The Subcellular Location of the Gene 1 Product of Cauliflower Mosaic Virus Is Consistent with a Function Associated with Virus Spread

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SUMMARY

Using immunogold cytochemistry, plasmodesmata have been identified as subcellular locations of the protein product (P1) of cauliflower mosaic virus gene 1 in infected turnip tissue. Thin sections, from tissue adjacent to chlorotic local lesions on systemically infected fully expanded leaves, were probed with antiserum to a protein product derived from a lacZ-gene 1 fusion, and with control sera. Modified plasmodesmata between infected mesophyll cells, and plasmodesmata in the end walls of phloem parenchyma cells were specifically labelled with anti-P1 serum. In the former case, the position of the label suggested that P1 was extracellular and had formed a structural component of the modified plasmodesmata. Cell walls at the corners of cells in both healthy and infected tissue were also labelled. Anti-P1 serum also reacted with nuclei and the leaf cuticle, in both healthy and infected tissue. These observations provide strong circumstantial evidence that P1 is involved in the cell-to-cell movement of cauliflower mosaic virus.

INTRODUCTION

The protein product of cauliflower mosaic virus (CaMV) gene 1 (P1) has been detected immunologically in extracts from infected turnips using antisera raised against lacZ-gene 1 fusion products as a family of polypeptides with Mr of 46000 (46K), 42K and 38K (Harker et al., 1987) or 45K and 35K (Young et al., 1987). Cell fractionation (Harker et al., 1987) indicated that the polypeptides were located in a high speed pellet enriched with CaMV replication complexes, although Young et al. (1987) reported that P1-specific polypeptides were loosely associated with virus particles and were restricted to the insoluble fraction of tissue homogenates also enriched for virus inclusion bodies. Neither of these studies, however, gave any definitive evidence for the function of the product(s) of gene 1. In a further study Martinez-Izquierdo et al. (1987) found a single 41K gene 1 product in virus inclusion bodies but not in preparations of virus particles.

Amino acid sequence comparisons between the deduced translation product of gene 1 and other proteins have identified similarities with ribonucleotide reductase, the ATP-binding site of some kinases (Martinez-Izquierdo et al., 1987) and some non-structural proteins from other plant viruses (Hull et al., 1986; Hull & Covey, 1985; Richins et al., 1987). The relationship between P1 and the protein kinase activity associated with virus particles and implicated in particle maturation (Menissier-de Murcia et al., 1986), has been resolved since the CaMV kinase activity and virus particles were both separable from P1 using CsCl purification (Young et al., 1987; Martinez-Izquierdo et al., 1987). Sequence similarity with other viral proteins is seen most clearly in comparisons between P1 and the equivalent proteins from other caulimoviruses, carnation etched ring virus (CERV; Hull et al., 1986) and figwort mosaic virus (Richins et al., 1987) and, to a lesser extent, between P1 and tobacco mosaic virus (TMV) 30K protein (Hull & Covey, 1985) which controls cell-to-cell spread of TMV in infected tissues (Leonard & Zaitlin, 1982; Deom et al., 1987; Meshi et al., 1987). Immunogold cytochemistry, used to identify the
subcellular location of the TMV 30K protein (Tomenius et al., 1987), showed that it accumulated in plasmodesmata in infected tobacco leaves, an observation consistent with a role in virus spread. Similarly, immunogold labelling has shown the association of a non-structural protein (P3) of alfalfa mosaic virus, a protein also considered to be involved in controlling cell-to-cell spread, with the middle lamella of cell walls (Stussi-Garaud et al., 1987).

This paper describes experiments in which electron microscopy and immunogold cytochemistry were used to investigate the subcellular location of the CaMV gene 1 protein(s) in tissue samples from turnip leaves systemically infected with CaMV.

METHODS

Plants and virus inoculation. CaMV Cabb B-JI was maintained in, and purified from, Brassica rapa L. cv. Just Right (turnip) as described by Hull et al. (1976). For inoculation, extracts of systemically infected tissue homogenized in 100 mm-sodium phosphate pH 7.0 were rubbed onto the second leaves of young turnip plants. Plants were maintained in a glasshouse at 20 °C ± 5 °C with a 16 h photoperiod supplemented with GE solarcolour sodium lighting.

Processing of leaf tissue. Twenty days post-inoculation, systemically infected leaf tissue showing local chlorotic lesions was sampled for electron microscopy. Tissue samples were taken from the edge of individual lesions and processed in parallel with equivalent pieces of healthy (mock-inoculated) tissue. Tissue samples from turnip leaves systemically infected with CaMV.

Immunoctytochemistry. Sections were reacted with rabbit polyclonal serum to a lacZ–gene 1 fusion protein (anti-P1), preimmune serum from the same rabbit or anti-β-galactosidase serum (anti-β-gal) (Harker et al., 1987); all except anti-β-gal were used at a dilution of 1/100, anti-β-gal was used at 1/250. Also, anti-P1 serum was repeatedly passed through a Sepharose 4B affinity column to which the gel-purified anti-β-gal serum fraction was used as a further control; this preparation was designated anti-β-gal. Tissue sections were examined in a JEOL JEM 1200EX electron microscope.

Tissue sections were incubated overnight at 4 °C in the primary antiserum diluted in either 'polyclonal blocking buffer' A [0.2 M-Tris–HCl pH 7.4, 1% v/v Tween 20, 0.1% w/v bovine serum albumin (BSA)] or B [buffer A but with 2.5% w/v milk powder ('Marvel', Cadbury's, Birmingham, U.K.) instead of BSA]. They were then washed seven times in distilled water and finally, for immunogold labelling, incubated for 1 to 3 h at room temperature in a 1:20 dilution of goat anti-rabbit IgG antiserum conjugated to 15 nm gold (Janssen Auroprobe EM GAR G15; Life Sciences Products, Janssen Pharmaceuticals, Oxford, U.K.). After extensive washing in distilled water, sections were stained as described above.

Partial purification of the lacZ–gene 1 fusion protein and preparation of an affinity column. The lacZ–gene 1 fusion protein produced in Escherichia coli F11recA (Harker et al., 1987) was purified using a procedure based upon the insolubility of lacZ fusion products (Stanley, 1983). E. coli F11recA was grown overnight in the presence of 140 μg/ml isopropyl-β-D-thiogalactopyranoside and 200 μg/ml carbenicillin and the cells were harvested by centrifugation. After washing at 4 °C, twice in fresh medium and once in distilled water, the bacteria, in water, were disrupted by sonication on ice and the soluble material was collected by centrifugation at 30000 g for 20 min. To ensure complete disruption, the pellet was sonicated, on ice, twice more and the insoluble pellet was washed with distilled water. The final pellet was heated at 100 °C for 5 min in sample buffer (5% 2-mercaptoethanol, 10% glycerol, 2% SDS, 46 mm-Tris–HCl pH 6.8, 0.01% bromophenol blue) and the dissolved proteins were separated in a 10% preparative SDS–polyacrylamide gel (Laemmli, 1970). The gel was chilled to 4 °C and soaked in 0.5 M-KCl (Xiong et al., 1982), and the fusion protein was excised as a gel slice and electro-eluted after the gel had been ground to a fine powder in liquid N2. Electro-elution and coupling of the protein to cyanogen bromide-activated Sepharose 4B (Pharmacia) were done in 0-1 M-NaHCO3, 0.5 M-NaCl pH 8.3 and 1% SDS to maintain solubility.

For the preparation of anti-P1 − 1 serum, the IgG fraction from P1 antiserum was passed repeatedly through a 2 ml fusion protein affinity column and the unbound material was retained. The retention of anti-P1 antibodies by the column was checked by probing Western blots of subcellular fractions from infected turnips enriched for P1 (Harker et al., 1987) with total anti-P1 IgG and with anti-P1 − 1. Recovery of specific anti-P1 IgG antibodies from the column was attempted by elution with 0.1 M-glycine–HCl at pH 2.0, pH 2.5 or pH 3.0 and assessed, after neutralization of the eluate, by Western analysis as above, but in all cases recovery was poor.
RESULTS

Sections of infected mesophyll tissue contained electron-dense inclusion bodies with embedded CaMV particles characteristic of caulimovirus infections. Virus particles were also frequently seen free in the cytoplasm and in modified plasmodesmata (Fig. 1). The modifications to plasmodesmata were similar to those reported for CaMV-infected Chinese...
Fig. 2. Location of gold label on sections of healthy (a, b, c) and infected (d, e, f) tissue treated with anti-P1 serum (a, b, c, d), anti-P1 - 1 serum (e) or preimmune serum (f). Cell walls at the extremities of cell junctions (arrowed) were labelled only by specific anti-P1 antibodies (a, b, c, d); plasmodesmata (p) in healthy tissue (b) were not labelled; labelling of modified plasmodesmata (mp) was not observed in the absence of P1 antibodies (e, f). Bar markers represent 500 nm.
cabbage leaves (Conti et al., 1972; Bassi et al., 1974), dahlia mosaic virus (DMV)-infected zinnia leaves (Kitajima & Lauritis, 1969), CERV-infected Saponaria vaccaria and Dianthus caryophyllus tissues (Lawson & Hearon, 1974). These modifications were characterized by an enlarged diameter of the inter-symplastic channel and the appearance of a finely granular tubular structure within a cell wall extension which together formed a papilla extending up to 800 nm towards either or both of two neighbouring cells (Fig. 1). Virus particles were observed within these tubular structures (Fig. 1, bottom panel) and in some cases appeared to be gathered at one end, presumably entering or exiting the enlarged channel. In the tissue areas examined, all plasmodesmata appeared to be modified although only a few contained particles. This is in contrast to observations made for DMV in zinnia and suggests that in CaMV-infected turnip leaves the tubular structures are not a transient modification triggered by the presence of the particle as was suggested by Kitajima & Lauritis (1969) for DMV.

Sections from healthy or infected tissue that had been incubated with the preimmune (Fig. 2) or anti-β-gal sera, or incubated without the primary antibody, showed a scattering of gold labelling without any specific localization.

Incubation of sections of infected and healthy tissue with anti-P1 or anti-P1 IgG resulted in localization of gold label over several subcellular areas, the leaf cuticle (Fig. 3), cell walls at the extremities of cell junctions (Fig. 2) and nuclei. Infection-specific localization of P1 was restricted to the modified plasmodesmata of mesophyll cells (Fig. 4) and phloem parenchyma (Fig. 5). The absence of similarly localized label in sections treated with preimmune serum from the same rabbit (Fig. 2), or anti-β-gal, indicated that there was a wide distribution of P1 and P1-related proteins in healthy tissue. To investigate this more rigorously a series of control experiments was undertaken.

For the blocking of non-specific binding of antibodies to tissue sections, Tween 20, BSA and gelatine were routinely used. As an alternative, sections of healthy and infected tissue were treated with primary and secondary antibodies in the presence of 2.5% (w/v) milk powder. This
Fig. 4. Immunocytochemical labelling of P1 in systemically infected leaves. Plasmodesmata were specifically labelled with anti-P1; transverse (solid arrows) and longitudinal sections (open arrows) of modified plasmodesmata indicate that P1 is probably extracellular. Bar markers represent 200 nm.
Fig. 5. Association of P1 with the end walls (ew) of phloem parenchyma in small vascular tissues of infected leaves. Gold label was seen in the vicinity of plasmodesmata (p) in the end walls but not exclusively within the wall or the cytoplasm. Bar markers represent 500 nm.

Anti-P1 serum had been raised by injecting the 'gel-purified' product of the lacZ fusion gene directly into rabbits (Harker et al., 1987), a procedure which inevitably led to the antigen being contaminated with some high Mr E. coli proteins, a potential source of ambiguity in these experiments. The insoluble nature of lacZ fusion proteins (Stanley, 1983) meant that the direct control experiment based upon competition from purified antigen for the specific binding of anti-P1 was not possible. An alternative approach for demonstrating the specificity of the observed binding of anti-P1 was to use a negative control based upon the removal of specific P1 antibodies from anti-P1. For this, separation of specific P1 antibodies from the serum by affinity chromatography was required. Purification of the lacZ–gene 1 fusion product from contaminating soluble proteins was achieved by extensive washing of insoluble E. coli extracts and gel electrophoresis; the purified product was used to prepare an affinity column for the selection of specific anti-P1 IgG antibodies. Western blotting of P1-containing extracts with antiserum fractionated on the fusion protein affinity column showed that specific anti-P1 IgG was selectively removed from preparations of total anti-P1 IgG. This serum, depleted in anti-P1 IgG, was designated anti-P1 - 1.

Both anti-P1 and anti-P1 - 1 labelled the cuticle of infected and healthy leaves (Fig. 3) and therefore demonstrated a reaction not specific to P1. Sometimes cell walls at the extremities of cell junctions were labelled in sections from both healthy and infected tissues treated with anti-P1 but not when sections were treated with anti-P1 - 1 (Fig. 2). Labelling of plasmodesmata was
not seen in healthy tissue treated with either anti-P1 or anti-P1-1, or in infected tissue treated with anti-P1-1 (Fig. 2).

After incubation with the anti-P1 serum, sections from infected tissue showed gold label clearly associated with modified plasmodesmata (Fig. 4). In both transverse and longitudinal sections of these plasmodesmata (Fig. 4), the antibody appeared to have reacted with components of the wall surrounding the tubular structure rather than with the tube itself. Longitudinal sections through the small vascular tissues of the leaf lamina were labelled around the end walls of phloem parenchyma (Fig. 5). In contrast to that over plasmodesmata between adjacent mesophyll cells (Fig. 4), this label was not restricted to extracellular structures but was seen both over the wall and over the neighbouring cytoplasm (Fig. 5). Also, the characteristic modifications to plasmodesmata were not seen in the end walls of phloem parenchyma although relatively few clear transverse sections of these structures were examined. Surprisingly, P1 was only rarely associated with free virus particles or inclusion bodies despite reports to the contrary based upon localization by subcellular fractionation (Young et al., 1987; Martinez-Izquierdo et al., 1987), although P1 inside virus particles would probably not have been detected.

DISCUSSION

Sections of CaMV-infected turnip tissue showed modifications of the plasmodesmata between mesophyll cells characteristic of caulimovirus infections. The alterations included an increased diameter of the intersymplastic channel sufficient to allow virus particles to pass through and, in many cases, virus particles were observed within the channel. The presence of virus particles free in the cytoplasm and en route between cells argues strongly that such particles mediate the spread of infection in these tissues, although it cannot be excluded that other forms of nucleoprotein, or nucleic acid, may also be involved, particularly as isolates of tobacco rattle virus (Lister, 1966) or cassava latent virus (Stanley & Townsend, 1986) that lack coat protein can infect plants systemically.

Initially, the reaction of the anti-P1 serum with sections of healthy and infected tissues identified several structures as locations for P1-specific proteins, although two were later identified as structures that reacted non-specifically. By increasing the specificity of the immunochemical reaction, using skimmed milk as a blocking agent, nuclei were excluded as sites of accumulation of P1-related proteins. Nuclei have been implicated as sites for accumulation of the TMV 30K or related proteins (Watanabe et al., 1986) although for the immunocytochemical localization of the TMV 30K protein, a distinction was made between the nuclear reaction and the infection-specific reaction with plasmodesmata on the basis of antibody dilution (Tomenius et al., 1987).

P1-related protein(s) was sometimes seen associated with cell walls at the triangle formed between two cells in contact and an intercellular space, in both healthy and infected tissues. The significance of this is not yet understood but the observation could presumably represent the location of host proteins with some epitopes in common with P1. A similar location was observed for TMV 30K protein in infected tissue (Tomenius et al., 1987).

The subcellular location of P1 was identified as being around modified plasmodesmata between mesophyll cells and at the ends of phloem parenchyma cells in small vascular elements. This provides strong circumstantial evidence for the involvement of CaMV P1 in the systemic spread of infection by movement from cell to cell and, at least locally, through small vascular bundles. The necessity for some modification of plasmodesmata to allow the local passage of macromolecules during virus infection has long been recognized (Atabekov & Dorokhov, 1984), whereas the spread of infectious agents over longer distances through the vascular tissues has received less attention. The association of P1 with the phloem parenchyma indicates that a similar mechanism may operate at the two sites permitting CaMV to enter and move through the peripheral vascular system. However, a modification equivalent to that seen for plasmodesmata between mesophyll cells was not observed and the detailed location of P1 at the end walls was both intracellular and extracellular whereas it appeared to be extracellular around modified plasmodesmata.
The mechanism by which P1 may facilitate virus movement in the leaf mesophyll is not at present understood although its association with the cell wall matrix, close to the modified plasmodesmata, may indicate a role as a structural component rather than a protein that actively transports virus, although it cannot be excluded that P1 was relocated by modification of the cell wall after an early association with plasmodesmata. The strikingly characteristic modification of plasmodesmata in plants infected with caulimoviruses may suggest that the mechanism of cell-to-cell spread may vary between different virus groups. In this context, it is interesting that among the caulimoviruses whose DNA has been sequenced P1 shows extensive similarity in amino acid sequence throughout the central region of the protein, but not at the termini (Hull et al., 1986; Richins et al., 1987). In contrast, the regions of similarity between CaMV P1 and TMV 30K are more limited, and occur in a short central region and at the carboxy terminus (Hull & Covey, 1985; Hull et al., 1986). In TMV-infected plants, plasmodesmata are not so extensively modified as those shown here and, although the 30K protein appears to be localized to plasmodesmata, its location differs from that of P1, in being mainly within the desmotubule (Tomenius et al., 1987). Complementation of spread between viruses in different groups has been reported (Taliansky et al., 1982) but not for caulimoviruses and tobamoviruses. Further comparative studies on the subcellular location of spread proteins from different viruses will greatly assist in our understanding of the phenomenon of virus movement in plants.

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REFERENCES


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