Different Helper Component Mutations Associated with Lack of Aphid Transmissibility in Two Isolates of Potato Virus Y

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ABSTRACT

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Two potato virus Y (PVY) isolates, PVY-0 NAT and PVY-1, were characterized as aphid-nontransmissible in plant-to-plant transmission tests. Membrane and sequential transmission tests determined that their lack of transmissibility was due to a defect in their helper component (HC) proteins. A comparative analysis of the HC genes of the nontransmissible isolates PVY-0 NAT and PVY-1 with a transmissible isolate, PVY-0, showed that a single amino acid substitution differentiated the HC of isolate PVY-0 NAT from that of isolate PVY-0. The substitution was located within a cysteine-rich region at the amino-terminal region of the protein, in the same position as mutations found in other HC-deficient potyviruses. However, the sort of exchange differed (Lys to Glu for potato virus C and for isolates R5A and Connecticut of zucchini yellow mosaic virus; Lys to Asn for isolate PVY-0 NAT). On the other hand, the HC of isolate PVY-1 had two amino acid substitutions; one of them (Gly to Asp) was also within the cysteine-rich region, and the other (Ser to Gly) was in the carboxy-terminal region of the protein. These aphid-nontransmissible isolates presented different HC-related properties. While the HC protein was detected in partially purified preparations obtained from PVY-0 NAT-infected plants, it could not be detected in equivalent preparations obtained from PVY-1-infected plants, even though the presence of an HC in these plants was demonstrated by serological means. In addition, the HC of this viral isolate showed an electrophoretic behavior distinct from those shown by the HCs of other PVY isolates. The relationships between the nucleotide sequence data and these HC-related properties are discussed.

Potato virus Y (PVY) is the type species of the genus *Potyvirus*. Virions are composed of a single positive-sense RNA helically surrounded by about 2,000 copies of the coat protein (CP), with a viral-encoded protein (VPg) covalently linked at the 5′ terminus of the RNA and a 3′ poly(A) tail. The viral RNA codes for a single polyprotein that is autoproteolytically processed to generate the mature products (1,11,24).

Most potyviruses are nonpersistently transmitted by aphids in nature (13). So far, two viral proteins are known to be involved in the transmission process: the viral CP and a nonstructural protein, the helper component (HC) (2,4,5,6,13,23). Although the role of these two proteins is not yet clear, artificial membrane feeding experiments have demonstrated that aphids need to acquire the HC prior to, or in combination with, the virions for transmission to take place (13). The biologically active HC is thought to be an oligomer soluble protein (29,31).

A few aphid-nontransmissible potyviruses with HC deficiencies have been reported thus far (14,15,19,30). In addition, several HC mutants have been obtained from full-length clones of tobacco vein mottling virus (TVMV) and of tobacco etch virus (TEV) (2,3,10). Interestingly, accumulation of virions and expression of symptoms in plants infected with these mutants are affected. In contrast, naturally occurring HC-defective isolates induce wild-type symptoms. Nucleotide sequence analysis of the genes coding for defective HCs in natural isolates, as well as data from mutants generated by site-directed mutagenesis, have identified single amino acid substitutions that abolish the biological activity of this protein in transmission (2,3,14,16,22,30).

In the present work, the lack of transmissibility by aphids of two PVY isolates had been assigned to a defect in their HCs. When analyzed, the nucleotide sequence data of their HC genes showed that they exhibited different mutations. The HCs of these nontransmissible isolates showed differences in their purification recoveries and electrophoretic behavior.

MATERIALS AND METHODS

Virus isolates and host plants. All viruses used were provided by F. Ponz (CIT-INIA, Madrid). PVY-N is a French potato isolate of known genomic sequence (25). Isolates PVY-0, PVY-0 NAT, and PVY-1 originated from Spanish field-grown pepper plants (20), and were kept in the greenhouse through a series of mechanical passages in tobacco (*Nicotiana tabacum* L. cv. Xanthinc) and pepper (*Capsicum annuum* L. cv. Yolo Wonder) plants. For this study, all isolates were maintained in tobacco plants by mechanical or aphid inoculation.

Virus and HC purification. Virions and HC preparations were obtained from leaves of systemically infected tobacco plants, 14 to 21 days after inoculation according to standard procedures (21,29). The partial purification of the HC protein included a sucrose gradient fractionation step. After their purification, HC preparations were shown to be virus free (data not shown) by membrane acquisition transmission tests (see below). Purified virions and HC preparations were analyzed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 5.0 to 12.5% gradient gels (18).

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Serology. All plants involved in the transmission experiments were tested for infection by double antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) (8), using a kit of monoclonal antibodies (MAbs l0E3 and l0E3-Biotin conjugated) to PVY CP (27). A rabbit polyclonal antibody (PAb) to PVY HC (HC-PAb) was used for serological detection of HC protein (7).

Aphid transmissions. Transmission tests were carried out at room temperature under fluorescent white light, using tobacco (*N. tabacum* cv. Xanthi-nc) as the assay plant. Groups of 40 aphids (*Myzus persicae* Sulzer), previously starved in glass vials for 2 to 3 h, were placed on leaves or artificial membranes and allowed to probe for 10 min. Ten aphids were transferred to each assay plant and allowed to stay overnight before being killed with an insecticide. Three types of transmission tests were performed.

Plant-to-plant transmission. Aphids were allowed to probe on young leaves of infected tobacco plants inoculated 10 to 20 days prior to the test, and were then placed on assay plants.

Membrane acquisition transmission. Aphids were allowed to feed through stretched Parafilm membranes on purified virus (200 µg/ml) diluted in an HC preparation (1 ml of HC preparation obtained from 100 g of fresh leaf tissue) containing 20% (wt/vol) sucrose. Homologous and heterologous virus/HC combinations were assayed.

			Position
C1 (+)	5'-CGAG*AAGCTT*CATAGTGCGTGG-3'	*HindIII*	963
C2 (-)	5'-CGAG*GAATTC*CAGCTGGTTGTC-3' A	*EcoRI*	1622
C5 (+)	5'-TGTA*AAGCTT*GTCCGCCAAAGC-3'	*HindIII*	1596
C6 (-)	5'-TCT*GAATTC*CCAAGTTCAGGGC-3'	*EcoRI*	2421
Primers used for sequencing			
C3 (+)	5'-TTGAGGACTGCGGTAGG-3'		1144
C4 (-)	5'-GTGCTTGCTGCTTCTCC-3'		1402
C7 (+)	5'-TGATGATGGTTGAGCC-3'		1936

Nt sequence identical (+) or complementary (-) to the virus sequence

Fig. 1. Primers used in the cloning and/or nucleotide sequence determination of the helper component genes.

Sequential transmission. Aphids were allowed to feed through stretched Parafilm membranes on HC preparations containing 20% sucrose prior to being placed for an additional 10-min acquisition access period on a leaf taken from a plant infected with a nontransmissible isolate. As a control, this leaf was also used in a plant-to-plant transmission test.

Western blot analysis. Extracts from virus-infected tobacco leaves were electrophoresed in discontinuous SDS-PAGE gradient gels (4.5% stacking gel, 5 to 15% resolving gel) followed by electroblotting to nitrocellulose membranes (32). After blotting, membranes were blocked with 5% (wt/vol) skim milk powder and incubated first with HC-PAb (1 µg/ml), and then with a goat anti-rabbit peroxidase-conjugated antibody (1 µg/ml).

Leaf extracts were obtained by grinding fresh leaf tissue (1:10 wt/vol) either in phosphate-buffered saline (PBS) (0.15 M NaCl and 0.015 M sodium phosphate [pH 7.4]), or in PBS plus 2% 2-mercaptoethanol (2-ME). Extracts were clarified at 3,000 × *g* for 15 s in a microcentrifuge. In PBS-prepared extracts, 2-ME was added to the supernatant aliquots to 1% (vol/vol), in order to obtain equivalent final concentrations of 2-ME using either extraction buffer. Samples were added with SDS (2%) and glycerol (10%) (vol/vol), and were boiled for 5 min prior to electrophoresis.

Time-course analysis of HC accumulation in leaf. Indirect (antigen coating) ELISA tests (9) using HC-PAb were performed to compare the kinetics of accumulation of PVY-0, PVY-0 NAT, and PVY-1 HCs in a tobacco leaf during systemic infection. For each virus, 15 plants were mechanically inoculated on two adjacent leaves with equal amounts of infected plant extracts (20 µl/leaf). At 2, 4, 6, 10, or 14 days postinoculation, the leaf above those inoculated was collected, weighed, and frozen at –80°C. Three leaves from three different plants at each period were sampled. The entire frozen leaf was ground in PBS (1:5 wt/vol) with 0.05 M phenylmethylsulphonylfluoride (PMSF). The extract was clarified in a microcentrifuge (3,000 × *g* for 15 s), diluted 1:1 with 0.05 M Na-carbonate buffer (pH 9.6), and analyzed by indirect ELISA using the HC-PAb. Additionally, a mixture of equal amounts (100 µl) of the three extract supernatants corresponding to the same time interval postinoculation was analyzed by indirect ELISA and by Western blotting using a MAb against the CP of PVY (MAb 10E3).

Plates were coated (100 µl/well) overnight at 4°C, incubated for 2 h at 30°C with either HC-PAb or MAb l0E3 (1 µg/ml) in

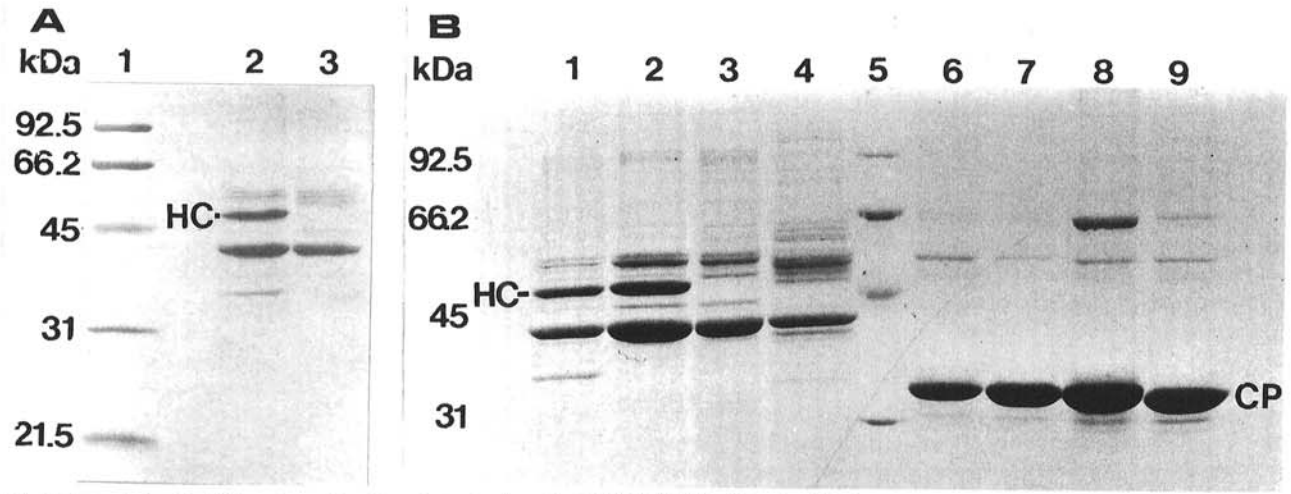

Fig. 2. A, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% gel) of helper component (HC) preparation obtained from PVY-0 infected tobacco (lane 2) and an equivalent fraction obtained from healthy tobacco (lane 3). Lane 1, molecular weight markers. **B,** SDS-PAGE (12.5% gel) of virion and HC preparations used in membrane acquisition transmissions. Lanes 1 to 4: HC preparations obtained from plants infected with isolates PVY-0, PVY-0 NAT, PVY-1, and PVY-N, respectively. The position of HC protein is marked. Lanes 6 to 9: Purified PVY-0, PVY-0 NAT, PVY-1, and PVY-N virions, respectively. The coat protein position is marked. Lane 5: Molecular weight markers. Both gels stained with Coomasie Blue.

PBS plus 0.2% Tween (PBS-T) and 0.3% skim milk powder, and finally incubated with goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated antibody, respectively. The assay was developed with four wells per sample to obtain each value.

Cloning and sequencing of HC genes. DNA fragments corresponding to the genes that code for the HC protein of isolates PVY-0, PVY-0 NAT, and PVY-1 were obtained by reverse transcription of viral RNA with avian myeloblastosis virus reverse transcriptase followed by polymerase chain reaction (PCR) amplification, using the primers shown in Figure 1. PCR products were cloned into *Eco*RI/*Hin*dIII-digested pT3T7 or pUC 18 plasmids for sequencing. To determine each nucleotide position, at least two clones were sequenced by the dideoxynucleotide chain termination method (26). The exchanges found were also verified by direct sequencing of PCR products as described for the Fmol DNA sequencing system (Promega Corp., Madison, WI). The nucleotide sequences obtained were assigned to nucleotide positions in the genome according to the sequence of isolate PVY-N (25). The deduced amino acid sequences were aligned with other potyviral HC sequences using the PILE UP program (12).

RESULTS

Analysis of purified virions and HC preparations. The four isolates, PVY-0, PVY-0 NAT, PVY-1, and PVY-N, induced similar symptoms in tobacco: vein clearing and systemic mosaic. Electrophoretic analysis of HC preparations obtained from infected plants showed a band with a molecular weight of about 50 kDa, corresponding to the HC protein (Fig. 2A, lane 2). This band was not detected in equivalent preparations from healthy plants (Fig. 2A, lane 3). Several other bands corresponding to plant proteins were also present (Fig. 2A). The amount of HC protein detected in PVY-0 and PVY-0 NAT HC preparations were similar to each other, but higher than that found in PVY-N HC preparations. Despite repeated attempts, purification of PVY-1 HC was unsuccessful, and the 50-kDa HC band could not be detected in any of these preparations (Figs. 2B and 3).

In contrast, virion purification yields were very similar for the four isolates, and ranged from 0.9 to 2 mg/100 g of fresh leaf tissue. When analyzed electrophoretically, the viral CP was detected as a major band of about 35 kDa, except for isolate PVY-N, which showed a slightly different mobility (33 kDa) (Fig. 2B, lanes 6 to 9).

Aphid transmissions. While plant-to-plant transmission of isolates PVY-0 and PVY-N was highly successful, transmission of isolates PVY-0 NAT and PVY-1 was negative in all attempts (Table 1). To determine whether viral CPs or HCs were involved in this lack of transmissibility, a series of membrane and sequential transmission experiments were performed.

In membrane acquisition tests, high rates of virus transmission were obtained when the purified virions of any of the four isolates were combined with PVY-0 or PVY-N HC preparations. In contrast, PVY-0 NAT HC preparations did not assist aphid transmission of either the homologous virus or the other three isolates, suggesting that a defect in the HC protein caused the lack of virus transmission (Table 2). As expected, PVY-1 HC preparations, in which the HC protein was not detected, did not assist any of the viruses tested in transmission (data not shown).

In sequential transmission tests, aphids transmitted both nontransmissible isolates, PVY-0 NAT and PVY-1, from infected plants, only if they were previously allowed to feed on a preparation containing active HC (Table 3). The control plant-to-plant transmissions remained negative (Table 3). This indicated that in both isolates the viral CPs efficiently mediated transmission, and suggested that, for PVY-1 also, a defect in the HC protein was the reason for its lack of transmissibility.

Western blot analysis of the HC protein in leaf extracts. HCs of three isolates, including that of PVY-1 which could not be purified, were detected in leaf extracts of infected plants by Western blot analysis (Fig. 4). Surprisingly, in all cases the HC protein generated two different electrophoretic banding patterns, depending on the extraction procedure. When a sulfhydryl reducing agent (2-ME) was present in the extraction buffer (PBS-ME), only the 50-kDa HC band was detected (Fig. 4, lanes 1 to 3). However, when the extraction buffer was PBS and 2-ME was

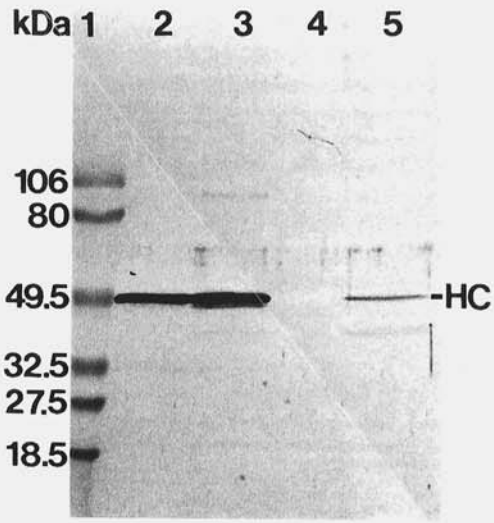

Fig. 3. Western blot analysis (10% gel) of helper component (HC) preparations obtained from plants infected with isolates PVY-0, PVY-0 NAT, PVY-1, and PVY-N (lanes 2 to 5, respectively). Lane 1: prestained molecular weight markers.

TABLE 1. Efficiency of plant-to-plant transmission of four isolates of potato virus Y (PVY) by aphids

Experiment	Transmission of virus isolates of			
	PVY-0	PVY-0 NAT	PVY-1	PVY-N
1	8/8[a]	0/9	0/18	4/6
2	6/6	0/18	0/9	9/9
3	9/9	0/9	0/9	8/9
4	5/9	0/9	0/8	3/8
5	6/8			6/6
Total	34/40	0/45	0/44	30/38

[a] Values are the number of infected plants over total number of plants employed in each experiment. Ten aphids per assay plant were used.

TABLE 2. Efficiency of aphid transmission of four purified isolates of potato virus Y (PVY) in the presence of homologous and heterologous helper component (HC)

Source of HC	Experiment	Purified virions			
		PVY-0	PVY-0 NAT	PVY-N	PVY-1
PVY-0	1	9/9[a]	9/9	9/9	8/8
	2	9/9	8/8	9/9	7/8
	3	3/3	8/8	9/9	8/8
	4	6/6		8/8	
	Total	27/27	25/25	35/35	23/24
PVY-0 NAT	1	0/9	0/9	0/9	0/8
	2	0/9	0/8	0/18	0/8
	3	0/9	0/8		0/8
	Total	0/27	0/25	0/27	0/24
PVY-N	1	8/8	8/8	12/15	7/8
	2	12/18	16/16	8/9	8/8
	3			5/9	7/8
	Total	20/26	24/24	25/33	22/24

[a] Values are the number of infected plants over total number of plants employed in each experiment. Ten aphids per assay plant were used.

added later, several bands with apparent sizes ranging from 100 to more than 200 kDa were recognized by the HC-PAb, in addition to the 50-kDa HC band (Fig. 4, lanes 4 to 6). In both cases, several minor bands with molecular weights smaller than 50 kDa were also present, possibly corresponding to degraded HC.

Extracts from PVY-0-, PVY-0 NAT-, and PVY-1-infected leaves are also shown in Figure 4, half of a leaf extracted by each procedure. In the case of PBS-prepared extracts, isolates PVY-0 and PVY-0 NAT gave identical HC banding patterns, similar to the one produced by isolate PVY-N (data not shown). In comparison, the banding pattern given by PVY-1 HC showed several differences; the 50-kDa HC band was either undetected or present in trace amounts, a band comparable with the 150-kDa oligomer was missing, and most of the HC accumulated in bands of higher molecular weight (Fig. 4, lane 6). These patterns were preserved among different plants and in different leaves within a plant (data not shown).

HC accumulation in the leaf. The kinetics of HC accumulation in a single tobacco leaf, measured by indirect ELISA, showed an increase in the amount of HC with time during the period tested (Fig. 5A). ELISA readings were very similar for PVY-0- and for PVY-0 NAT-infected leaves. In contrast, readings for PVY-1-infected leaves showed lower relative values at 10 and 14 days postinoculation (Fig. 5A). Interestingly, the kinetics of accumulation of the viral CPs, measured by indirect ELISA and by Western blotting, were similar among the three viruses (Fig. 5B).

Comparative analysis of the HC genes. For the three isolates, the putative HC gene was 1,368 nucleotides in length (Fig. 6). The nucleotide sequence of the HC gene of isolate PVY-0 NAT differed from that of isolate PVY-0 in two nucleotides, at positions 1,186 and 1,543. Only one of them induced an amino acid exchange (position 334, Lys to Asn). It was located in the same position of mutations found in other HC-deficient potyviruses. The HC gene of isolate PVY-1 differed from that of PVY-0 in two nucleotides, at positions 1,140 and 2,149, resulting in two amino acid substitutions (position 319, Gly to Asp, and 639, Ser to Gly) (Fig. 6). The former was located also within the above mentioned cysteine-rich cluster. The latter was located in the carboxy-terminal region of the protein (Fig. 6).

TABLE 3. Efficiency of aphid transmission in sequential assays of two naturally aphid-nontransmissible isolates of PVY

PVY-0 NAT-infected plants			PVY-1-infected plants		
Control plant-to-plant	Aphids fed first on solutions with HC		Control plant-to-plant	Aphids fed first on solutions with HC	
	PVY-0 HC	PVY-N HC		PVY-0 HC	PVY-N HC
0/9[a]	7/9	...	0/6	4/6	...
0/4	8/8	...	0/4	3/4	...
0/4	2/4	...	0/9	4/4	...
0/9	...	5/9	0/9	...	8/9
0/9	...	6/9	0/6	...	7/9
0/4	...	4/4	0/6	...	6/6
	...	...	0/4	...	3/6
	...	...	...	...	3/4
Total:					
0/39	17/21	15/22	0/48	11/14	27/34

[a] Values are the number of infected plants over total number of plants employed in each experiment. Ten aphids per assay plant were used.

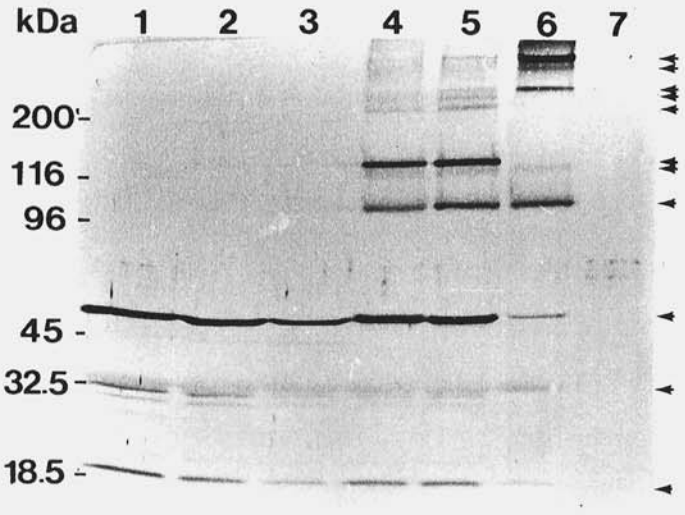

Fig. 4. Western blot analysis (5 to 15% gradient gel) of extracts from infected leaves, using rabbit polyclonal antibody to PVY helper component (HC-PAb). Half of a leaf extracted in phosphate-buffered saline (PBS) plus 2-mercaptoethanol (2-ME) (lanes 1 to 3) and the other half in PBS (lanes 4 to 7). Extracts from PVY-0- (lanes 1 and 4), PVY-0 NAT- (lanes 2 and 5), and PVY-1- (lanes 3 and 6) infected plants, respectively. Lane 7: extract from a healthy plant. Arrows show the major bands recognized by the antibody to the HC.

DISCUSSION

Two PVY isolates, PVY-0 NAT and PVY-1, were nontransmitted by aphids in plant-to-plant transmission tests. However, with

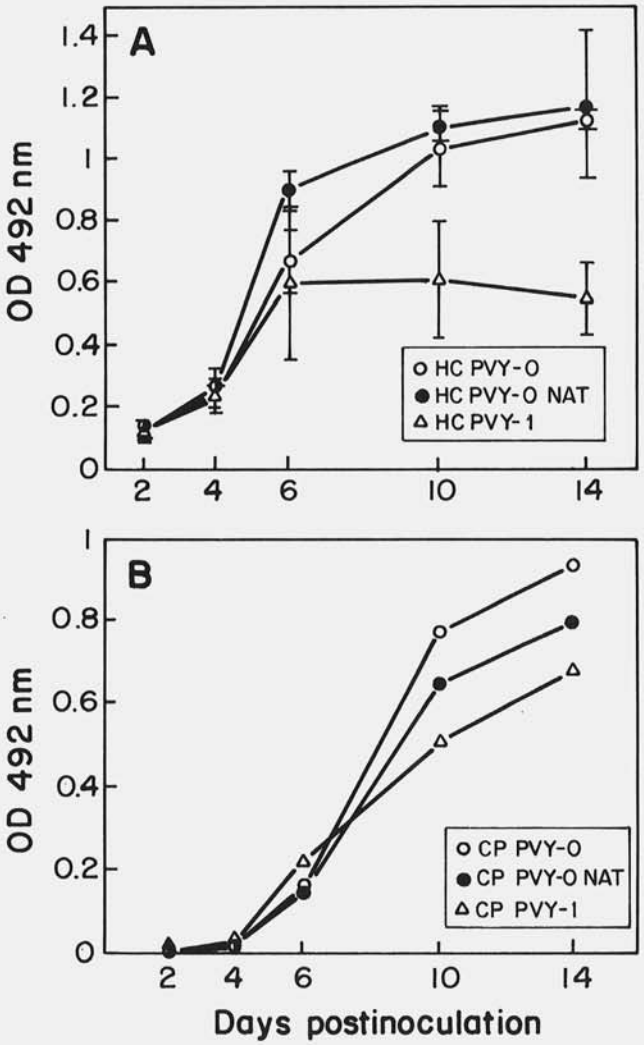

Fig. 5. A, Kinetics of helper component (HC) accumulation in leaf during systemic infection with PVY-0, PVY-0 NAT, or PVY-1, estimated by indirect enzyme-linked immunosorbent assay (ELISA), using rabbit polyclonal antibody to PVY helper component (HC-PAb). Each data point represents the average ELISA optical density readings of three leaves. Vertical bars delimit 95% confidence intervals. **B,** Kinetics of coat protein (CP) accumulation measured by indirect ELISA using monoclonal antibody (MAb) 10E3.

both isolates, virus transmission could be achieved if aphids were allowed to acquire biologically active HC either prior to or simultaneously with the virions, in sequential or membrane tests, respectively. This indicated that viral CPs were functionally active for transmission, and suggested that a deficiency in HC was responsible for the lack of viral transmissibility. In fact, PVY-0 NAT HC preparations did not assist transmission of purified virions from PVY isolates known to be naturally transmissible, indicating the defective characteristic of its HC. This kind of evidence could not be presented for isolate PVY-1, because of our inability to purify its HC using the same purification protocol. Nevertheless, serological detection of HC in PVY-1-infected plants excluded the possibility that the lack of plant-to-plant transmission was caused by low levels of this HC protein. This, together with the results of sequential tests, strongly suggested that isolate PVY-1 was also HC-defective.

Although both isolates PVY-0 NAT and PVY-1 induced wild-type symptoms in tobacco, similar to those induced by the transmissible isolate PVY-0, they presented different HC-related properties. Isolate PVY-0 NAT differed from isolate PVY-0 only in the lack of HC biological activity in transmission. The amounts of HC in preparations obtained from plants infected with isolates PVY-0 or PVY-0 NAT were very similar, suggesting that the alteration in PVY-0 NAT HC did not adversely affect its purification yield. This is the case of the previously described HC-defective isolate potato virus C (PVC), which also induces wild-type symptoms and whose HC can be readily purified (30). Thus, it is not surprising to discover that the unique amino acid exchange that differentiates the HC of isolate PVY-0 NAT from that of isolate PVY-0 is in the same position (amino acid 334) as the mutation hypothesized to cause the lack of biological activity of PVC HC (3,30). They differed, however, in the type of amino acid exchange. In the HC of isolate PVY-0 NAT, a noncharged residue (Asn) was substituted for a highly basic one (Lys). In the case of PVC and of the HC-defective isolates R5A and Connecticut of zucchini yellow mosaic virus (ZYMV) (14,15), a negatively charged residue (Glu) is substituted for a basic one (Lys). Results from site-directed mutagenesis analysis in TVMV suggest that a highly basic residue is needed in this particular position to preserve HC activity and that the activity would be abolished even by weakly basic residues (3).

The HC of isolate PVY-1 showed, by contrast, some properties that differentiate it from other PVY HCs. In addition to the lack of biological activity, we were not able to purify PVY-1 HC following a method successfully used with other PVY isolates. Furthermore, it presented a particular electrophoretic behavior. The comparative analysis of the HC genes of isolates PVY-1 and PVY-0 showed two amino acid substitutions. One of them, located at position 639, was an exchange between noncharged residues (Gly to Ser). The other was located near the amino terminus of the protein, at position 319, within a cysteine-rich region that has been suggested to be related to the biological activity of the protein in transmission (3). A negatively charged Asp residue was substituted for a noncharged, highly conserved Gly residue. These amino acids have very different hydropathic indexes (17) and the exchange could alter biological properties of the protein. This could explain the inability to recover the protein in the soluble fraction in the final steps of the purification process, perhaps due to aggregation.

On the other hand, the three HC genes sequenced here had a Val residue at position 509 that appeared to be independent of the isolates' transmissibility. This supports the assumption that this residue is not related to the lack of activity of PVC HC (29).

The finding that the HC protein has two electrophoretic banding patterns is novel. The multiple HC bands obtained when analyzing plant extracts elaborated under nonreducing conditions would represent protein polymers and it is possible that the cysteine-rich region of the protein is involved in its formation. As

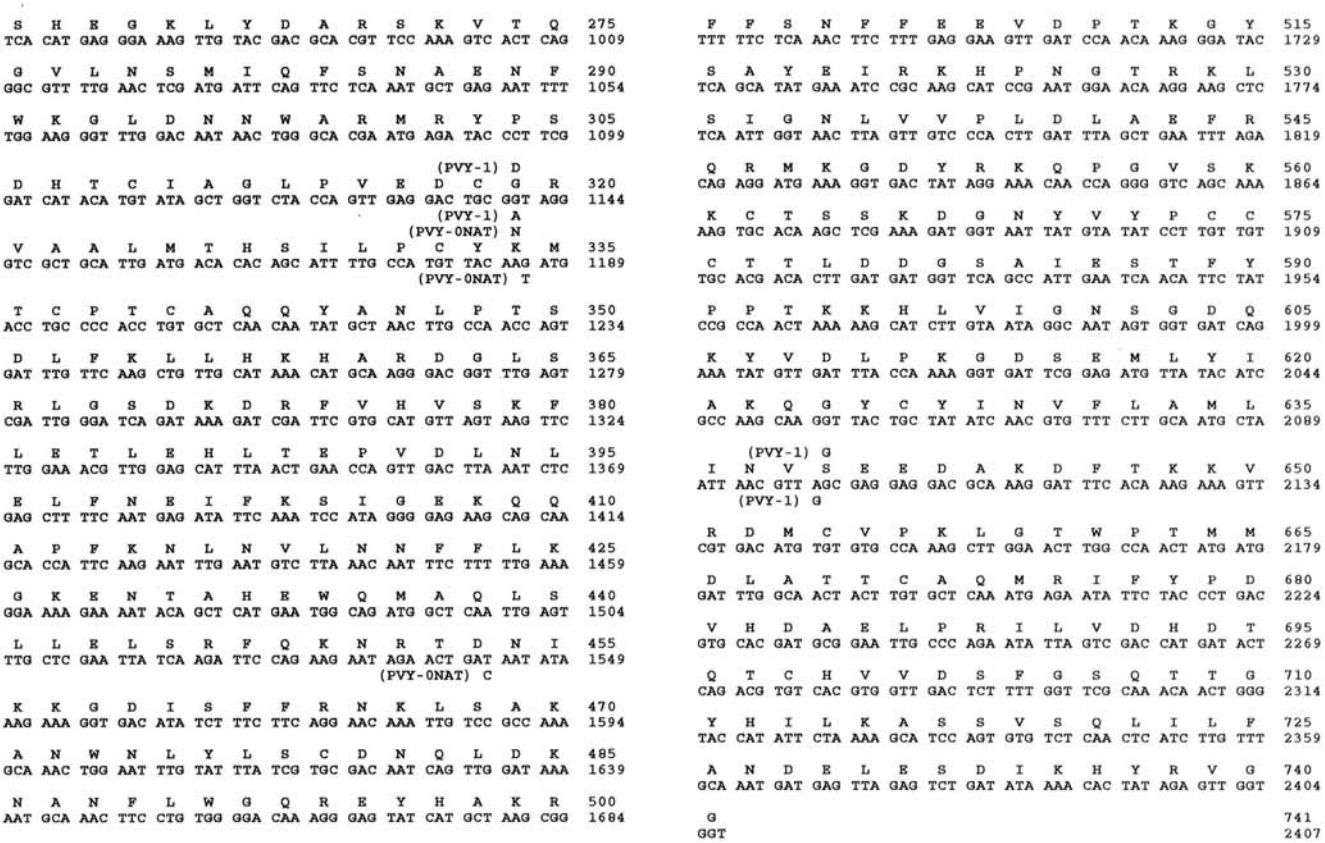

Fig. 6. Nucleotide sequence of the helper component (HC) genes of isolates PVY-0, PVY-0 NAT, and PVY-1, and deduced amino acid sequences. The sequence shown is that of PVY-0 HC. Nucleotide (below) or amino acid (above) differences in the HC sequences of isolates PVY-0 NAT and PVY-1 are indicated.

it has been pointed out, this region has similarities with the zinc-finger motifs (25) found in proteins with nucleic acid binding properties or in some proteins that may aggregate (28). In this regard, it is remarkable that the electrophoretic pattern of PVY-1 HC, which shows a mutation within this region, differs from those of other PVY HCs. Whether or not this electrophoretic behavior of the HC is specific to this protein and reflects an interactive property with biological significance in the viral transmission process is a subject of our current research.

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