Genetic Variation in Polypeptide Maps of Two *Globodera rostochiensis* Pathotypes

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ABSTRACT

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The protein composition of females of *Globodera rostochiensis* was analyzed with two-dimensional gel electrophoresis (2-DGE) followed by a sensitive silver stain. Standardization of the protein extraction from young females resulted in reproducible protein profiles, which were independent of host genotypes. Protein patterns of nematodes reared on potato and tomato were indistinguishable. Isoelectric focusing within the pH range 5–7 and nonequilibrium pH gradient electrophoresis of basic proteins followed by sodium dodecyl sulfate electrophoresis resolved approximately 720 protein spots per population. Comparison of two *G. rostochiensis* populations, classified as pathotype Ro_1 and Ro_5, revealed 680 invariant and 39 variant protein spots. Twenty-three variants seemed to be the result of amino acid substitutions that altered net charge and involved 11 putative loci. The underlying mechanisms of the remaining 16 variants were unclear.

Pathotypes of the potato cyst nematodes *Globodera rostochiensis* (Woll.) Behrens and *G. pallida* (Stone) Behrens are defined by their ability to overcome genes for resistance derived from *Solanum tuberosum* spp. *andigena* Juz. and Buk., *S. kurtzianum* Bitt. and Wittm., and *S. vernei* Bitt. and Wittm. (20). In Europe, eight pathotypes are presently recognized, five within *G. rostochiensis* (Ro_1-Ro_5) and three within *G. pallida* (Pa_1-Pa_3) (20). Several authors have suggested that most genes mediating resistance and virulence might operate on the basis of a gene-for-gene relationship (15,16,43).

The fundamental processes involved with the induction and maintenance of syncytia, and the specific mechanisms underlying virulence, are unknown. Second-stage larvae invade the roots and, adjacent to the xylem vessels, induce multinucleate transfer cells (17,18), which serve as feeding sites. In the incompatible combinations the syncytia remain small and are often accompanied by a necrotic reaction (14), resulting in a too limited amount of food for the females to develop. It has been suggested that, similar to other sedentary nematodes (2,3), protein substances in the saliva of the potato cyst nematodes are responsible for pathogenicity (15).

A major obstacle to biochemical research on obligate organisms, such as potato cyst nematodes, is the limited amount of biological material that can be obtained. Therefore, methods with high sensitivity are needed. Microelectrophoresis, for instance, has been used to study single nematodes (6,36) or even parts of them (35). However, its application is limited when dealing with complex protein mixtures.

Two-dimensional gel electrophoresis (2-DGE), as originally described by O'Farrell (29), combined with a silver stain (24,28) or autoradiography (22,29), is a powerful research tool. 2-DGE is able to resolve more than 100 gene products from minute amounts of crude nematode homogenates (1,8). In this study we investigated the potential of 2-DGE in monitoring patterns of gene expression and genetic variability in potato cyst nematodes.

MATERIALS AND METHODS

Populations. *G. rostochiensis* population MIER, classified as pathotype Ro_1 (Ro_1-M), was supplied by I. C. Miller and I. J. Bakker. *G. rostochiensis* population H, classified as Ro_5 (Ro_5-H), was obtained from H. J. Rumpenhorst. The former population was collected at Wageningen, The Netherlands, and the latter at Harmerz, W. Germany. Populations were maintained on *S. tuberosum* f. sp. *tuberosum* L. 'Eigenheimer.'

The virulence characteristics of the populations were estimated by testing their reproductive ability on the differentials used in the international pathotype scheme of Kort et al (20). *S. tuberosum* f. sp. *tuberosum* was represented by the commercial cultivar Eigenheimer, susceptible to all pathotypes. *S. tuberosum* f. sp. *andigena* was replaced by the commercial cultivar Saturna, which has the resistance gene H_1, derived from CPC 1673 (20). The number of females developed on the differentials was expressed as a percentage of the number developed on the susceptible cultivar Eigenheimer. These percentages were used as an indication for the number of virulent individuals in the populations. For population Ro_1-M these percentages were 0% for *S. tuberosum* spp. *andigena* CPC 1673, 0% for *S. vernei* 62.33.3, and 1% (0.2) for *S. vernei* 58.1642/4. For population Ro_5-H the percentages were 84.6% (3.7) for *S. tuberosum* f. sp. *andigena* CPC 1673, 14.8% (1.6) for *S. vernei* 62.33.3, and 30% (9) for *S. vernei* 58.1642/4. (The figures in parenthesis are the standard deviations.) Values for the first two differentials were calculated from the number of females that developed on roots of sprouts grown on water agar (26). The percentages for the latter were calculated from females developed on potatoes grown in pots.

With regard to the genetics of *G. rostochiensis*, it is noted that potato cyst nematodes are diploid organisms with a haploid chromosome number of nine (7). No clear evidence is available for sex determining heteromorphic chromosomes (7). Moreover, sex determination seems epigenic (25).

Preparation of protein samples. The cultivars Eigenheimer and Mentor, susceptible to both pathotypes, were inoculated with approximately 200 cysts and grown in 1-L pots filled with sandy loam and placed in a growth chamber at 18 C and 16 hr daylight. Populations were also reared on *Lycopersicum esculentum* L. 'Moneymaker.'

Females were collected in a small glass mortar and rinsed with 10 mM Tris-HCL, pH 7.4 (3×200 µl). Total protein samples were prepared by homogenizing 100 females in 60 µl of 10 mM Tris-HCL, pH 7.4, and 5% (v/v) 2-mercaptoethanol, and subsequently saturated with 64 mg of urea. Soluble proteins were obtained by homogenizing 200 females in 90 µl of 10 mM Tris-HCL, pH 7.4, and 5% (v/v) 2-mercaptoethanol. The homogenate was centrifuged for 10 min at 105,000 *g*, and the supernatant (60 µl) was saturated with 64 mg of urea. All protein samples were stored at −80 C until use.

Females collected at a certain time after inoculation are always a mixture of white and yellow, small and large individuals. To exclude undesirable variations, the nematodes were handpicked under a dissecting microscope. Only healthy-looking, white, full-grown females were selected. As a standard the nematodes were

harvested 33–45 days after inoculation. Sampling errors due to genetic variations between individuals were negligible, because protein samples were prepared by homogenizing 100 or more individuals.

Two-dimensional gel electrophoresis. Chemicals for isoelectric focusing and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA), except for ampholines pH range 5–5.5 and Nonidet P-40, which were obtained from Serva (D-69 Heidelberg, W. Germany) and Sigma Chemical Co. (St. Louis, MO), respectively.

Thirteen μl of medium consisting of 2% ampholines pH range 3–10, 8% ampholines pH range 5–7, 10% (w/v) Nonidet P-40, 25% (v/v) 2-mercaptoethanol was added to the thawed sample. The sample was centrifuged at 105,000 *g* for 20 min. Protein determinations were made according to Bradford (4) by measuring the shift in extinction of Coomassie Brilliant Blue at 595 nm.

Isoelectric focusing in the pH region 5–7 was essentially as described by O'Farrell (29) with the following modifications. Gels were polymerized in glass tubing (160 × 2 mm inside diameter). Samples (25–50 μl) were loaded without prefocusing. The remaining space in the tube above the sample was filled with a solution with the same buffer composition as the sample; i.e., 4.75 mM Tris-HCL, pH 7.4, 8.9 M urea, 1% (w/v) Nonidet P-40, 0.8% ampholines pH range 5–7, 0.2% ampholines pH range 3–10. In this way variations caused by application of different sample volumes were minimized. Isoelectric focusing was performed according the following schedule: 30 min, 100 V; 30 min, 200 V; 15 hr, 300 V; 3 hr, 400 V. Electrophoresis was toward the anode, with the basic reservoir on top and the acidic reservoir at the bottom. Equilibrium electrophoresis within the pH range 5–5.5 was carried out by replacing the ampholines pH range 5–7 with an equal amount of ampholines pH range 5–5.5.

Nonequilibrium pH gradient electrophoresis (30) was done by using the same polymerization mixture as for isoelectric focusing within the pH region 5–7. Electrophoresis was toward the cathode and was terminated after 4 hr at 400 V.

After isoelectric focusing the gels were equilibrated for 10 min in a SDS buffer (29) and subjected to a discontinuous SDS gel system (21) with a 12% (w/v) acrylamide separation gel (1.5-mm thickness; Bio-Rad model 220 slab gel appartus). After nonequilibrium pH gradient electrophoresis, a 15% (w/v) acrylamide separation gel was used. SDS electrophoresis was performed with a constant current of 25 mA.

The isoelectric points (pI) of the proteins were estimated by measuring the pH gradient with a pH contact electrode (Bio-Rad). The standard deviations of these pI values averaged 0.5 pH unit. Apparent molecular weights were estimated by using phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme as reference proteins (Bio-Rad low molecular weight standard solution).

Staining. The silver staining procedure described by Oakley (28) was modified as follows. Step 1, gels were fixed for 30 min in a solution containing 50% (v/v) methanol and 10% (v/v) acetic acid. Step 2, gels were stored overnight in 5% (v/v) methanol with 7% (v/v) acetic acid. Step 3, gels were soaked for 1 hr in 7% (v/v) glutaraldehyde. Step 4, gels were rinsed for 24 hr in distilled water (4 × 400 ml). Step 5, gels were complexed with silver for 1 hr in an ammoniacal silver solution containing 0.075% (w/v) NaOH, 1.2% (v/v) NH_4OH, 0.32% (w/v) $AgNO_3$, 300 ml per gel. Step 6, gels were washed in distilled water for 15 min (3 × 500 ml). Step 7, gels were transferred to a clean container and immersed in a solution containing 0.001% (w/v) citric acid and 0.002% (v/v) formaldehyde. The proteins became visible after approximately 15 min. Protein profiles were compared visually by superimposing the original gels on a bench viewer illuminated with fluorescent tubes. The number of replicates per object ranged from 4 to 20.

RESULTS

Variations in hatch and development were major obstacles in obtaining highly standardized protein samples. The time at which the nematodes were harvested varied from 33 to 45 days after inoculation. These variations were mainly caused by differences in the age of the tubers and cysts. Analysis of total protein extracts with 2-DGE within the pH range 5–7 showed that our protein extraction was not influenced by these variations. White, full-grown females harvested at 33, 36, 40, and 45 days after inoculation gave identical protein profiles. The protein patterns were also not influenced by the host genotypes. Females reared on the potato cultivars Mentor and Eigenheimer and the tomato cultivar Moneymaker revealed no noticeable differences.

Classification of variant protein spots. Despite the high standardizaton of the 2-DGE procedure, several proteins varied in intensities between repeated experiments or were even absent in some experiments. The main source of variation was found in the silver staining procedure. During the course of this study, we tested several silver staining methods (23,24,28,34,46), but obtained no better results than with the procedure described here. In this study only those variants were recorded that were consistently different between the two populations.

Comparison of the 2-DGE protein patterns of populations Ro_1-M and Ro_5-H revealed 680 invariant and 39 variant protein spots (Table 1). The variants were divided in three groups: variants that seemed to be the result of isoelectric point changing amino acid substitutions (IP-variants) (Fig. 1, A and B); variants characterized by the presence or absence of a protein (PA-variants) (Fig. 1C);

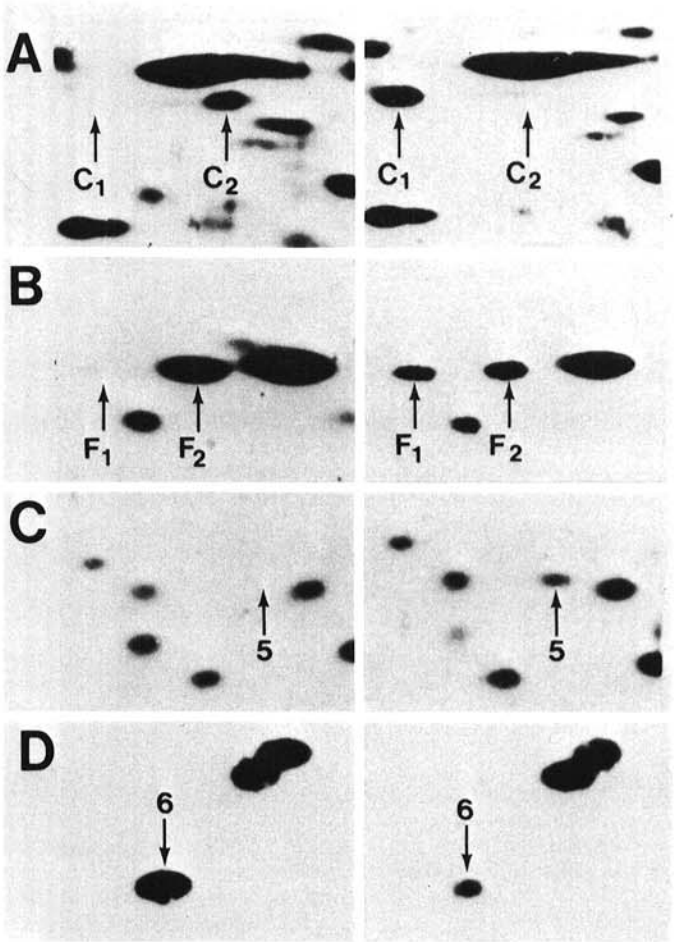

Fig. 1. Examples of the types of variant protein spots detected by the comparison of two-dimensional gel electrophoresis (2-DGE) protein patterns of *Globodera rostochiensis* populations Ro_1-M (left) and Ro_5-H (right). **A** and **B**, Variations having the characteristics expected from amino acid substitutions that alter the isoelectric point of a protein. The isoelectric point variants (IP-variants) are assigned capital letters referring to the putative loci and numbers referring to the alleles. **C** and **D**, Variants for which no putative corresponding allele product could be traced are marked with arabic numbers. Differences expressed by presence/absence data (**C**) and differences in concentration (**D**) were combined within one class of variants, the nonisoelectric point variants (NIP-variants).

and variants expressed by differences in concentration (C-variants) (Fig. 1D).

The corresponding IP-variants were designated as such because they had the characteristics expected from protein variations caused by mutations leading to net charge change (5,19,22, 32,37,39,44). The corresponding IP-variants had a moderate difference in isoelectric point, similar molecular weight, and seemed to be produced in similar quantities for each haploid set of chromosomes within each individual. Corresponding IP-variants were assumed to be encoded by alleles at the same locus. The IP-variants were assigned capital letters referring to the putative loci and numbers referring to the alleles. This interpretation was also supported by the color of the proteins. The color of the approximately 700 proteins studied here ranged from red (±5%), reddish brown (±70%), blackish brown (±5%), brownish gray (±5%), to gray (±15%). All corresponding IP-variants had identical colors, which is expected from proteins differing in a small number of amino acid residues (27).

In this study the IP-variant loci manifested themselves in two ways. The first group consisted of IP-variant loci that were monomorphic in both populations (Table 1: loci *A*, *C*, *D*, *G*, *H*, and *J*). These IP-variants had similar spot sizes and intensities in populations Ro_1-M and Ro_5-H (e.g., Fig. 1A). The second group consisted of IP-variant loci that were monomorphic in population in Ro_5-M and polymorphic in Ro_5-H (Table 1: loci *B*, *E*, *F*, *I*, and *K*). An important clue in recognizing these IP-variants as such was that the protein quantity of each of the allele products in Ro_5-H was smaller than that of the corresponding allele product in Ro_1-M (e.g., Fig. 1B). This behavior was expected from mutations resulting in a net charge change. The sum of the protein quantities of the allele products from a polymorphic locus should be equal or at least close to the protein quantity of the corresponding allele product from a monomorphic locus.

The remaining variants for which no putative corresponding allele products could be traced were designated as C- or PA-variants (Fig. 1, C and D). The C- and PA-variants were combined within one class of variants, the nonisoelectric point variants (NIP-variants). The NIP-variants were designated with an arabic number (Figs. 1, 2, 3; Table 1).

2-DGE, pH range 5–7. Total protein analysis resolved 393 invariant protein spots and 11 IP-, five PA-, and three C-variants (Fig. 2; Table 1). 2-DGE of soluble proteins revealed six IP- and two PA-variants and 79 invariant protein spots, which were not detectable in total protein samples (Table 1). Proteins with an isoelectric point higher than the proteins V, W, X, and Y were not examined because of the relative poor resolution and reproducibility in this area (Fig. 2).

Scrutiny of the total protein patterns of different generations revealed no disparities with the results presented here. Total protein patterns from three and two generations of Ro_1-M and Ro_5-H, respectively, were identical.

2-DGE, pH range 5–5.5. Analysis of the soluble protein fraction demonstrated that C-variant 3 was actually composed of two variant protein spots (Table 1).This type of 2-DGE resolved, in addition, one PA- and one C-variant not traced with 2-DGE, pH range 5–7 (Table 1).

2-DGE, basic proteins. Nonequilibrium pH gradient electrophoresis of soluble proteins resolved six IP-variants, three PA-variants, and approximately 170 invariant proteins not detected with 2-DGE, pH range 5–7. The majority of these variant and invariant proteins probably had an isoelectric point above 6.8. This was estimated by using the proteins V, W, X, and Y as references. These proteins, resolved both with electrophoresis within the pH range 5–7 (Fig. 2A) and with nonequilibrium pH gradient electrophoresis (Fig. 3A), had isoelectric points of 6.86, 6.92, 6.86, and 7. The reference proteins were easily recognized in both systems by their relative abundance and color. Proteins V, W, and X were reddish brown and Y was gray. It was puzzling that the IP-variants I_1 and I_2 (Fig. 3), which had lower electrophoretic mobilities toward the cathode than protein V, were not detected with 2-DGE within the pH range 5–7 (Fig. 2, A and B).

The basic high molecular weight proteins had a low

TABLE 1. Invariant and variant proteins detected by the comparison of populations Ro_1-M and Ro_5-H

2-DGE system		Isoelectric point variants[a]											Nonisoelectric point variants[a]							
Total protein		*A*1	*A*2	*B*1	*B*2	*C*1	*C*2	*D*1	*D*2	*E*1	*E*2	*E*3	1	2	3[b]	4	5	6	7	8
pH range 5–7;	Ro_1-M	+	−	+	−	−	+	−	+	−	+	−	−	±	±	−	−	+	−	−
393[c]	Ro_5-H	−	+	±	±	+	−	+	−	±	−	±	+	+	+	+	+	±	+	+
	M.W.[d]	59	59	54	54	46	46	38	38	36	36	36	60	58	57	46	37	25	23	19
	pI[e]	6.8	6.94	6.49	6.53	6.78	6.9	6.62	6.7	6.54	6.64	6.76	5.6	5.54	5.5	6.5	6.4	6.05	5.56	6.6
	color[f]	r.b.	r.b.	r.b.	r.b.	r.b.	r.b.	r.b.	r.b.	b.b.	b.b.	b.b.	g.	g.	g.	g.	r.b.	r.b.	b.b.	r.b.
Soluble protein		*F*1	*F*2	*G*1	*G*2	*H*1	*H*2						9	10						
pH range 5–7;	Ro_1-M	−	+	−	+	−	+						+	+						
237 (79)[c]	Ro_5-H	±	±	+	−	+	−						−	−						
	M.W.[d]	70	70	44	44	22	22						36	30						
	pI[e]	6.24	6.28	6.46	6.52	6.2	6.26						6.46	6.5						
	color[f]	r.b.	r.b.	r.b.	r.b.	r.b.	r.b.						b.b.	r.b.						
Soluble protein													3a[b]	3b[b]	11	12				
pH range 5–5.5;	Ro_1-M												±	±	−	±				
125 (8)[c]	Ro_5-H												+	+	+	+				
	M.W.[d]												57	57	28	24				
	pI[e]												5.48	5.51	5.42	5.41				
	color[f]												g.	g.	g.	g.				
Soluble protein		*I*1	*I*2	*J*1	*J*2	*K*1	*K*2						13	14	15					
basic[g];	Ro_1-M	+	−	+	−	+	−						+	+	−					
295 (170)[c]	Ro_5-H	±	±	−	+	±	±						−	−	+					
	M.W.[d]	52	52	34	34	27	27						46	28	28					
	color[f]	r.b.	r.b.	r.b.	r.b.	b.g.	b.g.						r.b.	r.b.	r.b.					

[a] The presumptive loci and alleles of the isoelectric point variants (IP-variants) are designated with capital letters and arabic numbers, respectively. The nonisoelectric point variants (NIP-variants) are assigned arabic numbers. The symbols + and − indicate the presence or absence of a protein spot; ± points to a lower concentration when compared with the corresponding protein in the opposite population. For the latter three 2-DGE systems, only those variants are listed that were not detected with the previous system(s).

[b] NIP-variant 3 is composed of two proteins.

[c] The total number of invariant proteins resolved. In parenthesis, the number of invariant proteins not detected in the previous 2-DGE system(s).

[d] Molecular masses in kilodaltons.

[e] Isoelectric points.

[f] r.b., reddish brown; b.b., blackish brown; b.g., brownish gray; and g., gray.

[g] The majority of the variant and 170 invariant proteins resolved with nonequilibrium pH gradient electrophoresis have a pI > 6.8.

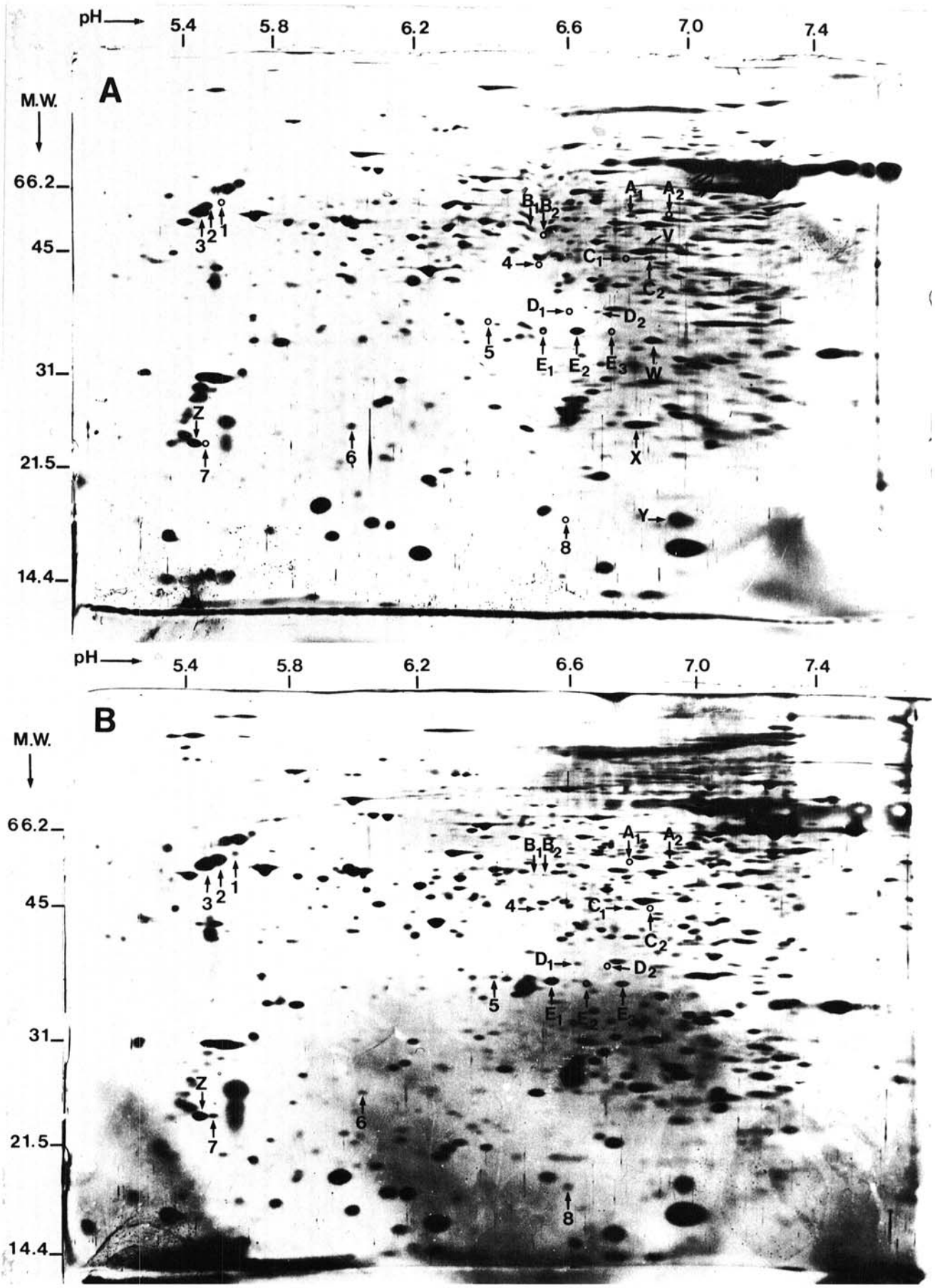

Fig. 2. 2-DGE within the pH range 5–7 of total protein (25 μg) from *G. rostochiensis* populations Ro_1-M (**A**) and Ro_5-H (**B**). The variant proteins that discriminate population Ro_1-M from Ro_5-H are indicated as described for Figure 1. Open circles indicate the absence of a protein spot. A number of minor variant proteins (e.g., locus B) are only visible on the original gel patterns. Proteins V, W, X, Y, and Z are referred to in the text. Molecular masses are given in kilodaltons.

DISCUSSION

Various electrophoretic techniques have been used to study the potato cyst nematode *G. rostochiensis* (9,10,11,12,31,33, 40,41,42,45). The number of proteins detected in these studies ranged from 10 to 70. Conventional disc electrophoresis of females showed marked variations between protein patterns of different populations (11,12,41,42). Many of those variations appeared not to be genetically determined and were ascribed to the comparison of females differing in physiological stage (12). Because large quantities of synchronized females are difficult to obtain, several investigators switched to electrophoresis of second-stage larvae and eggs and achieved more reproducible protein profiles (9,10,12,31,40). So far the most extensive report dealt with isoelectric focusing of eggs resolving 40 major protein bands and 23 enzymes (10). Intraspecific variation within *G. rostochiensis* was observed at one enzyme locus (10).

In this study the sample preparation was highly standardized by a careful selection of uniform females under a dissecting microscope. In this way variations among independent experiments caused by differences in age or condition of the nematodes were minimized. Unlike other investigators (12), we observed no noticeable effect of the host genotypes. Protein patterns of females reared on potato and tomato were indistinguishable.

IP- and NIP-variants. Codominant alleles coding for proteins differing in one or more net charge changing amino acids are a prominent group among the variants detected by enzyme electrophoresis (13) and 2-DGE (5,19,22,32,37,39,44). In view of these reports and the characteristics of the IP-variants, it seems reasonable to assume that the corresponding IP-variants are the products of alleles at the same locus. Evaluation of the variants detected in previous 2-DGE studies (5,19,22,32,37,39,44) shows that it is unlikely that other types of genetic variation will give rise to protein variations having the characteristics of the IP-variants. Of course, our results provide no conclusive evidence, and one should always be aware of exceptions.

Eleven loci were involved with the 23 IP-variants. Population Ro_1-M appeared monomorphic for all IP-variant loci, whereas Ro_5-H was polymorphic for five loci and monomorphic for six loci (Table 1). However, not necessarily all individuals in populations Ro_1-M and Ro_5-H needed to be homozygous for these monomorphic loci. The 2-DGE protein patterns represented the average protein composition of 100 individuals or more, and hence, alleles present in low frequencies may remain unnoticed. This may especially occur with alleles producing small quantities of protein (e.g., Fig.2, A and B, loci *B* and *D*).

The methodology used here allowed no clear-cut distinction between the C-variants and the PA-variants. Proteins classified by us as C-variants may have been the result of presence/absence data in individuals. The absence in one genotype (e.g., null alleles) and the presence in the other will result in a C-variant in case populations Ro_1-M and Ro_5-H contain unequal proportions of these genotypes. The majority of the NIP-variants, five C-variants, and 11 PA-variants were probably genetically determined. Patterns from three subsequent generations of population Ro_1-M and two of Ro_5-H provided no evidence that they were caused by a variable expression of the genotype.

The genetic background of the NIP-variants was unclear. A number of NIP-variants may, similar to the IP-variants, be the result of net charge changing amino acid substitutions. For example, protein Z and NIP-variant 7 (Fig. 2, A and B) may be encoded by alleles at the same locus, but not recognized as such, because protein Z was recorded as invariant. A quantitative difference between protein Z in populations Ro_1-M and Ro_5-H, which was equal to the quantity of NIP-variant 7, was not detectable with this system.

Some NIP-variants may also be generated by mutations in the regulatory sequences of the genome and structural genes, which influence the synthesis, processing, and degradation of other proteins. In this situation one mutation may result in more than one NIP-variant.

Virulence. Populations Ro_1-M and Ro_5-H differ widely in their capability to overcome various genes for resistance present in potato. Speculation on relationships between the IP- and NIP-variants in the females and genes for (a)virulence is not yet feasible. We are investigating segregation patterns of virulence genes and the IP- and NIP-variants.

In theory, it may be possible to trace proteins encoded by genes for virulence with 2-DGE. However, there are several points of concern. First, it is not known whether genes for (a)virulence are translated into proteins and, if so, whether they are in sufficient amounts to be detectable. Second, not all genetic variation is visible on a 2-DGE pattern. Only about 30% of the amino acid

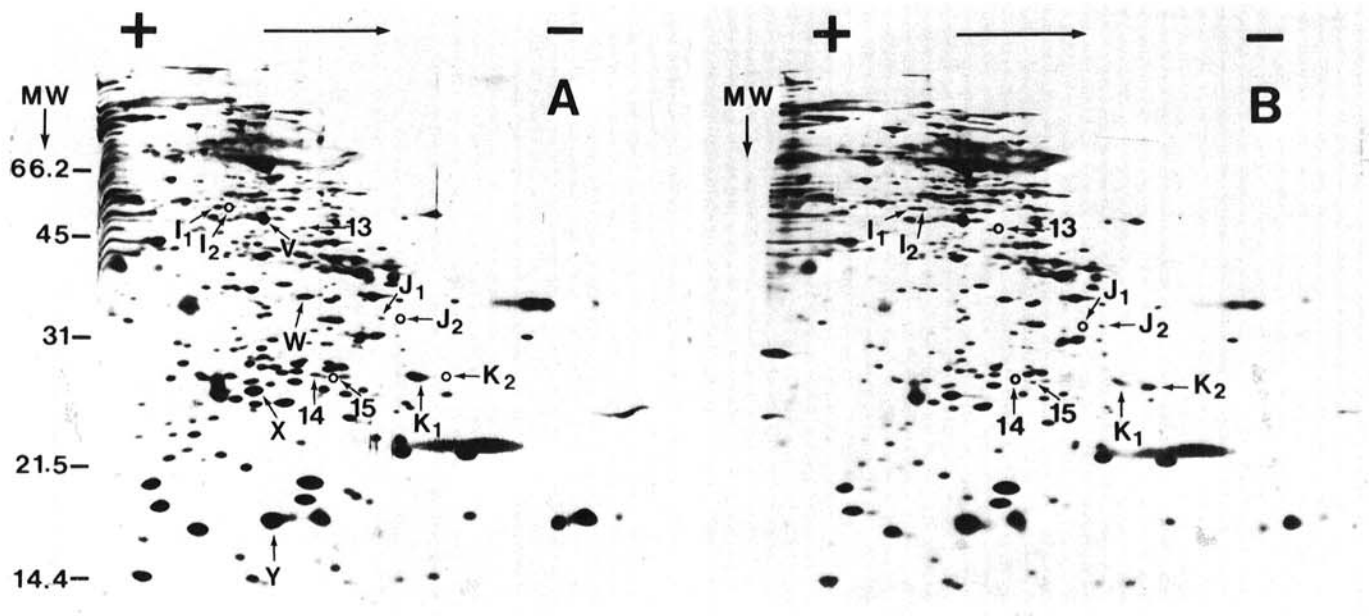

Fig. 3. 2-DGE of basic soluble proteins (35 μg) of *G. rostochiensis* populations Ro_1-M (**A**) and Ro_5-H (**B**). In the first dimension the proteins were separated by nonequilibrium pH gradient electrophoresis. The sample was applied at the anode. The proteins that discriminate population Ro_1-M from Ro_5-H are marked as described for Figure 1. Open circles indicate the absence of a protein spot. The proteins V, W, X, and Y are referred to in the text. Molecular masses are given in kilodaltons.

substitutions results in a displacement in the isoelectric focusing dimension. Furthermore, minor variations in protein concentration (e.g., those that might be caused by differences in regulatory genes influencing the synthesis of structural gene products) cannot be discerned with current 2-DGE techniques. Third, genes for virulence may not be expressed in young females, but only in the larval stages. Studying the different stages of development may therefore be necessary.

Despite these points of concern, 2-DGE remains a promising approach in elucidating the molecular processes of virulence and resistance, as well as other basic mechanisms involved with the induction and maintenance of syncytia. Taking the 2,000 proteins produced by the thoroughly studied nematode *Caenorhabditis elegans* (38) as a reference, we estimate that the 700 proteins analyzed here represent 15–30% of the proteins encoded by the entire *G. rostochiensis* genome. Probably this figure can be increased by assaying eggs, the various larval stages, and males.

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