The 2b protein of cucumber mosaic virus is essential for viral infection of the shoot apical meristem and for efficient invasion of leaf primordia in infected tobacco plants

Anurag Sunpapao, Takashi Nakai, Fang Dong, Tomofumi Mochizuki and Satoshi T. Ohki

Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan

It has been reported previously that a 2b protein-defective mutant of the cucumber mosaic virus (CMV) Pepo strain (Δ2b) induces only mild symptoms in systemically infected tobacco plants. To clarify further the role of the 2b protein as an RNA silencing suppressor in mosaic symptom expression during CMV infection, this study monitored the sequential distribution of Δ2b in the shoot meristem and leaf primordia (LP) of inoculated tobacco. Time-course histochemical observations revealed that Δ2b was distributed in the shoot meristem at 7 days post-inoculation (p.i.), but could not invade shoot apical meristem (SAM) and quickly disappeared from the shoot meristem, whereas wild-type (Pepo) transiently appeared in SAM from 4 to 10 days p.i. In LP, Δ2b signals were detected only at 14 and 21 days p.i., whereas dense Pepo signals were observed in LP from 4 to 18 days p.i. Northern blot analysis showed that small interfering RNA (siRNA) derived from Δ2b RNA accumulated earlier in the shoot meristem and LP than that of Pepo. However, a similar amount of siRNA was detected in both Pepo- and Δ2b-infected plants at late time points. Tissue printing analysis of the inoculated leaves indicated that the areas infected by Pepo increased faster than those infected by Δ2b, whereas accumulation of Δ2b in protoplasts was similar to that of Pepo. These findings suggest that the 2b protein of the CMV Pepo strain determines virulence by facilitating the distribution of CMV in the shoot meristem and LP via prevention of RNA silencing and/or acceleration of cell-to-cell movement.

INTRODUCTION

RNA silencing, which is based on sequence-specific degradation of RNA (Ding & Voinnet, 2007), is a key antiviral mechanism in plants (Ratcliff et al., 1997, 1999). To counteract this host defence mechanism, many RNA and DNA plant viruses encode suppressor proteins (Anandalakshmi et al., 1998; Brigneti et al., 1998; Voinnet et al., 1999) that inhibit various steps of the RNA silencing process (Voinnet, 2005). Many viral suppressor proteins have been characterized as viral pathogenicity factors (Brigneti et al., 1998; Voinnet et al., 1999) and as determinants of systemic viral infection (Kasschau & Carrington, 2001; Yelina et al., 2002; Havelda et al., 2003; Deleris et al., 2006; Diaz-Pendon et al., 2007).

The 2b protein of cucumber mosaic virus (CMV) has been shown to be one such suppressor protein (Brigneti et al., 1998). Guo & Ding (2002) demonstrated that 2b restricts the long-distance spread of RNA silencing, and Goto et al. (2007) suggested that 2b interferes with the RNA silencing pathway by binding to small interfering RNA (siRNA). Furthermore, Zhang et al. (2006) showed that 2b interacts directly with Argonaute1, a component of the antiviral RNA-induced silencing complex. Earlier studies suggested that 2b controls systemic viral movement, and a lack of 2b protein was reported to be associated with reduced pathogenicity (Ding et al., 1995a, b). In addition, the 2b protein of CMV strain Fny was found to influence viral movement within leaves and throughout the whole plant (Soards et al., 2002). Finally, Diaz-Pendon et al. (2007) used silencing-defective Arabidopsis ecotypes to demonstrate that the silencing-suppressive activity of 2b is required to establish systemic CMV infection and is an essential contributor to virulence.

In a previous study, we studied a 2b-defective mutant (Δ2b) of the Pepo CMV strain and found that Δ2b induces only mild symptoms in tobacco plants, contrasting with the severe mosaic symptoms of wild-type Pepo (Ryang et al., 2004). Because the expression of mosaic symptoms has been implicated in virus distribution in leaf primordia (LP) of infected plants (Hosokawa et al., 1990), we hypothesized that 2b mediates virus distribution in...
developing tissues, including the shoot apical meristem (SAM) and LP. We showed previously that Pepo could infect SAM cells, but the shoot meristem tissues recovered from the Pepo infection (Mochizuki & Ohki, 2004, 2005). Recently, a requirement for the 16K suppressor protein of tobacco rattle virus (TRV) for SAM invasion by TRV was reported (Martin-Hernández & Baulcombe, 2008). This report supported our hypothesis that 2b facilitates SAM invasion by CMV. However, the distribution of Pepo and Δ2b in these tissues has not yet been clarified. To elucidate the role of 2b in CMV distribution in developing tissues, we compared the sequential distribution of Pepo and Δ2b in the shoot meristem and LP, as well as in inoculated leaves. We found that Pepo 2b is essential for the viral infection of SAM, for the efficient invasion of LP and for efficient cell-to-cell movement in infected tobacco plants.

METHODS

Plant materials, CMV and inoculation. The Pepo strain of CMV (Pepo) was originally obtained from Cucurbita pepo in Japan (Osaki et al., 1973). Δ2b is a modified Pepo strain that lacks translation of an intact 2b protein (Ryang et al., 2004). Five- to seven-leaf-stage tobacco plants (Nicotiana tabacum cv. Xanthi-nc) were inoculated with the viruses. The largest leaf was mechanically inoculated with 50 μl purified Pepo or Δ2b (50 μg ml⁻¹ per plant, and the inoculated plants were grown in a greenhouse at 24–30 °C.

Histochemical observations. Procedures for the preparation of shoot meristem and LP sections from inoculated tobacco plants, immunohistochemistry using anti-CMV IgG and in situ hybridization using a digoxigenin (DIG; Roche Diagnostics)-labelled RNA probe complementary to the conserved 3' untranslated region (3'-UTR) sequence of the four CMV positive-sense RNAs (Saitoh et al., 1999) were conducted using methods described previously (Mochizuki & Ohki, 2004). The stained sections were examined with a BX-50 light microscope (Olympus).

RNA analysis. Total RNA was extracted from inoculated tobacco tissues using TRI Reagent (Sigma) according to the manufacturer’s instructions. For detection of CMV genomic RNAs, 1 μg total RNA was loaded onto a 1.5% denaturing agarose gel. The procedures for blotting onto Hybond-N⁺ membrane (GE Healthcare Biosciences), hybridization with DIG-labelled RNA probes complementary to the conserved 3'-UTR sequence and detection of signals were performed as described previously (Kobori et al., 2002).

For siRNA detection, 20 μg total RNA was dissolved in an equal volume of 100% formamide and loaded onto a 15% acrylamide gel containing 7 M urea. The low-molecular-mass RNAs were transferred onto a Hybond-NX membrane (GE Healthcare Biosciences) by electroblotting. Hybridization was performed using DIG-labelled, full-length CMV RNAs complementary to the CMV negative-sense RNAs transcribed from Pepo CMV clones (Saitoh et al., 1999). The procedures for hybridization and detection of signals were performed as described previously (Mochizuki & Ohki, 2004).

Protein analysis. The procedures for protein extraction, SDS-PAGE, blotting onto nitrocellulose membranes and detection of CMV coat protein (CP) were performed as described previously by Saiga et al. (1998).

Protoplast inoculation. Tobacco leaves peeled at a lower epidermal layer were incubated for 1 h in a solution of 1.2% Cellulase Onozuka RS (Yakult Pharmaceutical), 0.2% Macerozyme (Yakult Pharmaceutical) and 0.6 M mannitol (pH 5.8). The protoplasts were collected and washed three times in 0.6 M mannitol (pH 5.8). Inoculation with purified CMV was carried out as described previously (Mochizuki & Ohki, 2005). The inoculated protoplasts were incubated at 24 °C in the dark. The accumulation of CMV RNA and CP was examined at 10 and 24 h post-inoculation (p.i.) according to the methods described above.

Tissue printing analysis. Leaf tissue samples were brushed with Celite to remove the epidermis. The brushed leaves were pressed directly onto a nitrocellulose membrane (Bio-Rad) that had been treated with 0.2 M CaCl₂ prior to blotting. The membrane was blocked with 3% skimmed milk and 0.05% Tween 20 in Tris-buffered saline (TTBS). CMV CP was detected in tissue prints using a specific antibody. The primary antibody was diluted 1:3000, and an alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody was diluted 1:10000 in TTBS and incubated at 4 °C overnight. Immunoreactive areas were visualized using a colour substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt and nitrotetrazolium blue chloride.

RESULTS

Involvement of 2b protein in efficient CMV distribution in the shoot meristem and LP

To assess the role of 2b protein in CMV infection of the shoot meristem and LP, the sequential distribution of Pepo and Δ2b in these tissues was compared. Five shoot meristem tissue samples and three LP 2–3 mm in length from different plants were sampled at 4, 7, 10, 14, 18 and 21 days p.i. The distribution of Pepo and Δ2b was observed by immunohistochemical microscopy and in situ hybridization.

Fig. 1(a) shows the distribution of Pepo and Δ2b in the shoot meristem. Pepo CP signals were detected in most shoot meristem tissues at 4–10 days p.i., and dense signals were detected within the tunica and corpus zones of SAM cells at 4 and 7 days p.i. The Pepo signals gradually decreased at 14 days p.i., and no signals were detected at 18 days p.i. However, some signals were still detectable in the cells below the SAM and within primordia (Fig. 1a, upper panels). Conversely, Δ2b CP signals were first detected in the shoot meristem at 7 days p.i., but signals were not detected in the SAM. Furthermore, Δ2b signals disappeared in the shoot meristem at 10 days p.i. and were barely detectable in the shoot meristem at 18 days p.i. (Fig. 1a, lower panels). CMV CP signals were not observed in the shoot meristem from uninfected tobacco samples (Fig. 1c). A similar distribution of Δ2b positive-sense viral RNA (vRNA) was observed by in situ hybridization (data not shown).

Fig. 1(b) shows typical images of Pepo and Δ2b distribution in LP from 4 to 21 days p.i. At 4 days p.i., Pepo CP signals were detected in the major veins of LP, and signals were detected in the surrounding tissues at 7 days p.i. By 10–21 days p.i., the Pepo signals had spread throughout the LP, with the strongest signals at 14 days p.i. The Pepo signals gradually decreased from 18 to 21 days
p.i. (Fig. 1b, upper panels). In the Δ2b-inoculated LP, a few Δ2b CP signals were detected at 14 and 21 days p.i. only (Fig. 1b, lower panels, arrows). CMV CP signals were not detectable in the LP from uninfected tobacco samples (Fig. 1d).

**Accumulation of vRNA and siRNA in the shoot meristem and leaf primordia**

Because CMV 2b protein acts as a silencing suppressor, the RNA silencing activity directed against Pepo or Δ2b in developing tissues was assessed by comparing the amounts of siRNA derived from vRNA, which can be used as a general indicator of RNA silencing against the virus (Hamilton & Baulcombe, 1999). After inoculation with Pepo or Δ2b, three shoot meristem tissue samples (3 mm in length) and 36 LP of a similar size were sampled from different plants at 4, 7, 10, 14, 18 and 21 days p.i. The amounts of vRNA and siRNA in the tissues were examined by Northern blot analysis. The experiments were repeated five times.

In shoot meristem, the amount of Pepo vRNA peaked at 7 and 10 days p.i. but decreased at 14 days p.i. By 18–21 days
p.i., the amount of Pepo vRNA was modestly restored (Fig. 2a). The earliest Pepo siRNA accumulation was detected at 7 days p.i., and Pepo siRNA accumulated at a high level until 21 days p.i. (Fig. 2a), whereas CMV distribution in the shoot meristem decreased from 14 to 18 days p.i. (Fig. 1a). These results correlated with the results of a previous study (Mochizuki & Ohki, 2004). In Δ2b-infected shoot meristem, large amounts of Δ2b vRNA were detected at 7 and 10 days p.i., but accumulation decreased from 14 to 18 days p.i. Δ2b siRNA was faintly detected at 4 days p.i., peaked at 7 and 10 days p.i. and was detected consistently from 10 to 21 days p.i. Although the Δ2b CP signals were detected only at 7 days p.i. with histochemical observations (Fig. 1a, lower panels), the accumulation of Δ2b vRNA in the shoot meristem was detected at 7 and 10 days p.i. (Fig. 2a). To confirm the accumulation of Δ2b CP in the shoot meristem, Western blot analysis was conducted. Accumulation of Δ2b CP in the shoot meristem was detected at 4 days p.i. The amount of Δ2b CP peaked at 7 and 10 days p.i. but declined at 14 days p.i. (Fig. 2b), similar to the Northern blot results.

In the LP (Fig. 2c), Pepo vRNA was first detected at 4 days p.i. and was detected at all subsequent time points. Pepo siRNA was detected at low levels at 7 days p.i. and was consistently detected from 10 to 21 days p.i. In the LP of Δ2b-inoculated tobacco at 4 days p.i., low levels of vRNA 3 and 4 were detected. The accumulation of Δ2b vRNA reached a maximum at 10 days p.i. and was consistently detected up to 21 days p.i. However, the amount of Δ2b vRNA was lower than that of Pepo. High levels of Δ2b siRNA was detected at 7 days p.i. in LP and consistently accumulated up to 21 days p.i. (Fig. 2c).

**The 2b protein affects the efficiency of cell-to-cell movement of CMV in inoculated leaves**

We monitored the accumulation of CP and vRNA of Pepo and Δ2b in tobacco using protoplast techniques. Although the level of Pepo vRNA was lower than that of Δ2b at 10 h p.i., the amount of Pepo and Δ2b in the inoculated protoplasts after 24 h was similar (Fig. 3). The level of Δ2b CP was slightly lower than that of Pepo (Fig. 3). On the
other hand, another experiment showed that the level of Δ2b CP was slightly higher than that of Pepo (data not shown). We concluded that the difference between experiments was within the experimental error range and that the 2b protein of Pepo has little effect on CMV replication at the single-cell level.

Next, the efficiency of cell-to-cell movement of Pepo and Δ2b was assessed by tissue printing analysis. The largest leaves of each tobacco plant were inoculated mechanically using cotton swabs to apply Pepo or Δ2b at 100 μg ml⁻¹ in 0.5 cm diameter spots. The spread of CMV infection from the spot-inoculated area was observed at 2, 4 and 6 days p.i. for Pepo and at 4, 6 and 8 days p.i. for Δ2b. Pepo signals were detected around the inoculated points at 2 days p.i., and at 4 days p.i. the virus had colonized within the inoculated points and surrounding tissues. Finally, dense Pepo signals were detected in most of the leaf at 6 days p.i. (Fig. 4, upper panels). In contrast, Δ2b signals were first detected at 6 days p.i., which is later than those for Pepo, and more signals were detected around the inoculated spot at 8 days p.i. (Fig. 4, lower panels). CMV CP signals were not observed in uninfected tobacco leaves (Fig. 4, Healthy).

DISCUSSION

To clarify the role of the 2b suppressor protein in the establishment of systemic infection by CMV, we compared the sequential distribution of Pepo and Δ2b in developing tissues. Histochemical observations revealed that the 2b protein is essential for SAM invasion by CMV (Fig. 1a) and for efficient viral infection of LP (Fig. 1b). Northern blot analysis showed that siRNA from Δ2b genomic RNA accumulated earlier than that of Pepo in these tissues (Fig. 2). We also showed by tissue printing analysis that the 2b protein facilitated cell-to-cell movement of CMV (Fig. 4).

SAM in plants is known to be virus-free due to its capacity to evade infection (Hull, 2002). It has been reported that an RNA-mediated surveillance system regulates RNA signaling and protects the shoot apex from viral invasion (Foster et al., 2002). RNA silencing also has been suggested to be an inhibitory mechanism of SAM against virus infection. The RNA-dependent RNA polymerase (RDR6), which is an integral part of the RNA silencing machinery, is involved in the exclusion of virus from the apical growing points in Nicotiana benthamiana inoculated with potato virus X (PVX) or tobacco mosaic virus (TMV) (Qu et al., 2005; Schwach et al., 2005). Conversely, some viruses, including the CMV Pepo strain used in our study, have been found in the SAM of wild-type host plants (Sheffield, 1942; Walkey & Webb 1968; Roberts et al., 1970; Lin & Langenberg, 1984; Mochizuki & Ohki, 2004). Our results showed that lack of the 2b suppressor protein leads to a deficiency of SAM invasion by CMV and an early reduction of CMV infection in shoot meristem (Fig. 1a). TRV also transiently invades SAM cells, and the 16K suppressor protein of TRV is required for SAM invasion by TRV (Martín-Hernández & Baulcombe, 2008). In contrast to TMV and PVX, TRV transient accumulation in SAM is unaffected by down-regulation of RDR6. Similar to the 16K suppressor protein of TRV, the 2b suppressor of CMV was necessary for SAM invasion, suggesting that suppression of RNA silencing by the 2b protein is required for transient CMV infection of SAM cells; however, we did not determine whether RDR6 was involved in anti-CMV RNA silencing in the shoot meristem.

Our histochemical observations demonstrated that efficient distribution of Δ2b in the developing tissues was inhibited by lack of the 2b protein (Fig. 1a, b). However, distinct accumulation of Δ2b vRNA was detectable from 7 to 18 days p.i. in the shoot meristem and LP by Northern blot analysis. Although it is unclear why a large amount of Δ2b vRNA was detectable in these tissues, one explanation is that Northern blot analysis might detect vRNA in the phloem solution in sieve elements of vascular systems, whereas histochemical microscopy would not detect the virus in the sieve elements because of the difficulty of fixing the materials in the liquids in sieve elements. We hypothesize that Δ2b particles can move a long distance through the phloem solution but that unloading from the vascular cells might be inhibited. The requirement for a
suppressor protein for long-distance viral transport in specific tissues has been reported. Havelda et al. (2003) reported that distribution of p19-defective *Cymbidium* ringspot virus was restricted in vascular tissues in systemic leaves of inoculated *N. benthamiana*. Furthermore, Deleris et al. (2006) showed that P38-deficient turnip crinkle virus (TCV) did not unload from vascular tissues, and the dsRNA-specific RNase Dicer-like protein 4 helped prevent the vascular exit of the TCV mutant, implicating RNA silencing as a key control mechanism of viral long-distance transport across a specific boundary. These findings support our hypothesis that the 2b protein interferes with RNA silencing in the vascular cells of developing tissues to establish CMV infection. Indeed, we found that a large amount of Δ2b siRNA accumulated at 7 days p.i. in the shoot meristem and LP, which was faster than the accumulation of Pepo siRNA (Fig. 2a, c). In addition, abundant Δ2b siRNA accumulated even when the amount of Δ2b vRNA was less (Fig. 2a, 14 and 18 days p.i. for Δ2b). In contrast, Pepo siRNA levels consistently paralleled the accumulation of Pepo vRNA (e.g. Fig. 2a, 14 days p.i., less vRNA correlated with less siRNA). The Δ2b vRNA unloaded from the phloem solution may have degraded immediately in the vascular cells.

Tissue printing analysis of the inoculated leaves provided evidence that the 2b protein of Pepo was involved in the efficiency of cell-to-cell movement (Fig. 4). It is possible that the 2b protein of Pepo encourages CMV spread in the shoot meristem and LP by facilitating cell-to-cell movement. Soards et al. (2002) reported that the diameter of the infection site in leaves caused by a Δ2b version of the Fny strain [Fny Δ2b expressing green fluorescent protein (GFP)] was similar to that caused by wild-type Fny–GFP. However, the ratio of infected epidermal cells to infected mesophyll cells differed between Fny Δ2b and Fny–GFP. The authors suggested that the 2b protein plays a role in tissue- or cell-specific cell-to-cell movement of CMV. They also reported that Fny Δ2b was less able to spread systemically than the wild-type virus and that it accumulated at lower levels in inoculated and upper uninoculated tissues. These results are similar to our result that Pepo Δ2b infection in uninoculated SAM and primordia was restricted.

We conclude that the 2b protein of Pepo is essential for efficient invasion of the SAM and LP of infected tobacco plants. Following Pepo infection, some fully expanded tobacco leaves display mosaicism, whereas other leaves recover and are symptomless (Ohki et al., 1990), known as cycling symptom expression. There is some evidence to indicate that uninfected cells appear developmentally in the LP, that Pepo is distributed over almost the whole area of the leaf (e.g. LP of Pepo at 10 and 14 days p.i. in Fig. 1b) and that this is followed by the development of mosaic leaves (F. Dong and others, unpublished data). The uninfected LP (Fig. 1b, Pepo at 21 days p.i.) might develop as symptomless recovered leaves. Δ2b invaded the LP only weakly (Fig. 1b, lower panels), which suggests that reduced infection of LP by Δ2b results in attenuation of Δ2b. Diaz-Pendon et al. (2007) reported that the 2b protein was dispensable for inducing disease symptoms. However, the silencing suppressor activity of 2b was required for the establishment of CMV infection, indicating an indirect role of 2b in virulence (Diaz-Pendon et al., 2007). Our results also suggest that the RNA silencing function of the 2b protein might indirectly promote mosaic symptom development by affecting the kinetics of viral distribution in developing tissues and/or by facilitating cell-to-cell movement.

**REFERENCES**


