Effect of the alfalfa mosaic virus movement protein expressed in transgenic plants on the permeability of plasmodesmata

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Symplastic transport of different sized fluorescent probes has been assessed in leaf epidermal cells of transgenic Nicotiana plants expressing the movement protein (MP) of alfalfa mosaic virus (AMV). In both N. tabacum and N. benthamiana, the size exclusion limit (SEL) of plasmodesmata increased from \( M_r \) 1000, which represents the commonly accepted limit, to over 4·4K. However, in control plants, movement of a 3K probe was seen in 11 to 22% of the injections, indicating that plasmodesmata may on occasion allow the passage of molecules larger than was previously thought. The increase of SEL due to the presence of the AMV MP, although significant, remains insufficient to permit the passage of viral particles and the possibility of other mechanisms involved in viral cell-to-cell spread is discussed.

To invade the inoculated tissue, most plant viruses move from cell to cell via a symplastic pathway, most likely through plasmodesmata [for reviews see Hull (1989), Maule (1991), Deom et al. (1992)]. Plasmodesmata are dynamic, gatable channels that traverse the cell wall, providing a means for intercellular communication. Fluorescent tracers up to only \( M_r \) 1000 move symplastically in dye-coupling experiments (Erwee & Goodwin, 1985; Derrick et al., 1990). Wolf et al. (1989) reported the same result concerning the gating capacity of tobacco plasmodesmata, suggesting an effective pore diameter of 0·73 nm. The observed functional limit of plasmodesmata is therefore well below the size of viral agents (diameter from 10 to 30 nm for most viruses) (Hull, 1989). Plasmodesmata must be functionally altered to facilitate viral cell-to-cell movement. A virus-encoded protein called movement protein (MP) has been implicated in this process. The principal evidence for this is a reported increase of the size exclusion limit (SEL) of plasmodesmata, to between 10K and 17K, in transgenic plants expressing the tobacco mosaic virus (TMV) MP (Wolf et al., 1989).

In the case of alfalfa mosaic virus (AMV), a 32K protein, designated P3, appears to be involved in viral cell-to-cell spread (Dore et al., 1991). In this context, we have investigated the symplastic transport of fluorescent dyes to estimate the gating capacity of plasmodesmata putatively modified in transformed plants expressing P3. This estimation was made in plant lines obtained from three hosts of AMV (Nicotiana tabacum cv. Xanthi, cv. Xanthi NN and N. benthamiana) to assess whether the effect of P3, if any, was a general phenomenon.

Transgenic plants of N. tabacum cv. Xanthi, genotype nn or genotype NN, were obtained as previously described (Erny et al., 1992). In the case of N. benthamiana, the binary vector pBI 121.1 (Clonetech) was chosen for plant transformation. The P3 gene was inserted into the pBI plasmid, replacing the gene coding for \( \beta \)-glucuronidase. The procedure leading to plant transformation and regeneration essentially followed that given by Jefferson et al. (1987). Plants expressing the P3 protein are referred to as S3, N3 and B3 for N. tabacum nn, N. tabacum NN and N. benthamiana, respectively.

In each series, P3 was assayed by Western blotting as in Erny et al. (1992). The plant exhibiting the highest production of P3 was selected and its progeny was used for the microinjection experiments. In each offspring however, 20 to 40% of the plants did not express the viral protein at a detectable level and these plants were then discarded; the level of P3 expression in the remaining plants was homogeneous and similar to that observed in regenerated plants by Erny et al. (1992). The amount of P3 in transgenic plants was at least equivalent to that found in systemically infected leaves. It was estimated to be around 100 ng/g of fresh material by quantitative Western blot assay (Berna et al., 1986), using Escherichia coli-produced P3 (Schoumacher et al., 1992a) as standard (data not shown). Controls were represented by plants transformed with non-recombinant vectors.
For dye-coupling experiments, half matured leaves (the third or the fourth leaf down from the apex) were excised from 8- to 12-week-old plants. The plant material was coded to ensure a 'blind' experimental procedure. Fluorescein isothiocyanate-labelled dextrans (F-dextrans) with Mr values of 3K (Molecular Probes Inc) and of 4.4K (Sigma) were dissolved in water to a concentration of 2 to 5 mM and the dye integrity was checked by thin-layer chromatography. Prior to injection, F-dextran dye was mixed equally with a small blue-fluorescing probe, cascade blue hydrazide (triptophum salt, Molecular Probes Inc.). This molecule (Mr 664) was used as an internal control to assess whether the injection occurred in the vacuolar or in the cytosolic compartment. Filamented glass microcapillaries (GD-1, 1 mm x 90 mm; Narishige) pulled using a microelectrode puller (Narishige model PP-83), were backfilled with the chosen combination of dyes. The leaves to be injected were fixed, abaxial side up, on a Petri plate and covered with distilled water. Micro-injections were performed using a Narishige micro-manipulator (model MO-204). Probes were injected into leaf epidermal cells under the control of a pressure injection unit (model PLI-II, Medical Systems Corp.). Injections of probes were observed using a Nikon Microphot SA microscope equipped for epifluorescence. Filter sets used were for fluorescein: excitation 450 to 490 nm, dichroic mirror 510 nm, emission 520 nm; for cascade blue: excitation 365 nm, dichroic mirror 520 nm, emission 450 nm.

Positive dye movement was only scored for injections where fluorescence from both the F-dextran and the cascade blue probes was seen within 5 min in epidermal cells surrounding the injected one. As the Mr of the cascade blue allows it to diffuse freely through plasmodesmata (Ding et al., 1992), no movement of this dye was recorded after vacuolar injection (about 30% of the total number of injections) and the result was then discounted. At least six to nine cytoplasmic injections were made for each plant.

Table 1 summarizes the results as the percentage of the cytoplasmic injections that showed movement of the F-dextran probes out of the injected cells. In control plants, no movement of the largest molecule (4-4K) was observed. Using the 3K tracer, however, the fluorescein label spread into the neighbouring cells in 11 to 22% of injections (depending on the species). Indeed, Wolf et al. (1989) reported movement of a 3-9K dye in 14% of injections (one of seven injections) into tobacco mesophyll cells; they nevertheless considered this figure negligible. Terry & Robards (1987) showed that a 1-1K F-dextran probe could move symplastically in nectary trichome cells of Abutilon striatum, in more than 70% of the cases. The conductivity of plasmodesmata was proposed to be slightly greater than for the other higher plants. Together with the present results, this implies that for some plants or some tissues at least, the plasmodesmal SEL might be higher than the widely accepted figures of 0-8K to 1K (Wolf et al., 1989; and references therein), most probably lying between these values and about 3K. Intensive testing with fluorescent probes of Mr values between 1K and 3K would resolve this issue.

In plants expressing the AMV MP (S3, N3 or B3), the 3K F-dextran probe rapidly and freely moved out of the injected cell (Table 1). With the 4-4K tracer, the fluorescent label was found to spread into adjacent cells in 24% of the cases in S3 plants, whereas dye mobility was noticed in one of two injections for both N3 and B3 plants.

These results lead us to conclude that P3 modifies the gating capacity of plasmodesmata in a similar way in all the plants we studied. The AMV MP alters the plasmodesmal permeability increasing the SEL to over 4-4K, thus causing a modification functionally equivalent to that observed after trichome cell infection with tobacco rattle virus (Derrick et al., 1992). Nevertheless, this represents a relatively minor functional alteration in comparison to that induced by the TMV MP, which enables the passage of 10K probes in non-vascular tissue (Ding et al., 1992). One has to take into account, however, that in the latter work, the designation 'non-vascular tissue' did not include the epidermis for which no specific data were presented. Epidermal cells are indeed thought to constitute a different symplastic domain (Erwee & Goodwin, 1985).

Based on a calculation of the Stokes radii of the F-dextran probes (Peters, 1986), the effective pore diameter of plasmodesmata would increase to approximately 2.2 nm in the presence of the AMV MP. This value remains within the limits of the physical width (2.5 nm) established for the cytoplasmic annulus in natural tobacco plasmodesmata (Ding et al., 1992). This suggests that P3 affects the regulation of molecular transport through plasmodesmata without greatly modifying their structure, which is consistent with observations made by electron microscopy on transgenic plans (O. Rohfritsch, unpublished). The modification of the plasmodesmal permeability is still insufficient to permit the passage of viral particles. Other mechanisms must be involved in this process. It is worth noting that another function for the viral MP has been proposed. It has been shown that the TMV MP binds single-stranded nucleic acids in vitro (Citovsky et al., 1990) and furthermore, electron microscopic studies reveal that these MP–nucleic acid complexes are long, unfolded and very thin (less than 1.5 to 2.0 nm in diameter) (Citovsky et al., 1992). Thus the MP might interact with both the plasmodesmata and the viral genome to promote viral cell-to-cell spread. It
Table 1. Mobility of fluorescent dyes in epidermal cells of transformed Nicotiana plants*

<table>
<thead>
<tr>
<th>Fluorescent probe</th>
<th>Genotype nn</th>
<th>Genotype NN</th>
<th>N. benthamiana</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>S3</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N. tabacum cv. Xanthi</td>
</tr>
<tr>
<td>F-dextran (3K)</td>
<td>22 (7 plants)</td>
<td>95 (7 plants)</td>
<td>14 (3 plants)</td>
</tr>
<tr>
<td>F-dextran (44K)</td>
<td>0 (5 plants)</td>
<td>24 (5 plants)</td>
<td>0 (5 plants)</td>
</tr>
</tbody>
</table>

* Data represent the percentage of cytoplasmic injections (six to nine per plant) that showed movement of the given probe within 5 min after injection. The number of plants which were tested is indicated in parentheses.

should be noted that P3 can also bind single-stranded nucleic acids (Schoumacher et al., 1992a, b). Nevertheless, it has not yet been demonstrated that the resulting complexes are unfolded and thin enough to be compatible with the modified effective pore diameter reported here, and that the nucleic acid-binding ability of the MP actually reflects an in vivo process.

In this context, it seems important to identify the MP domain(s) required for plasmodesmal interactions. For this purpose, contiguous in-frame deletions have been made along the whole sequence of P3 and the resulting MP constructs were used for plant transformation. Further microinjection studies will be done on these genetically modified plant lines. Nevertheless, the results presented in this paper already provide further evidence supporting a role for the viral MP in increasing the SEL of plasmodesmata.

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References


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