Evidence that Heteroencapsidation Between Two Potyviruses Is Involved in Aphid Transmission of a Non-Aphid-Transmissible Isolate from Mixed Infections

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ABSTRACT

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A non-aphid-transmissible isolate of zucchini yellow mosaic virus, ZYMV-NAT, which possesses a transmission-deficient capsid protein, was regularly transmitted by aphids from plants infected concomitantly by this virus and a transmissible isolate of papaya ringspot virus type W (PRSV-E2). This phenomenon was reproduced in in vitro acquisition experiments by mixing virions purified from plants co-infected by ZYMV-NAT and PRSV-E2, with PRSV-E2 helper component (HC) preparations. In contrast, ZYMV-NAT was not transmitted regardless of the HC used, when purified ZYMV-NAT virions were used either alone or mixed with purified PRSV-E2 virions. Immunosorbent electron microscopy experiments using the decoration technique were done with crude or purified extracts from singly or doubly infected plants. In extracts from plants infected by only one virus, particles were either fully decorated or not decorated when homologous or heterologous antisera were used, respectively. In extracts from plants infected by the two viruses, a third type of particle presenting irregular decorations was frequently observed. These particles that showed a phenotypic mixing were also revealed by the two-sites enzyme-linked immunosorbent assay technique: high absorbance values were observed at $A_{405\ nm}$ with extracts from plants with a mixed infection, while no or weaker absorbance was obtained with extracts from single infections alone or mixed. It is concluded that ZYMV-NAT aphid transmission, in vivo from plants doubly infected by this virus and PRSV-E2 or in vitro from preparations containing purified virions from these doubly infected plants, occurred through heteroencapsidated particles and was mediated by PRSV-E2 HC.

Transmission by aphids of a normally non-aphid-transmissible isolate when in a mixed infection with a transmissible virus, is a well known phenomenon for different groups of viruses transmitted by aphids in a persistent, semi-persistent, or nonpersistent manner (24,25,33,35). The mechanisms involved in the case of persistent viruses, and particularly for barley yellow dwarf virus (BYDV), have been elucidated; dependent transmission has been associated with heterologous encapsidation, a mechanism by which the nucleic acid of the nontransmissible isolate is encapsidated within a capsid protein shell consisting of capsomers of the transmissible isolate (transcapsidation or genomic masking) (5), or of both isolates (phenotypic mixing) (12).

For nonpersistent viruses and particularly for potyviruses, the situation is different because two distinct proteins coded for by the virus genome are involved in aphid transmission: the helper component (HC) and the coat protein (CP) (29,30). Therefore, loss of aphid transmissibility may result from deficiencies in either one of these proteins. Recent comparisons of amino acid sequences of these two proteins, conducted for aphid and non-aphid-transmissible isolates, revealed that changes of only one or two amino acids in either of the proteins could lead to loss of aphid transmissibility (2,9,36).

In the case of HC-deficient isolates, transmission of a nontransmissible isolate may occur whenever a functional HC is provided. This transmission may be observed after aphid sequential probing, first on a plant infected by a transmissible virus, and subsequently on plants infected by a nontransmissible one (14,29), or in in vitro acquisition tests when purified virions of the nontransmissible isolate are mixed with HC from a transmissible isolate (16). This form of assistance probably also occurs in plants doubly infected by an HC-deficient isolate and a transmissible one (13,18). However, this functional complementation cannot explain the transmission of nontransmissible isolates producing efficient HC, but with a transmission-deficient CP. This is the situation reported by Hobbs and McLaughlin (11) who showed that a non-aphid-transmissible isolate of bean yellow mosaic virus (BYMV) with a transmission-deficient CP was transmissible when in a mixed infection with an aphid-transmissible isolate of pea mosaic virus (PMV). Therefore another mechanism must be involved in assisting aphid transmission of potyviruses.

Zucchini yellow mosaic virus (ZYMV) and papaya ringspot virus (PRSV) are two potyviruses that cause severe losses in cucurbit crops in many parts of the world (22,31). Several isolates of these viruses have been described that are poorly or non-aphid-transmissible. Loss of transmissibility could be related either to the production of an inactive HC (ZYMV-PAT) (15,16) or to modifications in the amino acid sequence of a triplet asp-ala-gly located near the capsid protein N-terminus (ZYMV-NAT) (9,10).

We report here that the non-aphid-transmissible isolate of ZYMV (ZYMV-NAT), which is transmission-deficient on the CP, is transmitted when in a mixed infection with an aphid-transmissible isolate of PRSV, and we provide evidence that this phenomenon occurs through heteroencapsidation.

MATERIALS AND METHODS

Virus isolates. The non-aphid-transmissible isolate of ZYMV (ZYMV-NAT) was obtained from B. Raccah, ARO, the Volcany center, Israel, and has been previously described (1); it produces active HC but is transmission deficient on the CP (9,10).

The aphid-transmissible isolate of PRSV type watermelon (PRSV-E2) has been isolated in France and was described previously (19).

Plant inoculations. Melon plants (*Cucumis melo* L. 'Védrantais') were first mechanically inoculated on one cotyledon with PRSV-E2 by the standard method used in our laboratory (17). Some of the plants and noninoculated ones were mechanically inoculated subsequently with ZYMV-NAT on the alternate cotyledon, 2 or 3 days later. This sequential inoculation procedure has been used to avoid competition phenomena that can occur

when ZYMV is co-inoculated with other potyviruses (6, and Lecoq, *unpublished*). Plants were used, 10–15 days after the second inoculation, as sources for plant-to-plant aphid transmission experiments, or for virus and HC purifications.

Virus and HC purifications. Viruses and HC were separately purified from plants infected with either PRSV-E2 or ZYMV-NAT only, or from plants co-infected with both isolates. The purifications were done as previously described (20).

Aphid transmission experiments. All transmissions were performed using *Myzus persicae* Sulzer reared on healthy pepper plants as previously described (17). Plant-to-plant transmissions were carried out as follows: after a 1- to 3-h starvation period, groups of aphids were deposited on detached infected leaves for a 1- to 2-min acquisition access period. Those found in the probing position were transferred carefully to healthy plants. For each treatment, 10 test plants with three aphids per plant, and 20 test plants with one aphid per plant were used.

A second set of aphids was used for sequential acquisition experiments. Aphids were singly deposited first on a leaf infected by an isolate for 1 min, then transferred to a leaf infected by the other virus for 1 min, before being transferred to healthy test plants. Ten test plants with three aphids per plant were used for each treatment.

For in vitro acquisition experiments, groups of starved aphids were allowed a 10-min acquisition access period to test solutions through a stretched Parafilm membrane (20,32). The test solutions contained 80 μg of purified virus per milliliter, fresh HC prepared within the same day, and 20% sucrose. For each treatment, 10 test plants with 10 aphids per plant, and 35 test plants with one aphid per plant were used. Aphids were allowed to feed on test plants for at least 2 h before being killed by an insecticide. The plants were observed for symptom development for 15–20 days after exposure to the aphids.

All test plants found infected by ZYMV after aphid transmission were used to inoculate mechanically *C. melo* 'WMR29' plants, which are resistant to PRSV (3), to recover ZYMV alone. After 10 days incubation, the WMR29 plants were checked as virus sources for aphid transmission to determine the transmissibility of the ZYMV subcultures.

Enzyme-linked immunosorbent assay (ELISA). Double antibody sandwich (DAS) ELISA (4) was used to identify the viruses present in each test plant developing virus symptoms after the aphid transmission tests. Polyclonal antisera against ZYMV-E9 and PRSV-E2 obtained in our laboratory (19,21) were used to prepare IgG and alkaline phosphatase-conjugated IgG. No, or only slight, heterologous reactions were noticed between ZYMV and PRSV when using these reagents in DAS-ELISA, allowing an easy differentiation of plants with single or double infections. Virus concentrations in source leaves used for aphid transmission experiments were estimated also by DAS-ELISA. The absorbance ($A_{405\ nm}$) readings of plant extracts were compared to those obtained with purified virus preparations at known concentrations and diluted in healthy plant sap, at the same dilutions as those of infected plant extracts.

In two-sites ELISA (TS-ELISA), IgG specific for one virus was used in the plate-coating step to trap virus, whereas conjugated IgG specific for the second virus was used to react with the trapped virus. To limit nonspecific fixation of viral particles to the plates, the following modifications were applied to our standard DAS-ELISA protocol: after the plate-coating step, the plates were blocked with 0.5% bovine serum albumin (BSA) at 37 C for 2 h; plant extractions were made in 0.01 M phosphate buffer, pH 7, and the incubation of plant extracts in plates lasted for 1.5 h at room temperature.

Immunosorbent electron microscopy (ISEM). The technique used was derived from Derrick's method (8) combined with the decoration step as described by Milne and Luisoni (23). Grids covered with a pyroxiline membrane were incubated for 20 min at 37 C with a mixture of ZYMV and PRSV antisera at appropriate dilutions (1:3,200 and 1:6,400 for PRSV and ZYMV antisera, respectively). The grids were washed twice in phosphate buffer (0.06 M, pH 7.2) for 10 min, placed on drops of virus preparations and left for 2 h at room temperature. Two types of virus preparations were used in the ISEM experiments: crude extracts prepared by grinding leaves used for aphid transmission in 4 vol (w/v) of phosphate buffer and diluted 1:30, and purified virus preparations at 1:300 dilution. Afterwards, the grids were rinsed with 10 drops of double-distilled water, drained, and deposited on a drop of antiserum (PRSV 1:30 or ZYMV 1:60). After incubation for 20 min at 37 C, the grids were washed with 10 drops of double-distilled water, drained and negatively stained with 1 drop of 1% ammonium molybdate, pH 7. They were finally drained and dried before being observed with a Phillips CM10 electron microscope.

RESULTS

Plant to plant transmission experiments. Aphid transmission of ZYMV-NAT was not observed from melon plants infected only by this virus (Table 1), or if aphids were allowed to probe first on PRSV-E2-infected plants and subsequently on ZYMV-NAT-infected plants (data not shown). In contrast, transmission of ZYMV-NAT occurred from plants with mixed infection by PRSV-E2 and ZYMV-NAT (Table 1). Transmission rates for ZYMV-NAT were 34% with three aphids per test plant and 19% (46 plants among 240 inoculated) when one aphid was deposited per test plant. Frequently, test plants were found to be co-infected by ZYMV-NAT and PRSV following aphid transmissions, but ZYMV-NAT was also transmitted alone, particularly when only one aphid was deposited per test plant (25 plants among 240 inoculated), which limits the chance of multiple inoculations. The leaves used as sources of virus for aphid acquisition tests had similar concentrations of each virus whether the plants had single or double infections (Table 1).

When ZYMV transmission was detected, mechanical inoculation to WMR29 plants permitted the separation of ZYMV from PRSV. No ZYMV transmission by aphids was observed from the WMR29 plants, confirming that they were infected by ZYMV-NAT and consequently that it was ZYMV-NAT that was transmitted from doubly infected plants and not an aphid-transmissible ZYMV contaminant.

In vitro acquisition experiments. Four experiments were done to determine the role of the source of HC and virions in the transmissibility of ZYMV-NAT, and the results have been combined (Table 2). No transmission was observed when either purified viruses or HC were omitted from the feeding solutions. When HC of either PRSV-E2 or ZYMV-NAT was mixed with PRSV-E2 virions, transmission of PRSV-E2 occurred. The transmission rate was high when the homologous HC was used (66%), whereas it was very low with ZYMV-NAT HC (2%). In contrast, no ZYMV transmission was observed when ZYMV-NAT virions purified from plants with single infections were used, either alone

TABLE 1. Aphid transmission of a non-aphid-transmissible isolate of zucchini yellow mosaic virus (ZYMV-NAT) from plants with single or mixed infections with an aphid-transmissible isolate of papaya ringspot virus (PRSV-E2) and estimated virus concentrations in source leaves

Virus in source plant[a]	Virus concentrations[b]		Transmission[c]	
	PRSV	ZYMV	PRSV	ZYMV
PRSV-E2	34 a	...	24/30	0/30
ZYMV-NAT	...	209 b	0/30	0/30
PRSV-E2 + ZYMV-NAT	26 a	100 b	83/120	41/120

[a] Aphids were allowed a 1-min acquisition access period on infected leaves before being transferred by groups of three to each test plant.

[b] Virus concentration (expressed in μg/g of infected tissue) were estimated by comparing the absorbance (A_{405nm}) readings obtained in DAS-ELISA with extracts from source leaves (diluted 1:500) with the A_{405nm} of known concentration of purified virus in healthy plant sap at the same dilution. Numbers followed by the same letter did not differ significantly ($P = 0.05$) by analysis of variance.

[c] Transmission of PRSV or ZYMV was assessed by DAS-ELISA and expressed as the number of infected plants over the number of plants inoculated.

or artificially mixed with PRSV-E2 purified virions regardless of the HC used.

Transmission of ZYMV-NAT was observed only when the HC was from PRSV-E2 and when purified virions were from plants co-infected by ZYMV-NAT and PRSV-E2. Under these conditions, the rate of transmission of ZYMV-NAT was 16% when 10 viruliferous aphids were deposited per test plant. Similar to the observations made in the plant-to-plant transmission experiments, ZYMV-NAT could be transmitted alone, but this occurred only when one aphid was deposited per test plant (two plants among 450 inoculated).

To investigate a possible involvement of the source of HC in the transmission of ZYMV-NAT, mixtures of HC or HC extracted from doubly infected plants were also tested. The results were similar to those observed with PRSV-E2 HC. ZYMV-NAT was transmitted only if purified viruses originated from doubly infected plants and the HC solution contained HC from PRSV-E2.

The presence of ZYMV-NAT in the plants that reacted positively in DAS-ELISA for ZYMV was confirmed by transfers to WMR29 from which aphids were not able to transmit ZYMV.

ISEM. Observations were made on grids prepared either with crude extracts from infected plants or with purified viruses; results were similar in both cases. In extracts from ZYMV-NAT- or PRSV-E2-infected plants, or in purified preparations of each isolate, all particles were either completely decorated when the homologous antiserum was used in the decoration step (Fig. 1A and Fig. 2A, F), or not decorated, when the heterologous antiserum was used (Fig. 1B and Fig. 2B, E).

In plants co-infected by ZYMV-NAT and PRSV-E2, or in purified virus preparations from these plants, three types of particles were always observed: some were homogeneously decorated, some were not decorated as described above, and a third type was only partially decorated (Fig. 1C and Fig. 2D, H). This third type of particle was observed in all preparations from 12 different plants with mixed infections and represented a total of 18.2% of 1,283 particles observed on 24 grids. These particles could either have long or short sections decorated by the antiserum used. When a mixture of the two antisera was used for the decoration step, all particles appeared completely decorated. When purified virions from single infections were mixed in a 1:1 ratio and used for decoration, partially decorated particles were never observed (Fig. 2C, G).

Two-sites ELISA. The TS-ELISA was used concurrently with DAS-ELISA to compare preparations from melons singly or doubly infected by ZYMV-NAT and PRSV-E2, and artificial mixtures (1:1) of preparations from singly infected plants. In DAS-ELISA, high absorbance values were obtained at 405 nm with extracts from plants infected by the homologous virus or by both viruses, or with artificial mixtures of extracts from singly infected

TABLE 2. Aphid transmission of purified virions of an aphid-transmissible isolate of papaya ringspot virus (PRSV-E2) and of a non-aphid-transmissible isolate of zucchini yellow mosaic virus (ZYMV-NAT) in the presence of helper component (HC) extracts

Aphid feeding source[a]		Transmission[b]	
HC	Virus	NAT	E2
NAT[c]	...	0/40	0/40
NAT	NAT	0/50	0/50
NAT	(E2/NAT)	0/50	0/50
NAT	E2+NAT	0/50	1/50
NAT	E2	0/50	3/50
E2	...	0/40	0/40
E2	NAT	0/40	0/40
E2	(E2/NAT)	8/50	33/50
E2	E2+NAT	0/50	24/50
E2	E2	0/50	30/50
(E2/NAT)	...	0/40	0/40
(E2/NAT)	NAT	0/40	0/40
(E2/NAT)	(E2/NAT)	4/50	24/50
(E2/NAT)	E2+NAT	0/50	20/50
(E2/NAT)	E2	0/40	19/40
E2+NAT	...	0/40	0/40
E2+NAT	NAT	0/40	0/40
E2+NAT	(E2/NAT)	1/50	16/50
E2+NAT	E2+NAT	0/40	15/40
E2+NAT	E2	0/50	9/50
...	NAT	0/40	0/40
...	E2	0/40	0/40
...	(E2/NAT)	0/40	0/40

[a] Aphids were allowed a 10-min acquisition access period before being transferred by groups of 10 on healthy test plants. Feeding solutions contained a mixture of HC preparation, 20% sucrose, and 80 μg/ml of purified viruses. When either HC or virus was omitted, it was replaced by an equal volume of a respective buffer.

[b] Transmission of PRSV or ZYMV was assessed by DAS-ELISA and expressed as the number of plants infected over the number of plants inoculated (cumulated data from four independent experiments).

[c] NAT or E2: HC or virus purified from plants singly infected by ZYMV-NAT or PRSV-E2 respectively; E2 + NAT: mixture in equal amounts of HC or purified virus preparations from plants infected by PRSV-E2 or ZYMV-NAT, respectively; (E2/NAT): HC or virus purified from plants doubly infected by PRSV-E2 and ZYMV-NAT.

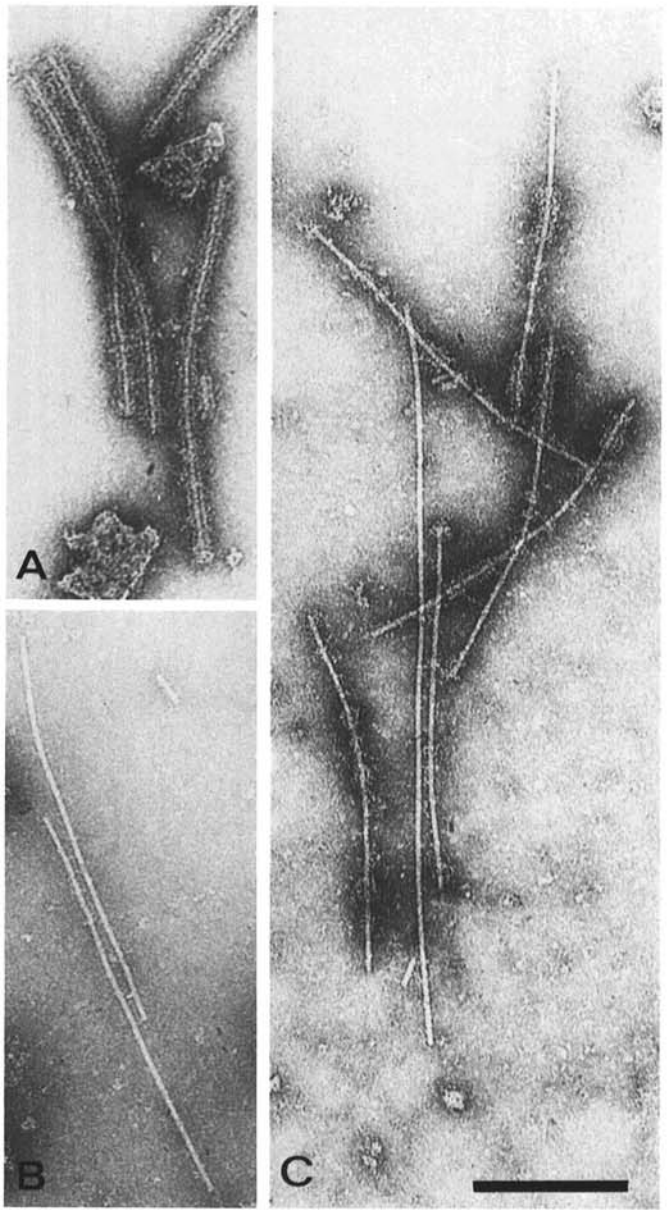

Fig. 1. Electron micrographs of extracts from plants infected **A,** by papaya ringspot virus (PRSV-E2); **B,** by zucchini yellow mosaic virus (ZYMV-NAT), or **C,** by both of these viruses. Grids were coated with a mixture of PRSV and ZYMV antisera and trapped particles were decorated with PRSV antiserum. Numerous partially decorated particles were observed in extracts from doubly infected plants (C). Bar = 300 nm.

plants (Table 3). In contrast, no reactions or only very slight reactions were observed with extracts from the heterologous virus. When TS-ELISA was used, high absorbances were observed with extracts from plants infected by the two viruses, whereas no or weaker reactions were detected with extracts from single infections or with mixtures of these extracts. The weak reactions that were still detected with extracts from plants singly infected by the virus homologous to the conjugated IgG used suggest that some non-specific binding to the plates did occur despite the use of BSA as a blocking agent.

DISCUSSION

Loss of aphid transmissibility is a relatively common phenomenon for potyviruses, and it may occur because of deficiencies of either the CP or the HC proteins. For ZYMV-NAT, which

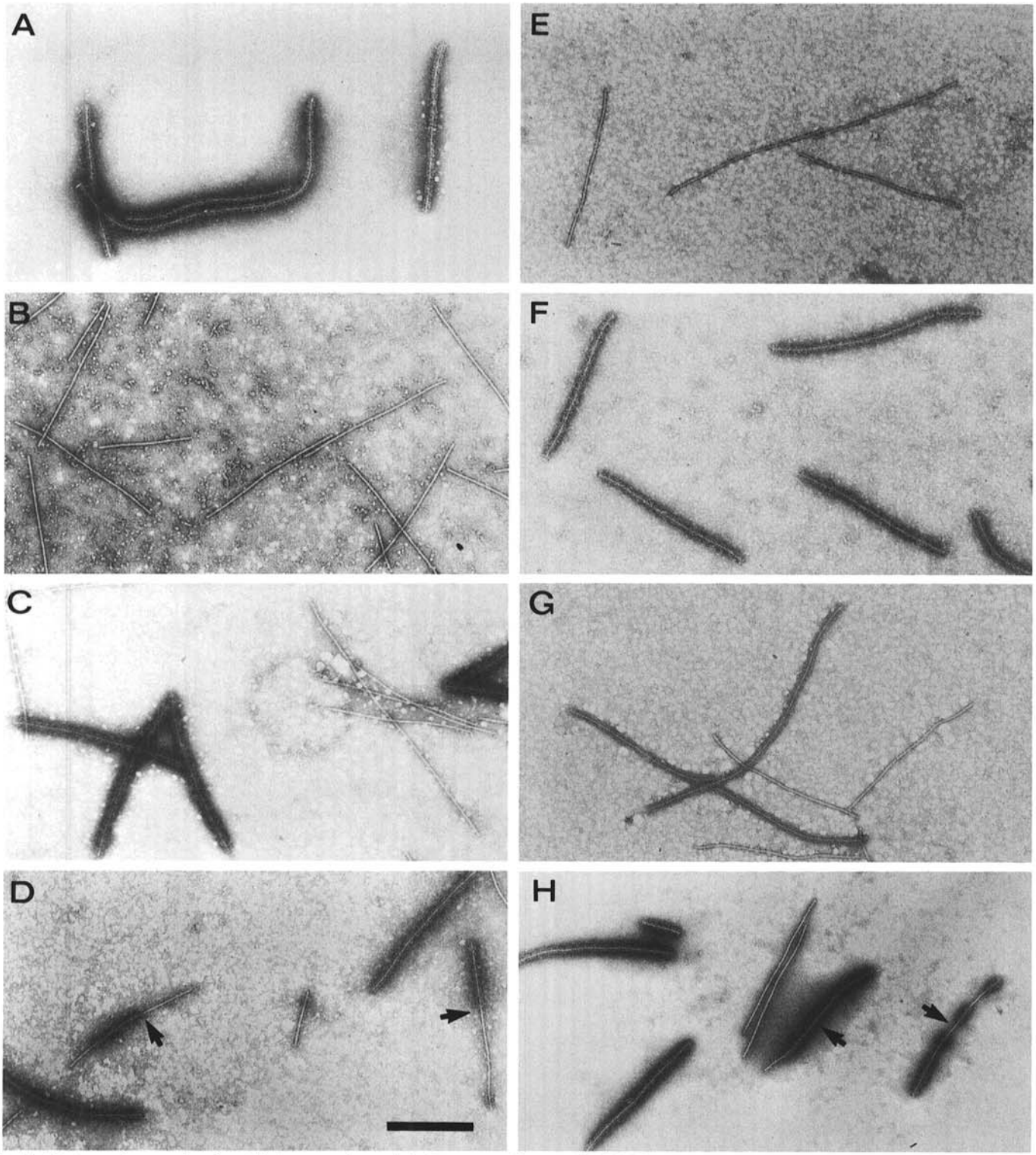

Fig. 2. Electron micrographs of purified preparations of **A, E,** papaya ringspot (PRSV-E2); **B, F,** zucchini yellow mosaic (ZYMV-NAT) viruses; **C, G,** a 1:1 mixture of these virus preparations; or **D, H,** a purified preparation from plants co-infected by PRSV-E2 and ZYMV-NAT. Grids were coated with a mixture of PRSV and ZYMV antisera, and trapped particles were decorated with PRSV (A–D) or ZYMV (E–H) antisera. In artificial mixtures, particles were either decorated or not decorated (C, G), whereas in preparations from doubly infected plants some particles appeared partially decorated (arrows) regardless of which antiserum was used for decoration (D, H). Bar = 400 nm.

TABLE 3. Absorbance values obtained at 405 nm (A_{405nm}) in double antibody (DAS) or two-sites (TS) enzyme-linked immunsorbent assay (ELISA) with extracts from plants with single or mixed infections with papaya ringspot virus (PRSV-E2) and zucchini yellow mosaic virus (ZYMV-NAT)[a]

Plant extracts containing the virus indicated[b]	DAS-ELISA[c]		TS-ELISA[c]	
	ZYMV IgG ZYMV Conjugate	PRSV IgG PRSV Conjugate	ZYMV IgG PRSV Conjugate	PRSV IgG ZYMV Conjugate
None	0.009 (0.005)[e]	0.005 (0.004)	0.006 (0.005)	0.007 (0.005)
NAT[d]	1.655 (0.285)	0.012 (0.006)	0.009 (0.005)	0.067 (0.007)
E2	0.018 (0.004)	0.412 (0.078)	0.157 (0.047)	0.015 (0.005)
(E2/NAT)	1.462 (0.178)	0.528 (0.115)	0.453 (0.063)	0.512 (0.064)
E2+NAT	1.451 (0.194)	0.330 (0.065)	0.087 (0.024)	0.089 (0.014)

[a] A_{405nm} was read after 150 min of substrate incubation at 20 C.
[b] Systemically infected leaves were ground in 0.01 M phosphate buffer, pH 7 (1:10, w/v) 10–15 days after inoculation.
[c] Immunoglobulins G were used at 0.5 μg/ml and 1 μg/ml of concentrations for ZYMV and PRSV, respectively; IgG conjugated with alkaline phosphatase were used at dilutions 1:2,000 and 1:1,000 for ZYMV and PRSV, respectively.
[d] NAT or E2: extracts from plants singly infected by ZYMV-NAT or PRSV-E2, respectively; E2 + NAT: mixture in equal amounts of extracts from plants infected by PRSV-E2 and ZYMV-NAT, respectively; (E2/NAT): extracts from plants doubly infected by PRSV-E2 and ZYMV-NAT.
[e] Results are expressed as mean absorbance values for 25 extracts for each treatment, followed by standard deviation in parentheses.

produces a functional HC (1), loss of aphid transmissibility is due to a single amino acid change in the coat protein (9).

Aphid transmission of ZYMV-NAT was not observed from melons singly infected by this isolate but was regularly observed from plants doubly infected with an aphid-transmissible isolate of PRSV. To investigate this phenomenon, different possible interactions between these two viruses were successively studied. The failure of aphids to transmit ZYMV-NAT virions when mixed with PRSV-E2 HC or HC prepared from doubly infected plants, indicates that PRSV-E2 HC does not mediate aphid transmission of ZYMV-NAT. Increase in ZYMV-NAT multiplication in co-infected plants was not responsible for its aphid transmission because similar concentrations of ZYMV-NAT were present in singly and doubly infected plants.

In in vitro acquisition experiments, ZYMV-NAT transmission occurred only when purified virions originated from plants doubly infected and when the HC preparations contained PRSV-E2 HC. These observations led us to speculate that ZYMV-NAT was present, in doubly infected plants, in an aphid-transmissible form.

This hypothesis was confirmed by ISEM experiments, which revealed the frequent occurrence of particles only partially decorated in crude or purified preparations from doubly infected plants. These particles were never observed in extracts from singly infected plants nor in mixtures of the two viruses purified separately, indicating that they were not artifacts occurring when the two viruses were present in the same preparation. These particles were not further detected when a mixture of the two antisera was used in the decoration step, indicating that they were indeed constituted with CP from both viruses. These particles, presenting a phenotyping mixing, resemble those observed in other cases of potyvirus double infections (7,26,27).

Another line of evidence for the occurrence of this type of particle in doubly infected plants was brought by the TS-ELISA, which revealed in these plants particles that were trapped by IgG specific to one virus and that reacted strongly with conjugated IgG specific to the second virus.

Failure of the ZYMV-NAT HC to mediate the transmission of the heteroencapsidated particles may result from the specificity of the virus-HC interaction. Indeed ZYMV-NAT HC was found to be very efficient in mediating aphid transmission of several ZYMV isolates (1,16), whereas in our experiments it was very inefficient in mediating PRSV-E2 transmission. This confirms previous observations of the low efficiency of ZYMV HC to assist transmission of PRSV (20).

These results led us to the conclusion that ZYMV-NAT transmission from plants doubly infected by ZYMV-NAT and PRSV-E2, or from preparations containing virions purified from these plants, is occurring through heteroencapsidated particles, containing RNA from ZYMV-NAT and partially or totally encapsidated by CP from PRSV-E2. Transmission of these particles is mediated through PRSV-E2 HC. We have not yet determined, however, whether ZYMV-NAT RNA is transmitted through the phenotypically mixed particles that we observed in ISEM or through transcapsidated particles (i.e., particles constituted of ZYMV-NAT RNA and only PRSV-E2 CP). This will be further investigated.

Heteroencapsidation has been shown to be involved in the transmission of BYDV isolates by different aphid species that are normally not vectors (12,34). Here we provide evidence that a heteroencapsidation mechanism may also be involved in the transmission of a non-aphid-transmissible potyvirus. Aphid transmission of nontransmissible isolates from mixed infections has been reported for several potyviruses (11,28); it would be interesting to verify whether this also occurred through heteroencapsidation.

If heteroencapsidation occurs naturally in crops or weed reservoirs when potyviruses are in mixed infections, which is a common situation in the fields, this phenomenom may have a considerable epidemiological importance. Indeed it could explain the transmission and the maintenance in the environment of non-aphid-transmissible isolates. More significant may be the change in vector specificity, in a similar way to that described for BYDV. A transmissible potyvirus could be disseminated by an aphid species that is not recognized as an usual natural vector of this virus: such a situation has been reported for soybean mosaic virus isolates differing in vector specificity when in mixed infection (28).

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