Characterization of a Zucchini Yellow Mosaic Virus Isolate with a Deficient Helper Component

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ABSTRACT

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Two isolates of zucchini yellow mosaic virus (ZYMV) were found to be either highly (HAT) or poorly (PAT) aphid-transmissible. The inefficient transmission of ZYMV-PAT was independent of the virus source plant or aphid species used. The ZYMV-PAT and ZYMV-HAT, from which the former was derived, did not differ in their host range or serological properties. Purified virions of either ZYMV-HAT or ZYMV-PAT were readily transmitted by aphids when crude or concentrated helper component (HC) from ZYMV-HAT was provided, but were not transmitted when mixed with crude or concentrated HC from ZYMV-PAT. It is concluded that ZYMV-PAT HC is responsible for the defect in aphid transmissibility of this isolate. However a component serologically related to HC was detected both in ZYMV-PAT-infected plants or in ZYMV-PAT RNA in vitro translation products, indicating that an ineffective form of HC is produced by ZYMV-PAT. Furthermore, in in vitro acquisition experiments conducted with ZYMV-PAT and a non-aphid-transmissible isolate (ZYMV-NAT) deficient in the coat protein, high levels of transmission were observed only in the combination containing purified virions of ZYMV-PAT and HC from ZYMV-NAT. This provided additional proof for the deficiencies in the HC for ZYMV-PAT and in the coat protein for ZYMV-NAT.

Zucchini yellow mosaic virus (ZYMV), a member of the potyviruses, has been associated in the last decade with very severe economical losses in cucurbit crops in many parts of the world (18,20,22,23). As a typical potyvirus, ZYMV is transmitted in a nonpersistent manner, and requires a helper component (HC) protein encoded by the viral genome for its transmission by aphids (10,16).

Isolates of ZYMV are highly variable and may differ in serological properties, symptomatology, host range, and capacity to overcome resistance genes (15,18,22; Lecoq, *unpublished data*). Isolates differing in aphid transmissibility have also been reported (1,13).

Complete or partial loss of aphid transmissibility has been described for several potyviruses and this phenomenom seems to be relatively common for this group of viruses (19). Although assays for in vitro virus acquisition by aphids were conducted only in a few cases, most often it was possible to associate loss of aphid transmissibility with changes in viral coat protein (1,11,21). Harrison and Robinson (8) have compared the nucleotide sequence of the N'-terminal end of the coat protein gene of several potyviruses and found mutations in the triplet asp-ala-gly in non-aphid-transmissible isolates. More recently Atreya et al (2) demonstrated that a point mutation replacing the gly with asp on that triplet in the tobacco vein mottling virus (TVMV) coat protein induced loss of aphid transmissibility, confirming the key role of this short amino acid sequence in aphid transmission.

Lack of transmissibility can also be derived as a result of a deficiency in the helper gene of the potyvirus. Circumstantial evidence was brought by Kassanis and Govier (12) suggesting that the potato virus C (PVC) isolate of potato virus Y (PVY) was deficient in the HC activity. Thornbury et al (26) confirmed this suggestion by in vitro transmission experiments. Comparison of the amino acid sequence derived from the nucleotide sequence of the HC gene showed 24 amino acid differences between PVC and PVY HC proteins. However, comparisons conducted with the amino acid sequence of five other aphid-transmissible potyviruses revealed only two amino acid changes that were specific to PVC (26).

We report in the present study the characterization of a poorly aphid-transmissible (PAT) variant of ZYMV for which loss of

aphid transmissibility appears to be related to a change in the biological activity of the HC rather than to a change in the coat protein properties. A preliminary report of part of this work has been presented (13).

MATERIALS AND METHODS

Virus isolates. The highly aphid-transmissible isolate ZYMV-E15 has been described previously (17); in this study we will refer to it as ZYMV-HAT. The poorly aphid-transmissible isolate was obtained during the summer 1983 from a melon plant mechanically inoculated with ZYMV-HAT. This plant exhibited typical symptoms of infection by ZYMV-HAT, but was found in several attempts ineffective as a virus source for aphids. The ZYMV-PAT subculture was obtained from this plant, after three single local lesion transfers on *Chenopodium amaranticolor* Coste et Reyn. and *C. quinoa* Willd. followed by an increase in melon.

The ZYMV-NAT is a non-aphid-transmissible isolate that was obtained in Israel, following a series of mechanical inoculations; transmission tests indicated that lack of aphid transmissibility depended on the coat protein (1). The nucleotide sequence and the derived amino acid sequence of this isolate was described by Gal-On et al (5). This isolate has on the coat protein the triplet asp-thr-gly instead of the triplet asp-ala-gly, which is present in the aphid-transmissible isolate (6).

Mechanical inoculations were done by the standard methods used in our laboratories (14,23).

Serological assays. Serological assays were conducted either by the slide precipitin method (15), by the sodium dodecyl sulfate (SDS)-immunodiffusion technique (24), or by the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (3). The antiserum against ZYMV-HAT and those used in the in vitro translation experiments were prepared as previously described (17,28), whereas the TVMV-HC antiserum was donated by T. P. Pirone.

Virus purification. The method previously described (16) was used to purify the three virus isolates from infected melon (*Cucumis melo* L. 'Védrantais') or squash (*Cucurbita pepo* L. 'Small Sugar Pumpkin') 3–4 wk after inoculation. In purifications intended for RNA extraction, the final Cs_2SO_4 density gradient centrifugations were made as described by Xiong et al (28). Virus concentrations were determined spectrophotometrically by using an estimated extinction coefficient $E^{0.1\%}_{260nm} = 2.5$.

Partial purification of the HC. Crude HC-containing preparations were obtained by a method described previously (16). Three to four weeks after inoculation 3.5 g of infected leaves was carefully ground with a mortar and pestle in 10 ml of 0.3 M K_2HPO_4, pH 9. The homogenate was strained through gauze and the filtrate was centrifuged at 5,000 *g* for 10 min. The resulting supernatant was centrifuged at 145,000 *g* for 4 h in a Beckman R 50Ti rotor (Beckman Instruments, Fullerton, CA). The upper part of the supernatant was then carefully collected and used as a soluble fraction containing HC. These fractions, hereafter referred to as HC, were devoid of infectious virus particles and found to be effective in mediating virus transmission in a previous study (16).

To obtain concentrated HC preparations, 30% ammonium sulfate was added to the supernatant; after incubation for 1 h at 4 C, the mixture was centrifuged at 8,000 rpm for 10 min. The resulting pellet was resuspended in one fifth of the original volume of 20 mM K_2HPO_4, pH 7.2, buffer and homogenized with a cell grinder before centrifugation at 8,000 rpm for 10 min. The supernatant was dialyzed overnight at 4 C, before the addition of sucrose to a final concentration of 20%.

Aphid transmission experiments. Colonies of *Myzus persicae* Sulzer and *Aphis gossypii* Glover were reared as previously described (14) on melon and pepper plants, respectively.

Plant to plant transmissions were conducted as follows: after a 1- to 3-h starvation period, groups of 10–15 aphids were deposited on infected leaves for a 1- to 2-min acquisition period. Aphids found to be in probing position were carefully transferred to healthy plants with a camel's hair brush.

For in vitro acquisition experiments, groups of starved aphids were allowed a 10-min acquisition access period to the test solution through a stretched Parafilm membrane, according to a method described previously (16,25). In a first series of experiments, the test solutions contained 40 μg of purified virus per milliliter, fresh HC prepared within the same day, and 20% sucrose. Five aphids were placed on each of five or 10 melon test plants at the first leaf stage. In subsequent experiments, the test solutions contained 80 μg of purified virus per milliliter, concentrated HC, and 20% sucrose. Ten aphids were placed on each test plant.

Aphids were allowed to remain on test plants at least 2 h before being killed by an insecticide spray or fumigation. Plants were subsequently maintained for 3–4 wk in an insect-proof greenhouse.

In vitro translation experiments. The RNA was obtained from freshly purified virus preparations, after incubation in RNA dissociation buffer (0.2 M Tris, 2 mM EDTA, 2% SDS, pH 9) for 2 min at 60 C and separation by linear-log sucrose density gradient centrifugation as described by Dougherty and Hiebert (4). Translations of ZYMV RNAs in rabbit reticulocyte lysate (RRL) were performed as described by Dougherty and Hiebert (4). Twelve micrograms of RNA was used in 180 μl of RRL translation mixture. The reaction mixtures were incubated at 30 C for 90 min, and the reactions were terminated by adding 3 vol of Laemmli dissociation buffer (62.5 mM Tris, pH 6.8, 2% SDS [w/v], 4% [v/v] 2-mercaptoethanol, and 10% [v/v] glycerol). In vitro translation products were identified by immunoprecipitation with different potyviral protein antisera and by 7.5–15% SDS-polyacrylamide gradient gel electrophoresis (SDS-PAGE) as described by Hiebert and Purcifull (9).

RESULTS

Biological and serological properties of ZYMV-PAT. The ZYMV-PAT was not transmitted by *M. persicae* or *A. gossypii* when three viruliferous aphids were deposited per test plant (Table 1), whereas ZYMV-HAT was very efficiently transmitted. A possible effect of the host plant used as virus source in the poor transmissibility of ZYMV-PAT was also examined; neither the plant species used as a virus source nor the stage of infection greatly modified the transmission rates observed previously (Table 2).

Occasional transmissions of ZYMV-PAT were noticed, particularly when the number of vectors per plant was increased (Table 1). The nature of the resulting virus isolates was examined by aphid transmission tests; they always were identified as ZYMV-PAT according to their transmission rates and were not contaminations by an aphid-transmissible isolate. Probability of

TABLE 1. Transmission of two isolates of zucchini yellow mosaic virus (ZYMV) by two aphid species and mechanical inoculation

Means of transmission[a]	Number of aphids per test plant	ZYMV-HAT[b]	ZYMV-PAT[b]
Myzus persicae	1	17/30[c]	0/30[d]
	3	26/30	0/30
	5	30/30	0/30
	10	30/30	1/30
Aphis gossypii	3	27/30	0/30
Mechanical inoculation		30/30	30/30

[a] For aphid transmission, aphids were allowed a 1-min acquisition access period on infected melon Védrantais plants before being transferred to melon Védrantais test plants; for mechanical inoculation, infected melon tissue was macerated in 0.03 M Na_2HPO_4 containing 0.2% Na-diethyldithiocarbamate (1:4, w/v). Activated charcoal (75 mg/ml) and 400-mesh Carborundum (75 mg/ml) were added before rubbing extracts on cotyledons of melon Védrantais test plants.

[b] ZYMV-HAT, highly aphid-transmissible or ZYMV-PAT, poorly aphid-transmissible.

[c] Numerator = number of plants infected; denominator = number of plants inoculated.

[d] Plants infected following aphid transmission of ZYMV-PAT were individually checked and found to be inefficient virus sources for vector transmission.

transmission by a single *M. persicae* was estimated to be 0.004 and 0.446 for ZYMV-PAT and ZYMV-HAT, respectively, using the Gibbs and Gower transformation (7) and cumulated data of transmission to 165 test plants with three aphids per plant for each isolate.

A comparison of the symptomatology of the two isolates did not show marked differences, with one exception for cucurbit plants with old infections: plants infected by ZYMV-PAT often showed some level of recovery. Inoculation of a series of differential melon lines possessing the *Fn* or *Zym* genes (15) showed that both isolates belong to the same pathotype as described previously for ZYMV-E15 (HAT) (15).

A polyclonal antiserum prepared against ZYMV-HAT was used in SDS-immunodiffusion tests, and ZYMV-PAT reacted by forming a single precipitation line that fused without spurring with that of ZYMV-HAT. In slide precipitation tests, both isolates had identical titers (1:1,024) against the same bleeding of an antiserum to ZYMV-HAT; no residual reaction was observed when this antiserum was cross-absorbed with a partially purified virus preparation of ZYMV-PAT.

Virus yields after purifications and virus-specific infectivities were estimated to determine the possibility that differences in virus concentrations in plants or in virus stability may be responsible for the poor transmission of ZYMV-PAT. Generally higher virus yields were obtained from plants infected by ZYMV-PAT than from plants infected by ZYMV-HAT (means of 79 and 31 mg/kg for three independent purifications, respectively). No significant difference was observed in the specific infectivity of purified virus preparations (40 μg/ml) tested on *C. amaranticolor* (means of 43 and 45 local lesions per leaf for ZYMV-HAT and ZYMV-PAT, respectively, for three independent experiments).

In vitro acquisition experiments. To determine the reasons for the poor aphid transmissibility of ZYMV-PAT, different combinations of purified virus or HC were tested by in vitro acquisition experiments (Table 3). In the absence of either HC or purified viruses, no transmission occurred. Also, in all treatments in which the HC was derived from plants infected by ZYMV-PAT, no transmission occurred. On the other hand, high rates of transmission were observed when the HC was derived from plants infected by ZYMV-HAT, and purified virus was ZYMV-HAT or ZYMV-PAT (Table 3). To confirm that ZYMV-PAT had been effectively transmitted from mixtures with ZYMV-HAT HC, the efficiency of these infected plants as sources of virus for aphids was tested. They all proved to be very poor sources confirming that they had been infected by ZYMV-PAT.

Experiments were also conducted using concentrated HC preparations, higher concentrations of purified virus, and a larger number of aphids per test plant. Results were similar to those obtained in the first set of experiments (Table 4).

In vitro complementation for aphid transmission between a non- and a poorly aphid-transmissible isolate. To demonstrate that the loss of aphid transmissibility in ZYMV-PAT and ZYMV-NAT is a result of different deficiencies, in vitro virus acquisition experiments were conducted with different combinations of purified virus or HC (Table 5). Transmission only occurred in one combination, when virions of ZYMV-PAT were mixed with the ZYMV-NAT HC, confirming that ZYMV-NAT produces an active HC, whereas ZYMV-PAT possesses a functional coat protein.

In vivo and in vitro synthesis of HC by ZYMV-PAT. Experiments were conducted to determine whether ZYMV-PAT-infected plants produced HC. An HC serologically related protein was detected in ZYMV-PAT-infected plants using an antiserum to TVMV-HC prepared by Thornbury and Pirone (27) which cross reacts with ZYMV-HC (10). In two independent experiments conducted in DAS-ELISA with four and six replicates for each treatment, infected plant extracts diluted 1:100 gave respective

TABLE 2. Aphid transmission of two isolates of zucchini yellow mosaic virus (ZYMV) from different sources[a]

Source[b]	Weeks after inoculation	ZYMV-HAT[c]	ZYMV-PAT[c]
Melon (inoculated leaf)	3	25/30[d]	2/30[e]
Melon	3	24/30	0/30
	8	24/30	0/30
	14	24/30	0/30
Zucchini squash	3	23/30	0/30
Cucumber	3	22/30	0/30

[a] Aphids (*Myzus persicae*) were allowed a 1-min acquisition access period on infected leaves before being transferred to test plants.
[b] Unless otherwise indicated, sources of virus were systemically infected leaves from plants that had been mechanically inoculated.
[c] ZYMV-HAT, highly aphid-transmissible or ZYMV-PAT, poorly aphid-transmissible.
[d] Numerator = number of plants infected; denominator = number of plants inoculated.
[e] Plants infected following aphid transmission of ZYMV-PAT were individually checked and found to be inefficient virus sources for vector transmission.

TABLE 3. Aphid transmission of two purified isolates of zucchini yellow mosaic virus (ZYMV) in the presence of extracts containing helper component (HC) of these isolates

Virus[a]	HC	Transmission[b]
ZYMV-HAT	ZYMV-HAT	33/40[c]
ZYMV-HAT	ZYMV-PAT	0/40
ZYMV-PAT	ZYMV-HAT	31/40[d]
ZYMV-PAT	ZYMV-PAT	0/40
ZYMV-HAT		0/20
ZYMV-PAT		0/20
	ZYMV-HAT	0/20
	ZYMV-PAT	0/20

[a] Solutions contained a combination of virions (40 μg/ml), HC-containing extracts, and 20% sucrose. When either HC or virus were omitted they were replaced by an equal volume of buffer (0.3 M K_2HPO_4, pH 9, and 0.02 M potassium phosphate buffer, pH 7.4, respectively). ZYMV-HAT = highly aphid-transmissible; ZYMV-PAT = poorly aphid-transmissible.
[b] Aphids (*Myzus persicae*) were allowed a 10-min acquisition access period before being transferred by groups of five to melon test plants.
[c] Numerator = number of plants infected; denominator = number of plants inoculated (cumulated data from four independent experiments).
[d] Plants infected following aphid transmission of ZYMV-PAT were individually checked and found to be inefficient virus sources for vector transmission.

TABLE 4. Aphid transmission of two purified isolates of zucchini yellow mosaic virus (ZYMV) in the presence of concentrated preparations of helper component (HC) of these isolates

Virus[a]	HC	Transmission[b]
ZYMV-HAT	ZYMV-HAT	24/30[c]
ZYMV-HAT	ZYMV-PAT	0/30
ZYMV-PAT	ZYMV-HAT	28/30[d]
ZYMV-PAT	ZYMV-PAT	0/30
ZYMV-HAT		0/25
ZYMV-PAT		0/25
	ZYMV-HAT	0/25
	ZYMV-PAT	0/25

[a] Solutions contained a combination of virions (80 μg/ml), concentrated HC preparations, and 20% sucrose. When either HC or virus were omitted, they were replaced by an equal volume of buffer (0.3 M K_2HPO_4, pH 9, and 0.02 M potassium phosphate buffer, pH 7.4, respectively). ZYMV-HAT = highly aphid-transmissible; ZYMV-PAT = poorly aphid-transmissible.
[b] Aphids (*Myzus persicae*) were allowed a 10-min acquisition access period before being transferred by groups of 10 to melon test plants.
[c] Numerator = number of plants infected; denominator = number of plants inoculated (cumulated data from three independent experiments).
[d] Plants infected following aphid transmission of ZYMV-PAT were individually checked and found to be inefficient virus sources for vector transmission.

mean absorbance values at 405 nm (A_{405nm}) of 0.35 and 0.37 for ZYMV-HAT, and 0.2 and 0.22 for ZYMV-PAT. Healthy plant extracts and purified virion preparations (0.5 μg/ml) gave A_{405nm} values of 0.06 and 0.02, respectively. In the second experiment, ZYMV-HAT-infected plant extracts diluted 1:200 yielded an A_{405nm} value of 0.21, equivalent to that observed with ZYMV-PAT at a 1:100 dilution.

To further investigate the synthesis of a HC-related protein by ZYMV-PAT, in vitro translation experiments were done using RNA preparations from ZYMV-HAT and ZYMV-PAT. No differences were observed in the pattern of the proteins synthetized in vitro in the RRL by the two strains either before or after immunoprecipitation by antisera to papaya ringspot virus type watermelon amorphous inclusion protein, ZYMV coat or cylindrical inclusion proteins, tobacco etch virus nuclear inclusion proteins a and b (Fig. 1), or by an antiserum to TVMV-HC (data not shown).

TABLE 5. In vitro complementation for aphid transmission of non- (NAT) and poorly (PAT) aphid-transmissible isolates of zucchini yellow mosaic virus (ZYMV)

Virus[a]	HC	Transmission[b]
ZYMV-NAT	ZYMV-NAT	0/30[c]
ZYMV-NAT	ZYMV-PAT	0/30
ZYMV-PAT	ZYMV-NAT	25/30[d]
ZYMV-PAT	ZYMV-PAT	0/30
ZYMV-NAT		0/25
ZYMV-PAT		0/25
	ZYMV-NAT	0/25
	ZYMV-PAT	0/25

[a]Solutions contained a combination of virions (80 μg/ml), concentrated HC preparations, and 20% sucrose. When either HC or virus were omitted they were replaced by an equal volume of buffer (0.3 M K_2HPO_4, pH 9, and 0.02 M potassium phosphate buffer, pH 7.4, respectively).
[b]Aphids (*Myzus persicae*) were allowed a 10-min acquisition access period before being transferred by groups of 10 to melon test plants.
[c]Numerator = number of plants infected; denominator = number of plants inoculated (cumulated data from three independent experiments).
[d]Plants infected following aphid transmission of ZYMV-PAT were individually checked and found to be inefficient virus sources for vector transmission.

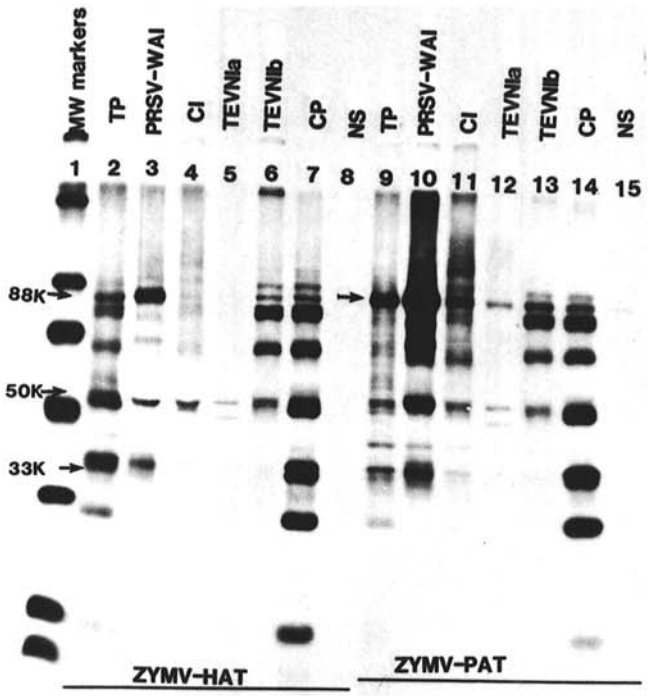

Fig. 1. Analysis of the in vitro translation products of zucchini yellow mosaic virus (ZYMV) RNA of isolates that were highly (HAT) or poorly (PAT) aphid-transmissible. Lane 1, MW markers from top to bottom, 200K, 97K, 67K, 45K, 29K, 17K, and 14K; lane 2, TP, total products of ZYMV-HAT translation. ZYMV-HAT translation products immunoprecipitated by antiserum to papaya ringspot virus (PRSV) type watermelon (-W) amorphous inclusions (AI), lane 3; to ZYMV cylindrical inclusions (CI), lane 4; to tobacco etch virus nuclear inclusion a (TEVNIa), lane 5; or b (TEVNIb), lane 6; to ZYMV coat protein (CP), lane 7. Lane 8, immunoprecipitation with normal serum (NS). Lane 9, TP of ZYMV-PAT. ZYMV-PAT translation products immunoprecipitated by antiserum to PRSV-WAI, lane 10; to ZYMVCI, lane 11; to TEVNI a and b, lanes 12 and 13; and to ZYMVCP, lane 14. Lane 15, immunoprecipitation with NS. The translation conditions in the rabbit reticulocyte lysate and the analyses of products were as outlined in Xiong et al (28). The molecular weights of some products are given on the left.

DISCUSSION

The ZYMV-PAT isolate is poorly transmitted by different aphid species, from different host species, and at different times after inoculation, in conditions in which the ZYMV-HAT isolate is transmitted efficiently. Poor aphid transmissibility cannot be associated with the nature of the source plant or with the experimental conditions in which the transmission tests were conducted, but is rather an intrinsic property of the isolate. When the two isolates were purified simultaneously, generally higher yields were obtained for ZYMV-PAT ruling out the possibility that a lower virus multiplication was responsible for the low transmission rates observed. Similar specific infectivities for purified virus preparations of the two isolates also suggested that there are no major differences in virus stability in vitro.

In vitro acquisition experiments, using homologous and heterologous combinations of purified virus preparations and crude or concentrated HC preparations allowed us to conclude that loss of aphid transmissibility of ZYMV-PAT is associated with a modification of the HC activity of this isolate. No transmission occurred when purified ZYMV-HAT virus particles were mixed with ZYMV-PAT HC, whereas ZYMV-HAT virus particles were very efficiently transmitted when mixed with homologous HC. This demonstrates that ZYMV-PAT HC is not functional for mediating the transmission of a highly transmissible ZYMV isolate. In similar experiments in which ZYMV-HAT HC was mixed with purified ZYMV-PAT virions, a high level of transmission was observed for ZYMV-PAT, indicating that this isolate is readily transmissible whenever a functional HC is provided.

The ZYMV-PAT is apparently producing a protein which is serologically related to HC. This has been seen in plants which were inoculated by ZYMV-PAT and in in vitro translation products of ZYMV-PAT RNA. However, our ELISA data may suggest that there is a lower concentration of HC in ZYMV-PAT-infected plants than in ZYMV-HAT-infected plants; on the other hand, the use of concentrated ZYMV-PAT HC did not lead to transmission, ruling out the possibility that the poor aphid transmissibility of ZYMV-PAT could be due to a lower HC concentration in crude extracts. The difference observed in A_{405nm} values obtained in ELISA may as well reflect serological differences in the HC proteins from the two isolates. If a mutation leading to a difference in the amino acid sequence of the HC protein is involved in the loss of aphid transmissibility of ZYMV-PAT, this change may also modify the HC epitopes.

The ZYMV-PAT and PVC are so far the only potyvirus isolates for which there is direct evidence that the HC activity alone is responsible for the loss of aphid transmissibility. Although few attempts were made to characterize the nature of lack of aphid transmissibility, it seems that the occurrence of HC deficient isolates is less common than the occurrence of coat protein deficient isolates (1,11,21).

Loss of aphid transmissibility of several potyviruses has been associated with repeated transfers through mechanical inoculation (16); for ZYMV-PAT we have not been able to trace back exactly when the modification from a transmissible to a non-transmissible form occurred, but it occurred after not more than five successive transfers. This suggests that poorly or non-aphid-transmissible isolates may have a selective advantage that allows them to

compete successfully over aphid-transmissible ones in this experimental context, although in nature this may not be the case.

Poorly or non-aphid-transmissible potyvirus isolates may be of practical interest for plant breeders, because loss of aphid transmissibility does not apparently modify the virus pathogenicity. Such isolates would permit inoculation of large numbers of plants, with limited risks of unwanted spread of the virus within the greenhouses. Furthermore, poorly and non-aphid-transmissible isolates of ZYMV deficient on the HC function or with a modified coat protein offer a unique model for understanding the interactions leading to the transmission of potyviruses by aphids.

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