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Detection of PR 1-type Proteins in *Amaranthaceae*, *Chenopodiaceae*, *Graminae* and *Solanaceae* by Immunoelectroblotting

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SUMMARY

Pathogenesis-related (PR) proteins (M_r 14000 to 18000) serologically related to PR 1a protein from *Nicotiana tabacum* cv. Xanthi-nc were detected in infected plants or salicylic acid-treated plants of maize, barley, tomato, potato, *Solanum demissum*, *Gomphrena globosa* and *Chenopodium amaranticolor* using affinity-purified antibodies in immunoelectroblots. Only in *Go. globosa* was a protein related to PR 1a found in a healthy plant.

Pathogenesis-related (PR) proteins (Antoniw *et al.*, 1980), also known as b-proteins, were first identified in leaves of tobacco plants infected with tobacco mosaic virus (TMV) (Gianinazzi *et al.*, 1970; Van Loon & Van Kammen, 1970). It has since been shown that PR proteins are induced by other viruses, by bacteria and by fungi (Kassanis *et al.*, 1974; Ahl *et al.*, 1981; Gianinazzi *et al.*, 1980). PR proteins have also been found in many pathogen-infected plant species, including other *Nicotiana* spp., tomato, potato, cowpea, cucumber, celery, citron, *Gomphrena globosa* and *Gynura aurantiaca* (for review, see Redolfi, 1983). Therefore PR proteins are probably an important response to pathogen infection. The tobacco PR proteins have been the most intensively studied (for review, see Antoniw & White, 1983) and have been shown to be closely associated with the resistance to further infection induced by infection with TMV (Kassanis *et al.*, 1974). In tobacco, PR proteins and resistance are also induced by certain chemicals, e.g. polyacrylic acid (Gianinazzi and Kassanis, 1974), ethephon (Van Loon, 1977) and salicylic acid and related compounds (White, 1979).

The PR 1a, 1b and 1c proteins of tobacco cv. Xanthi-nc have been purified and shown to be a family of proteins with similar mol. wt. and amino acid compositions (Antoniw *et al.*, 1980). An antiserum to PR 1a protein was used to show that the PR 1a, 1b and 1c proteins, but not the other eight PR proteins in cv. Xanthi-nc, are closely related serologically (Antoniw *et al.*, 1985). Ahl *et al.* (1985) showed that the PR proteins b0, b1 and b3 in *N. sylvestris*; b2 in *N. tomentosiformis*, and b1″ in *N. glutinosa* and *N. debneyi* are also serologically related to the PR 1 group of proteins in tobacco cv. Xanthi-nc. White (1983) reported the detection by ELISA of proteins related serologically to PR 1a in cowpea and potato following either treatment with salicylic acid or virus infection. Salicylic acid treatment of *Go. globosa* induced an increase in a protein related serologically to PR 1a and the presence of the PR 1a-related protein in potato was confirmed in double diffusion tests.

The p14 PR protein produced in viroid-infected tomato plants is also serologically related to the PR 1 group of tobacco (Nassuth & Sänger, 1986). A second, unrelated, family of serologically interrelated PR proteins, b4, b5 and b6b, has also been found in tobacco cv. Xanthi-nc (Fortin *et al.*, 1985).

In this paper we describe the use in immunoelectroblot tests of an affinity-purified antiserum prepared to the PR 1a protein from tobacco cv. Xanthi-nc to identify serologically related PR 1-

type proteins in other plants, including for the first time some from monocotyledonous families.

Plants were grown in a sand–peat compost in a glasshouse at 17 to 20 °C and had supplementary light during the winter. *N. tabacum* cv. Xanthi-nc and *Solanum demissum* Y type were inoculated with TMV and potato virus Y (PVY) respectively when 8 to 12 weeks old. Two-week-old plants of *S. tuberosum* cv. Désirée and 6-week-old *Chenopodium amaranticolor* were inoculated with potato virus X (PVX), and leaves with many well distributed necrotic lesions were harvested 1 to 2 weeks later.

Zea mays (maize) plants were inoculated at the two-leaf stage with brome mosaic virus (BMV) and the resulting necrotic areas were removed for extraction after 7 days.

Six week-old *Go. globosa* and tomato plants cv. Moneymaker were placed in trays, watered with 2·5 mM-salicylic acid and harvested after 7 days. Control plants were treated with distilled water.

Seedlings of barley cv. Georgi were inoculated with *Erysiphe graminis* (mildew) on emergence and harvested 3 weeks later at the same time as control plants.

PR 1a protein was purified as described by Antoniw *et al.* (1980). Protein extractions at neutral pH were done by crushing leaf tissue in a mortar and pestle in 1 ml/g of SDS–PAGE sample buffer (125 mM-Tris–HCl, 10% SDS, 10% 2-mercaptoethanol, 10% glycerol, pH 6·8), filtering the extract through cheesecloth, and boiling it for 2 min.

Leaf material was extracted at acid pH by trituration in 1 ml/g of extraction buffer (84 mM-citric acid, 32 mM-Na_2HPO_4, 6 mM-L-ascorbic acid pH 2·8) in a pestle and mortar and then centrifuged for 5 min at 8000 ***g***. The supernatant fraction was applied to a column (25 × 1 cm) of Sephadex G-50 (fine) equilibrated in resuspension buffer (50 mM-Tris–HCl, 1 mM-EDTA pH 8·0) at 25 °C and the fractions containing protein, as judged by the A_{280} were pooled and concentrated by freeze-drying. Samples were mixed with 1 to 2 vol. SDS–PAGE sample buffer and boiled for 2 min. Denatured samples were electrophoresed in 1·5 mm 4·5% stacking/12·5% resolving polyacrylamide gels prepared according to Laemmli (1970). Samples of 20 to 50 µl were electrophoresed at constant current.

Electroblotting was done in a Bio-Rad Trans-Blot Cell. Resolving gels were soaked in transfer buffer (375 mM-Tris, 92 mM-glycine, 20% methanol pH 8·3) and laid on wetted nitrocellulose (0·45 µm pore), clamped into a holder and immersed in the transfer tank. Blotting was overnight at 25 to 60 V. Duplicate electroblots were routinely stained for total protein with 0·1% amido black in 45% methanol : 10% acetic acid. Duplicate gels were stained with 0·1% Coomassie Brilliant Blue R250 in 40% methanol : 10% acetic acid.

Indirect enzyme-assisted immune staining of specific polypeptides in neutral pH extracts and acid extracts was done essentially as described by Rybicki & von Wechmar (1982). Blots were blocked in 3% bovine serum albumin in 10 mM-Tris–HCl, 150 mM-NaCl, pH 7·4 (blocking buffer). Affinity-purified antiserum to PR 1a from tobacco cv. Xanthi-nc was incubated in blocking buffer with blots in closed containers on a shaker at room temperature for 2 to 2·5 h. Blots were washed for 10 min on a shaker with three changes of 100 mM-Tris–HCl, 2·5 M-NaCl, pH 7·5. Immobilized PR 1a antibody was reacted with Protein A conjugated to horseradish peroxidase diluted 1 : 1000 and immobilized conjugate was detected with 4-chloro-1-naphthol (0·5 mg/ml in a 1 : 5 mixture of methanol and 50 mM-Tris–HCl, 200 mM-NaCl, pH 7·4 containing 0·015% H_2O_2).

Excised strips of nitrocellulose with a specific horizontal band of PR 1a protein, localized by immune staining of a vertical edge lane of a blot, were processed for affinity purification of antibody as described by Olmsted (1981) and Rybicki (1984, 1986). Strips were blocked, incubated in diluted antiserum, washed and then the bound antibody was eluted with 0·1 M-glycine–HCl pH 2·9, which was then neutralized by the addition of 0·1 M-NaOH . The procedure was repeated three times and the pooled antibody preparations were dialysed against water and concentrated by lyophilization.

Affinity-purified antibodies to the PR 1a protein from tobacco cv. Xanthi-nc were used in immunoblots to detect PR 1a and serologically related proteins in various plant extracts. Neither acid nor neutral pH extracts from healthy tobacco cv. Xanthi-nc reacted in blots (Fig. 1, lanes 2 and 4 respectively). Using ELISA, Antoniw *et al.* (1985) were able to detect small amounts (1

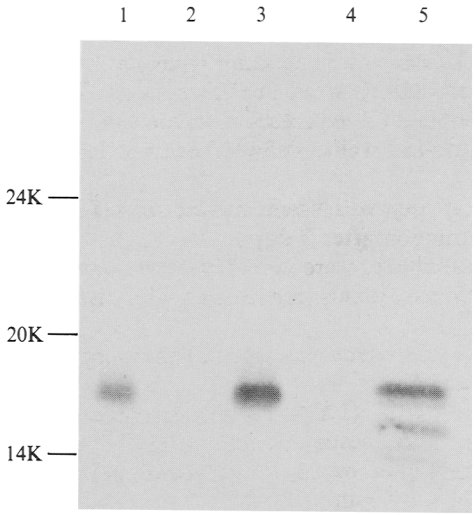

Fig. 1

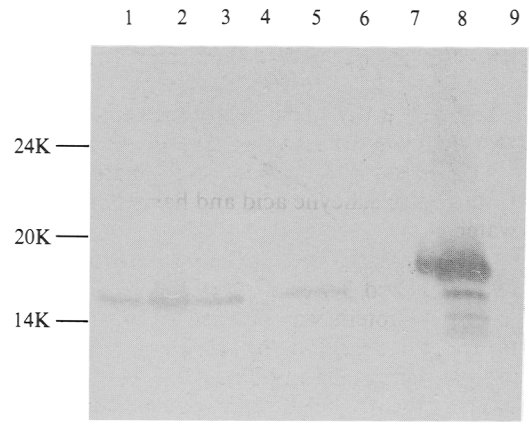

Fig. 2

Fig. 1. Immunoblot of PR 1a-related proteins. Lane 1, purified PR 1a protein from Xanthi-nc; lane 2, low pH extract from healthy Xanthi-nc; lane 3, low pH extract from TMV-infected Xanthi-nc; lane 4, neutral pH extract from healthy Xanthi-nc and lane 5, neutral pH extract from TMV-infected Xanthi-nc. Mol. wt. markers were α-lactalbumin (14K), soybean trypsin inhibitor (20K) and PMSF-treated trypsinogen (24K) and migrated to the indicated positions.

Fig. 2. Immunoblot of PR 1a-related proteins. Low pH extracts from: lane 1, *Go. globosa*; lane 2, *Go. globosa* treated with salicylic acid; lane 3, *S. demissum* infected with PVY; lane 4, *S. demissum*; lane 5, tomato treated with salicylic acid; lane 6, tomato; and lane 7, purified PR 1a protein from Xanthi-nc. Neutral pH extracts from: lane 8, Xanthi-nc infected with TMV and lane 9, Xanthi-nc. Mol. wt. markers were as for Fig. 1.

ng/g of leaf) of PR 1a protein in healthy plants, but were unable to detect such small quantities using immunoblots.

Both neutral and acid extracts from TMV-infected plants contained a protein of approximate M_r 17000 that reacted strongly with PR 1a antiserum (Fig. 1, lanes 5 and 3). This protein comigrated with purified PR 1a protein from tobacco cv. Xanthi-nc (lane 1). The mol. wt. of PR 1a has been estimated at approximately 14500 by analytical; ultracentrifugation and amino acid analysis (Antoniw *et al.*, 1980). However, PR 1 proteins 1a, 1b and 1c behave anomalously during electrophoresis in denaturing conditions and comigrate with an apparent mol. wt. of approximately 16500 (Pierpoint, 1986).

The neutral extract from TMV-infected plants (Fig. 1, lane 5) gave additional bands of lower mol. wt. which were not present in the acid extract (lane 3). These bands did not appear when pre-immune serum was used or when serum was omitted altogether, suggesting that they are not caused by non-specific reactions or by natural plant peroxidases but are either breakdown products of PR 1a which are an artefact of extraction at this pH, or PR 1-like proteins which are soluble at pH 7·0 but insoluble at low pH.

Fig. 2 shows that a protein serologically related to PR 1a occurred in an acid extract from *S. demissum* Y type leaves in which PVY infection had been localized (lane 3) but not in healthy leaves (lane 4). This protein had a similar mol. wt. (15000) to a reactive protein found in tomatoes treated with salicylic acid (lane 5), and is probably identical to the P14 protein induced in tomato by infection with potato spindle tuber viroid. P14 is also serologically related to PR 1 proteins in tobacco cv. Samsun NN and *N. glutinosa* (Nassuth & Sänger, 1986). The nucleotide sequence of the mRNA of the tomato P14 protein is not known but the 60% homology in the amino acid sequence may account for its serological relationship to the tobacco PR 1 proteins (Cornelissen *et al.*, 1986).

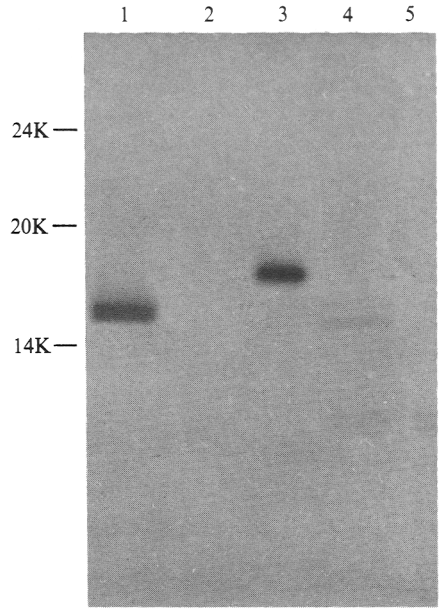

Fig. 3

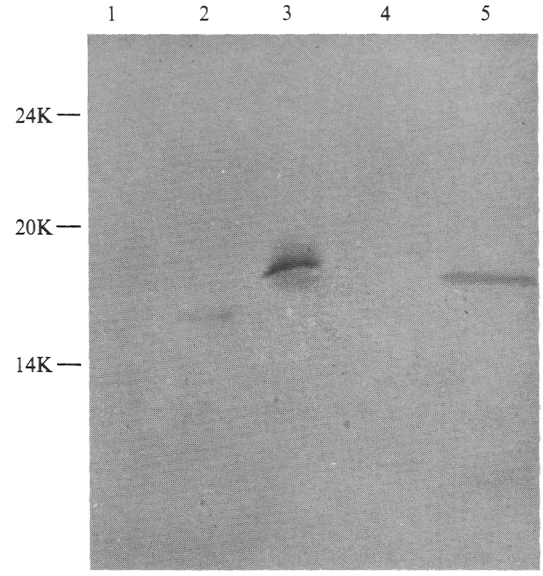

Fig. 4

Fig. 3. Immunoblot of PR 1a-related proteins. Neutral pH extracts from: lane 1, *C. amaranticolor* infected with PVX; lane 2, *C. amaranticolor*; lane 3, purified PR 1a protein from Xanthi-nc; lane 4, potato infected with PVX and lane 5, potato. Mol. wt. markers were as in Fig. 1.

Fig. 4. Immunoblot of PR 1a-related proteins. Neutral pH extracts from: lane 1, barley; lane 2, barley infected with mildew; lane 3, purified PR 1a protein from Xanthi-nc; lane 4, maize and lane 5, maize infected with BMV. Mol. wt. markers were as for Fig. 1.

Untreated plants of *Go. globosa* also contained a M_r 15000 protein (Fig. 2, lane 1) that reacted with the antiserum to PR 1a and which increased in amount when *Go. globosa* plants were treated with salicylic acid (lane 2). Pennazio & Redolfi (1980) showed that, when sprayed onto leaves of *Go. globosa*, acetylsalicylic acid induced resistance to tomato bushy stunt virus but did not induce the synthesis of new proteins. Their failure to detect a new protein associated with resistance can be explained if such a protein was constitutively produced in healthy plants but increased upon acetylsalicylic acid treatment as is the case with the PR 1a-related protein. White *et al.* (1986) have shown that in acetylsalicylic acid-treated tobacco plants, both the concentration of PR protein and resistance increases with increasing acetylsalicylic acid concentration.

Fig. 3 shows an immunoblot of neutral extracts from PVX-infected and healthy potato (lanes 4 and 5) and from *C. amaranticolor* locally infected with PVX (lane 1). A healthy *C. amaranticolor* extract and purified PR 1a protein are in lanes 2 and 3. PVX-infected potato had a PR 1a-related protein with a mol. wt. of approximately 14500 and is probably the PR 1a-related protein in potato detected by ELISA and double diffusion tests by White (1983). Large amounts of a M_r 15000 reactive protein were detected in *C. amaranticolor* infected with PVX.

Neutral pH extracts of healthy and mildew-infected barley (lanes 1 and 2) and healthy and BMV-infected maize (lanes 4 and 5) are shown in Fig. 4. BMV-infected maize (lane 5) contained a reactive protein with a similar mol. wt. (17000) to purified PR 1a (lane 3). Infection of barley cv. Georgi with a compatible strain of mildew induced the plant to produce a M_r 15000 protein related to PR 1a. This is the first report of PR 1-type proteins or PR proteins of any sort in monocotyledonous plants. Their existence in both monocotyledonous and dicotyledonous plants and the retention of their serological relationship during evolution indicates an important, if as yet undefined role for PR proteins. PR 1-type proteins were not found in any healthy extracts

other than that from *Go. globosa* but this plant's constitutive production of PR 1 proteins may be similar to that seen in the intraspecific hybrid between *N. glutinosa* and *N. debneyi* (Ahl *et al.*, 1985). The use of immunoblots together with specific antisera to PR proteins purified from tobacco should help in both the detection of PR proteins in a wide range of plants, and the identification of serologically related families of PR proteins. The technique is simple, sensitive and specific: PR 1a-related proteins were detected in unconcentrated extracts of all plants tested and their mol. wt. were estimated by reference to stained duplicate blots or gels. Moreover, because detection is by means of indirect immunoassay, a wider range of related proteins can be detected than by use of the equally sensitive double antibody sandwich-ELISA technique (Rybicki & von Wechmar, 1982).

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