Induction of Bean PR-4d-Type Protein in Divergent Plant Species After Infection with Tobacco Ringspot Virus and Its Relationship with Tobacco PR-5

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ABSTRACT

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A pathogenesis-related (PR) protein, immunologically related to the 21-kDa Pinto bean PR-4d protein, was induced in 20 plant species from six dicotyledonous families after infection with tobacco ringspot virus. This protein was also induced in lima bean plants upon treatment with mercuric chloride, 2-chloroethylphosphonic acid, and benzoic acid. PR-4d protein was present in most plant species that developed necrotic symptoms, but there was no correlation between its induction and the types of symptoms produced. The Pinto bean PR-4d and Samsun NN tobacco PR-5 proteins were reciprocally immunoreactive. Gel diffusion tests showed that Pinto PR-4d and tobacco PR-5 proteins are related but not identical. The bean PR-4d and tobacco PR-5 proteins are examples of structurally homologous but functionally diverse proteins that are induced in plants in response to physiological and parasitic stresses.

Additional keywords: thaumatin-like protein, tissue necrosis, Western blotting

The production of symptoms following infection by plant pathogens is accompanied, in many cases, by an enhanced accumulation of a class of host proteins termed pathogenesis-related (PR) proteins (1,25). PR proteins induced in tobacco (*Nicotiana tabacum* L.) leaves upon infection by tobacco mosaic virus (TMV) have been studied most intensely (3,10,11,21,26) and serve as prototypes for this class of proteins. The most abundant PR proteins in Samsun NN tobacco are acidic in nature and, based upon similarities in their molecular weights, amino acid compositions, and serological properties, are classified into five distinct groups (26): 1) PR-1a, PR-1b, and PR-1c; 2) PR-2, PR-N, and PR-O; 3) PR-P and PR-Q; 4) PR-R; and 5) PR-S. Similar sets of proteins are induced in Xanthi-nc tobacco after TMV infection and are equivalent to those produced in Samsun NN tobacco (11,21,26).

The induction of PR proteins is not an exclusive pathogen-specific response, because similar proteins also are synthesized under conditions of abiotic stress, such as chemical injury or osmotic shock, and during senescence (1,25). Obviously, the activation of genes coding for proteins is the response of the plants to biotic or abiotic stress, and under normal growth conditions, these genes are quiescent or are expressed at low levels. Several PR proteins have been identified as chitinases or glucanases (2,3,17,18,25). Consequently, there is considerable interest (4,6,13) in engineering these proteins as plant defense molecules for combating pathogens and pests.

An acidic 21-kDa protein, termed PR-4d, is induced in the primary leaves of the bean (*Phaseolus vulgaris* L.) cultivar Pinto, reacting hypersensitively to infection by tobacco ringspot virus (TRSV), southern bean mosaic virus, bean pod mottle virus, and TMV; it is absent from the comparable healthy leaves (14,15,23). The Pinto PR-4d protein is secreted into the intercellular fluid (23), as are several tobacco PR proteins (20,25,26). PR-4d is nonglycosylated and possesses low mobility ($R_f = 0.25$) in alkaline nondenaturing polyacrylamide gels. In preliminary observations (23), it was indicated that PR-4d is induced in *P. vulgaris* cultivars showing virus-induced localized necrosis but not in cultivars that develop mosaic or mottle symptoms. These results indicated that PR-4d protein is involved directly in tissue necrotization. TRSV infects a large variety of leguminous and nonleguminous species (8), producing a spectrum of symptoms that includes mosaic, mottle, ring spots, and local or systemic necrosis. The objective of this study was to ascertain if proteins related to bean PR-4d are induced in divergent plant species upon TRSV infection and if there is any relationship between their induction and the types of symptoms produced. We report that a PR-4d-type protein is induced in many plant species upon TRSV infection, but no correlation exists between its induction and symptom specificity. The bean PR-4d protein shows a strong immunological relationship with Samsun NN PR-5 protein, which is equivalent to the Xanthi-nc PR-R or tobacco thaumatin-like (TL) protein (11,21,26).

MATERIALS AND METHODS

Plants were raised in Promix BX (Premier Brands, New Rochelle, NY) in a greenhouse maintained between 25 and 29 C. TRSV (a soybean isolate) was propagated in cowpea, *Vigna unguiculata* (L.) Walp. 'California Blackeye.' TRSV inoculum consisted of purified virions (20 μg/ml) (16) or a 1:10 extract (1 g of tissue per 10 ml of 20 mM sodium phosphate buffer, pH 7.0) of cowpea leaves clarified by centrifugation (15,600 *g*, 10 min) and contained Celite (50 mg/ml). The primary leaves of 2-wk-old leguminous seedlings, cotyledonary leaves of cucurbits, and four to five fully expanded leaves of vigorously growing 3- to 5-wk-old plants of other species were inoculated with TRSV. These inocula induced between 80 and 90 lesions per primary leaf of lima bean, *P. lunatus* L. 'Nemagreen,' which when coalesced (6–7 days postinoculation) covered 80–90% of the leaf surface. TMV-U1 was propagated in *N. tabacum* 'Samsun' and purified according to Gooding and Hebert (9). A week prior to inoculation of *N. tabacum* or *N. glutinosa* L. with TMV virions (20 μg/ml), 20- to 25-cm-tall plants were trimmed to four to six leaves by removing the two youngest and one or two older

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leaves. At this TMV concentration, about 250–300 lesions were induced per leaf and over 80% of the surface was covered with the coalesced lesions at 5–6 days postinoculation.

PR-4d protein was purified from the local-lesion-bearing primary Pinto bean leaves according to the previously described procedure (15), except that the initial extraction was done in 100 mM sodium acetate-acetic acid buffer, pH 5.0. Following preparative polyacrylamide gel electrophoresis and electroelution, PR-4d was concentrated with Minicon B-15 concentrators (Amicon Corporation, Danvers, MA) and then stored at −20 C. Tobacco Samsun NN PR proteins were extracted and purified similarly (15), except that the acetone treatment was omitted. The various tobacco proteins were identified after the preparative nondenaturing electrophoresis (10), recovered from the excised gel fragments, and then concentrated. The isolated tobacco PR proteins were submitted to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (12) for ascertaining their purities and molecular weights. Our estimates of the molecular weights of tobacco PR proteins were similar to those reported elsewhere (10,26).

Protein concentration was estimated with the Bradford reagent (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as the standard. Antiserum to PR-4d was prepared in a New Zealand white rabbit and the antibody titer, estimated by gel diffusion, was 1:20. Purified PR-4d protein at a concentration of 3 μg/ml was readily detected by this procedure.

Leaf samples for serological detection of PR-4d protein were selected and processed as follows. From plant species in which only local necrotic lesions developed, the directly inoculated leaves were harvested between 6 and 9 days after inoculation. In cases such as cucumber, in which the appearance of local lesions was followed by systemic mosaic symptoms, the samples were obtained separately from the inoculated and systemically invaded leaves (25–30 days postinoculation). When symptoms other than necrosis developed, the inoculated leaves and those invaded systemically (30–40 days postinoculation) and showing distinctive symptoms were pooled to serve as a composite sample. Comparable leaf samples were obtained from the mock-inoculated plants. Harvested leaves were deribbed, cut into small segments, weighed, and stored at −17 C. These samples were allowed to thaw in 50 mM sodium acetate buffer, pH 5.0, containing 0.01% β-mercaptoethanol and 6 mM ascorbic acid (1 g of tissue per milliliter of buffer), homogenized, strained through cheesecloth, and clarified by centrifugation (15,600 g, 10 min). A 200-μl sample (undiluted or appropriately diluted) was heat-denatured in SDS and then electrophoresed in SDS-containing polyacrylamide gel slabs (12). The proteins were transferred to nitrocellulose membrane by electroblotting (28). Immunodetection was performed with anti-PR-4d-serum and goat anti-rabbit IgG horseradish peroxidase conjugate followed by treatment with 4-chloro-1-naphthol (Bio-Rad Laboratories). Samples from the chemically treated lima bean cultivar Nemagreen were analyzed similarly. The use of PR-4d IgGs purified by ammonium sulfate precipitation (27) or by chromatography on CM Affigel Blue (Bio-Rad Laboratories) offered no advantage over the use of the unfractionated antiserum in the immunodetection tests. Extracts of plant species that initially scored negative for PR-4d were treated with 60% ammonium sulfate, and the precipitated proteins were dissolved in 1/20th the original volume and retested for PR-4d presence by immunoblotting. Rabbit preimmune serum served as the control.

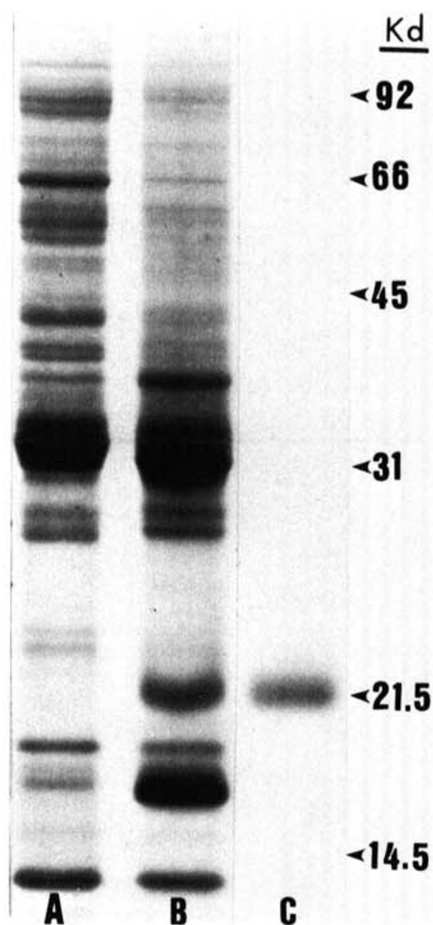

Fig. 1. Denaturing electrophoretic analysis of proteins (soluble at pH 5.0) from primary leaves of Pinto bean infected with tobacco ringspot virus and healthy leaves and of the purified PR-4d protein. Total protein (150 μg) from healthy (A) and infected leaves (B) and 10 μg of purified PR-4d protein (C) were analyzed with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using Tris-glycine-SDS buffer (pH 8.3) and then stained with Coomassie Brillant Blue. The positions of the molecular weight marker proteins are indicated along the right margin.

RESULTS

Denaturing electrophoresis of Pinto bean PR-4d protein. Purified PR-4d protein migrates as a single band in the denaturing gels with an apparent molecular weight of 21,000 (Fig. 1C). Extract from TRSV-infected Pinto bean leaves, but not that from healthy leaves, contains an abundant quantity of a protein that comigrates with the purified PR-4d (Fig. 1B and C).

Immunodetection of PR-4d-type protein in TRSV-infected plants. Observations concerning PR-4d induction in relation to the types of symptoms produced in the various plant species are summarized in Table 1.

PR-4d was not detected in the leaves of mock-inoculated plant species except in sugar beet, which contained trace quantities of the protein; however, the PR-4d amount was increased markedly (more than 20-fold) in the TRSV-infected leaves.

In most legumes, PR-4d was also detectable by the gel diffusion method, but in the nonlegumes, it was detectable by Western blotting only. The presence of TRSV coat antigen in all of the above-mentioned species was confirmed with immunodiffusion.

Induction of PR-4d-type protein with chemicals. Treatment with mercuric chloride, ethephon, and benzoic acid induced PR-4d protein in leaves of lima bean cultivar Nemagreen (Table 2). Trace amounts of this protein also were detected in plants sprayed with indole-3-acetic acid and abscisic acid, but not in plants treated with cupric chloride. Considerable necrosis developed on leaves treated with mercuric chloride or cupric chloride, but other chemicals caused no visible damage.

Immunological relationship between bean PR-4d and tobacco PR-5 proteins. Leaf extracts from TMV-infected *N. glutinosa* or *N. tabacum* 'Samsun NN,' but not from the comparable uninfected plants, reacted strongly with antiserum to bean PR-4d in the Western blots (Fig. 2A–E). Of the various tobacco PR proteins tested individually, only PR-5 reacted with anti-PR-4d-serum (Fig. 2F–I). No reaction was indicated between tomato P14 protein and anti-PR-4d-serum in the Western blots (data not presented).

The Pinto PR-4d and tobacco PR-5 proteins were reciprocally immunoreactive (Fig. 3, panels II and III). There was no serological cross-reactivity between PR-1a and tobacco PR-5 or Pinto PR-4d protein (Fig. 3, panel I). When immunoblots of the extracts of selected TRSV-infected plant species that contained PR-4d (Table 1, *Gomphrena globosa, Chenopodium amaranticolor*, and lima bean cultivar Nemagreen) were reacted with antisera to tobacco PR-1 or PR-2, no reaction was observed.

A gel diffusion test (Fig. 4) also showed that Pinto bean PR-4d and tobacco PR-5 proteins are serologically related. However, lack of confluence of the precipitin bands indicated that these proteins are not identical.

DISCUSSION

The Pinto bean PR-4d-type proteins were induced in most plant species that developed necrosis following TRSV infection. However, there was no correlation between the types of symptoms produced and the presence of this protein. For instance, PR-4d was present in lima bean cultivars Nemagreen and Dixie; whereas the former reacted with the production of local lesions, mosaic or mottle-type symptoms were produced in the latter. Further, PR-4d was synthesized in *Tetragonia expansa* and *P. polyanthus* leaves that developed only mosaic or mottle-type symptoms. Lack of any correlation between PR-4d induction and types of symptoms produced was also evident in lima bean plants treated with chemicals (Table 2). Apparently, PR-4d production and tissue necrosis are independent events. TRSV infection of *Cucurbita pepo, Pisum sativum,* and *Vicia faba* resulted in a rapid lethal necrosis with no PR-4d production. Possibly, as a consequence of rapid collapse and cell death, PR-4d failed to accumulate in these species to a detectable level or was not synthesized at all.

Infection of *P. vulgaris* 'Saxa' with alfalfa mosaic virus or treatment with mercuric chloride induces four acidic PR proteins ranging in molecular weights from 17,000 to 33,500 (7). One of these proteins, Saxa PR-4, shows some resemblance to Pinto PR-4d and tobacco PR-5 (23,25) proteins; Saxa PR-4 is rich in cystine (10.7%) and low in histidine (1.7%). However, the aspartic acid content of Saxa PR-4 (7%) is only one half that of Pinto PR-4d (23) or tobacco PR-5 (25), despite the fact that Saxa PR-4 has a larger mass (33.5 kDa). Further, Saxa PR-4 possesses a greater mobility (R_f = 0.65) in the alkaline non-denaturing gels than Pinto PR-4d (23) or tobacco PR-5 (10) proteins. Like Pinto PR-4d, Saxa PR-4 shows no serological relationship with tobacco PR-1 (7). Recently, Awade et al (2)

TABLE 1. Symptoms produced and presence or absence of bean PR-4d-type protein in plants infected with tobacco ringspot virus (TRSV)

Plant species	Symptoms	Presence or absence of PR-4d protein[a]
Family Aizoaceae		
Tetragonia expansa (New Zealand spinach)	Generalized chlorosis and yellow ring spots; nonnecrotic	+
Family Amaranthaceae		
Gomphrena globosa	Bright red necrotic lesions followed by systemic mosaic	+
Family Chenopodiaceae		
Beta vulgaris (sugar beet)	Local red necrotic lesions followed by leaf curling and systemic necrosis	+
Chenopodium amaranticolor	Brown necrotic local lesions	+
C. quinoa	Light brown necrotic lesions	+
Family Compositae		
Calendula officinalis	Mosaic, ring spots, and etching; nonnecrotic	−
Family Curcurbitaceae		
Cucumis sativus (cucumber)	Necrotic lesions followed by yellow mosaic	+
Cucurbita pepo (pumpkin)	Necrotic local lesions followed by rapid systemic necrosis	−
Family Leguminosae		
Canavalia ensiformis (jack bean)	Dark brown local lesions	+
Phaseolus acutifolius (Tepary bean)	Circular dark brown local lesions	+
P. angularis (azuki bean)	Mosaic or mottle; non-necrotic	+
P. coccineus	Mild mottle and chlorosis; nonnecrotic	−
P. lunatus cv. Dixie (lima bean)	Brilliant yellow mosaic; nonnecrotic	+
P. lunatus cv. Nemagreen[b]	Dark brown or purple local lesions and systemic necrosis	+
P. polyanthus	Mild mosaic; nonnecrotic	+
P. trichocarpus	Local necrotic lesions and systemic necrosis	+
P. vulgaris cv. Pinto	Local necrotic lesions; systemic mosaic and necrosis	+
Pisum sativum cv. Wando (pea)	Local necrotic lesions followed by systemic necrosis and rapid collapse	−
Vicia faba cv. Fava Bush (broad bean)	Local and systemic necrosis, rapid wilting, and death	−
Vigna aconitifolia	Dark brown necrotic lesions and systemic mottle	+
V. marina	Purple local lesions and systemic mosaic	+
V. radiata	Brown necrotic local lesions	+
V. unguiculata (cowpea)	Dark brown local lesions and systemic necrosis	+
Family Malvaceae		
Abelmoschus esculentus (okra)	Bright yellow mosaic; nonnecrotic	−
Family Solanaceae		
Datura stramonium	Necrotic local lesions and mosaic	+
Nicotiana glutinosa	Yellow ring spots and mottle; nonnecrotic	−
N. tabacum cv. Samsun NN	Mild mosaic and chlorosis	−
Petunia hybrida	Necrotic lesions and systemic mosaic	+

[a]Ascertained by Western blotting procedure. Following denaturing electrophoresis, proteins were electroblotted to nitrocellulose membrane. Immunodetection was by using PR-4d antiserum (rabbit polyclonal; dilution 1:500) and goat anti-rabbit IgG horseradish peroxidase conjugate (dilution 1:2,000) followed by treatment with 4-chloro-1-napthtol. + = Presence; − = absence.

[b]Bean PR-4d protein was also detected in the following lima bean cultivars, all of which reacted with local lesion production upon TRSV infection: Burpee Bush, Cangreen Bush, Florida Butter, Fordhook No. 242, Henderson Bush, Jackson Wonder, King of the Garden, and Sieva Pole.

reported that Saxa PR-4 possesses chitinase activity and accumulates in the intercellular fluid; additionally, they identified two new Saxa PR proteins (28 kDa and 30 kDa) that possess 1,3-β-glucanase activity and serologically cross-react with tobacco PR-2 and maize PRm-6b proteins.

Our results demonstrate that Pinto PR-4d and tobacco PR-5 proteins are reciprocally immunoreactive. In preliminary tests, a distinctive but very faint reaction was observed between tobacco PR-5 (TL) protein and anti-PR-4d-serum in Western blots of nondenaturing gels, and this was the only protein in the intercellular tobacco fluid with which any reaction was evident; further, upon denaturing with SDS and β-mercaptoethanol, the reactivity of this protein with anti-PR-4d-serum was enhanced five- to 10-fold (W. S. Pierpoint, *personal communication*). Apparently, reduction of the disulfide bonds exposed additional sites on tobacco PR-5 (TL) protein for interacting with PR-4d antibodies. These results provide an independent corroboration of the serological relatedness of bean PR-4d and tobacco PR-5 proteins. The reactivity of the sweet-tasting protein thaumatin with antiserum to Xanthi-nc tobacco PR-R (= Samsun NN PR-5) is similarly enhanced when denatured with dithiothreitol (5).

No reaction was observed between tobacco PR-1, PR-2, or PR-4 protein and bean anti-PR-4d-serum in the immunoblotting tests. Further, tomato P14, which is serologically related to tobacco PR-1 (19), gave no reaction with anti-PR-4d-serum. Our results indicating the lack of a serological relationship between tobacco PR-5 and tobacco PR-1 proteins are similar to the findings of Kauffmann et al (11); however, an immunological relationship between these two proteins was reported by Van Loon et al (26).

Although PR proteins are induced under a variety of stress situations, a degree of specificity must exist in the recognition of a stress by plants and subsequent synthesis of these proteins. This is indicated by the observation that whereas infection of Samsun NN tobacco by TMV results in the synthesis of a PR-4d-type protein, infection by TRSV does not. Further, in *C. amaranticolor* and *G. globosa*, infection by TRSV caused induction of Pinto PR-4d-type proteins but not of tobacco PR-1 group proteins; however, infection of *C. amaranticolor* by potato virus X and salicylic acid treatment of *G. globosa* lead to the production of tobacco PR-1-type proteins (28). Similarly, of the various metal salts tested for induction of PR proteins in tobacco, only those of barium and manganese were effective (1). These observations suggest that the mechanism or mechanisms responsible for adaptation to biotic and abiotic stimuli in plants involve differential gene expression (24).

Considerable sequence homology exists between Samsun NN tobacco PR-5 and thaumatin, osmotin (a salt-adapted tobacco protein), and a maize protein possessing trypsin and α-amylase inhibiting activities (5,21,22). Tobacco PR-5 does not possess any inhibitory activity against α-amylase, trypsin, and several other proteases (11,21), nor is it a 1,3-β-glucanase (B. Fritig, *personal communication*). Consequently, the biological function of tobacco PR-5 protein remains undefined. We have detected (23) 1,3-β-

TABLE 2. Induction of bean PR-4d-type protein in chemically treated lima bean cultivar Nemagreen plants[a]

Chemical	Symptoms	Presence or absence of PR-4d protein[b]
Mercuric chloride	Chlorosis and necrotic spots	+
Cupric chloride	Extensive foliar necrosis	−
Indole-3-acetic acid	No visual symptoms	Trace amount
Ethephon	No visual symptoms	+
Abscisic acid	No visual symptoms	Trace amount
Benzoic acid	No visual symptoms	+

[a]The primary leaves of 2-wk-old plants were sprayed daily for 4 days with aqueous solutions (adjusted to pH 7.0, if necessary) of mercuric chloride (0.01%), cupric chloride (0.01%), benzoic acid (0.01%), abscisic acid (0.01%), or indole-3-acetic acid (100 μg/ml). A solution of 1 mg/ml of ethephon (2-chloroethylphosphonic acid) was rubbed on the leaves daily for 4 days. Leaves were harvested 5 days after the last treatment, thoroughly washed with water, dried, and stored at −20 C.

[b]Detected by Western blotting procedure using anti-PR-4d-serum and goat anti-rabbit IgG horseradish peroxide conjugate immunodetection system as described in footnote a of Table 1. + = Presence; − = absence.

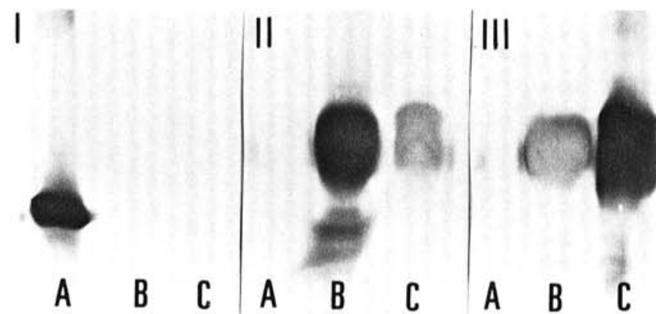

Fig. 3. Serological cross-reactivities between bean PR-4d and tobacco PR-5 (thaumatin-like) proteins. Approximately 10 μg each of purified tobacco PR-1a, our preparation (A); tobacco PR-5 (B); and bean PR-4d (C) were used in this Western blot test. The primary antibodies used were tobacco PR-1 (panel I), tobacco PR-5 (panel II), and bean PR-4d (panel III). The second antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase, and 4-chloro-1-naphthol served as the color developer.

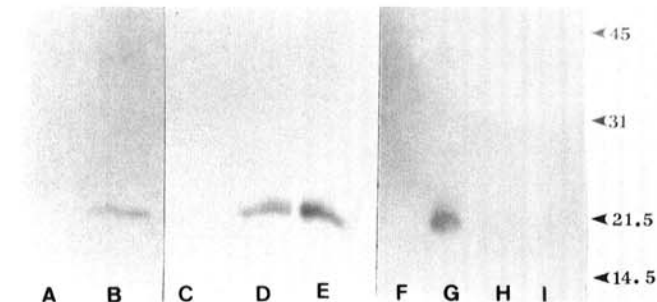

Fig. 2. Immunodetection of Pinto bean PR-4d-related proteins in plants infected with tobacco mosaic virus and its relationship with tobacco PR-5. The samples were analyzed by the Western blotting procedure using anti-PR-4d-serum and goat anti-rabbit IgG horseradish peroxide conjugate as described in footnote a of Table 1. Leaf extracts from healthy *Nicotiana glutinosa* (A), infected *N. glutinosa* (B), healthy *N. tabacum* 'Samsun NN' (C), and infected *N. tabacum* 'Samsun NN' (D); purified bean PR-4d, 2 μg (E); purified tobacco PR-4, 2 μg (F); tobacco PR-5, 2 μg (G); tobacco PR-2, 2 μg (H); and tobacco PR-1a, provided by A. Asselin, 2 μg (I).

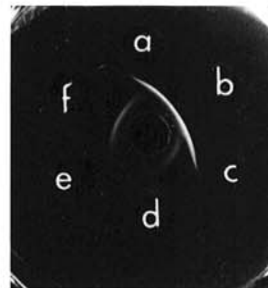

Fig. 4. Detection of tobacco PR-5-type protein by immunodiffusion in leaf extracts of virus-infected plants. The gel plates were prepared with 1% agarose in 20 mM phosphate buffer, pH 7.0. Each well was charged with a 30-μl sample, and the plates were maintained at 25 C. Central well: tobacco PR-5 (thaumatin-like) antiserum, undiluted. Peripheral wells: leaf homogenates, 1 g tissue in 1 ml of 20 mM phosphate buffer, pH 7.0, from healthy Samsun NN tobacco (a), Samsun NN infected with tobacco mosaic virus (b), Pinto bean infected with tobacco ringspot virus (c), healthy Pinto bean (d), healthy lima bean cultivar Nemagreen (e), and Nemagreen infected with tobacco ringspot virus (f).

glucanase activity associated with bean PR-4d protein. Pinto bean PR-4d and tobacco PR-5 typify proteins that are functionally diverse but structurally homologous. A sequence of five to seven amino acids is sufficient to define an antigenic site in proteins, and a common sequence of six amino acids is sufficient for serological cross-reactivity (27). Induction of proteins related structurally to PR-4d and tobacco PR-5 in divergent plant species signifies similar homeostatic adjustments in response to the biotic or abiotic stimuli. Our results extend observations that proteins analogous to the tobacco PR-5 group are synthesized in unrelated species upon viral infection or chemical injury, besides those of the tobacco PR-1 and PR-2 groups (20,28).

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