The 3a protein from cucumber mosaic virus increases the gating capacity of plasmodesmata in transgenic tobacco plants

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The 3a protein, encoded by RNA 3 of cucumber mosaic virus (CMV), is the putative movement protein of viral progeny in infected plants. An analysis of transgenic tobacco plants constitutively expressing the CMV 3a protein showed that the protein is accumulated in leaves at every stage of development. In fully expanded leaves the protein is immunodetectable mostly in a cell-wall-enriched fraction. Dye-coupling experiments using fluorescent-dextran probes were performed on fully expanded leaves to study the modifying effect of CMV 3a protein on the gating capacity of plasmodesmata. Movement of fluorescein-isothiocyanate-labelled dextran with a mean molecular mass of 10000 Da, and an approximate Stokes' radius of 2.3 nm, was detected between cells of the 3a protein transgenic plants, but not in the control plants. These results are consistent with the idea that the CMV 3a protein is involved in the modification of plasmodesmata and, therefore, in the cell-to-cell spread of the virus.

Plant viruses move from cell-to-cell via plasmodesmata, dynamic channels that extend through cell walls and provide cytoplasmic continuity between adjacent cells. This cytoplasmic continuity is not uniform but rather is restricted to symplastic domains, suggesting that not all plasmodesmata function in the same manner (Robards & Lucas, 1990). Whether the extent of viral spread is affected by the presence of these symplastic domains is presently unknown.

The size exclusion limit of plasmodesmatal transport is greatly exceeded by the diameter of virus particles or even of folded viral nucleic acids. So, some modification of plasmodesmatal gating is required for cell-to-cell movement of plant viruses to occur. This is an active process seemingly mediated by virally encoded movement proteins (MP), which have been detected in the cell-wall fractions of virus-infected and MP-expressing transgenic plants (reviewed in: Hull, 1989; Maule, 1991; Deom et al., 1992; McLean et al., 1993). Electron microscopy has revealed structural modifications in the form of tubular extensions from plasmodesmata in plants infected by different members of the comovirus and caulimovirus groups, which otherwise move as viral particles (reviewed in Hull, 1989). Recently, it has been reported that the MPs of cauliflower mosaic virus (CaMV) (Perbal et al., 1993) and cowpea mosaic virus (CPMV) (Wellink et al., 1993) are responsible for inducing the tubular structures. Although tobacco mosaic virus (TMV) and alfalfa mosaic virus (AMV) MPs do not induce such marked structural modifications of plasmodesmata, it has been shown by fluorescence-dye-coupling experiments that these MPs are able to increase the plasmodesmatal size exclusion limit in transgenic plants (Wolf et al., 1989; Poirson et al., 1993).

In the case of the cucumovirus group, it is not known whether cell-to-cell movement takes place as viral particles, or as nucleoproteins as has been proposed for TMV and AlMV (reviewed in: Hull, 1989; van der Kuyl et al., 1991). The presence of virion-like particles of cucumber mosaic virus (CMV) and the related tomato aspermy virus (TAV) has been reported in the secondary plasmodesmata of infected cells, with otherwise non-obvious structural modifications (Lawson & Hearon, 1970; Martelli & Russo, 1985).

The genome of CMV, the type member of the group, consists of three single-stranded RNAs of messenger polarity. RNAs 1 and 2 encode proteins 1a and 2a, which are involved in viral replication. RNA 3 encodes the 3a protein and the coat protein (CP). The CP is translated
from the subgenomic RNA 4 (reviewed in Palukaitis et al., 1992). Results obtained using deletion and insertion mutants transcribed from cDNA clones of RNA 3 suggest that the 3a protein is involved in the cell-to-cell movement of the virus, although it is not clear if the CP is also involved in this mechanism since a functional CP is required for efficient RNA replication and/or accumulation (Suzuki et al., 1991; Boccard & Baulcombe, 1993).

To gain further insight into the mechanism governing the cell-to-cell movement of CMV, we have analysed the distribution and subcellular localization of the putative CMV MP in transgenic tobacco plants expressing the CMV 3a protein, and used dye-coupling experiments to study its effects on the gating capacity of plasmodesmata.

Leaf discs of *Nicotiana tabacum* L. cv. Xanthi nica plants were transformed using standard methods (Horsch et al., 1985) with *Agrobacterium tumefaciens* LBA 4404 harbouring the recombinant plasmid pGAMP 3a (Fig. 1), which contains the 3a gene from CMV-24 (Garcia-Luque et al., 1983) as well as 86 nucleotides of the 5' leader sequence and 89 nucleotides from the 3' intergenic region of RNA 3. The gene was cloned into the SacI site of a modified plant expression vector, pMJD 82 (kindly donated by Dr M. Dowson Day), between an enhanced CaMV 35S promoter flanked with the TMV omega leader sequence, and the NOS terminator sequence. For our purposes, the *NcoI* site present in the multicloning site of pMJD 82 was eliminated to avoid interference with the CMV 3a gene initiation codon, and an *EcoRI* site was created at the 5' side of the expression cassette.

To construct pGAMP 3a, the *EcoRI* fragment of the resultant plasmid was introduced into pGUS, a binary plasmid derived from plasmid pGA 492 (Ann, 1986) in which the GUS gene and the promoter and terminator flanking sequences from plasmid pBI 221.1 (Clontech) had been introduced previously. The recombinant binary plasmid pGAMP 3a was transferred to *A. tumefaciens* LBA 4404 by direct transformation.

The amino acid sequence of the CMV-24 3a protein, as deduced from the nucleotide sequence, is identical to that from CMV-Fny (Owen et al., 1990). The accumulation of the 3a protein in leaf tissue from transgenic plants was assessed by Western immunoblot hybridization, using a polyclonal rabbit antiserum raised against purified *E. coli*-expressed CMV-24 3a protein (unpublished results). For these analyses, total plant protein extracts were obtained as described by Erny et al. (1992), and subcellular fractionation was done essentially as described by Godefroy-Colborn et al. (1986).

Briefly, leaves were powdered under liquid nitrogen and lysed in 2 ml of grinding buffer (GB: 10 mM-KCl, 5 mM-MgCl₂, 0.4 mM-sucrose, 10% glycerol and 10 mM-2-mercaptoethanol in 100 mM-Tris–HCl buffer, pH 7.5) per gram of tissue. The slurry was filtered through Miracloth (Calbiochem) by centrifugation at 1000 g. The filtrate was centrifuged at 1000 g to give the 1000 g pellet, and the supernatant was further centrifuged at 30000 g to give a 30000 g pellet and the supernatant (S fraction). After two washes with GB buffer, the Miracloth residue was extracted twice with GB buffer containing 2% Triton X-100 (GBT) and filtered as above. The resulting filtration residue is referred to as the cell-wall (CW) fraction, and the combined Triton-soluble filtrates correspond to the CWT fraction. Both the 1000 g and 30000 g pellets were washed twice with GBT, and further fractionated by centrifugation to give the pellet fractions (P1 and P30, respectively), and the corresponding supernatant fractions (P1T and P30 T, respectively). The proteins present in the S, CWT, P1T and P30T fractions were precipitated with 4 vol of acetone, and recovered by centrifugation. Each of the subcellular fractions thus obtained were resuspended in 1 ml of electrophoresis sample buffer (ESB: 4.5% SDS, 9 M-urea and 7.5% 2-mercaptoethanol in 75 mM-Tris–HCl, pH 6.8) per gram of tissue, and heated at 95 ºC for 10 min, prior to being analysed. The CWT and CWT fractions were incubated at 37 ºC for 30 min, and then at 95 ºC. By these means, three independent transgenic lines (lines 43, 44 and 78).
or even higher than that present in systematically infected leaves during CMV infection (Fig. 2). Subcellular localization of the 3a protein was assessed in young and mature leaves by subcellular fractionation and western immunoblot hybridization. Fig. 3(b) shows that the 3a protein was mainly located in the cell-wall-enriched fraction of mature leaves. In young leaves (Fig. 3a), a significant amount of the 3a protein was also detected in a crude membrane/organellar fraction and a soluble fraction, but it disappeared from these fractions as the leaves continued to age. The dependence of the subcellular distribution of the 3a protein upon the developmental stage of the tissue resembles more closely the situation in transgenic plants for the TMV MP (Deom et al., 1990) than that for AlMV MP, which is mostly found in the cell-wall fraction (Enny et al., 1992). It remains to be determined whether the differences observed are due to differential modifications of the protein or to different mechanisms of action.

For dye-coupling experiments, we selected plants from lines 43, 44 and 78, all of which show high expression levels of 3a protein. As controls we used transformed plants with vector pGUS. Mature leaves excised from plants at the 18 to 20 leaf stage were selected for microinjection which was performed as described previously (Poirson et al., 1993). Saturated aqueous solutions of fluorescein-isothiocyanate-labelled dextrans (F-dextrans) with average molecular masses of 4400 Da and 10000 Da (Sigma) were used. Dye integrity was checked by thin-layer chromatography. A blue-fluorescing probe, 8-methoxyxylene-1,3,6-trisulfonic acid, trisodium salt (MPTS; Molecular Probes Inc.), with a molecular mass of 538 Da, was used as an internal control. This small molecule, like its analogue cascade blue (Derrick et al., 1990), does not diffuse through membranes but moves freely through plasmodesmata, thus differentiating between cytoplasmic and vacuolar injections. Each F-dextran dye was mixed equally with MPTS prior to injection. Leaves to be injected were attached, abaxial side up, to a Petri dish and covered with 0.3 M-sorbitol. Probes were injected into leaf trichome cells and the movement of dyes was observed using a Nikon Microphot SA microscope equipped for epifluorescence.

Microinjections were only scored when the MPTS was observed to move to the adjacent cell, indicating that the injection had entered the cytoplasmic compartment. Several individual plants for each transgenic line were tested, with at least 12 cytoplasmic microinjections being scored for each line. The score was taken as positive when the MPTS and the F-dextran probes both moved from the injected cell to the adjacent one in the trichome within 4 min.

The results of the dye-coupling experiment are summarized in Table 1. In control plants, no movement

![Fig. 2. Detection of CMV 3a protein in transgenic and CMV-infected tobacco plants. Whole-leaf protein extracts equivalent to 10 mg of tissue were separated on an SDS-13% polyacrylamide gel and blotted onto nitrocellulose. Samples correspond to mature leaves from two different tobacco plants from transgenic lines 43 (lanes 1 and 2), 44 (lanes 3 and 4) and 78 (lanes 5 and 6), and from non-transgenic tobacco plants systemically infected with CMV-24 at 4 weeks post-inoculation (lane 7), and mock-inoculated tobacco plants (lane 8). The Western blot was incubated with anti-3a serum and goat anti-rabbit immunoglobulin G-peroxidase. Molecular mass markers are indicated.](image)

![Fig. 3. Western immunoblot detection of 3a protein in subcellular fractions obtained from young (a) and fully expanded (b) leaves of transgenic tobacco plants (line 76). An amount of extract corresponding to 10 mg of tissue was analysed for each fraction. Samples CW, P1 and P30 correspond to the Triton-insoluble fractions: CW, cell-wall-enriched fraction; P1, 1000 g pellet; P30, 30000 g pellet. CWT, P1T and P30T indicate the corresponding Triton X-100-soluble fractions. S is the 30000 g supernatant.](image)
Table 1. Mobility of fluorescent probes through the symplastic domain of leaf trichome cells of transformed Nicotiana tabacum L. cv. Xanthi ne plants*

<table>
<thead>
<tr>
<th>Probe</th>
<th>Line 43</th>
<th>Line 44</th>
<th>Line 78</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-dextran 4400 Da</td>
<td>100% (15)</td>
<td>95% (25)</td>
<td>100% (14)</td>
<td>12% (32)</td>
</tr>
<tr>
<td>F-dextran 10000 Da</td>
<td>33% (14)</td>
<td>30% (18)</td>
<td>45% (12)</td>
<td>0% (22)</td>
</tr>
</tbody>
</table>

* Data represent the percentage of cytoplasmic injections in which the specified fluorescent probe moved between adjacent trichome cells within 4 min. At least three different plants of each line were tested. Values in parentheses represent number of injections.

of the larger F-dextran (10000 Da) was ever detected. However, in 12% of injections the F-dextran of 4400 Da was observed to move into the neighbouring cell. The absence of fluorescence spread in all injected plants indicates that the dye movement detected was not due to the polydisperse size distribution of the Sigma F-dextrans.

This observation is consistent with previous reports that the plasmodesmal size exclusion limit is occasionally higher than commonly reported (800 to 1000 Da) for a variety of tissues (Wolf et al., 1989; Poirson et al., 1993; Kempers et al., 1993; compare with Terry & Robards, 1987). The increase in plasmodesmal permeability in a subset of normal cells may reflect physiological conditions that enhance communication between cells within a symplastic domain.

In all transgenic plants expressing the CMV 3a protein, the F-dextran of 4400 Da average size moved rapidly from the basal injected cell to the upper cells of the trichome. In addition, the fluorescent probe of 10000 Da was seen to spread into adjacent cells in 30 to 45% of the injections into plants from all three lines tested (Table 1).

These results show that in the three lines of transgenic plants tested, the presence of the putative MP from CMV, principally in the cell wall, correlates with modified gating capacity of plasmodesmata. It increases the plasmodesmal size exclusion limit in many cases to over 10000 Da, the mean size of the larger dextrans used in this study. A 10000 Da limit would be equivalent to a Stokes’ radius of approximately 2-3 nm. CMV MP thus causes a modification that is functionally equivalent to those induced by TMV and red clover necrotic mosaic virus MPs, which enable the spread of probes of between 9.4 and 17.2 kDa in non-vascular tissues (Wolf et al., 1989; Fujiwara et al., 1993). A size exclusion limit in the region of 2.3 nm would not in itself be sufficient to allow the cell-to-cell passage of intact virus particles (29 nm diameter), although it would allow the passage of unfolded nucleoprotein complexes (1.5 to 2 nm diameter) such as those formed in vitro by TMV MP (Citovsky et al., 1992). This assumes that the CMV MP is able to form this type of complex, and that such complexes are functional in vivo. As pointed out by Derrick et al. (1993), it is also possible that the plasmodesmatal size exclusion limit might be increased further during the intercellular passage of the infectious unit, as the result of specific interactions between viral components and host factors.

The presence of the CMV 3a protein in the cell-wall fraction of transgenic tobacco plants, and its ability to increase the plasmodesmatal size exclusion limit, are consistent with the properties of other plant viral MPs, and suggest strongly that the 3a protein is involved in the cell-to-cell movement of CMV.

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References


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