Subcellular localization of the 28 kDa protein of the triple-gene-block of bamboo mosaic potexvirus

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Open reading frame 2 of the bamboo mosaic potexvirus (BaMV) genome encodes a 28 kDa protein, the first of the ‘triple-gene-block’ of BaMV which is believed to play a role in cell-to-cell movement of the virus in host plants. The 28 kDa protein was expressed in Escherichia coli and polyclonal antiserum was raised in a rabbit. Western blot analyses showed that the 28 kDa protein was associated mainly with components in the cell wall and 30000 g pellet fractions of a BaMV-infected leaf homogenate. Immunogold electron microscopy of infected leaf tissues revealed that the 28 kDa protein was associated with electron-dense crystal-line bodies (EDCBs) in the cytoplasm and nuclei. Nuclear EDCBs were found closely associated with nucleoli. Gold-labelled EDCB-like structures were also detected in the cytoplasm, but not within nuclei, in protoplasts up to 48 h post-inoculation. No specific labelling of the 28 kDa protein was found within any cytoplasmic structures or within cell walls.

Bamboo mosaic potexvirus (BaMV), which primarily infects members of the Bambusoideae, has a single-stranded positive-sense RNA genome that encodes five major open reading frames (ORFs) (Lin et al., 1994). Proteins encoded by BaMV ORFs 2, 3 and 4 share features with the products of the corresponding ‘triple-gene-block’ (TGB) of other potexviruses. Mutational analysis of the genome of white clover mosaic potexvirus (WCIMV) has revealed the requirement of the TGB for systemic spread of the virus in host plants (Beck et al., 1991). The potexvirus TGB has counterparts in the genomes of other viruses such as carla-, some furo-, and hordeiviruses, the products of which are also essential for virus movement (Rupasov et al., 1989; Gilmer et al., 1992; Petty & Jackson, 1990). However, it remains unclear what role each of the proteins plays in virus movement.

The cell-to-cell movement of plant viruses seems to occur by passage through plasmodesmata, facilitated by viral protein (Maule, 1991; Deom et al., 1992). The P30 protein of tobacco mosaic virus (TMV) induces an increase in the gating limit of plasmodesmata (Wolf et al., 1989) and possesses non-specific single-stranded RNA-binding activity (Citovsky et al., 1990, 1992). The movement proteins of comoviruses (Shanks et al., 1989; Van Lent et al., 1990), caulimoviruses (Linstead et al., 1988) and nepoviruses (Wieczorek & Sanfacon, 1993) form tubular structures extending from plasmodesmata in which virus particles have been detected. Different from both of the above, proteins encoded by ORF2 of potato virus X (PVX) and foxtail mosaic potexvirus (FMV) are associated predominantly with cytoplasmic inclusions (Davies et al., 1993; Rouleau et al., 1994). No such proteins were detected in plasmodesmata or in tubular structures.

The 28 kDa protein encoded by ORF2 of BaMV has high amino acid sequence similarity with both the 26 kDa and 25 kDa homologues of FMV and PVX, respectively (Lin et al., 1994). The 25 kDa protein of PVX was detected mainly in the insoluble P1 and P30 fractions of infected tissue homogenates (Davies et al., 1993). The 26 kDa protein of FMV was found predominantly in the soluble fraction (S30) (Rouleau et al., 1994). In the present study, we have determined the subcellular localization of the 28 kDa protein in BaMV-infected tissues.

Two BaMV isolates, BaMV-O (Lin et al., 1994) and BaMV-S were used; the latter is a mutant derived from BaMV-O. BaMV-S showed a better replication rate and faster systemic movement in Nicotiana benthamiana than BaMV-O (Y.-H. Hsu, unpublished). ORF2 cDNA of BaMV-O was amplified from plasmid pBL29 (Lin et al., 1994) by PCR and cloned into pET8c to form pJP1. Escherichia coli BL21 (DE3) harbouring pJP1 was used to overproduce the 28 kDa protein according to the method of Chang & Doi (1990). To partially purify the 28 kDa protein, harvested cells were sonicated in lysis buffer (10 mM Tris–HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 10% glycerol) and centrifuged at 10000 g for 5 min at 4 °C. The overexpressed 28 kDa protein in the pellet was washed several times with lysis buffer containing 1 M...
C. quinoa protein. Leaf homogenates from infected BaMV-S (Fig. 1a) were fractionated into cell wall fraction (CW) from the filtrate. The latter was subjected to differential centrifugation to obtain a 1000 g pellet (P1), a 30,000 g pellet (P30) and a supernatant (S30) fraction. The CW fraction was resuspended in homogenization buffer containing 1% Triton X-100, stirred for 60 min at 4 °C and filtered through a cellulose filter to separate proteins which were loosely bound to the cell debris (Scw) and those that were insoluble and precipitated in the cell wall fraction (Pcw). Each fraction was analysed immunologically for the presence of the 28 kDa protein. The 28 kDa protein was detected mainly in the CW fraction of the BaMV-S-infected leaf homogenate of C. quinoa (Fig. 1b) and in CW and P30 fractions of N. benthamiana (Fig. 1c). Some of the 28 kDa protein in the CW fraction was soluble in homogenization buffer containing 1% Triton (Fig. 1b, c, lane Scw). Only trace amounts of the 28 kDa protein could be observed in P1 and S30 fractions. The presence of 28 kDa protein in CW and P30 fractions suggests that the cell wall and subcellular organelles are the major compartments for the protein. Alternatively, the presence of this protein in the cell wall fraction may imply that most of the 28 kDa protein is in aggregates which are either retained on the cellulose filter during filtration, or sedimented during centrifugation due to low solubility. The presence of BaMV 28 kDa protein in the insoluble fractions of leaf homogenates of systemic and local lesion hosts is different from the corresponding ORF2 products of both PVX and FMV (Davies et al., 1993; Rouleau et al., 1994). It seems that the BaMV 28 kDa protein is less soluble than its PVX and FMV counterparts. This is consistent with the fact that little of the overexpressed 28 kDa protein was soluble in a buffer in which the 26 kDa protein of FMV could be dissolved (Rouleau et al., 1994).

For immunoelectron microscopy, BaMV-O-infected green bamboo (Bambusa oldhamii) and C. quinoa leaves, and barley protoplasts were processed as previously described (Lin & Chen, 1991), except that protoplasts were first embedded in 1% low-melting-point agarose in culture medium before processing. The isolation and inoculation of barley protoplasts with BaMV-O RNA were performed as previously described (Lin et al., 1992). Ultra-thin sections were treated with 200-fold diluted rabbit anti-28 kDa protein serum or anti-BaMV capsid protein serum, followed by treatment with gold-labelled goat anti-rabbit IgG (Lin & Chen, 1991).

BaMV induced the formation of a large number of electron-dense crystalline bodies (EDCBs), besides virus aggregates, in the cytoplasm or nuclei of infected cells of fully expanded bamboo leaves. The EDCBs were labelled with anti-28 kDa protein serum followed by immunogold (Fig. 2a). Very often, the nuclear EDCBs (with immunogold label) were associated with the nucleoli (Fig. 2a, b). Of the 29 nuclei in which both

NaCl and electro-eluted after SDS-PAGE in a 12% gel. Part of the purified protein was transferred to a PVDF membrane and the N-terminal 10 amino acids were determined to be MDNRTIDLLT (ABI model-476 amino acid sequencer), the same as those predicted for the 28 kDa protein encoded by ORF2 of BaMV-O (Lin et al., 1994). Antiserum against gel-purified 28 kDa protein was raised in a New Zealand white male rabbit. The antiserum reacted specifically with a single protein in Chenopodium quinoa leaves infected with either BaMV-S (Fig. 1a, lane 2) or BaMV-O (data not shown). No such protein was detected in uninfected plant tissue (Fig. 1a, lane 1).

Procedures for subcellular fractionation of the 28 kDa proteinin plant tissue were modified from a previous report by Rouleau et al. (1994). BaMV-S-infected N. benthamiana or C. quinoa leaves were collected 6 days post-inoculation (p.i.). The leaf homogenate was filtered through a cellulose filter (Schleicher and Schuell, no. 334151) to separate the insoluble cell wall fraction (CW) from the filtrate. The latter was subjected to differential centrifugation to obtain a 1000 g pellet (P1), a 30,000 g pellet (P30) and a supernatant (S30) fraction. The CW fraction was resuspended in homogenization buffer containing 1% (v/v) Triton X-100, stirred for 60 min at 4 °C and filtered through a cellulose filter to separate proteins which were loosely bound to the cell debris (Scw) and those that were insoluble and precipitated in the cell wall fraction (Pcw). Each fraction was analysed immunologically for the presence of the 28 kDa protein. The 28 kDa protein was detected mainly in the CW fraction of the BaMV-S-infected leaf homogenate of C. quinoa (Fig. 1b) and in CW and P30 fractions of N. benthamiana (Fig. 1c). Some of the 28 kDa protein in the CW fraction was soluble in homogenization buffer containing 1% Triton (Fig. 1b, c, lane Scw). Only trace amounts of the 28 kDa protein could be observed in P1 and S30 fractions. The presence of 28 kDa protein in CW and P30 fractions suggests that the cell wall and subcellular organelles are the major compartments for the protein. Alternatively, the presence of this protein in the cell wall fraction may imply that most of the 28 kDa protein is in aggregates which are either retained on the cellulose filter during filtration, or sedimented during centrifugation due to low solubility. The presence of BaMV 28 kDa protein in the insoluble fractions of leaf homogenates of systemic and local lesion hosts is different from the corresponding ORF2 products of both PVX and FMV (Davies et al., 1993; Rouleau et al., 1994). It seems that the BaMV 28 kDa protein is less soluble than its PVX and FMV counterparts. This is consistent with the fact that little of the overexpressed 28 kDa protein was soluble in a buffer in which the 26 kDa protein of FMV could be dissolved (Rouleau et al., 1994).

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Fig. 2. Thin sections of expanded (a, b and c) or rolled young (d) leaves of green bamboo (B. oldhamii) infected with BaMV-O, fixed, embedded and treated with anti-BaMV 28 kDa protein serum (a, b and d) or anti-BaMV capsid protein serum (c), followed by gold-labelled goat anti-rabbit IgG. Abbreviations used: C, BaMV-specific electron-dense crystalline bodies (EDCBs); Ch, chloroplast; Cy, cytoplasm; CW, cell wall; Nu, nucleus; Nuo, nucleolus; St, starch grain; V, virions; Va, vacuole. Bars represent 500 nm (a) and 200 nm (b, c and d), respectively.
EDCBs and nucleoli were clearly visible, 24 had their nucleoli associated with EDCBs. Most label for the 28 kDa protein was found in association with EDCBs and no label was found within any of the cytoplasmic structures or within the cell wall (Fig. 2a). BaMV particles either aggregated in the cytoplasm (Fig. 2a) or associated with cytoplasmic EDCBs were not labelled with anti-28 kDa protein serum. On the other hand, they were labelled with anti-BaMV capsid protein serum (Fig. 2c). Neither antiserum labelled uninfected cells adjacent to the infected one of the same section or cells from uninfected leaves (data not shown). Antisera pre-absorbed with healthy plant proteins or without pre-absorption gave similar results.

The cytopathological effects, such as virus aggregates and formation of EDCBs, were also observed in most infected cells from rolled young bamboo leaves. However, EDCB-like bodies of varying electron density and irregular morphology were observed both in the nuclei and cytoplasm of early infected cells. They were also labelled with anti-28 kDa protein serum (Fig. 2d).

Immunoelectron microscopy of BaMV-O-inoculated C. quinoa leaves 7 days p.i. revealed essentially similar 28 kDa protein-labelling results as those observed in infected bamboo cells (data not shown). However, for barley protoplasts, inclusion bodies labelled with anti-28 kDa protein serum began to appear in the cytoplasm 24 h p.i. (Fig. 3a). EDCB-like structures were observed in the cytoplasm of protoplasts harvested 48 h p.i. (Fig. 3b). No 28 kDa protein or EDCB-like structures were detected within nuclei in protoplast samples up to 48 h p.i.

The BaMV '28 kDa inclusions' appear structurally distinct from the lamellar inclusions or the intertwined fine filaments, with which the 25 kDa ORF2 of PVX and the 26 kDa ORF2 of FMV are associated, respectively (Davies et al., 1993; Rouleau et al., 1994). The EDCBs induced by BaMV-infection are unique. Since they occur in several hosts besides the natural host, bamboo, and have been reported by different laboratories (Kitajima et al., 1977; Lin & Chen, 1991), it is unlikely that they are artefacts. The 28 kDa protein was associated with EDCBs occurring in both the cytoplasm and nuclei of infected cells (Fig. 2). Similarly, the 25 kDa lamellar inclusion structures of PVX had both cytoplasmic and nuclear locations (Davies et al., 1993), whereas the 26 kDa inclusions of FMV were detected only in the cytoplasm and never within the nuclei (Rouleau et al., 1994).

The proximity of virus particle aggregates to the ORF2 homologue-associated cytoplasmic inclusions in PVX-, FMV- (Davies et al., 1993; Rouleau et al., 1994) and now BaMV-infected tissues (Fig. 2) raises the possibility that these inclusions are the active pools of ORF2 proteins. The ORF2 protein inclusions might be sites where processing reactions required for virus transport take place. This hypothesis is supported by the fact that BaMV RNA was detected within EDCBs by in situ hybridization using a BaMV genomic RNA 3'-end-specific probe (Lin et al., 1993). RNA-binding activity
has been observed for the insoluble aggregates of cauliflower mosaic caulimovirus P1 protein (Thomas & Maule, 1995) and the 66 kDa cytoplasmic inclusion of tamarillo mosaic potyvirus, which also possesses NTPase and RNA-helicase activities (Eagles et al., 1994). If this is also true for the ORF2 products of potexviruses, the apparent abundance of these proteins in inclusions in infected tissue would be more reasonable. However, it may also be possible that the EDCBs we observed, as well as those for PVX and FMV, are repositories for excess amounts of ORF2 products in infected plants, as suggested by Citovsky et al. (1993). Therefore, the location of 28 kDa protein does not necessarily reflect the area in which it has a function.

Recently, the cell-to-cell movement of PVX has been shown to be associated with an increase in the size-exclusion limit of plasmodesmata (Angell et al., 1996). The 25 kDa protein in the lamellar inclusions of PVX-infected tissue may be an indirect factor responsible for plasmodesmatal modification. Our observation of the high frequency of association of the EDCBs with nucleoli, which has not been reported previously, suggests indirect and other functions for the 28 kDa protein of BaMV.

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References


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