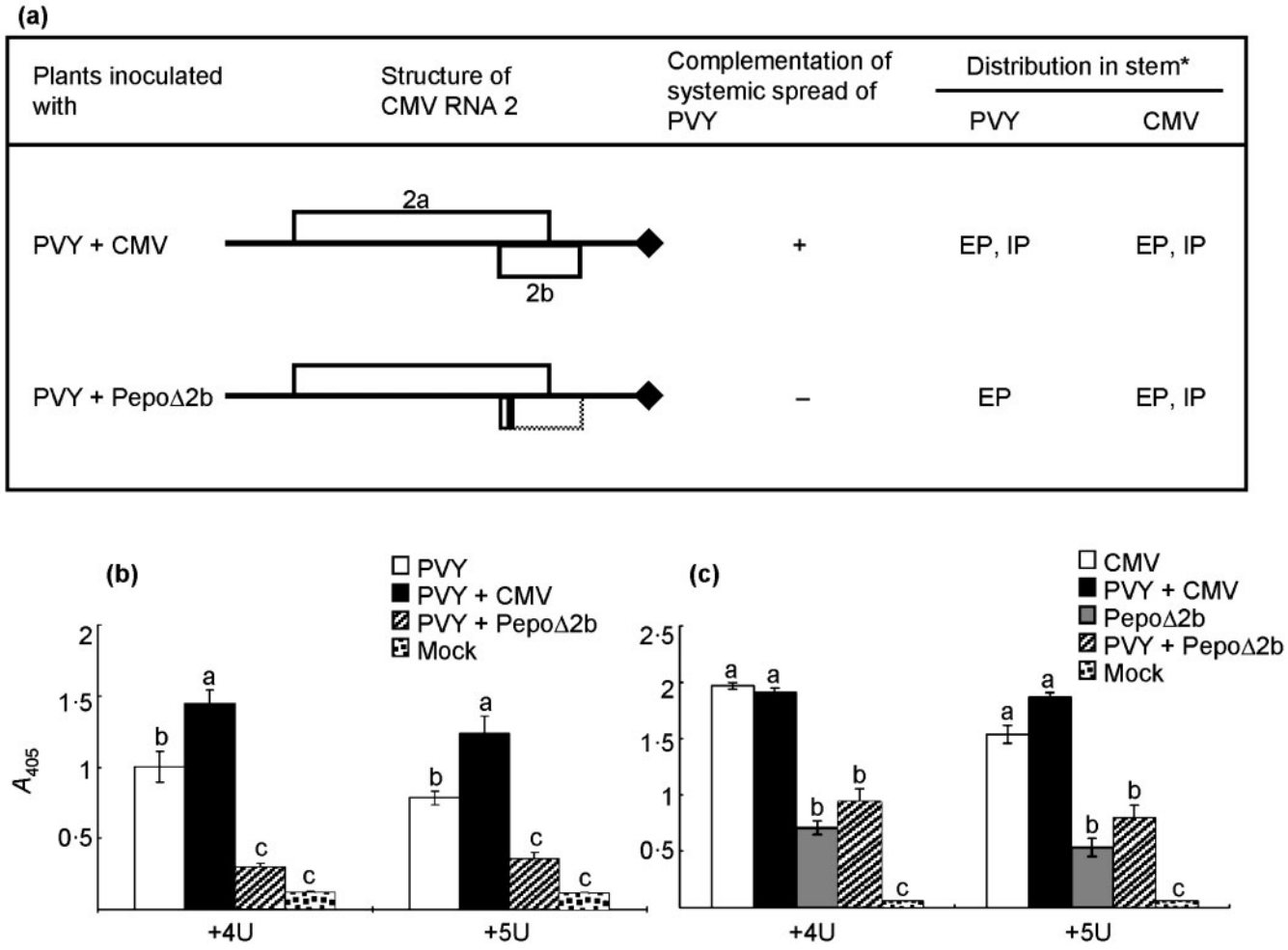

Fig. 5. Systemic spread of PVY in *N. tabacum* plants doubly infected with PVY and CMV or PepoΔ2b. (a) Schematic structure of CMV RNA 2 mutant. Each structure contains either the wild-type 2b ORF of CMV or PepoΔ2b in CMV RNA 2. +, Systemic spread of PVY effectively occurred; –, systemic spread of PVY did not occur fully as in singly infected plants. *The distribution of PVY or CMV was determined by tissue immunoblot analysis of stem sections of *N. tabacum* plants. EP, External phloem; IP, internal phloem. (b) Accumulation of PVY in systemically infected leaves that were infected singly with PVY, or doubly with PVY and CMV or PepoΔ2b. (c) Accumulation of CMV in systemically infected leaves that were infected singly with either CMV or PepoΔ2b, or doubly with PVY and CMV or PepoΔ2b. Accumulation of PVY or CMV was determined by ELISA. Each bar represents the mean ELISA value (A_{405}) obtained from six individual plants. Different letters represent a significant difference of the means at $P=0{\cdot}05$, according to Bonferroni's multiple-range test. Error bars represent SD. Systemically infected leaves were sampled at 15 days p.i. U, Upper infected leaves (+4 and +5 leaves above inoculated leaves).

younger tissues in doubly infected plants. As PVY cannot use this pathway fully, it does not move up the stem to young tissues in singly infected plants.

The mechanism by which CMV alleviates the limitation of PVY systemic spread can be explained by the ability of the CMV 2b protein to suppress host factors that are involved in the defence system, as the 2b protein suppresses RNA silencing (Brigneti *et al.*, 1998). It has already been shown to be important in overcoming the phloem restriction of a luteovirus, which could also be related to RNA silencing (Ryabov *et al.*, 2001). We showed that double infection with PepoΔ2b, a CMV mutant that does not translate the 2b protein, did not assist systemic spread of PVY (Fig. 5b). Another possible mechanism is that PVY may lack a factor for systemic spread in *N. tabacum* and that this is complemented by CMV in doubly infected plants. PepoΔ2b would possibly not complement this defect, as it might not produce a sufficient amount of complementing factor, i.e. protein 1a, 2a, 3a or CP (Taliansky & García-Arenal, 1995); this is because PepoΔ2b accumulates at lower levels than CMV (Fig. 5c). However, this is unlikely. PVY accumulation in plants doubly infected with PepoΔ2b was significantly lower than that in singly infected plants (Fig. 5b). In

the absence of functional 2b protein, other CMV factor(s) may impede PVY multiplication in plants doubly infected with PVY and PepoΔ2b.

RNA silencing is a natural regulatory mechanism in which particular RNAs are targeted and destroyed in a sequence-specific manner (Ratcliff *et al.*, 1997, 1999; Voinnet *et al.*, 1999; Voinnet, 2001; Waterhouse *et al.*, 2001). Ding *et al.* (2003) suggested that plants have evolved a powerful mechanism to traffic selected macromolecules in the symplasmic pathway: systemic transport of a protein or RNA would be regulated at multiple checkpoints, including phloem entry, transport and exit. RNA silencing may be hyperactivated in cells that control access to the phloem (Marathe *et al.*, 2000), especially near and in the internal phloem cells, and may be activated to suppress rapid systemic spread of a virus as an adaptive defence mechanism. The ability of a virus to move within infected tissues has been suggested to depend on its ability to block the systemic signalling that is generated by RNA silencing (Vance & Vaucheret, 2001; Voinnet, 2001; Baulcombe, 2002). CMV 2b protein autonomously enters, and probably translocates through, the phloem tissues (Guo & Ding, 2002). Consistent with these suggestions, we consider that CMV 2b protein acts as a suppressor protein, blocking the systemic, mobile signals that are associated with silencing against PVY. PVY then accumulates in the internal phloem tissue and is subsequently transported rapidly and systemically in doubly infected plants.

PVY isolate T01 readily infected *N. benthamiana* systemically, but not *N. tabacum* cv. Xanthi-nc. One possible mechanism is that the movement factor of PVY T01 may interact with a host factor that is different in *N. benthamiana* and *N. tabacum* cv. Xanthi-nc. Several viruses spread systemically in *N. benthamiana*, but are limited to the inoculated leaves of other hosts. For example, TMV with a mutation in the 30 kDa movement protein and *Odontoglossum ringspot virus* spread systemically in *N. benthamiana*, but not in *N. tabacum* (Hilf & Dawson, 1993; Waigmann *et al.*, 2000). Another possibility is that HC-Pro of PVY T01 may manipulate the suppressor function of systemic spread in *N. benthamiana*, but not in *N. tabacum*. HC-Pro of *Plum pox virus* (PPV) can strongly enhance the pathogenicity of *Potato virus X* in the systemic host of PPV, but it does not intensify the symptoms in *N. tabacum* that is not infected systemically by PPV (Sáenz *et al.*, 2002). HC-Pro is also involved in systemic spread and virulence of potyviruses. This function could be due to the silencing of suppressor activity (Kasschau & Carrington, 2001; Voinnet, 2001).

We conclude that the compensation by CMV strain Pepo of the inhibition of PVY T01 transport in the phloem is related to a restriction of the host defence mechanism by the 2b protein of CMV. However, we did not assess systemic spread of other PVY strains or combinations with other CMV strains. Our present results and more such data should provide important insights to help further exploration of the defence mechanism(s) against plant viruses in phloem tissue.

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