Discrimination Between Common and Necrotic Strains of Potato Virus Y by Denaturing Isoelectric Focusing

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ABSTRACT

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Purified virions from seven isolates of the common strain (PVY^O) and six isolates of the tobacco veinal necrotic strain (PVY^N) of potato virus Y were examined by denaturing carrier-ampholyte isoelectric focusing (CA-IEF). All gave a similar pattern of one major and either two or three minor bands. The pattern of all PVY^N isolates was identical and the coat protein was acidic (pI = 6.1 in all cases), whereas the PVY^O isolates could be divided into three distinct groups, all of which were more alkaline (pI = 6.8, 7.2, and 7.4). Isoelectric focusing followed by electroblotting and immunodetection showed the bands to be of viral origin. SDS-PAGE gave a single major band of 29.5 kDa in all cases. Isolation of virions from either potato or tobacco gave the same results. The isoelectric points of the coat proteins of 28 PVY isolates, calculated from published sequences, were significantly higher for isolates of PVY^O than for those of PVY^N. These findings suggest that CA-IEF may be used to discriminate between the common and necrotic strains of potato virus Y and that the PVY^N strain may be more homogeneous than the PVY^O strain.

Additional keywords: Nicotiana tabacum, potyvirus.

As agricultural pathogens, viruses are second only to fungi, and of the plant viruses, the largest and economically most important group is the family Potyviridae. Including four genera and over 180 definitive and possible members, this family accounts for 30%, and possibly up to 50%, of all known plant viruses (30,36). Of these, the genus *Potyvirus* (named after the type-species potato virus Y [PVY]) is the best characterized (2). Like other potyviruses, PVY virions are long, flexuous rods composed of a monopartite, single-stranded, messenger-sense, approximately 10 kb long, RNA molecule with a 5′-terminal genome-linked protein, encapsidated by about 2,000 units of a 266–267 amino acid coat protein (20).

The large and growing number of potyviruses has led to considerable problems with their identification and classification, since classical criteria such as host range, symptomatology, cross-protection, serology, and inclusion body morphology no longer suffice to distinguish among them (30). The growing need for additional parameters for characterizing these viruses has been discussed at length by Shukla and Ward (30). PVY has three recognized strains: PVY^O (common strain), PVY^N (tobacco veinal necrosis strain), and PVY^C (stipple streak strain) (4). In turn, well over 30 different isolates classified as either PVY^O or PVY^N are now known (32,34,35). Recent outbreaks of PVY^N in North America (14,31) and the attendant seed potato certification problems have further highlighted the need for additional means of rapidly detecting and categorizing these viruses. In particular, it has become necessary to readily distinguish between PVY^O and PVY^N.

While nucleotide and amino acid sequencing data provide the ultimate information for identification and classification, more rapid and simple means are required to screen the limited set of PVY isolates afflicting potatoes in North America. Currently, identification of PVY^N is based primarily on the induction of veinal necrotic symptoms in tobacco, which is time-consuming. Enzyme-linked immunosorbent assays (ELISA, DIBA) utilizing monoclonal antibodies (14,32) and nucleic acid hybridization (12) have been used with varying degrees of success. ELISA has a high success rate but the specificity of the monoclonal antibodies leads to the failure to detect some PVY isolates. Nucleic acid hybridization currently is limited by the absence of an adequately discriminating probe.

Denaturing isoelectric focusing is capable, under optimal conditions, of resolving proteins that differ by only one amino acid (21) and has been used in the classification of avian strains in the genus *Coronavirus* (22). Sequence comparisons of the coat proteins of several PVY isolates show that the N-termini vary while the C-terminal two-thirds are conserved and that the strains PVY^O and PVY^N can be grouped on this basis (35). We report here that the isoelectric points of the coat proteins of virions from the strains PVY^O and PVY^N are, on average, significantly different.

MATERIALS AND METHODS

Plants and viruses. Seven PVY^O isolates (isolates 1, 3, 5, 7, MA667, MA927, and TGB) and six PVY^N isolates (N27, P579, Q9, TU619, TU660, and Y^N138) were used in this study and have been described previously (15,32). The isolates were from Prince Edward Island (P579, Y^N138), New Brunswick (isolates 1, 3, 5, 7, and N27), Quebec (Q9), British Columbia (MA667, MA927), California (TU619, TU660), and Scotland (TGB). All Canadian isolates cause slight rolling of leaves in the potato cv. Jemseg, while both Californian isolates cause severe necrosis of leaves and tuber ring necrosis symptoms in Jemseg (15). All were propagated by mechanical transmission in *Nicotiana tabacum* L. 'Samsun' growing under greenhouse conditions as previously described (32).

Virus purification. The purification protocol reported for potato virus A (33) was used with minor modifications. Specifically, following the CsCl isopycnic centrifugation step, the viruses were recovered by side puncture of the tubes, diluted with PE (50 mM sodium phosphate, 10 mM EDTA), and pelleted by centrifugation for 1.5 h at 40,000 rpm in a SW 50.1 rotor (Beckman, Palo Alto, CA). The viruses were resuspended in PE, and the concentration was determined by absorption at 260 nm using an extinction coefficient of 2.9 (1 mg/ml, 1 cm light path) (4).

Isoelectric focusing gel electrophoresis. The sample buffer of Mayer et al (13) was used with the following modifications: Triton X-100 was replaced with the equivalent NP40 (BDH, Poole, UK), 2-D Pharmalyte was replaced with Ampholine pH 3.5–10 (Pharmacia, Piscataway, NY), and the high-protein-content protease inhibitor mix of leupeptin and α-2 macroglobulin was replaced with leupeptin (2 μM), pepstatin A (2 μM), and PMSF (400 μM) obtained from Boehringer Mannheim (Laval, Québec, Canada). The purified virus preparations were diluted with the buffer and loaded at 2 μg per lane.

Isoelectric focusing was performed in 0.75-mm-thick T5–C4 gels containing 2% ampholytes (Ampholine pH 3.5–10.0), 8 M urea, 2% NP40, and 10% glycerol, on an FBE-3000 flatbed apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden). Whatman No. 1 filter paper electrodes, positioned 7 cm apart and dampened with either 1.0 M sodium hydroxide or 1.0 M phosphoric acid, formed the cathode and anode, respectively (21). Nitrogen was used to displace CO_2 (5). Focusing was for 90 min at a constant 17 W (0.12 W/cm^2) with typical final steady state conditions of approximately 9 mA and 2,000 V. Actual temperature at the gel surface was monitored throughout the run with thermocouples (Type E, Omega Engineering thermometer, model 450 AET, Stamford, CT) adhered directly onto the gel. The temperature of the gel midway between the electrodes was maintained at 25 C ± 1.5 C. The pH gradient in the gel after the run was determined with a calomel surface pH electrode (Cole Parmer, model 5998-27 EEO, Chicago, IL) coupled to a digital pH meter (Orion Research, model 701A, Cambridge, MA). The gels were fixed and stained according to Garfin (7). Crocein Scarlet (also known as Biebrich Scarlet) was obtained from the Coleman & Bell Co. (Norwood, OH).

Immunoblotting. After isoelectric focusing, the gels were conditioned in a denaturing buffer containing SDS and 2-mercaptoethanol (13), and the proteins were then electroblotted to nitrocellulose. Membranes were probed with a purified polyclonal rabbit anti-PVY γ-globulin preparation using standard procedures, followed by the visualization of the coat protein bands with an anti-rabbit alkaline phosphatase conjugate (Sigma, St. Louis, MO) and the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega, Madison, WI).

Isoelectric point determinations. Experimental isoelectric points were determined by interpolation using the surface pH measurements obtained above. Theoretical isoelectric points were calculated from the published amino acid sequences below, using the program ISOELECTRIC of the University of Wisconsin GCG program package version 7.3 (6).

The coat protein sequences used for isoelectric point calculations were taken from Sudarsono et al (34) and van der Vlugt et al (35). Some of the isolates were found in both articles but with different names; however, each isolate was considered only once. The unpublished coat protein sequences of isolates PVY^N N27 and PVY^O Y139 (GenBank accessions U09508 and U09509, respectively) were also used.

Molecular weight determinations. The molecular weights of the PVY coat proteins were estimated by SDS-PAGE using the discontinuous buffer system of Laemmli (11). Gels were stained with Coomassie Brilliant Blue R-250. Molecular weights were calculated from the published amino acid sequences (35) with the GCG program PEPTIDESORT (6).

RESULTS

Isoelectric focusing and SDS-PAGE. To determine the isoelectric points of the coat proteins, the purified viruses of 13 isolates of PVY (seven PVY^O and six PVY^N) were focused in carrier ampholyte-generated pH gradients in the presence of 8 M urea and 2% NP40 (Fig. 1). All isolates produced one major band and variable numbers of minor bands. The isolates belonging to PVY^O had coat proteins with higher isoelectric points than those of PVY^N. The isoelectric points of the major species are given in Table 1. All the PVY^N isolates had the same pI and produced an identical banding pattern with three minor bands, whereas the PVY^O group banding was more heterogeneous. The PVY^O group contained three subgroups: 1) isolates 1, 5, TGB, and MA927; 2) isolate MA667; and 3) isolates 3 and 7. The PVY^O and PVY^N groups are similar in that the minor bands form an alkaline doublet and an acidic singlet, except isolates 3 and 7, which gave an alkaline singlet, relative to the major species. However, the difference between the isoelectric points of the major and minor bands is greater for PVY^O than for PVY^N. In other experiments (*data not shown*), a PVY^O isolate, isolate 6, also gave the same pattern as isolates 3 and 7. These various patterns were obtained with both Ampholine and Pharmalyte carrier ampholytes, indicating that the bands were not due to spurious carrier ampholyte-protein interactions. The patterns were also virus- and not host-specific, as demonstrated by purifying viruses from both tobacco and potato plants (*data not shown*). Electroblotting coupled with immunological detection of the focused proteins showed that they were of viral origin and not host contaminants (Fig. 2).

To determine if the differences in isoelectric point of the major species were due to proteolytic degradation, the viruses were analyzed by SDS-PAGE (Fig. 3). In all cases, the major species migrated as a 29.5-kDa molecule in close agreement with the value of 29.9 kDa calculated from a sequenced PVY coat protein gene (26). All the PVY^O also gave one minor 27-kDa band, except TGB, where the lower molecular weight band was quite strong. The relative intensity of this band in TGB appeared to remain constant during storage of the purified virus. In the case of PVY^N, two minor bands occurred with mobilities slightly greater or slightly less than that of the minor component in PVY^O.

Comparison of the isoelectric points of PVY^O and PVY^N coat proteins deduced from the amino acid sequence. The theoretical

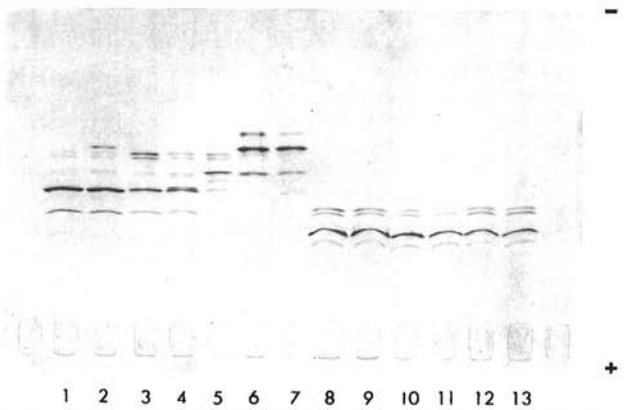

Fig. 1. Analysis of purified PVY isolates by denaturing isoelectric focusing. Lanes 1–13 are: isolate 1, isolate 5, TGB, MA927, MA667, isolate 7, isolate 3, TU619, TU660, Q9, N27, Y^{N}138, and P579. Lanes 1–7 are PVY^O and lanes 8–13 are PVY^N. The pH gradient in the gel was 3.5–10. The anode is at the bottom.

TABLE 1. Isoelectric points of PVY^O and PVY^N isolates determined by isoelectric focusing

Strain	Isolate	pI
PVY^O	PVY-1	6.8
	PVY-5	6.8
	PVY-TGB	6.8
	PVY-MA927	6.8
	PVY-MA667	7.2
	PVY-3	7.4
	PVY-6	7.4
	PVY-7	7.4
PVY^N	PVY-N27	6.1
	PVY-P579	6.1
	PVY-Q9	6.1
	PVY-TU619	6.1
	PVY-TU660	6.1
	PVY-Y^{N}138	6.1

isoelectric points for the coat proteins of 28 PVY isolates were calculated from the published amino acid sequences (34,35) using the GCG program ISOELECTRIC (Table 2). In most cases the pI for the PVY^O isolates was higher than that for the PVY^N isolates. Only four PVY^O isolates (PVY-D, PVY-Is, PVY-10, and PVY-43) had pI values lower than the highest PVY^N value calculated.

Both the theoretical and experimental isoelectric points were determined for one isolate of PVY^O, PVY-Y139 (GenBank accession U09509) (M. Singh and R. P. Singh, *unpublished*), and one isolate of PVY^N, PVY-N27 (accession U09508) (A. Dhar and R. P. Singh, *unpublished*). The experimental and theoretical pI values of 6.9 and 7.07 for PVY-Y139, and the corresponding values of 6.1 and 6.27 for PVY-N27, are in reasonable agreement, thereby providing an internal standard for comparison.

DISCUSSION

Detailed amino acid sequence comparisons of the coat protein of several PVY isolates showed that these data not only unambiguously identified the individual isolates but also discriminated between the PVY^O and PVY^N strains (35). These sequence data also suggested that IEF of viral coat proteins might be used to discriminate between isolates, particularly if only a limited set of isolates is involved. Unexpectedly, the results suggested that IEF may furthermore aid in distinguishing between strains.

The difference in the pI of the major species of the coat proteins of various isolates is probably due to sequence differences and not degradation. The results obtained by SDS-PAGE show that all coat proteins are of the same size; however, it is to be expected that limited exoproteolysis would not be revealed by this method. The mobility of the major band was that of a 29.5-kDa species in good agreement with the calculated molecular weight of 29.9 kDa (A. Dhar and R. P. Singh, *unpublished*). Previously reported molecular weights of 33–34 kDa (8,16) were determined by the method of Weber and Osborn (37). Shukla et al (26) obtained a value of 32 kDa using the method of Laemmli (11). The minor bands observed in SDS-PAGE have been reported previously (9,10,16) and are probably due to degradation during storage rather than in situ modification by host cell proteases (28). These degradation products would account for some of the minor bands observed in the higher resolution IEF gels. It has also been suggested that some of the heterogeneity observed in SDS-PAGE may be due to charge isomers (8). The similarity of the minor bands in the PVY^O and PVY^N groups, both by IEF and SDS-PAGE, suggests a similar tertiary structure for the coat protein for the members within each group. Experiments involving limited tryptic digestion of virions followed by IEF peptide profiling support this idea (*data not shown*).

The difference in pI between the PVY^O and PVY^N strains determined experimentally by IEF (Table 1) was also found by calculating the pI of several PVY isolates using available sequence data. An estimate of the difference in pI between these two strains was obtained with an unpaired t test of the combined values for all PVY^O and all PVY^N in Tables 1 and 2. The mean pIs of PVY^O and PVY^N were 7.1 ± 0.37 (SD) and 6.4 ± 0.31, respectively, and significantly different ($t = 5.8$).

The difference in pI between the PVY strains has both practical and biological implications. The results shown in Figure 2 suggest a screening method for crude samples. With use of a commercially precast ultra-high resolution immobilized pH gradient gels and a three-electrode IEF device (21), up to 100 samples can be focused,

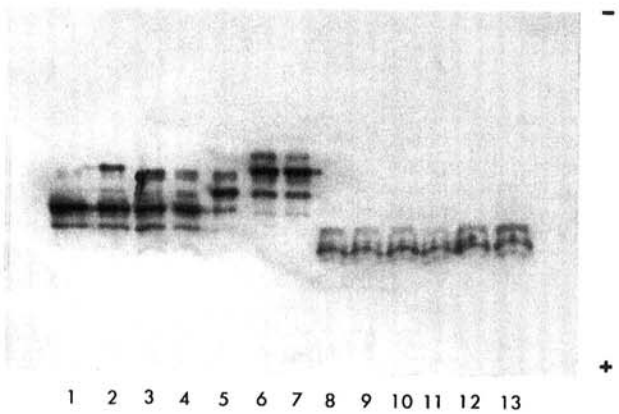

Fig. 2. Analysis of purified PVY isolates by denaturing isoelectric focusing and Western blotting. Lanes 1–13 are: isolate 1, isolate 5, TGB, MA927, MA667, isolate 7, isolate 3, TU619, TU660, Q9, N27, Y^N138, and P579. Lanes 1–7 are PVY^O and lanes 8–13 are PVY^N.

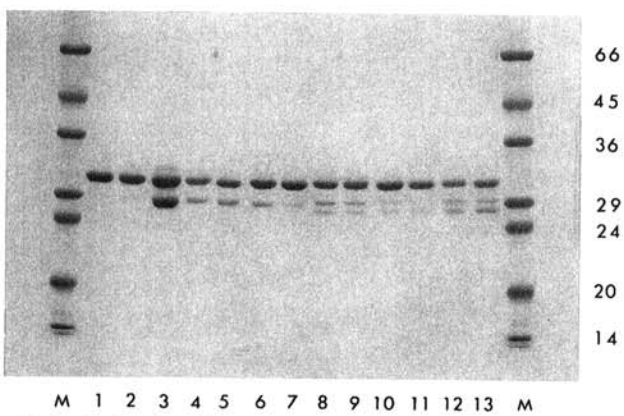

Fig. 3. Analysis of purified PVY isolates by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Lanes 1–13 are: isolate 1, isolate 5, TGB, MA927, MA667, isolate 7, isolate 3, TU619, TU660, Q9, N27, Y^N138, and P579. Lanes 1–7 are PVY^O and lanes 8–13 are PVY^N. The molecular weight values of the standards in kilodaltons are indicated on the right.

TABLE 2. Isoelectric points of PVY^O and PVY^N isolates calculated from the amino acid sequence[a]

Strain	Isolate	pI
PVY^O	PVY-43	6.38
	PVY-Is	6.52
	PVY-10	6.52
	PVY-D	6.52
	PVY-O3	6.79
	PVY-MsNr	7.07
	PVY-Y139	7.07
	PVY-Nl3	7.11
	PVY-Us	7.11
	PVY-Ch	7.11
	PVY-O4	7.11
	PVY-O	7.16
	PVY-NsNr[b]	7.16
	PepMoV	7.53
	PVY-O2	7.54
	PVY-O1	7.54
	PVY-Potato US	7.72
PVY^N	PVY-N27	6.27
	PVY-Nl2	6.27
	PVY-Europe H	6.28
	PVY-T	6.52
	PVY-Nl1	6.52
	PVY-Ru	6.63
	PVY-Go16	6.79
	PVY-Jp	6.79
	PVY-Hu	6.79
	PVY-Fr[c]	6.79
	PVY-Chilean	6.81

[a] All sequences were taken from Sudarsono et al (34) and van der Vlugt et al (35) except PVY-N27 and PVY-Y139, accessions U09508 and U09509, respectively.

[b] Lethal to young tobacco plants but actually closely related to the PVY^O strain (34).

[c] Produces PVY^N symptoms but classified as a PVY^O by van der Vlugt et al (35).

electroblotted, and then detected with polyclonal anti-PVY γ-globulin. While clearly not as rapid as automated ELISA, IEF data provide an alternate means for categorizing viral isolates and may serve as a preliminary guide for more in-depth investigations.

The difference in pI may also be of biological significance. The coat protein is thought to have a role in vector transmission (1) and possibly cell attachment (23). The N-terminus of the coat protein, which is exposed on the surface (27,29), is likely to be involved in these interactions. More significantly, the coat protein has also been implicated in host specificity, cross-protection, symptom production, and virulence (25). Amino acid substitutions in the coat proteins of a number of viruses have been correlated with symptom expression (3,17–19,24). In light of this, it would not be surprising if significant differences in the charge of a facet of the major viral protein could influence the interaction with the host and hence the outcome of the infection.

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