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Synthesis and Localization of Pathogenesis-Related Proteins in Tobacco

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The PR1 family of pathogenesis-related proteins from tobacco (*Nicotiana tabacum* L.) leaves is induced by a variety of pathogenic and chemical agents and is associated with resistance to tobacco mosaic virus. The majority of the PR1 proteins did not copurify with mesophyll protoplasts (the major cell type of the leaf) isolated from tobacco mosaic virus-infected *N. tabacum* cv. Xanthi-nc leaves. However, these isolated protoplasts were capable of synthesizing and selectively secreting the PR1 proteins. Using monoclonal antibodies for immunofluorescence microscopy, we localized these proteins to the extracellular spaces predominantly in regions adjacent to viral lesions as well as in xylem elements of infected leaves.

Infection of tobacco leaves with tobacco mosaic virus (TMV) results in one of two distinct disease processes that depend upon both the genetic background of the host and the viral strain involved. Many tobacco cultivars undergo a "systemic" infection in which TMV spreads from the original point of entry to many parts of the host with the potential of causing widespread damage, especially in younger leaves. In contrast, some tobacco cultivars are able to restrict (localize) the spread of TMV to a small zone of tissue around the point of entry where a necrotic lesion later appears. In tobacco, this "hypersensitive" reaction is accompanied by the induction throughout the plant of acquired resistance: the appearance of smaller and often fewer necrotic lesions in response to a second exposure to TMV (16, 17). Virus localization and the induction of acquired resistance are accompanied by the synthesis of abundant amounts of pathogenesis-related (PR) proteins. The production of these proteins may be part of a general defense mechanism against pathogenic attack, since their synthesis can also be induced by certain bacteria and fungi (9, 19). Alternatively, their induction by a number of chemical agents, such as acetylsalicylic acid (20), suggests that they are synthesized by the plant in response to stress.

The polypeptides of the PR1 gene family, PR1a, PR1b, and PR1c, with molecular weights of approximately 15,000, are biochemically and genetically the best characterized PR proteins (1, 3, 6, 13). Their synthesis is regulated predominantly at the level of mRNA accumulation and occurs on membrane-bound polysomes (5, 6, 11). The observation that these proteins can be extracted from TMV-infected leaves by vacuum infiltration suggests that they accumulate in the intercellular fluid of leaves (15), but elucidation of their function(s) has been hindered by uncertainty regarding their precise location within infected or chemically treated leaf tissue.

TMV (type strain) was used to infect leaves of *Nicotiana tabacum* cv. Xanthi-nc, which possesses the *N* gene or allele for the localization of TMV (18). Proteins extracted from infected whole leaf tissue or from mesophyl protoplasts prepared from this tissue were subjected to immunoblot analysis with a combination of polyclonal rabbit antisera against PR1 and the large subunit (LSU) of ribulose-1,5-bisphosphate carboxylase. Although the LSU was enriched in the mesophyll protoplast preparation as expected, less than 20% of the PR1 proteins found in the whole leaf preparation copurified with the mesophyll protoplasts (Fig. 1). Similar results were obtained with mesophyll protoplasts isolated from leaves in which PR protein synthesis had been induced by treatment with acetylsalicylic acid (data not shown). These data indicate that PR1 proteins do not accumulate in leaf mesophyll cells. Likely explanations are that mesophyll cells are not sites of PR protein synthesis or that these cells synthesize and then export PR proteins. To distinguish between these possibilities, we found it necessary to determine whether leaf mesophyll cells were able to synthesize and secrete PR proteins.

Mesophyll protoplasts were isolated (7) from Xanthi-nc tobacco leaves 6 days after infection with TMV. The protoplasts (approximately 10^4) were incubated in 500 μl of 0.55 M sorbitol–10 mM 2-(*N*-morpholino) ethanesulfonic acid (pH 5.5)–500 μCi of L-[^{35}S]methionine for 5 h at 22°C under illumination with white light at an approximate intensity of 160 to 200 microeinsteins m^{-2} s^{-1}. After being labeled, the intact protoplasts were separated from the reaction supernatant by centrifugation at 150 × *g* for 2 min. The pelleted mesophyll protoplasts and the reaction supernatant (secreted fraction) were brought up to equal volumes with RIPA–1% sodium dodecyl sulfate (SDS) (4), heated at 65°C for 5 min, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) without (Fig. 2A) or with (Fig. 2B) prior immunoprecipitation with anti-PR1 serum (5). The mesophyll protoplasts synthesized the PR1 polypeptides, but these proteins accumulated predominantly in the secreted fraction (Fig. 2). The presence of the PR1 proteins in the reaction supernatant was not the result of nonspecific leakage from partially disrupted protoplasts, since the supernatant and the pelleted protoplasts exhibited distinct patterns of labeled proteins. Synthesis and secretion of the PR1 proteins were also observed with mesophyll protoplasts from acetylsalicylic acid-treated leaves but not mock-inoculated (abraded) leaves (data not shown). While these

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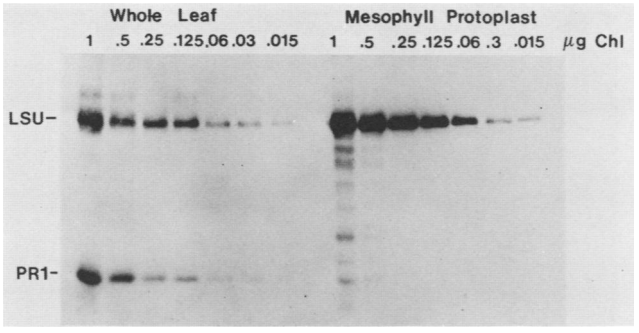

FIG. 1. Immunoblot analysis of PR1 protein and LSU accumulation in whole leaf tissue and mesophyll protoplasts from TMV-infected tobacco leaves. Xanthi-nc tobacco plants were grown as described previously (5) and infected with 10 μg of TMV ml^{-1}; Carborundum was used to abrade the upper surfaces of the leaves. Mesophyll protoplasts were prepared (7) from leaves 6 days after infection. Proteins were extracted from mesophyll protoplasts and whole leaves in SDS-PAGE sample buffer with a Dounce homogenizer. Since virtually all of the chlorophyll (Chl) in the leaf is located within the mesophyll cells, chlorophyll content can be used to normalize for the amount of mesophyll cell protein extracted from whole leaf tissue versus isolated mesophyll protoplasts. Protein extracts were subjected to SDS-PAGE (numbers above each lane indicate micrograms of chlorophyll loaded per lane) and immunoblot analysis with a mixture of anti-PR1 (5) and anti-LSU (4) polyclonal antisera. Unlike the LSU, PR1 proteins did not copurify with the mesophyll protoplasts.

results suggest that the mesophyll cells of TMV-infected or chemically treated tobacco leaves were able to synthesize and subsequently secrete PR1 proteins, they do not exclude other cell types as potential sites of PR1 protein synthesis. The mesophyll protoplast preparations themselves were slightly contaminated (<5%) with epidermal cells, which could have contributed to some of the PR1 protein synthesis.

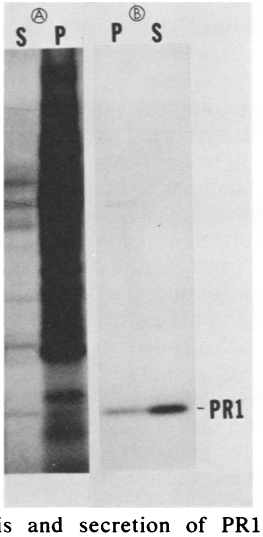

FIG. 2. Synthesis and secretion of PR1 polypeptides by mesophyll protoplasts isolated from TMV-infected leaves. Mesophyll protoplasts were labeled with [^{35}S]methionine. After being labeled, the reaction supernatant (secreted fraction, S) and intact protoplasts (P) were separated by centrifugation and analyzed by SDS-PAGE without (A) or with (B) prior immunoprecipitation with anti-PR1 serum. The difference between the two sets of newly synthesized polypeptides found in the supernatant versus pelleted protoplast fractions (A) shows that PR1 protein secretion is a specific process.

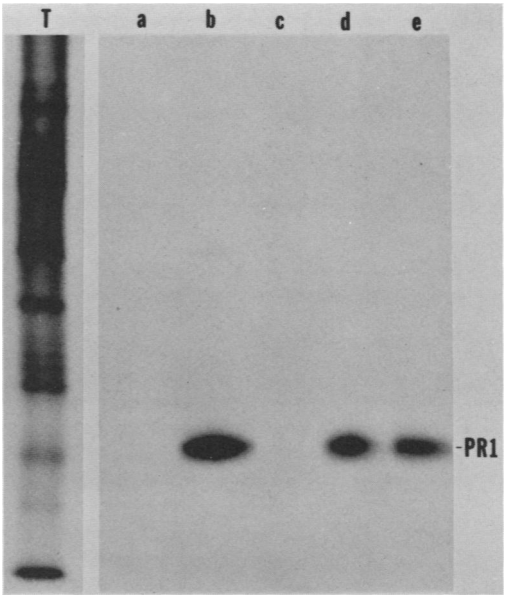

FIG. 3. Characterization of PR1-specific monoclonal antibodies. [^{35}S]methionine-labeled soluble proteins extracted from Xanthi-nc tobacco leaves (5) 6 days after infection with TMV were subjected to immunoprecipitation with preimmune rabbit serum (lane a), polyclonal anti-PR1 rabbit serum (5) (lane b), nonreactive (control) mouse ascites fluid (lane c), anti-PR1 monoclonal antibody 3-3G.1 (lane d), and anti-PR1 monoclonal antibody 2-11G.5 (lane e). Total soluble proteins (lane T) and immunoprecipitated polypeptides were analyzed by SDS-PAGE. Immunoprecipitations with monoclonal antibodies were carried out largely as described for rabbit-derived polyclonal antisera (4, 5), except that after the initial immunoreaction rabbit anti-mouse immunoglobulin G (Sigma) was added to the reaction mixture 30 min prior to the addition of Formalin-fixed *Staphylococcus aureus* cells (4). Both poly- and monoclonal anti-PR1 antibodies recognized the same major polypeptide band (PR1). The monoclonal antibody preparations were specific for PR1 proteins and did not contain the minor contaminating activities found in polyclonal anti-PR1 sera (5).

The extracellular location and distribution of these secreted proteins were determined by immunofluorescence (IF) microscopy. Since the polyclonal anti-PR1 rabbit serum used in the above-described experiments also contains some minor contaminating activities (5), it was unsuitable for these studies. Therefore, two independent BALB/c mouse-derived hybridoma lines were isolated by standard methodology (12), using purified PR1a as antigen. These two lines, 3-3G.1 and 2-11G.5, produced monoclonal antibodies that reacted with PR1 proteins in immunoprecipitation (Fig. 3) and immunoblot reactions as well as in an enzyme-linked immunosorbent assay (data not shown).

For IF microscopy, Xanthi-nc tobacco leaf tissues were fixed in 10% formaldehyde–150 mM sodium phosphate (pH 7.4) for 24 h, dehydrated, and embedded in paraffin wax. After being deparaffinized and rehydrated, 10-μm sections were reacted sequentially at 37°C for 30 min each with ascites fluid (diluted 1:50) derived from anti-PR1 or control hybridoma-induced tumors and then with fluorescein-conjugated sheep anti-mouse immunoglobulin G (diluted 1:50; Sigma Chemical Co.) before being viewed with a Zeiss IM35 microscope equipped with epifluorescence optics.

IF microscopy of cross sections through TMV-infected Xanthi-nc leaves (6 days postinfection) indicated that PR1 proteins accumulated predominantly in the extracellular

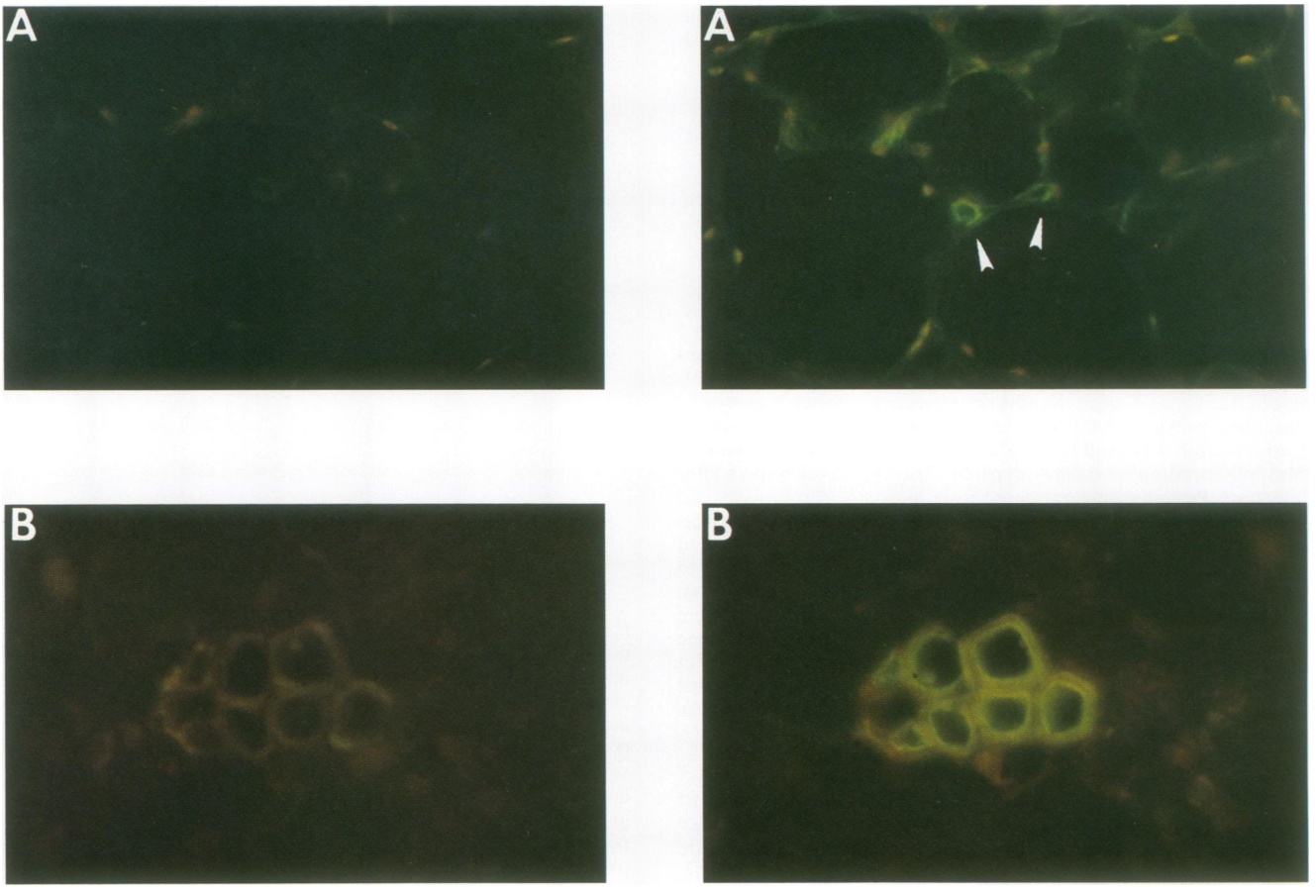

FIG. 4. Immunocytological detection of PR1 proteins in TMV-infected tobacco leaf tissue. (A) Two successive sections through a group of mesophyll cells from a TMV-infected leaf were stained with a nonreactive mouse monoclonal antibody preparation (left) or the PR1-specific monoclonal antibody 3-3G.1 (right). Specific PR1 protein signals were localized in the extracellular spaces around and between (arrowheads) mesophyll cells. Magnification, 400×. (B) Two successive sections through a vascular bundle in TMV-infected leaf tissue stained with a nonreactive mouse monoclonal antibody (left) or the PR1-specific monoclonal antibody 3-3G.1 (right). PR1 proteins accumulated in the xylem and appeared to be concentrated at the periphery of the lumen. Magnification, 400×.

spaces between and around mesophyll cells (Fig. 4A). The extracellular signal was strongest at the edges of viral lesions and decreased with distance from the lesions. These proteins also accumulated within the xylem of infected leaves at sites proximal to the lesions and at distances from the lesions at which no extracellular signal was apparent (Fig. 4B). Controls with uninfected leaves or monoclonal antibodies without anti-PR1 activity gave negative results. In addition, positive control experiments with anti-LSU serum resulted in localization of fluorescence specifically in the chloroplasts, showing that all parts of the tissue were accessible to antibodies (data not shown). However, the IF data had to be carefully and cautiously analyzed because of the autofluorescence of certain leaf structures, particularly the cell walls of the vascular tissue and the chloroplasts.

The distribution of the PR1 polypeptides observed by IF microscopy with either of the two PR1-specific monoclonal antibodies was consistent with the finding of Antoniw and White (2) with an enzyme-linked immunosorbent assay that PR1 protein concentrations are highest at the peripheries of lesions and diminish rapidly with distance. However, the appearance of PR1 proteins within the xylem was unexpected. Since the xylem consists of dead cells incapable of synthesizing new proteins, the PR1 proteins presumably entered the xylem from the intercellular fluid via the xylem pits (pores). It is not apparent from this study whether PR1 proteins can be transported over considerable distances through the xylem (e.g., from leaf to leaf), but grafting experiments by Gianinazzi (8) argue against such long-distance movement.

The high concentration of PR1 proteins in the zone adjacent to the viral lesions is consistent with the potential role of PR1 proteins in limiting TMV infections (2). However, given their location in the extracellular spaces and xylem, it is difficult to envisage any direct interaction with TMV, since this virus is believed to spread through the plant by passing from cell to cell via plasmodesmata and over longer distances through the phloem (reviewed in reference 14).

Nevertheless, the distribution and location of the PR1 proteins suggest at least four potential functions. First, these proteins may have an interferonlike activity which induces changes in surrounding healthy tissue (10). Second, the PR1 proteins may alter the extracellular environment in such a way as to inhibit viral replication within the cells. Third, they may have no effect on TMV replication itself but may be induced as part of a generalized response to plant disease, perhaps functioning against other pathogens which spread via the intercellular fluid or xylem or both. Fourth, they may not play a direct role in the resistance response but may rather function as stress proteins which help to limit the

damage caused by pathogens or the host's own responses to the pathogens or both. This last potential role is consistent with their induction by either chemicals or pathogens.

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