Enhanced Cytochemical Detection of Viral Proteins and RNAs Using Double-Sided Labeling and Light Microscopy

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ABSTRACT

We have developed a double-sided labeling technique for detecting viral proteins or RNAs in plastic-embedded leaf tissue by immunocytochemistry or in situ hybridization, respectively, and light microscopy. The signal from the target was enhanced by double-sided labeling when compared with single-sided labeling because sections were submerged in labeling solutions with both sides accessible to antibodies or complementary RNAs. The additional label was visible during microscopic analysis. Background signal was decreased since the tissue was probed and washed under conditions where folds and creases in the tissue were minimized. This technique uses the same equipment and chemicals as for single-sided labeling, and thus adjustments for reagent expenditures are not necessary. The procedure should be applicable to animal and plant tissue.

INTRODUCTION

Immunocytochemistry and in situ hybridization (ISH) have been successfully used to determine the location of plant or viral proteins and plant or viral RNAs, respectively, in various plastic-embedded plant or animal tissues using light microscopy (1,4,10,14,16,18). Conventionally, sections are analyzed after being mounted on glass slides precoated with an adhesive, such as poly-L-lysine or gelatin, and thus are labeled on only one side [i.e., single-sided labeling (SSL)]. This thereby limits the amount of target labeled and the sensitivity of the assay. The target may therefore be difficult to detect when at low levels, such as, during the early stages of viral infection or in tissue infected with a low titer virus. Also, SSL may lead to undesirable background signal due to reagents trapped in bubbles, creases or folds in the attached section. Bubbles, creases or folds can

also lead to out-of-focus areas in micrographs. In an effort to improve detection of viral proteins or RNAs in plastic-embedded leaf tissue beyond that achieved with SSL, we have developed double-sided labeling (DSL); a technique not previously reported for use with the light microscope.

MATERIALS AND METHODS

Plants and Viruses

Nicotiana tabacum L. cv. Xanthi nn, *Nicotiana benthamiana* and *Arachis hypogaea* L. (peanut) cv. Okrun were used as host plants. Purified U1 strain of tobacco mosaic tobamovirus (U1-TMV) (12), sunn-hemp mosaic tobamovirus (SHMV; a gift from Dr. C.M. Deom, University of Georgia) and peanut stripe potyvirus (PStV) (3) were used as inocula. Tobacco plants were grown and inoculated as previously described (4). Peanut and *N. benthamiana* plants were grown in 11- or 16-cm pots, respectively, in a greenhouse. At 4 weeks post-planting, two fully expanded trifoliate leaves of each peanut plant or two fully expanded leaves of each *N. benthamiana* plant were inoculated with an extract prepared from PStV-infected *N. benthamiana* leaves or from SHMV-infected *N. benthamiana* leaves, respectively. The inoculated plants were then grown as described (4).

Fixation and Embedding

For detection of TMV and PStV, tissues (6 × 3 mm) were randomly sampled from healthy or virus-infected leaves at various days post-inoculation. All tissues were fixed in 3% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. PStV- or TMV-infected tissue and requisite uninfected control tissue were fixed for 3 h at 4°C. SHMV-infected tissue and requisite uninfected control tissue were fixed in a microwave as previously described (4). The fixed tissues were washed 3 times for 15 min each in 0.1 M cacodylate buffer and partially dehydrated in a graded series of ethanol (30%, 50% and 70%; 30 min each). They were then embedded in LR White resin (London Resin Company, Hampshire, UK) as previously described (4).

Antisera and Biotinylated-RNA Probe

Polyclonal antiserum against TMV was prepared by injecting a rabbit with purified virus. Polyclonal antiserum against SHMV was a gift from Dr. C.M. Deom and antiserum against PStV was from Dr. D.V.R. Reddy (ICRISAT, Andhra Pradesh, India). All antisera were used without further purification. TMV plus strand-specific, biotinylated-RNA probe, complementary to nucleotides 1–3332 of the TMV genome, was prepared from a cDNA clone of the masked strain of TMV behind an SP6 promoter (7) using the MAXIscript™ in vitro transcription kit for the synthesis of large quantities of unlabeled RNA as described by the manufacturer (Ambion, Austin, TX, USA) but with the final concentrations of dCTP and biotin-14-CTP (Life Technologies, Gaithersburg, MD, USA) at 0.25 mM and 1.0 mM, respectively.

Immunocytochemistry

Semi-thin (2 µm) sections were cut, using glass knives on a Reichert-Jung 2050 microtome (Cambridge Instruments GmbH, Nussloch, Germany) and transferred using small needles onto drops of distilled water (4–5 sections per drop) within wells of 12-well Teflon®-coated glass slides (Cel-Line, Newfield, NJ, USA). After removing distilled water under a dissection microscope with a Pasteur pipet drawn out to a point, the sections were allowed to air-dry for approximately 10 min at room temperature (RT). A drop (30 µL) of blocking solution [2% bovine serum albumin (BSA) and 0.01% Tween 20 in 0.1 M phosphate buffer, pH 7.0] was added to each well, and the sections were incubated for 1 h at 37°C. During the incubation, sections slowly detached from the glass, and both sides were bathed with the solution. The wells had not been pre-coated with adhesive (e.g., poly-L-lysine), and thus the detachment occurred. The sections remained submerged and in contact with the blocking solution, in part, due to the hydrophilic nature of LR White (13). Access of solutions to both sides of a section attached to a glass slide by means of poly-L-lysine is less likely to occur. All subsequent additions and

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removals of solutions were done with pointed glass pipets under a dissection microscope to avoid aspirating the sections during manipulations. After the blocking solution was removed, 20 μ L of antiserum [1:100 dilution of SHMV antibody (Ab), 1:500 dilution of TMV Ab and 1:1000 dilution of PSTV Ab] in blocking solution with 1.0% BSA were added to each well, and the sections were incubated overnight at 4°C. The antiserum solution was removed, and the sections were washed 5 times by adding, incubating for 5 min and removing a washing solution [0.02 M Tris buffer containing 0.5 M NaCl and 0.01% Tween 20 (TTBS), pH 7.5]. The sections were incubated in the blocking solution for 30 min and then for 30 min in gold-conjugated (1 nm) goat polyclonal IgG to rabbit IgG (BioCell Research Laboratories, Cardiff, UK) diluted 1:100 in 1% BSA solution (20 μ L per well). The sections were washed 5

times in washing solution as described above and were then transferred individually onto drops of distilled water in wells of clean 12-well Teflon-coated slides, washed 5 times in distilled water for 2 min per wash and then incubated in 20 μ L per well of silver enhancing solution (Aurion, Wageningen, The Netherlands) for 5–10 min in the dark on ice. The sections were washed 5 times in distilled water and then dried individually onto poly-L-lysine-coated glass slides. After counterstaining for approximately 1 min in 0.01% toluidine blue, the sections were air-dried, examined and photographed with a Nikon Microphot-FX microscope equipped with a Nikon camera either as bright-field or dark-field images (Nikon, Melville, NY, USA). Through this procedure, folds or creases in the tissue were minimized due mainly to the greatly reduced time of incubation in solutions after attachment to poly-L-

lysine-coated slides. Control samples were prepared and analyzed either by labeling sections from the same virus-infected tissues using the SSL technique described previously (4) with virus-specific antibody or by labeling sections from healthy tissues using the DSL technique with virus-specific antibody. Control samples were then incubated with gold-conjugated (1 nm) goat polyclonal IgG to rabbit IgG and then silver-enhanced as above.

In Situ Hybridization

Sections were cut, transferred to distilled water and air-dried in wells of Teflon-coated slides as described for the immunocytochemical analyses. As for the immunocytochemical analyses, the sections were not attached to the slide with poly-L-lysine until after the probing and washing steps (see Immunocytochemical section). The sections were covered with prehybridiza-

tion solution (11) containing 0.01% Tween 20 (20 μ L per well) and incubated for 1 h at 42°C. The prehybridization solution was removed, and the sections were incubated in hybridization solution (ca. 400 ng RNA probe in 1 mL prehybridization solution, 20 μ L per well) for 8 h at 42°C. The sections were washed 5 times for 5 min each, in TTBS, incubated in the same blocking solution used for the immunocytochemical studies for 30 min, followed by an overnight incubation in a 1:100 dilution of goat anti-biotin IgG (Sigma Chemical, St. Louis, MO, USA) in 1% (wt/vol) BSA at 4°C. The sections were washed 5 times as above and incubated in gold-conjugated (1 nm) rabbit polyclonal IgG to goat IgG (BioCell Research Laboratories) diluted 1:100 in a 1% (wt/vol) BSA solution for 30 min at

RT. The sections were then washed, silver-enhanced, washed, dried and counterstained as described for immunocytochemical analysis. Control samples consisted of sections from healthy tissues probed with the biotinylated complementary strand to TMV RNA, TMV-infected tissues probed with a biotinylated 250-bp complementary strand to a mouse β -actin gene (Ambion), both using the DSL technique, or TMV-infected tissues probed with the biotinylated complementary strand to TMV RNA using the SSL technique. Sections analyzed by SSL were air-dried onto poly-L-lysine-coated glass slides. The sections were covered with prehybridization solution (15 μ L per section) and incubated for 1 h at 42°C. All subsequent steps were similar to those for DSL except that sections were incubated with 150 μ L of solution per slide instead of 20 μ L per well as for

the DSL procedure, and washes were done in a glass staining jar. The SSL procedure was devised to mimic the DSL procedure as closely as possible to allow comparisons.

RESULTS AND DISCUSSION

Immunocytochemistry

In early experiments using various viruses and host plants, we found that if a viral protein or RNA did not accumulate to a certain level in cells, either because it was early in the infection cycle or because the virus normally produced low levels of progeny, detection by SSL-immunocytochemistry and light microscopy could be difficult (e.g., Figure 1B). Several techniques, including the use of ultrasmall gold particle probes (8) and epipolarized light

Figure 1. Accumulation of SHMV in cells from inoculated *N. benthamiana* leaf tissue at 14 days post-inoculation. Sections were analyzed for SHMV accumulation by DSL or SSL immunocytochemistry using a polyclonal antibody against SHMV. A) A section analyzed using the DSL technique. B) A section from the same harvested tissue used for (A) and analyzed using the SSL technique. C) A section from healthy *N. benthamiana* leaf tissue analyzed using the DSL technique. Arrows indicate regions with virus accumulation. Bar = 20 μ m.

Figure 2. Accumulation of PSTv in cells from systematically-infected *A. hypogaea* cv. Okrun leaf tissue at 10 days post-inoculation. Sections were analyzed for PSTv accumulation by DSL- or SSL-immunocytochemistry using a polyclonal antibody against PSTv. A) A section analyzed using the DSL technique. B) A section from the same harvested tissue used for (A) and analyzed using the SSL technique. C) A section from healthy Okrun leaf tissue analyzed using the DSL technique. Arrows indicate regions with virus accumulation, and arrowheads indicate regions with background signal. Bar = 11 μ m.

Figure 3. Accumulation of TMV in cells from inoculated *N. tabacum* cv. Xanthi nn leaf tissue at 3 days post-inoculation. Sections were analyzed for TMV accumulation by DSL- or SSL-immunocytochemistry using a polyclonal antibody against TMV. A) A section analyzed using the DSL technique. B) A section from the same harvested tissue used for (A) and analyzed using the SSL technique. C) A section from healthy Xanthi nn leaf tissue analyzed using the DSL technique. Closed arrows indicate regions with virus accumulation. Open arrows indicate folded or creased regions of the section that are out of focus in the micrograph. Bar = 40 μ m.

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microscopy (5), were used to improve the detection, but the increase in the signal from the target was often accompanied by an increase in background noise (data not shown). SHMV-infected leaf tissue from *N. benthamiana* embedded in LR White resin was used to demonstrate the increase in signal and accuracy obtained using the DSL technique vs. the SSL technique for a low titer virus. Using DSL-immunocytochemistry, much stronger signal was seen in individual cells than that observed using SSL-immunocytochemistry (compare Figure 1, A and B). Similar results were obtained with PSTV in *N. benthamiana* (data not shown) or *A. hypogaea* (Figure 2) and with TMV in *N. tabacum* (Figure 3). PSTV does not accumulate to such high titers as TMV in the respective plants. We also found during SSL-immunocytochemistry that uneven adhesion of a section to the glass slide caused accumulation of labeling reagents in areas

between the section and slide and resulted in high background signal (compare Figure 2, A and B). In addition, section folds often occurred during SSL-immunocytochemistry, which resulted in out-of-focus micrographs (compare Figure 3, A and B).

In Situ Hybridization

ISH is a powerful technique for examining gene expression in individual cells (17). A major problem with ISH is keeping the sections attached to the slide during the hybridization and washing procedures (19). Various slide-coating materials, including 0.01% poly-L-lysine (Sigma Chemical) (4), Vectabond (Vector Laboratories, Burlingame, CA, USA) (19), 0.4% Formvar and 3-aminopropyltriethoxysilane (both from Sigma Chemical) (9), have been tried in our previous experiments and none gave 100% retention of sections on slides (data not shown). Even when using those coating materials that

performed best in our studies for tissue retention on slides, folding and creasing of sections were often a problem for the SSL-ISH. In DSL-ISH, viral RNAs are labeled before the sections are attached to the slides, and no section loss occurred during washing steps with this procedure. In addition, the background signal was low because probing and washing took place with sections not attached to a fixed support, thereby avoiding trapping of spurious signal underneath sections. DSL-ISH using TMV-infected tissues probed with biotinylated RNA complementary to TMV RNA gave strong labeling signal compared with SSL-ISH and fewer problems associated with section folding (Figure 4).

DSL has been used for electron microscopic studies (2,6,15) but not for light microscopic studies. The DSL technique developed for light microscopy differs from the procedure for electron microscopy in that the section

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Figure 4. Accumulation of TMV plus strand RNA in cells from inoculated *N. tabacum* cv. Xanthi nn leaf tissue at 3 days post-inoculation. Sections were analyzed for TMV plus strand RNA accumulation by DSL- and SSL-ISH using a biotinylated-RNA probe complementary to nucleotides 1–3332 of the TMV genome. A) A section probed using the DSL technique. B) A section from the same harvested tissue used for (A) above and probed using the SSL technique. C) A section from healthy Xanthi nn leaf tissue probed using the DSL technique. Arrows indicate regions with viral RNA accumulation and arrowheads indicate folded regions that are out of focus. Bar = 50 µm.

is not attached to a solid support (i.e., the grid), and both sides of the section are probed at one time rather than sequentially. The DSL-immunocytochemistry and DSL-ISH techniques reported here do not require additional equipment or chemicals and provide more sensitive and accurate results than SSL for routine light microscopic studies. The procedure should be applicable to animal as well as plant tissue.

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