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Antoniw, J. F., Ritter, C. E., Pierpoint, W. S. and Vanloon, L. C. 1980. Comparison of Three Pathogenesis-related Proteins from Plants of Two Cultivars of Tobacco Infected with TMV . *Journal of General Virology*. 47 (1), pp. 79-87.

The publisher's version can be accessed at:

- <https://dx.doi.org/10.1099/0022-1317-47-1-79>

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Comparison of Three Pathogenesis-related Proteins from Plants of Two Cultivars of Tobacco Infected with TMV

By J. F. ANTONIW,¹ C. E. RITTER,² W. S. PIERPOINT¹
AND L. C. VAN LOON²

¹*Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts AL5 2JQ, U.K. and*

²*Department of Plant Physiology, Agricultural University, Wageningen, The Netherlands*

(Accepted 5 October 1979)

SUMMARY

A procedure is described for purifying the b_1 , b_2 and b_3 proteins from leaves of *Nicotiana tabacum* cv. Xanthi-nc, a cultivar reacting hypersensitively to tobacco mosaic virus (TMV). All three proteins consist of a single polypeptide, have similar mol. wt. of about 14 200 but differ in charge. In contrast, the b_4 protein differs from b_1 to b_3 in both charge and size. The same procedure purifies the proteins IV, III and II from TMV-infected leaves of *N. tabacum* cv. Samsun NN. The Samsun NN proteins IV to II resemble the b_1 to b_3 proteins from Xanthi-nc in electrophoretic mobility in polyacrylamide gels and in mol. wt.; b_1 , IV and III have similar amino acid compositions.

It is suggested that these proteins be called pathogenesis-related proteins (PRs) and a unified system of nomenclature is proposed which groups similar proteins.

INTRODUCTION

Nicotiana tabacum cv. Xanthi-nc or Samsun NN plants infected with certain viruses produce at least four host proteins that cannot be found in uninfected plants (Kassanis *et al.* 1974; Van Loon, 1975). These pathogenesis-related proteins, when observed in Xanthi-nc, are called b_1 to b_4 in order of decreasing electrophoretic mobility in polyacrylamide gels (Gianinazzi *et al.* 1970); those in extracts from Samsun NN are called I to IV in order of increasing mobility (Van Loon & Van Kammen, 1970). The electrophoretic properties of the two groups of proteins are superficially similar but they have not been critically compared.

There are two conflicting explanations for the differences between the proteins from Xanthi-nc, both based on the electrophoretic examination of partially purified preparations. Gianinazzi *et al.* (1977) reported that b_1 , b_2 and b_3 co-migrated in the presence of SDS as a single band with an estimated mol. wt. of 16 000, whereas b_4 migrated as a single band of mol. wt. 29 000; they suggested that b_2 and b_3 were polymers of b_1 . Rohloff & Lerch (1977), however, using similar experimental procedures, reported that b_1 and b_2 co-migrated in the presence of SDS with an estimated mol. wt. of 13 400, whereas b_3 and b_4 dissociated to give three components with mol. wt. of 35 500, 60 000 and 64 500; electrophoresis in gels of different concentrations of acrylamide, analysed as described by Hedrick & Smith (1968), led these authors to conclude that b_1 and b_2 are one pair of charge isomers and b_3 and b_4 are another pair. In an effort to resolve these conflicting views, we have re-examined

the electrophoretic behaviour of these proteins and have also compared the proteins produced by the two cultivars of tobacco.

METHODS

Plant material. *Nicotiana tabacum* cv. Xanthi-nc and Samsun NN plants were grown in 13 cm diam. pots using a sand-peat compost, in a glasshouse at 20 to 25 °C with supplementary lighting during the winter months. When the plants were about 8 weeks old the leaves were dusted with carborundum and inoculated with a solution of type-strain TMV (10 µg/ml) using a gauze pad.

Protein extraction. Seven days after inoculation, leaves covered with local lesions were removed, deribbed and homogenized with an equal volume (v/w) of extraction buffer in a Kenwood blender at high speed for 2 min. The extraction buffer (buffer A; Antoniw & Pierpoint, 1978) contained 84 mM-citric acid, 32 mM- Na_2HPO_4 , 14 mM-2-mercaptoethanol and 6 mM-L-ascorbic acid at a final pH of 2.8. The homogenate was filtered through four layers of muslin and centrifuged at 10000 g for 15 min. The supernatant was used as the crude protein solution.

Protein separation. For gel filtration, preparative (84 × 5 cm) and analytical (140 × 1 cm) columns of Sephadex G-50 (fine) were prepared and equilibrated in buffer C (50 mM-tris-HCl, 1 mM-EDTA, 3 mM-2-mercaptoethanol, final pH 8.0 at 25 °C) as described by Antoniw & Pierpoint (1978).

For ion-exchange chromatography, DEAE-cellulose (DE-52, Whatman) was equilibrated in buffer C using the manufacturer's recommended procedure and poured into a column (12 × 2.5 cm).

Up to 200 ml of crude protein solution was chromatographed on the preparative Sephadex column. The b_2 and b_3 proteins eluted in the same fractions as the b_1 protein and were pooled and adsorbed on to the column of DEAE-cellulose. The b proteins were eluted with a linear gradient of 0 to 300 mM-NaCl in buffer C and the fractions containing b_1 , b_2 and b_3 proteins were pooled and concentrated by lyophilization. After dissolving in a small volume of buffer C and dialysing against the same buffer, the proteins were finally purified by gel filtration on the analytical Sephadex column.

Polyacrylamide gel electrophoresis (PAGE). Proteins were analysed in 10% (w/v) polyacrylamide gels at pH 8.9, or in gels containing 0.1% sodium dodecyl sulphate (SDS) at pH 7.2; the gels were stained for protein using Coomassie brilliant blue G250, destained, photographed and scanned using a Beckman model 25 spectrophotometer (Antoniw & Pierpoint, 1978).

Crude protein solutions were also analysed, following extensive dialysis against buffer C, by electrophoresis on gels of 8.75, 10.0, 11.25, 12.5, 13.75 and 15.0% (w/v) acrylamide using the pH 8.9 buffer system. These gels were stained for protein, scanned at 610 nm and the log Rf of each protein band was plotted against the acrylamide concentration as described by Hedrick & Smith (1968). The lines of best fit for each set of points were calculated by the method of least squares using a computer programme which also used the F-test to determine whether there was a significant difference at the 5% level of confidence between the slopes of the straight lines obtained for all pairs of proteins (Antoniw *et al.* 1979). Moreover the programme compared the data for any two proteins to see if, within the accuracy of the data, they could be represented by a single straight line.

Ultracentrifugation. This was done in an MSE Centriscan 75 ultracentrifuge. The protein samples were dialysed extensively against buffer C and the mol. wt. was determined on triplicate samples of 0.15 mg protein/ml by the low speed sedimentation equilibrium method (MSE Technical Publication no. 73) at 20 °C and 25000 rev/min. The density of

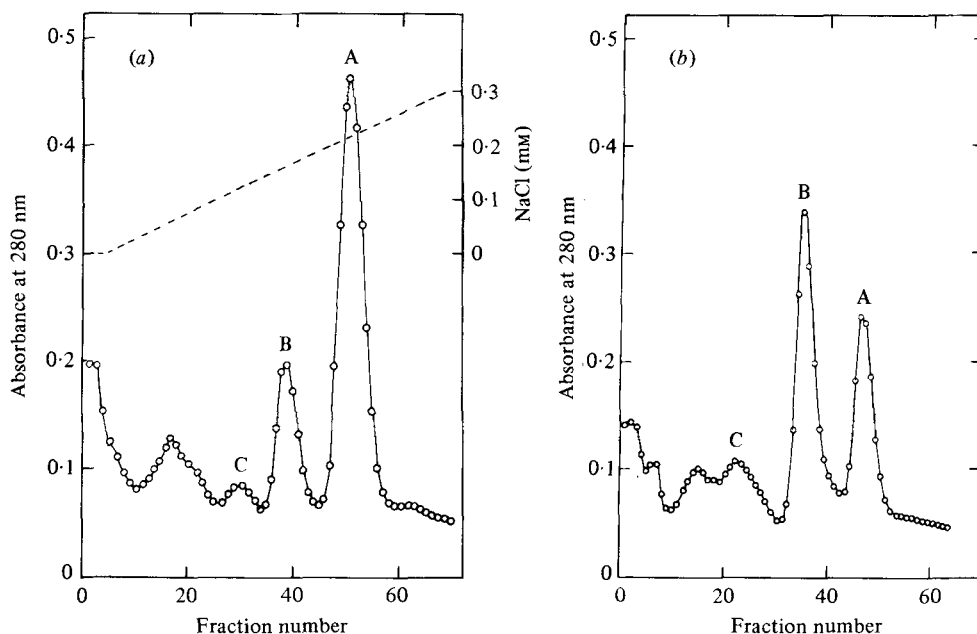


Fig. 1. Chromatography of crude protein solutions derived from TMV-infected leaves of tobacco cultivars. Crude protein extracts of (a) Xanthi-nc and (b) Samsun NN were absorbed on to a column (12×2.5 cm) of DEAE-cellulose and eluted in 8 ml fractions with a linear gradient of NaCl (---) as described in Methods. Peaks of eluted protein labelled A, B and C in (a) correspond to b_1 , b_2 and b_3 and those in (b) correspond to IV, III and II respectively.

the buffer was determined pycnometrically. The partial specific volumes of the proteins were calculated from their amino acid compositions, where known, as described by Cohn & Edsall (1943) or assumed to be the same as that of the b_1 protein.

Protein hydrolysis and analysis. Samples of protein were dialysed against distilled water and then lyophilized. Duplicate samples were hydrolysed under nitrogen in 6M-HCl containing 10 mM-phenol for 24, 48 and 72 h at 110 °C. The samples were analysed with a Technicon amino acid analyser. Protein composition was expressed as residues of each amino acid per 100 residues; the average standard deviation of these values between analyses of b_1 done at different times was ± 1 residue.

RESULTS

Purification of b proteins from Xanthi-nc

The b_1 protein was purified by Antoniwi & Pierpoint (1978) from TMV-infected Xanthi-nc tobacco by a process involving extraction at pH 2.8, gel filtration on Sephadex G-50 (fine) and adsorption on to DEAE-cellulose. The b_2 and b_3 proteins co-purified with b_1 up to the stage where b_1 was specifically eluted from the DEAE-cellulose column with 200 mM-NaCl. This procedure was modified so that after the sample was applied, protein was eluted by a linear NaCl gradient in buffer C. The eluate had an absorbance profile shown in Fig. 1(a), and examination of the separated fractions by PAGE showed that peaks A, B and C consisted of almost pure b_1 , b_2 and b_3 respectively. These were further purified by gel filtration on the analytical column of Sephadex G-50 (fine), after which each protein fraction gave a single band on PAGE both in the absence (Fig. 2) or presence (Fig. 3) of SDS.

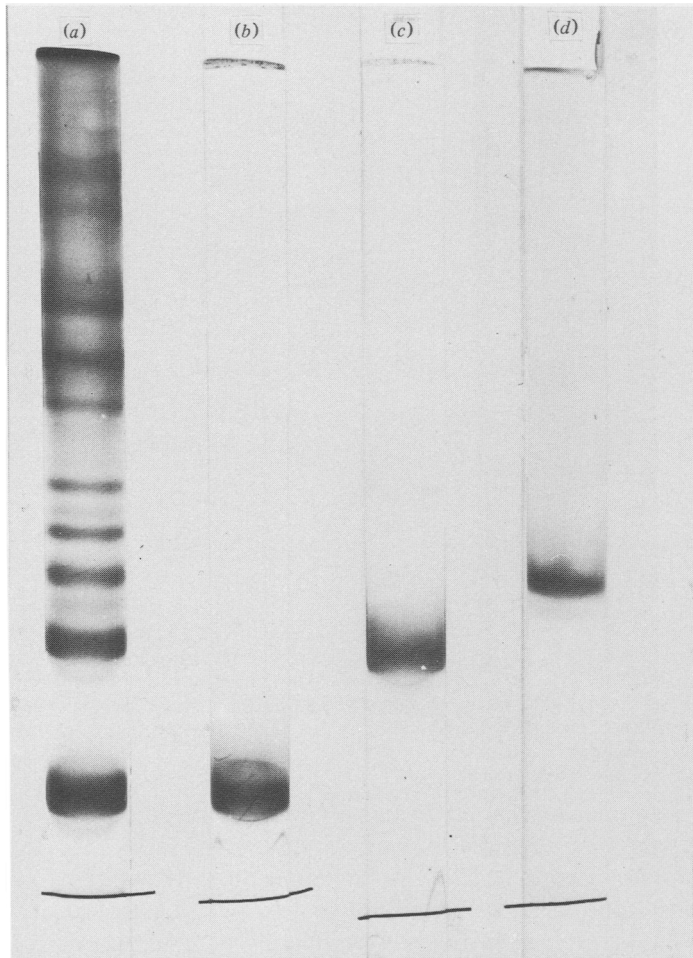


Fig. 2. Gel electrophoresis of extracts of TMV-infected leaves of Xanthi-nc tobacco and of *b* proteins purified from that extract. (a) Crude protein solution, (b) purified b_1 , (c) purified b_2 and (d) purified b_3 . Gel was of 10% polyacrylamide at pH 8.9.

Mol. wt. of the b proteins

The three proteins eluted similarly from the gel filtration columns ($K_{av} = 0.25 \pm 0.01$) and co-migrated on PAGE in the presence of SDS (Fig. 3). This suggests that they have similar mol. wt. which were estimated to be around 15000 (see also Antoniw & Pierpoint, 1978).

Low speed sedimentation equilibrium analysis of the purified *b* proteins confirmed the mol. wt. estimates. The plots of $\log c$ against R^2 were linear and the mol. wt. calculated from the slopes of the graphs gave average values of b_1 as 14200 ± 250 (five replicates), b_2 as 14200 ± 150 (three replicates) and b_3 as 14100 ± 130 (two replicates).

Purification of components IV to II from Samsun NN and comparison with b proteins

Electrophoresis of mixtures of crude protein extracts of infected Xanthi-nc and Samsun NN in 10% acrylamide gels showed that b_1 co-migrated with component IV, b_2 with III, b_3 with II and b_4 with I (Fig. 4) suggesting that the proteins from these two sources are

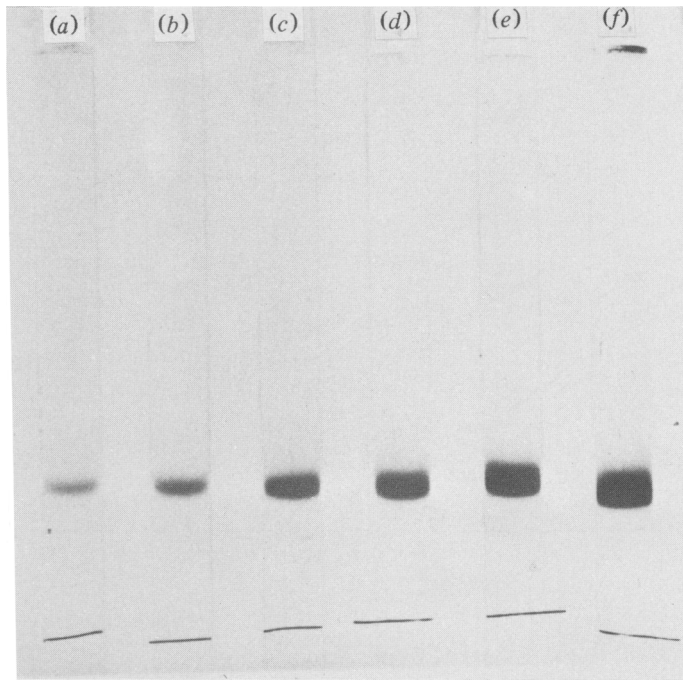


Fig. 3. Gel electrophoresis of purified b proteins in the presence of SDS. (a) b_1 , (b) b_2 , (c) b_3 , (d) $b_1 + b_3$, (e) $b_2 + b_3$, and (f) $b_1 + b_2 + b_3$. Gels were of 10% polyacrylamide containing SDS (0.1%) at pH 7.2.

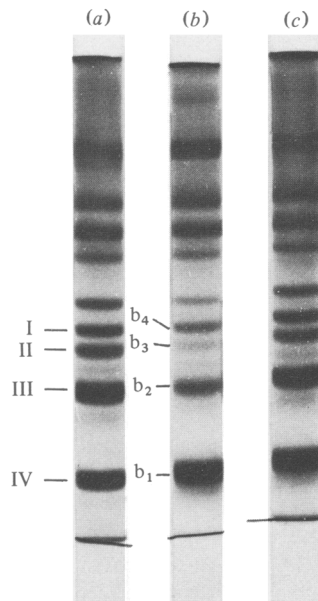


Fig. 4. Electrophoresis of extracts of TMV-infected leaves of Xanthi-nc and Samsun NN in polyacrylamide (10%) gels. (a) Crude protein solution from Samsun NN, (b) crude protein in solution from Xanthi-nc and (c) a mixture of crude protein from both cultivars.

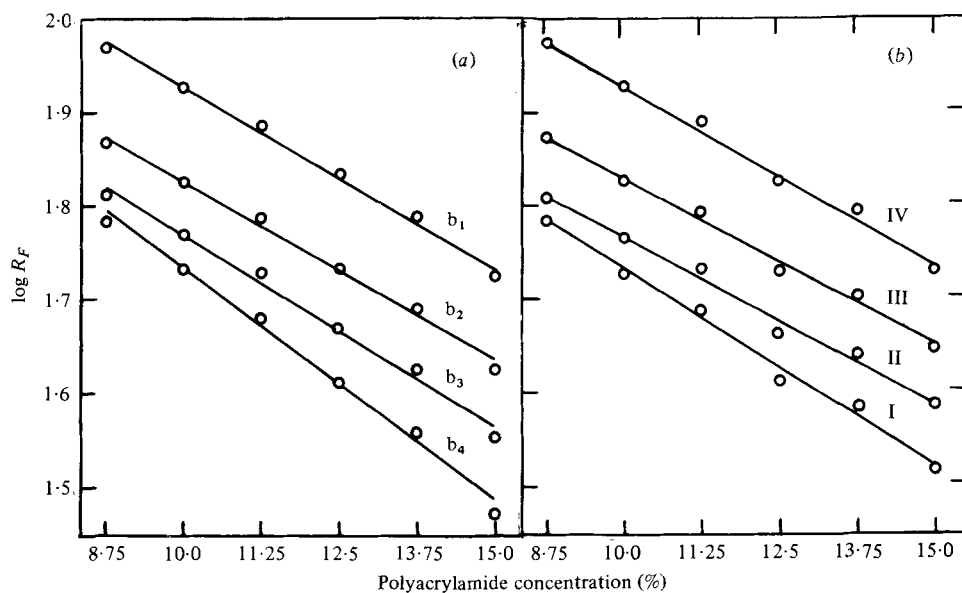


Fig. 5. Data from the electrophoresis in polyacrylamide gels of (a) the proteins b_1 to b_4 in crude extracts of Xanthi-nc and (b) proteins IV to I in crude extracts of Samsun NN, plotted as described by Hedrick & Smith (1968).

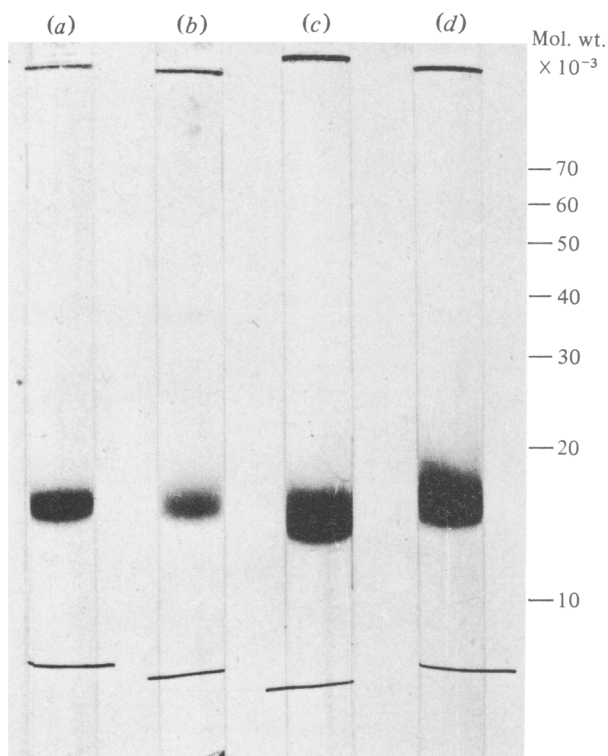


Fig. 6. Gel electrophoresis of purified proteins from Samsun NN tobacco in the presence of SDS. (a) protein IV, (b) protein III, (c) protein II and (d) a mixture of IV, III and II. Electrophoresis was in 10% polyacrylamide gels containing 0.1% SDS at pH 7.2. The scale on the right is derived from the migration of marker proteins: bovine serum albumin, ovalbumin, cytochrome *c* and TMV coat-protein.

Table 1. Amino acid composition of virus-induced proteins (mol/100 mol)

	b ₁ (Xanthi-nc)	IV (Samsun NN)	III (Samsun NN)
Aspartic acid	15.5	15.0	15.8
Threonine	4.1	3.6	4.0
Serine	6.6	4.7	4.8
Glutamic acid	13.4	14.6	13.0
Proline	3.5	3.2	4.2
Glycine	9.0	9.0	9.6
Alanine	11.2	11.3	10.0
Valine	9.4	12.0	13.1
Methionine	0.9	0.9	1.0
Isoleucine	0.6	0.0	0.9
Leucine	4.2	4.4	4.4
Tyrosine	8.1	8.8	7.9
Phenylalanine	0.9	0.7	0.8
Histidine	4.0	5.2	4.6
Lysine	2.1	1.7	2.6
Arginine	3.6	3.7	3.2

the same. Electrophoretic patterns of crude protein extracts from Xanthi-nc and Samsun NN in gels of different concentrations of acrylamide, were analysed by plotting $\log R_F$ of the bands of induced protein against acrylamide concentration (Fig. 5). Statistical analysis of the data for these plots indicated that the slopes of the lines for b₁, b₂ and b₃ from Xanthi-nc were not significantly different from one another, thus confirming that these three proteins have similar mol. wt. and are separated electrophoretically because of their different charges. The slope of the line for the b₄ protein however showed that its mol. wt. was significantly larger than those of the other proteins. The same analysis for the induced proteins from Samsun NN also indicated that components IV, III and II have similar mol. wt. and different charges, whereas that of component I is significantly larger. Furthermore the statistical analysis indicated that there was no significant difference between the lines representing b₁ and component IV, b₂ and III, and b₃ and II respectively. The lines for b₄ and I did not appear to be identical. However it is difficult to locate precisely the relatively faint b₄ and I bands as there are other proteins present in this region of the gel.

The purification procedure used for the b proteins was applied to extracts of TMV-infected Samsun NN leaves and yielded purified preparations of IV, III and II (Fig. 1). Analysis by PAGE in the presence of SDS again showed that each of the proteins IV to II gave a single band and that they co-migrated with b₁ to b₃ (Fig. 6). Proteins IV to II also eluted in similar volumes from the analytical gel filtration column ($K_{av} = 0.25 \pm 0.01$). Low speed sedimentation equilibrium analysis of the purified proteins on the analytical ultracentrifuge provided estimates of the mol. wt. of component IV as 15100 ± 160 (three replicates), III as 15000 ± 30 (three replicates) and II as 14100 ± 300 (three replicates).

The amino acid compositions of IV and III are similar, and only differ appreciably from b₁ in their content of serine and valine (Table 1).

DISCUSSION

Our evidence suggests that b₁, b₂ and b₃ from Xanthi-nc each consist of a single polypeptide chain of similar size, which are 'charge isomers' in the terminology of Hedrick & Smith (1968). The b₄ protein has not yet been purified to the state where its peptide composition can be determined with certainty, but it clearly differs from the other three proteins in both charge and size (Fig. 5). These conclusions agree with the suggestion

of Rohloff & Lerch (1977) that b_1 and b_2 are charge isomers, but do not agree with their suggestion that b_3 contains three polypeptides and is a charge isomer of b_4 . Nor do they confirm the suggestion of Gianinazzi *et al.* (1977) that b_2 and b_3 are polymeric forms of b_1 . Our evidence also demonstrates that components IV to I from Samsun NN are related to each other in the same way that the b proteins are and, moreover, each one is electrophoretically similar to the corresponding protein from Xanthi-nc. The similarity in amino acid composition of b_1 , IV and III (Table 1) is consistent with this conclusion.

Because the proteins induced during pathogenesis and related situations in Xanthi-nc tobacco are very similar to those induced in Samsun NN, it is desirable to unify the nomenclature of these proteins. The fact that three of the proteins have enough in common to constitute a 'family', suggests a system of nomenclature similar to that used for histones (Rasmussen *et al.* 1962); this would emphasize 'family' relationships, and possibly functional relationships also, and would be flexible enough to accommodate other proteins of this type that have been recognized (Van Loon & Ritter, 1978; Antoniw & White, 1980). We propose therefore, that these proteins, coded for by the host plant but induced only in pathological or related situations, be called pathogenesis-related proteins or PRs, and that for each plant species or cultivar, they be numbered according to their mobility in PAGE. Thus the fastest migrating component, b_1 from Xanthi-nc tobacco becomes Xanthi-nc PR-1. However, all other PRs which resemble PR-1 in migrating as a single band with identical electrophoretic mobility in SDS gels and having similar mol. wt. should be regarded as members of the same family and called, in order of decreasing mobility, PR-1a, PR-1b, etc. Thus b_1 , b_2 and b_3 from Xanthi-nc, and IV, III and II from Samsun NN become PR-1a, PR-1b and PR-1c, for each cultivar respectively. Since b_4 and I differ from the PR-1 proteins in both size and charge, they are dissimilar and are thus designated Xanthi-nc PR-2 and Samsun NN PR-2, respectively.

The purified preparations of b_1 , b_2 and b_3 from Xanthi-nc and IV, III and II from Samsun NN are currently being analysed to find the molecular basis of the differences between the proteins from one cultivar and the extent of the similarities between the proteins from the two different cultivars.

We thank EMBO for providing a short-term fellowship to C.E.R., Mr P. B. Norbury for technical assistance, Maureen M. Leggatt for the amino acid analyses and Mr R. F. White for TMV (type strain).

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(Received 20 July 1979)