Distribution of Barley Stripe Mosaic Virus Protein in Infected Wheat Root and Shoot Tips

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SUMMARY

Post-sectioning immunogold–silver staining (IGSS) specifically detected barley stripe mosaic virus (BSMV) protein in Lowicryl- or Araldite-embedded plant tissues at the light microscope level. Electron microscopy of adjacent sections showed that BSMV particles were present where the IGSS reaction was positive. BSMV protein was localized in or near the apical meristems of root and shoot tips of systemically infected wheat. Infected cells were distributed in a mosaic pattern.

INTRODUCTION

Most plant viruses do not infect the meristematic cells of rapidly growing plants. Thus, for example, meristem tip culture often yields vegetatively propagated, virus-free plants. However, some plant viruses do infect apical initials of root or shoot meristems and infection of these cells has usually been confirmed by electron microscopic examination or infectivity assay. Tobacco ringspot virus (TRSV), but not tobacco mosaic virus (TMV), was detected in the terminal 500 μm of bean roots (Crowley et al., 1969; Atchison & Francki, 1972) and observed in apical initials of tobacco shoot meristems (Roberts et al., 1970). Tobacco rattle virus (TRV) and potato virus X (PVX) were identified in the meristematic zones of shoot apices and root tips, respectively (Kitajima & Costa, 1969; Appiano & Pennazio, 1972). Walkey & Webb (1968) found several viruses in the apices of various hosts. However, Smith & Schlegel (1964) were unable to detect clover yellow mosaic virus (CYMV) in the distal 0.4 mm sections of broad bean root tips. Thus, in several combinations of virus and host there appears to be a ‘virus-free’ zone of variable length in plants near the shoot or root meristems.

Fluorescent antibody staining has been used to detect the presence, distribution and accumulation of virus protein in infected plants (Cremer & van der Veken, 1964; Hosokawa & Mori, 1983). However, a problem has been that autofluorescence of the plant cell reduces the sensitivity of immunofluorescent labelling (Cremer & van der Veken, 1964), and the resulting insensitivity of the procedure has prevented its wide use. The effect of autofluorescence can be reduced by using a fluorescent probe detectable in the 546 to 590 nm range (Hapner & Hapner, 1978).

Recently, a promising immunogold silver staining (IGSS) method (Holgate et al., 1983) was reported in which immunoglobulin adsorbed onto colloidal gold is localized at antigenic sites and subsequently enhanced by precipitating silver onto the gold to give particles visible by light microscopy. The method not only overcomes problems of autofluorescence of plant cells, but has been reported to be up to 200-fold more sensitive than the standard immunoperoxidase method (Holgate et al., 1983). Furthermore, it is possible to see gold-labelled substances in semithin sections of resin-embedded tissue at the light microscope level (Danscher & Norgaard, 1983). Thus, with the IGSS method presented here, tissues routinely processed for ultrastructural investigation are also suitable for light microscopic immunolocalization of viral protein in Lowicryl- or Araldite-embedded plant tissues. This paper describes the distribution of barley stripe mosaic virus (BSMV) protein in or near apical meristems of systemically infected wheat plants.
METHODS

Viruses. BSMV (type strain, ATCC no. pv43) and TMV (common strain, ATCC no. pv135) were maintained in wheat (*Triticum aestivum* L. cv. Michigan Amber) and Turkish tobacco (*Nicotiana tabacum* L. cv. Xanthi), respectively.

Sampling of root and shoot tips. One-week-old seedlings of wheat were mechanically inoculated with sap from infected plants. Infected plants showing systemic mosaic symptoms 7 to 10 days after inoculation were removed from pots. After a thorough and gentle wash in running water, roots were excised and plants were transferred to 1:10 dilutions of Hoagland's solution (Hoagland & Arnon, 1950) in continuous light at 22 °C. Eight to 10 days later, the terminal 4 mm of newly grown root tips were harvested. Preliminary results showed that very few roots of BSMV-infected wheat plants contained virus in the distal 2 mm of root tips. Therefore, in all subsequent experiments, the 2 to 4 mm portion of roots behind the tip were first examined for the presence of virus by immunosorbent electron microscopy (ISEM) (Derrick, 1973; Roberts et al., 1982). Carbon-backed collagen-covered grids were precoated with BSMV-specific antiserum diluted 1:500 in 0.1 M-phosphate buffer pH 7.0 for 1 min, and drained by touching with a filter paper strip. Root pieces were chopped thoroughly with a razor blade in distilled water and drained antibody-coated grids were floated on the drop for 5 min and subsequently negatively stained with 2% phosphotungstic acid, pH 7.2. Only the terminal 2 mm of tips of roots found to be infected with BSMV were processed.

Shoot tips, including the meristematic region 2 mm above the roots, were cut from infected seedlings showing mosaic symptoms 7 to 12 days after inoculation and processed immediately.

Tissue preparation. Infected and healthy shoot tip pieces (1 × 1 mm) were fixed and embedded as previously described (Lin & Langenberg, 1983). After fixation with 1% cold glutaraldehyde in 0.1 M-phosphate-citrate buffer pH 7.2, overnight at 5 °C, tissues were divided into two samples. One was dehydrated with an ascending methanol series and embedded in Lowicryl HM20 at low temperature (Lin & Langenberg, 1983). The other was post-fixed with 1% OsO₄ for 1 h at room temperature, dehydrated with acetone, and embedded in Araldite 502.

Root caps were removed from root tips after fixation to facilitate complete penetration of the plastic and the 2 mm root tips were cut in half lengthwise. All root tips fully infiltrated with Lowicryl or Araldite were flat-embedded between pieces of 1 inch-square gel bond (FMC, Rockland, Me., U.S.A.) or carbon-coated slides (Langenberg et al., 1972).

Preparation of antisera and gold–IgG complexes. Antibodies against BSMV (anti-BSMV) and TMV (anti-TMV) were elicited in rabbits by intramuscular injection of 5 mg purified virus in Freund's complete adjuvant (Ball, 1964). Titres of 1/512 in double diffusion tests were reached against virus at 2 μg/ml 6 weeks after the initial antigen injection.

The preparation of gold-labelled goat anti-rabbit IgG complexes has been described previously (Lin & Langenberg, 1983).

Immunogold–silver staining (IGSS). Immunogold–silver staining (Holgate et al., 1983) was modified as follows: 0.3 to 1 μm-thick Lowicryl HM20- or Araldite 502-embedded tissue sections were floated on a drop of 4% paraformaldehyde on glass slides coated with Haupt’s solution (Johansen, 1940) and dried overnight at 37 °C. Slides were immersed in saturated sodium hydroxide in absolute ethanol for 20 to 30 min and washed in three changes of absolute ethanol (Dellorto et al., 1982). Sections were then treated with undiluted normal goat serum for 5 min to block non-specific binding, drained and treated with diluted (1:5000) rabbit anti-BSMV in 0.15 M-NaCl, 0.1 M-phosphate pH 7.0 (PBS), containing 5% (v/v) normal goat serum for 30 min. Sections were then washed three times for 10 min in PBS and then for 5 min with undiluted goat serum. Sections were stained with gold-labelled goat anti-rabbit IgG (*A*s₂₅₅ = 0.5 to 1.0) for 1 h and washed in PBS and deionized distilled water for 30 min each. All steps were at room temperature unless specified otherwise. Silver enhancement of gold label was with a solution containing gum arabic, hydroquinone and silver lactate adjusted to pH 3.5 by addition of citrate buffer (Danscher, 1981).

Controls were similarly processed healthy tissue, or infected tissue processed using diluted (1:5000) anti-TMV in place of anti-BSMV, or both. Immunostained sections were examined under a Zeiss or Nikon phase-contrast microscope. All bar markers represent 50 μm.

Electron microscopy. Thin sections of Lowicryl HM20- or Araldite 502-embedded tissues showing silver to gold interference colours were mounted on 200-mesh nickel grids covered with carbon-backed collodion film, stained with uranyl acetate and lead citrate and examined in a Zeiss EM 10A electron microscope. Some sections were immunostained for BSMV using the two-step staining procedure using gold-labelled goat anti-rabbit IgG complexes following rabbit anti-BSMV serum (Lin & Langenberg, 1983).

RESULTS

Immunogold–silver staining

When the modified IGSS method was used to stain glutaraldehyde-fixed and Lowicryl HM20-embedded semithin sections after etching with saturated sodium hydroxide, BSMV protein in
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**Fig. 1**
Fig. 1. Semithin section of BSMV-infected leaf, treated with anti-BSMV at a concentration of 1:5000, followed by gold-labelled goat anti-rabbit IgG complexes (Absorbance = 0.5 to 1.0) and the silver precipitation reaction, showing positive black silver staining over the cytoplasm of infected cells. Bar marker represents 50 μm.

**Fig. 2**
Fig. 2. Control section of BSMV-infected leaves, treated as in Fig. 1 except that anti-TMV at a 1:5000 dilution replaced anti-BSMV. There is no cytoplasmic staining and little or no non-specific background staining. Bar marker represents 50 μm.

Infected cells stained intensely (Fig. 1; Figs. 2 to 5 show similarly prepared light micrographs). The best dilutions of primary antiserum for the demonstration of BSMV protein by the IGSS method were from 1:1000 to 1:50000; the staining intensity declined when more dilute primary antisera (e.g. 1:200000) were used. Control sections were not stained when anti-TMV was used at dilutions of 1:1000 to 1:50000 (Fig. 2).

The specific immunostaining of viral protein by this procedure was unaffected by secondary fixation with osmic acid after etching of the Araldite sections. No difference in immunostaining was found between etched sections of Lowicryl HM20 and Araldite 502. However, no specific staining was detected when sections were stained with immunogold alone (i.e. without silver precipitation enhancement), even after etching of the sections.

**Immunocytochemical staining of infected wheat root tips**

The presence of BSMV in infected root tips varied greatly from one root to another. BSMV was rarely detected in sections of the terminal 2 mm of root tips of systemically infected wheat seedlings by immunostaining. Plants regenerated new roots about 3 days after transfer to Hoagland's solution and newly emerged roots provided the best source for the detection of BSMV in root tips. However, few or no young terminals contained detectable BSMV 4 or 5 days after transfer and ISEM showed that root tips were more likely to contain virus 8 to 10 days after transfer. Virus was not always present; in different experiments 6 of 21, 1 of 14, 7 of 24, 0 of 17,
Fig. 3. Longitudinal section (0.3 μm thick) along the axis of the apical meristem of a BSMV-infected root tip, treated as in Fig. 1, showing the uneven distribution of BSMV proteins in the meristematic region. Bar marker represents 50 μm.

Fig. 4. Longitudinal section of BSMV-infected root tip in the zone of elongation, treated as in Fig. 1, showing the specific staining in some of the nuclei (small arrows). Large arrow points towards root tip. Bar marker represents 50 μm.
11 of 27 and 5 of 15 roots contained virus. The higher the concentration of BSMV detected in the 2 to 4 mm region, the greater was the chance of finding BSMV particles in the terminal 2 mm. Thus, marking individual root tips during tissue preparation proved helpful. Therefore, 2 to 4 mm sections from root tips were first examined for BSMV by ISEM before processing for sectioning and only virus-containing root terminals and healthy controls were processed for immunohistochemical staining studies.

Light microscopic examination of longitudinal sections of 2 mm root terminals indicated that meristematic cells were located in approximately the terminal 0-5 mm of the roots, excluding the root cap. Meristematic cells were much smaller than root cap cells, contained few vacuoles, were almost completely filled with dense cytoplasm, and contained nuclei with prominent nucleoli. Behind the meristematic cells, the cortical and epidermal cells were very large and highly vacuolated whereas those in the stele were smaller and had a prominent cytoplasm. Using anti-BSMV at a concentration of 1:5000, the majority of cells showing specific black silver staining were identified as the highly vacuolated cells in the cortex and epidermis in the zone of elongation (Fig. 3). In most root tips BSMV infection was detected in cells first when they were in the zone of initiation. In some infected root tips, viral protein was located within meristematic cells at the periphery of the quiescent centre, but it was never detected within those quiescent cells. BSMV protein was often detected in the root caps and cells adjacent to the stele on one or on both sides. The stele cells of most infected roots, as far back as 2 mm within the maturation zone, were virus-free but some xylem cells were stained with silver. Positively staining cells were present in a mosaic pattern. Most of the silver staining of infected cells was of the cytoplasm and of some nuclei (Fig. 4). Controls were not stained.

Immunocytochemical staining of infected wheat shoot tips

Light microscopic examination of longitudinal sections of shoot tips showed shoot apex (SA), leaf primordia (LP) and immature leaves (IL) (Fig. 5). BSMV protein was not detected by IGSS in the apical meristem and youngest leaf primordia of systemically infected shoot tips 7 to 12 days post-inoculation. The youngest leaf stained was about 0-7 mm away from the apical meristem (Fig. 5). The inset in Fig. 5 shows silver over the cytoplasm of infected cells; no such silver was observed in control sections or uninoculated tissue sections. Mature cells of the leaf sheath also contained detectable BSMV protein. The distribution of BSMV in infected wheat shoot tips, like that in root tips, varied greatly from one tip to another.

When transverse sections of undeveloped leaves, from the crown region 2 mm above the roots, were examined, the cytoplasm of older leaf cells in the outer layers stained intensely. However, virus was only sometimes present in the innermost leaves, at the height of the 2 mm meristematic region.

Electron microscopy

When ultrathin sections immediately adjacent to sections described above for light microscopy were examined by electron microscopy, aggregates of BSMV particles were seen in all places that showed positive IGSS staining. Few or no BSMV particles were observed in positively staining nuclei of root tips as shown in Fig. 4 (small arrows). However, when ultrathin sections were immunostained with gold–IgG complexes, large numbers of gold particles were found over nuclei of infected cells (Fig. 6), but not over those of healthy tissue (not shown). In regions of sections that did not show positive IGSS, no BSMV virions (or protein) were observed. Thus, the IGSS method correlates well with electron microscopy.

DISCUSSION

The present study shows that BSMV protein can be visualized in semithin sections of infected plant tissues following fixation and processing for electron microscopy, provided that sections are etched prior to the IGSS reaction. In plastic semithin sections, silver precipitation enhancement was essential in order to detect small quantities of viral protein in infected plant tissues, such as meristematic cells. Thus, once tissues are preserved and embedded for typical electron microscopy, semithin and ultrathin sections can be cut from the same sample block for
Fig. 5. Longitudinal section of a BSMV-infected shoot tip, treated as in Fig. 1, showing the occurrence of BSMV proteins in an immature leaf. Bar marker represents 50 µm. Inset shows specific staining at a higher magnification. Bar marker represents 40 µm. SA, Shoot apex; LP, leaf primordia; IL, immature leaf.
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Fig. 6. Electron micrograph of BSMV-infected wheat root cell immunostained for BSMV protein with rabbit anti-BSMV followed by gold-labelled goat anti-rabbit IgG showing positive label over cytoplasmic and nuclear BSM virions (arrows) and diffuse chromatin in nucleus (N). Bar marker represents 350 nm.

Histological or immunocytochemical studies. Semithin sections can be utilized for screening a large piece of tissue. Based on the results obtained, ultrathin sections can then be used for ultrastructural studies.

The fixatives used in this study, glutaraldehyde alone or followed by osmic acid, preserved fine structure and permitted immunostaining. Although evidence has shown that post-fixation with osmic acid reduces antigenicity severely (Roth et al., 1981; Bendayan, 1982), this effect seems to be reversed by the etching of Lowicryl and Araldite sections.

The patchy distribution of virus particles in systemically infected tissues is well known for many viruses. It appears that BSMV is also distributed unevenly in infected root and shoot tips. Considerable variation was found both in the size of BSMV-free regions near the apical meristems and in the pattern of infection of zones of cells perpendicular to the axis of the apical meristem. This is shown both in infected root and shoot tips (Fig. 3 and 5). The distribution of BSMV near the apical meristems is a mosaic pattern. This may explain why only a proportion of meristem tip cultures yield virus-free plants.

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REFERENCES


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