Acidic and Basic Extracellular Pathogenesis-Related Leaf Proteins from Fifteen Potato Cultivars

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ABSTRACT

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Fifteen potato cultivars (Avon, Bintje, Cherokee, Green Mountain, Hudson, Jemseg, Kennebec, Nooksack, Norchip, Norland, Sable, Saco, Sebago, Superior, and Yukon Gold) produced necrotic local lesions on leaves inoculated with high concentrations (20 mg/ml) of U_2 tobacco mosaic virus. Proteins in the intercellular fluid extracts from inoculated leaves were characterized by native and denaturing polyacrylamide gel electrophoresis, gel filtration, and chromatofocusing. The presence of glycoproteins was also tested. Nine acidic (apparent isoelectric points [pI] 3.95–5.60), low molecular weight (28,200–40,700) soluble proteins, and six major basic proteins (apparent pI 7.2–9.2, M_r 14,100–33,300) were found in addition to several minor ones. Four acidic proteins with R_f 0.54 (M_r 38,900, apparent pI 3.95), 0.56 (M_r 40,700, apparent pI 4.10), 0.59 (M_r 40,700, apparent pI 4.0), and 0.62 (M_r 40,700, apparent pI 3.95) could be used as genetic markers to identify cultivars. No acidic proteins were observed that were equivalent to the b_1, b_2, and b_3 (pathogenesis-related la, b, c) proteins found in various *Nicotiana* species. Three acidic peroxidases (glycoproteins) (R_f 0.09, 0.39, and 0.42) were identified.

Additional key words: polypeptides, *Solanum tuberosum*, stress.

Pathogenesis-related (PR) proteins, also named b proteins, can be detected in several plants subjected to various types of stress (2,9,15,20). Soluble acidic, low molecular weight protease-resistant proteins from *Nicotiana* sp. are best characterized (9,14,17). These proteins, newly synthesized in response to stress (10,11), accumulate in the intercellular fluid (IF) of foliar tissue (2,16). Except possibly for peroxidase isozymes (17), the exact functions of these extracellular stress proteins are still unknown (2,13,20). Biochemical and serological studies have shown groups of related proteins in various *Nicotiana* species (1,9,14). However, except for tomato (13), there is no comparative study of PR proteins in the other Solanaceae.

The lack of information on potato PR proteins prompted us to characterize extracellular PR proteins in various potato cultivars known to react hypersensitively to tobacco mosaic virus (TMV) infection (5,12). This stress was chosen because it is one of the best factors known for inducing large amounts of extracellular PR proteins (2,20).

MATERIALS AND METHODS

Viruses, plants, and intercellular fluid extracts. Strains of TMV (U_1, U_2, U_4, U_6, and Cc) and tomato mosaic virus were increased and purified as described (3). Virus-free (indexed) potato tubers were obtained from Dr. G. Banville (Station Les Buissons, MAPAQ, Québec). Cultivars used were: Avon, Bintje, Cherokee, Green Mountain, Hudson, Jemseg, Kennebec, Nooksack, Norchip, Norland, Sable, Saco, Sebago, Superior, and Yukon Gold. Growth conditions, virus inoculations, and IF extractions were as previously described (16), except that virus concentrations of 20 mg/ml were used for inoculations of potato leaflets. IF extracts were used immediately or frozen at −20 C. Tobacco (*Nicotiana tabacum* L. 'Xanthi-nc') was used as an indicator plant.

Polyacrylamide gel electrophoresis (PAGE). Protein analysis was done by disc PAGE in 10% gels for acidic proteins (Davis system) (7) or in 15% gels for basic proteins (Reisfeld system) (19). Molecular weights were determined using a two-dimensional PAGE system (16) involving native conditions in the first dimension and denaturing lithium dodecyl sulfate (LiDS) in the second dimension. Polyacrylamide linear gradient (8–16%) gels were used in the second dimension to improve molecular weight determinations (16) and to counteract the electrophoretic retardation of nonreduced glycoproteins (17). Gels were stained with Coomassie Blue followed by silver nitrate (16).

Gel filtration chromatography. Molecular weight determinations by gel filtration were carried out using a 120- × 1-cm Sephadex G-75 (Pharmacia Fine Chemicals, Dorval, Québec) column. IF was extracted in 0.1 M neutral sodium phosphate buffer, and chromatography was done with the same buffer. Elution profiles of 1-ml samples were compared with those of standard proteins (low molecular weight gel filtration calibration kit, Pharmacia Fine Chemicals). The flow rate was at 40 ml/hr and 1.3 ml fractions (2 min) were collected and analyzed by polyacrylamide gel electrophoresis.

Peroxidase activities. Peroxidase activities were tested in native polyacrylamide gels (17).

Chromatofocusing. Determination of apparent isoelectric points (pI) of acidic proteins was done with a 30- × 1-cm column of exchanger (PBE 94, Pharmacia Fine Chemicals) following manufacturer's instructions. The flow rate was adjusted at 40 ml/hr using a peristaltic pump. The column was equilibrated with 0.025 M histidine-Cl buffer, pH 6.2, and 5 ml of eluent (an eightfold dilution of Polybuffer 74, Pharmacia Fine Chemicals) adjusted to pH 4.0 with HCl, was used. Samples (4 ml) were extracted in histidine buffer. Fractions of 4 ml (6 min) were collected and their pH measured immediately. The run ended when the pH stabilized around 3.9 and fractions were analyzed by native PAGE. The resulting gradient lasted about 4.5 hr (total of 180 ml). Determinations of pIs of basic proteins were done using the PBE

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118 exchanger (Pharmacia Fine Chemicals) in 0.025 M triethylamine-Cl buffer, pH 11.0, as the starting buffer and a 1:45 dilution of Pharmalyte, pH 8–10.5 (Pharmacia Fine Chemicals) (adjusted to pH 7.0 with HCl), as eluent.

Concanavalin A chromatography and affinity electrophoresis. Chromatography on concanavalin A-Sepharose 4B (Sigma Chemicals, St. Louis, MO) was as described (17). Affinity electrophoresis was done with the native PAGE system for acidic proteins except that glycerol was used instead of sucrose in the sample buffer. Before sample application, concanavalin A-Sepharose 4B slurry was inserted in the sample slot (2-mm bed). Comparison of affinity and regular, nonaffinity electrophoresis was made by using two adjacent slots on the same gel.

TABLE 1. Occurrence and relative intensity of four acidic cultivar-dependent[a] proteins after polyacrylamide gel electrophoresis analysis of intercellular fluid proteins from potato infected with U_2 tobacco mosaic virus

	Band position (R_f)			
Cultivar	0.56	0.58	0.61	0.64
Avon	+[b]	+	+	−
Bintje	++	+	−	+
Cherokee	+	−	+	+
Green Mountain	++	++	++	++
Hudson	++	−	++	+
Jemseg	+	−	+	+
Kennebec	+++	++	−	++
Nooksack	+++	++	−	++
Norchip	−	++	−	++
Norland	+	−	+	+
Sable	+	−	+	−
Saco	−	+	−	+
Sebago	++	+++	+	+
Superior	−	++	++	++
Yukon Gold	−	+	−	+

[a] Concentrations of proteins varied among plants within a cultivar, but relative concentrations of proteins with R_f 0.56–0.64 were rather constant.
[b] Symbols: + = faint band; ++ = band easily detected; +++ = intense band; − = no visible band (after staining with Coomassie Blue only).

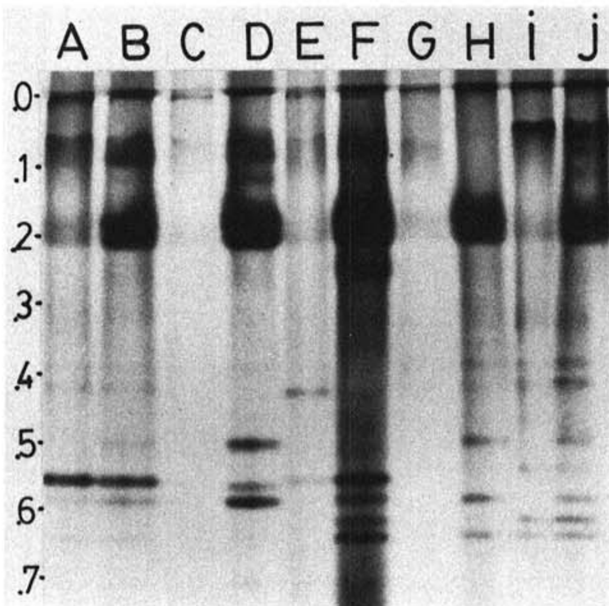

Fig. 1. Electrophoretic patterns of intercellular fluid acidic proteins from potato (*Solanum tuberosum* L.). Intercellular fluid proteins from cultivar Kennebec (A,B), Sebago (C,D), Green Mountain (E,F), Norchip (G,H), and Superior (I,J) were extracted 14 days after mock inoculation (A,C,E,G,I) or inoculation with U_2 tobacco mosaic virus (B,D,F,H,J). Proteins were separated in a 10% polyacrylamide gel under native conditions (Davis system) and stained with Coomassie Blue followed by silver nitrate. Numbers on the left indicate migrations relative to bromophenol blue.

RESULTS

Local lesion production and virus restriction. All TMV strains at high concentration (at least 10 mg/ml) gave local lesions on potato leaflets. However, all potato cultivars inoculated with U_2-TMV showed about 10 times more lesions than those inoculated with the other strains. Concentrations of 20 mg/ml (U_2-TMV) were necessary to consistently produce 10–50 local lesions per leaflet. Necrotic lesions resembled TMV lesions on hypersensitive tobacco. Depending on environmental conditions and age of the leaf, yellowing and abscission of infected leaves occurred. Lesions efficiently limited virus spread in all cultivars for all TMV strains except for cultivar Green Mountain, where systemic spread was detected in noninoculated leaves by using tobacco indicator plants.

IF proteins. New acidic and basic proteins were identified after PAGE analysis of TMV-infected potato IF foliar extracts compared with healthy ones (Figs. 1 and 2). Small amounts of IF proteins were also detected in healthy plants as for tobacco (9). Analysis of potato acidic proteins (Fig. 1) revealed a pattern very different from the one with tobacco (16), and fast-migrating proteins ($R_f \simeq 0.60$), like some well-known tobacco IF proteins, were absent in potato extracts.

Acidic extracellular protein patterns of infected potato cultivars were identical except for four proteins (R_f 0.56–0.64) (Fig. 1 and Table 1). The presence of two other protein bands of lower migration rate (R_f 0.24 and 0.49) was also cultivar dependent. However, these proteins were not consistently observed in all plants of a given cultivar.

Accumulation of basic proteins after localized TMV infection was also observed (Fig. 2), but no difference could be found among cultivars.

Molecular weights and isoelectric points. The molecular weights of the major IF proteins were determined by gel filtration chromatography and 2-D gel electrophoresis (Figs. 3 and 4 and Tables 2 and 3). Molecular weights estimated by gel electrophoresis varied between 14,100 and 40,700, comparing well to tobacco PR proteins (16). Molecular weights determined by both methods are close enough to exclude aggregation of these proteins except possibly for one acidic (R_f 0.09) and one basic (R_f 0.78) protein.

Apparent isoelectric points of acidic proteins, estimated by chromatofocusing (Table 2), were near 4.0 for proteins with R_f 0.56–0.64. A similar range of low pIs was also found for some PR tobacco proteins (18). The other acidic proteins also had an apparent pI below 6.0. Apparent isoelectric points of basic proteins (Table 3) were between 7.2 and 9.2, which is lower than pI 10.7 of p14 tomato PR protein (13).

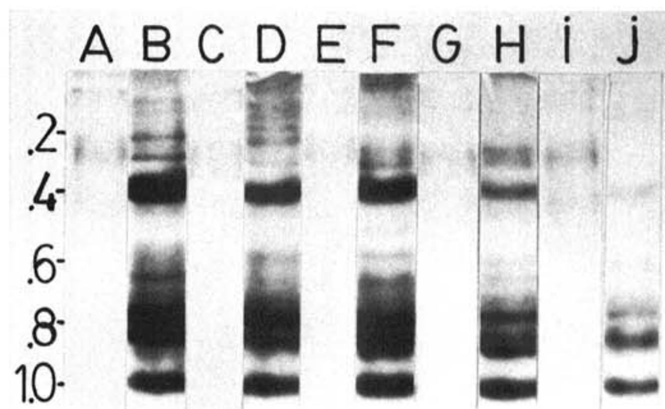

Fig. 2. Electrophoretic patterns of intercellular fluid basic proteins from potato (*Solanum tuberosum* L.). Intercellular fluid proteins from cultivar Kennebec (A,B), Sebago (C,D), Green Mountain (E,F), Norchip (G,H), and Superior (I,J) were extracted 14 days after mock inoculation (A,C,E,G,I) or inoculation with U_2 tobacco mosaic virus (B,D,F,H,J). Proteins were separated in a 15% polyacrylamide gel under native conditions (Reisfeld system) and stained as described in Figure 1. Numbers on the left indicate migrations relative to the lowest major band.

Peroxidases and glycoproteins. Most plant peroxidases, including potato peroxidases, are glycoproteins (8). Glycoproteins were identified by affinity chromatography and affinity electrophoresis using concanavalin A (Fig. 5). With both techniques, acidic proteins with R_f 0.39–0.42 and R_f 0.09 behaved like glycoproteins. Peroxidase activities were tested in native polyacrylamide gels, and enzyme activity was localized in the same zones. Moreover, the same bands were detected when glycoproteins were separated by chromatofocusing followed by native PAGE and tested for peroxidase activities (apparent pIs of 4.85 and 4.45).

DISCUSSION

Most potato cultivars are highly resistant to TMV (5), and large concentrations of TMV were needed to infect potato. Upon TMV infection, 14 potato cultivars reacted hypersensitively and Green Mountain was systemically infected. This type of reaction has been previously observed with cultivar Green Mountain (4). Stress by necrotic local lesions was accompanied by accumulation of extracellular proteins in potato leaflets. Among several IF proteins, peroxidase isozymes were identified (17 and Fig. 5). Molecular weights of IF potato proteins were low (<40,700) and compared well to molecular weights of previously described PR proteins (20). Groups of proteins could be tentatively identified. In addition to peroxidases, acidic proteins with R_f 0.18 and 0.21 shared similar physical properties (Table 2). Another group could involve four acidic proteins with R_f 0.56–0.64 and molecular weights around 40,000 and differing only by their apparent isoelectric points (Table 2). With basic proteins, proteins with R_f 0.83–1.00 have close molecular weights and could represent another group. The use of serology and sequence data will be needed to show homologies among these poteins.

Acidic proteins with R_f 0.56–0.64 are most interesting as their accumulation is cultivar-dependent (Table 1). These proteins could be used as genetic markers to identify cultivars.

New potato proteins (M_r 12,000 and 16,000) were detected upon infection with citrus exocortis viroid (6). These proteins seem similar to proteins of tomato (*Lycopersicon esculentum* Mill.) infected with the same viroid. One protein with a molecular weight of 14,225 was studied in tomato infected with potato spindle tuber viroid (13). Except for its isoelectric point, this protein shares

TABLE 2. Characteristics of acidic intercellular fluid proteins from potato[a] infected with U_2 tobacco mosaic virus

Relative migration (R_f)[b]	Molecular weights (× 10^{-3})		Apparent pI[e]
	Gel filtration[c]	PAGE[d]	
0.09	64.0	38.0	4.85
0.18	16.5	28.2	5.60
0.21	16.5	29.5	5.00
0.39	39.5	33.1	4.45
0.42	39.5	31.6	4.45
0.56	26.0	38.9	3.95
0.58	26.0	40.7	4.10
0.61	26.0	40.7	4.00
0.64	26.0	40.7	3.95

[a] Potato cultivar Kennebec was used except for protein with R_f 0.61 (cultivar Hudson).
[b] In 10% polyacrylamide gel electrophoresis under native conditions (Davis system).
[c] Determined by Sephadex G-75 filtration.
[d] Determined by lithium dodecyl sulfate denaturing linear gradient (8–16%) gel electrophoresis.
[e] Determined by chromatofocusing.

TABLE 3. Characteristics of the major basic intercellular fluid proteins from potato[a] infected with U_2 tobacco mosaic virus

Relative migration (R_f)[b]	Molecular weights (× 10^{-3})		Apparent pI[e]
	Gel filtration[c]	PAGE[d]	
0.37	18.6	22.9	7.2
0.37	23.4	27.9	8.4
0.78	58.1	33.3	8.9
0.83	14.8	15.2	8.6
0.85	14.8	14.7	8.4
1.00	14.8	14.1	9.2

[a] Potato cultivar Kennebec was used.
[b] In 15% polyacrylamide gel electrophoresis under native conditions (Reisfeld system) where R_f was calculated by comparison with the lowest major band position (see Fig. 2).
[c] Determined by Sephadex G-75 filtration.
[d] Determined by lithium dodecyl sulfate denaturing linear gradient (8–16%) gel electrophoresis.
[e] Determined by chromatofocusing.

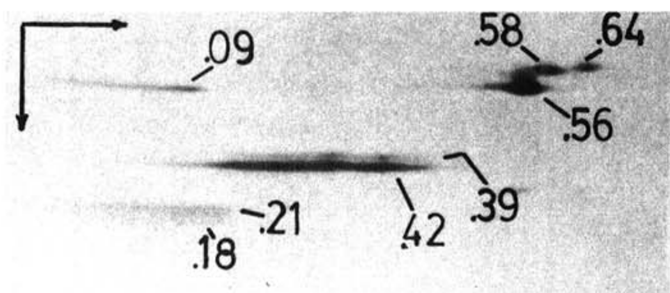

Fig. 3. Two-dimensional electrophoretic pattern of intercellular fluid acidic proteins of U_2 tobacco mosaic virus-infected *Solanum tuberosum* L. 'Kennebec.' Separation of intercellular fluid proteins was in a 10% polyacrylamide gel under native conditions (Davis system) in the first dimension (horizontal arrow) followed by a lithium dodecyl sulfate denaturing linear gradient (8–16%) polyacrylamide gel in the second dimension (vertical arrow). Proteins were extracted and stained as described in Figure 1. Protein bands are identified with their R_f from the first dimension gel (see Fig. 1). Molecular weights are shown in Table 2.

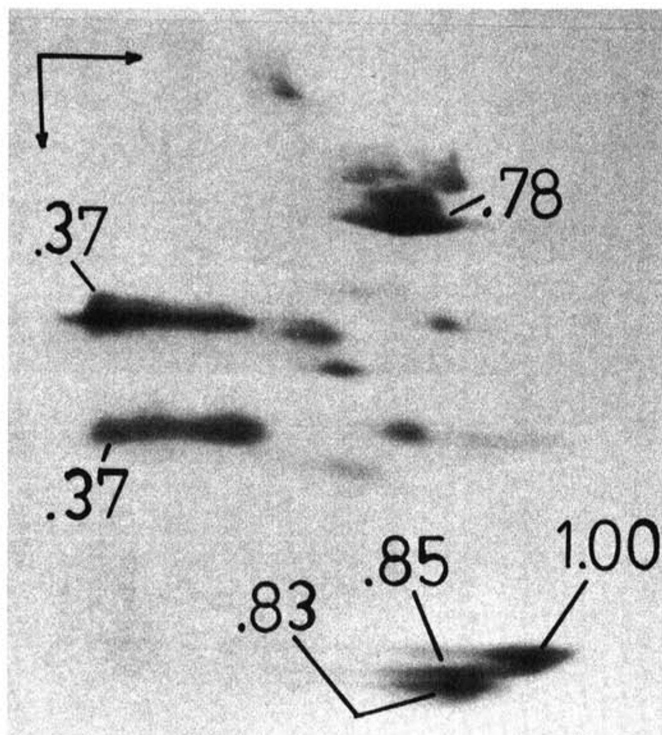

Fig. 4. Two-dimensional electrophoretic pattern of intercellular fluid basic proteins of U_2 tobacco mosaic virus-infected *Solanum tuberosum* L. 'Kennebec.' Separation of intercellular fluid proteins was in a 15% polyacrylamide gel under native conditions (Reisfeld system) in the first dimension (horizontal arrow) followed by a lithium dodecyl sulfate denaturing linear gradient (8–16%) polyacrylamide gel in the second dimension (vertical arrow). Proteins were extracted and stained as described in Figure 1. Protein bands are identified with their R_f from the first dimension gel (see Fig. 2). Molecular weights are shown in Table 3.

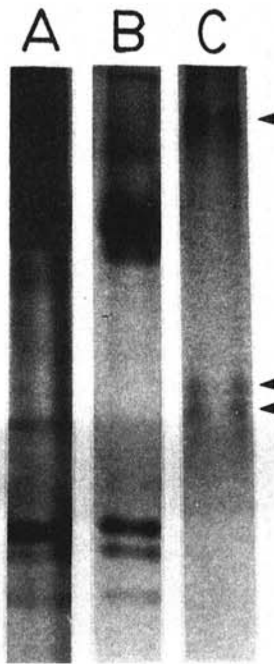

Fig. 5. Electrophoretic patterns of intercellular fluid glycoproteins of U_2 tobacco mosaic virus-infected *Solanum tuberosum* L. 'Kennebec.' Intercellular fluid extract was subjected to native polyacrylamide gel electrophoresis for acidic proteins (Davis system) (A) or to concanavalin A-affinity electrophoresis in the same gel (B). Proteins adsorbed onto a concanavalin A column (C) were also analyzed. Staining was as described in Figure 1 and photographs were overexposed to show the faint glycoprotein bands. Arrows indicate position of glycoproteins.

sequence homology and several other characteristics with tobacco b_1 protein. The pI of this tomato PR protein is 10.7 (13), whereas that of tobacco is around 4.0 (18). In our experiments, such an acidic protein was not detected in potato and the tobacco b_1 equivalent in potato could well be a basic protein like tomato p14. Analysis of basic proteins by 2-D PAGE indicated that proteins with molecular weights near 14,000 were present in TMV-infected potatoes (Fig. 4). Overall, our results indicate that hypersensitive potato and tobacco react similarly to TMV by producing numerous extracellular proteins. This phenomenon is probably not restricted to this plant family as other distantly related species also accumulate extracellular proteins (16). This phenomenon seems related to various types of nonspecific stress of green tissue in the plant kingdom (2). The biological significance of this phenomenon remains to be elucidated.

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